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RESEARCH ARTICLE

In ovo temperature manipulation differentially influences limb musculoskeletal development in two lines of chick embryos selected for divergent growth rates

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SUMMARY

Selective breeding has led to diverging phenotypic evolution in layer and broiler chickens through genomic and epigenetic modifications. Here we show that *in ovo* environmental manipulation differentially influences embryonic limb muscle phenotype in these two breeds. We demonstrate that raising incubation temperature from 37.5 to 38.5°C between embryonic days (ED) 4 and 7 increased motility and body mass in both layer and broiler embryos. In layers, this was accompanied by gastrocnemius muscle hypertrophy, increased fibre and nuclei numbers and a higher nuclei to fibre ratio (ED18), preceded by increased hindlimb Myf5 (ED5–8), Pax7 (ED5–10), BMP4 (ED6–9) and IGF-I (ED9–10, ED18) mRNAs. In broilers, the same temperature treatment led to reduced gastrocnemius cross-sectional area with fewer fibres and nuclei and an unchanged fibre to nuclei ratio (ED18). This was preceded by a delay in the peak of hindlimb Myf5 expression, increased Pax7 (ED5, ED7–10) and BMP4 (ED6–8) but reduced IGF-I (ED8–10) mRNAs. Rather than promoting myogenesis as in layer embryos, the temperature treatment promoted gastrocnemius intramuscular fat deposition in broilers (ED18) preceded by increased hindlimb PPARγ mRNA (ED7–10). The treatment increased tibia/tarsus bone length as well as femur cross-sectional area in both breeds, but femur length and bone to cartilage ratio in the femur and tibia/tarsus were only increased in treated layers (ED18). We conclude that *in ovo* temperature manipulation differentially affected the molecular regulation of hindlimb myogenic, adipogenic and growth factor expression in broiler and layer embryos, leading to differential changes in muscle phenotype. The underlying interactive mechanisms between genes and the environment need further investigation.

Key words: Myf5, Pax7, IGF-I, muscle, bone, chick embryo, movement, temperature.

INTRODUCTION

Domesticated chickens, *Gallus gallus domesticus* (Linnaeus 1758), have been extensively selected for different phenotypic traits through successive breeding of their major common ancestor, wild *Gallus gallus* (Arthur and Albers, 2003; Eriksson et al., 2008; Rubin et al., 2010). Selection pressures have been placed on fast post-hatch growth rates and breast meat yield in broilers and on egg-laying yield in layers (Arthur and Albers, 2003). The underlying molecular mechanisms that have led to diverging phenotypic evolution in these two breeds are not fully characterised but a whole-genome sequencing study has shown that genomic polymorphisms, nucleotide deletions and selective sweeps overlapping genes that control growth, appetite and metabolic regulation may be involved (Rubin et al., 2010). In addition to genomic differences, evidence suggests that the embryonic milieu and, more specifically, the egg-yolk environment also contribute to phenotypic differences *via* epigenetic mechanisms (Ho et al., 2011). In support of the epigenetic control of phenotypic diversity, several studies have shown that manipulating egg incubation temperature can affect musculoskeletal development in birds (Hammond et al., 2007; Maltby et al., 2004) but also in fish (Albokhadaim et al., 2007) and reptiles (Booth, 1998). This implies that diverging domestic chicken phenotypes emerged from complex synergistic interactions between genomic and epigenetic alterations (Ho et al., 2011).

In light of this, a given environmental factor such as egg incubation temperature may differentially interact with the embryonic milieu and the epigenetic regulation of respective genomes, leading to diverging phenotypic changes in developing broiler and layer embryos. Besides the basic scientific interest in studying breed-specific phenotypic responses to environmental manipulation, there are important animal welfare implications from this work. Intense selection for fast post-hatch growth rate has led to increased incidence of leg deformities and lameness in broilers, causing major welfare concerns (Arthur and Albers, 2003; Kestin et al., 2001). Any attempt to improve broiler leg embryonic development *via* simple manipulation of egg incubation temperature may improve post-hatch leg strength and postural stability, which may help address some animal welfare ethical concerns.

To understand how environmental manipulations may influence limb development and subsequent phenotype, it is important to determine how this development, including that of myogenesis, is regulated. It is well characterised that during embryonic development, limb skeletal muscles originate from ventrolateral epithelial cells of the somatic dermomyotome that detach and migrate into the limb buds (for a review, see Buckingham et al., 2003). Delamination and migration of muscle progenitor cells is controlled by the tyrosine kinase receptor c-Met and its ligand hepatocyte growth/scatter factor (HGF) (Dietrich et al., 1999), whose expression is inhibited by bone morphogenesis protein (BMP)-4 in posterior limb mesenchyme (Scaal et al., 1999). BMP-4 has also been shown to promote the proliferation of muscle precursor cells by preventing their premature differentiation (Amthor et al., 1999). Once somatic progenitor cells

reach the limb, they begin to express the myogenic regulator factors MyoD and Myf5 and undergo rapid proliferation (Buckingham et al., 2003). Evidence suggests that Myf5 actions are more targeted towards myoblast specification and proliferation whereas MyoD prepares myoblasts for differentiation and myogenin activates the differentiation programme leading to the expression of muscle-specific contractile proteins (Buckingham et al., 2003; Ishibashi et al., 2005). In chicken, muscle fibre formation occurs in two waves. Primary fibres form between embryonic days (ED) 4 and 7 followed by a second wave of myoblast proliferation and differentiation to form secondary fibres between ED8 and 16 (Crow and Stockdale, 1986; Lee et al., 2004). Muscle fibre formation ends by hatch (ED21) (Smith, 1963), after which muscle growth and regeneration occurs through the activation of undifferentiated muscle precursor cells known as satellite cells (Moss, 1968). Pax7 has been shown to play an essential role in embryonic satellite cell specification as well as in their survival during the perinatal period (Buckingham, 2007).

Myogenesis is also regulated by growth factors, among which insulin-like growth factor (IGF)-I has been shown to exert time-dependent actions; namely, initially stimulating myoblast proliferation then promoting cell cycle withdrawal and myogenesis *via* inhibition and then activation of myogenin expression through (Erk)1/2 signalling (Adi et al., 2002). IGF-I also promotes myofibre growth and protein synthesis through the phosphoinositide 3-kinase (PI3K)/Akt signalling cascade (Rommel et al., 2001) as well satellite cell activation (Charge and Rudnicki, 2004).

Further evidence indicates that muscle development is linked to skeletogenesis. It has been postulated that embryonic bone growth drives muscle growth through mechanical stimulation (Hall and Herring, 1990). This is supported by reports showing that muscle stretch augments the expression of myogenic regulatory factors (Lowe and Alway, 1999) and IGF-I, leading to fibre hypertrophy (Goldspink, 1999). Conversely, embryonic muscular activity plays an essential role in bone and joint development, although the underlying mechanisms involved are poorly understood (Hall and Herring, 1990; Pitsillides, 2006). For instance, embryonic paralysis using neuromuscular blocking agents reduces skeletal growth by reducing embryonic movement *in ovo* and thereby the load imposed on the bones by muscle contraction (Hall and Herring, 1990). Furthermore, drug-induced flaccid muscle paralysis leads to limb deformities, reduced longitudinal bone growth and greater cartilage to bone ratio, indicative of reduced osteogenesis in chick embryos (Lamb et al., 2003).

