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Immediate and protracted transcriptional response of muscle tissue to transient variation of incubation temperature in broilers

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Abstract

Eggshell temperature is a critical factor, which can possibly influence immediate and/or long-term gene transcription and expression. Manipulation of temperature (low 36.8°C and high 38.8°C relative to control 37.8 °C) at two specific periods of embryonic development day (ED7-10 or ED10-13) was carried out and microarray-based gene expression profiles were explored in hind-limb and breast muscles. The overall results show tissue-specific patterns of transcriptional changes depending on temperature, day of the treatment and indicate immediate and long-term responses to the aforementioned treatment factors. Interestingly, the high temperature modification at early ED7-10 showed a profound immediate response based on the number of differentially expressed genes (DEGs), while the effect the low temperature was observed more at late ED10-13 compared to early ED7-10. Moreover, the low temperature modification at ED10-13 relative to ED7-10 and compared to other treatment conditions resulted in more DEGs at the day 35 post-hatch sampling stage regarded as long-term effects and implying an involvement of epigenetic mechanisms. microRNA (miRNA) expression analysis was performed to understand post-transcriptional regulation. *In silico* functional analysis of DEGs and differential miRNAs suggests that acute response to high temperature at ED7-10 for both muscle tissue types elevated mRNA transcripts related to cell maintenance, organismal development, and survival ability such as FABP1 in hind-limb and SMAD3 in breast muscle. Down-regulation of miR-199a-5p, miR-1915, miR-638 in hind-limb muscle and up-regulation of miR-133 in breast muscle were treatment specific and positively influence size of body and myogenesis, respectively. Low temperature condition at ED 10-13 affected reduction on programmed cell death and possibly gained mass of skeletal muscle in hind-limb samples (e.g. NR1H3), while pathways of accelerated cardiovascular system, skeletal-muscular, and connective tissue development were over-represented via the RUNX2 gene in breast. Interestingly, down regulation of let-7, miR-93 and miR-130c in breast was associated with diminished size of bone in type II muscle. Thermal interventions during incubation initiate immediate and delayed transcriptional responses that are specific for timing and direction of treatment. For miRNAs the study shows substantial immediate alterations, whereas late miRNA response was small. The mechanisms mediating considerable phenotypic plasticity contribute to the biodiversity and broaden the basis for managing poultry populations.

Kurzzusammenfassung

Die Eierschalentemperatur ist ein sehr sensibler Faktor, dessen Beeinflussung potentielle Auswirkungen auf die unmittelbare und/oder langfristige Genexpression in verschiedenen Geweben bewirken kann. Die vorliegende Arbeit befasst sich mit der Manipulation der Brutbedingungen beim Haushuhn durch die gezielte Erhöhung und Verringerung der Inkubationstemperatur (36,8°C und 38,8°C) relativ zur Kontrolle (37,8°C) in zwei Entwicklungsstadien („embryonal day“; ED7-10 oder ED10-13). Effekte auf Mikroarray-basierte Expressionsprofile in Proben des Oberschenkel- und Brustmuskelgewebes wurden untersucht. Die Ergebnisse zeigten, dass in Abhängigkeit der Inkubationstemperatur und des embryonalen Zeitraums der Behandlung, Veränderungen in den gewebespezifischen Expressionsmustern sowohl auf Ebene der embryonalen Entwicklung als auch im adulten Stadium nachgewiesen werden konnten. Diese Veränderungen deuteten auf unmittelbare und langfristige Anpassungen an die Variation der Brutfaktoren hin. Die Erhöhung der Inkubationstemperatur im Zeitraum ED7-10 zeigte anhand der Anzahl differenziell exprimierter Gene (DEGs) deutliche und unmittelbare Effekte. Die Auswirkungen der Temperaturverringerung waren vor allem zum Zeitpunkt ED10-13 zu beobachten. Zudem resultierte die Temperaturverringerung an ED10-13 in einer erhöhten Anzahl an DEGs zum 35. Lebenstag im Vergleich zu den anderen getesteten Brutbedingungen und impliziert die Manifestation von Langzeiteffekten unter Beteiligung epigenetischer Mechanismen. Um die zu Grunde liegende posttranskriptionale Regulation von Genen zu beleuchten, wurden holistische micro RNA (miRNA) Expressionsanalysen durchgeführt. Die Ergebnisse unterstrichen die wichtige Rolle der miRNAs an der Regulation der Genexpression in der unmittelbaren Antwort auf die Variation der Inkubationstemperatur. Im adulten Stadium, representiert durch den 35. Lebenstag, spielt die Dynamik der miRNA-Expression offensichtlich eine geringere Rolle in der Steuerung der transkriptionellen Antwort. Funktionelle *in silico* Analysen der DEGs und der differentiell ausgelenkten miRNAs deuteten darauf hin, dass akute Anpassungen auf erhöhte Inkubationstemperaturen während ED7-10 in beiden Muskelgeweben zur verstärkten Abundanz von mRNA-Transkripten (z.B. FABP1 im Oberschenkel und SMAD3 im Brustmuskel) mit Bezug zur Zellerhaltung, Organismusentwicklung und Überlebensfähigkeit führten. Zudem zeigten sich behandlungsspezifische Herabregulationen von miR-199a-5p, miR-1915, miR-638 im

Oberschenkel sowie die Heraufregulation von miR-133 im Brustmuskel mit positivem Einfluss auf Körpergröße bzw. Myogenese. Individuen die verringerten Temperaturen an ED10-13 ausgesetzt waren, lieferten auf transkriptioneller Ebene Anzeichen für verminderten programmierten Zelltod und reduzierte Gewichte des Oberschenkelmuskels (z.B. NR1H3), während Signalwege des kardiovaskulären Systems, der Skelettmuskulatur und der Bindegewebsentwicklung überrepräsentiert waren (z.B. RUNX2). Des Weiteren war die Herabregulation von let-7, miR-93 und miR-130c im Brustmuskel assoziiert mit quantitativen Parametern der Knochengröße und der Typ-II-Muskulatur. Die Variation der Bruttemperatur führt zu unmittelbaren und persistenten transkriptionellen Antworten, die für den Zeitpunkt und die Richtung der Temperaturvariation spezifisch sind. Hinsichtlich der miRNAs zeigt die Studie substantielle sofortige Auslenkungen durch die experimentellen Bedingungen, während die nachweisbaren Langzeiteffekte gering sind. Die Mechanismen, welche die deutliche phänotypische Plastizität ermöglichen, tragen zur Biodiversität bei und erweitern die Basis der Geflügelhaltung und - züchtung.

Contents

CHAPTER I

General introduction

1 General introduction

1.1 Broiler chicken and myogenesis

Production and consumption of poultry meat are growing at a global level considering the demand from many parts of the world. In EU-28 countries, the broiler market is expected to continuous grow because of increasing domestic demand (USDA, 2014). Imported un-cooked broiler meats are necessary for meeting the demand in Europe. Brazil and Thailand remain the largest suppliers, especially Thai salted and frozen broiler cuts (USDA, 2014). The *per capita* consumption of poultry in Germany has exceeded beef meat since 1997 (LEL-LFL Bayern, 2013). Meat type poultry was developed from red jungle fowl (*Gallus gallus*) while were selected for meat purpose as a domestic subclass named broiler (*Gallus gallus domesticus*) (Ka-Shu Wong et al., 2004). Characteristic of broilers are white feathers, yellowish skin, high growth performance and food efficiency. The production time has been reduced a half time of traditional species to only five to seven weeks (McKee, 2003). The benefit of poultry to others species are nutritional properties, competitive prices production, and lack of cultural free obstacles. Concerning nutritional benefit broiler meat provides low-fat meat without intramuscular fat. White meat (i.e. breast muscle) and red meat (i.e. hind-limb muscle) contain 1.3 % and 7.3 % fat, respectively (Mountney, 1989). Moreover, the quantity of unsaturation fatty acids and low sodium and cholesterol levels even emphasize the advantage to consumer's lifestyle like eating clean diet (Reno, 2007) to professional body builder (Kleiner et al., 1994).

Selection of high growth rates, maximize feed efficiency and increase amount of muscling e.g. increasing fiber diameters are ultimate aims of broiler production (Werner and Wicke, 2007). Whereas focusing to improve muscle characteristic, an intensive hypertrophic growth could cause multiple problems to animals such as leg disorder, deep pectoral myopathies (green muscle disease), white striping and negative meat quality like reduce water holding capacity during processing and storage (PSE-like condition) (Petracci and Cavani 2011; Maltby et al. 2004). To lessen the chance of pathological process but still have positive influence on muscle yield, increasing the total muscle fiber number (MFN) is a promising condition (Werner and Wicke, 2007). The procedure can be done by selective breeding or optimizing environment during myogenesis. Because

MFN is determined during either myogenesis or early post-hatch process, manipulation of environment must be done along with embryogenesis (Halevy et al., 2006).

Mechanisms of myogenesis start with paraxial mesoderm which is pre- stem myogenic cells continuously segmented into somites. The split of somites caused new formations including dermatomes, myotomes, syndetomes and sclerotomes. The dermatomyotome (combination between dermato and myotome) is formed from premyoblastic cells. In maturing somites, premyoblastic cells differentiate into four myogenic cells including myotomal cells, embryonic myoblasts, fetal myoblasts and satellite cells. Myotomal cells and embryonic myoblasts will proliferate and differentiate into primary muscle fibers and become myoblasts and then fuse to myotubes. The fetal myoblasts will fuse to scaffold myotubes as secondary muscle fibers. The adult myoblasts from satellite cells are critical for muscle regeneration and self-renewal (reviewed from Sobolewska et al., 2011). Balancing between proliferation and differentiation are essential to embryonic muscle growth (Werner and Wicke, 2007). Considering myogenesis in cell cycle, the process consists of four different periods; G1, S, G2, and M. During these stages, the critical period is in G1-S phase transition because myoblast could progress to S-phase or exit to G0-stage. In case of G1-S phase, it initiates next DNA replication processes that cause increasing of MFN by mitotic activity of myoblast. The G1-G0 phase leads to differentiation of myoblast to myotubes and mature myofibers (Heywood et al., 2005).

Muscle in broiler can be characterized into five differences types; I, IIA, IIB, IIIA and IIIB (McKee, 2003). The first three “Twitch” muscle classification was done by differentiating of speed contraction, oxidative capacity and glycolytic metabolism (Peter et al., 1972). Type I fiber is visualized by its “red” color. The characteristic in slow-contracting, high myoglobin, mitochondria, and capillaries supporting high oxidative metabolism (Lawrie, 2006). Localization of this muscle type is for example the soleus muscle which enables stable activity such as walking and standing (Hník et al., 1985). Moreover, due to higher fat and iron contains, type I fiber develop stronger flavor than type II fiber. Type II “white” fibers are a fast-contracting fibers with high glycogen content for glycolytic metabolism. Type II fibers are capable of short bursts activity in “Fight or Flight” situation (Petracci and Cavani, 2011). The “white” meats are the majority of muscle in broiler and can be divide into subtypes; IIA and IIB (Peter et al. 1972). Type IIA fibers have mixed oxidative-glycolytic fibers which are suitable for fast-moving and repetitive action with more endurance such as the sartorius (red) muscle.

Type IIB fibers contain higher levels of ATP and glycogen. These fast-contracting fibers are more easily exhausted. It was found in pectoral muscle (breast muscle), posterior *latissimus dorsi* and satorius (white). Type III are a slow-tonic "intermediate" fibers containing two subtypes (IIIA and IIIB). The position was found in plantaris and anterior *latissimus dorsi* which always stay in contracted position (Richardson and Mead, 1999).

1.2 Incubation and hatchery

Even though broilers are selected breeds for meat production, the proper hatchering and husbandry are necessary for survival ability and high-quality chick. Now, a fertile commercial egg from provider should take 21 embryonic days (ED) for full hatching time. After receiving the fertile eggs from broiler breeder, the eggs will be transferred to hatcheries, which are controlled responsible for incubation and hatching. The incubation chamber or setter will provide temperature, humidity, air velocity which are adjustable for an appropriate development of the embryo. The setter also rotates the position of eggs to proper orientation to prevents the embryo from sticking to shell membranes and reducing embryo heat production (COBB, 2013b). After ED18th, eggs are transferred to the hatchery tray which provides an environment for the final stage of an embryo without an orientation. At ED21st, newly-hatched chicks are breaking or “pipping” their shell. Subsequently to hatching process, chicks will be transported to grow-out farms while controlling the environment for brooding around 14 days (COBB, 2013a). The study of embryo development was demonstrated by Hamburger and Hamilton, 1951. The complete 46 HH stages show the progress from pre-streak to the newly born chick. Along the different phases of development, the characteristic is becoming noticeable especially on myogenesis process. In broilers, the muscle fibers are formed into two importance phases; primary and secondary muscle fiber. Between ED4th to ED7th (HH-24 to HH-31), primary fibers which are core fibers are formed and then transformed to myotubes. Following the scaffold formation, smaller secondary muscle fibers are smaller and arranged around the primary muscle fiber until ED15th (HH-41) (Miller and Stockdale, 1987). Significant abundance of fetal myoblasts was found between ED8th to ED12th (Stockdale, 1992). At finishing off the secondary phase, the adult myoblasts will convert to a primary source of myogenic precursors in the postnatal muscle formation depending on morphology and localization of myofibers (Hartley et al., 1992; Mauro, 1961; Schultz and McCormick, 1994). During this research, the

developmental stages of late primary fiber formation (ED7th-ED10th) and early secondary fiber formation (ED10th-ED13th) are addressed (Figure 1.1).

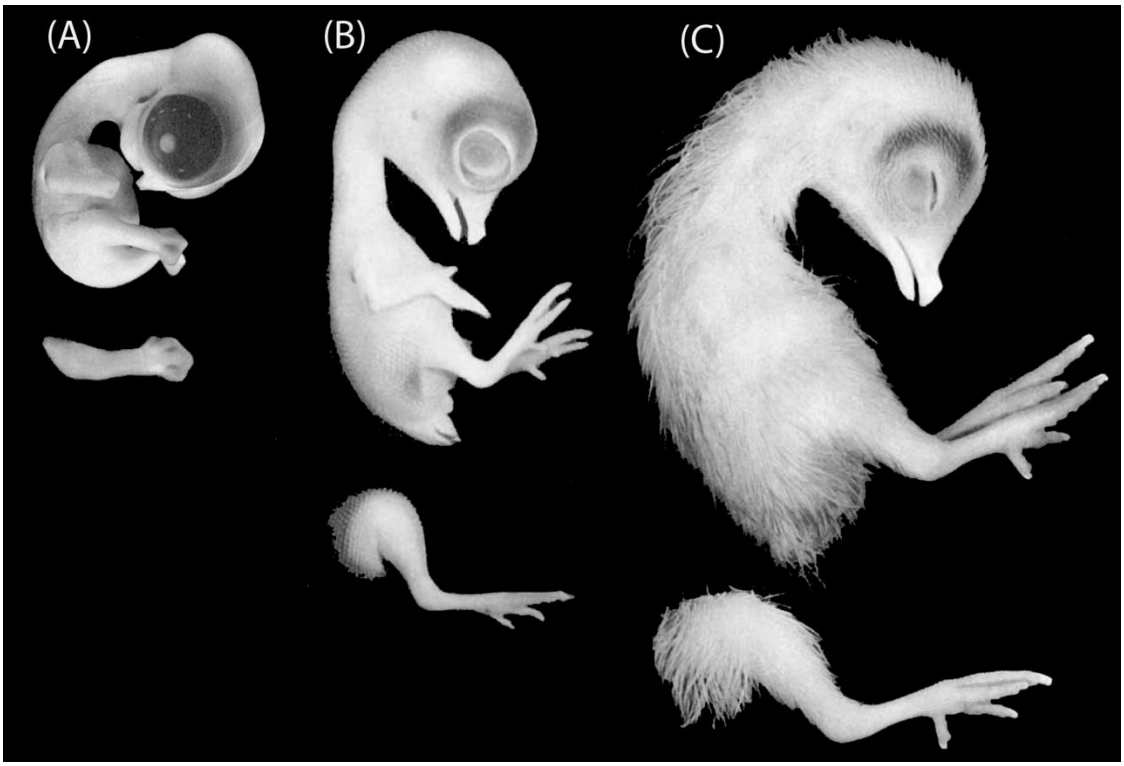

Figure 1.1 Development of chicken embryo from ED7 (A), ED10 (B) and ED13 (C) with additional hind muscle (below). Phenotype on ED7 (A) showed the development of web between 1st and 2nd digits, margin of wing and anterior tip of mandible beak. At ED10 (B), wing and leg are consistently much longer including 3rd toe, beak, primordium of comb, labial groove and uropygial gland. From ED13 (C), nostrils, down feathers, egg tooth, comb, wattles and scales on legs are formed (Hamburger and Hamilton, 1951).

1.3 Temperature manipulation

One of a well-established physical factors during incubation is temperature which induces embryo development, survival ability and long-term performance (Sobolewska et al., 2011;Lourens et al., 2005). During the incubation, chicken embryos use egg shell as protection, source of calcium for skeletal development, for gas and water exchange through egg shell pores. The developmental processes are stimulated by eggshell temperature. To manipulate eggshell temperature, a variation of temperature and incubation period could cause acute and long-term response by the animal. Previous

researchers have shown multiple responses in avian thermoregulation, mortality, hatchability and post-hatch characteristic (Table 1.1). For example, increasing the incubation temperature positively influences breast meat yield of featherless broilers (Hadad et al., 2014), enlarge muscle fiber in turkey (Maltby et al., 2004), but also has been associated with live weight reduction in the same animal (Krischek et al., 2013). lower incubation temperature significantly elevated female embryo mortality in Australian Brush-turkey (Eiby et al., 2008) and reduced growth rates of wood duck (Durant et al., 2010).

In broilers, the study of incubation temperature manipulation revealed possible influences on many characteristics. For instance, feather development which has essential roles in broiler thermoregulation and skin protection are affected by modulation of incubation temperature. The variation of feather follicle is various body regions (Dahlke et al., 2008). The follicle density in breast was decreased in early-low late-high (LH; 36.9 °C - 39.7 °C) treatment when compare with standard high incubation temperature (Scott et al., 2015). Leksrisompong et al. (2007) have reported the effect of elevated incubation temperature during late development (ED14-20) which had effects in additional hatching time requirement, reduce weight of heart, body, gizzard, and small intestines on hatching day. Also, the chick has pale and whiter color when compared with control. This phenotypic change was a result from unwell developed nutrition absorption pathways are confirmed by Barri et al. (2011). The result showed deeper crypts in jejunum and relatively high expression of nutrient transporter mRNA (PepT1 and DOH6) in elevated incubation temperature treatment (Barri et al., 2011). The response to early low incubation temperature (ED0-ED10) showed decelerated development *in-ovo* then stunted post-hatch growth leading to reduced embryonic weight, hatchability, and early chick quality. The adverse effect remained and caused by reduced fillet, carcass and breast meat yields at adult stage (Joseph et al., 2006). Werner et al., 2008 showed that increased temperature between ED7 to ED10 positively influenced slaughter and breast muscle weights in broiler males, but did not affect meat quality.

Table 1.1 Literature of incubation temperature manipulation in various avian models

Organism	Temperature manipulation	Response	Reference
Australian-brush turkey	Increase	Increased sex-specific mortality in males	Eiby et al., 2008
	Decrease	Increased sex-specific mortality in females	
Broiler	Increase	Decreased follicle density in breast	Scott et al., 2015
		Decreased heart, BW, gizzard, and small intestines at hatching day	Leksrisompong et al., 2007
		Increased hatching time	
		Chick has white color	
		Deeper crypts in the jejunum	Barri et al., 2011
		Increased nutrient transporter mRNA	
		Increased slaughter and breast weights	Werner et al., 2008
		Increased breast weight in both gender	Piestun et al., 2013
		Increased relative weight in pectoralis	Collin et al., 2007
	Decrease	Decreased embryonic weight, hatchability	Joseph et al., 2006
		Decreased slaughter and breast weights	
Featherless broiler	Increase	Increased breast meat yield	Hadad et. al., 2014
Malleefowl	Decrease	Increased incubation period	Booth, D.T. 1987
		Increased total energy expenditure	
Turkey	Increase	Increased muscle fiber	Maltby et al., 2004
		Decreased live weight	Krischek et al., 2013
Wood ducks	Decrease	Increased incubation duration	Hepp et al., 2006
		Decreased ducklings wet and dry mass	
		Decreased growth rates	Durant et al., 2010

1.4 Molecular regulation

During chicken myogenesis, multiple genes and proteins are coordinated and expressed. The process of determining mesodermal progenitor and differentiating myogenic cells result in hyperplasia and functional contractile muscle (Oksbjerg et al., 2004). In early myogenesis, dermomyotome development (pre-segmented of dermatome and myotome) is facilitated by wnt and Sonic hedgehog (SHH) proteins. The expressions of Pax3 (Paired Box 3), Pax7 (Paired Box 7) and Lbx1 (Ladybird Homeobox 1) is localized in migrating cells in dermomyotomes and then initiates myogenic regulatory

factors (MRFs) expression which are stimulates “muscle and limb buds” formation (reviewed from Werner and Wicke, 2007). Myogenesis is mainly influenced by MRFs which is tissue specific (class II) transcription factors including MyoD1 (myogenic differentiation 1), Myf5 (myogenic factor 5), myogenin and MRF4 (myogenic regulatory factor 4) (figure 1.2). MRFs contain a basic helix-loop-helix (bHLH) domain and maintain conversion of the cells into myogenic lineage. The presence of Mef2 (myocyte enhancer factor) facilitates functional MRFs. While MyoD and Myf5 regulate skeletal muscle lineages determination, Myogenin and MRF4 are focused on muscle differentiation (Berkes and Tapscott, 2005; Chen and Goldhamer, 1999; Sławińska et al., 2013). For example, myoblasts that exit the mitotic cell cycle (G1-G0 transition) show up-regulation of Myogenin and MRF4 but down-regulation of MyoD and Myf5 in turkey (Liu et al., 2005). The study on myogenic expression gene could help to understand the mechanism and regulation which might influence mitotic activity by environment manipulation.

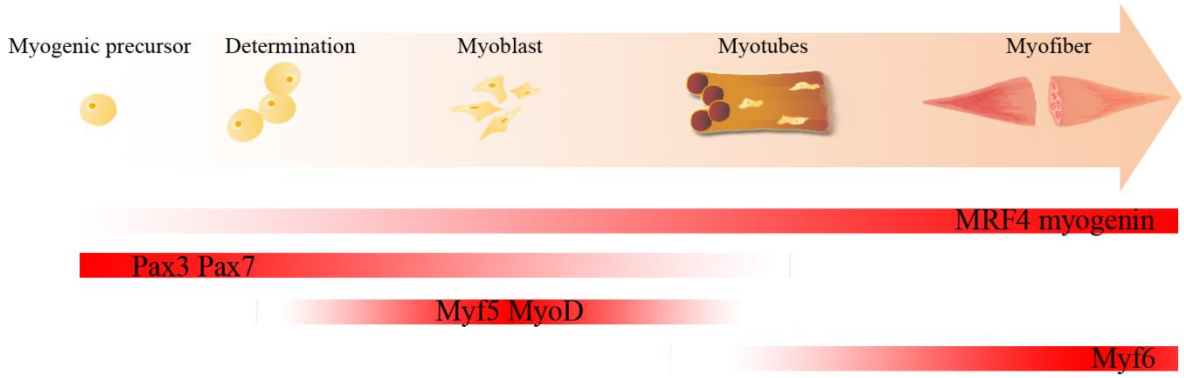

Figure 1.2 Myogenesis and transcriptomic response; presence of genes differ within the development as shown by the intensity of colors (modified from Hettmer and Wagers, 2010).

1.5 Expression profiling and gene regulation tools

Gene expression profiles are useful in the study of the effects of stress, pathogen interaction and other quantitative traits. For understanding expression patterns and regulation processes in broilers, multiple techniques are broadly used including mRNA microarrays and microRNA arrays which provide a vast amount of information about transcriptional production and regulation. In this the study, expression profiling is based

on the Affymetrix Chicken Gene 1.0 ST Array and the GeneChip® miRNA 3.0 Array. Both arrays have their benefits and disadvantages.

1.5.1 Affymetrix Chicken Gene 1.0 ST Array

Up to 1.4 million different oligonucleotide probes are placed on each microarray. The design of 25 oligonucleotides is located in a specific area of the array which is called a probe cell. Multiple probe cells merge into new probe sets ready for evaluations at both transcripts (gene) and exon levels, which allow the study of transcript variants, alternative promoter usage, alternative splicing and alternative transcript termination. Chicken Gene 1.0 ST Array is a high-density exon array designed based on "galGal3" build version. The array contained 439,582 probes within 18,214 gene-level probe sets. An advantage of the exon array is the high transcript coverage by covering the entire length of the gene, whole-transcriptome analysis with 3'-biased free expression designs and high data reproducibility (Affymetrix, 2016b).

1.5.2 GeneChip miRNA 3.0 Array

In 1993, lin-4, the first MicroRNAs (miRNAs) has been reported as a small complementary RNA (Lee et al., 1993). Since then, the study of miRNAs has been an attractive topic for gene regulation. miRNAs are single-stranded, small non-coding RNAs (ncRNAs) with approximately 20-24 nucleotides (nt) length that regulate gene expression. Their functions are partially or perfect binding to complementary sequences at 3'untranslated regions (UTR), coding sequences and 5′UTR of target messenger RNAs (mRNAs) as a process of post-transcriptional regulation (Almeida et al., 2011). The estimation of miRNA activities reveals that more than 60% of human protein translation of coding genes is affected (Friedman et al., 2008). To study gene expression in the post-transcriptionally process, the GeneChip miRNA 3.0 Array was selected. This miRNA array provides benefits from various perspectives like comprehensive coverage of all mature miRNA sequences in miRBase release 17 and snoRNAbase V.3, streamlined analysis for all species on one array, rich information and the low minimum requirement for total RNA (130 ng). The GeneChip miRNA 3.0 Array is capable of studying the role of small non-coding RNAs including protein translation inhibition, alternative splicing regulation, ribosomal RNA processing and mRNA degradation. In 153 organisms (19,724

probe sets) including 544 miRNA in chicken are well annotated (Affymetrix, 2016a). For further information, the specifications of two array chips are summarized in Table. 2.

Shifting the incubation temperature at specific periods of myogenetic development could contribute to the gene expression efficiency and thus affect broiler meat production without the costs of decreased meat quality. This thesis contains three parts of research; to analyze expression profiles of type I muscle (breast muscle), type IIB muscle (breast muscle) and gene regulation via microRNA profiling as shown in figure 1.3.

Table 2 Specifications of Chicken Gene 1.0 ST Array and GeneChip miRNA 3.0 Array

Platform specification	**Chicken Gene 1.0 ST Array**	**GeneChip miRNA 3.0 Array**
Array type	Exon array	miRNA array
Database build	galGal3	miRBase v17
Signal correlation coefficient	>0.99	
Reproducibility (inter- and intra-lot)		>0.95
Transcripts detected at 1.3 amol in 130 ng		0.85
Dynamic range	~3 logs	>3 logs
Total RNA input required	50–500 ng	130−1,000 ng
Probe feature size	5 μm	11 μm
Background probes	Antigenomic set	
Poly-A controls	dap, lys, phe, thr	
Hybridization controls	bioB, bioc, bioD, creX	
Probe length	Up to 25-mer	Up to 25-mer

1.6 Experimental design

The general strategy taken in this study is shown in Figure 1.3.

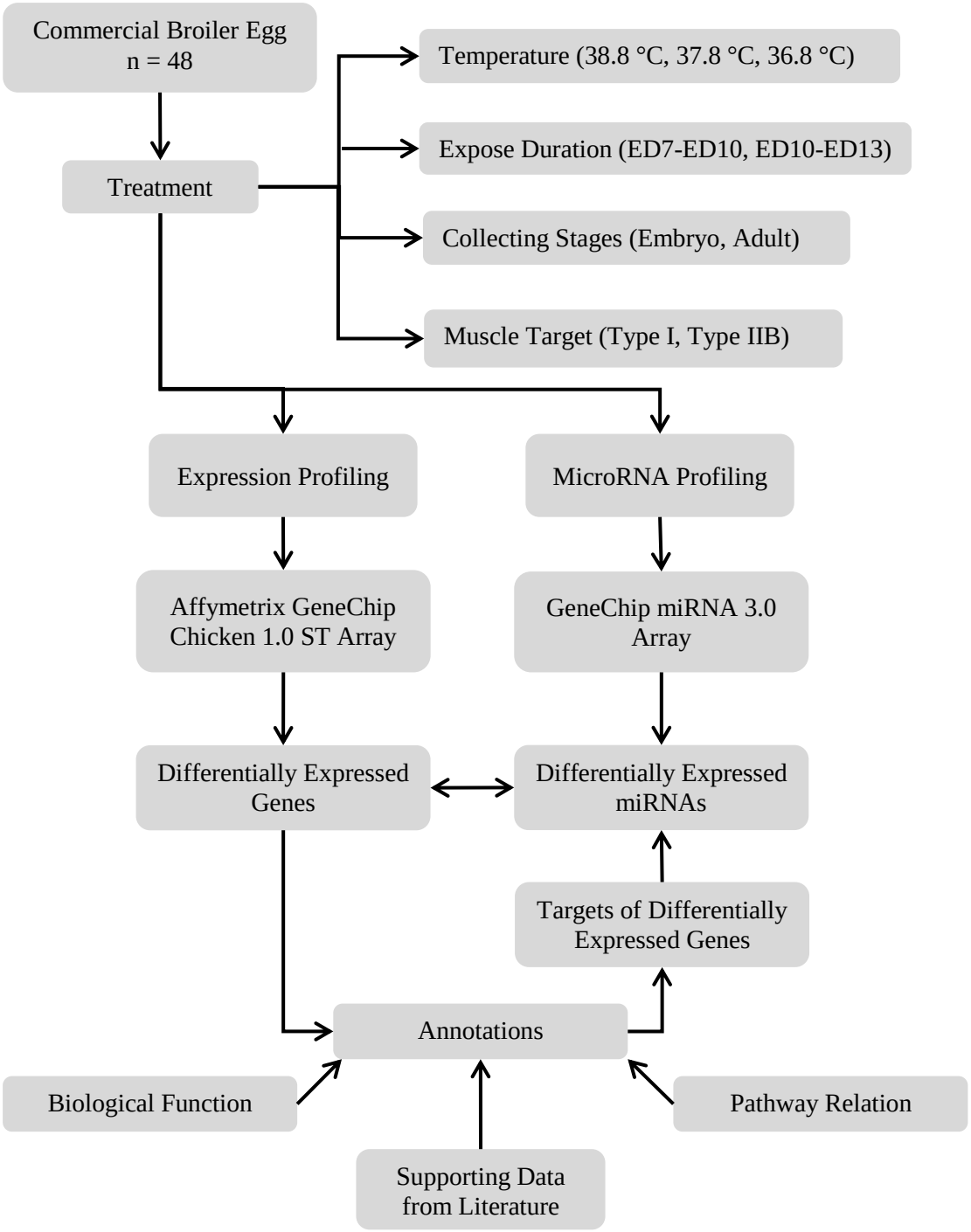

Figure 1.3 Pipeline overview of this study

1.7 Aims

There is experimental evidence that modification of the incubation temperature affects postnatal muscle growth in poultry. The molecular mechanisms of effects of modulated incubation temperatures on prenatal / *in-ovo* muscle development and postnatal growth are still unclear.

Against this background, the study aims to identify the molecular pathways of myogenesis which affect characteristics of muscle structure, muscle fiber number and types, as well as muscle growth and meat quality. Acute, transient and long-term changes in the expression profiles of muscle tissues caused by the modified incubation conditions are analysed and interpreted in view of phenotypic changes. Answering the questions of which genes and which functional networks are regulated due to the experimental conditions in the context of the observed phenotypic effects provides candidate genes for traits of muscle growth and therefore prospects for the development of DNA markers for these traits. Genes found to be differentially expressed due to variable incubation temperature that cause phenotypic alterations are involved in pathways relevant to the respective traits and are potential candidate genes. Therefore the modulation of gene expression by varying the incubation temperature in broilers during the *in-ovo* development was analysed.

The following objectives have been identified:

1. Perform transcriptome analysis of hind and breast muscle at an embryo and mature stages in broilers.
2. Determine acute- and delayed transcriptome responses to increasing or decrease of incubation temperature and at early and late in-*ovo* development.
3. Identify biological pathways involved in and candidate genes associated with muscle traits

Publication

CHAPTER II

Immediate and long-term transcriptional response of hind muscle tissue to transient variation of incubation temperature in broilers

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Abstract

Background: In oviparous species accidental variation of incubation temperatures may occur under natural conditions and mechanisms may have evolved by natural selection that facilitate coping with these stressors. However, under controlled artificial incubation modification of egg incubation temperature has been shown to have a wide-ranging impact on post-hatch development in several poultry species. Because developmental changes initiated *in-ovo* can affect poultry production, understanding the molecular routes and epigenetic alterations induced by incubation temperature differences may allow targeted modification of phenotypes.

Results: In order to identify molecular pathways responsive to variable incubation temperature, broiler eggs were incubated at a lower or higher temperature (36.8 °C, 38.8 °C) relative to control (37.8 °C) over two developmental intervals, embryonic days (E) 7–10 and 10–13. Global gene expression of M. gastrocnemius was assayed at E10, E13, and slaughter age [post-hatch day (D) 35] (6 groups; 3 time points; 8 animals each) by microarray analysis and treated samples were compared to controls within each time point. Transcript abundance differed for between 113 and 738 genes, depending on treatment group, compared to the respective control. In particular, higher incubation temperature during E7-10 immediately affected pathways involved in energy and lipid metabolism, cell signaling, and muscle development more so than did other conditions. But lower incubation temperature during E10-13 affected pathways related to cellular function and growth, and development of organ, tissue, and muscle as well as nutrient metabolism pathways at D35.

