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Influence of incubation temperature on embryonic Days 4–7 on gene expression, *M. pectoralis* and *M. gastrocnemius* microstructure, and tibia characteristics in Ross 308 Broilers

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ABSTRACT

The incubation temperature can influence chicken bone and muscle development, and breast and leg muscles may respond differently to changes therein. Ross 308 broiler eggs were incubated at 36.5°C, 37.5°C (control), 38.5°C and 39.0°C on embryonic days 4–7. *M. pectoralis* and *M. gastrocnemius* were sampled at hatching and 5 weeks post-hatching, and differences in gene expression, microstructure and energy metabolism were examined. Tibia strength was tested, and gait scoring was performed. Broiler performance was similar at slaughter age, but initial growth was slowed by the 36.5°C treatment. The 38.5°C treatment reduced the *M. gastrocnemius* weight and shifted the *M. pectoralis*/*M. gastrocnemius* ratio. All treatments reduced *M. gastrocnemius* glycogen content relative to the control. The expression of single myogenesis-related genes was altered in the *M. pectoralis* by 36.5°C. The results indicate that the incubation temperature influences broiler muscle energy metabolism, and that the temperature of 38.5°C hampers leg muscle development.

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Breast muscle; bone strength; gait score; embryogenesis; histology

Introduction

Modern commercial broiler chickens have been selected for efficient and fast growth, reducing the time required to reach a suitable weight and conformation for slaughter (Zuidhof et al., 2014; Tallentire et al., 2016). The dramatic increase in these chickens' growth has made them resource-sparing sources of high-quality protein, but has come with compromises in animal welfare. The anatomical properties of these animals (large breast muscles and short, widely spaced legs) place high degrees of stress on their locomotor systems during rapid growth, leading to distinct gait dynamics and a predisposition for gait impairment at the age of slaughter (Paxton et al., 2013). About one-fifth to one-quarter of broiler chickens have abnormal gaits that cause pain (Caplen et al., 2013; Kittelsen et al., 2017; Granquist et al., 2019; Riber et al., 2021).

Such welfare problems could be mitigated during the incubation period, as all chicken organ systems, including the muscles and bones, develop in the egg. The muscles develop from three 'generations' of myoblasts, which proliferate at specific intervals and differentiate into myofibers. Embryonic myoblasts proliferate on embryonic days (EDs) 4–7, followed by foetal myoblasts on EDs 8–12 (Miller & Stockdale, 1987; Biressi et al., 2007). Thereafter, adult myoblasts are present and can proliferate after hatching when stimulated, for example by muscle damage (Morgan & Partridge, 2003; Jankowski et al., 2020). The first steps of bone development occur on day 4 of embryogenesis, but this development, including organisation and mineralisation, is completed only after hatching (Caplan, 2007).

Our review of the literature revealed that changes in the incubation temperature during early, mid-term and late embryogenesis can induce changes in muscle and bone development in broiler chickens (Kettrukat et al., 2023). Previous studies have shown that higher temperatures in the first week of incubation affect leg muscle proliferation and structure in layer and broiler chickens, but these analyses were performed with embryos (Hammond et al., 2007; Al-Musawi et al., 2012). Improved walking ability has been observed with an increase in the incubation temperature during week 2 (Guz et al., 2020) and a decrease in this temperature on EDs 10–18 (Ipek & Sozcu, 2016). The incubation temperature affects the locomotor system on various

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levels; relative to a decreased temperature, an increased temperature in the second week of incubation increases the activities of metabolic muscle enzymes (Krischek et al., 2016, 2018). The application of a temperature of 38.8°C for 8 h/day on EDs 10–14 increased the expression of myogenesis-associated genes (Yalcin et al., 2022). Furthermore, changes in the incubation profile, such as the use of a lower starting temperature, can improve bone ash in newly hatched broilers (Groves & Muir, 2014).