In light of the above, we hypothesized that during embryonic development, environmental factors such as temperature manipulation differentially interact with the embryonic milieu and the epigenetic regulation of respective genomes to produce diverging phenotypic changes in broiler and layer embryos. To test this hypothesis, we subjected layer and broiler eggs to a temperature treatment that has been shown to induce phenotypic changes in limb muscle and bone of egg-layer embryos (Hammond et al., 2007) and compared muscular phenotypic changes in both breeds. We also measured embryonic motility, bone growth and the embryonic expression of genes known to regulate myogenic and adipogenic differentiation to determine whether changes in these parameters contributed to the muscular phenotypic changes observed.

MATERIALS AND METHODS

Animals

A total of 144 fertile chicken eggs were used in this study, 72 of which were of either egg-laying (white leghorn; Joice and Hill Poultry Ltd, Norfolk, UK) or meat-broiler (Ross, Faccenda Hatchery, Essex, UK) breed. Before the start of the study, a high-precision Squirrel Logger (Grant Instruments Ltd, Cambridge, UK) was used to monitor and carefully map the temperatures within two identical LMS 301 forced draft incubators (Wolf Laboratories, York, UK). The logger consisted of 10 probes that were placed throughout the incubator, with each probe recording the temperature at 5 min intervals. If any positions within the incubators deviated by more than 0.3°C from the set temperature, at any time over a 3 day period, they were excluded. During the study, the temperatures continued to be monitored and any eggs judged to have experienced a temperature deviation that was out of the set temperature range ±0.3°C were removed. At the start of the study, each incubator housed an equal number of randomly selected layer and broiler eggs. Eggs were placed horizontally on fibre egg trays and incubated in the same manner: 37.5°C, 60–70% relative humidity, daily 90 deg rotations and darkness.

Treatment regime

On ED4, half of the eggs from each breed, i.e. 36 layer and 36 broiler eggs were randomly selected and incubated at a higher temperature, 38.5°C, until ED7 inclusively (Fig. 1). All other incubation conditions remained the same. On ED8, eggs incubated at 38.5°C were returned to the incubator set at control temperature (37.5°C). Embryos were therefore categorised into one of four groups: layer control (LC), layer treated (LT), broiler control (BC) and broiler treated (BT).

Physical activity *in ovo* and tissue sampling

Embryonic physical activity *in ovo* was measured over a 5 min period using previously described methods (Al-Musawi et al., 2011;

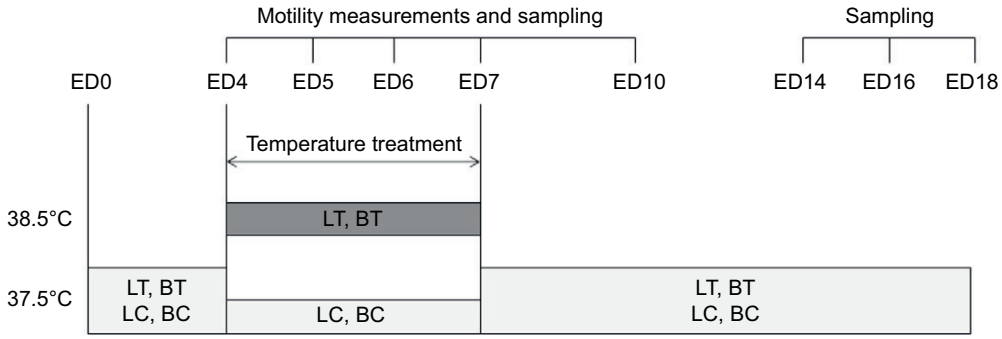

Fig. 1. Experimental design. White leghorn layer (L) and Ross broiler (B) eggs were incubated either at 37.5°C (LC and BC) or 38.5°C (LT and BT) on embryonic days (ED) 4–7. All eggs were incubated at the control temperature (37.5°C) from ED0 to 3 and from ED8 to 18.

Table 1. Real-time PCR primers in the 5′ to 3′ direction

Target	Accession number	Forward primer	Reverse primer
Myf5	NM_001030363	GCAGCCACTATGAGGGAGAG	GTCCCGGCAGGTGATAGTAG
IGF-I	NM_001004384	CCCAGAAACACTGTGTGGTG	ATTCCCTTGTGGTGTAAGCG
Pax7	NM_205065.1	ACTGCGACAAGAAGGAGGAA	CTCTTCAAAGGCAGGTCTGG
PPARγ	NM_001001460.1	TCGCATCCATAAGAAAAGCA	CTTCTCCTTCTCCGCTTCTG
BMP4	NM_205237.2	GAGAGGAGCCTCCAGAGAT	GCTGAGGTTGAAGACGAAGC

Hammond et al., 2007). Measurement of total body movements was carried out in ED5–10 chick embryos, using four embryos per ED and per group (96 eggs in total). In brief, an egg was randomly removed from the incubator, placed in an insulated support to prevent cooling, and 'windowed'. A LED light source (which does not emit heat) was placed close to the blunt end of the egg to illuminate the inside and aid in observation. Amnion contractions, which promote a swinging motion of the embryo, were excluded (Oppenheim, 1966). Immediately after motility measurements, embryos were removed from their eggs, separated from their yolk sacs and decapitated prior to embryonic body mass (head included) measurements. The right hindlimb was then removed from each of these embryos and stored in a RNA stabilization solution (RNAlater®, Ambion, Cambridgeshire, UK) at –20°C for subsequent quantitative real-time RT-PCR.

Body masses were measured again on ED14, 16 and 18 (four eggs per group per ED; 48 eggs in total). On ED18 (3 days prior to hatch), leg gastrocnemius muscles and bones were sampled. The right gastrocnemius muscle was dissected and stored in RNAlater® at –20°C pending quantitative real-time reverse transcription (RT)-PCR analysis. Both the left gastrocnemius muscle and left femur were snap frozen in OCT mounting compound (VWR International Ltd, Lutterworth, UK) in liquid-nitrogen-cooled isopentane pending histological examination. The whole right hindlimb skeleton (femur, tibia and tarsus) was cleaned of adhering tissue and stored in acetone for 4 days for the removal of fat prior to histological staining.

Quantitative real-time RT-PCR

Total RNA purification, reverse transcription (RT) and real-time PCR were carried out on frozen hindlimb (ED5–10) and gastrocnemius (ED18) tissues, using protocols previously published (Al-Musawi et al., 2011). Unless otherwise stated, all reagents were purchased from Sigma UK Ltd (Poole, UK). Total RNA was purified using a standard acid guanidium thiocyanate-phenol-chloroform extraction method (Chomczynski and Sacchi, 1987). The resulting RNA pellet was re-suspended in 50 µl of nuclease-free water. Potential genomic DNA contamination was removed using a RNase-free DNase kit (RQ1; Promega UK Ltd, Southampton, Hampshire, UK). RNA quantity and quality was evaluated using the NanoDrop® ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA) and integrity was verified by formaldehyde gel electrophoresis. First strand cDNA synthesis was performed using the QuantiTect Reverse Transcriptase kit (QIAGEN Ltd, Sussex, UK) using 1 µg of total RNA in a volume of 20 µl according to manufacturer's instructions. All reactions were carried out simultaneously with the same master mix to prevent variability and amplification inefficiencies between samples.