Conclusion: Shifts in incubation temperature provoke specific immediate and long-term transcriptional responses. Further, the transcriptional response to lower incubation temperature, which did not affect the phenotypes, mediates compensatory effects reflecting adaptability. In contrast, higher incubation temperature triggers gene expression and has long-term effects on the phenotype, reflecting considerable phenotypic plasticity.

Keywords: Gene expression, Pathway analysis, *In-ovo* development, Poultry, Microarray

Introduction

Chickens and other birds are homeotherms that require that their body temperatures are maintained within a limited range during pre and post-hatch processes [1]. Altering the temperature range during the critical developmental periods may cause only minor morphological differences, or could even produce lethal events. Since under natural conditions unpredictable periods may occur when incubation temperatures are unfavorable, natural selection could have promoted traits and mechanisms that provide resilience against such exogenous factors and that are reflected by immediate, acute or long-term, delayed responses (Du and Shine, 2015). Shifts in the incubation temperature of eggs under controlled experimental conditions have been shown to impact post-hatch development in several bird species. However, results of previous studies are inconsistent. For example, a higher incubation temperature was concluded to positively affect breast meat yield in featherless broilers [2] and muscle fiber size in turkey [3], but was associated with body weight loss in live chicken [4]. Similarly, lower incubation temperature was indicated to have a prolonged effect on female embryo mortality in Australian Brush-turkey [5], but reportedly reduced growth rates of wood duck [6]. Thus, the effects of incubation temperature changes on post-hatch development remain unclear. In particular, there is a lack of studies addressing the response to exogenous physical effects on the level of gene expression that will promote the understanding of the underlying compensatory, adaptive and regulatory process that might be associated with the treatment.

The *in-ovo* development of birds offers a valuable model in which to study environmental effects on myogenesis. Indeed, the identification of shifts in muscle and growth traits facilitates the detection of candidate genes for these traits. During avian myogenesis, the muscle fibers are formed in two phases. The primary muscle fiber, which is a core fiber, transforms to a myotube between the 4th and 7th embryonic days (E). Next, secondary muscle fibers, which are smaller and derive from myoblasts, arrange around the primary muscle fiber as a scaffold, proceeding until E15 [7]. Fetal myoblasts are most abundant between E8 and E12 [8]. After the secondary phase, depending on morphology and localization of the myofibers, the adult myoblasts will transform and become the primary source of myogenic precursors for postnatal muscle formation [9–11]. During both critical stages, temperature manipulation may cause differential expression of genes to produce phenotypic changes. Previous studies showed that

elevated incubation temperature over E7-10 positively influenced carcass traits in broiler males, but did not affect meat quality [12]. Thus, shifting the incubation temperature during targeted periods of *in-ovo* development could contribute to the improvement of the efficiency of broiler meat production, without sacrificing meat quality. This study addresses the transcriptomic response of skeletal muscle tissue to transient reduction and elevation of incubation temperature at early (E7-10) and late (E10-13) secondary muscle fiber development. Microarray expression profiles of treated samples were compared to those of the respective controls immediate after the treatment periods (E7-10; E10-13) and also later at slaughter (Fig. 1). The results have implication for the molecular foundation of potential impact on meat production traits and also provide insight into the mechanisms involved in the resilience against low and the phenotypic plasticity against high incubation temperature.

Materials and methods

Animals and tissue collection

As outlined in Fig. 1, hatching eggs of a commercial broiler line (Cobb-Vantress Inc., Siloam Springs, USA) were randomly assigned to the following experimental groups: H10 and L10, which were subjected to higher (38.8 °C) or lower (36.8 °C) incubation temperature, respectively, between E7-10; and H13 and L13, which were subjected to the same temperature shifts, respectively, but between E10-13. During the rest of the incubation period, all eggs were incubated at 37.8 °C, like the control group (C10, C13). Samples were collected immediately at the end of the treatment periods at E10 and E13, respectively, and in addition at post-hatch at day 35. The hatchlings were reared in barn system and fed a standard diet ad libitum until day 35 (D35; slaughter). Samples of hind tissues (M. gastrocnemius) were collected and immediately stored in liquid nitrogen. Embryonic samples taken at ED10 and ED13 as well as samples of D35 were sexed and for each experimental group (C10, H10, L10 and C13, H13, L13) at each time point (E10 or E13, respectively plus D35) samples, balanced for sex, were selected for gene expression analyses with 8 samples per treatment (Fig. 1). The recording of zoo-technical and biochemical traits was performed at the end of the respective treatment periods [53]. Increased incubation temperature led to slight but significant differences in body weight and mitochondrial respiratory capacity, whereas decreased incubation temperature only had subtle effects on a few parameters (Additional file 6). The study

was approved by the institutional Animal Welfare Committees and was conducted according to the guidelines of the German Law of Animal Protection.

RNA isolation

Total RNA of frozen individual tissue samples was isolated with Tri-Reagent-extraction (Sigma-Aldrich, Taufkirchen, Germany) according to manufacturer's protocol. DNase treatment and a column-based purification using the RNeasy Mini Kit (Qiagen, Hilden, Germany) were also performed according to manufacturers' protocols. To check RNA integrity, samples were visualized on 1 % agarose gels containing ethidium bromide. RNA concentration was determined by spectrometry with a NanoDrop ND-1000 spectrophotometer (PEQLAB, Erlangen, Germany). The absence of DNA contamination was confirmed by using the RNA as a template in standard PCR to amplify fragments of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene. To prevent degradation, all RNAs were stored at −80 °C until further use.

Expression microarray

500 ng of total RNA was reverse-transcribed into cDNA with the Ambion WT Expression Kit (Life Technologies GmbH, Darmstadt, Germany). Biotin-labeled cRNA targets were made using the Affymetrix GeneChip WT Terminal Labeling Kit (Affymetrix, Santa Clara, CA, USA). Fragmented biotin-labeled cRNAs were hybridized onto Chicken Gene 1.0 ST Arrays (Affymetrix), which contains 18,214 probe-sets. After staining and washing, the arrays were scanned and raw data were obtained with the Affymetrix GCOS 1.1.1 software.

Normalization and statistical analysis

For expression data analysis raw data (cel-files) obtained by Affymetrix GCOS 1.1.1 software of all arrays were used as input files for the Affymetrix Expression Console for subsequent normalization and estimation of expression levels. Quantitative expression levels of transcripts were estimated using PLIER algorithm (Probe Logarithmic Intensity Error) and using DABG (detection above background) to evaluate detection by combining probe-level p-values to generate probe cell intensity values at exon level. All data were deposited in a MIAME-compliant database, the National Center for Biotechnology Information Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo; accession number: GSE76670). All "present" values (default settings with detection p-values of ≤ 0.04) were selected and integrated within gene-level annotation. To extract the

outlying and nonspecific results, criteria on standard deviation (SD ≥ 0.16) and means (m ≥ 2.5) were applied using "genefilter" in R (www.r-project.org). Changes in transcript abundance were determined by analysis of variance (JMP Genomics, SAS-Institute) considering individual and combined effects of temperature, treatment period and gender and slaughter weight. Sex was excluded from the statistical model due to marginal effects. The final model included fixed effects of temperature, treatment period and interactions. Slaughter weight was included as covariate for the 35 days post-hatched time course. Comparisons of treated samples (L and H) to controls (C) within the respective time points (E10, E13, D35) were considered. Transcripts with significant differences of abundance at p-values ≤ 0.05 were selected and queried for pathways analysis. At pre-hatch stages p ≤ 0.05 equals FDR adjusted p-values of q ≤ 0.18; at D35 p ≤ 0.05 corresponding q-values ranged between 0.35 and 0.70.

Real time quantitative RT-PCR (qPCR)

For validation of microarray data, the gene expression of three genes was determined by Real-time quantitative PCRs using the same D35 samples used for microarray analyses. The assays were done in duplicate in volumes of 10 µl using the LightCycler 480 SYBR Green I Master Kit (Roche), on a LightCycler 480 Real-Time PCR System (Roche Diagnostics GmbH, Germany). The temperature profiles comprised an initial denaturation step at 95 °C for 10" and 40 cycles consisting of denaturation at 95 °C for 15", annealing at 60 °C for 10" and extension at 72 °C for 15". The amplified genes were GAPDH and ACTB as well as FGA, NR43A and AHSG (Additional file 7), where the first two were used as reference genes to account for variation of cDNA amounts after reverse transcription by calculating a normalization factor. Target genes were selected because of their redundant assignment to different but related biofunctions. For all the assays threshold cycles were converted to copy numbers using a standard curve generated by amplifying serial dilutions of an external PCR standard (10^7 - 10^2 copies). After completion of amplification protocol all samples were subjected to melting curve analyses and gel electrophoresis. Primers were obtained from Sigma-Aldrich, Germany.

Pathway analyses and major categories

Least-squares means of expression level and fold changes including "UP" and "DOWN" regulation among the tissues were estimated. Annotation data for Affymetrix Chicken Genome Arrays were obtained from the producer (Affymetrix Chicken Genome

Array annotations release 34). Ingenuity Pathway Analysis was used for functional annotation estimation of association between dataset and pathway. Differentially expressed genes (DEGs) were analyzed referring to Ingenuity Pathways Knowledge Base (IPKB). Biological and canonical pathways were identified from the IPKB library. Significance was considered based on Fisher's exact test p-values adjusted for multiple testing using the Benjamini-Hochberg correction procedure. Cut-off criteria were set to corrected BH p-values ≤ 0.05 for canonical pathways and for biofunctions, respectively. The variation of pathways was assigned, and we focused on the top most affected biological functions related to tissue development and myogenesis. All pathways were grouped into new categories based on criteria concerning the major roles in comprehensive biological routes on organismal, organ, tissue, cell or molecular levels. All biological functions were categorized in eight major groups (gr. 1 – gr. 8) as follows: cell maintenance, proliferation, differentiation, and replacement (gr. 1); organismal organ and tissue development (gr. 2); nutrient metabolism (gr. 3); genetic information and nucleic acid processing (gr. 4); molecular transport (gr. 5); cell signaling and interaction (gr. 6); small molecule biochemistry (gr. 7); and response to stimuli (gr. 8). "Activated" and "deactivated" genes were assigned by positive and negative Z-scores, predicting the activation state of related transcription regulators. Significant pathways that were altered with *in-ovo* temperature modifications were clustered and visualized by heatmap. Genes assigned to major categories, as defined below, and with Z-scores were selected to derive IPA networks. Top network results were displayed covering related DEGs with annotation from NCBI reference sequence base [54].

Results

Global gene expression pattern of chicken hind muscle The chicken gene 1.0 ST array contains 165,815 probesets representing 20,828 transcripts encoding for 18,214 genes. After quality filtering and normalization, probesets representing 8,909 transcripts were subjected to further analyses. Analysis of variance was used to identify differentially expressed genes (DEGs) by comparing gene expression levels of treatment group (*in-ovo* temperature modification) against the control. The number of DEGs for each comparison at embryonic stages and at D35 is shown in Table 1. At the embryonic stage, higher temperature during E7-10 versus control (H10ΔC, 38.8 °C) significantly altered the expression of 738 genes compared to other treatment conditions (Table 1). Lower

temperature during E10-13 versus control (L13ΔC, 36.8 ° C) affected more genes than did low temperature during E7-10 (389 vs 140). Long-term effects of the *in-ovo* temperature modification were investigated at D35. Lower temperature in the early and late treatment period (L10ΔC and L13ΔC) resulted in a high number of DEGs at D35 (693 and 288, respectively), whereas higher temperature produced fewer DEGs at D35 (167 and 247, respectively) (Table 1). In addition, the majority of DEGs were downregulated in embryonic stage, but were up-regulated at D35, as shown in Table 1. The direction of regulation of FGA, NR4A3 and AHSG, exemplarily chosen as to represent genes assignment to several pathways, as indicated by microarrays and qPCR were consistent. The correlation coefficients were highly significant and ranged between 0.71 and 0.84. Taken together, the qPCR analyses indicated a reproducible analysis.

Distinct response to temperature alteration by time and direction

A comparison of the DEGs between treatment conditions showed that most DEGs were unique for each condition, e.g., 685 and 366 DEGs for H10ΔC and L13ΔC in the embryonic stages (Fig. 2a), and 516 and 216 DEGs for L10ΔC and L13ΔC at D35 (Fig. 2b), respectively. Some DEGs were shared between two conditions, including those common either to L10 and L13 (10 and 45 at embryonic stage and D35) or to H10 and H13 (17 and 9 at embryonic stage and D35), which were almost exclusively consistently regulated. There were only a few DEGs that were common across more than 2 conditions. Comparisons of DEGs from identical treatments in embryonic and D35 samples (Fig. 2c) revealed 55 common DEGs in total for the 4 treatments. Of these, 5 up and 14 down-regulated transcript ids were regulated in the same direction in both embryonic stages and at D35. However, most of the common DEGs were regulated in opposite directions by stage, e.g., up-regulation in embryos and down-regulation at D35. A list of common DEGs with fold-change and p-value is available in Additional file 1.

Pathway analysis

To understand the underlying biology and identify relevant pathways, DEGs were analyzed using the Ingenuity Pathway Analysis software tools (IPA, Ingenuity Systems Inc., Redwood City, USA). All DEGs lists were separated into up or down-regulated genes for each comparison (temperature modification vs control). All significant biological pathways associated with p-values and gene members are available in Additional files 2, 3, 4 and 5. The pathway analysis approach is effective for handling a list of DEGs, and generates a list of biological terms/ pathways. To encompass most

pathways affected by all treatment factors (temperature modification, embryonic stage, and growth stage), we grouped 52 and 49 significant pathways derived from embryos and D35 broiler, respectively, into eight major categories of interest: group (gr.) 1, cell maintenance, proliferation, differentiation and replacement; gr.2, organismal, organ, and tissue development; gr.3, nutrient metabolism; gr.4, genetic information and nucleic acid processing; gr.5, molecular transport; gr.6, cell signaling and interaction; gr.7, small molecule biochemistry; and gr.8, response to stimuli. Overall, biological pathways involved in cell growth (gr.1) and tissue development (gr.2) were affected by modification of incubation temperature both in embryos and at D35 based on the number of pathways, as shown in Fig. 3. Activation states of upstream regulators were further analyzed for the dataset based on the Z-score calculation from Ingenuity Pathway Analysis (IPA) (Additional files 2-5).

In particular, a higher temperature during E7-10 (H10ΔC; Fig. 3a, rows 1&2) altered pathways involved in lipid metabolism, cell signaling, energy metabolism, muscle development and function, and small molecule biochemistry, more so than did other conditions in embryos (Fig. 3a, rows 3–8). Z-scores indicate that H10ΔC condition tended to activate several pathways related to nutrient metabolism (gr.3) and small molecule biochemistry (gr.7) (Additional file 2). A lower temperature (L10ΔC and L13ΔC; Fig. 3a, rows 5–8) affected pathways related cell maintenance, proliferation, differentiation and replacement (gr.1) and organismal, organ, and tissue development (gr.2). L13ΔC tended to suppress cellar processes related to cell death, thus promoting maintenance in the major category cell maintenance, proliferation, differentiation and replacement (gr.1) and to activate developmental processes in mesoderm and muscle (gr.2, organismal, organ, and tissue development) (Additional file 3).

Using all DEGs obtained for H10ΔC in embryos, a network was generated covering 19 DEGs. FABP1 (fatty acid binding protein 1), PPARA (peroxisome proliferator activated receptor alpha), and PPARGC1A (peroxisome proliferator-activated receptor gamma, coactivator 1 alpha) are highly connected genes in the network and related to energy production, lipid metabolism and small molecule biochemistry (Fig. 4a). For L13ΔC, the generated network was related to suppressed cell death and survival but stimulated cell growth and digestive developmental processes, including genes GPI (glucose-6-phosphate isomerase), NR1H3 (nuclear receptor subfamily 1, group H, member 3), and SRF (serum response factor) (Fig. 4b).

At D35, decreased incubation temperature during E10-13 (L13ΔC; Fig. 3b, rows 7&8) strongly changed pathways related to cellular function and growth development of organs, tissue and muscle as well as nutrient metabolism pathways (Fig. 3b). For L10ΔC (Fig. 3b, rows 5&6) a considerable number of genes were affected that belong to Ingenuity biological functions related to organismal, organ, and tissue development (gr.2). Notably, according to Z-scores L10ΔC tended to exhibit inhibitory effect on genetic processing categories (gr.4; Genetic information and nucleic acids), whereas L13ΔC was more likely to activate most categories (Additional file 4). For H13ΔC (Fig. 3b, rows 3&4) broadly the same molecular routes were shifted, however in opposite direction (Fig. 3b). For H10ΔC, no trends of activation or inhibition of pathways were obvious (Additional file 5).

The network established for L10ΔC contained genes of top pathways including gene expression, cellular function and maintenance, and organismal development (Fig. 5a). Highly connected genes included HDAC4 (histone deacetylase 4), TBP (TATA Box Binding Protein), MYOD1 (myogenic differentiation 1), and SOX6 (sex determining region Y-box 6) that are related to inactivation of transcription and muscle cell differentiation. For L13ΔC, activation of pathways related to proliferation, differentiation, and development at the cell, tissue, and organ levels was predicted. Accordingly, the consistently increased transcript abundances revealed a network (Fig. 5b). The involved genes for nutrition metabolism included APOA1 (apolipoprotein A1), GFPT1 (glutamine fructose-6-phosphate transaminase 1) and proliferation of muscle development included APOD (apolipoprotein D), and DES (desmin).

Discussion

This study demonstrates that transcriptomic and pathway regulation changes occur in broiler embryos and at D35 as a result of temperature manipulation during early (E7-10) and later (E10-13) development stage. Evidence was reported that early elevated incubation temperature positively influenced growth traits, but did not affect meat quality [12]. Indeed the chicken analyzed here showed slight but significant increase in body weight when transiently incubated at higher temperature, whereas decreased incubation temperature did not affect body weight.

Immediate effects observed in-ovo

Embryonic days 7–10 and 10–13 cover the final stage of primary muscle formation and start of secondary muscle fiber formation, respectively [7]. During myogenesis, multiple transcripts have major roles in regulating muscle development, such as BCK (B isoform of creatine kinase) [13], cTnT (chicken cardiac troponin T) [14], Mstn (myostatin), and MyoD (myogenic differentiation 1) [15]. Previous research showed many regulated genes expressed during myogenesis being sensitive to incubation temperature manipulation.

Immediate response to early high temperature treatment (H10ΔC)

Due to our experiment, the major impacts on the transcriptome resulted from early high (H10ΔC) and late low (L13ΔC) temperature shifts, with the majority of DEGs being up regulated. The H10ΔC comparison indicated that pathways involved in lipid metabolism, energy production, oxidation and beta-oxidation of fatty acid were activated. In this network, 19 up-regulated DEGs were represented, including FABP1, PPARA, and PPARGC1A. FABP1 and PPARA act in fatty acid uptake, metabolism, and intracellular transportation of lipids metabolism, cell proliferation, cell differentiation and respiration as well as inflammation responses [16]. A previous study showed that L-FABP in chicken had higher expression in fat-type chicken at 3, 5, and 7 weeks old (p-value ≤ 0.05), and is linked to abdominal fat deposition and high lipogenesis rate [16]. Moreover, a previous study showed shifts of expression of AMPK-PPARA pathway genes due to thermal conductions [17]. PPARGC1, regulates energy metabolism, muscle fiber specialization and adaptive thermogenesis [18–20]. A previous study reported that single nucleotide polymorphisms (SNPs) in chicken PPARGC1A are significantly related to abdominal fat weight without growth trait effects [21]. Moreover, PPARGC1A (PGC-1α) which was influenced by cold stress (4 °C from D28 to D38) in chicken could influenced a change in fiber type distribution and phenotype [20]. Exemplarily, modulated expressions of these genes reflect shifts of biological functions related to growth and metabolism.

The results demonstrate immediate shifts of transcript abundance due to manipulation of incubation temperature. During E7-10, high temperature (38.8 °C) manipulation influenced mainly lipid (FABP1, PPARA) and energy production (PPARGC1) pathway. Accordingly, changes of body, liver, and heart weight were evident [22]. Moreover, activity of mitochondrial respiration (state-3-pyruvate/ma late and state-3-succinate/rotenone) and enzyme activities (glycogen phosphorylase, lactate

dehydrogenase, and cytochrome oxidase) were elevated [22]. Thus, H10 treatment could influence lipid production and metabolism and also promoted phenotypic change.

Immediate response to late low temperature treatment (L13ΔC)

At later development (E10-13), low temperature had a greater impact on the transcriptome (L13ΔC). The biological functions affected were predicted to affect cellular processes balancing differentiation, proliferation and maintenance. In contrast, high temperature treatment down regulated the development of cytoplasm and vasculogenesis, but increased inflammation and cell death. The L13ΔC network related to cell death and survival and cellular growth and proliferation. Candidate DEGs included GPI, NR1H3, and SRF, involved in metabolic, proliferation, and differentiation pathways. In fact GPI encodes a member of the glucose phosphate isomerase protein family, involved in glucose metabolism [23]. In chicken, GPI is up-regulated in muscle development [24]. NR1H3 belongs to the NR1 subfamily of the nuclear receptor superfamily (synonym: liver X receptor alpha), which are key regulators of macrophage function, inflammation, and lipid homeostasis in differentiating chondrocytes [25, 26]. In chicken, NR1H3 is considered a key regulator of fatty acid homeostasis [27] and cholesterol homeostasis [25]. SRF encodes a ubiquitous nuclear protein that stimulates cell proliferation and differentiation. In chicken embryo, SRF expression is restricted primarily to striated muscle cell lineages, which increased mass of nuclear and activating alpha actin gene activity [28].

Later in E10-13, lower temperature (36.8 °C) was associated with shifts of pathways towards balancing anabolic and catabolic pathways, which is in line with phenotypic change being slight and non-significant [22]. Enzyme activity (cytochrome oxidase) and mitochondrial respiration (state-3-pyruvate/malate) were lower than at normal condition. It was suggested that lower temperature at late treatment might decelerate embryonic activity.

Long-term effects observed at D35

In-ovo shifts of thermal conditions had long term effects on the transcriptome observed at D35. Higher or lower incubation temperature has also been shown to impact postnatal development in avian species [3, 6, 29]. Because the egg shell temperature is sensitive to environmental change, it can directly impact developmental processes as well [30, 31]. Acute temperature modulation at the late embryonic stage was previously suggested to cause long-term transcriptomic changes, but few studies demonstrated an

ongoing effect. A recent report showed embryonic temperature manipulation affected thermoregulatory mechanisms [32]. Another study found that periodic incubation temperature change between 37.8 °C and 39.5 °C from E16-18 initiated acute (E17) and late term (D13 post-hatch) positive effects on diameter of myofibers and muscle cell proliferation in chicken [1]. These findings might have been resulted from modifications to the stress response and thermogenesis by the increased temperature from E7-16, resulting in reduced oxygen consumption, heart rate, and egg shell temperature. These changes directly affected broiler embryo growth and development [33]. Similarly, another study showed that temperature manipulation caused a high density of blood vessels in the chorioallantoic membrane during embryogenesis [34]. In our studies, long-term transcriptomic changes were due to low temperature treatments (L10ΔC and L13ΔC conditions) primarily leading to downregulation. IPA analysis indicated that increasing the incubation temperature to 38.7° may influence cell cycle and skin development at the early time point (E7-10). After that, high temperature E10-13 treatment tended to activate apoptosis in cell development but deactivated cardiovascular system and body trunk. Effects on metabolic process showed a reduction of carbohydrate metabolism, synthesis. Furthermore, a negative effect still remained for concentration of lipid and acyl glycerol.

Long-term response to early low temperature treatment (L10ΔC)

The lower incubation temperature resulted in more DEGs in both early and late treatments. Early low temperature (L10ΔC) tended to activate pathways involved in organismal development and cell proliferation but strongly suppress transcriptional process. All significant pathways including gene expression, cellular function and maintenance, and organismal development formed a network. Candidate genes included HDAC4, MYOD1, and SOX6, which are related to inactivation of transcription and muscle cell differentiation.

Previous research showed HDAC4 was associated in modulating cell growth and differentiation by controlling histone deacetylase activity, which alters chromosome structure and affects transcription factor access to DNA [35, 36]. A negative effect of HDAC4 overexpression is down-regulation of cardiac muscle gene expression and leads to inhibition of cardio myogenesis [37]. Normally HDAC4 was found in neuromuscular junction especially in myonuclei of fast oxidative skeletal muscle fibers [38, 39]. Down regulated of this gene suggested multiple transcriptional abnormalities including cardiac hypertrophy [40] and influence to MYOD1 expression. MYOD1 encodes a protein that

belongs to a basic helix-loop-helix family of transcription factors and the myogenic factors subfamily. Generally, MYOD1 acts in muscle cell differentiation by inducing cell cycle arrest. During pre-gastrulating epiblast in chicken, MYOD1 can induce skeletal muscle lineage self-renewal and differentiation [41]. Moreover, MYOD1 also works with the downstream effector VGL-2 in skeletal myogenesis [42]. Another DEGs which suggested to downregulate in cardiac and skeletal muscle is SOX6, a member of the SOXD gene family, encodes functional domains including a DNA binding domain (the HMG box) and two coiled-coil domains [43]. The encoded protein is a transcriptional activator and critical role in cartilage development and mesenchymal differentiation [44]. Moreover, SOX6 is well known to function as a transcriptional suppressor of slow fiber-specific genes [45, 46].

Lowered incubation temperature had large effects on postnatal expression in terms of number of transcripts with shifted abundance. Manipulation of early treatment (E7-10) led to down regulation in transcriptional processes and muscle cell differentiation. Moreover, cardio (HDAC4) and skeletal (MYOD1 and SOX6) myogenesis were negatively affected. The phenotype of D35 chicken exposed to lower temperature showed a slight nonsignificant reduction of carcass and leg compared to the control group; higher incubation temperature led to increased weights (Additional file 6).

Long-term response to late low temperature treatment (L13ΔC)

Late low temperature treatment (L13ΔC) was predicted to activate pathways in cellular and organismal development including cell survival, development of body trunk, contractility of cardiac muscle, and proliferation of mammary epithelial cells, but to have a negative effect on size of body and muscle cell pathways. In metabolism, elevated uptake and metabolism of lipid and carbohydrate, together with small molecule biochemistry like oxidation of fatty acid, tended to reduce concentration of lipid. Inflammatory response was also predicted to be suppressed. The IPA network highlighted the activation of tissue development, skeletal and muscular disorders, and cell-to-cell signaling. Selection of Fold change (FC) related in every major category revealed a set of candidate genes: APOD, APOA1, DES, and GFPT1.

APOD encodes a component of high-density lipoprotein (HDL) with a high degree of homology to plasma retinol binding protein and lipocalins. During late chicken embryogenesis, the expression of APOD is enriched among subsets of central nervous system (CNS) neurons then again in skin during developing of feather [47]. The

molecular function involved lipoprotein metabolism, as shown by APOA1, HDL, and LDL in the network Fig. 5b. APOA1 is the major protein component of high-density lipoprotein in plasma. It promotes cholesterol efflux from tissues to the liver for excretion, and is a cofactor for lecithin cholesterol acyl transferase (LCAT), which is responsible for the formation of most plasma cholesteryl esters. APOA1 is negatively correlated with aging and influences muscle development in Thai indigenous chicken [48]. Desmin (DES) encodes a muscle-specific class III intermediate filament. Homopolymers of this protein form a stable intra-cytoplasmic filamentous network connecting myofibrils to each other and to the plasma membrane. It maintains the structural integrity of highly solicited skeletal muscle and is important to other biological processes including muscle contraction and development, especially in heart contraction [49–51]. GFPT1 controls the flux of glucose into the hexosamine biosynthetic pathway, providing building blocks for the glycosylation of proteins and lipids [52]. The product of this gene catalyzes the formation of glucosamine 6-phosphate, which participates in carbohydrate biosynthesis and apoptosis regulation. GFPT1 is expressed in many tissues including skeletal muscle and heart [52]. Network connection revealed discreet interaction between GFPT1 and APOA1. Low-incubation temperature at late treatment (L13) had an impact on multiple DEGs and pathways in both embryo stages and at D35. However, these transcriptomic changes were not associated with significant phenotypic changes compared to the control (Additional file 6). The transcriptional response to lower incubation temperature appears to mediate compensatory effects that indicate a considerable adaptability. In nature transient reduction of incubation temperature during natural brooding happens. Accordingly, regulatory mechanism evolved in birds that enable the emergence of normal phenotypes. In contrast, higher incubation temperature triggers gene expression and has long-term effects on the phenotype. Elevated temperature is not likely in natural brooding, consequently not compensatory mechanisms evolved. Phenotypic changes associated with increased incubation temperature display metabolic plasticity of chicken.

Conclusions

Our experiment shows that manipulation of incubation temperature immediately effected transcriptomic changes and influenced the long-term expression. In parallel the results on growth, carcass, meat quality and mitochondrial respiratory

activities indicate effects of transient variation of incubation temperature as well [22, 53]. The observations indicate the successful activation of compensatory mechanisms in adaptation to lowered temperature and phenotypic plasticity in response to elevated temperature. Further investigations of the mechanism behind these regulatory processes including epigenetic modifications provide the perspective to improve resistance to environmental changes without much effect on growth performance [32]. Moreover, numerous genes which play important roles in metabolic pathways and which showed changed expression due to shifted incubation temperature represent candidate genes for further genetic improvement in terms of resilience against temperature shifts or in terms of increased muscle growth without affecting meat quality.

Figure titles and Tables

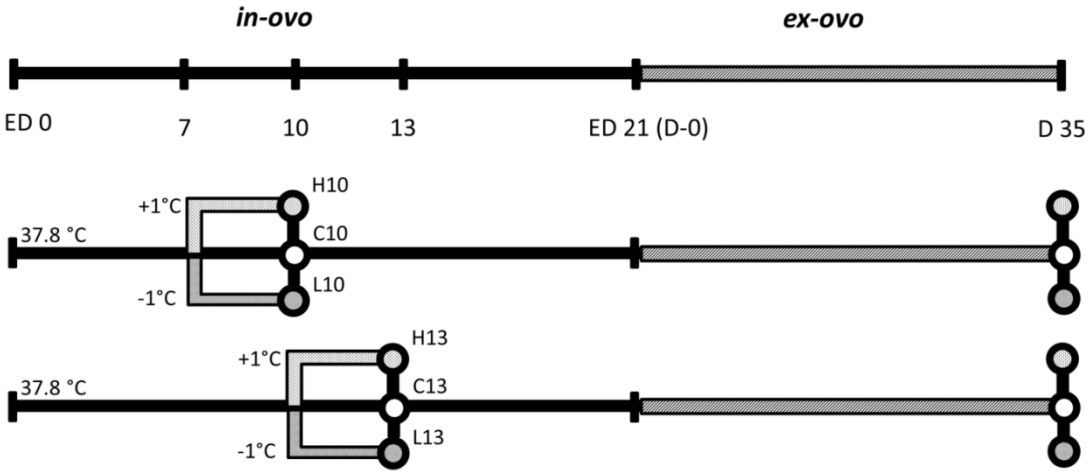

Figure 1 Experimental design; indicating the two periods of modulated incubation temperatures at E7-10 and E10-13 and the time points of samplings (circles; n=8 samples at a time).

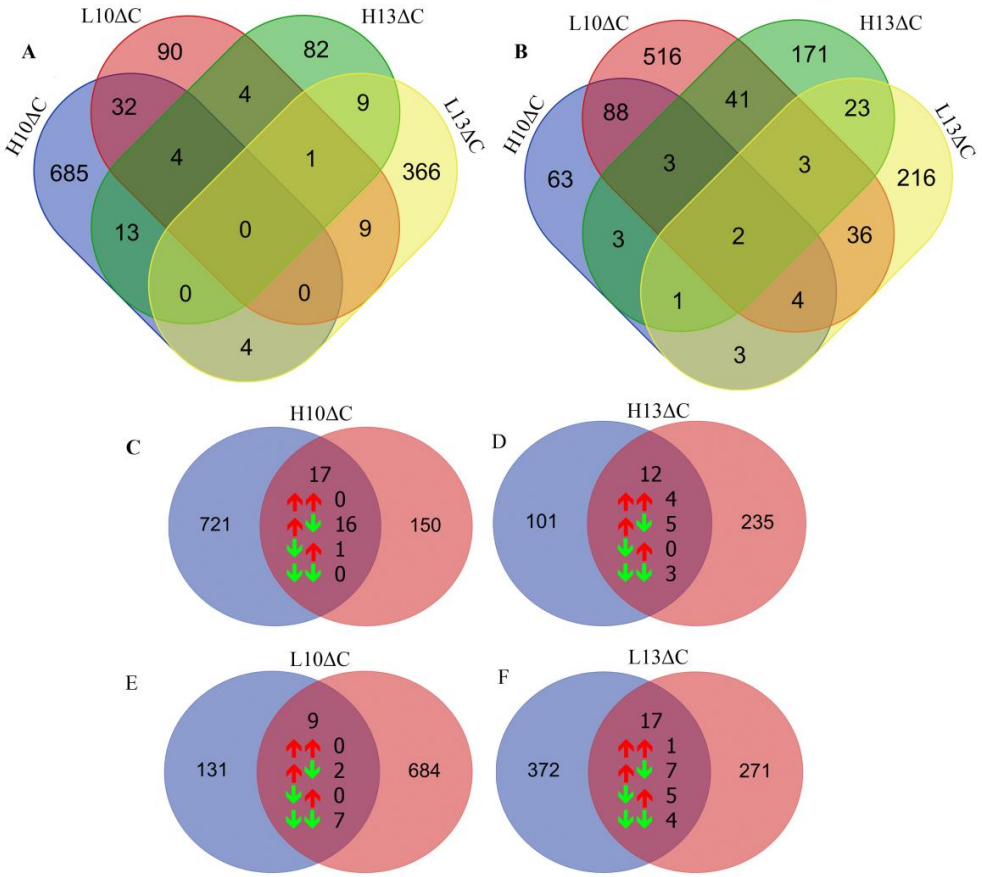

Figure 2 Venn diagrams; displaying numbers of differentially expressed genes for each treatment condition relative to control. Comparisons between treatment conditions at embryonic stages (**A)** at D 35 stage (B) and between embryonic and D 35 stage after the same treatments (C) (blue embryonic, red D 35 chicken).