We showed previously that the breast and leg muscles of broiler chickens respond differently to temperature manipulation in week 1 of incubation, leading to a shift in the pectoralis major (*M. pectoralis*) to gastrocnemius (*M. gastrocnemius*) ratio (Oksbjerg et al., 2019). In the present study, we changed the incubation temperature on EDs 4–7 and assessed *M. pectoralis* and *M. gastrocnemius* development at multiple levels (weight, gene expression, microstructure and metabolism) at hatching and 5 weeks thereafter. We also assessed bone size and strength and walking ability and performance to comprehensively investigate the influence of the early incubation temperature on locomotor system development in broiler chickens. We expected to observe differences in the responses of the breast and leg muscles and in responses to different temperatures, as both fast-twitch and slow-twitch muscle fibres form during this incubation period (Crow & Stockdale, 1986). In other words, we hypothesised that the responses of the *M. pectoralis* and *M. gastrocnemius* would differ according to their fibre composition (98% type IIB and mixed, respectively) (Ono et al., 1993). Whereas previous studies of the effects of temperature manipulation on muscle microstructure and metabolism have had an economic, meat quality – related focus (Krischek et al., 2013; Janisch et al., 2015), this study focusses on locomotor system development in relation to broiler chickens' walking ability and welfare, addressed in few previous studies (Ipek & Sozcu, 2016; Oksbjerg et al., 2019). The improvement of these parameters via changes in the incubation temperature would provide a cost-efficient solution for intensive commercial rearing systems.

Materials and methods

Animals and incubation procedure

This experiment was performed in accordance with the Danish Veterinary and Food Administration's animal welfare requirements (LBK 253, 08/03/2013). In total, 420 broiler eggs of the strain Ross 308 were obtained from a parent flock aged 37 weeks. On arrival, the eggs were numbered, examined for damage (e.g. cracks) and weighed. The heaviest and lightest eggs were excluded, and the rest were divided into 8 batches of 45 eggs each with approximately the same combined weight. VirkonS solution (1%; Pharmaxim AB, Helsingborg, Sweden) was sprayed onto the eggshells for disinfection. Then, the eggs were kept at a 45° angle at room temperature for 40 h, during which time they were turned twice. At setting, the eggs were placed in eight Rcom MX-50 incubators (Autoelex Co. Ltd., Gyeongsangnam-do, Korea), which automatically maintained the temperature and humidity and turned them every hour. Two temperature loggers (Stowaway TidbiT; Onset Computer Corporation, Bourne, MA, USA) were placed in each incubator.

The incubation protocol is presented in Figure 1. Four temperature treatments (36.5°C, 37.5°C (control), 38.5°C and 39.0°C) were applied to two incubators each on EDs 4–7. To monitor the temperature and humidity, the incubators' water tanks were checked every other day and refilled when necessary, and the shell temperatures of five pre-selected eggs per incubator were measured using an ear thermometer (Thermoscan 9; Braun, Kronberg, Germany) and documented every other day.

On day 12 post-setting, candling of the eggs was performed. Eggs with no sign of a living embryo were removed and opened. Infertile eggs were separated from embryonic deaths. On day 18 post-setting, the remaining eggs were transferred to a hatcher with a maintained temperature of 37.5°C and humidity of 65%. Before transfer, the eggs were candled again to exclude those with no sign of a living embryo.

Sampling of newly hatched chicks

To enable the matching of hatched chicks to their eggs, the eggs were separated by a wooden grid after transfer to the hatcher. Hatched chickens with dried feathers were taken out of the incubator in the morning on days 20, 21 and 22 post-setting, and thus were not of exactly the same age. Thus, we refer to the number of days post-setting in the Materials and Methods section and the age of chicks hatched on day 21 in subsequent sections for better readability. The hatchability values (percentages of chicks hatched among all fertile eggs) were 80% (36.5°C), 81% (37.5°C), 63% (38.5°C) and 47% (39.0°C). The sexes were not equally distributed; the percentages of males were 55% (36.5°C), 47% (37.5°C), 52.3% (38.5°C) and 39.5% (39.0°C). All chicks were wing-marked and weighed. Half of the chicks were allocated to eight 1.65-m^2 floor pens according to the incubators in which they hatched, and the other half were sacrificed for sampling.