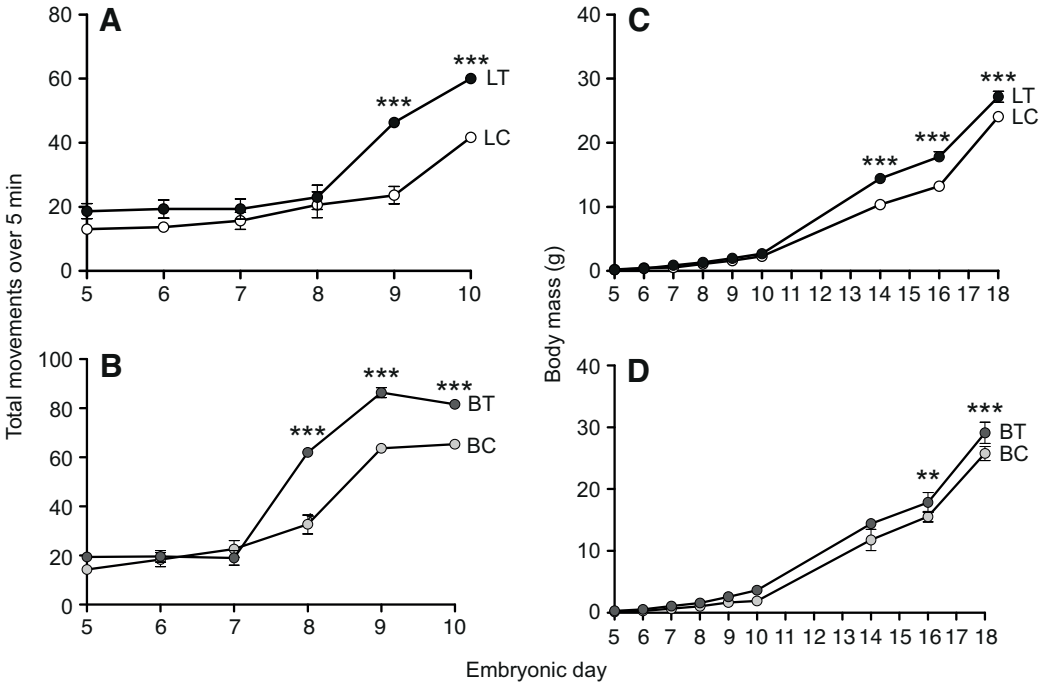

Fig. 2. Embryonic physical activity and body growth measurements. (A,B) Total number of *in ovo* body movements over a 5 min period on ED5–10 in layer and broiler embryos incubated at either 37.5°C (LC and BC) or 38.5°C (LT and BT) during ED4–7. (C,D) Body mass (g) of layer and broiler embryos on ED5–18 incubated at either 37.5°C (LC and BC) or 38.5°C (LT and BT) during ED4–7. Results are presented as means ± s.e.m. (N=4 embryos per temperature per breed per day); asterisks represent significant differences (**P<0.01; ***P<0.0001) between incubation temperatures within breeds at each embryonic stage.

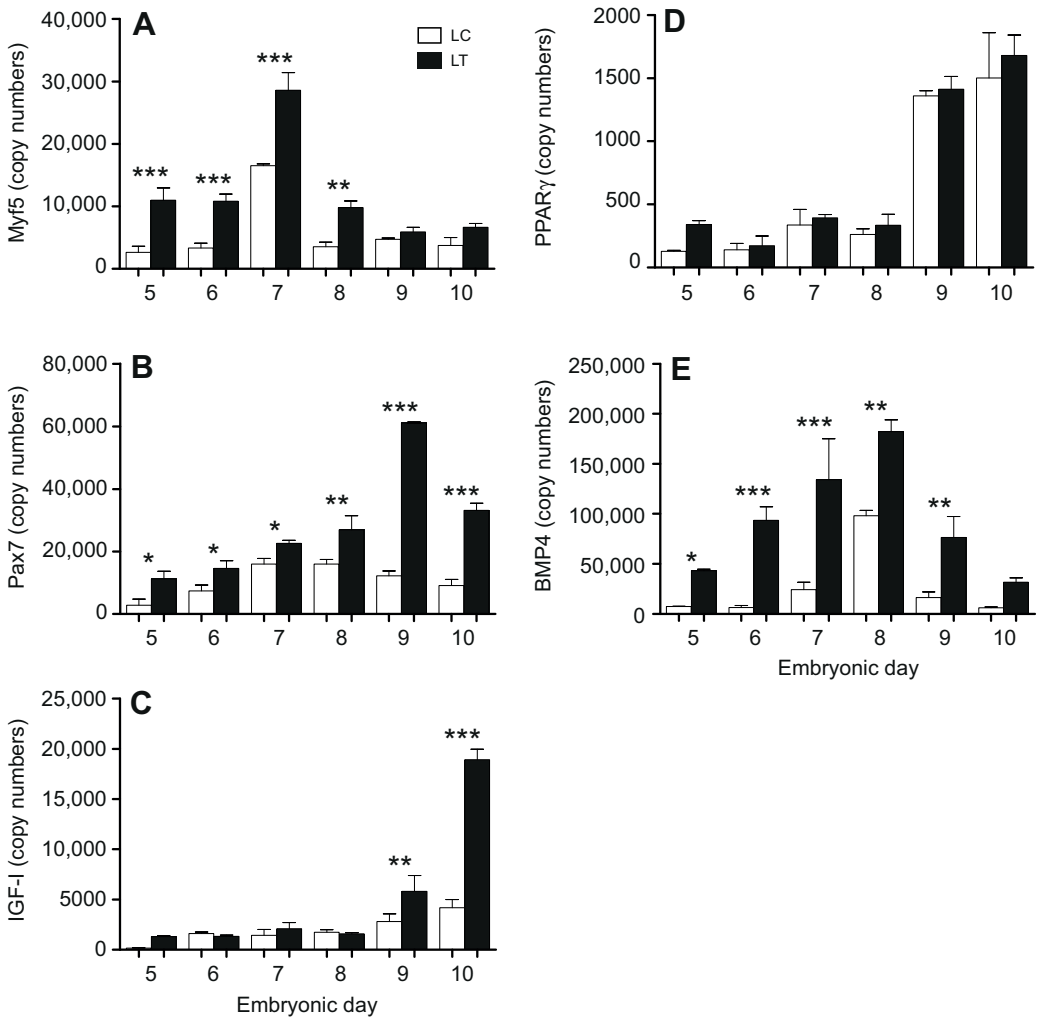

Fig. 3. Real-time PCR analyses on 1 μg of total RNA extracted from the hindlimb of layer embryos aged ED5–10, incubated at either 37.5°C (LC) or 38.5°C (LT) during ED4–7. The mRNA expressions (copy numbers) for (A) Myf5, (B) Pax7, (C) IGF-I, (D) PPARγ and (E) BMP4 are shown. Results are presented as means ± s.e.m. (N=4 embryos per temperature per day); asterisks represent significant differences (*P<0.05; **P<0.01; ***P<0.0001) between incubation temperatures within each embryonic stage.