Figure 3 Significant pathways altered by *in-ovo* thermal modifications; in **A**: embryonic stage and **B**: D 35 stage. DEGs associated with each comparison (treatment vs control) are separated into up-regulation (U) or down-regulation (D). Thermal modification treatments: increase (H) or decrease (L) incubation temperature during E7-10 (H10 and L10) or E10-13 (H13 and L13). Significant pathways (IPA defined) are grouped into eight major categories of interest; group (gr.)1 cell maintenance proliferation differentiation and replacement, gr.2 organismal organ and tissue development, gr.3 nutrient metabolism, gr.4 genetic information and nucleic acid processing, gr.5 molecular transport, gr.6 cell signalling and interaction, gr.7 small molecule biochemistry, and gr.8 response to stimuli and associated. The –log (*p*-value) associated with significant pathways (Fisher' exact test) are plotted in green (small) to red (large)

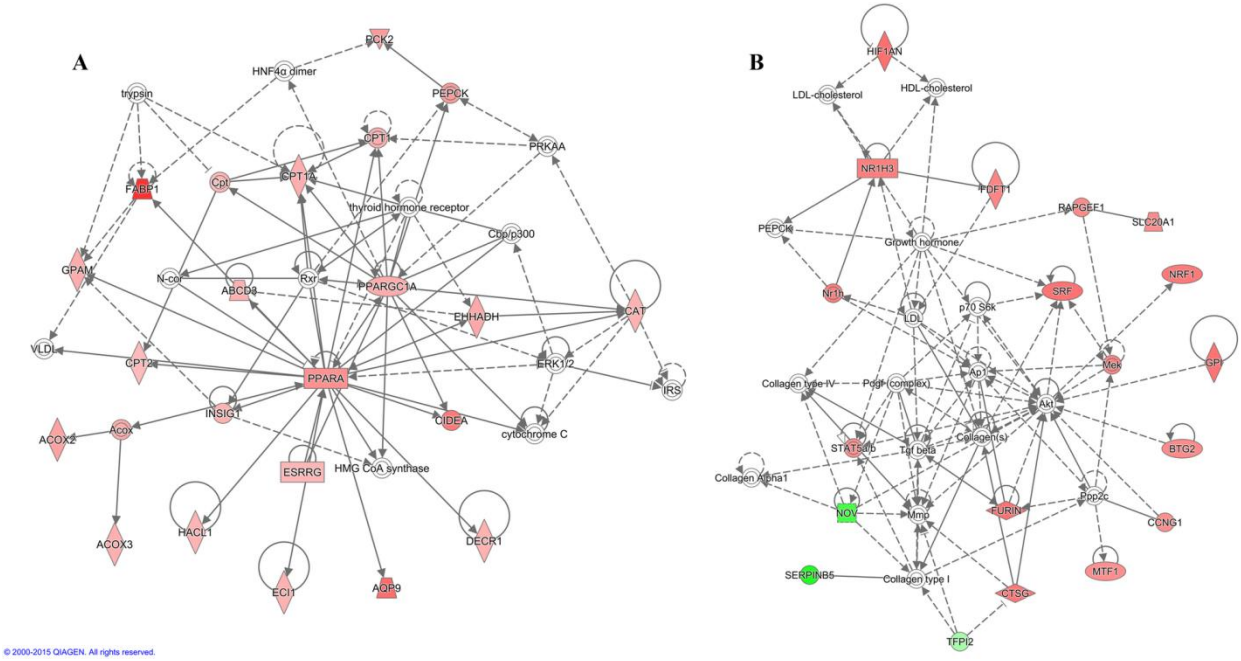

Figure 4 Functional networks derived from sets of DEG obtained for H10ΔC (a) and L13ΔC (b) at embryonic stage. Based on the Ingenuity KnowledgeBase a network of up regulated genes was derived for H10ΔC indicating activated energy production, lipid metabolism and small molecule biochemistry. For L13ΔC a network was found implying deactivated cell death and survival, but activated cellular growth and proliferation pathways. Red and green indicate up- and down-regulation; network shapes indicate various classes of network components; line and arrows indicate undirected and directed interactions

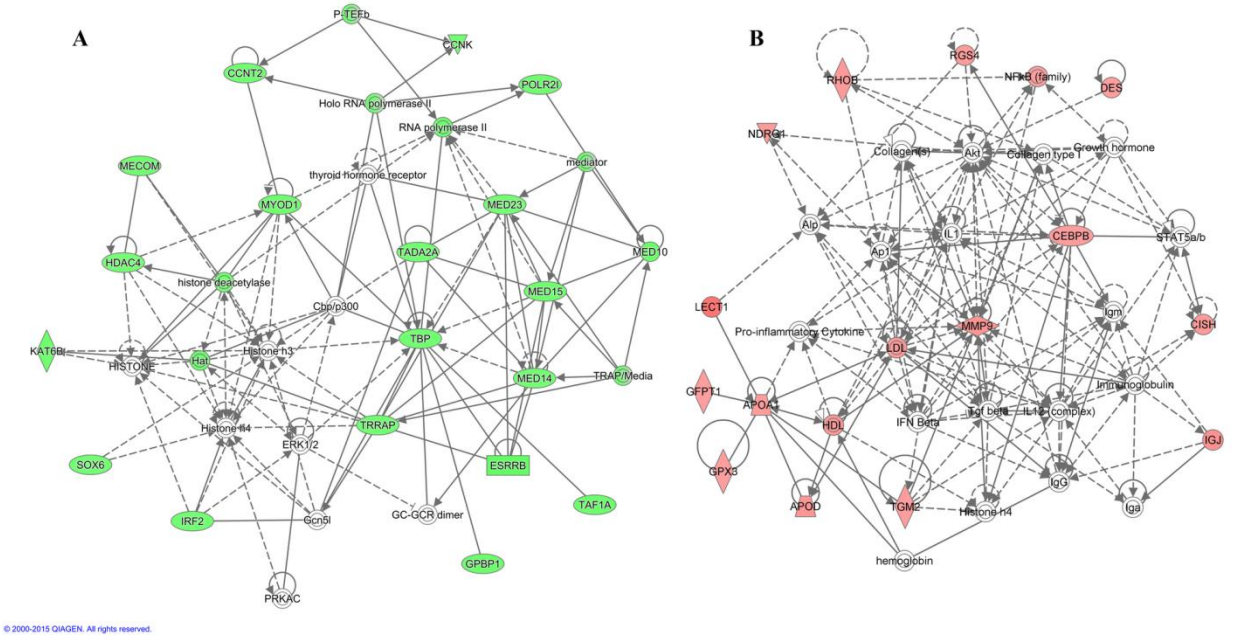

Figure 5 Functional networks derived from sets of DEG obtained for L10ΔC (a) L13ΔC (b) at D35. Based on the Ingenuity KnowledgeBase a network of down regulated genes was derived for L10ΔC indicating deactivated gene expression, cellular function and organismal development networks. For L13ΔC a network was found implying deactivated tissue development, skeletal and muscular disorders and cell-to-cell signaling pathways.

Red and green indicate up- and down-regulation; network shapes indicate various classes of network components; line and arrows indicate undirected and directed interactions.

Table 1 Differentially expressed genes (DEGs); between each *in-ovo* thermal modification condition relative to the control separated for embryonic or D 35 stages (*p*-value ≤ 0.05).

	Treatment (ΔC)	Probe sets	DEGs	Regulation	
				Up	Down
Embryo	H10 - C10	812	738	662	76
	H13 - C13	176	113	88	25
	L10 - C10	169	140	34	106
	L13 - C13	503	389	258	131
D 35	H10 - C10	217	167	35	132
	H13 - C13	332	247	108	139
	L10 - C10	768	693	104	589
	L13 - C13	330	288	123	165

Additional files

Additional file 1: Table S1. Common DEGs at embryonic stages and D35. Table S2. Common DEGs of H10 and H13 or L10 and L13 at embryonic stages. Table S3. Common DEGs of H10 and H13 or L10 and L13 at D35. (DOCX 41 kb)
Additional file 2: Assignment of DEGs to major categories, and biological functions obtained at embryonic stage for early treatment; H10UΔC, H10DΔC, L10UΔC and L10DΔC. (DOCX 21 kb)
Additional file 3: Assignment of DEGs to major categories, and biological functions obtained at embryonic stage for late treatment; H13UΔC, H13DΔC, L13UΔC and L13DΔC. (DOCX 23 kb)
Additional file 4: Assignment of DEGs to major categories, and biological functions obtained at D35 for early treatment; H10UΔC, H10DΔC, L10UΔC and L10DΔC (DOCX 22 kb)
Additional file 5: Assignment of DEGs to major categories, and biological functions obtained at D35 for late treatment; H13UΔC, H13DΔC, L13UΔC and L13DΔC. (DOCX 23 kb)
Additional file 6: Body weight, carcass weight and weight of hind muscles of broilers of the experimental groups used for expression analyses. (DOCX 19 kb)
Additional file 7: Primers used for quantitative real-time PCR (qPCR) (DOCX 15 kb)

Abbreviations

ΔC: 'delta' control, difference treatment versus control; D35: post-hatch days 35, slaughter date; DEGs: differentially expressed genes; E7, E10, E13: embryonic days 7th, 10th and 13th respectively; gr.1: major category group 1 cell maintenance, proliferation, differentiation and replacement; gr.2: major category group 2 organismal, organ, and tissue development; gr.3: major category group 3 nutrient metabolism; gr.4: major

category group 4 genetic information and nucleic acid processing; gr.5: major category group 5 molecular transport; gr.6: major category group 6 cell signaling and interaction; gr.7: major category group 7 small molecule biochemistry; gr.8: major category group 8 response to stimuli; H10: embryos were incubated at high temperature (38.8 °C) at embryonic days 7–10 and at control temperature (37.8 °C) at remaining time before and after; H13: embryos were incubated at high temperature (38.8 °C) at embryonic days 10–13 and at control temperature (37.8 °C) at remaining time before and after; IPA: Ingenuity Pathway Analysis; L10: embryos were incubated at low temperature (36.8 °C) at embryonic days 7–10 and at control temperature (37.8 °C) at remaining time before and after; L13: embryos were incubated at low temperature (36.8 °C) at embryonic days 10–13 and at control temperature (37.8 °C) at remaining time before and after.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Conceived and designed the experiments: CK MW KW. Performed the experiments: WN CK SJ MW SP KW. Participated in sampling: WN RB CK SJ. Analyzed the data: WN NT EM SP KW. Drafted and wrote the manuscript: WN KW. Reviewed and approved the final manuscript: WN NT EM RB CK SJ SP KW.

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Reference

1. Piestun Y, Harel M, Barak M, Yahav S, Halevy O: Thermal manipulations in late-term chick embryos have immediate and longer term effects on myoblast proliferation and skeletal muscle hypertrophy. J Appl Physiol. 2008; 106:233–240.
2. Hadad Y, Cahaner A, Halevy O: Featherless and feathered broilers under control versus hot conditions. 2. Breast muscle development and growth in pre- and posthatch periods. Poult Sci. 2014; 93:1076–1088.
3. Maltby V, Somaiya A, French NA, Stickland NC: In ovo temperature manipulation influences post-hatch muscle growth in the turkey. Br Poult Sci. 2004; 45:491–498.
4. Krischek C, Gerken M, Wicke M: Effects of a higher incubation temperature between embryonic day 9 and 12 on growth and meat quality characteristics of turkeys. Br Poult Sci. 2013; 54:5–11.
5. Eiby YA, Wilmer JW, Booth DT: Temperature-dependent sex-biased embryo mortality in a bird. Proc R Soc B Biol Sci. 2008; 275:2703–2706.
6. Durant SE, Hepp GR, Moore IT, Hopkins BC, Hopkins WA: Slight differences in incubation temperature affect early growth and stress endocrinology of wood duck (Aix sponsa) ducklings. J Exp Biol. 2010; 213:45–51.
7. Miller JB, Stockdale FE: What muscle cells know that nerves don't tell them. Trends Neurosci. 1987; 10:325–329.
8. Stockdale FE: Myogenic cell lineages. Dev Biol. 1992; 154:284–298.

9. Hartley RS, Bandman E, Yablonka-Reuveni Z: Skeletal muscle satellite cells appear during late chicken embryogenesis. Dev Biol. 1992; 153:206–216.
10. Mauro A: Satellite cell of skeletal muscle fibers. J Biophys Biochem Cytol. 1961; 9:493–495.
11. Schultz E, McCormick KM: Skeletal muscle satellite cells. Rev Physiol Biochem Pharmacol. 1994; 123:213–257.
12. Werner C, Wicke M: Increasing the temperature at a certain period during the incubation of broiler eggs improves the carcass and breast yields without a negative impact on the meat quality. Arch Anim Breed. 2008;11–16.
13. Lyons GE, Mühlebach S, Moser A, Masood R, Paterson BM, Buckingham ME, Perriard JC: Developmental regulation of creatine kinase gene expression by myogenic factors in embryonic mouse and chick skeletal muscle. Dev Camb Engl. 1991; 113:1017–1029.
14. Tidyman WE, Sehnert AJ, Huq A, Agard J, Deegan F, Stainier DYR, Ordahl CP: In vivo regulation of the chicken cardiac troponin T gene promoter in zebrafish embryos. Dev Dyn. 2003; 227:484–496.
15. Gabriel J, Alves H, Do Rosário M, Secatto A, Coutinho L, Macari M: Abundance of MyoD and myostatin transcripts in chicken embryos submitted to distinct incubation temperatures and timing exposures. Braz J Biol. 2011; 71:563–564.
16. Zhang Q, Shi H, Liu W, Wang Y, Wang Q, Li H: Differential expression of L-FABP and L-BABP between fat and lean chickens. Genet Mol Res. 2013; 12:4192–4206.
17. Zhang Z, Bi M, Yao H, Fu J, Li S, Xu S: Effect of cold stress on expression of AMPKalpha-PPARalpha pathway and inflammation genes. Avian Dis. 2014; 58:415–426.
18. Lin J, Wu H, Tarr PT, Zhang C-Y, Wu Z, Boss O, Michael LF, Puigserver P, Isotani E, Olson EN, Lowell BB, Bassel-Duby R, Spiegelman BM: Transcriptional co-activator PGC-1 alpha drives the formation of slow-twitch muscle fibres. Nature. 2002; 418:797–801.
19. Shu JT, Xu WJ, Zhang M, Song WT, Shan YJ, Song C, Zhu WQ, Zhang XY, Li HF: Transcriptional co-activator PGC-1α gene is associated with chicken skeletal muscle fiber types. Genet Mol Res. 2014; 13:895–905.
20. Ueda M, Watanabe K, Sato K, Akiba Y, Toyomizu M: Possible role for avPGC-1α in the control of expression of fiber type, along with avUCP and avANT mRNAs in the skeletal muscles of cold-exposed chickens. FEBS Lett. 2005; 579:11–17.
21. Wu GQ, Deng XM, Li JY, Li N, Yang N: A potential molecular marker for selection against abdominal fatness in chickens. Poult Sci. 2006; 85:1896–1899.
22. Krischek C, Janisch K, Naraballobh W, Brunner R, Wimmers K, Wicke M: Alteration of the incubation temperature between embryonic day 7 and 13 influences weights and mitochondrial respiratory and enzyme activities in breast and leg muscles of broiler embryos. Mol Reprod Dev. 2015; In press.
23. Rengaraj D, Lee SI, Yoo M, Kim TH, Song G, Han JY: Expression and Knockdown Analysis of Glucose Phosphate Isomerase in Chicken Primordial Germ Cells. Biol Reprod. 2012; 87:57–57.
24. Hallböök F, Persson H, Barbany G, Ebendal T: Development and regional expression of chicken neuroleukin (glucose-6-phosphate isomerase) messenger RNA. J Neurosci Res. 1989; 23:142–151.
25. Gentili C, Tutolo G, Pianezzi A, Cancedda R, Descalzi Cancedda F: Cholesterol secretion and homeostasis in chondrocytes: a liver X receptor and retinoid X receptor heterodimer mediates apolipoprotein A1 expression. Matrix Biol J Int Soc Matrix Biol. 2005; 24:35–44.

26. Song C, Hiipakka RA, Kokontis JM, Liao S: Ubiquitous receptor: structures, immunocytochemical localization, and modulation of gene activation by receptors for retinoic acids and thyroid hormones. Ann N Y Acad Sci. 1995; 761:38–49.
27. Demeure O, Duby C, Desert C, Assaf S, Hazard D, Guillou H, Lagarrigue S: Liver X receptor regulates fatty acid synthase expression in chicken. Poult Sci. 2009; 88:2628–2635.
28. Croissant JD, Kim J-H, Eichele G, Goering L, Lough J, Prywes R, Schwartz RJ: Avian Serum Response Factor Expression Restricted Primarily to Muscle Cell Lineages Is Required for α-Actin Gene Transcription. Dev Biol. 1996; 177:250–264.
29. Barri A, Honaker CF, Sottosanti JR, Hulet RM, McElroy AP: Effect of incubation temperature on nutrient transporters and small intestine morphology of broiler chickens. Poult Sci. 2011; 90:118–125.
30. Hulet R, Gladys G, Hill D, Meijerhof R, El-Shiekh T: Influence of Egg Shell Embryonic Incubation Temperature and Broiler Breeder Flock Age on Posthatch Growth Performance and Carcass Characteristics. Poult Sci. 2007; 86:408–412.
31. Joseph NS, Lourens A, Moran ET: The effects of suboptimal eggshell temperature during incubation on broiler chick quality, live performance, and further processing yield. Poult Sci. 2006; 85:932–938.
32. Loyau T, Bedrani L, Berri C, Métayer-Coustard S, Praud C, Coustham V, Mignon-Grasteau S, Duclos MJ, Tesseraud S, Rideau N, Hennequet-Antier C, Everaert N, Yahav S, Collin A: Cyclic variations in incubation conditions induce adaptive responses to later heat exposure in chickens: a review. animal. 2015; 9:76–85.
33. Piestun Y, Halevy O, Yahav S: Thermal manipulations of broiler embryos--The effect on thermoregulation and development during embryogenesis. Poult Sci. 2009; 88:2677–2688.
34. Pinchuck S, Meiri N, Yahav S: The effect of thermal manipulation, during early embryogenesis, on angiogenesis and thermo-tolerance in broilers. Wageningen, The Netherlands; 2011;44–45.
35. Backs J, Song K, Bezprozvannaya S, Chang S, Olson EN: CaM kinase II selectively signals to histone deacetylase 4 during cardiomyocyte hypertrophy. J Clin Invest. 2006; 116:1853–1864.
36. Guan Y, Chen Q, Yang X, Haines P, Pei M, Terek R, Wei X, Zhao T, Wei L: Subcellular relocation of histone deacetylase 4 regulates growth plate chondrocyte differentiation through Ca2+/calmodulin-dependent kinase IV. AJP Cell Physiol. 2012; 303:C33–C40.
37. Karamboulas C, Swedani A, Ward C, Al-Madhoun AS, Wilton S, Boisvenue S, Ridgeway AG, Skerjanc IS: HDAC activity regulates entry of mesoderm cells into the cardiac muscle lineage. J Cell Sci. 2006; 119:4305–4314.
38. Cohen TJ, Barrientos T, Hartman ZC, Garvey SM, Cox GA, Yao T-P: The deacetylase HDAC4 controls myocyte enhancing factor-2-dependent structural gene expression in response to neural activity. FASEB J. 2009; 23:99–106.
39. Tang H, Macpherson P, Marvin M, Meadows E, Klein WH, Yang X-J, Goldman D: A Histone Deacetylase 4/Myogenin Positive Feedback Loop Coordinates Denervation-dependent Gene Induction and Suppression. Mol Biol Cell. 2009; 20:1120–1131.
40. Wang Z, Qin G, Zhao TC: HDAC4: mechanism of regulation and biological functions. Epigenomics. 2014; 6:139–150.
41. Gerhart J, Neely C, Elder J, Pfautz J, Perlman J, Narciso L, Linask KK, Knudsen K, George-Weinstein M: Cells that express MyoD mRNA in the epiblast are stably committed to the skeletal muscle lineage. J Cell Biol. 2007; 178:649–660.

42. Bonnet A, Dai F, Brand-Saberi B, Duprez D: Vestigial-like 2 acts downstream of MyoD activation and is associated with skeletal muscle differentiation in chick myogenesis. Mech Dev. 2010; 127:120–136.
43. Hagiwara N: Sox6, jack of all trades: A versatile regulatory protein in vertebrate development. Dev Dyn. 2011; 240:1311–1321.
44. Tagariello A: Balanced translocation in a patient with craniosynostosis disrupts the SOX6 gene and an evolutionarily conserved non-transcribed region. J Med Genet. 2006; 43:534–540.
45. Hagiwara N, Yeh M, Liu A: Sox6 is required for normal fiber type differentiation of fetal skeletal muscle in mice. Dev Dyn. 2007; 236:2062–2076.
46. von Hofsten J, Elworthy S, Gilchrist MJ, Smith JC, Wardle FC, Ingham PW: Prdm1- and Sox6-mediated transcriptional repression specifies muscle fibre type in the zebrafish embryo. EMBO Rep. 2008; 9:683–689.
47. Ganfornina MD, Sanchez D, Pagano A, Tonachini L, Descalzi-Cancedda F, Martinez S: Molecular characterization and developmental expression pattern of the chicken apolipoprotein D gene: Implications for the evolution of vertebrate lipocalins. Dev Dyn. 2005; 232:191–199.
48. Teltathum T, Mekchay S: Proteome Changes in Thai Indigenous Chicken Muscle during Growth Period. Int J Biol Sci. 2009; 5:679–685.
19. Li Z: Desmin Is Essential for the Tensile Strength and Integrity of Myofibrils but Not for Myogenic Commitment, Differentiation, and Fusion of Skeletal Muscle. J Cell Biol. 1997; 139:129–144.
50. Paulin D, Li Z: Desmin: a major intermediate filament protein essential for the structural integrity and function of muscle. Exp Cell Res. 2004; 301:1–7.
51. Otten E, Asimaki A, Maass A, van Langen IM, van der Wal A, de Jonge N, van den Berg MP, Saffitz JE, Wilde AAM, Jongbloed JDH, van Tintelen JP: Desmin mutations as a cause of right ventricular heart failure affect the intercalated disks. Heart Rhythm. 2010; 7:1058–1064.
52. Zoltowska K, Webster R, Finlayson S, Maxwell S, Cossins J, Muller J, Lochmuller H, Beeson D: Mutations in GFPT1 that underlie limb-girdle congenital myasthenic syndrome result in reduced cell-surface expression of muscle AChR. Hum Mol Genet. 2013; 22:2905–2913.
53. Janisch S, Sharifi AR, Wicke M, Krischek C: Changing the incubation temperature during embryonic myogenesis influences the weight performance and meat quality of male and female broilers. Poult Sci. 2015; 94:2581–2588.
54. Pruitt KD, Brown GR, Hiatt SM, Thibaud-Nissen F, Astashyn A, Ermolaeva O, Farrell CM, Hart J, Landrum MJ, McGarvey KM, Murphy MR, O'Leary NA, Pujar S, Rajput B, Rangwala SH, Riddick LD, Shkeda A, Sun H, Tamez P, Tully RE, Wallin C, Webb D, Weber J, Wu W, DiCuccio M, Kitts P, Maglott DR, Murphy TD, Ostell JM: RefSeq: an update on mammalian reference sequences. Nucleic Acids Res. 2014; 42:D756–D763.

CHAPTER III

Transient shifts of incubation temperature reveal immediate and long-term transcriptional response in chicken breast muscle underpinning resilience and phenotypic plasticity

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Abstract

Variations in egg incubation temperatures can have acute or long-term effects on gene transcription in avian species. Altered gene expression may, in turn, affect muscle traits in poultry and indirectly influence commercial production. To determine how changes in eggshell temperature affect gene expression, incubation temperatures were varied [36.8°C (low), 37.8°C (control), 38.8°C (high)] at specific time periods reflecting two stages of myogenesis [embryonic days (ED) 7–10 and 10–13]. Gene expression was compared between interventions and matching controls by microarrays in broiler breast muscle at ED10 or ED13 and post-hatch at day 35. Early (ED7-10) high incubation temperature (H10ΔC) resulted in 1370 differentially expressed genes (DEGs) in embryos. Ingenuity pathway analysis revealed temporary activation of cell maintenance, organismal development, and survival ability genes, but these effects were not maintained in adults. Late high incubation temperature (ED10-13) (H13ΔC) had slightly negative impacts on development of cellular components in embryos, but a cumulative effect was observed in adults, in which tissue development and nutrition metabolism were affected. Early low incubation temperature (L10ΔC) produced 368 DEGs, most of which were down-regulated and involved in differentiation and formation of muscle cells. In adults, this treatment down-regulated pathways of transcriptional processes, but up-regulated cell proliferation. Late low temperature incubation (L13ΔC) produced 795 DEGs in embryos, and activated organismal survival and post-transcriptional regulation pathways. In adults this treatment activated cellular and organ development, nutrition and small molecule activity, and survival rate, but deactivated size of body and muscle cells. Thermal interventions during incubation initiate immediate and delayed transcriptional responses that are specific for timing and direction of treatment. Interestingly, the transcriptional response to transiently decreased incubation temperature, which did not affect the phenotypes, prompts compensatory effects reflecting resilience. In contrast, higher incubation temperature triggers gene expression and has long-term effects on the phenotype. These mechanisms of considerable phenotypic plasticity contribute to the biodiversity and broaden the basis for managing poultry populations.

Introduction

In homeotherms like birds, pre- and post-hatch development occurs only within a limited range of body temperature [1]. *In-ovo*, temperature is a critical factor affecting embryo development. Changes in eggshell temperature or inadequate timing of the proper temperature can result in morphological changes, through altered gene expression, that is fatal or produces long-term alterations in development. Under natural conditions and from the evolutionary point of view, one could argue that due to the likelihood of variation of brooding temperature under natural conditions mechanisms should have evolved to promote resilience against these unpredictable environmental factors. Indeed, in birds parent brooding causes more stable conditions than for many reptile species with shallow nests [2]. There are differences in nest temperatures varying from about 30°C to 40°C among avian species [3]. In unattended periods the nest temperature may drop considerably [4,5]. Therefore mechanisms to cope with transient lowered temperature likely exist. Such coping mechanisms may still exist in commercial broiler lines even after long-term artificial selection under highly controlled conditions.

In fact, under controlled artificial conditions in poultry production, improvement of productivity and resilience, for example to hot climates, are major issues; accordingly most research has focused on increased incubation temperature. For instance, a slightly higher egg incubation temperature has been associated with positive effects on breast meat yield (% of BW) of featherless broiler chicken [6] and muscle fiber development in turkey [7]. However, higher incubation temperature can also produce lower body weight [8]. Divergent outcomes may be attributable to differences in the intensity and duration of incubation temperature changes. Nonetheless, understanding how these changes affect development is crucial for identifying any long-term consequences and potential application of variation of incubation temperature in poultry breeding.

Also during *in-utero* development of mammalian species, including human, aberration of body temperature due to maternal fever may impact the post-natal life. For example, maternal fever significantly increases the risk of autism and developmental delay in humans [9]. In fact, embryonic and fetal development are periods of rapid growth and cell differentiation and pre-determination of later life. Adverse environmental conditions during embryonic and fetal development provoke an adaptive response, which may lead to both persistently biased responsiveness to extrinsic factors and permanent consequences for the organismal phenotype [10,11]. The *in-ovo* development of the poultry is an ideal model for studying the impact of exogenous (physical) effects and

analysing mechanisms of gene-environment interactions taking place during embryonic development and potentially affecting later life time development.

Avian myogenesis occurs in two phases during embryo development. First, between embryonic days (ED) 4 and 7, primary muscle fibers are formed from myoblasts. Next, from ED7-15, secondary muscle fibers develop around the scaffold of primary muscle fibers [12]. Fetal myoblasts are most abundant between ED8 and 12 [13]. Primary fibers represent a heterogeneous population that are committed to becoming fast (white), mixed fast/slow, and mainly slow (red) fibers, whereas secondary fibers belong to the fast myogenic lineage, i.e. the two developmental phases of myogenesis give rise to different myofibers [14]. Breast muscle is a valuable meat product in chicken. *Musculus pectoralis* consists of type II, white muscle fibers, with fast-contracting properties and high glycogen content for glycolytic metabolism. Changes in incubation temperature occurring during the transition from primary to secondary muscle fiber formation may induce gene expression changes in the embryo that result in altered muscle phenotypes. Elevated incubation temperature between ED7 and 10 positively influences slaughter and breast muscle weights in broiler males. In contrast, there was no effect on hind muscle weight, with represents a red muscle mainly consisting of type I fibers. Moreover, there was no negative effect on meat quality [15].

Manipulation of incubation temperature at specific periods may offer a method by which to improve the efficiency of broiler meat production by altering gene expression during myogenesis. This study sought to identify immediate and late transcriptomic responses in a white muscle (*Musculus pectoralis*) breast muscle following changes in incubation temperature. The identification of differentially expressed genes and their functional annotation to pathways and networks offers insight into the physiological mechanism that has evolved to cope with lower and higher incubation temperatures including those relevant to improve muscularity and heat tolerance.

Materials and Methods

Sample Collection

Animal care procedure followed the guidelines of the German Law of Animal Protection and the experimental protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of the Department of Animal Sciences of the University of Goettingen, Germany and the Leibniz Institute for Farm Animal Biology. Commercial broiler line eggs (Cobb-Vantress Inc., Siloam Springs, Arkansas, USA) were randomly

selected and divided into 6 experimental groups (total number of eggs 1001) (Fig 1). Three groups had early intervention, and incubation conditions of these groups over ED7 to 10 were as follows: 1) high temperature of 38.8°C and 65% relative humidity (RH) (group H10); 2) control temperature of 37.8°C and 55% RH (C10), which equals the conditions before and after the intervention; and 3) low temperature of 36.8°C and 55% RH (L10). The remaining 3 groups had late intervention, and incubation conditions of these groups over ED10 to 13 mimicked those of the early groups, with high (H13), control (C13), and low (L13) temperatures. At ED10 and ED13 subsets of each group were obtained and breast muscles were prepared and stored for subsequent analyses. In addition to the eggs for the collection of embryo samples at ED10 or ED13, respectively, a set of eggs was treated the same way in parallel. Except for the specific treatment periods at ED7-10 or ED 10–13 these eggs were incubated at 37.8°C, 55% RH until 3 days before hatching, when RH was increased to 65% until hatch. After hatch chicks were fed a standard diet ad libitum until slaughter age at day 35 (D35). Broilers were slaughtered at the experimental poultry abattoir of the Department of Animal Sciences of the University of Goettingen, Germany, by electronarcosis (0.12 A, 5 to 10 sec) followed by exsanguination according to German animal welfare laws and regulations. Zoo-technical and biochemical traits were recorded. Breast tissue samples (*M. pectoralis*) were collected in liquid nitrogen at slaughter (D35). Embryonic samples taken at ED10 and ED13 as well as samples of D35 were sexed and for each experimental group (C10, H10, L10 and C13, H13, L13) at each time point (ED10 or ED13, respectively, plus D35) samples, balanced for sex, were selected for gene expression analyses with 8 samples per treatment (Fig 1).

RNA Preparation

Total RNA of individual tissue samples (n = 88) was isolated by Tri-Reagent extraction (Sigma-Aldrich, Taufkirchen, Germany). DNase treatment and column-based purification using the RNeasy Mini Kit (Qiagen, Hilden, Germany) were used to ensure purity. Quality of RNA was checked using 1% agarose gels containing ethidium bromide. Concentration of RNA was also detected by spectrometry with a NanoDrop ND-1000 spectrophotometer (PEQLAB, Erlangen, Germany). Additionally, the absence of DNA contamination was verified by using the RNA as template in standard PCR amplifying fragments of the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene. All RNAs were stored at −80°C until further use.

Microarray Data Processing

cDNA was generated by reverse-transcription of 500 ng of total RNA with the Ambion WT Expression Kit (Life Technologies GmbH, Darmstadt, Germany). Biotin-labeled cRNA targets were identified with Affymetrix GeneChip WT Terminal Labeling Kit (Affymetrix, Santa Clara, CA, USA). Fragmented biotin-labeled cRNAs were hybridized on Chicken Gene 1.0 ST Arrays (Affymetrix) covering 439,582 probe-sets representing 18,214 gene level probe-sets. Following staining and washing protocols, the arrays were scanned by Affymetrix GCOS 1.1.1 software for raw results with official annotation (galgal3 build 34).