From each of the sacrificed chicks, the *Mm. pectorales* attached to the sternum and the *Mm. gastrocnemii* were

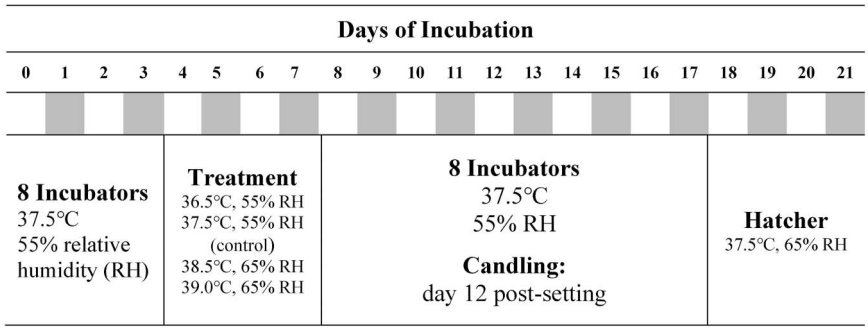

Figure 1. Incubation procedures and treatments. RH, relative humidity.

removed and weighed. Samples were taken for histological analysis by covering in TissueTek OCT (Sakura Finetek USA Inc., Torrance, CA, USA) and freezing in isopentane cooled by liquid nitrogen. Samples for RT-qPCR were frozen in liquid nitrogen. The samples were placed in cryotubes (Nunc; Thermo Fisher Scientific Inc., Waltham, MA, USA) and kept in liquid nitrogen until storage at −70°C. The yolk sac was removed and weighed, and the sex of the chick was noted according to the gonads.

Rearing

The chicks allocated to the floor pens were raised on commercial starter feed (Optima Mini Start; DLG, Fredericia, Denmark). On day 10 post-hatching (31 days after setting), the feed was changed to a growth formula (Optima SAL Medio; DLG), the size of each floor pen was adjusted according to the number of the chickens in it (ca. 0.075 m^2/chicken), and the chickens were weighed. Feed efficiency (kilogram feed/kilogram body weight) was recorded at the pen level from days 1–10 and days 10–34 post-hatching. The average feed efficiency values were 1.20 (36.5°C), 1.23 (37.5°C), 1.23 (38.5°C) and 1.29 (39.0°C) from day 1–10 and 1.39 (36.5°C), 1.39 (37.5°C), 1.30 (38.5°C) and 1.44 (39.0°C) from day 10–34.

Gait scoring

On day 33 post-hatching (day 54 post-setting), the gaits of all chickens were scored as described by Kestin et al. (1992). In each pen, a separate area was created with a fence and individual chickens were placed in it. Chickens that did not walk voluntarily were encouraged gently by touching their tail feathers or tapping the fencing behind them.

Sampling of 5-week-old chickens

On days 34 and 35 post-hatching (days 55 and 56 post-setting), all remaining chickens were euthanised with CO_2 and weighed. Samples for histological and RT-qPCR analyses were taken from the *M. pectoralis* and *M. gastrocnemius* as described above. Samples for the measurement of the glycogen content were taken from both muscles in a manner similar to RT-qPCR sample collection. The right tibia was removed from each chicken, the attached muscle tissue was roughly removed, and the bone was stored at −20°C. The sex of each chicken was noted according to the gonads.

Measurement of tibia characteristics

The tibiae were thawed, and the periost with attached muscle remnants and cartilage was removed using a knife. Then, the bone length and weight were determined. The bone strength was measured as described by Oksbjerg et al. (2019) using a texture analyser (TMS Touch SE-no 13–1067–02, Spectronic CamSpec Ltd, Leeds, United Kingdom). Briefly, a breaking curve was generated, and the distance to fracture (millimetres), slope of the force increase (Newtons/millimetre), peak force (Newtons), tibia diameter (millimetres) and work [area under the curve (AUC); Newtons*millimetre] were determined.