Primer sequences and Entrez accession numbers are outlined in Table 1. Primers spanning more than one exon (whenever possible) were designed using Primer-3 Web-Software (Whitehead Institute for Biomedical Research, Cambridge, MA, USA) and manufactured by MWG-Biotech (Ebersberg, Germany). Real-time RT-PCR assays were carried out using the QuantiTect SYBR Green kit (QIAGEN Ltd). Reactions were carried out in duplicate, consisting of either 1 μl sample cDNA or serially diluted DNA standard (target sequence of interest). Samples were processed using the MJ Research Chromo4 real-time PCR detector (Bio-Rad Laboratories Ltd, Hampshire, UK) with the following thermal cycling conditions: 95°C for 15 min followed by 35 amplification cycles of 94°C for 15 s, 54°C for 25 s and 72°C for 15 s. MJ Research Opticon 3.1 software was used to generate standard curves with a correlation coefficient (r^2) greater than 0.98. Gene expression data were normalised to total RNA (1 μg starting material) according to Ramakers et al. (Ramakers et al., 2003) and are presented as copy numbers. The specificity and purity of amplicons were verified using melting curves, agarose gel electrophoresis and DNA sequencing using the Applied Biosystems 3730 DNA analyzer (Geneservice Ltd, Oxford, UK).

Muscle histology

Transverse (10 μm) cryo-sections were taken at the mid-belly region of the snap-frozen left gastrocnemius muscles (ED18) using a Bright Crysotat at –20°C (Bright Instruments, Huntingdon, UK). Sections were adhered to Superfrost slides (Fisher Scientific, Loughborough, UK), air dried and stored at –80°C pending histological examination. Prior to staining, frozen slides were allowed to thaw for 30 min at room temperature. Some sections were stained with 0.1% Mayer's haematoxylin and 1% eosin for quantification of total apparent muscle fibre number, nuclei number and average fibre cross-sectional area (CSA) (Heywood et al., 2005). Other sections were stained with Sudan Black B for quantification of the intramuscular fat deposit area (Bancroft and Stevens, 1996).

Bone histology

Transverse 10 μm cryo-sections were taken at the mid-shaft of the left femur at ED18. Sections were stained in 0.1% Mayer's haematoxylin and 1% eosin for quantification of cortical CSA.

Following acetone storage, whole right hindlimb skeletons (femur, tibia and tarsus) at ED18 were fixed in 96% ethanol for 2 days and then stained with 0.005% Alizarin Red S and 0.015% Alcian Blue (GURR®, Hopkin and Williams, Essex, UK) for assessment of mineralised bone and cartilage area, respectively. Bones were stored in 100% glycerol. For assessment of longitudinal bone growth, bone lengths were measured using a dissecting microscope, calibrated using a 1 mm graticule.

Photography and image analysis

Light photomicrographs were taken using a Leica light microscope and images were analysed using the Kontron KS300 image

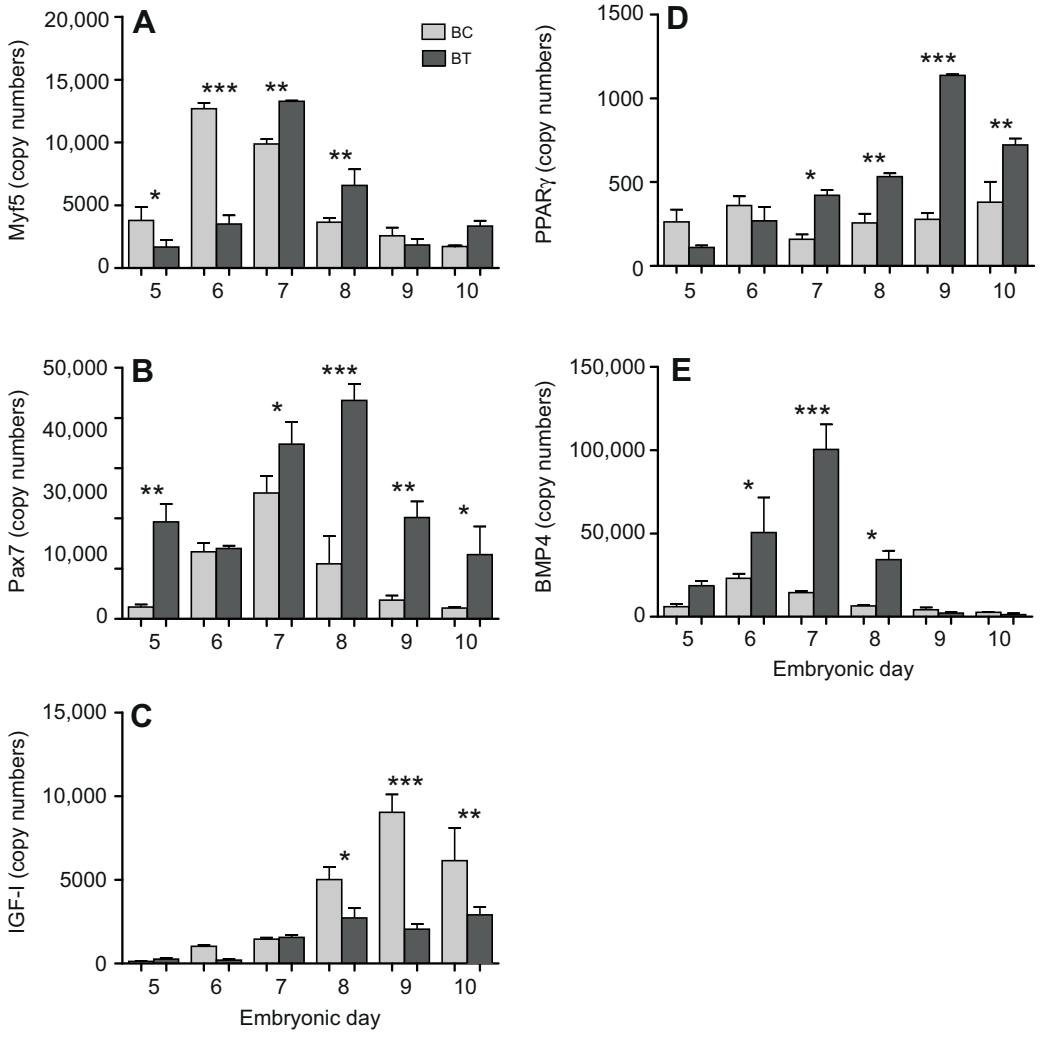

Fig. 4. Real-time PCR analyses on 1 μg of total RNA extracted from the hindlimb of broiler embryos aged ED5–10, incubated at either 37.5°C (BC) or 38.5°C (BT) during ED4–7. The mRNA expressions (copy numbers) for (A) Myf5, (B) Pax7, (C) IGF-I, (D) PPARγ and (E) BMP4 are shown. Results are presented as means ± s.e.m. (N=4 embryos per temperature per day); asterisks represent significant differences (*P<0.05; **P<0.01; ***P<0.0001) between incubation temperatures within each embryonic stage.

analysis software (Zeiss, Oberkochen, Germany). For measurement of the entire gastrocnemius muscle CSA (ED18), low-magnification pictures (×25) were taken. For myofibre and nuclear quantification, high-magnification pictures (×400) were taken, choosing four frames at random from each muscle section. The total number of fibres and nuclei was counted in each of the frames and the mean number is presented as the total apparent fibre number in the entire muscle CSA (such that over 40% of the whole muscle section was counted). The CSA of ~200 fibres in each of the four frames was measured and the mean fibre area calculated (such that over 50% of the fibres in a frame were measured).

For direct comparison of intramuscular fat staining, four random frames were captured (×400) from each muscle section and the area of staining was represented as a percentage of entire muscle CSA.

For quantification of femur CSA, i.e. cortical thickness (ED18), low-magnification pictures (×25) were taken and the outer bone area was subtracted from the medullary cavity.