Statistical Analysis

Affymetrix Expression Console Software was used to normalize and quantify transcript expression by using the PLIER (Probe Logarithmic Intensity Error) algorithm together with DABG (detection above background), which joins probe-level p-values to create probe cell intensity values at the exon level. All data were deposited in an MIAME-compliant database, the National Center for Biotechnology Information Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo; accession number: GSE76670). Default thresholds were used to assign a "present" call (detection p-values < 0.04); present calls were synchronized with gene-level annotation. Filtering outlier and nonspecific results were done by using "genefilter" in R (www.r-project.org) considering standard deviations of normalized expression values at the gene-level. Analysis of variance was applied to detect transcriptional changes between treatment groups using the Mixed procedure in JMP Genomics (JMP Genomics 5, SAS Institute) considering individual and combined effects of temperature, treatment period and gender and slaughter weight. Sex was excluded from the statistical model due to marginal effects. The final model included fixed effects of temperature, treatment period and interactions. Slaughter weight was included as covariate for the 35 days post-hatch time course. Transcripts with significant differences of abundance at p-values 0.05 were selected and queried for pathways analysis. At pre-hatch stages $p < 0.05$ equals FDR adjusted p-values of $q < 0.15$; at D35 $p < 0.05$ corresponding q-values ranged between 0.2 and 0.7. Moreover, we have previously shown consistency of microarray expression data with real time qPCR data using hind muscle tissue of the same animals. In fact, we obtained significant correlation coefficients ranging between 0.71 and 0.84 [16]. Annotated genes with different transcript abundances are termed "differentially expressed genes (DEGs)"; higher (lower) abundance in treated group vs. control groups is termed up-regulated (down-regulated).

Pathway Mining

Differentially expressed genes (DEGs) with p-values and fold changes were subjected to pathway analysis using the Ingenuity Pathway Analysis software (IPA, QIAGEN Redwood City, USA). Networks, biological pathways, and gene functions of the DEGs were extracted from the IPA Knowledge Base. Significant canonical pathways and biological functions (Fisher's exact test, p-values < 0.05) were further adjusted for multiple testing (Benjamini-Hochberg) [17]. To simplify the interpretation of complexed biological networks, significant biological functions and pathways were aggregated into eight `major categories´ (sc. 1 – sc. 8): cell maintenance, proliferation, differentiation, and replacement (sc. 1); organism organ and tissue development (sc. 2); nutrient metabolism (sc. 3); genetic information and nucleic acid processing (sc. 4); molecular transport (sc. 5); cell signaling and interaction (sc. 6); small molecule biochemistry (sc. 7); and response to stimuli (sc. 8). Furthermore, a state of pathway regulation was indicated by "activated" with a positive Z-score or "deactivated" with a negative Z-score (IPA). Genes used to generate biological networks were selected from pathways with a significant Z-score. Legend of network shapes and relationships are available in S1 Fig.

Results

Effects on Breast-Muscle Transcriptome

The chicken gene 1.0 ST array contains 165,815 probe-sets representing 20,828 transcripts encoding for 18,214 genes. After data pre-processing and filtering, 8,317 entries (gene level) passed to downstream analyses. In this study, we aimed to identify the effects of embryonic incubation temperature on transcriptional changes of the muscle tissue. We hypothesized that the effects may depend on time-windows of the embryonic development more specifically the development of muscle cells. We also speculated that the effects may have an influence on muscle development post-hatch. Therefore, transcriptional profiles of the breast muscle were compared between treatment conditions and control at the embryonic (*in-ovo*) and adult (post-hatch) stages using analysis of variance. Our results showed that high temperature (38.8°C) treatment during E7-10 profoundly changed the transcriptional profile compared to the same thermal treatment during E10-13 in terms of the number of DEGs (1370 vs. 365) as shown in Table 1. On the other hand, low temperature (36.8°C) treatment showed smaller effects during the same embryonic stage (E7-10) as well as the later stage of E10-13. Long-term effects of changing embryonic incubation temperature were also shown in adult stage (35 days post-

hatch) with pronounced effects observed in the low thermal treatment group during E10-13 (Table 1). The further information on q-value is represented in S1 Table.

Immediate and Long-Term Effects

To identify immediate and long-term effects of the embryonic incubation temperature on changes of the breast muscle transcriptional profile, we extracted common and unique DEGs from different treatment conditions as well as from the two developmental stages. Firstly, DEGs from each treatment condition compared to control were mined separately for embryonic (Fig 2A) or adult (Fig 2B) samples. Overall results revealed that the number of unique DEGs for each treatment condition is greater than that of common DEGs, suggesting that changing of the incubation temperature at a particular time-window of embryonic development affects different gene-sets and pathways. Further extraction of common DEGs between embryonic and adult samples for each treatment condition are shown in Fig 2C–2F. About 3% of the DEGs were common between the embryonic and adult samples across treatment conditions, while a majority of DEGs were unique for each stage and thermal treatment combination. The present results suggest complex biological processes and gene regulation may involve long-term effects of changing incubation temperature as well as possible environmental interactions.

To address the short- and long-term regulation of transcript expression following *in-ovo* temperature modification, DEG lists within the same treatment condition (H10, H13, L10, or L13) were compared between embryonic stages (ED10 or ED13) and D35 (Fig 2C–2F). The number of common DEGs between the two stages ranged from 22 to 42 (average 7%). Of 126 common transcripts in total (120 unique genes), 30 up and 36 down-regulated genes showed the same direction of shifting between stages (S2 Table). For groups exposed to lower temperature at either ED7-10 or ED10-13 the majority (22 out of 34 and 20 out of 28, respectively) of the DEGs found in embryonic and D35 samples showed the identical direction of change of transcript abundances compared to the matched controls; for L10ΔC most DEGs are down-regulated whereas for L13ΔC they are up-regulated. Of the 42 common DEGs of the H10ΔC at embryonic stages and D35 30 showed higher abundance in the treated samples compared to controls at embryonic stage, but lower abundance in treated than in untreated samples of D35. For H13ΔC no trends are obvious. Lowered incubation temperature leads to a higher proportion of genes that are consistently modulated over the lifetime from embryonic stages to D35; higher incubation temperature, in particular at ED7-10 results in a considerable number of genes that are diametrically shifted in immediate and late response.

Pathway Mining

To gain insight into molecular mechanisms underlying the thermal change effects, DEGs from each treatment (6 conditions) and stage (embryos and adults) were subjected to a knowledge base enrichment analysis for significant biological functions and pathways using IPA. Altogether, 92 and 115 biological functions were significantly enriched for DEGs derived from embryos and adult breast-muscle samples, respectively. To summarize and simplify the results, significant bio-functions were aggregated into eight major categories based on related ontology terms. The results are shown in Fig 3 and detailed information is accessible in S3–S6 Tables. During early (ED7-10) muscle development, high embryonic incubation temperature affected biological processes including cell growth, tissue and organ development, nutrient metabolism, and cell signaling at higher degree (based on number of DEGs, bio-functions and statistically significant threshold) compared to the low temperature treatment. However, during late (ED10-13) muscle development, low temperature affected more of the aforementioned biological processes than did high temperature treatment (Fig 3). Prediction of pathway regulation as "activation" or "inactivation" based on Z-score (IPA) suggested that high incubation temperature at ED7-10 led to a shifting of cell maintenance, proliferation, differentiation, and replacement (sc. 1), and organism organ and tissue development (sc. 2) towards increased formation of cells, tissues and organs and decreased apoptosis, necrosis and death (S3 Table). Interestingly, DEGs derived from the high temperature group during early muscle development (ED7-10) revealed gene networks related to skeletal muscle development and function of which SMAD3 (smad family member 3) functions as an down-regulated hub gene, highly connected with other genes in the network (Fig 4A). High incubation temperature during ED10-13 tended to hamper formation of filaments, cytoskeleton and cytoplasma (S4 Table).

Long-term effects of thermal changes during embryonic development on biological processes were detected in adult samples, but less pronounced than observed in the embryos (immediate effects). Interestingly, low embryonic incubation temperature treatment seems to have long-term effects on cell growth and tissue development at later age. Low incubation temperature during ED10-13 affected most of genes related to organismal survival and post-translational modification. For L13ΔC a network related to skeletal-muscular and connective tissue development was derived (Fig 4B) with RUNX2 (runt-related transcription factor 2) as the highly connected hub-gene. L10ΔC led to inactivation of pathways related to muscle cell formation and differentiation (S3 Table).

At D35, L10ΔC revealed most DEGs, however, L13ΔC affected more pathways than did early treatment (S5 and S6 Tables). Lower incubation temperature during ED10-13 (L13UΔC) significantly influenced pathways related to five major categories including cell maintenance, organismal and tissue development, nutrient metabolism, molecular transport, and small molecule biochemistry (sc.1, 2, 3, 5, and 7). Z-scores show that L13UΔC condition led to activation of most pathways that are related to anabolic functions, whereas the biofunction organismal death is strongly inactivated (S6 Table). For L10ΔC, 19 transcripts formed a network for gene expression, cellular function, and cell signaling pathways (Fig 5A). MED24 (mediator complex subunit 24), TBP (TATA box binding protein), and TRRAP (transformation/transcription domain associated protein) were identified as top candidate genes in this network. For L13ΔC, the network included organ, embryonic, and skeletal-muscular system development and function (Fig 5B). The 17 main transcripts included candidate genes of the myosin-myogenin group (MYH2, MYL3, and MYOG).

The 22 durable down-regulated DEGs that are due to lower incubation temperature at ED7-10 (Fig 2D) and that are common in immediate and late response belong to gene expression pathways, and show negative Z-scores indicating deactivation. The functional network comprising these genes displays pathways of cell-to-cell signaling and interaction, cell death and survival, cell signaling, molecular transport, and vitamin and mineral metabolism (S2A Fig). For cold treatment at ED10-13 those 20 DEGs that were consistently up-regulated in short-term and long-term were related to proliferation and cell death, with Z-scores indicating deactivation of the first and activation of the second (Fig 2F). The functional network based on these DEGs is related to cell death and survival, cellular development, cellular growth and proliferation, protein synthesis, connective tissue disorders, as well as organismal injury and abnormalities (S2B Fig). For H10ΔC functional annotation analysis of 30 genes being immediately up-regulated at ED 10 but down-regulated at D35 (Fig 2C) revealed opposing regulation of biofunctions related to amino acid metabolism and hyperplasia including formation of type II myofibers.

Discussion

The study aimed to identify genes and pathways that are immediately and lately shifted due to decreased and increased incubation temperature at two phases of myogenesis. Temperature adjustments at specific developmental time points could influence muscle development and potentially impact commercial meat production. In

fact, modulation of *in-ovo* development with impact on post-hatch growth has been demonstrated in a number of experiments, which differ in terms of direction and extent of temperature change and the time period. Hammond et al. (2007) [18] and Lourens et al. (2005) [19] showed that higher incubation temperatures during the first week of *in-ovo* development increases total embryo mass; however no effect on post-hatch was found [19]. We previously reported that the exact conditions used here, i.e. high temperature (38.8°C) between ED 7 to 10 or ED 10 to 13 results in higher body weights of 35 d old broilers compared to broilers from normal (37.8°C) or lower (36.8°C) temperature conditions within these periods [20]. Increment of body weight was attributable to increased size of breast muscle rather than hind muscle [15]. The treatment intervals coincide with secondary fiber development. Treatments may thus have stronger impact on the breast muscle than on the hind muscle with the former mainly consisting of type II fibers originated from secondary fibers and the later mainly consisting of type I fibers originated from earlier developing primary fibers. Moreover, ED10 and ED13 embryos of the low temperature group had slightly but significantly lower weights than the control group at the respective time points; higher incubation temperature slightly but non-significantly increased the embryo weight (S3 Fig) [21]. Mitochondrial respiratory activity was lower in low temperature group at ED10 and ED13; higher incubation temperature led to higher mitochondrial respiratory rates at ED13. For metabolic enzymes only subtle mostly non-significant effects were found at the embryonic stages [21]. Accordingly, the D35 chickens analyzed here showed slight but significant increases in body or carcass weight when transiently incubated at higher temperature at ED7-10 or ED 10–13, whereas decreased incubation temperature did not affect either body weight or carcass weight (S4 Fig). Breast muscle weight of the 35 days old broilers was highest in the H10 group and lowest in the L13 groups. While these two extremes differed significantly, there were no significant differences among the other groups and no deviation from the respective controls.

The results suggest that transcriptional regulation taking place immediately *in-ovo* and in long-term after hatch displays mechanism that mediate resilient coping with low incubation temperature, whereas higher incubation temperature provokes phenotypic plasticity. We suspect that resilience to low temperature has been evolved by natural selection in bird species and still exists in commercial broiler lines. The phenotypic plasticity associated with higher incubation temperature offers perspectives for targeted modulation of traits relevant in poultry production by modulating incubation temperature.

Incubation Temperature Has Immediate Effects on Gene Transcription at Embryonic Stages

Incubation temperature changes at both stages of myogenesis (ED7-10 and ED10-13) result in immediate changes to breast muscle gene transcription. In particular, higher incubation temperature during early myogenesis and lower temperature during later myogenesis promote the up-regulation of many transcripts. The expression pattern of ED10 and ED13 were distinct, indicating the developmental stage has a larger effect than incubation temperature.

Immediate Response to Early Modulation of Incubation Temperature

For early treatment, high temperature (H10ΔC) activated pathways in cell maintenance and organismal development, and especially affected survivability pathways. In contrast, low temperature (L10ΔC) down-regulated the differentiation and formation of muscle cells. The latter may contribute to the slightly lower body weight of ED10 embryos in the low temperature group. But since also liver and heart weight were significantly reduced in L10 compared to C10, low temperature seems to have a general quietening effect on development, not specific to myogenesis. Higher temperature did not provoke significant differences of weights (body, liver, and heart) compared to ED10 control embryos. Moreover, activity of mitochondrial respiration (state-3-pyruvate/malate and state-3-succinate/rotenone) and enzyme activities (glycogen phosphorylase, lactate dehydrogenase, and cytochrome oxidase) was shifted by low and high temperature with inhibiting and activating effects on metabolic processes, respectively [21]. The high breast muscle weight of D35 broilers of the H10 group is in line with the increased expression of genes of proliferative pathways, particularly at the early hyperplastic phase of secondary fibers formation, that become white fibers, the major proportion of fibers in the M. pectoralis. The up-regulated transcripts following high temperature treatment formed a network in tissue development and connective tissue and skeletal muscle system development and function. SMAD3 is the hub gene in that network. SMAD3 is a transcription factor involved in the regulation of growth factor expression including transforming growth factor and connective tissue growth factors that are relevant to many developmental processes including myogenesis. The regulatory effect in myoblast was shown recently [22]. Moreover, SMAD 3 is involved in myostatin signaling during myogenesis. Analyzes of differential expression of wild and myostatin knockout mice revealed that many DEG exhibited an SMAD3 binding motif [23]. In our study SMAD3 was down-regulated in muscle growth promoting hypertrophic conditions.

Immediate Response to Late Modulation of Incubation Temperature

During later myogenesis (ED10-13), lower temperature produced more transcriptomic changes than did higher temperature. These conditions up-regulated pathways related to organismal survival and post-translational modification. Further prediction via Z-score revealed a potential prevention of organismal death and an increase in development of cardio-vascular system, size of body, and metabolism of carbohydrate but an inactivation of cellular proliferation and differentiation. In result L13ΔC treatment is associated with reduced *in-ovo* growth, but has no impact on adult body weight. Higher temperature late treatment affected a few pathways. The possibility of regulation along this process tended to inhibit cytoplasm development and formation of cytoskeleton and filaments. In the L13ΔC network, pathways affecting cardiovascular system, skeletal-muscular, and connective tissue development are interconnected via RUNX2. RUNX2 plays a central role in osteoblastic differentiation and skeletal morphogenesis [24]. In chicken embryos, overexpression of RUNX2 produces multiple phenotypes including joint fusions, expansion of carpal elements, and shortening of skeletal elements [24]. On the other hand, inactivation of RUNX2 results in a disruption in chondrocyte differentiation, vascular invasion, osteoclast differentiation, and periosteal bone formation as seen by severe shortening of the limbs [24]. Down-regulation of RUNX2 under low temperature conditions might suppress myogenesis process and embryo differentiation. This is in line with the reduced embryo body weight compared to control as well as enzyme activity (cytochrome oxidase) and mitochondrial respiration (state-3-pyruvate/malate) in the L13 group [21].

Incubation Temperature Has Long-Term Effects on Gene Transcription at D35

In-ovo temperature manipulation also produced long-term transcriptional changes, as demonstrated by differential gene expression in adult chickens. Our findings add support to previous research that demonstrated that increased and/or decreased incubation temperature affects post-hatch growth in avian species [1,25,26,27,28].

Long-Term Response to Early Modulation of Incubation Temperature

In adults, the highest number of genes with different transcript abundance compared to control was induced following low-temperature incubations at ED7-10 (L10ΔC). However, this did not lead to significant changes in either body weight or breast muscle weight of 35 day old broilers. Up- and down-regulated DEGs were related to cell cycle and nucleic acid processing with Z-scores indicating activation and deactivation, respectively. The DEG revealed a network related to gene expression,

cellular function, and cell signaling pathways and covering genes belonging to the RNA polymerase II apparatus like MED14, MED15, MED23, MED24, TBP, and TRRAP along with this treatment. Increased incubation temperature at ED7-10 did not lead to shifts of expression in broilers that reveal any prominent pathways in terms of significance and Z-scores. MED24, a mediator or transcriptional coactivator, interacts with RNA polymerase II and promotes formation of a transcriptional pre-initiation complex [29]. Further, several related genes, like MED10, MED14, MED15, and MED23, were represented in the network. Downregulation of MED24 may alter RNA polymerase II activity and lead to abnormal transcription/translation of genes. Similarly, TBP (TATA-binding protein) interacts with transcription factor IID (TFIID), which binds to the core promoter to position RNA polymerase II properly [30]). Two forms of TBP mRNA are expressed in chicken [31]. Disruption of TBP causes phenotypic abnormalities with delayed mitosis and induced apoptosis [32]. Finally, TRRAP is a phosphoinositide 3-kinase-related kinase (PIKK) family member involved in transcription and DNA repair [29]. TRRAP is essential for early development, particularly for the mitotic checkpoint and regular cell cycle progression [33]. Thus, downregulation of genes following L10ΔC treatment may affect global gene transcription *in-ovo*.

Long-Term Response to Late Modulation of Incubation Temperature

High temperature treatment during ED10-13 tended to suppress tissue development pathways, especially body size. Furthermore, nutrition metabolism, quantity, and synthesis of carbohydrates and lipids were also suppressed. In contrast, low-temperature treatment during ED10-13 tended to activate transcripts of trophic pathways and function while pathways related to cell death and apoptosis were reduced. In the nutrition group, metabolism of lipids was activated, but fat accumulation was deactivated, which may reduce concentrations of fatty acid components. In the network analysis, activation of organ, embryonic and skeletal-muscular system development were significant pathways with RAF1 and actin being hub genes. Actin represents an abundant protein with fundamental function in muscle tissue and the abundance of its transcript is linked with molecules that are not differentially expressed. Myosins are actin-based motor proteins that function in skeletal muscle contraction [34]. The proper function of both myosin heavy chain (MYH2) and myosin light chain (MYL3) are necessary to accompany actin filaments during eukaryotic motility processes [29]. Moreover, myogenin (MYOG), a muscle-specific transcription factor, is essential for developing functional skeletal muscle [35]. In chicken, MYOG affects muscle fiber trait specification [36]. Thus, upregulation of these genes could enhance muscle cell and contraction

processes. RAF1, which links also T-actin, is a MAP3K, i.e. a higher order kinase affecting the ERK-pathway via MEK1 and MEK2. The serine/threonine specific protein kinases, ERK1 and ERK2 are involved in control of gene expression and by this have effects on cell and tissue formation. Only recently it was shown in mice that the ERK1/2 pathways is essential for the maintenance of adult muscle fibers and the link of the nerval and muscle system [37]. Also in chicken the ERK1/2 MAP is known to be involved in protein synthesis pathways particularly in myoblast cells [38]. Modulated incubation temperatures had significant effects on adult transcriptomes, and led to subtle but still significant phenotypic differences as long as increased temperature is concerned, i.e. increased body and breast muscle weight due to high incubation temperature. Lowered incubation temperature led to shifts of expression of an even higher number of genes but was not associated with significant phenotypic changes of day 35 broilers.

Conclusion

Increasing as well as decreasing of incubation temperature at two stages (ED7-10 and ED10-13) affected the abundance of numerous transcript immediately and in the long-term. Functional annotation indicated that these genes are assigned to biofunctions related to cell formation and survival tending to be promoted, whereas metabolic pathways were less modulated. However, the sets of modulated genes were mostly specific to the different treatments. The fact that increased incubation temperature increased organismal growth but lowered temperature did not affect the phenotype at D35 suggest that the shifts of expression associated with low temperature represent molecular routes promoting resilience to the treatment. In contrast, elevated incubation temperature conditions the organisms for increased growth. The altered expression displays the molecular pathways that mediate the phenotypic plasticity. The results have implications in terms of natural selection and the development of mechanisms to cope with advise conditions and in terms of deriving strategies to improve poultry breeding. Epigenetic temperature acclimatization might alter body growth and enrich poultry resistance to various environmental effects. It would be important to address the epigenetic changes at the molecular level in future studies.

Figure titles and Tables

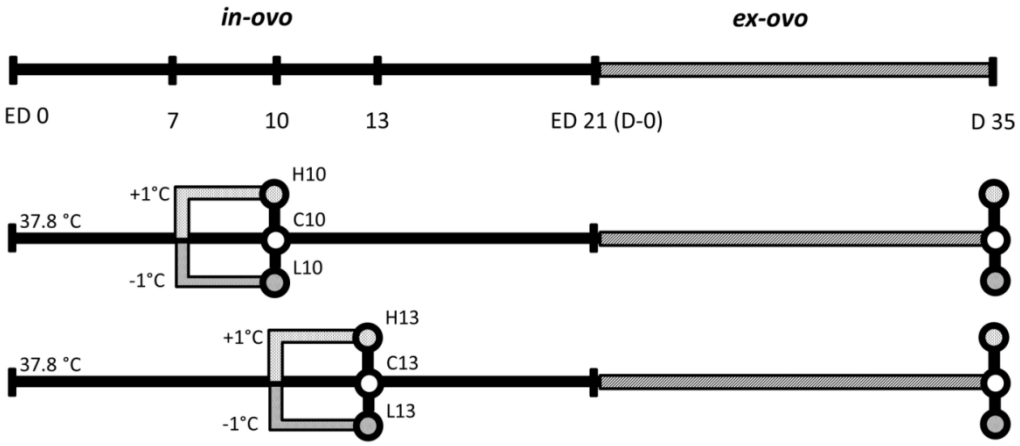

Fig 1. The experimental design shows the timeline and parameters for treatment. Each circle indicates the time point for sample collection (88 samples; n = 8 per treatment with adult controls n=8 in total). H, high temperature; L, low temperature; C, control; ED, embryonic day.

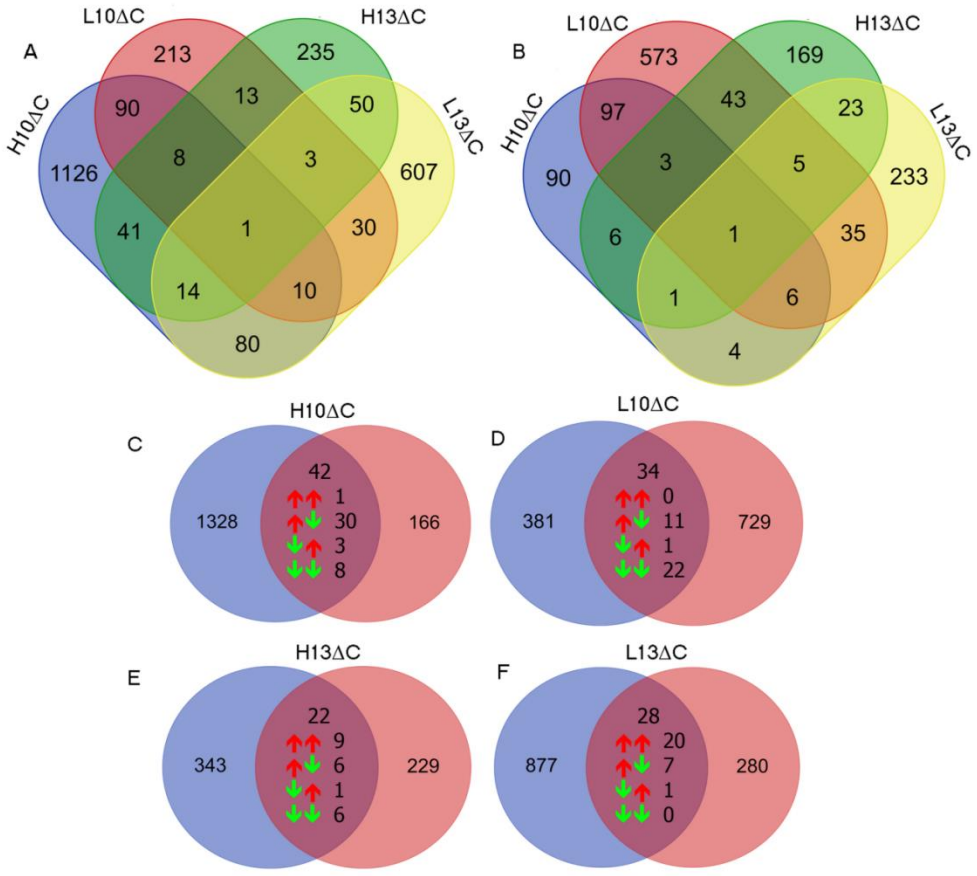

Fig 2. Differentially expressed genes for each treatment condition relative to controls within embryonic (A) stages and at D35 (B) and between embryonic stages and D35 within the same treatments (C-F) (blue embryonic, red adult). Intersection areas show numbers of common DEGs and their direction of regulation in each stage

Fig 3. Significant pathways affected by differential gene expression at embryonic stages and D35. Up (U) or Down (D) regulation of DEG associated with each comparison (versus control) are separated within each thermal modification: increase (H) or decrease (L) in incubation temperature during ED7-10 (H10 and L10) or ED10-13 (H13 and L13). Significant pathways are grouped into eight super-categories (sc.): sc.1 cell maintenance, proliferation, differentiation, and replacement; sc.2 organismal organ and tissue development; sc.3 nutrient metabolism; sc.4 genetic information and nucleic acid processing; sc.5 molecular transport; sc.6 cell signaling and interaction; sc.7 small molecule biochemistry; and sc.8 response to stimuli and associated. The –log (*p* values) related with significant pathways (Benjamini Hochberg corrected) are plotted in green (small) to red (large).

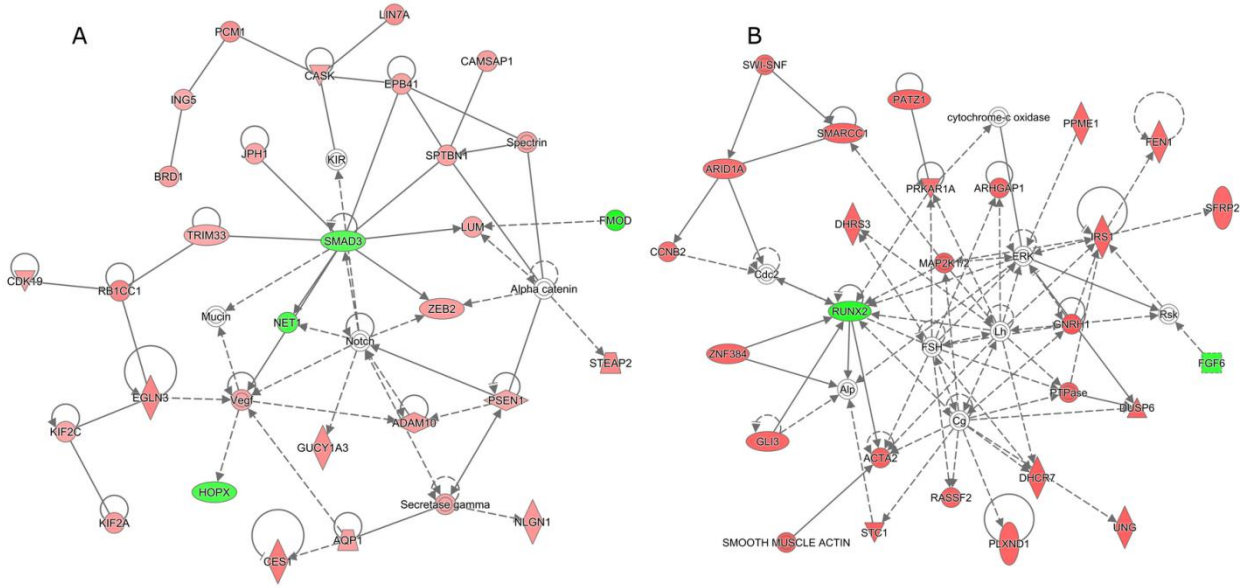

Fig 4. Major gene networks at ED10 and ED13. For H10ΔC a network related to tissue development and skeletal muscle development and function was derived (A). For L13ΔC a network related to skeletal-muscular and connective tissue development was derived (B). Red color, up-regulated; Green color, down-regulated.

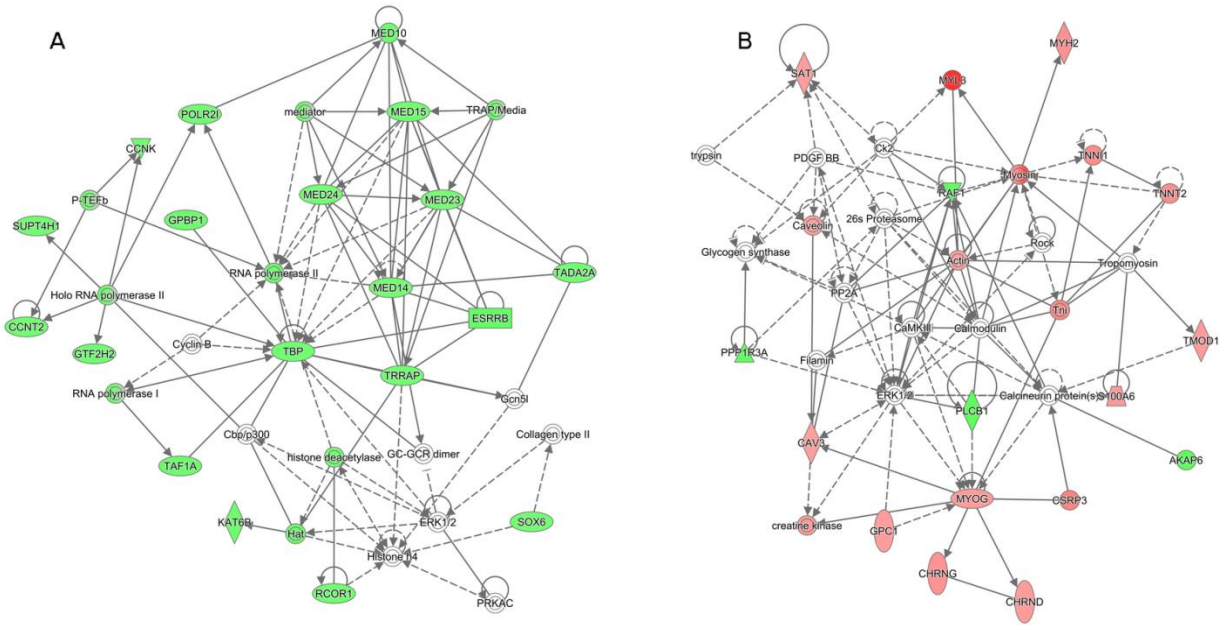

Fig 5. Major gene networks at D35. For L10ΔC a network related to gene expression, cell signaling and cellular function and maintenance pathways was derived (A). For L13ΔC a network related to organ, embryonic and skeletal-muscular system development and function was derived (B). Red color, up-regulated; Green color, down-regulated.

Table 1. Numbers of differentially expressed probes sets and respective genes (DEGs) revealed by comparisons between each *in-ovo* thermal modification condition and the time-matched control separated for embryonic stages *or* at D35 (*p*-value ≤ 0.05*)**.**

	Treatment	Probe-sets	DEG	Regulation	
				Up	Down
Embryo	**H10ΔC**	1484	1370	1090	280
	H13ΔC	470	365	308	57
	L10ΔC	415	368	97	271
	L13ΔC	905	795	763	32
D35	**H10ΔC**	262	208	32	176
	H13ΔC	325	251	121	130
	L10ΔC	846	761	74	687
	L13ΔC	349	308	160	148

*q<0.15 at embryonic stages; q= 0.2-0.7 at D35

Supporting Information

S1 Fig. IPA legend of network shapes and relationships.
(source http://ingenuity.force.com/ipa/articles/Feature_Description/Legend). (TIF)

S2 Fig. Major gene networks derived from genes consistently regulated at embryonic stages and D35. For L10ΔC a network related to cell-to-cell signaling and interaction, nervous system development, and cell survival pathway was derived (A). For L13ΔC a network related to cell death and survival, cellular development, and cellular growth and proliferation was derived (B). Red color, up-regulated; Green color, down-regulated. (TIF)

S3 Fig. Body weight after exposure to H (High 38.8°C), C (Control 37.8°C), and L (Low 36.8°C) temperature at the end of intervention, ED10 and ED13, respectively (adapt from [17]). (DOCX)

S4 Fig. Body, carcass and breast muscle weights at day 35 after *in-ovo* exposure to H (High 38.8°C), C (Control 37.8°C), and L (Low 36.8°C) temperature at ED7-10 and ED10-13. (DOCX)

S1 Table. Numbers of differentially expressed probes sets at p 0.05 and corresponding q-value for the variance components. The comparisons between each *in-ovo* thermal modification condition and the time-matched control separated for embryonic stages or post-hatch D35. (DOCX)

S2 Table. DEGs common to both embryonic stages and D35. (DOCX)

S3 Table. Assignment of DEGs to biological functions (major categories and Ingenuity-bio-functions) (p 0.05) obtained at embryonic stage for early treatment; H10UΔC, H10DΔC, L10UΔC and L10DΔC. (DOCX)

S4 Table. Assignment of DEGs to biological functions (major categories and Ingenuity-bio-functions) (p 0.05) obtained at embryonic stage for late treatment; H13UΔC, H13DΔC, L13UΔC and L13DΔC. (DOCX)

S5 Table. Assignment of DEGs to biological functions (major categories and Ingenuity-bio-functions) (p 0.05) obtained at adult stage for early treatment; H10UΔC, H10DΔC, L10UΔC and L10DΔC. (DOCX)

S6 Table. Assignment of DEGs to biological functions (major categories and Ingenuity-bio-functions) (p 0.05) obtained at adult stage for late treatment; H13UΔC, H13DΔC, L13UΔC and L13DΔC. (DOCX)

Reference

1. Piestun Y, Harel M, Barak M, Yahav S, Halevy O. Thermal manipulations in late-term chick embryos have immediate and longer term effects on myoblast proliferation and skeletal muscle hypertrophy. J Appl Physiol. 2008; 106: 233–240. doi: 10.1152/japplphysiol.91090.2008 PMID: 19023019

2. Du W-G, Shine R. The behavioural and physiological strategies of bird and reptile embryos in response to unpredictable variation in nest temperature: Thermal response of reptile and bird embryos. Biol Rev. 2015; 90: 19–30. PMID: 24593133

3. Webb DR. Thermal Tolerance of Avian Embryos: A Review. The Condor. 1987; 89: 874. doi: 10.2307/ 1368537

4. Lill A. Nest Inattentiveness and Its Influence on Development of the Young in the Superb Lyrebird. The Condor. 1979; 81: 225. doi: 10.2307/1367621

5. Jia C-X, Sun Y-H, Swenson JE. Unusual Incubation Behavior and Embryonic Tolerance of Hypothermia by the Blood Pheasant (*Ithaginis cruentus*). The Auk. 2010; 127: 926–931. doi: 10.1525/auk.2010. 09254

6. Yair Hadad, Cahaner Avigdor, Halevy Orna. Featherless and feathered broilers under control versus hot conditions. 2. Breast muscle development and growth in pre- and post-hatch periods. Available: http://ps.oxfordjournals.org/content/93/5/1076.long.