Histological analysis

The frozen samples were cut to 10 µm using a cryostat (Leitz 1720 digital, Leitz, Stuttgart, Germany), mounted on glass slides and stored at −20°C until staining. Six samples per sex and incubation treatment were randomly chosen and stained with hematoxylin and eosin (H&E) for the measurement of muscle fibre diameter, nicotinamide adenine dinucleotide – tetrazolium reductase (NADH–TR) for the examination of muscle fibre type and Oil Red O for the measurement of the intramuscular fat percentage. The samples obtained at hatching were stained only with H&E. H&E staining was performed according to Dubowitz and Sewry (2007); Mayer's hematoxylin was first applied to stain

the nuclei blue, and eosin solution was then applied to stain the cytoplasm pink. For NADH-TR staining, the slides were incubated in a NADH – nitro blue tetrazolium (NBT) solution (450 µM NADH, 978 µM NBT) at 37°C for 30 min. This solution stains oxidative muscle fibres dark blue, glycolytic fibres light blue and intermediate fibres medium blue. Oil Red O working solution (0.5% Oil Red O solution in isopropanol diluted to 0.3% with distilled water) was used to stain lipids red. The staining quality was suitable for four to six samples per group and sex.

For analysis, the samples from hatchlings and adult chickens were placed under a light microscope with 40×/0.75 and 10×/0.30 objectives, respectively, and three random fields per specimen were photographed using the Infinity Capture software (version 6.0.0; Lumenera Corporation, Ottawa, Canada). Images of the H&E – and NADH-TR – stained slides were analysed using the ImageJ software (version 1.54f, National Institutes of Health, Bethesda, MD, USA). The muscle fibres' cross-sectional area, density (number per square millimetre) and type proportions were determined. The Oil Red O – stained slides were analysed using the MultiScan Base software (version 18.03; Computer Scanning System II, Warsaw, Poland). The percentages of red staining on four images per specimen were calculated. After staining, not all samples were of suitable quality for analysis, which left four to six samples per group and sex for the statistical analysis, as specified in Table 3.

Glycogen content measurement

Subsamples (25 mg) of the samples prepared for glycogen content measurement were obtained. The total glycogen content was determined using the method described by Sterten et al. (2010). Briefly, the glycogen was hydrolysed by the addition of 1 M HCl and heated in a water bath at 95°C for 2 h. The liquid phase was frozen and stored at –80°C until further analysis. The absorbance was then measured using an ABX Pentra 400 auto-analyser (Horiba, Kyoto, Japan). The glucose residue, as a measure of the glycogen content, was calculated using the following formula:

$$\left[\frac{\mu mol}{g}\right] = \frac{glucose\ residues\left(\frac{\mu mol}{l}\right) * dilution\ volume * \frac{1000mg}{g}}{muscle\ sample\ weight\ in\ mg}.$$

Gene expression analysis

RNA was extracted from the samples taken for gene expression analysis using an acid-guanidinium-phenol – based reagent/chloroform/isopropanol method (Simms et al., 1993). Prior to isolation, the samples were homogenised in a tissue homogeniser (TissueLyser LT; Qiagen). RNA integrity was checked on a 1.2% agarose gel stained with ethidium bromide – free nucleic acid (SimplySafe; EURx Sp. z o. o., Gdansk, Poland), and the purity and concentration were assessed using a NanoDrop™ spectrophotometer (Thermo Fisher Scientific). cDNA was generated from 900 ng purified RNA using the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions. qPCR was performed using the iTaq™ kit (Bio-Rad Laboratories) on a StepOnePlus real-time PCR system (Applied Biosystems, Waltham, MA, USA). Briefly, the reaction mixture consisted of 500 nM each of the forward and reverse primers, 100 nM probe, 1× iTaq Universal Supermix and 90 ng template. The cycling parameters were 50°C for 2 min, 95°C for 10 min (for initial deactivation of the cDNA polymerase) and 40 cycles at 95°C for 1 min and 60°C for 1 min.