Statistical analysis

Data were analysed using GraphPad Prism version 5.00 (GraphPad Software, LA Jolla, CA, USA). Pair-wise comparisons were carried out between either LC and LT embryos or BC and BT embryos on various EDs using one-way ANOVA. Provided a significant F-ratio (P<0.05) was obtained, *post hoc* Bonferroni tests were performed to determine treatment effects for each ED. Area under the curve analyses were performed on embryonic motility data using GraphPad Prism. Numerical values are presented as means ± s.e.m. of four replicate embryos.

RESULTS

Physical activity *in ovo*

Analysis of *in ovo* body movements was carried out from ED5 to 10 (Fig. 2A,B). LT embryos were more motile than LC embryos on ED9 (twofold, P<0.0001) and ED10 (1.4-fold, P<0.0001) whereas BT embryos moved more than BC embryos on ED8 (1.9-fold, P<0.0001), ED9 (1.4-fold, P<0.0001) and ED10 (1.3-fold, P<0.0001). Area under the curve analysis between ED5 and 10 revealed that although broilers were more active than layers, the temperature treatment promoted a greater increase in activity in layer embryos (46%) than in broilers (34%).

Body mass

Embryos were weighed from ED5 to 10 and then again on ED14, 16 and 18 (Fig. 2C,D). LT embryos were heavier than their control counterparts on ED14 (1.4-fold, P<0.0001), ED16 (1.3-fold, P<0.0001) and ED18 (1.1-fold, P<0.0001). BT embryos were heavier than their controls on ED16 (1.1-fold, P<0.01) and ED18 (1.2-fold, P<0.001).

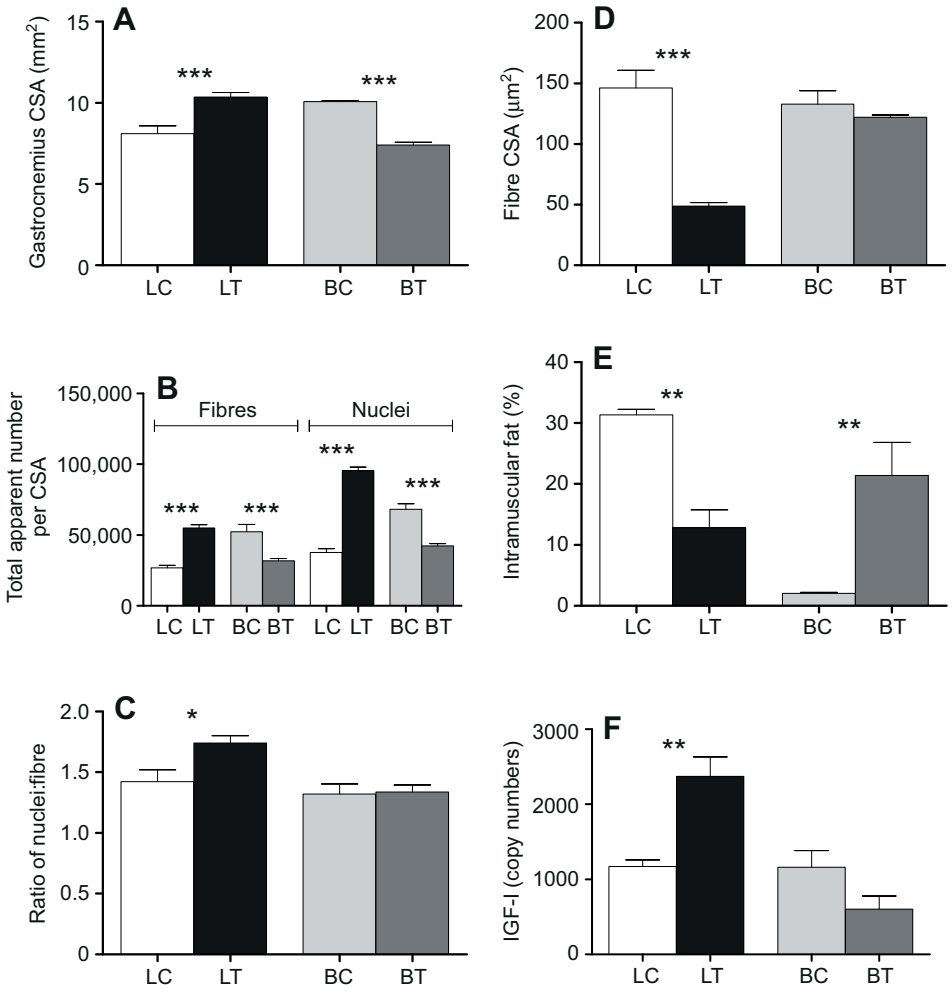

Fig. 5. Gastrocnemius muscle cellular characteristics and IGF-I expression at ED18. (A) Gastrocnemius muscle cross-sectional area (CSA), (B) total apparent fibre and nuclei numbers per CSA, (C) nuclei to fibre ratios, (D) average fibre CSA, (E) intramuscular fat area and (F) IGF-I mRNA expression in the gastrocnemius muscles of layer and broiler embryos incubated at either 37.5°C (LC and BC) or 38.5°C (BC and BT) during ED4–7. Results are presented as means ± s.e.m. (N=4 embryos per temperature per breed per day); asterisks represent significant differences (*P<0.05; **P<0.01; ***P<0.0001) between incubation temperatures within breeds on ED18.

Gene expression levels in the layer hindlimb

Analysis of hindlimb mRNA levels was carried out between ED5 and 10 (Fig. 3). Myf5 mRNA expression peaked on ED7 in LC and LT embryos (Fig. 3A). Myf5 levels were higher in LT on ED5 (4.2-fold, P<0.0001), ED6 (3.3-fold, P<0.0001), ED7 (1.7-fold, P<0.0001) and ED8 (2.8-fold, P<0.01) but were statistically similar on ED9 and 10 (Fig. 3A). Pax7 mRNA levels (Fig. 3B) were higher in LT embryos on each of the days tested, with the greatest differences observed on ED9 (fivefold, P<0.0001) and ED10 (3.6-fold, P<0.0001). IGF-I mRNA levels increased in LT embryos on ED9 (twofold, P<0.01) and ED10 (4.5-fold, P<0.0001) but were unaffected by the temperature treatment on ED5–8 (Fig. 3C). The temperature treatment did not affect PPARγ transcript levels at any on the embryonic stages examined (Fig. 3D). BMP4 mRNA levels peaked on ED8 in both LC and LT embryos (Fig. 3E) and were higher in LT embryos on ED6 (15-fold, P<0.0001), ED7 (5.5-fold, P<0.0001), ED8 (twofold, P<0.01) and ED9 (4.6-fold, P<0.01).

Gene expression levels in the broiler hindlimb

Myf5 mRNA expression peaked on ED6 in BC and 1 day later (ED7) in BT embryos (Fig. 4A). The mRNA levels of Myf5 were reduced in BT embryos on ED5 (2.3-fold, P<0.05) and ED6 (3.6-fold, P<0.0001) but were higher on ED7 (1.3-fold, P<0.01) and ED8 (1.8-fold, P<0.01). Pax7 mRNA levels peaked on ED7 and 8 in BC and BT embryos, respectively (Fig. 4B). Pax7 levels were higher in BT embryos on all of the embryonic stages examined, with the exception of ED6 (Fig. 4B). The greatest observed difference was on ED8, where BT levels were fourfold greater (P<0.0001) than in BC embryos. The mRNA levels of IGF-I increased sharply in the later embryonic stages examined in BC embryos, namely ED8–10, but did not increase to the same extent in the BT group (Fig. 4C). As a result, IGF-I levels were lower in BT embryos on ED8 (1.8-fold, P<0.05), ED9 (4.5-fold, P<0.0001) and ED10 (twofold, P<0.01). The mRNA levels of PPARγ (Fig. 4D) were higher in BT embryos on ED7 (2.7-fold, P<0.05), ED8 (twofold, P<0.01), ED9 (fourfold, P<0.0001) and ED10 (1.9-fold, P<0.01).