7. Maltby V, Somaiya A, French NA, Stickland NC. In ovo temperature manipulation influences post-hatch muscle growth in the turkey. Br Poult Sci. 2004; 45: 491–498. doi: 10.1080/00071660412331286190 PMID: 15484723

8. Krischek C, Gerken M, Wicke M. Effects of a higher incubation temperature between embryonic day 9 and 12 on growth and meat quality characteristics of turkeys. Br Poult Sci. 2013; 54: 5–11. doi: 10. 1080/00071668.2013.763901 PMID: 23444849

9. Zerbo O, Iosif AM, Walker C, Ozonoff S, Hansen RL, Hertz-Picciotto I. Is maternal influenza or fever during pregnancy associated with autism or developmental delays? Results from the CHARGE (CHild-hood Autism Risks from Genetics and Environment) study. J Autism Dev Disord. 2013; 43:25–33. PMID: 22562209

10. Bateson P, Barker D, Clutton-Brock T, Deb D, D'Udine B, Foley R, et al. Developmental plasticity and human health. Nature 2004; 430:419–421. PMID: 15269759

11. Oster M, Murani E, Metges CC, Ponsuksili S, Wimmers K. High- and low-protein gestation diets do not provoke common transcriptional responses representing universal target-pathways in muscle and liver of porcine progeny. Acta Physiol (Oxf). 2014; 210:202–14.

12. Miller JB, Stockdale FE. What muscle cells know that nerves don't tell them. Trends Neurosci. 1987; 10: 325–329. doi: 10.1016/0166-2236(87)90089-0

13. Stockdale FE. Myogenic cell lineages. Dev Biol. 1992; 154: 284–298. PMID: 1426639

14. Miller JB, Stockdale FE. Developmental regulation of the multiple myogenic cell lineages of the avian embryo. J Cell Biol. 1986; 103: 2197–2208. PMID: 3782296

15. Werner C, Wicke M. Increasing the temperature at a certain period during the incubation of broiler eggs improves the carcass and breast yields without a negative impact on the meat quality. Arch Anim Breed. 2008; 11–16.

16. Naraballobh W, Trakooljul N, Muráni E, Brunner R, Krischeck C, Januisch S, Wicke M, Ponsuksili S, Wimmers K. Immediate and long-term transcriptional response of hind muscle tissue to transient variation of incubation temperature in broilers. BMC Genomics. 2016; 17:323. doi: 10.1186/s12864-016-2671-9 PMID: 27142659

17. Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J. Roy. Statist. Soc. Ser. B 57, 289–300 (1995).

18. Hammond CL, Simbi BH, Stickland NC. In ovo temperature manipulation influences embryonic motility and growth of limb tissues in the chick (Gallus gallus). J Exp Biol. 2007; 210: 2667–2675. doi: 10.1242/ jeb.005751 PMID: 17644681

19. Lourens A, van den Brand H, Meijerhof R, Kemp B. Effect of eggshell temperature during incubation on embryo development, hatchability, and posthatch development. Poult Sci. 2005; 84: 914–920. PMID: 15971530

20. Janisch S, Sharifi AR, Wicke M, Krischek C. Changing the incubation temperature during embryonic myogenesis influences the weight performance and meat quality of male and female broilers. Poult Sci. 2015; 94: 2581–2588. doi: 10.3382/ps/pev239 PMID: 26316339

21. Krischek C, Janisch S, Naraballobh W, Brunner R, Wimmers K, Wicke M. Altered incubation temperatures between embryonic Days 7 and 13 influence the weights and the mitochondrial respiratory and enzyme activities in breast and leg muscles of broiler embryos. Mol Reprod Dev. 2016; 83: 71–78. PMID: 26599350

22. Córdova G, Rochard A, Riquelme-Guzmán C, Cofré C, Scherman D, Bigey P, et al. SMAD3 and SP1/ SP3 Transcription Factors Collaborate to Regulate Connective Tissue Growth Factor Gene Expression in Myoblasts in Response to Transforming Growth Factor β. J Cell Biochem. 2015; 116: 1880–1887. PMID: 25727481

23. Yang X, Koltes JE, Park CA, Chen D, Reecy JM. Gene Co-Expression Network Analysis Provides Novel Insights into Myostatin Regulation at Three Different Mouse Developmental Timepoints. Zhou Y, editor. PLOS ONE. 2015; 10: e0117607. doi: 10.1371/journal.pone.0117607 PMID: 25695797

24. Stricker S, Fundele R, Vortkamp A, Mundlos S. Role of Runx Genes in Chondrocyte Differentiation. Dev Biol. 2002; 245: 95–108. doi: 10.1006/dbio.2002.0640 PMID: 11969258

25. Joseph NS, Lourens A, Moran ET. The effects of suboptimal eggshell temperature during incubation on broiler chick quality, live performance, and further processing yield. Poult Sci. 2006; 85: 932–938. PMID: 16673774

26. Loyau T, Bedrani L, Berri C, Métayer-Coustard S, Praud C, Coustham V, et al. Cyclic variations in incubation conditions induce adaptive responses to later heat exposure in chickens: a review. animal. 2015; 9: 76–85. doi: 10.1017/S1751731114001931 PMID: 25118598

27. Piestun Y, Halevy O, Yahav S. Thermal manipulations of broiler embryos—The effect on thermoregulation and development during embryogenesis. Poult Sci. 2009; 88: 2677–2688. doi: 10.3382/ps.2009-00231 PMID: 19903968

28. Pinchuck S, Meiri N, Yahav S. The effect of thermal manipulation, during early embryogenesis, on angiogenesis and thermo-tolerance in broilers. Wageningen, The Netherlands; 2011. pp. 44–45.

29. Pruitt KD, Brown GR, Hiatt SM, Thibaud-Nissen F, Astashyn A, Ermolaeva O, et al. RefSeq: an update on mammalian reference sequences. Nucleic Acids Res. 2014; 42: D756–D763. doi: 10.1093/nar/ gkt1114 PMID: 24259432

30. Hernandez N. TBP, a universal eukaryotic transcription factor? Genes Dev. 1993; 7: 1291–1308. PMID: 8330735

31. Yamauchi J, Sugita A, Fujiwara M, Suzuki K, Matsumoto H, Yamazak i T, et al. Two Forms of Avian (Chicken) TATA-Binding Protein mRNA Generated by Alternative Polyadenylation. Biochem Biophys Res Commun. 1997; 234: 406–411. doi: 10.1006/bbrc.1997.6653 PMID: 9177284

32. Um M, Yamauch i J, Kato S, Manley JL. Heterozygous Disruption of the TATA-Binding Protein Gene in DT40 Cells Causes Reduced cdc25B Phosphatase Expression and Delayed Mitosis. Mol Cell Biol. 2001; 21: 2435–2448. doi: 10.1128/MCB.21.7.2435-2448.2001 PMID: 11259592

33. Herceg Z, Hulla W, Gell D, Cuenin C, Lleonart M, Jackson S, et al. Disruption of Trrap causes early embryonic lethality and defects in cell cycle progression. Nat Genet. 2001; 29: 206–211. doi: 10.1038/ ng725 PMID: 11544477

34. Cooper GM. The Cell: A Molecular Approach [Internet]. 2nd ed. Sunderland (MA): Sinauer Associates; 2000. Available: http://www.ncbi.nlm.nih.gov/books/NBK9961/.

35. Asakura A, Fujisawa-Sehara A, Komiya T, Nabeshima Y, Nabeshima Y. MyoD and myogenin act on the chicken myosin light-chain 1 gene as distinct transcriptional factors. Mol Cell Biol. 1993; 13: 7153– 7162. PMID: 8413304

36. Wang Q, Liu Y-P, Jiang X-S, Yang C-W, Du H-R, Qiu M-H, et al. [Correlation analysis of relationships between polymorphisms of high quality chicken myogenin gene and slaughter and meat quality traits]. Yi Chuan Hered Zhongguo Yi Chuan Xue Hui Bian Ji. 2007; 29: 1089–1098.

37. Seaberg B, Henslee G, Wang S, Paez-Colasante X, Landreth GE, Rimer M. Muscle-Derived Extracellular Signal-Regulated Kinases 1 and 2 Are Required for the Maintenance of Adult Myofibers and Their Neuromuscular Junctions. Mol Cell Biol. 2015; 35: 1238–1253. doi: 10.1128/MCB.01071-14 PMID: 25605336

38. Duchêne S, Audouin E, Crochet S, Duclos MJ, Dupont J, Tesseraud S. Involvement of the ERK1/2 MAPK pathway in insulin-induced S6K1 activation in avian cells. Domest Anim Endocrinol. 2008; 34: 63–73. PMID: 17166687

CHAPTER IV

miRNAs regulate acute transcriptional changes in broiler embryos in response to modification of incubation temperature

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Abstract

microRNAs are post-transcriptional regulators that play critical roles in diverse biological processes. We hypothesized that miRNAs may be involved in regulating transcriptome responses to changes in embryonic incubation temperature in chickens, a process that may have important implications for broiler meat production. Therefore, we conducted comparative transcriptome profiling of miRNAs to examine altered expression in breast and hind muscle of embryos and day 35 chickens experiencing high (38.8°C), control (37.8°C), or low (36.8°C) embryonic incubation temperature during embryonic day (ED) 7–10 or ED10–13. The results revealed differential expression of some miRNAs due to modification of embryonic incubation temperature in muscle type-specific and developmental stage-specific manners. The immediate effects of thermal change observed in embryos were considerable compared to long-term effects in chickens at day 35 post-hatch, which were subtle. Upregulation of miR-133 in breast muscle and downregulation of miR-199a-5p, miR-1915, and miR-638 in hind muscle post-ED7–10 high-temperature treatment were functionally associated with myogenesis and body size. The effects of ED10–13 low-temperature treatment were also observed in downregulation of let-7, miR-93, and miR-130c. Several differential miRNAs were functionally linked to nutrition metabolism, thermogenesis, and apoptosis. These results provide insight into the dynamics of miRNA expression at variable embryonic incubation temperatures and indicate a major regulatory role of miRNAs in acute responses to modified environmental conditions.

Keywords: chicken; incubation temperature; microRNA; expression; microarray; muscle

Introduction

Embryonic incubation temperature is a key factor for optimal physiological and developmental processes that may a have long-term influence on adult chickens. Incubation temperature profoundly influences physiological responses via alteration of biochemical reaction rates and protein structures as well as catalytic enzyme functions (Tattersall et al., 2012). Within a limited range, it is critical for broilers to optimize body temperature during pre- and post-hatch processes (Piestun et al., 2008).

Manipulation of incubation temperature during specific stages of development can result in immediate transcriptomic changes in embryos, although changing temperature beyond critical thresholds can be lethal. Previous studies demonstrated that high temperatures during embryonic day (ED) 7–10 positively associate with improvement of slaughter and breast muscle weights in male broilers, but do not influence meat quality (Werner and Wicke, 2008). Our previous experiments showed acute and long-term transcriptomic changes with temperature manipulation during muscle fiber formation. Also, thermal incubation treatments influence several biological functions and pathways depending on stage of muscle fiber development (Naraballobh et al., 2016). Hence, manipulation of embryonic incubation temperature may have implications in broiler meat production.

Several studies have indicated that temperature changes impact not only transcriptional changes, but also post-transcriptional regulation in diverse species. In aquatic ectotherms Atlantic cod (*Gadus morhua*), changing incubation temperature during the early somite stage can have significant long-term effects on microRNA (miRNA) activities in juvenile pituitary, gonad, and liver tissues (Bizuayehu et al., 2015). Another marine species, Senegalese sole (*Solea senegalensis*), induces dynamic expression of several miRNAs during early development at lower incubation temperatures (15°C) (Campos et al., 2014). After entering deep cold torpor, lined ground squirrels (*Ictidomys tridecemlineatus*) and little brown bats (*Myotis lucifugus*) have reduced miR-106b expression, which is associated with lower body temperature during hibernation and is involved in regulation of hypoxia inducible transcription factor-1α (HIF-1α) in animals' skeletal muscle and liver (Maistrovski et al., 2012). Further, heat stress alters expression of several miRNAs in primary cultured human small airway epithelial cells (Potla et al., 2015). Altogether, this evidence demonstrates that miRNAs have evolutionarily conserved roles in diverse biological processes, including temperature control.

miRNAs are conserved, non-coding RNAs of approximately 17–22 nucleotides in length that are involved in RNA silencing (cleavage) and post-transcriptional regulation in most, if not all, eukaryotes (Bartel, 2004). Biogenesis of miRNAs involves transcription as long primary transcripts (pri-miRNAs), which are processed to pre-miRNAs and then to mature miRNAs that are ultimately loaded selectively onto the RNA-inducing silencing complex (RISC) to become functional. miRNAs play important roles in numerous biological processes, such as developmental timing, cell death, and cell proliferation. Animal miRNAs partially or perfectly bind target sequences generally at the 3' untranslated regions (3'-UTR) of target genes (Kim et al., 2009). In general, an individual miRNA can regulate hundreds or thousands of target genes, and a single gene can be targeted by several miRNAs. These complex relationships pose a challenge to obtaining discrete results in miRNA studies.

We investigated potential miRNAs involved in regulation of transcriptome responses to modification of embryonic incubation temperature during early (ED7–10) or late (ED10–13) muscle fiber development of broiler-type chickens. In addition to traditional in silico target prediction, we complemented the assignment of miRNA–mRNA relationships and determination of functionally relevant miRNAs derived from this study by using correlation analyses between expression of differentially expressed miRNAs and previous mRNA expression data from the same samples. Potential target genes were further analyzed for biological functions and pathways using enrichment analysis of Ingenuity.

Materials and methods

Design and sample collection

We used hatching eggs from a commercial broiler line (Cobb-Vantress Inc., Siloam Springs, USA) and equally randomly assigned 1,001 hatching eggs to 6 experimental groups. All environmental conditions were comparable for all groups except experimental thermal profile. Incubation (machine) temperature was maintained at 37.8°C with 55% relative humidity (RH) until three days prior to hatch, at which time RH was adjusted to 65% until hatching. The experimental thermal profile was modified for groups 1–3 during early development (ED7–10) and for groups 4–6 during late muscle development (ED10–13) by adjusting temperature to 38.8°C (high temperature), 37.8°C (control), or 36.8°C (low temperature). The following experimental groups were

established: 1) 38.8°C, 65% RH, ED7–10 (H10); 2) 37.8°C, 55% RH, ED7–10 (C10); 3) 36.8°C, 55% RH, ED7–10 (L10); 4) 38.8°C, 65% RH, ED10–13 (H13); 5) 37.8°C, 55% RH, ED10–13 (C13); 6) 36.8°C, 55% RH, ED10–13 (L13).

For each group, breast muscle (*M. pectoralis*) and hind muscle (*M. gastrocnemius*) tissue samples were collected at the respective embryonic stages (ED10 or ED13) and at D35 post-hatch. Tissue samples were immediately dissected, snap frozen in liquid nitrogen, and stored at –80°C until use. Post-hatch chicks were fed a standard diet *ad libitum* until slaughter at D35. Zootechnical and biochemical traits were examined as previously described (Janisch et al., 2015). All animals were sexed, and 6–8 sex-balanced animals per experimental group at ED10, ED13, or D35 were used for expression analyses. Study design and sample collection procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the Department of Animal Sciences of the University of Goettingen, Germany and the Leibniz Institute for Farm Animal Biology and conducted according to the guidelines of the German Law of Animal Protection and the "EU Directive 2010/63/EU for animal experiments".

Small RNA isolation

Total RNA was isolated from individual samples (6 samples x 6 treatment groups x 2 muscle tissues at ED10 or ED13; 8 samples x 6 treatment groups x 2 muscle tissues at D35) using Tri-Reagent (Sigma-Aldrich, Taufkirchen, Germany), and the small RNA fraction was retained using miReasy and RNeasy MinElute Cleanup kits (Qiagen, Hilden, Germany) with an on-column DNase treatment according to the manufacturer's protocol. RNA integrity was assessed by an approximate 2:1 ratio of 28S and 18S rRNA bands on gel electrophoresis. Total RNA concentration was determined using a NanoDrop ND-1000 spectrophotometer (PEQLAB, Erlangen, Germany). Additionally, absence of trace DNA contamination was verified by PCR amplification of *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*) in RNA samples. All RNA samples were stored at −80°C until use.

microRNA expression

Small RNA fractions (200 ng) were used for sample preparation using a FlashTag BioTin RNA labeling kit (Affymetrix, Santa Clara, CA, USA). Fragmented biotin-labeled cRNAs were further hybridized for 16 hours to an Affymetrix GeneChip miRNA 3.0 Array containing 19,724 probe-sets designed from 153 species based on miRBase version 17. After staining and washing on an Affymetrix Fluidics Station 450, arrays were

scanned on an Affymetrix G3000 Gene Array Scanner. Raw data were pre-processed using Affymetrix GCOS 1.1.1 software.

Data processing and statistical analysis

Raw probe signal intensity was pre-processed and normalized using Perfect Matched and Detection Above Background features of Affymetrix Expression Consol software. Data were submitted to the MIAME-compliant database Gene Expression Omnibus (accession number: GSE83703-GSE83704) accessible via the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/geo). Differential expression of miRNAs was computed by analysis of variance (JMP Genomics, SAS-Institute, Cary, NC, USA). Independent calculation was performed for each tissue. Fixed effects of temperature, treatment period, and their interactions were modeled in statistical tests. For analysis in D35 samples, slaughter weight was used as covariance in the statistical model. Differentially expressed miRNAs were identified by comparing treatment groups and corresponding controls for ED7–10 or ED10–13. Significance threshold was set at $p \leq 0.05$, controlled for multi-hypothesis testing by False Discovery Rate (FDR) correction.

Functional miRNAs and potential target genes

To identify functional miRNAs and potential target genes, we integrated miRNA expression data from the present study and mRNA expression data of samples from our previous publication using correlation analysis and target prediction (Figure 1). Firstly, correlation analyses between signal intensities of differentially expressed miRNAs and all mRNA probe-sets were calculated. All significant negative correlations between miRNA–mRNA pairs were retained ($p \leq 0.05$; 5% FDR). Secondly, all differentially expressed miRNAs were scanned for potential target genes against all available chicken mRNA sequences in the NCBI database using Target Scan software (Agarwal et al., 2015). Predicted targets were further filtered using RNA Hybrid software (Rehmsmeier, 2004) with an energy threshold cut-off of ≤–25 kcal/mole. A "functional" miRNA was defined as a differentially expressed miRNA that negatively correlated with mRNA transcriptional level and was predicted as a binding site on the respective target gene candidates.

Pathway analysis

Differentially expressed miRNAs and their potential targets were mined for biological functions and gene regulatory networks using Ingenuity Pathway Analysis (Ingenuity Systems Inc., Redwood City, CA, USA). Statistical significance was determined based on Fisher's exact test ($p \leq 0.05$). Significant biological pathways for

each list of miRNAs and target genes were aggregated into new major categories based on shared GO subterms to simplify results, while maintaining a comprehensive view of biological processes.

Eight major biological functional groups were defined: (1) cell maintenance, proliferation, differentiation, and replacement; (2) organismal, organ, and tissue development; (3) nutrient metabolism; (4) genetic information and nucleic acid processing; (5) molecular transport; (6) cell signaling and interaction; (7) small molecule biochemistry; and (8) response to stimuli. Significant pathways were further considered "activated" or "deactivated" based on positive or negative Ingenuity Z-scores, respectively. Selected genes were used to generate regulatory networks based on best Z-score, *p*-value, and biological functions related to tissue development and myogenesis.

Result

Differentially expressed miRNAs

Although the Affymetrix GeneChip miRNA 3.0 array contains multiple mature miRNAs from diverse species that may resemble a chicken miRNA family, we treated each mature miRNA probe-set as an entity (feature) in statistical tests and then aggregated significant probe-sets into unique mature miRNAs. The number of differential probe-sets and mature miRNAs (unique miRNAs) for each tissue type, temperature treatment condition, and sampling stage are summarized in Table 1.

Several differentially expressed mature miRNAs were detected in almost all comparisons at a statistical threshold of $p < 0.05$ and FDR-adjusted $p < 0.18$, except for D35 breast muscle (Table 1). To include all miRNAs that may be biologically meaningful, we relaxed the threshold for D35 breast muscle to consider all significant tests with $p < 0.05$ and FDR-adjusted $p < 0.71$. Overall, hundreds of miRNAs were differentially expressed across comparisons of temperature treatments, tissue types, and sampling stages, with a comparable number of upregulated and downregulated miRNAs. These results suggest that dynamic miRNA changes may regulate transcriptional alterations due to modification of embryonic incubation temperature.

Functional miRNAs and potential target genes

Integrating miRNA expression data with previous gene expression profiles of matched samples (www.ncbi.nlm.nih.gov/geo; accession number: GSE76670) using correlation analysis revealed "functional" miRNAs with negatively correlated miRNA–

mRNA relationships. From our previous study, we found that increasing temperature from 37.8°C to 38.8°C during ED7–10 (H10) and decreasing temperature from 37.8°C to 36.8°C during ED10–13 (L13) resulted in considerable immediate transcriptomic changes (based on the abundance of differentially expressed genes) in embryos, while decreasing temperature during ED7–10 (L10) as well as ED10–13 (L13) showed large long-term effects in D35 chickens (Naraballobh et al., 2016). Therefore, we focused on these treatment conditions.

Numbers of miRNA–mRNA pairs, covering potential miRNA-targeted genes negatively correlated with miRNAs and also predicted as miRNA binding sites, and unique miRNAs are presented in Table 2. Overall, embryos had higher numbers of potential miRNA–mRNA relationships, ranging from 104 miRNA–mRNA pairs for L13 in hind muscle to 941 pairs for H10 in breast muscle, compared to D35 chickens (2 pairs for L13 in hind muscle and 19 pairs for L10 in breast muscle).

For H10, 421 unique genes were negatively correlated with 40 miRNAs and 200 unique genes were predicted as target candidates for 38 miRNAs in breast and hind muscles. Number of miRNA–mRNA pairs in breast muscle was higher than in hind muscle during the embryonic stage. A considerable number of miRNAs and potential targets were also identified in L13 in both muscle types. Only up to 10 miRNA–mRNA pairs were found among treatment conditions and muscle types at D35 (Table 2).

Differential miRNAs and potential target genes were further used to generate a hierarchical clustering based on expression level to demonstrate an overall negative correlation between miRNA and mRNA expression levels (Figure 2). Altogether, these results suggest that miRNAs may play an essential regulatory role on the immediate transcriptome response to modification of incubation temperature.

Pathway analysis

To functionally link miRNAs to the physiological effects of modification of embryonic incubation temperature, differentially expressed miRNAs and potential target genes were analyzed using Ingenuity and its Knowledge Base software. To simplify and comprehend the resulting complex biological pathways and key genes, we aggregated significant terms and pathways into “major functional categories” based on shared terms and keywords of those pathways. In addition, we used the Z-scores generated by Ingenuity algorithms, which can predict tendency of “pathway activation” or “pathway deactivation” defined by positive or negative scores, respectively. Pathways in significant

functional categories for each treatment condition at embryonic stages are summarized in Table 3 and Supplementary Tables S1 and S2.

In breast muscle of embryos, H10 showed activation of pathways related to cell maintenance and proliferation, organismal and tissue development, and nutrient metabolism, while L13 showed stimulated cell maintenance and proliferation, organismal and tissue development, genetic information and nucleic acid processing, cell signaling, and interaction and response to stimuli. Hind muscle of embryos showed deactivation of cell maintenance and proliferation, while L13 affected cell maintenance, proliferation, and differentiation pathways. Detailed information of pathway analyses can be found in Supplementary Tables S1 and S2. Functional miRNAs obtained from H10 and L13 that were highly associated with various target genes and that therefore were related to at least 4 out of 8 major categories are provided in Tables 4 and 5.

Regulatory networks predicting potential physiological effects

Representative miRNA–mRNA regulatory networks were modeled for H10 (Figure 3) and L13 (Figure 4) treatment in breast and hind muscles. The networks integrate miRNAs, potential target genes, and Ingenuity biofunctions and display an enrichment of miRNA–mRNA pairs related to major functional categories of cell maintenance, proliferation, and differentiation as well as tissue and organ development (Supplementary Tables S1 and S2).

Representative miRNA–mRNA regulatory networks demonstrate complex connectivity and relationships between the two molecular features. Sets of miRNAs target several genes that assemble into biological pathways and hence regulate these pathways. For example, miRNAs derived from H10 revealed activation of cytoskeletal organization and inhibition of cytoskeletal formation, demonstrating the overall fine tuning and balancing impact of miRNA post-transcriptional regulation (Figure 3A and 3C). We also observed activation of pathways involved in white blood cell quantity, vasculogenesis, and thermogenesis as well as stimulation of body size. Pathways involved in reduced organismal death and perinatal death are shown in Figure 3B and 3D.

Breast muscle that experienced reduced incubation temperature during ED10–13 showed stimulation of pathways related to proliferation, activity, formation, differentiation, and homeostasis of white blood cells. Regulated pathways included reduced growth of neurity (Figure 4A). In hind muscle, we identified pathways involved in activation of apoptosis and cell survival (Figure 4C). In breast muscle, we identified

pathways related to white blood cell quality and development of body trunk. We also identified deactivation of organismal death and bone size (Figure 4B) and, in hind muscle, inhibition of growth of connective tissue (Figure 4D). Additional information for all pathways derived from miRNA–mRNA relationships indicated from integrated data analysis is available in Supplementary Figures S1–S4.

Discussion

Accumulating evidence suggests that modification of embryonic incubation temperature can result in phenotype variations of D35 chickens, such as adaptation to environmental conditions like heat stress. We have previously reported that changing incubation temperature during embryonic myogenesis influences weight gain and meat quality of broilers (Janisch et al., 2015). Further, we have demonstrated that both increasing and decreasing incubation temperature (1°C from the control, 37.8°C) immediately affects transcriptome profiles of embryonic muscle and associates with transcriptional changes of muscle of D35 chickens, indicating potential long-term effects of embryonic incubation temperature (Naraballobh et al., 2016). This study now establishes posttranscriptional regulation by miRNAs in the above phenomenon.

Indeed, we found many differentially expressed miRNAs after thermal incubation treatments at the embryonic stage, compared to only a few differential miRNAs in D35 chickens. These results suggest that miRNAs play a major role in acute regulation of gene expression, especially in response to environmental circumstances during embryonic development, when thermoregulatory systems are not yet fully functional. It may also explain subtle changes of miRNAs and mRNAs in D35 chickens associated with embryonic thermal treatment. However, mechanisms other than miRNA regulatory networks seem to be more important for long-term transcriptional changes in response to *in-ovo* thermal treatment.

For functional analysis, we focused on two thermal treatment conditions: H10 (high temperature during ED7–10) and L13 (low temperature during ED10–13), based on differentially expressed target mRNAs from previous reports (Naraballobh et al., 2016). Because studies of miRNAs have relied on target prediction, software tools based on different algorithms could have varied results. We therefore combined miRNA–mRNA correlation analysis and target prediction approaches to identify and simultaneously validate functional miRNAs and their potential target genes. We combined two

approaches to predict potential miRNA target genes, Target Scan and RNA Hybrid. This combined approach is not only based on conservation of miRNA seed match, but also minimum free energy hybridization of RNAs. Further, we analyzed *in silico* biological functions of the identified differential miRNAs in regulatory contexts with their potential target genes using Ingenuity analysis software and Knowledge Base and further linked them to gene ontology (GO)-based functional categories and physiological effects using the Ingenuity Z-score approach.

H10: High temperature during ED7–10

Within each major category, networks linking target mRNAs, miRNAs, and biofunctions display the multi-connectivity of these elements (Figures 3 and 4 for major categories 1 and 2; Supplementary Figure S1–S4 for remaining major categories). While most knowledge about the functional role of miRNAs comes from studies of pathological conditions, in particular cancer, our results provide evidence for miRNAs' role in ontogenetic proliferation and differentiation processes. In fact, H10 treatment consistently shifts expression of miRNAs related to these cellular developmental processes at *in-ovo* stages. Moreover, H10 treatment promotes pathways related to organismal survival and carbohydrate metabolism. In particular, thermogenesis, which is involved in thermoregulation, is important in breast muscle but not hind muscle.

In breast muscle, two differential miRNAs, miR-138 and miR-3017a, targeted genes that were enriched for major category 2 and predicted for activating thermogenesis. Among those target genes was *CSPG4*, which is known to be involved in vasculogenesis. Interestingly, 12 miRNAs, including miR-133, miR-199, and miR-212, were associated with genes in major category 3 that are related to metabolism and synthesis of carbohydrates: upregulated *ADRB2*, *CX3CL1*, and *PPP1R3B*; and downregulated *CHPF* and *CHST3*. While *PPP1R3B* was found in liver and skeletal muscle tissues, it is also involved in regulating glycogen synthesis by forming a glycogen-targeting subunit for phosphatase PP1.

In hind muscle, differentially expressed miRNAs, including miR-199a-5p, miR-1915, and miR-638, were related to 14 genes in major category 2, which is associated with accumulated body size and reduced perinatal death, including *CUL4B*, *ITSN1*, *MLL5*, and *MYH11*. MYH11 is a smooth muscle myosin belonging to the myosin heavy chain family that shares features of ATP hydrolysis, actin binding, and potential for kinetic energy transduction. Alternative splicing of *MYH11* generates different isoforms

during muscle cell development that might indicate cancer (Sebestyen et al., 2015). *MYH11* was upregulated and correlated with miR-971 in breast muscle as well as miR-1915, miR-199a-5p, and miR-638 in hind muscle. Further, the results show that hydrolysis of carbohydrates mediated by genes such as *MTM1*, *NT5E*, *PLCB1*, and *MGLL*, which are related to nutrition metabolism in major category 3, are targeted by miRNAs shifted at H10 in hind muscle (miR-199a-5p, miR-212, and miR-222).

Interestingly, both tissues from the H10 group resulted in stimulation of major category 1, organization of cytoskeleton and cytoplasm, which is linked to several miRNA-targeted genes, including *CDKN1B*, *MTUS1*, *PIK3R1*, *PLXNB2*, and *SYK*. Especially *PIK3R1*, which is represented in multiple functional categories, was predicted to be a potential target of miR-739 in breast muscle and miR-2861, miR-3960, and miR-4592 in hind muscle. In addition, several target genes were associated with improved survival in major category 2 by deactivating organismal death and perinatal death in both breast and hind muscle. Overall, H10 treatment effects tended to favor improvement of body weight and organs compared to low temperature treatment, which is in line with previous observations (Krischek et al., 2016).

miR-133, known as a muscle-specific miRNA also called "myomiR," was upregulated in breast muscle along with miR-199a-5p, miR-1915, and miR-638, which were further selected to model miRNA–mRNA regulatory networks. miR-133, together with miR-1 and miR-206, expressed in cardiac and skeletal muscle can impact muscle proliferation, myotube formation, and differentiation (Koning et al., 2012; Wang, 2013). However, Chen et al. (2006) showed that upregulation of miR-133 was associated with myoblast proliferation but reduced cell differentiation. Regulation of miR-133 itself is initiated at the level of pri-miRNA processing (Ge and Chen, 2011). In chickens, miR-133a and miR-1a have been reported as stimulatory factors in late-stage development in response to myogenin (Wang et al., 2012).

miR-199a-5p is a member of the miR-199 family, which is involved in multiple roles, including stem cell differentiation, embryo development, and cardiomyocyte protection (Gu and Chan, 2012). Upregulation of miR-199a-5p is related to pathological processes of fibroblast proliferation (Lino Cardenas et al., 2013), whereas downregulation of miR-199a-5p involves control of angiogenic responses (Chan et al., 2012). Moreover, miR-199a-5p has a critical role in *WNT2*-mediated regulation of proliferation and differentiation processes in smooth muscle hypertrophy (Hashemi Gheinani et al., 2015).