The primer and probe sequences are displayed in Table 1. The primers and probes for ribosomal protein lateral stalk unit P0 (RPLP0), lactate dehydrogenase (LDHA) and vascular endothelial growth factor A were designed by Young and Rasmussen (2020). The primer and probe sequences for insulin-like growth factor 1 (IGF-1), paired box protein 7 (PAX7), myogenin (MyoG) and citrate synthase (CS) were designed using the Primer Express software (version 3.0.1; Thermo Fisher Scientific) based on chicken-specific sequences, and produced by LGC Biosearch Technologies (Lystrup, Denmark).

The results were analysed by relative quantification using the ΔΔCt method, with RPLP0 serving as the reference gene (Livak & Schmittgen, 2001). ΔΔCt values were compared between groups for each muscle and sampling timepoint (hatching and slaughter).

Statistical analysis

The statistical analysis was performed using the R software (version 4.3.1, The R Foundation for Statistical Computing, Vienna, Austria) and R studio (version 2023.9.1.494, Posit PBC, Boston, MA, USA). A generalised linear model including the fixed variables of temperature and sex was created, and the variables' effects were assessed using two-way ANOVA and the 'car' package. The interaction of temperature and sex was not significant for most parameters and was thus left out of the models, resulting in the following:

$$Y_{ij} = \mu + A_i + B_j + e_{ij}$$

where Y_{ij} is the dependent variable, µ is the overall mean, A_i and B_j are the fixed effects of temperature and sex and e_{ij} is the error term. Data from cases with significant interaction are presented in separate figures. P values < 0.05 were

Table 1. Primer and probe sequences used in gene expression analysis by RT-qPCR.

Gene	Primer Forward	Primer Reverse	Probe	Reference
Ribosomal protein lateral stalk unit P0 (RPLP0)	ACACGATGATGCGCAAAGC	CACGTTCCCACGGATGTGA	AACCCCGCCTTGGAGAAGCTGCT	Young and Rasmussen (2020)
Insuline-like growth factor 1 (IGF-1)	GCTGCCGGCCCAGAA	ACGAACTGAAGAGCATCAACCA	CACTGTGTGGTGCTGAG	own design
Paired box protein 7 (PAX7)	CCGTGTTCAGGTGTGGTTCA	TTGCACCCGCCTGCTT	CAACCGACGAGCAAGA	own design
Myogenin (MyoG)	GGAGAAGCGGAGGCTGAAG	GCAGAGTGCTGCGTTTCAGA	AGGTGAACGAAGCCT	own design
Citrate Synthase (CS)	CGACCCGCAGTTCATTGAG	GACGTTGCCCCCTTCGT	TGATGCGCCTCTACC	own design
Lactate Dehydrogenase A (LDHA)	ACGCCGGCAGTACACCAT	GCATGAGCGTGCTCCTCTTT	TCTCAAGGATCATCTCATCCACAATGTCCA	Young and Rasmussen (2020)
Vascular endothelial growth factor A (VEGFA)	GATGTGTACAACGTCACGATGGA	CTGTAAGAAGCTCATGTGCGCTAT	TCGCAAGAATTAAACCCCATCAGAGTCAGC	Young and Rasmussen (2020)

considered to be significant. The data are presented as estimated marginal means, obtained with the 'emmeans' R package, with standard errors of the mean. Post-hoc Tukey's tests were performed using the 'multcomp' package. The hatchability, sex ratio and feed efficiency were not analysed statistically due to the small numbers of observations (two incubators/pens per temperature).

The gene expression data were analysed by calculating the differences between means of the log(fold-change) values of the control and respective treatment groups, and corresponding confidence intervals. A difference between the control and treatment groups was considered to be significant when the confidence interval did not encompass 0 (Nolan & Bustin, 2013).

Results

Performance

Data on the performance parameters, including muscle weights and muscle ratios, are provided in Table 2. At hatching, the absolute and yolk-free body weights were similar between sexes and among treatment groups. On day 10 and thereafter, the body weight and average daily gain (ADG) were significantly greater in male than in female chickens. Up to day 10, the body weight and ADG tended to be lower in the 36.5°C group than in the 38.5°C group ($P = 0.07$). At slaughter age, body weights were similar in all groups.