BMP4 mRNA levels peaked on ED6 and 7 in BC and BT embryos, respectively (Fig. 4E). BMP4 mRNA levels were higher in BT embryos on ED6 (2.2-fold, P<0.05), ED7 (sevenfold, P<0.0001) and ED8 (5.2-fold, P<0.05).

Gastrocnemius muscle cellularity and fat content

At ED18, the gastrocnemius muscle CSA was increased (1.3-fold, P<0.0001) in LT embryos compared with LC embryos but reduced (1.4-fold, P<0.0001) in BT embryos compared with BC embryos (Fig. 5A). The total apparent number of muscle fibres was increased in LT embryos (twofold, P<0.0001) but was reduced in BT embryos (1.6-fold, P<0.0001) compared with their respective controls; in a similar fashion, the total apparent number of myonuclei was increased in LT embryos (2.5-fold, P<0.0001) but reduced in BT embryos (1.6-fold, P<0.0001; Fig. 5B). As such, the ratio of nuclei

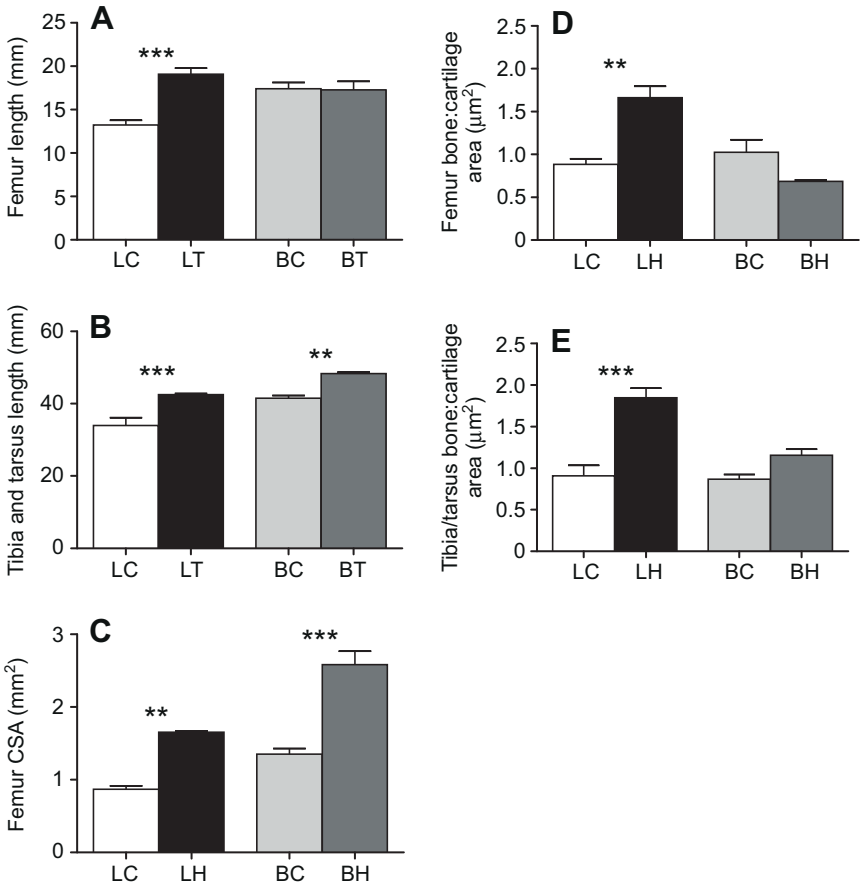

Fig. 6. Limb skeletogenesis characteristics at ED18. (A) Femur length, (b) tibia and tarsus length, (C) femur CSA, (D) femur bone to cartilage ratio and (E) tibia and tarsus bone to cartilage ratio in layer and broiler embryos incubated at either 37.5°C (LC and BC) or 38.5°C (LT and BT) during ED4–7. Results are presented as means ± s.e.m. (N=4 embryos per temperature per breed per day); asterisks represent significant differences (**P<0.01; ***P<0.0001) between incubation temperatures within breeds on ED18.

to fibres was raised in LT embryos (1.2-fold, P<0.05) but was unaffected in BT embryos (Fig. 5C). Mean fibre CSA was reduced in LT embryos (threefold, P<0.0001) but no differences were observed between BC and BT embryos (Fig. 5D). The percentage of intramuscular fat was reduced in LT embryos (2.4-fold, P<0.01) yet markedly increased in BT embryos (10-fold, P<0.01) compared with their respective controls (Fig. 5E).

IGF-I mRNA levels in the gastrocnemius muscle

On ED18, IGF-I mRNA levels in the gastrocnemius muscle were higher (twofold, P<0.01) in LT embryos than in their controls (Fig. 5F). There were no significant differences in IGF-I mRNA levels between BC and BT embryos (Fig. 5F).

Leg bone morphology

Chick legs were sampled on ED18 for assessment of gross and histological parameters (Fig. 6). Following temperature treatment, the femur (Fig. 6A) was longer in LT embryos (1.5-fold, P<0.0001) as was the combined tibia/tarsus (1.3-fold, P<0.0001; Fig. 6B). Femur lengths did not vary between BC and BT embryos, yet the tibia/tarsus was longer in BT embryos (1.2-fold, P<0.01). The mid-femur CSA (Fig. 6C) was increased in both LT (1.9-fold, P<0.01) and BT (1.9-fold, P<0.0001) embryos compared with their respective controls.

The ratio of mineralised bone to cartilage area in the femur was higher in LT embryos than controls (1.9-fold, P<0.01), yet there was no difference in broilers (Fig. 6D). Similarly, the bone to cartilage ratio in the tibia/tarsus was higher in LT embryos than controls (twofold, P<0.0001) but no differences were observed in broilers (Fig. 6E).

DISCUSSION

Phenotypic diversity in domestic chickens and specialisation into meat-producing broilers and egg-producing layers is believed to result from complex synergistic interactions between genomic alterations and variations in the egg-yolk environment (Ho et al., 2011; Rubin et al., 2010). This implies that *in ovo* environmental manipulation may differentially influence the epigenetic regulation of the broiler and layer genomes, leading to diverging phenotypic changes. We hypothesized that a temperature manipulation protocol that has been shown to promote limb musculoskeletal development in layer embryos (Hammond et al., 2007) would induce different phenotypic changes in broiler embryos.