Human miR-1915 is expressed in regulated adult renal progenitor cells prior to stemness and repair (Sallustio et al., 2013). Increasing miR-1915 expression improves differentiation from tubular cells into adipocyte-like and epithelial-like cells, while downregulation of miR-1915 could cause CD133 overexpression, which is an important marker of renal progenitors (Sallustio et al., 2013). *BCL2* is a miR-1915 target that shows a negative correlation and modulated multidrug resistance by increasing drug sensitivity in human colorectal carcinoma cells (Xu et al., 2013). Moreover, tumor suppressor p53, which is altered in numerous human cancers, induces expression of miR-1915 to target *BCL2* translation in response to DNA damage and stimulated apoptosis (Nakazawa et al., 2014), suggesting that downregulation of miR-1915 could reduce apoptotic cell death.

miR-638 regulates human vascular smooth muscle cell proliferation and migration to promote development of vascular pathologies. By targeting the NOR1/cyclin D pathway, miR-638 has been proposed as an alternative treatment for vascular proliferative diseases (Li et al., 2013). Recently, upregulated miR-638 has been associated with inhibited proliferation and promoted myeloid differentiation in acute myeloid leukemia cells by targeting cyclin-dependent kinase 2 (Lin et al., 2015).

Overall, for H10, shifts of miRNA expression mainly affects pathways related to cell survival, angiogenesis and vascularization and also more specific pathways of myogenesis.

L13: Low temperature during ED10–13

At the level of biofunctions, L13 treatment affects pathways related to cellular and organismal development via processes of proliferation, differentiation, and death. This is similar to H10 conditions; however, largely different miRNAs and target genes are shifted, indicating that alternative pathways are addressed to keep conditions close to homeostasis.

Compared to H10 treatment, L13 had less differentially expressed genes. Low temperature treatment during ED10–13 (i.e., formation of secondary fibers during myogenesis to which structural myoblasts attach core muscle) activated several miRNAs that in turn regulated major categories 1 and 2. In breast muscle, major category 1 showed potential activation of 23 biological functions, such as cellular activity of formation and engulfment. Formation of cells was influenced by upregulation of *RNF2* and *TCF3* and downregulation of *RUNX2*. These genes were targeted by miR-130c, miR-263a-star, and

miR-312-5p. Moreover, other differentially expressed genes, including *JAM3*, *PATZ1*, *PICK1*, *SOAT1*, and *SRF*, were also associated with these miRNAs.

It is interesting that differential miRNAs from L13 treatment related to predicted tendency of reduced bone size. Target genes *NOTCH2*, *HIVEP3*, *AMER1,* and *RUNX2* were regulated by downregulation of let-7, miR-93, and miR-130c.

Furthermore in breast muscle at L13 eight differentially expressed genes and 16 differential miRNAs, including let-7, miR-92, and miR-93, belonged to major category 3, with biofunctions of uptake of carbohydrate and D-glucose.

For hind muscle, major category 1 relates to initiation of cell death and apoptosis via multiple differentially expressed genes, including downregulated *ADAMTS20* and *OSBP2* and upregulated *PRPS1*, *RCAN2*, and *SRF*. Multiple miRNAs, including miR-132, miR-138, miR-222, miR-271, miR-383, miR-1245b-3p, miR-2137, and miR-3042, are well correlated with this functional category. *IRS1*, *RAPGEF1*, *RUNX2*, and *SRF* are targeted by miR-130c, miR-271, and miR-2137. Further, an activated biological function in major category 8 was cell movement of neutrophils, which relates to *CTSG*, *PRKCQ*, *SRF*, and *TSC1*. These target genes are targeted by miR-138, miR-222, miR-271, and miR-383.

We have reported that L13 treatment is associated with low body weight of embryos compared to high temperature treatment (Krischek et al., 2016). Bone and muscle are highly associated during developmental stages (Daly et al., 2004), so reducing bone size could also correlate with low body weight.

Let-7 has been a major topic of discussion for functional roles of miRNAs. Let-7 is among seven miRNAs that play major roles in reducing proliferation during differentiation (Wang, 2013). Let-7 family members are associated with aging in humans and downregulation of cell cycle control, such as cellular proliferation and differentiation pathways (Drummond et al., 2011). Moreover, during myogenesis, let-7 can suppress Dicer and HMGA2, which have roles in adipogenesis and mesenchymal differentiation (Dröge and Davey, 2008). Interestingly, long non-coding RNA H19, possessing multiple let-7 binding sites, is proposed to prohibit let-7 from binding to other targets (Shenoy and Blelloch, 2014).

miR-93 functions as a tumor suppressor in breast cancer cell lines by regulating proliferation and differentiation states (Liu et al., 2012). miR-93 can potentially downregulate *AKT3*, which reduces proliferation and facilitates differentiation of

myoblasts in skeletal muscle development (Wei et al., 2013). Moreover, upregulation of miR-93 can reduce hypoxia-induced apoptosis in both endothelial and skeletal muscle cells and then improve perfusion recovery from hind limb ischemia in vitro and in vivo (Hazarika et al., 2013).

miR-130c is related to thermal regulation in various species, especially in aquatic ectotherms. Previous studies showed that Atlantic cod (*Gadus morhua*) have less miR-130c transcripts during early somite formation at 9.5°C incubation temperature (Bizuayehu et al., 2015). Another marine species, Senegalese sole (*Solea senegalensis*) vigorously expresses several miRNAs, including miR-130c, during early development (20-somite stage) at lower incubation temperature (15°C) (Campos et al., 2014).

Conclusion

In the present study, we have demonstrated that modification of embryonic incubation temperature immediately affects miRNA expression profiles of breast and hind muscles of chicken embryos and is associated with altered expression of miRNAs in D35 chickens. An integration analysis of miRNA data and previous matched-sample mRNA data revealed functional miRNAs and enabled assembly of miRNA–mRNA regulatory networks related to biological pathways and potential physiological effects.

Differential miRNAs and targeted mRNAs showed treatment condition specificity depending on embryonic time (ED7–10 or ED10–13), tissue type, and stage of development. The large repertoire of miRNA–mRNA pairs that are shifted in various experimental groups but that finally fine-tune similar biofunctions reflect a large functional biodiversity and resilience.

This study reveals substantial immediate alterations of miRNAs due to experimental environmental conditions, whereas long-term miRNA responses were minor. This indicates a major regulatory role of miRNAs in acute responses to modified environmental conditions. Other, likely epigenetic, effects that have more long-term relevance remain to be analyzed.

Figure titles and Tables

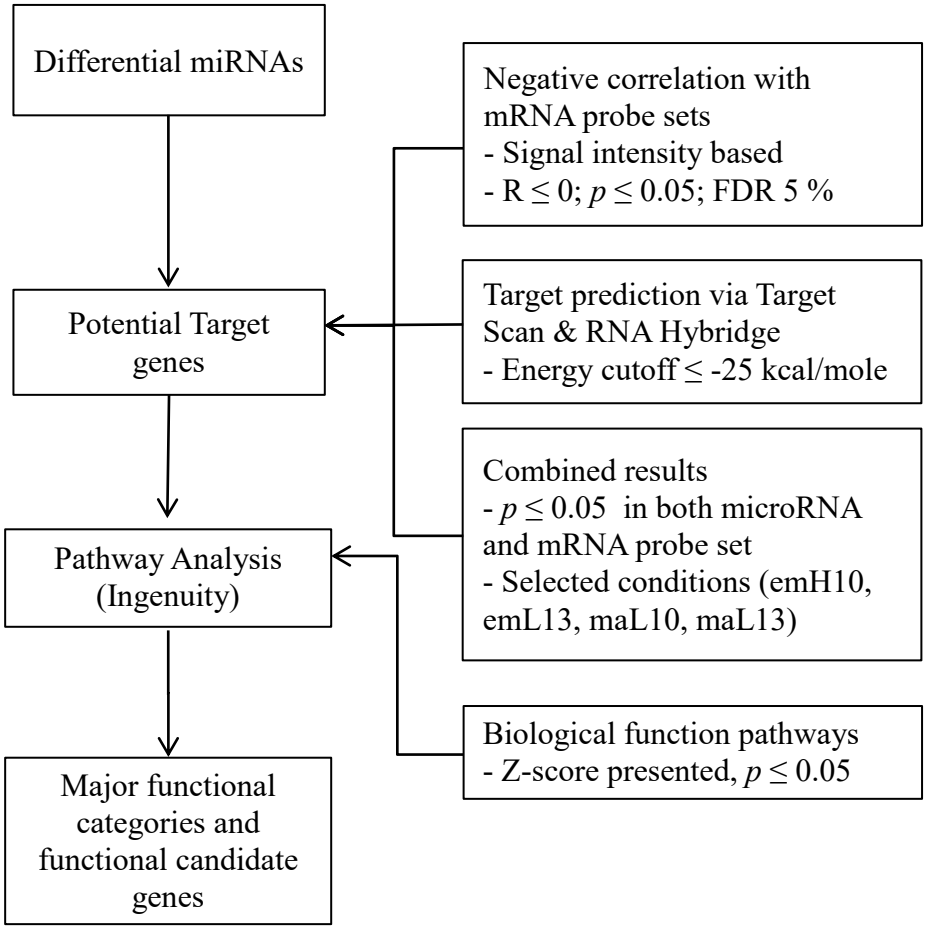

Figure 1 microRNA functional analysis pipeline

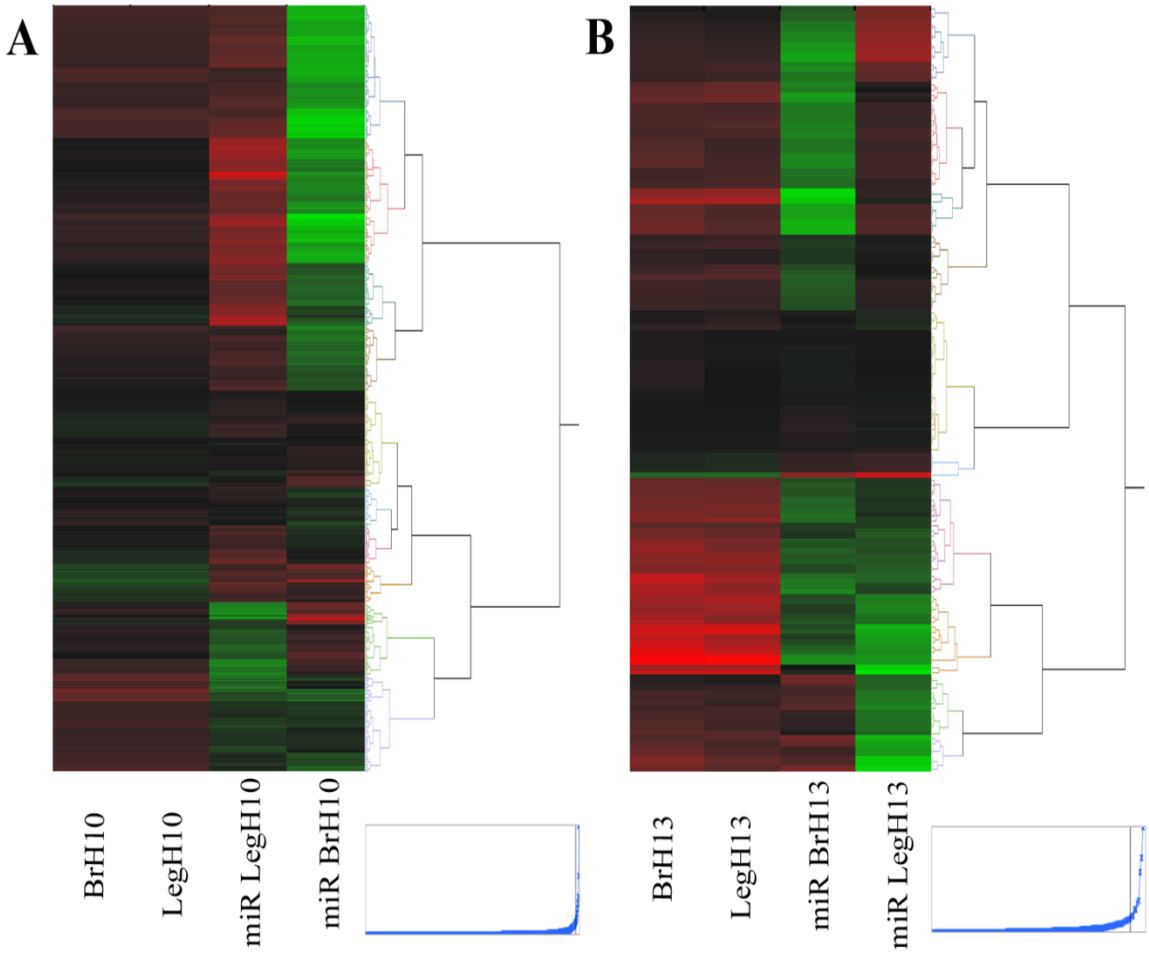

Figure 2. Expression-based (least-squares means) hierarchical clustering of differential miRNAs and potential mRNA targets derived from embryonic breast and hind muscle after H10 (A) or L13 (B) treatment.

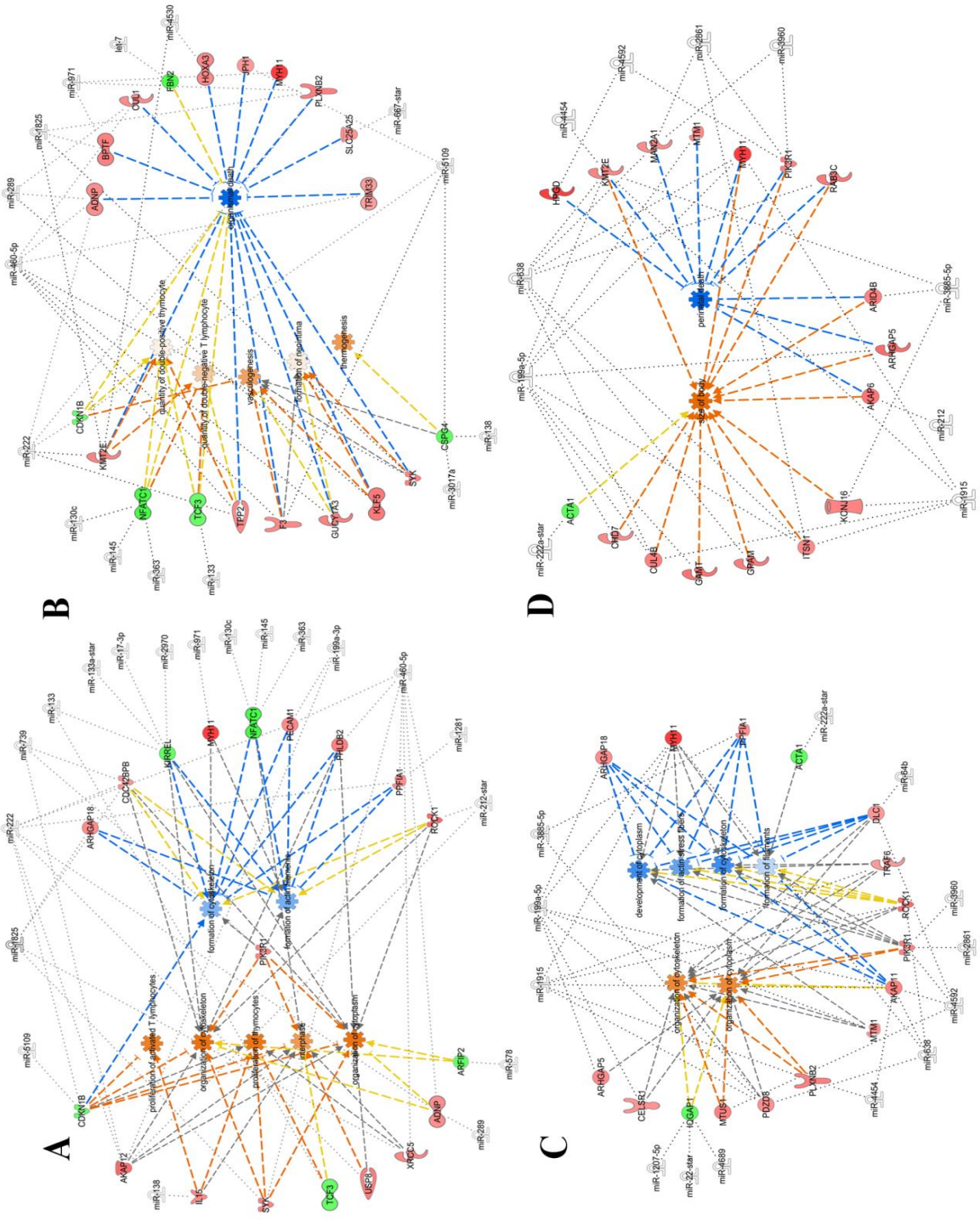

Figure 3. miRNA–mRNA regulatory networks. Representative gene regulatory networks derived from breast muscle (A and B) or hind muscle (C and D) of H10 group that are related to functional category group 1 (maintenance, proliferation, differentiation, and replacement of cells) (A and C) or group 2 (organ and tissue development) (B and D). Activated pathways are orange, while deactivated pathways are blue.

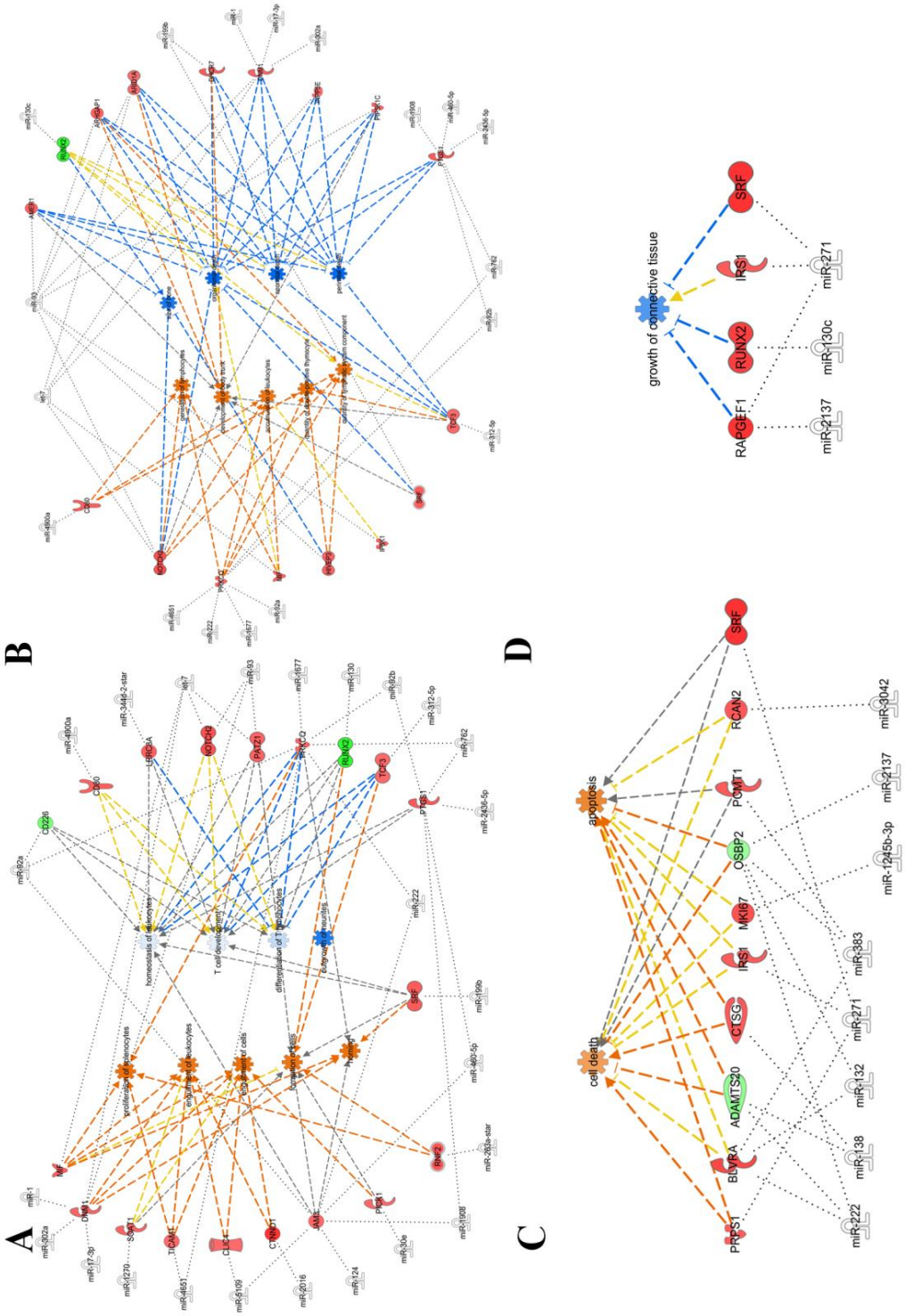

Figure 4. miRNA–mRNA regulatory networks. Representative gene regulatory networks derived from breast muscle (A and B) or hind muscle (C and D) of L13 group that are related to functional category group 1 (maintenance, proliferation, differentiation, and replacement of cells) (A and C) or group 2 (organ and tissue development) (B and D). Activated pathways are orange, while deactivated pathways are blue.

Table 1. Differentially expressed miRNAs ($p < 0.05$; FDR-adjusted $p < 0.18$)

	Treatment (ΔC)	Total[b] miRNAs (breast)	Unique[c] miRNAs (breast)	Regulation		Total[b] miRNAs (hind)	Unique[c] miRNAs (hind)	Regulation	
				Up	Down			Up	Down
Embryo	H10–C10	694	243	158	85	603	262	174	88
	H13–C13	380	160	71	88	362	211	123	88
	L10–C10	455	201	113	88	536	200	60	140
	L13–C13	733	316	204	112	401	224	90	134
D35[a]	H10–C10	148	107	74	33	98	88	31	57
	H13–C13	380	165	114	51	1328	419	176	243
	L10–C10	154	80	48	32	881	419	146	273
	L13–C13	165	85	69	16	1808	550	146	404

[a]Relaxed statistical significance threshold using $p < 0.05$ and FDR-adjusted $p < 0.71$ for breast muscle at D35 post-hatch.
[b]Number of probe-sets on the microarray (redundantly counting the same kind of miRNA from different species).
[c]Each kind of miRNA (unique sequences only counted once).

Table 2. Functional miRNAs and potential target genes for selected treatment conditions

Stage	Treatment	Tissue	miRNA-RNA pairs	mRNA targets	Unique miRNAs
Embryo	H10-C10	Breast	941	421	40
		Hind	444	200	38
	L13-C13	Breast	394	168	50
		Hind	104	49	25
D 35	L10-C10	Breast	19	10	1
		Hind	10	8	7
	L13-C13	Breast	7	6	5
		Hind	2	1	2

Table 3. Major categories of biological functions and ratios of numbers of Ingenuity biofunctions within each category with positive/negative Z-scores

Major categories	BrEmH10	LegEmH10	BrEmL13	LegEmL13
1—Cell maintenance, proliferation, differentiation, and replacement	8 : 2	2 : 4	23 : 4	2 : 0
2—Organismal, organ, and tissue development	5 : 1	1 : 1	11 : 4	1 : 0
3—Nutrient metabolism	4 : 0	2 : 0	2 : 0	
4—Genetic information and nucleic acid processing	2 : 0	2 : 0	5 : 0	
5—Molecular transport			2 : 0	
6—Cell signaling and interaction	1 : 1		4 : 0	
7—Small molecule biochemistry				
8—Response to stimuli			2 : 0	1 : 0

Table 4. Differential miRNAs targeting genes in significant pathways in embryonic breast and hind muscles affected by H10 treatment

Target	miRNA	*p*-value	FDR	Fold change (ΔC)	Regulation
Breast	miR-133	0.0484	0.169063	1.562238	Up
	miR-1825	0.0362	0.138553	1.836794	Down
	miR-199a-3p	0.0002	0.002251	1.466493	Down
	miR-212-star	0.0155	0.074994	2.272748	Up
	miR-222	0.0355	0.136636	1.410896	Up
	miR-289	0.0137	0.068449	1.634883	Up
	miR-4530	0.0476	0.167115	1.732465	Up
	miR-460-5p	0.0046	0.03024	1.845955	Down
	miR-5109	0.0364	0.138945	1.340839	Up
Hind	miR-1915	0.0262	0.122264	1.631923	Down
	miR-199a-5p	0.0368	0.153318	1.377803	Down
	miR-212	0.0138	0.078559	2.337939	Up
	miR-2861	0.0306	0.135907	1.682341	Down
	miR-3885-5p	0.0119	0.070698	1.723271	Down
	miR-3960	0.0133	0.076269	1.587999	Down
	miR-4454	0.0044	0.034992	1.609598	Down
	miR-4592	0.0003	0.004014	2.299092	Down
	miR-638	0.0054	0.040279	1.673629	Down

Table 5. Differential miRNAs targeting genes in significant pathways in embryonic breast and hind muscles affected by L13 treatment

Target	miRNA	*P*-value	FDR	Fold Change (ΔC)	Regulation
Breast	let-7	0.013	0.06576	1.8467	Down
	miR-130c	0.00001	0.00119	1.53561	Up
	miR-1677	0.0004	0.00476	2.01333	Up
	miR-17-3p	0.0158	0.07607	1.55538	Up
	miR-1908	0.0217	0.09616	1.85711	Down
	miR-199b	0.0004	0.00439	1.86844	Down
	miR-222	0.0017	0.01387	1.72226	Up
	miR-312-5p	0.048	0.16801	1.75657	Up
	miR-460-5p	0.0267	0.11173	1.59116	Down
	miR-4651	0.0198	0.08992	1.7233	Down
	miR-4900a	0.0058	0.0361	2.30027	Down
	miR-5109	0.0044	0.02921	1.51323	Down
	miR-762	0.0329	0.12961	1.56617	Down
	miR-92a	0.0015	0.01269	2.95471	Up
	miR-92b	0.0463	0.1638	1.59139	Down
	miR-93	0.0176	0.08216	1.55025	Down

Target	miRNA	*P*-value	FDR	Fold Change (ΔC)	Regulation
	miR-138	0.048	0.18149	1.38977	Up
	miR-2137	0.0047	0.03628	1.99394	Down
Hind	miR-222	0.0359	0.15105	1.44301	Up
	miR-271	0.0034	0.02838	2.74458	Down
	miR-383	0.001	0.01133	3.58192	Up

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Reference

Agarwal, V., Bell, G.W., Nam, J.-W., and Bartel, D.P. (2015). Predicting effective microRNA target sites in mammalian mRNAs. eLife **4**. doi:10.7554/eLife.05005

Bartel, D.P. (2004). MicroRNAs. Cell **116**: 281–297. doi:10.1016/S0092-8674(04)00045-5

Bizuayehu, T.T., Johansen, S.D., Puvanendran, V., Toften, H., and Babiak, I. (2015). Temperature during early development has long-term effects on microRNA expression in Atlantic cod. BMC Genomics **16**. doi:10.1186/s12864-015-1503-7

Campos, C., Sundaram, A.Y., Valente, L.M., Conceição, L.E., Engrola, S., and Fernandes, J.M. (2014). Thermal plasticity of the miRNA transcriptome during Senegalese sole development. BMC Genomics **15**: 525. doi:10.1186/1471-2164-15-525

Chan, Y.C., Roy, S., Huang, Y., Khanna, S., and Sen, C.K. (2012). The microRNA miR-199a-5p down-regulation switches on wound angiogenesis by derepressing the v-ets erythroblastosis virus E26 oncogene homolog 1-matrix metalloproteinase-1 pathway. J. Biol. Chem. **287**: 41032–41043. doi:10.1074/jbc.M112.413294

Chen, J.-F., Mandel, E.M., Thomson, J.M., Wu, Q., Callis, T.E., Hammond, S.M., Conlon, F.L., and Wang, D.-Z. (2006). The role of microRNA-1 and microRNA-133 in skeletal muscle proliferation and differentiation. Nat. Genet. **38**: 228–233. doi:10.1038/ng1725

Daly, R., Saxon, L., Turner, C., Robling, A., and Bass, S. (2004). The relationship between muscle size and bone geometry during growth and in response to exercise. Bone **34**: 281–287. doi:10.1016/j.bone.2003.11.009

Dröge, P., and Davey, C.A. (2008). Do cells let-7 determine stemness? Cell Stem Cell **2**: 8–9. doi:10.1016/j.stem.2007.12.003

Drummond, M.J., McCarthy, J.J., Sinha, M., Spratt, H.M., Volpi, E., Esser, K.A., and Rasmussen, B.B. (2011). Aging and microRNA expression in human skeletal muscle: A microarray and bioinformatics analysis. Physiol. Genomics **43**: 595–603. doi:10.1152/physiolgenomics.00148.2010

Ge, Y., and Chen, J. (2011). MicroRNAs in skeletal myogenesis. Cell Cycle **10**: 441–448. doi:10.4161/cc.10.3.14710

Gu, S., and Chan, W.-Y. (2012). Flexible and versatile as a chameleon—sophisticated functions of microRNA-199a. Int. J. Mol. Sci. **13**: 8449–8466. doi:10.3390/ijms13078449

Hashemi Gheinani, A., Burkhard, F.C., Rehrauer, H., Aquino Fournier, C., and Monastyrskaya, K. (2015). MicroRNA miR-199a-5p regulates smooth muscle cell proliferation and morphology by targeting WNT2 signaling pathway. J. Biol. Chem. **290**: 7067–7086. doi:10.1074/jbc.M114.618694

Hazarika, S., Farber, C.R., Dokun, A.O., Pitsillides, A.N., Wang, T., Lye, R.J., and Annex, B.H. (2013). MicroRNA-93 controls perfusion recovery after hindlimb ischemia by modulating expression of multiple genes in the cell cycle pathway. Circulation **127**: 1818–1828. doi:10.1161/CIRCULATIONAHA.112.000860

Janisch, S., Sharifi, A.R., Wicke, M., and Krischek, C. (2015). Changing the incubation temperature during embryonic myogenesis influences the weight performance and meat quality of male and female broilers. Poult. Sci. **94**: 2581–2588. doi:10.3382/ps/pev239

Kim, V.N., Han, J., and Siomi, M.C. (2009). Biogenesis of small RNAs in animals. Nat. Rev. Mol. Cell Biol. **10**: 126–139. doi:10.1038/nrm2632

Koning, M., Werker, P.M.N., van Luyn, M.J.A., Krenning, G., and Harmsen, M.C. (2012). A global downregulation of microRNAs occurs in human quiescent satellite cells during myogenesis. Differentiation **84**: 314–321. doi:10.1016/j.diff.2012.08.002

Krischek, C., Janisch, S., Naraballobh, W., Brunner, R., Wimmers, K., and Wicke, M. (2016). Altered incubation temperatures between embryonic days 7 and 13 influence the weights and the mitochondrial respiratory and enzyme activities in breast and leg muscles of broiler embryos. Mol. Reprod. Dev. **83**: 71–78. doi:10.1002/mrd.22596

Li, P., Liu, Y., Yi, B., Wang, G., You, X., Zhao, X., Summer, R., Qin, Y., and Sun, J. (2013). MicroRNA-638 is highly expressed in human vascular smooth muscle cells and inhibits PDGF-BB-induced cell proliferation and migration through targeting orphan nuclear receptor NOR1. Cardiovasc. Res. **99**: 185–193. doi:10.1093/cvr/cvt082

Lin, Y., Li, D., Liang, Q., Liu, S., Zuo, X., Li, L., Sun, X., Li, W., Guo, M., and Huang, Z. (2015). miR-638 regulates differentiation and proliferation in leukemic cells by targeting cyclin-dependent kinase 2. J. Biol. Chem. **290**: 1818–1828. doi:10.1074/jbc.M114.599191

Lino Cardenas, C.L., Henaoui, I.S., Courcot, E., Roderburg, C., Cauffiez, C., Aubert, S., Copin, M.-C., Wallaert, B., Glowacki, F., Dewaeles, E., Milosevic, J., Maurizio, J., Tedrow, J., Marcet, B., Lo-Guidice, J.-M., Kaminski, N., Barbry, P., Luedde, T., Perrais, M., Mari, B., and Pottier, N. (2013). miR-199a-5p is upregulated during fibrogenic response to tissue injury and mediates TGFbeta-induced lung fibroblast activation by targeting caveolin-1. PLoS Genet. **9**: e1003291. doi:10.1371/journal.pgen.1003291

Liu, S., Patel, S.H., Ginestier, C., Ibarra, I., Martin-Trevino, R., Bai, S., McDermott, S.P., Shang, L., Ke, J., Ou, S.J., Heath, A., Zhang, K.J., Korkaya, H., Clouthier, S.G., Charafe-Jauffret, E., Birnbaum, D., Hannon, G.J., and Wicha, M.S. (2012). MicroRNA93 regulates proliferation and differentiation of normal and malignant breast stem cells. PLoS Genet. **8**: e1002751. doi:10.1371/journal.pgen.1002751

Maistrovski, Y., Biggar, K.K., and Storey, K.B. (2012). HIF-1α regulation in mammalian hibernators: Role of non-coding RNA in HIF-1α control during torpor in ground squirrels and bats. J. Comp. Physiol. B **182**: 849–859. doi:10.1007/s00360-012-0662-y

Nakazawa, K., Dashzeveg, N., and Yoshida, K. (2014). Tumor suppressor p53 induces miR-1915 processing to inhibit Bcl-2 in the apoptotic response to DNA damage. FEBS J. **281**: 2937–2944. doi:10.1111/febs.12831

Naraballobh, W., Trakooljul, N., Muráni, E., Brunner, R., Krischek, C., Janisch, S., Wicke, M., Ponsuksili, S., and Wimmers, K. (2016). Immediate and long-term transcriptional response of hind muscle tissue to transient variation of incubation temperature in broilers. BMC Genomics **17**. doi:10.1186/s12864-016-2671-9

Piestun, Y., Shinder, D., Ruzal, M., Halevy, O., Brake, J., and Yahav, S. (2008). Thermal manipulations during broiler embryogenesis: Effect on the acquisition of thermotolerance. Poult. Sci. **87**: 1516–1525. doi:10.3382/ps.2008-00030

Potla, R., Singh, I.S., Atamas, S.P., and Hasday, J.D. (2015). Shifts in temperature within the physiologic range modify strand-specific expression of select human microRNAs. RNA **21**: 1261–1273. doi:10.1261/rna.049122.114

Rehmsmeier, M. (2004). Fast and effective prediction of microRNA/target duplexes. RNA **10**: 1507–1517. doi:10.1261/rna.5248604

Sallustio, F., Serino, G., Costantino, V., Curci, C., Cox, S.N., De Palma, G., and Schena, F.P. (2013). miR-1915 and miR-1225-5p regulate the expression of CD133, PAX2 and TLR2 in adult renal progenitor cells. PLoS ONE **8**: e68296. doi:10.1371/journal.pone.0068296

Sebestyen, E., Zawisza, M., and Eyras, E. (2015). Detection of recurrent alternative splicing switches in tumor samples reveals novel signatures of cancer. Nucleic Acids Res. **43**: 1345–1356. doi:10.1093/nar/gku1392

Shenoy, A., and Blelloch, R.H. (2014). Regulation of microRNA function in somatic stem cell proliferation and differentiation. Nat. Rev. Mol. Cell Biol. **15**: 565–576. doi:10.1038/nrm3854

Tattersall, G.J., Sinclair, B.J., Withers, P.C., Fields, P.A., Seebacher, F., Cooper, C.E., and Maloney, S.K. (2012). Coping with thermal challenges: Physiological adaptations to environmental temperatures, in: Terjung, R. (Ed.), Comprehensive Physiology. John Wiley & Sons, Inc., Hoboken, NJ, USA.