Differential effect of incubation temperature on embryonic muscle phenotype

A previous study has shown that raising the incubation temperature of layer eggs by 1°C on ED4–7 led to increased embryonic limb myogenesis, characterised by increased gastrocnemius muscle mass, increased numbers of fibres and nuclei as well as increased nuclei to fibre ratio on ED18 (Hammond et al., 2007). In line with this previous report, the present study shows that the same temperature treatment increased gastrocnemius CSA as well as the number of fibres and nuclei on ED18, thereby confirming previous phenotypic changes in LT embryos and showing reproducibility. As hypothesized, the same temperature treatment protocol induced different phenotypic changes in the gastrocnemius muscle of BT embryos. Instead of promoting myogenesis as in LT, the treatment led to a reduction in gastrocnemius CSA accompanied by reduced fibre and nuclei numbers without affecting the nuclei to fibre ratio or average fibre CSA in ED18 BT embryos. To our knowledge, this

is the first study to show that the same environmental manipulation protocol promotes divergent phenotypic changes in embryos of two different chicken breeds.

A few studies have shown that environmental factors such as maternal nutrition in mammals (Bayol et al., 2005; Bedi et al., 1982) and egg incubation temperature in fish (Albokhadaim et al., 2007) and birds (Maltby et al., 2004) can influence muscle development and subsequent post-natal and/or post-hatching growth. However, very few studies have examined how these environmental factors affect the timing and level of expression of genes that regulate myogenesis. Myogenesis is regulated by transcription and growth factors whose expression levels can be modulated by manipulation of egg incubation temperature (Wilkes et al., 2001; Xie et al., 2001). In the present study, increased gastrocnemius muscle CSA and increased muscle fibre and nuclei numbers on ED18 in LT embryos were preceded by a rise in hindlimb expression of the myogenic regulatory factor Myf5 between ED5 and 8, specifically, around the time of primary fibre formation (ED4–7) (Crow and Stockdale, 1986). Given that Myf5 can direct myogenic cell specification in the absence of other myogenic factors (Valdez et al., 2000), our results are indicative of increased myogenic specification in LT hindlimb. Increased Myf5 mRNA was followed by increased expression of BMP-4 on ED6–9 and IGF-I mRNAs on ED9–10 and ED18 in LT. Although not entirely specific to myogenesis, BMP-4 and IGF-I have both been shown to regulate myogenic factor expression and thereby myogenic proliferation and differentiation (Adi et al., 2002; Amthor et al., 1999). Primary fibres are believed to play a scaffolding role for the formation of secondary fibres (ED8–16) (Crow and Stockdale, 1986; Lee et al., 2004); therefore, increased proliferation and myogenic commitment during primary fibre formation (Myf5) followed by a rise in growth factor expression (BMP-4 and IGF-I) help explain the subsequent myofibre hyperplasia observed on ED18 in LT embryos. In broiler embryos, the same temperature treatment led to impaired myogenesis on ED18. This was preceded by a shift in the peak of Myf5 mRNA levels from ED6 to ED7 without affecting expression levels. Given the well-characterised function of Myf5 in myogenic cell specification (Buckingham et al., 2003), unchanged Myf5 levels in the BT hindlimb indicate that myogenic cell specification was not increased. Although BMP-4 mRNA levels increased on ED6–8, IGF-I mRNA levels decreased from ED8 to 10 in BT embryos. From this we conclude that differences in the timing and level of Myf5 and IGF-I expression in the developing hindlimb help explain the subsequent phenotypic differences with regards to gastrocnemius CSA and numbers of fibres and nuclei on ED18 in layer and broiler embryos subjected to the same temperature manipulation protocol. Nevertheless, a multitude of signals regulate limb myogenesis, including fibroblast growth factors, sonic hedgehog, Wnt signalling and others (for a review, see Duprez, 2002). Therefore, it would be valuable to perform a transcriptome analysis of these tissues to obtain a more complete overview of the differential changes in gene expression and associated signalling pathways involved in such phenotypic changes.

The upstream mechanisms leading to changes in the expression of myogenic transcription and growth factors and the subsequent muscle phenotypic differences reported in this study are unknown. A potential mechanism may be the differential interactions between the high incubation temperature and the egg-yolk environment. A recent study has shown that there were differences in the yolk composition of layer and broiler eggs and that the egg-yolk environment influenced the morphological and physiological development of broiler and layer embryos; such changes may be mediated through differences in yolk thyroid hormone and testosterone (Ho et al., 2011). Higher incubation temperatures have been shown to affect yolk-sac utilisation and yolk-free body mass in chick embryos, but differences are dependent upon the protocol used. For instance, increasing egg incubation temperature by 1 to 1.5°C between ED14 and 20 leads to increased yolk-sac utilisation and greater body mass at hatch (Leksrisompong et al., 2007), whereas increasing the temperature to 38.5°C for 6 h daily between ED10 and 18 reduced yolk-sac utilisation (Yalcin et al., 2008). It is unclear whether these temperature manipulation protocols differentially affect egg-yolk utilisation in a breed-specific manner. In the present study, yolk composition and yolk-sac utilisation were not measured. However, we can speculate that the temperature treatment may differentially affect yolk-sac utilisation and the transfer of nutrients and hormones from the yolk to the developing layer and broilers embryos, thereby leading to the diverging muscle phenotypic changes reported. Environmentally induced phenotypic changes are believed to be mediated through epigenetic mechanisms (Ho et al., 2011). Therefore, genome-wide epigenetic studies combined with yolk composition and yolk-sac utilisation studies would provide clarification as to how temperature manipulation impacts on embryonic muscle phenotype in a breed-specific manner.

Possible mesenchymal cell commitment shift away from myogenesis in the treated broiler embryo hindlimb

An important observation made in the present study was that instead of stimulating myogenesis as in layers, the temperature treatment promoted intramuscular adiposity in the gastrocnemius muscle of broiler embryos on ED18. Concomitantly, intramuscular fat was reduced in temperature-treated layers. This indicates that the balance between muscle *versus* adipose differentiation was differentially shifted in the gastrocnemius muscle of these two breeds in response to the same temperature treatment.

Like myoblasts, adipocytes originate from mesenchymal stem cells (Bowers and Lane, 2007) and their differentiation into functional adipocytes is predominately regulated by peroxisome proliferative activated receptor gamma (PPAR)-γ (Wang et al., 2008). Furthermore, it has been suggested that PPARγ may be involved in the adipogenic conversion of satellite cells during intramuscular adipose tissue generation (Vettor et al., 2009). Interestingly, increased intramuscular adiposity in BT embryos at ED18 was preceded by increased hindlimb PPARγ mRNA levels between ED7 and 10 whereas those levels were unaffected in ED5–10 LT embryos. Given the essential role of PPARγ in adipocyte differentiation (Rosen et al., 1999), the upregulation of its transcript from ED7 to 10 may indicate that a greater proportion of mensenchymal stem cells in the developing limb committed to the adipogenic lineage in BT embryos. Given that increased intramuscular adiposity was concomitant with limb muscle atrophy and fibre hypoplasia, such a shift in adipogenic cell specification may have occurred at the expense of myogenesis in BT embryos. A possible effect of temperature manipulation on muscle *versus* adipose cell commitment is further supported by evidence that both myogenesis and adipogenesis are epigenetically regulated (Chen et al., 2011; Li et al., 2010; Saccone and Puri, 2010). Furthermore, the topic of muscle *versus* fat cell specification and differentiation has recently gained considerable interest with the discovery that brown adipocytes derive from Myf5-expressing cells and that the transcription factor PRD1-BF1-RIZ1 homologous domain containing 16 (PRDM16) controls the differentiation shift between brown fat *versus* muscle in mice (Seale et al., 2008). Brown adipose tissue transfers energy from food to heat and its development and

activity are affected by temperature, exercise and nutrition (Barbatelli et al., 2010; Cannon and Nedergaard, 2004; Xu et al., 2011). However, birds do not have brown adipose tissue, and no avian uncoupling protein (UCP)-1 gene homolog, the molecular signature of brown adipose tissue, has been identified (Saarela et al., 1991). Nevertheless, a study has shown that mesenchymal cells isolated from embryonic chick limb buds differentiate into brown adipocyte-like cells (Mezentseva et al., 2008), which express PPARγ. It is unclear whether these avian cells also express Myf5. Furthermore, a predicted mRNA sequence for avian PRDM16 has been submitted to the NCBI database (accession number XM_417551.2). We have attempted to measure expression levels of PRDM16 by real-time PCR with primers designed from the predicted avian sequence. Although specific transcript could be detected, the levels of expression were too low for accurate quantification (data not shown), thus no conclusions could be drawn.