Wang, X.G., Yu, J.F., Zhang, Y., Gong, D.Q., and Gu, Z.L. (2012). Identification and characterization of microRNA from chicken adipose tissue and skeletal muscle. Poult. Sci. **91**: 139–149. doi:10.3382/ps.2011-01656

Wang, X.H. (2013). MicroRNA in myogenesis and muscle atrophy: Curr. Opin. Clin. Nutr. Metab. Care **16**: 258–266. doi:10.1097/MCO.0b013e32835f81b9

Wei, W., He, H.-B., Zhang, W.-Y., Zhang, H.-X., Bai, J.-B., Liu, H.-Z., Cao, J.-H., Chang, K.-C., Li, X.-Y., and Zhao, S.-H. (2013). miR-29 targets Akt3 to reduce proliferation and facilitate differentiation of myoblasts in skeletal muscle development. Cell Death Dis. **4**: e668. doi:10.1038/cddis.2013.184

Werner, C., and Wicke, M. (2008). Increasing the temperature at a certain period during the incubation of broiler eggs improves the carcass and breast yields without a negative impact on the meat quality. Arch. Anim. Breed. **51**: 11–16.

Xu, K., Liang, X., Cui, D., Wu, Y., Shi, W., and Liu, J. (2013). miR-1915 inhibits Bcl-2 to modulate multidrug resistance by increasing drug-sensitivity in human colorectal carcinoma cells. Mol. Carcinog. **52**: 70–78. doi:10.1002/mc.21832

CHAPTER V

General discussion

General discussion

Incubation temperature is considered as an important physical factor that initiates embryonic development in broilers. To maximize productivity and improve the quality of chicken, proper incubation temperature is controlled in a specific duration and intensity. Though myogenesis is well studied, the relation between developmental processes and transcriptomic changes are yet to be connected and this may partially explain phenotypic plasticity. Previous study showed that manipulated incubation temperature at 38.8 °C between ED7 to ED10 could influence breast muscle weight in male broilers (Werner and Wicke, 2008). However, several factors like muscle-type specific, ED time-point and variation of temperature might influence transcripts as well. To this end, we used functional genomics approaches such as microarray based gene expression profile and IPA knowledgebase to explore genes and pathways involved in the physiological responding to modification of embryonic incubation temperature in both immediate and long-term consequences.

Immediate and long-term transcriptional response of hind muscle tissue to transient variation of incubation temperature in broilers

First, our studies covered broadly analysis of transcript regulation in type I muscle (hind-limb muscle). The characteristic of this muscle type is slow twitch oxidative fiber with high myoglobin and mitochondria. Responding to activities made them having high endurance to fatigue with slow contraction times. Our results showed that manipulation temperature between ED7-ED10-ED13 could have immediate and long-term influence the transcriptomic response (Naraballobh et al., 2016a). In details, the immediate response of DEGs occurred at higher temperature (38.8 °C) between ED7-ED10 (H10) and at lower temperature (36.8 °C) between ED10-ED13 (L13). H10 condition activated pathways concerned in lipid metabolism, energy production and also activated oxidation and beta-oxidation of fatty acid as well. During early stage of our experiment, the junction between ending of primary muscle fiber and starting of secondary muscle could be sensitive to elevated temperature. As a result, selected DEGs like FABP1, PPARA and PPARGC1A were up-regulated. FABP1 facilitated fatty acid uptake, metabolism and intracellular transportation of lipids (Zhang et al., 2013). PPARA increased quality and quantity of peroxisomes which is a subcellular organelle containing enzymes for lipid

metabolism and respiration. PPARGC1 influenced mitochondrial biogenesis and external physiological stimuli as well as muscle fiber specialization and adaptive thermogenesis (Lin et al., 2002; Shu et al., 2014; Ueda et al., 2005). Another immediate effect, L13 condition suggested that lower temperature could cause increase cell survival and cellular proliferation. Up-regulation transcripts of L13 condition were related to glycolytic gene activities (GPI) (Rengaraj et al., 2012), fatty acid homeostasis (NR1H3) (Demeure et al., 2009), and expression pattern of striated muscle cell lineages (SRF) (Croissant et al., 1996).

In adult broiler, long-term response represented an impact on lowering temperature (36.8 °C) but not in higher temperature (38.8 °C) of hind-limb muscle. The chronic effect was hypothesized and confirmed either by transcript or phenotypic changes by several researcher including us (Loyau et al., 2015; Piestun et al., 2008; Piestun et al., 2009; Pinchuck et al., 2011). Early low temperature (L10) trends to activate the pathway concerned with organismal development and cell proliferation but strongly suppress transcriptional process which leads to the deactivation in gene expression. Moreover, down-regulation of HDAC4, MYOD1 and SOX6 are related to inactivation of transcription and muscle cell differentiation. HDAC4 with lower expression promoted myogenesis but only in cardiac muscle (Karamboulas et al., 2006). MYOD1 as a member of MRFs was necessary to skeletal muscle lineage, self-renewing and differentiating (Gerhart et al., 2007). SOX6 functions as a transcriptional suppressor of slow fiber specific genes, and play a major role in cartilage development and mesenchymal differentiation (Tagariello 2006; Hagiwara et al., 2007; von Hofsten et al., 2008). L13 has predicted activating effects on pathways in cellular and organismal development; including cell survival, development of body trunk, contractility of cardiac muscle and proliferation of mammary epithelial cells but inhibiting effect on pathways related to size of body and muscle cells pathways. The impact on nutrition metabolism, uptake and metabolism of lipids and carbohydrates together with small molecule biochemistry like oxidation of fatty acid tended to reduce body fat. Inflammatory response also decreased in multiple regions of body (Naraballobh et al., 2016a). Up-regulated of DEGs were presented by APOD, APOA1, DES and GFPT1. The APOD gene encodes a component of high-density lipoprotein and works together with APOA1 which promotes cholesterol efflux and is negatively correlated with aging and influences muscle development (Teltathum and Mekchay, 2009). DES encodes a muscle-specific class III intermediate filament and maintaines the structural integrity of highly solicited skeletal muscle like

contraction of muscle (Li, 1997; Paulin and Li, 2004; Otten et al., 2010). Moreover, desmin-positive and myosin-negative myoblasts were reported as proliferating cells (Yablonka-Reuveni and Nameroff, 1990). GFPT1 is involved in carbohydrate biosynthesis and apoptosis regulation. The results show that the specific pattern of gene regulation by temperature manipulation at ED7-10 and ED10-13 are distinguishable in stage of development.

Transient shifts of incubation temperature reveal immediate and long-term transcriptional response in chicken breast muscle underpinning resilience and phenotypic plasticity

Second, we also performed transcriptomic change association in type IIb muscle. Breast muscle has primarily anaerobic metabolism for short bursts activities then fatigue. Fast twitch glycolytic fiber are characteristic by low myoglobin content and mitochondria. The type IIb muscle type usually displays in white meat. Our experiment showed that similar treatments in manipulation temperature and treatment period could intensively influence transcriptomic change as observed in hind muscle study (Naraballobh et al., 2016b). Even though similar pattern of transcripts appeared, quantity and quality of DEGs indicate less severe effects in breast muscle compared to hind muscle. Moreover, significant pathways and networks relations demonstrated both independent and some similar regulations compared with type I muscle. H10ΔC activated pathways in cell maintenance and organismal development especially on survivability. Network relation includes tissue development, connective tissue and skeletal muscle system development. These functions are activated according to the transcriptomic changes. For example SMAD3 is signal transducers and transcriptional modulators in deposition of intramuscular fat (IMF) and inhibiting adipogenesis in white adipose tissue (WAT) (Ye et al., 2014). Down-regulated of SMAD3 reduced neuroepithelial cell transforming and several DEGs including JPH1. JPH1 is important for construction of skeletal muscle triad junction and muscle contraction (Takeshima et al., 2000;Komazaki et al., 2002). Up-regulated in triad biogenesis maintained proper development of muscle contraction and prevented lethality shortly after birth (Al-Qusairi and Laporte, 2011). L13ΔC had several UP regulation pathways in organismal survival and post-translational modification. Due to the network, cardiovascular system, skeletal-muscular and connective tissue development were impacted. Our result showed that among DEGs only FGF6 and RUNX2 were down-regulated transcripts. RUNX2 could directly stimulate

gene expression, post-translational modification and protein-protein interactions (T.-F. Li et al., 2004). Moreover, a prominent role of RUNX2 concerns joint fusions, osteoblastic differentiation, and skeletal morphogenesis in chicken embryos (Stricker et al., 2002). Down-regulated of RUNX2 could result in an interruption of chondrocyte differentiation, vascular invasion and severe shortening of the limbs (Stricker et al., 2002).

Adult breast muscle at full development stage showed only very subtle transcriptional change in elevated temperature groups. Higher temperature treatment on ED10-13, it tended to deactivate size of body pathway and downward nutrition metabolism. L10ΔC activated pathway of ion homeostasis but deactivated transcriptional processes and inflammation. DOWN regulated networks included gene expression, cellular function, and cell signaling. DEGs like MED24, TBP and TRRAP were found with negative effects in transcriptional process regulation. As transcriptional coactivator complex, down regulated of MED24 and other complex subunit members degraded RNA polymerase II activity and lead to abnormal on transcript/translation process. TBP helps positioning RNA polymerase II at TATA box and delays mitosis if downregulation occurred (Um et al., 2001). TRRAP has a vital role for early development exclusively for mitotic checkpoint and regular cell cycle progression (Herceg et al., 2001). Hence, downward incubation of TRRAP could cause glioblastoma multiforme (GBM) and other cancer cells (Wurdak et al., 2010). Our experiment showed that L13ΔC treatment tended to activate transcripts in many categories including cellular and organ development, organismal development, nutrition metabolism and inflammatory response. Network connection concerned an activation of organ, embryonic and skeletal-muscular system development which were displayed by myosin-myogenin group (MYH2, MYL3, MYOG) and down-regulation of RAF1. Cholinergic receptor group in chicken showed that muscle receptor delta-subunit gene upstream of CHRND was activated during myotube formation (Wang et al., 1990) and CHRNG motif was localized then attached MyoD and myogenin fusion proteins (Jia et al., 1992). The myosin and myogenin complex, not only facilitated skeletal muscle contraction but the complex is also an important complement with actin filaments during eukaryotic motility processes (Cooper, 2000). MYOG was one of the molecular genetic markers to muscle fiber traits specification and mutation in chicken (Wang et al., 2007). RAF1 is a MAP3K, a higher order kinase affecting the ERK-pathway via MEK1 and MEK2. The serine/threonine specific protein kinases, ERK1 and ERK2 are involved in the control of gene expression and cellular formation. Latest study was found that the ERK1/2 pathways are essential for the maintenance adult muscle fibers and

protein synthesis pathways particularly in myoblast cells in mice and chicken respectively (Seaberg et al., 2015; Duchêne et al., 2008)

MiRNAs regulates dynamic transcriptional changes of broiler embryos in response to modification of the incubation temperature

Third, post-transcriptional response via miRNA is our relevant topic when addressing response to variable incubation temperature. We hypothesized about transcriptional regulation which could apprehend multiple processes with distinction at various time-points. Our results show significant high expression of target genes in embryonic stage more than in adult stages (Naraballobh et al., 2016c). This evidence confirmed miRNA expression which could be dominant express to a fixed period (Kang et al., 2013; (Xu et al., 2006). Various target genes were found at early high temperature (H10) and late low temperature (L13) treatment in both tissues. While mRNA-miRNA pairs were less abundance in L13 condition, contrast signals were noticeable in type II muscle. This evidence could support muscle specific regulation via miRNAs. The assumptions regarding biological functions of miRNA regulation are related to target gene activities. During H10 treatment, the activation of major categories gr.2 and gr.3 was an example of pathways specifically regulated in either muscle as thermogenesis and carbohydrate synthesis in breast muscle and size of body and hydrolysis of carbohydrate in hind muscle, respectively. Co-expression in both tissues was found for the activation of major category gr. 1 (organization of cytoskeleton and cytoplasm) and deactivation of major category gr.2 (organismal death and perinatal death). Accordingly, selection of related miRNA sets which regulated pathways like miR-133 (members of myomiRs), miR-199a-5p, miR-1915 and miR-638 were queried. Up-regulation of miR-133 was associated with myoblast proliferation but reduce cell differentiation (Chen et al., 2006; Koning et al., 2012). In chicken, miR-133a and miR-1a was regulated in late stages development and strongly response to myogenin (Wang et al., 2012). The biological function of miR-199a-5p showed multiple regulations on embryo development, cardiac myocytes and stem cell differentiation e.g. WNT2-mediated regulation of proliferative and differentiation processes in smooth muscle hypertrophy (Gu and Chan, 2012; Hashemi Gheinani et al., 2015). Down-regulation of miR-199a-5p controlled angiogenic responses (Chan et al., 2012). miR-1915 regulates adult renal progenitor cells prior to stemness and repair (Sallustio et al., 2013). Down-regulation of miR-1915 could be used as molecular markers of renal progenitors (Sallustio et al., 2013) and could be a factor for

reduction of apoptotic cell (Nakazawa et al., 2014). miR-638 was regulated in human vascular smooth muscle cells proliferation and migration which promoted vascular pathologies. The up-regulation of miR-638 could inhibit proliferation of myeloid differentiation in acute myeloid leukemia cells (Lin et al., 2015).

Another high impact treatment during embryonic stage was L13 condition. In breast muscle, activated major categories gr.1 (cellular activity of formation and engulfment), gr.2 (prevent apoptosis mechanism) and gr.3 (uptake of carbohydrate and D-glucose) were evident but deactivation of size of bone. In hind muscle, initiation of cell death and apoptosis in major categories (gr.1), degradation in growth of connective tissue (gr. 2), and stimulation in cell movement of neutrophils (gr.8) were unique regulations. Consequently, downward of bone size could be reason for interfered myogenesis. miRNA sets like let-7, miR-93 and miR-130c were potentially associated. let-7 led to reproducible reduction in proliferation (Wang, 2013). Moreover, let-7 suppressed Dicer and HMGA2 which have a role in adipogenesis and mesenchymal differentiation (Dröge and Davey, 2008). miR-93 served as a tumor suppressor in breast cancer cell lines (Liu et al., 2012). Down-regulation of miR-93 potentially promoted multiple stem cell regulatory genes like AKT3 and stimulated aortic vascular smooth muscle and cell tumorigenesis (Liu et al., 2012;Wei et al., 2013). miR-130c was related to thermal plasticity as shown in aquatic ectotherms (Campos et al., 2014; Bizuayehu et al., 2015). Up-regulation of miR-130c regulated MAPK and mTOR pathways which happened during early development at lower incubation temperature of Senegalese sole (Campos et al., 2014). The pathways are related to an inhibition of cell proliferation but stimulated differentiation process.

Future perspective

Multiple pathway regulations demonstrated that external conditions had large effects on transcript regulation. Even though microarrays are designed for high throughput result with high reliability and accuracy, the validation process is still needed. We performed confirmation analyses by real-time polymerase chain reactions (qPCR) via LightCycler480 system (Roche, Mannheim, Germany). The amplification was done according to manufacturer's instructions. The Pearson correlation between microarray and qPCR was calculated by $2^{-\Delta\Delta Ct}$ methods and normalization factors (Livak and Schmittgen, 2001). GAPDH and ACTB were used as common house-keeping genes for the data normalization. The validation process performed on randomly selected potential candidate gene. For example, FGA (Fibrinogen Alpha Chain), NR4A3 (Nuclear Receptor

Subfamily 4, Group A, Member 3), AHSG (Alpha-2-HS-glycoprotein) were selected as being involved in many pathways. Consistency of microarrays and qPCR was demonstrated. The Pearson correlation coefficients (r) were highly significant between 0.71 and 0.84. Generally, the qPCR analyses indicated a highly reproducible analysis.

To detech differentially expressed mRNA and miRNA, NGS (Next Generation Sequencing notably high-throughput sequencing) technology could serve as an alternative approval. NGS is a new DNA sequencing technology with robust and highest parallel sequencing. NGS introduced to whole-genomic solution and to coverage over common to rare read (Koboldt et al., 2013). NGS technology integrated to RNA-seq (RNA sequencing is also known as whole transcriptome shotgun sequencing (WTSS)) reveals the existence and the amounts of RNA and small RNA. Previous studies showed a high correlation between RNA-Seq and microarray technologies in transcriptome profiling (Kuhn et al., 2008). However, hybridization-based microarray might be problematic with cross-hybridization results, extremely expressed genes and genetic variants. RNA-Seq is a beneficial solution among these problems especially for miRNA validation (Kuhn et al., 2008). An option to validate miRNA targets are pull-down assays for miRNA-processing proteins that are identifying mRNAs which bound to specific proteins (van Rooij, 2011). The knockdown method by forcing an over-expression of specific miRNA also applies for the validation process, but it needs attention on off-target effects. Vice versa, the complementary method seem more beneficial by inhibiting selected mature miRNA function by using antisense oligoribonucleotides (ASO) (Kuhn et al., 2008). Whereas multiple methods could be used for transcriptomic profiling and validation processes, Microarrays are still a convenient choice for the majority of research studies in liability and cost efficiency.

Altogether, our studies showed that several transcripts and its regulations occurred due to manipulation of incubation temperature. The transcriptomic change appeared immediately after treatment and with long-term effects (no evidence via miRNA in adult stage). Our experiment showed that multiple factors including muscle-type, intensity of temperature changes and period of treatment time affected myogenesis and related pathways specifically. The phenotypic result demonstrated that phenotypic plasticity occurred at embryonic and adult stage. Phenotypic measurement at embryo stage showed that for H10 treatment heart, liver and body weights were higher than for L10 but not significant different from control. At later treatment in L13 body weight was significantly lower than in C13 and in H13 while liver weight was lower in L13. Long-term effect

were obvious at D35 for carcass, breast and hind muscle weights. The result showed that at D35, carcass and hind weights from L10 and L13 treatment were significantly lower than at higher temperature (H10 and H13) but there is no difference from control (C10 and C13). The breast muscle from L10 and L13 treatments were slightly lighter than H10 and H13 without any significant. The evidence on phenotypic plasticity supported transcriptomic change with possibility of post-transcriptional regulation. While incubation eggs were manipulated, multiple gene expressions were adjusted to maintain survivability. The consequence of treatment showed affected on major categories gr.1, 2 and 3. Our result demonstrated that during ED7-10 higher temperature treatment potentially influences multiple genes and pathways which regulated myogenesis. Both muscle types immediately response to stimulation but only type I muscle had phenotypic plasticity in adult. Activation of fatty acid uptake and metabolism associated with cell signaling and muscle development. Whereas lower temperature during ED10-13 influence prolongation of programmed cell death pathway. Both treatments showed zero difference on hatching and mortality rate. miRNA regulation in both muscles was concerned with the major categories 1 and 2. Epigenetic regulation on myomiRs like miR-133 was presented and associated with myoblast proliferation. Even though adult transcript activities were considerable in L10 and L13 treatment, phenotypic changes showed merely downward and non-significant differences in muscle type I and II respectively. Biological pathways and DEGs revealed that L10 treatment interfered gene expression process. Treatment on L13 has positively influenced pathways in cellular and organismal development, contractility of cardiac muscle but negative effects on size of body and muscle cells pathways. The post-transcriptional regulation was not found interfering enough during mature stage. Our study presents potentially pathways and transcript regulations due to temperature manipulation at specific periods. It supports previous study from Werner and Wicke, 2008 which is an initial elevated incubation temperature positively influenced phenotypic change with an impact on abundance of transcript and gene regulations. The selection of DEGs needs to be verified and further investigated in combination with more detailed phenotypic like for example histological. The optimized temperature at 38.8 °C between ED7-10 could positively influence phenotype but not with lower temperature treatment. Together with other environmental parameters like proper diet, transcriptomic profiling, and epigenomic studies can improve broiler husbandry and achieve high-quality broilers to support the global demand.

CHAPTER VI

Summary

6 Summary

The major aim of the study described in this thesis is to understand the molecular basis and to identify potential pathways and transcriptomic changes due to transient incubation temperature variation. Acute and long-term gene expressions were interrogated in both, muscle type I (hind-limb) and type II (breast). Based on transcriptomic changes, both types of muscles showed that at embryonic sampling stage, the modification of higher incubation temperature 38.8 °C at ED7-10 (H10) and lower incubation temperature 36.8 °C at ED10-13 (L13) are most effective conditions to cause immediate transcriptomic changes. For the adult sampling stage of D35-posthatch, lower temperature in both times points (36.8 °C at ED7-10 and ED10-13; L10 and L13) associated with most transcriptomic changes. The effects of thermal manipulation during embryonic development on miRNAs, a class of post-transcriptional regulators were also investigated. The expression profile of miRNAs was profoundly affected by the modification of incubation temperature at the embryonic sampling stage but only mild at the adult stage (D35 post-hatch) indicating immediate response of the miRNAs to environmental (temperature) changes of the embryos. In addition, the phenotypic record also showed that the high embryonic incubation temperature had an influence on higher body weight (embryo and D35 stage), carcass, and hind muscle weight (D35 stage) comparing to lower temperature ($p<0.05$) relative to control groups.

Pathway analysis showed an acute response of the H10 condition in both muscle tissue types in which elevated mRNA transcripts were enriched for functional categories of cell maintenance, organismal development, and survival ability (FABP1, PPARA, and PPARGC1A in hind muscle and SMAD3 in breast muscle). The results demonstrated an up-regulation of miR-133 in breast muscle and down-regulation of miR-199a-5p, miR-1915 and miR-638 in hind muscle that positively influence myogenesis and size of body, respectively. Acute response from L13 condition was predicted for reduced programmed cell death and possibly gained mass of skeletal muscle (GPI, NR1H3, and SRF) in hind-limb muscle while accelerated cardiovascular system, skeletal-muscular, and connective tissue development via RUNX2 in breast. Moreover, down-regulated expression of let-7, miR-93 and miR-130c was predicted to associate with diminished size of bone in type II muscle.

Long-term effects of incubation temperature showed subtle transcriptomic changes. At D35 post-hatch stage, L10 condition influenced cellular functions, organismal development (HDAC4, MYOD1, and SOX6 in hind muscle), gene expression and cell signaling (RNA polymerase II apparatus like MED24, TBP, and TRRAP in breast muscle). Interestingly, the L13 treatment condition was functionally predicted to influence activated nutrition metabolism and improved survival chance (APOD, APOA1, DES, and GFPT1 in hind muscle). Besides, activation of organ, embryonic and skeletal-muscular system development were significant pathways with RAF1 and actin being hub genes in breast muscle.

In summary, the studies described in this thesis used genomic approaches to identify sets of functional candidate genes, microRNAs and pathways that might influence myogenesis and animal development via the modification of embryonic incubation temperature. The results demonstrated several putative candidate genes, post-transcriptional regulation, and pathways, which are involved in cellular, organismal, and nutrition development in both, embryonic and D35 stage. The transcriptomic response reflected animal adaptability and phenotypic plasticity in response to treatment conditions. The regulation mechanisms involved in the modification of incubation temperature suggests an epigenetic adaptation, which may work along with metabolic mechanisms in controlling metabolic homeostasis in the long-term and improve poultry resistance to environmental changes without much effect on growth performance. The present results of the modification of embryonic incubation temperature may have an impact on field application on adaptation, phenotypic plasticity, and animal husbandry to improve the broiler production.

Zusammenfassung

Das wesentliche Ziel dieser Arbeit war die Aufklärung der molekularen Grundlagen und die Identifizierung von transkriptionellen Änderungen und Signalwegen die zur Adaptation an die vorrübergehende Variation der Bruttemperatur beitragen. Dafür wurden kurz- und langfristige Veränderungen in Proben der Oberschenkel- und Brustmuskulatur als repräsentative Gewebe mit vorrangigem Auftreten von Typ I bzw. Typ II Muskelfasern untersucht. Zum embryonalen Probenzeitpunkt konnte für beide Muskeltypen konnte gezeigten werden, dass sowohl die Erhöhung der Inkubationstemperatur an ED7-10 (H10) auf 38,8°C als auch die Temperaturerniedrigung an ED10-13 (L13) auf 36,8°C unmittelbare Veränderungen auf Transkriptomebene induziert. Dagegen bewirkte die Verringerung der Temperatur (36,8°C an ED7-10 und ED10-13; L10 und L13) die deutlichsten transkriptionellen Auslenkungen zum adulten Probezeitpunkt (35. Lebenstag). Zusätzlich wurden die Auswirkungen der Temperaturveränderungen während der Embryonalentwicklung auf Ebene der Expression von miRNAs, als potentielle post-transkriptionale Regulatoren, untersucht. Zum embryonalen Probezeitpunkt zeigten sich deutliche Unterschiede in den miRNA-Expressionsprofilen zwischen den Behandlungsgruppen. Dagegen waren im adulten Stadium (35. Lebenstag) nur geringfügige Unterschiede nachweisbar. Dies spricht für eine unmittelbare Initiation von Adaptationsprozessen auf Umweltreize (z.B. Temperatur) im Embryo. Zusätzlich zeigten die untersuchten phänotypischen Parameter, dass die Erhöhung der Bruttemperatur, im Vergleich zu den anderen getesteten Brutbedingungen, mit erhöhtem Gewicht (embryonal) einherging und sich auf die Masse von Oberschenkel- und Brustmuskel (embryonal und adult) auswirkte. Durch die Integration der Daten mit Hilfe von Signalweganalysen konnte gezeigt werden, dass in den H10 Individuen in beiden untersuchten Muskelgewebstypen funktionelle Kategorien mit Bezug zur Zellerhaltung, Organismusentwicklung und Überlebensfähigkeit angereichert waren (FABP1, PPARA und PPARGC1A im Oberschenkel und SMAD3 im Brustmuskel). Diese Signalwege repräsentieren molekulare Pfade mit potentiellen langfristigen Effekten auf den Phänotyp. Weiterhin zeigten die Ergebnisse eine Heraufregulation von miR-133 im Brustmuskel und die Herabregulationen von miR-199a-5p, miR-1915, miR-638 im Oberschenkel mit positivem Einfluss auf funktionelle Signalwege der Myogenese bzw. des Körperbaus. Unmittelbare transkriptionelle Antworten, induziert durch L13

Bedingungen, zeigten Anzeichen für verminderten programmierten Zelltod und eine erhöhte Masse an Skelettmuskulatur (GPI, NR1H3 und SRF) im Oberschenkel, während molekulare Pfade des Blutflusses sowie der Skelettmuskel- und Bindegewebsentwicklung im Brustmuskel überrepräsentiert waren (z.B. RUNX2). Des Weiteren war die Herabregulation von let-7, miR-93 und miR-130c im Brustmuskel assoziiert mit quantitativen Parametern der Knochengröße und der Typ-II-Muskulatur.

Hinsichtlich der langfristigen Auswirkungen der veränderten Inkubationstemperatur zeigten sich feine transkriptionelle Änderungen. Am 35. Lebenstag mündeten die Effekte der L10 Behandlung in der Beeinflussung zellulärer Funktionen, der Organismusentwicklung (HDAC4, MYOD1 und SOX6 im Oberschenkelmuskel) sowie der Genexpression und der zellulären Signaltransduktion (RNA-Polymerase II Komplex wie z.B. MED24, TBP, TRRAP im Brustmuskel). Funktionelle Vorhersagen, basierend auf den Expressionsdaten der L13 Behandlungsgruppe, deuteten auf einen beeinflussten Ernährungsstoffwechsel und verbesserte Muskelentwicklung hin (APOD, APOA1, DES und GFPT1 im Oberschenkelmuskel). Daneben zeigten sich auch signifikante Effekte auf die Entwicklung des Embryos und des Skelettmuskelsystems mit RAF1 und Aktin als zentrale Gene im Brustmuskel.

Im Rahmen dieser Arbeit wurden genombiologische Ansätze verfolgt, um funktionale Kandidatengene, miRNAs und Signalwege mit potentiellen Effekten auf Myogenese und Tierentwicklung zu identifizieren, die durch Modifikation der embryonalen Umwelt induziert werden. Die identifizierten molekularen Features und post-transkriptionellen Regulatoren zeigten sich sowohl embryonal als auch im adulten Stadium an der zellulären, organismischen und nutritiven Entwicklung beteiligt. Zudem reflektierten die transkriptionellen Auslenkungen die Anpassungsfähigkeit und phänotypische Plastizität als Antwort auf die embryonale Veränderung der Haltungs-Umwelt. Die beteiligten Regulationsmechanismen implizieren epigenetische Modifikationen, die im Zusammenspiel mit metabolischen Mechanismen an der langfristigen Aufrechterhaltung des metabolischen Gleichgewichts beteiligt sind und die Resistenzen des Geflügels gegenüber Umweltveränderungen verbessern ohne das Wachstum wesentlich zu beeinflussen. Damit liefern die vorliegenden Ergebnisse wesentliche Erkenntnisse zur Anpassungsfähigkeit und phänotypischen Plastizität von Broilern und bieten Möglichkeiten zur Verbesserung von Produktivität und Robustheit.

CHAPTER VII

Reference

References

Affymetrix. 2016a. ‘miRNA 3.0 Array’. http://www.affymetrix.com/support/technical/datasheets/mirna_3_datasheet.pdf.

———. 2016b. ‘Model & Applied Research Organisms Gene 1.0 ST Arrays’. http://www.affymetrix.com/support/technical/datasheets/model_org_gene_1_0_st_array_datasheet.pdf.

Almeida, Maria I., Rui M. Reis, and George A. Calin. 2011. ‘MicroRNA History: Discovery, Recent Applications, and next Frontiers’. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis* 717 (1–2): 1–8. doi:10.1016/j.mrfmmm.2011.03.009.

Al-Qusairi, Lama, and Jocelyn Laporte. 2011. ‘T-Tubule Biogenesis and Triad Formation in Skeletal Muscle and Implication in Human Diseases’. *Skeletal Muscle* 1 (1): 26. doi:10.1186/2044-5040-1-26.

Barri, A., C. F. Honaker, J. R. Sottosanti, R. M. Hulet, and A. P. McElroy. 2011. ‘Effect of Incubation Temperature on Nutrient Transporters and Small Intestine Morphology of Broiler Chickens’. *Poultry Science* 90 (1): 118–25. doi:10.3382/ps.2010-00908.

Berkes, Charlotte A., and Stephen J. Tapscott. 2005. ‘MyoD and the Transcriptional Control of Myogenesis’. *Seminars in Cell & Developmental Biology* 16 (4–5): 585–95. doi:10.1016/j.semcdb.2005.07.006.

Bizuayehu, Teshome Tilahun, Steinar D Johansen, Velmurugu Puvanendran, Hilde Toften, and Igor Babiak. 2015. ‘Temperature during Early Development Has Long-Term Effects on microRNA Expression in Atlantic Cod’. *BMC Genomics* 16 (1). doi:10.1186/s12864-015-1503-7.

Booth, D.T. 1987. ‘Effect of Temperature on Development of Mallee Fowl Leipoa Ocellata Eggs’. *Physiological Zoology* Vol. 60, No. 4 (Jul.-Aug., 1987): 437–45.

Campos, Catarina, Arvind YM Sundaram, Luisa MP Valente, Luis EC Conceição, Sofia Engrola, and Jorge MO Fernandes. 2014. ‘Thermal Plasticity of the miRNA Transcriptome during Senegalese Sole Development’. *BMC Genomics* 15 (1): 525. doi:10.1186/1471-2164-15-525.

Chan, Y. C., S. Roy, Y. Huang, S. Khanna, and C. K. Sen. 2012. ‘The MicroRNA miR-199a-5p Down-Regulation Switches on Wound Angiogenesis by Derepressing the v-Ets Erythroblastosis Virus E26 Oncogene Homolog 1-Matrix Metalloproteinase-1 Pathway’. *Journal of Biological Chemistry* 287 (49): 41032–43. doi:10.1074/jbc.M112.413294.

Chen, J. C., and D. J. Goldhamer. 1999. ‘Transcriptional Mechanisms Regulating MyoD Expression in the Mouse’. *Cell and Tissue Research* 296 (1): 213–19.