The possibility that temperature manipulation leads to a differential shift in mesenchymal cell commitment in favour of adipogenesis and at the expense of myogenesis in BT, while the opposite may occur in LT embryos, requires further investigation.

Temperature, embryonic motility and bone growth: differential outcome on myogenesis

It well documented that the developments of skeletal muscle and bone are coordinated. Evidence of this comes from previous studies in which chick embryos treated with paralytic drugs exhibited reduced longitudinal bone growth, reduced osteogenesis and limb deformities (Lamb et al., 2003). Conversely, bone growth is believed to promote muscle growth through stretch stimuli (Lowe and Alway, 1999). In the present study, the temperature treatment increased embryonic motility in both layer (ED9–10) and broiler (ED8–10) embryos but with greater magnitude in layer (46%) than in broiler embryos (34%). In line with previous suggestions that increased embryonic motility promotes limb musculoskeletal development (Hammond et al., 2007), increased activity in LT embryos was accompanied by a concomitant increase in limb bone growth and myogenesis. However, this did not apply to BT embryos, in which increased activity was accompanied by an increase in only two of the bone parameters measured (tibia tarsus length and femur CSA) and impaired gastrocnemius muscle development. Anatomically, the gastrocnemius muscle is anchored to the femur bone proximally and to the tarsometatarsus bone distally, and it thus runs alongside the tibia bone (Kardon, 1998; King and McLelland, 1984). Therefore, any increase in tibia length implies that the developing gastrocnemius muscle is undergoing stretch. Muscle stretch has been shown to augment the expression of myogenic regulatory factors (Lowe and Alway, 1999) and IGFI (Goldspink, 1999), leading to increased muscle mass and muscle fibre hypertrophy. Therefore, in LT embryos, the concomitant increase in tibia length and muscle Myf5 and IGF-I mRNAs implies that mechanical stretch may have mediated the increase in gastrocnemius muscle CSA, fibre hyperplasia and myogenic cell proliferation, observed on ED18 in response to the temperature treatment. In BT embryos, the same temperature treatment also increased tibia length, implying that the developing gastrocnemius muscle also experienced stretch. However, this mechanical stimulus did not result in increased gastrocnemius muscle development. Furthermore, neither Myf5 nor IGF-I mRNAs were increased in the BT hindlimb. Therefore, the developing gastrocnemius muscle of temperature-treated broiler embryos did not appear to respond to mechanical stimulation. This implies that the impact of increased embryonic activity on limb bone and muscle development may be differentially regulated in broiler and layer embryos.

Like myoblasts and adipocytes, osteoblasts originate from the mesenchyme (Chen et al., 2011). The myocyte enhancer factor-2 interacting transcriptional repressor (MITR) has been shown to act as a molecular switch to promote osteogenesis by inhibition of adipogenesis through inactivation of PPARγ transcriptional activity (Chen et al., 2011). Increased PPARγ in BT hindlimb could reflect reduced MITR activity, leading to reduced osteoblast specification. As a measure of osteogenesis, we calculated the bone to cartilage ratio as described by Lamb et al. (Lamb et al., 2003). Data showed that this ratio was unaffected in either the femur or tibia/tarsus bone of BT embryos whereas it increased in both bones in LT embryos. Although these data do not provide clear evidence of reduced osteogenesis in BT embryos, they show that the temperature treatment did not stimulate osteogenesis in BT embryos to the same extent as in LT embryos. Together with differential changes in PPARγ mRNA, this may indicate a differential cell commitment shift between osteogenic and adipogenic lineages in treated layer and broiler embryos. Therefore, a thorough examination of differential mesenchymal stem cell commitment shift into muscle, bone and fat in BT and LT embryos would be valuable.

Possible increase in satellite cell specification and implications for animal welfare

In both layer and broiler breeds, the temperature treatment led to increased Pax7 expression on all embryonic days examined in LT embryos and on all but ED6 in BT embryos. Pax7 is upregulated in proliferating muscle precursor cells and is required for satellite cell specification during embryogenesis as well as their post-natal survival (Buckingham and Relaix, 2007; Halevy et al., 2004; Mansouri et al., 1996; Seale et al., 2000). In the present study, increased Pax7 expression may therefore be indicative of increased satellite cell specification in both temperature-treated layer and broiler embryos. Given that increased muscle loading promotes satellite cell activation and fibre hypertrophy and that satellite cell deficiency is a limitation to skeletal muscle adaptation to loading (Adams, 2006), our findings indicate that the egg temperature treatment studied may help increase satellite cell supply. This may in turn increase the potential for leg muscle adaptation to loading while body mass increases rapidly post-hatch, thereby helping to improve postural stability in growing broiler chicks.

In contrast, it has been proposed that Pax7-expressing cells may contribute to intramuscular fat formation through upregulation of PPARγ (Vettor et al., 2009). Therefore, the concomitant upregulation of both PPARγ and Pax7 in temperature-treated broilers implies that some Pax7-expressing cells may differentiate into the adipogenic lineage and further contribute to increased intramuscular fat post-hatch. However, this possibility is disputable given that more recent evidence suggests that Pax7-positive cells are committed to the myogenic lineage and do not spontaneously adopt an adipogenic fate (Starkey et al., 2011).

Overall, the present data show that the temperature treatment studied impaired limb myogenesis in embryonic broilers and did not promote osteogenesis to the extent it did in layer embryos. Therefore, the potential benefits of the treatment on broiler post-hatch postural stability and welfare appear limited, but remain to be fully investigated.

Conclusions

This study shows that a transient increase in incubation temperature early in embryonic development produces differential phenotypic changes in the developing limbs of broiler and layer embryos. These phenotypic changes are accompanied by differential changes in

expression levels of genes involved in myogenic development and growth. Potential upstream genomic/epigenomic interactive mechanisms may be involved and require further investigation. It is also unclear whether such phenotypic differences will affect post-hatch development and growth and whether they will persist into adulthood. However, this *in ovo* model offers great potential for studying complex interactions between the embryonic environment, the genome and its epigenetic regulation and how such interactions mediate phenotypic evolution.

LIST OF ABBREVIATIONS

BC	broiler control
BMP	bone morphogenic protein
BT	broiler treated
ED	embryonic day
HGF	hepatocyte growth/scatter factor
IGF	insulin-like growth factor
LC	layer control
LT	layer treated
MITR	myocyte enhancer factor-2 interacting transcriptional repressor
PI3K	phosphoinositide 3-kinase
PPAR	peroxisome proliferative activated receptor
PRDM16	PRD1-BF1-RIZ1 homologous domain containing 16
RT-PCR	reverse transcription polymerase chain reaction

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