Chen, Jian-Fu, Elizabeth M Mandel, J Michael Thomson, Qiulian Wu, Thomas E Callis, Scott M Hammond, Frank L Conlon, and Da-Zhi Wang. 2006. ‘The Role of microRNA-1 and microRNA-133 in Skeletal Muscle Proliferation and Differentiation’. *Nature Genetics* 38 (2): 228–33. doi:10.1038/ng1725.

COBB. 2013a. ‘COBB Broiler Management Guide’. Cobb-Vantress, Inc. http://cobb-vantress.com/docs/default-source/guides/cobb-broiler-management-guide---english.pdf.

———. 2013b. ‘COBB Hatchery Management Guide’. Cobb-Vantress, Inc. http://cobb-vantress.com/docs/default-source/management-guides/hatchery_management_guide_englishAB158662CC0DBEA86B974859.pdf.

Collin, A., C. Berri, S. Tesseraud, F. E. Requena Rodón, S. Skiba-Cassy, S. Crochet, M. J. Duclos, et al. 2007. 'Effects of Thermal Manipulation during Early and Late Embryogenesis on Thermotolerance and Breast Muscle Characteristics in Broiler Chickens'. *Poultry Science* 86 (5): 795–800.

Cooper GM. 2000. *The Cell: A Molecular Approach*. 2nded. Sunderland (MA): Sinauer Associates; http://www.ncbi.nlm.nih.gov/books/NBK9961/.

Croissant, Jeffrey D., Jae-Hong Kim, Gregor Eichele, Lisa Goering, John Lough, Ron Prywes, and Robert J. Schwartz. 1996. 'Avian Serum Response Factor Expression Restricted Primarily to Muscle Cell Lineages Is Required for α-Actin Gene Transcription'. *Developmental Biology* 177 (1): 250–64. doi:10.1006/dbio.1996.0160.

Dahlke, Fabiano, Elisabeth Gonzales, Renato Luis Furlan, Adriano Cordeiro Gadelha, Paulo Sérgio Rosa, Régis Fernando Pastorelo Meurer, and Alex Maiorka. 2008. 'Folicular Development of Chicken Broiler Embryos of Different Genotypes, Exposed to Chronic Thermal Stress'. *Ciência Rural* 38 (8): 2314–20. doi:10.1590/S0103-84782008000800034.

Demeure, O., C. Duby, C. Desert, S. Assaf, D. Hazard, H. Guillou, and S. Lagarrigue. 2009. 'Liver X Receptor Regulates Fatty Acid Synthase Expression in Chicken'. *Poultry Science* 88 (12): 2628–35. doi:10.3382/ps.2009-00258.

Dröge, Peter, and Curt A. Davey. 2008. 'Do Cells Let-7 Determine Stemness?' *Cell Stem Cell* 2 (1): 8–9. doi:10.1016/j.stem.2007.12.003.

Duchêne, Sophie, Estelle Audouin, Sabine Crochet, Michel J. Duclos, Joëlle Dupont, and Sophie Tesseraud. 2008. 'Involvement of the ERK1/2 MAPK Pathway in Insulin-Induced S6K1 Activation in Avian Cells'. *Domestic Animal Endocrinology* 34 (1): 63–73. doi:10.1016/j.domaniend.2006.11.001.

Durant, S E, G R Hepp, I T Moore, B C Hopkins, and W A Hopkins. 2010. 'Slight Differences in Incubation Temperature Affect Early Growth and Stress Endocrinology of Wood Duck (Aix Sponsa) Ducklings'. *The Journal of Experimental Biology* 213 (1): 45–51. doi:10.1242/jeb.034488.

Eiby, Yvonne A., Jessica Worthington Wilmer, and David T. Booth. 2008. 'Temperature-Dependent Sex-Biased Embryo Mortality in a Bird'. *Proceedings of the Royal Society B: Biological Sciences* 275 (1652): 2703–6. doi:10.1098/rspb.2008.0954.

Friedman, R. C., K. K.-H. Farh, C. B. Burge, and D. P. Bartel. 2008. 'Most Mammalian mRNAs Are Conserved Targets of microRNAs'. *Genome Research* 19 (1): 92–105. doi:10.1101/gr.082701.108.

Gerhart, Jacquelyn, Christine Neely, Justin Elder, Jessica Pfautz, Jordanna Perlman, Luis Narciso, Kersti K. Linask, Karen Knudsen, and Mindy George-Weinstein. 2007. 'Cells That Express MyoD mRNA in the Epiblast Are Stably Committed to the Skeletal Muscle Lineage'. *The Journal of Cell Biology* 178 (4): 649–60. doi:10.1083/jcb.200703060.

Gu, Shen, and Wai-Yee Chan. 2012. 'Flexible and Versatile as a Chameleon—Sophisticated Functions of microRNA-199a'. *International Journal of Molecular Sciences* 13 (12): 8449–66. doi:10.3390/ijms13078449.

Hadad Yair, Avigdor Cahaner, and Orna Halevy. 2014. 'Featherless and Feathered Broilers under Control versus Hot Conditions. 2. Breast Muscle Development and Growth in Pre- and Posthatch Periods'. Accessed December 16. http://ps.oxfordjournals.org/content/93/5/1076.long.

Hagiwara, Nobuko, Michael Yeh, and Ann Liu. 2007. 'Sox6 Is Required for Normal Fiber Type Differentiation of Fetal Skeletal Muscle in Mice'. *Developmental Dynamics* 236 (8): 2062–76. doi:10.1002/dvdy.21223.

Halevy, O., S. Yahav, and I. Rozenboim. 2006. ‘Enhancement of Meat Production by Environmental Manipulations in Embryo and Young Broilers’. *World’s Poultry Science Journal* 62 (3): 485. doi:10.1017/S0043933906001103.

Hamburger, Viktor, and Howard L. Hamilton. 1951. ‘A Series of Normal Stages in the Development of the Chick Embryo’. *Journal of Morphology* 88 (1): 49–92. doi:10.1002/jmor.1050880104.

Hartley, R. S., E. Bandman, and Z. Yablonka-Reuveni. 1992. ‘Skeletal Muscle Satellite Cells Appear during Late Chicken Embryogenesis’. *Developmental Biology* 153 (2): 206–16.

Hashemi Gheinani, Ali, Fiona C. Burkhard, Hubert Rehrauer, Catharine Aquino Fournier, and Katia Monastyrskaya. 2015. ‘MicroRNA MiR-199a-5p Regulates Smooth Muscle Cell Proliferation and Morphology by Targeting WNT2 Signaling Pathway’. *Journal of Biological Chemistry* 290 (11): 7067–86. doi:10.1074/jbc.M114.618694.

Hepp, G. R., R. A. Kennamer, and M. H. Johnson. 2006. ‘Maternal Effects in Wood Ducks: Incubation Temperature Influences Incubation Period and Neonate Phenotype’. *Functional Ecology* 20 (2): 308–14. doi:10.1111/j.1365-2435.2006.01108.x.

Herceg, Z., W. Hulla, D. Gell, C. Cuenin, M. Lleonart, S. Jackson, and Z. Q. Wang. 2001. ‘Disruption of Trrap Causes Early Embryonic Lethality and Defects in Cell Cycle Progression’. *Nature Genetics* 29 (2): 206–11. doi:10.1038/ng725.

Hettmer, Simone, and Amy J Wagers. 2010. ‘Muscling in: Uncovering the Origins of Rhabdomyosarcoma’. *Nature Medicine* 16 (2): 171–73. doi:10.1038/nm0210-171.

Heywood, J. L., G. M. McEntee, and N. C. Stickland. 2005. ‘In Ovo Neuromuscular Stimulation Alters the Skeletal Muscle Phenotype of the Chick’. *Journal of Muscle Research and Cell Motility* 26 (1): 49–56.doi:10.1007/s10974-005-9007-8.

Hník, P., R. Vejsada, D. F. Goldspink, S. Kasicki, and I. Krekule. 1985. ‘Quantitative Evaluation of Electromyogram Activity in Rat Extensor and Flexor Muscles Immobilized at Different Lengths’. *Experimental Neurology* 88 (3): 515–28.

Jia, H. T., H. J. Tsay, and J. Schmidt. 1992. ‘Analysis of Binding and Activating Functions of the Chick Muscle Acetylcholine Receptor Gamma-Subunit Upstream Sequence’. *Cellular and Molecular Neurobiology* 12 (3): 241–58.

Joseph, N. S., A. Lourens, and E. T. Moran. 2006. ‘The Effects of Suboptimal Eggshell Temperature during Incubation on Broiler Chick Quality, Live Performance, and Further Processing Yield’. *Poultry Science* 85 (5): 932–38.

Kang, Li, Xinxing Cui, Yujie Zhang, Chunhong Yang, and Yunliang Jiang. 2013. ‘Identification of miRNAs Associated with Sexual Maturity in Chicken Ovary by Illumina Small RNA Deep Sequencing’. *BMC Genomics* 14 (1): 352. doi:10.1186/1471-2164-14-352.

Karamboulas, C., A. Swedani, C. Ward, A. S. Al-Madhoun, S. Wilton, S. Boisvenue, A. G. Ridgeway, and I. S. Skerjanc. 2006. ‘HDAC Activity Regulates Entry of Mesoderm Cells into the Cardiac Muscle Lineage’. *Journal of Cell Science* 119 (20): 4305–14. doi:10.1242/jcs.03185.

Ka-Shu Wong, Gane, Bin Liu, Jun Wang, Yong Zhang, Xu Yang, Zengjin Zhang, Qingshun Meng, et al. 2004. ‘A Genetic Variation Map for Chicken with 2.8 Million Single-Nucleotide Polymorphisms’. *Nature* 432 (7018): 717–22. doi:10.1038/nature03156.

Kleiner, Susan M., Terry L. Bazzarre, and Barbara E. Ainsworth. 1994. ‘Nutritional Status of Nationally Ranked Elite Bodybuilders’. *International Journal of Sport Nutrition* 4: 54–54.

Koboldt, Daniel C., Karyn Meltz Steinberg, David E. Larson, Richard K. Wilson, and Elaine R. Mardis. 2013. 'The Next-Generation Sequencing Revolution and Its Impact on Genomics'. *Cell* 155 (1): 27–38. doi:10.1016/j.cell.2013.09.006.

Komazaki, Shinji, Koichi Ito, Hiroshi Takeshima, and Hiroaki Nakamura. 2002. 'Deficiency of Triad Formation in Developing Skeletal Muscle Cells Lacking Junctophilin Type 1'. *FEBS Letters* 524 (1–3): 225–29. doi:10.1016/S0014-5793(02)03042-9.

Koning, Merel, Paul M.N. Werker, Marja J.A. van Luyn, Guido Krenning, and Martin C. Harmsen. 2012. 'A Global Downregulation of microRNAs Occurs in Human Quiescent Satellite Cells during Myogenesis'. *Differentiation* 84 (4): 314–21. doi:10.1016/j.diff.2012.08.002.

Krischek, C., M. Gerken, and M. Wicke. 2013. 'Effects of a Higher Incubation Temperature between Embryonic Day 9 and 12 on Growth and Meat Quality Characteristics of Turkeys'. *British Poultry Science* 54 (1): 5–11. doi:10.1080/00071668.2013.763901.

Kuhn, Donald E., Mickey M. Martin, David S. Feldman, Alvin V. Terry, Gerard J. Nuovo, and Terry S. Elton. 2008. 'Experimental Validation of miRNA Targets'. *Methods* 44 (1): 47–54. doi:10.1016/j.ymeth.2007.09.005.

Lawrie, Ralston A. 2006. *Lawrie's Meat Science*. 7. ed. Woodhead Publishing in Food Science, Technology and Nutrition. Boca Raton, Fla.: CRC Press.

Lee, Rosalind C., Rhonda L. Feinbaum, and Victor Ambros. 1993. 'The C. Elegans Heterochronic Gene Lin-4 Encodes Small RNAs with Antisense Complementarity to Lin-14'. *Cell* 75 (5): 843–54. doi:10.1016/0092-8674(93)90529-Y.

Leksrisompong, N., H. Romero-Sanchez, P. W. Plumstead, K. E. Brannan, and J. Brake. 2007. 'Broiler Incubation. 1. Effect of Elevated Temperature During Late Incubation on Body Weight and Organs of Chicks'. *Poultry Science* 86 (12): 2685–91. doi:10.3382/ps.2007-00170.

LEL-LFL Bayern. 2013. 'Agrarmärkte. Chapter 8 Vieh Und Fleisch'. In *Agrarmärkte*. München.

Li, Tian-Fang, Yufeng Dong, Andreia M Ionescu, Randy N Rosier, Michael J Zuscik, Edward M Schwarz, Regis J O'Keefe, and Hicham Drissi. 2004. 'Parathyroid Hormone-Related Peptide (PTHrP) Inhibits Runx2 Expression through the PKA Signaling Pathway'. *Experimental Cell Research* 299 (1): 128–36. doi:10.1016/j.yexcr.2004.05.025.

Li, Z. 1997. 'Desmin Is Essential for the Tensile Strength and Integrity of Myofibrils but Not for Myogenic Commitment, Differentiation, and Fusion of Skeletal Muscle'. *The Journal of Cell Biology* 139 (1): 129–44. doi:10.1083/jcb.139.1.129.

Lin, Jiandie, Hai Wu, Paul T. Tarr, Chen-Yu Zhang, Zhidan Wu, Olivier Boss, Laura F. Michael, et al. 2002. 'Transcriptional Co-Activator PGC-1 Alpha Drives the Formation of Slow-Twitch Muscle Fibres'. *Nature* 418 (6899): 797–801. doi:10.1038/nature00904.

Lin, Yi, Dengju Li, Qing Liang, Shangqing Liu, Xuelan Zuo, Lin Li, Xuemei Sun, Wenxin Li, Mingxiong Guo, and Zan Huang. 2015. 'miR-638 Regulates Differentiation and Proliferation in Leukemic Cells by Targeting Cyclin-Dependent Kinase 2'. *Journal of Biological Chemistry* 290 (3): 1818–28. doi:10.1074/jbc.M114.599191.

Liu, C., D. C. McFarland, and S. G. Velleman. 2005. 'Effect of Genetic Selection on MyoD and Myogenin Expression in Turkeys with Different Growth Rates'. *Poultry Science* 84 (3): 376–84.

Liu, Suling, Shivani H. Patel, Christophe Ginestier, Ingrid Ibarra, Rachel Martin-Trevino, Shoumin Bai, Sean P. McDermott, et al. 2012. 'MicroRNA93 Regulates Proliferation and Differentiation of Normal and Malignant Breast Stem Cells'. Edited by Jeffrey M. Rosen. *PLoS Genetics* 8 (6): e1002751. doi:10.1371/journal.pgen.1002751.

Livak, K J, and T D Schmittgen. 2001. 'Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2(-Delta Delta C(T)) Method'. *Methods (San Diego, Calif.)* 25 (4): 402–8. doi:10.1006/meth.2001.1262.

Lourens, A., H. van den Brand, R. Meijerhof, and B. Kemp. 2005. 'Effect of Eggshell Temperature during Incubation on Embryo Development, Hatchability, and Posthatch Development'. *Poultry Science* 84 (6): 914–20.

Loyau, T., L. Bedrani, C. Berri, S. Métayer-Coustard, C. Praud, V. Coustham, S. Mignon-Grasteau, et al. 2015. 'Cyclic Variations in Incubation Conditions Induce Adaptive Responses to Later Heat Exposure in Chickens: A Review'. *Animal* 9 (1): 76–85. doi:10.1017/S1751731114001931.

Maltby, V, A Somaiya, N A French, and N C Stickland. 2004. 'In Ovo Temperature Manipulation Influences Post-Hatch Muscle Growth in the Turkey'. *British Poultry Science* 45 (4): 491–98. doi:10.1080/00071660412331286190.

Mauro, A. 1961. 'Satellite Cell of Skeletal Muscle Fibers'. *The Journal of Biophysical and Biochemical Cytology* 9 (February): 493–95.

McKee, Shelly. 2003. 'Muscle Fiber Types in Broilers and Their Relationship to Meat Quality'. *Poultry Science*. http://www.poultryscience.org/docs/pba/1952-2003/2003/2003%20McKee.pdf.

Miller, Jeffrey Boone, and Frank E. Stockdale. 1987. 'What Muscle Cells Know That Nerves Don't Tell Them'. *Trends in Neurosciences* 10 (8): 325–29. doi:10.1016/0166-2236(87)90089-0.

Mountney, G. J. 1989. *Poultry Products Technology*. Food Products Press. https://books.google.de/books?id=3WOMrgEACAAJ.

Nakazawa, Kazuya, Nurmaa Dashzeveg, and Kiyotsugu Yoshida. 2014. 'Tumor Suppressor p53 Induces miR-1915 Processing to Inhibit Bcl-2 in the Apoptotic Response to DNA Damage'. *FEBS Journal* 281 (13): 2937–44. doi:10.1111/febs.12831.

Naraballobh, Watcharapong, Nares Trakooljul, Eduard Muráni, Ronald Brunner, Carsten Krischek, Sabine Janisch, Michael Wicke, Siriluck Ponsuksili, and Klaus Wimmers. 2016a. 'Immediate and Long-Term Transcriptional Response of Hind Muscle Tissue to Transient Variation of Incubation Temperature in Broilers'. *BMC Genomics* 17 (1). doi:10.1186/s12864-016-2671-9.

———. 2016b. 'Transient Shifts of Incubation Temperature Reveal Immediate and Long-Term Transcriptional Response in Chicken Breast Muscle Underpinning Resilience and Phenotypic Plasticity'. *PLoS ONE* 11(9): e0162485. doi:10.1371/journal.pone.0162485

———. 2016c. 'MiRNAs Regulates Dynamic Transcriptional Changes of Broiler Embryos in Response to Modification of the Incubation Temperature'. Reviewed by Gene.

Oksbjerg, Niels, Florence Gondret, and Mogens Vestergaard. 2004. 'Basic Principles of Muscle Development and Growth in Meat-Producing Mammals as Affected by the Insulin-like Growth Factor (IGF) System'. *Domestic Animal Endocrinology* 27 (3): 219–40. doi:10.1016/j.domaniend.2004.06.007.

Otten, Ellen, Angeliki Asimaki, Alexander Maass, Irene M. van Langen, Allard van der Wal, Nicolaas de Jonge, Maarten P. van den Berg, et al. 2010. 'Desmin Mutations

as a Cause of Right Ventricular Heart Failure Affect the Intercalated Disks'. *Heart Rhythm* 7 (8): 1058–64. doi:10.1016/j.hrthm.2010.04.023.

Paulin, D, and Z Li. 2004. 'Desmin: A Major Intermediate Filament Protein Essential for the Structural Integrity and Function of Muscle'. *Experimental Cell Research* 301 (1): 1–7. doi:10.1016/j.yexcr.2004.08.004.

Peter, James B., R. James Barnard, V. Reggie Edgerton, Cynthia A. Gillespie, and Kerstin E. Stempel. 1972. 'Metabolic Profiles of Three Fiber Types of Skeletal Muscle in Guinea Pigs and Rabbits'. *Biochemistry* 11 (14): 2627–33.

Petracci, Massimiliano, and Claudio Cavani. 2011. 'Muscle Growth and Poultry Meat Quality Issues'. *Nutrients* 4 (12): 1–12. doi:10.3390/nu4010001.

Piestun, Y., S. Druyan, J. Brake, and S. Yahav. 2013. 'Thermal Manipulations during Broiler Incubation Alter Performance of Broilers to 70 Days of Age'. *Poultry Science* 92 (5): 1155–63. doi:10.3382/ps.2012-02609.

Piestun, Y., O. Halevy, and S. Yahav. 2009. 'Thermal Manipulations of Broiler Embryos--The Effect on Thermoregulation and Development during Embryogenesis'. *Poultry Science* 88 (12): 2677–88. doi:10.3382/ps.2009-00231.

Piestun, Y., M. Harel, M. Barak, S. Yahav, and O. Halevy. 2008. 'Thermal Manipulations in Late-Term Chick Embryos Have Immediate and Longer Term Effects on Myoblast Proliferation and Skeletal Muscle Hypertrophy'. *Journal of Applied Physiology* 106 (1): 233–40. doi:10.1152/japplphysiol.91090.2008.

Pinchuck, S., N. Meiri, and S. Yahav. 2011. 'The Effect of Thermal Manipulation, during Early Embryogenesis, on Angiogenesis and Thermo-Tolerance in Broilers'. In , 44–45. Wageningen, The Netherlands.

Rengaraj, D., S. I. Lee, M. Yoo, T. H. Kim, G. Song, and J. Y. Han. 2012. 'Expression and Knockdown Analysis of Glucose Phosphate Isomerase in Chicken Primordial Germ Cells'. *Biology of Reproduction* 87 (3): 57–57. doi:10.1095/biolreprod.112.101345.

Reno, Tosca. 2007. *The Eat-Clean Diet*. National Book network.

Richardson, R. I., and G. C. Mead, eds. 1999. *Poultry Meat Science*. Poultry Science Symposium Series, v. 25. Wallingford, Oxon, UK: New York, NY, USA : CABI Pub.

Sallustio, Fabio, Grazia Serino, Vincenzo Costantino, Claudia Curci, Sharon N. Cox, Giuseppe De Palma, and Francesco P. Schena. 2013. 'miR-1915 and miR-1225-5p Regulate the Expression of CD133, PAX2 and TLR2 in Adult Renal Progenitor Cells'. Edited by Giovanni Camussi. *PLoS ONE* 8 (7): e68296. doi:10.1371/journal.pone.0068296.

Schultz, E., and K. M. McCormick. 1994. 'Skeletal Muscle Satellite Cells'. *Reviews of Physiology, Biochemistry and Pharmacology* 123: 213–57.

Scott, J., M. J. Da Costa, and E. O. Oviedo-Rondon. 2015. 'Incubation Temperature Profiles Affect Broiler Feathering'. *The Journal of Applied Poultry Research* 24 (1): 49–57. doi:10.3382/japr/pfu022.

Seaberg, Bonnie, Gabrielle Henslee, Shuo Wang, Ximena Paez-Colasante, Gary E. Landreth, and Mendell Rimer. 2015. 'Muscle-Derived Extracellular Signal-Regulated Kinases 1 and 2 Are Required for the Maintenance of Adult Myofibers and Their Neuromuscular Junctions'. *Molecular and Cellular Biology* 35 (7): 1238–53. doi:10.1128/MCB.01071-14.

Shu, J.T., W.J. Xu, M. Zhang, W.T. Song, Y.J. Shan, C. Song, W.Q. Zhu, X.Y. Zhang, and H.F. Li. 2014. 'Transcriptional Co-Activator PGC-1α Gene Is Associated with Chicken Skeletal Muscle Fiber Types'. *Genetics and Molecular Research* 13 (1): 895–905. doi:10.4238/2014.February.14.19.

Sławińska, Anna, Jadwiga Brzezińska, Maria Siwek, and Gabriela Elminowska-Wenda. 2013. 'Expression of Myogenic Genes in Chickens Stimulated in Ovo with Light and Temperature'. *Reproductive Biology* 13 (2): 161–65. doi:10.1016/j.repbio.2013.04.003.

Sobolewska, Adrianna, Gabriela Elminowska-Wenda, Joanna Bogucka, Michał Szpinda, Konrad Walasik, Marek Bednarczyk, and Monika Paruszewska-Achtel. 2011. 'Myogenesis--Possibilities of Its Stimulation in Chickens'. *Folia Biologica* 59 (3–4): 85–90.

Stockdale, F.E. 1992. 'Myogenic Cell Lineages'. *Dev Biol* 154: 284–98.

Stricker, Sigmar, Reinald Fundele, Andrea Vortkamp, and Stefan Mundlos. 2002. 'Role of Runx Genes in Chondrocyte Differentiation'. *Developmental Biology* 245 (1): 95–108. doi:10.1006/dbio.2002.0640.

Tagariello, A. 2006. 'Balanced Translocation in a Patient with Craniosynostosis Disrupts the SOX6 Gene and an Evolutionarily Conserved Non-Transcribed Region'. *Journal of Medical Genetics* 43 (6): 534–40. doi:10.1136/jmg.2005.037820.

Takeshima, Hiroshi, Shinji Komazaki, Miyuki Nishi, Masamitsu Iino, and Kenji Kangawa. 2000. 'Junctophilins'. *Molecular Cell* 6 (1): 11–22. doi:10.1016/S1097-2765(05)00005-5.

Teltathum, Tawatchai, and Supamit Mekchay. 2009. 'Proteome Changes in Thai Indigenous Chicken Muscle during Growth Period'. *International Journal of Biological Sciences* 5 (7): 679–85.

Ueda, Masatoshi, Kouichi Watanabe, Kan Sato, Yukio Akiba, and Masaaki Toyomizu. 2005. 'Possible Role for avPGC-1α in the Control of Expression of Fiber Type, along with avUCP and avANT mRNAs in the Skeletal Muscles of Cold-Exposed Chickens'. *FEBS Letters* 579 (1): 11–17. doi:10.1016/j.febslet.2004.11.039.

Um, M., J. Yamauchi, S. Kato, and J. L. Manley. 2001. 'Heterozygous Disruption of the TATA-Binding Protein Gene in DT40 Cells Causes Reduced cdc25B Phosphatase Expression and Delayed Mitosis'. *Molecular and Cellular Biology* 21 (7): 2435–48. doi:10.1128/MCB.21.7.2435-2448.2001.

USDA. 2014. 'EU-28, Poultry and Products Annual, Further Growth Expected for EU-28 Broiler Sector in 2015'. FR9161. United states department of agriculture, foreign agricultural service. http://gain.fas.usda.gov/Recent%20GAIN%20Publications/Poultry%20and%20Products%20Annual_Paris_EU-28_9-29-2014.pdf.

van Rooij, E. 2011. 'The Art of MicroRNA Research'. *Circulation Research* 108 (2): 219–34. doi:10.1161/CIRCRESAHA.110.227496.

von Hofsten, Jonas, Stone Elworthy, Michael J Gilchrist, James C Smith, Fiona C Wardle, and Philip W Ingham. 2008. 'Prdm1- and Sox6-Mediated Transcriptional Repression Specifies Muscle Fibre Type in the Zebrafish Embryo'. *EMBO Reports* 9 (7): 683–89. doi:10.1038/embor.2008.73.

Wang, Qiong, Yi-Ping Liu, Xiao-Song Jiang, Chao-Wu Yang, Hua-Rui Du, Mo-Han Qiu, and Qing Zhu. 2007. '[Correlation analysis of relationships between polymorphisms of high quality chicken myogenin gene and slaughter and meat quality traits]'. *Yi Chuan = Hereditas / Zhongguo Yi Chuan Xue Hui Bian Ji* 29 (9): 1089–98.

Wang, X. G., J. F. Yu, Y. Zhang, D. Q. Gong, and Z. L. Gu. 2012. 'Identification and Characterization of microRNA from Chicken Adipose Tissue and Skeletal Muscle'. *Poultry Science* 91 (1): 139–49. doi:10.3382/ps.2011-01656.

Wang, X. M., H. J. Tsay, and J. Schmidt. 1990. 'Expression of the Acetylcholine Receptor Delta-Subunit Gene in Differentiating Chick Muscle Cells Is Activated

by an Element That Contains Two 16 Bp Copies of a Segment of the Alpha-Subunit Enhancer'. *EMBO J.* 9(3): 783–790.

Wang, Xiaonan H. 2013. 'MicroRNA in Myogenesis and Muscle Atrophy': *Current Opinion in Clinical Nutrition and Metabolic Care* 16 (3): 258–66. doi:10.1097/MCO.0b013e32835f81b9.

Wei, W, H-B He, W-Y Zhang, H-X Zhang, J-B Bai, H-Z Liu, J-H Cao, K-C Chang, X-Y Li, and S-H Zhao. 2013. 'miR-29 Targets Akt3 to Reduce Proliferation and Facilitate Differentiation of Myoblasts in Skeletal Muscle Development'. *Cell Death and Disease* 4 (6): e668. doi:10.1038/cddis.2013.184.

Werner, Carsten, and Michael Wicke. 2007. 'How Is the Embryonic Development of the M. Pectoralis Superficialis in Turkey Embryos Influenced by the Incubation Temperature? Theoretical Background and Hypothetical Investigations'. *Archives Animal Breeding* 50 (08–14).

Werner, C., and M. Wicke. 2008. 'Increasing the Temperature at a Certain Period during the Incubation of Broiler Eggs Improves the Carcass and Breast Yields without a Negative Impact on the Meat Quality'. *Archives Animal Breeding*, no. 51: 11–16.

Wurdak, Heiko, Shoutian Zhu, Angelica Romero, Mihaela Lorger, James Watson, Chih-Yuan Chiang, Jay Zhang, et al. 2010. 'An RNAi Screen Identifies TRRAP as a Regulator of Brain Tumor-Initiating Cell Differentiation'. *Cell Stem Cell* 6 (1): 37–47. doi:10.1016/j.stem.2009.11.002.

Xu, Hongtao, Xiaobo Wang, Zhenglin Du, and Ning Li. 2006. 'Identification of microRNAs from Different Tissues of Chicken Embryo and Adult Chicken'. *FEBS Letters* 580 (15): 3610–16. doi:10.1016/j.febslet.2006.05.044.

Yablonka-Reuveni, Zipora, and Mark Nameroff. 1990. 'Temporal Differences in Desmin Expression between Myoblasts from Embryonic and Adult Chicken Skeletal Muscle'. *Differentiation; Research in Biological Diversity* 45 (1): 21–28.

Ye, Yaqiong, Shumao Lin, Heping Mu, Xiaohong Tang, Yangdan Ou, Jian Chen, Yongjiang Ma, and Yugu Li. 2014. 'Analysis of Differentially Expressed Genes and Signaling Pathways Related to Intramuscular Fat Deposition in Skeletal Muscle of Sex-Linked Dwarf Chickens'. *BioMed Research International* 2014: 724274. doi:10.1155/2014/724274.

Zhang, Q., H. Shi, W. Liu, Y. Wang, Q. Wang, and H. Li. 2013. 'Differential Expression of L-FABP and L-BABP between Fat and Lean Chickens'. *Genetics and Molecular Research* 12 (4): 4192–4206. doi:10.4238/2013.October.7.5.

Annex 1

List of abbreviations

ACTB	Actin, Beta
AHSG	Alpha-2-HS-glycoprotein
APOA1	Apolipoprotein A-I
APOD	Apolipoprotein D
ASO	Antisense oligoribonucleotides
cDNA	Complementary DNA
CHRND	Cholinergic Receptor, Nicotinic, Delta
CHRNG	Cholinergic Receptor, Nicotinic, Gamma
DEGs	Differential expression genes
DES	Desmin
DNA	Deoxyribonucleic acid
ED	Embryonical Day
FABP1	Fatty acid-binding protein 1
FC	Fold change
FDR	False discovery rate
FGF6	Fibroblast Growth Factor 6
GAPDH	Glyceroldehyde-3-phosphate dehydrogenase
GFPT1	Glutamine--Fructose-6-Phosphate Transaminase 1
GPI	Glucose-6-Phosphate Isomerase
Gr.1	Major categories group 1 Cell maintenance, proliferation differentiation and replacement
Gr.2	Major categories group 2 Organismal, organ and tissue development
Gr.3	Major categories group 3 Nutrient metabolism
Gr.4	Major categories group 4 Genetic information and nucleic acid processing
Gr.5	Major categories group 5 Molecular transport
Gr.6	Major categories group 6 Cell signaling and interaction
Gr.7	Major categories group 7 Small molecule biochemistry
Gr.8	Major categories group 8 Response to stimuli
H10(ΔC)	Treatment by increase temperature to 38.7 °C at Embryonical Day 7-10 (compare with control group)

H13(ΔC)	Treatment by increase temperature to 38.7 °C at Embryonical Day 10-13 (compare with control group)
HDAC4	Histone Deacetylase 4
HH	Hamburger and Hamilton stages (chicken embryo development)
IPA	Ingenuity Pathways Analysis
JPH1	Junctophilin 1
L10(ΔC)	Treatment by decrease temperature to 36.7 °C at Embryonical Day 7-10 (compare with control group)
L13(ΔC)	Treatment by decrease temperature to 36.7 °C at Embryonical Day 10-13 (compare with control group)
Lbx1	Ladybird Homeobox 1
Let-7	Let-7 microRNA precursor
MED	Mediator Complex Subunit
MFN	Muscle fiber number
miRNAs	Micro RNAs (miR)
MRFs	Myogenic regulatory factors
mRNAs	messenger RNAs
Myf5	Myogenic factor 5
MYH2	Myosin, Heavy Chain 2
MYL3	Myosin, Light Chain 3
MYOD1	Myogenic differentiation 1
MYOG	Myogenin (Myogenic Factor 4)
NGS	Next Generation Sequencing notably high-throughput sequencing technology
NR1H3	Nuclear Receptor Subfamily 1, Group H, Member 3
nt	Nucleotides
Pax	Paired Box gene
PCR	Polymerase chain reaction
PPARA	Peroxisome proliferator-activated receptor
PPARGC1A	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
qPCR	Quantitative real time RT-PCR
RNA	Ribonucleic acid

RNA-seq	RNA sequencing notably whole transcriptome shotgun sequencing (WTSS)
RUNX2	Runt-Related Transcription Factor 2
r-value	Correlation coefficients
SHH	Sonic hedgehog proteins
SMAD3	SMAD Family Member 3
SOX6	SRY (Sex Determining Region Y)-Box 6
SRF	Serum Response Factor
TBP	TATA Box Binding Protein
TRRAP	Transformation/Transcription Domain-Associated Protein
UTR	Untranslated regions
z-score (IPA)	Predicted pathway by gene expression pattern
ΔC	Compare versus control group

Annex 2

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