The *M. pectoralis* weight at slaughter age did not differ among groups. It was greater in males than in females, but this difference was not significant in relation to the body weight. The absolute and relative *M. gastrocnemius* weights were affected by the treatment. The absolute weight was lesser in the 38.5°C group than in the 36.5°C group, and intermediate in the control and 39.0°C groups. The *M. gastrocnemius* weight relative to the body weight was lesser in the 38.5°C group than in the control and 36.5°C groups. Similarly, the *M. pectoralis*/*M. gastrocnemius* ratio was highest, reflecting a lighter *M. gastrocnemius* in relation to the *M. pectoralis*, for the 38.5°C group, and this

Table 2. Performance data and muscle weights of female and male broiler chickens subjected to different incubations temperatures (36.5°C, 37.5°C, 38.5°C, 39.0°C) during embryonic day 4–7.

	Temperature (T)					Sex (S)			*p*-value	
	36.5°C	37.5°C	38.5°C	39.0°C	SEM	F	M	SEM	T	S
n (hatch)	33	35	19	16		50	53			
BW hatch [g]	48.1	47.8	48.0	46.9	0.71	47.3	48.1	0.44	0.53	0.17
Yolk-free BW [g]	41.5	41.3	41.2	40.0	0.64	40.7	41.3	0.37	0.271	0.208
M. gastrocnemius weight hatch [g]	0.135	0.136	0.132	0.122	0.0046	0.127	0.135	0.0026	0.060	0.032
n (up to 5 weeks)	34	38	29	22		65	58			
BW day 10 [g]	370	380	391	384	7.6	371	392	4.8	0.098	<0.01
BW day 35 [g]	2941	2850	2907	2820	49.0	2685	3074	30.6	0.16	<0.01
ADG day 0–10 [g]	32.2	33.3	34.4	33.7	0.74	32.3	34.5	0.47	0.069	<0.01
ADG day 10–35 [g]	100.8	96.9	98.8	95.8	1.74	90.4	105.7	1.09	0.088	<0.01
M. pectoralis weight, 5 weeks [g]	292	281	291	285	7.2	268	308	4.5	0.333	<0.01
M. pectoralis, % of BW	9.89	9.85	10.12	10.20	0.168	9.99	10.04	0.105	0.216	0.715
M. gastrocnemius weight, 5 weeks [g]	13.9a	13.4ab	12.9b	12.9ab	0.35	12.3	14.2	0.22	0.041	<0.01
M. gastrocnemius, % of BW	0.47a	0.47a	0.44b	0.46ab	0.024	0.46	0.47	0.015	<0.01	0.347
Ratio M. pectoralis/ M.gastrocnemius	21.2a	21.1a	23.1b	22.2ab	0.56	21.9	21.9	0.35	<0.01	0.873

F – female; M – male; BW – body weight; ADG – average daily gain; feed efficiency calculated as kg gain per kg feed on a pen-basis, Trt – Treatment, S – Sex; *p*-values below 0.05 were considered statistically significant

[a, b]LSM with different superscript are significantly different ($p < 0.05$); [†]Body weight at hatch based on data from both chicks that were killed at hatch and chicks that were raised to 5 weeks.

value was significantly higher than those for the control and 36.5°C groups.

Muscle microstructure and energy metabolism

The muscle fibre data are provided in Table 3 and Figure 2 and Figure 3. At hatching, significant interaction between treatment and sex was found for the *M. gastrocnemius* fibre size. Females had larger fibres than males in all groups except the 39.0°C group, in which the opposite was true (Figure 2). However, this difference disappeared in the growth phase, and the *M. gastrocnemius* fibre size was similar in all treatment groups and both sexes on day 35 post-hatching. From hatching to the age of 5 weeks, the cross-sectional area of the *M. gastrocnemius* fibres increased 30-fold. At 5 weeks, the *M. pectoralis* had a significantly larger fibre cross-sectional area and lesser fibre density than did the *M. gastrocnemius*. The fibre type proportions are shown in Figure 3. No significant effect of treatment or sex on the fibre composition of either muscle was observed at 5 weeks. The proportions differed between the *M. pectoralis* and *M. gastrocnemius*; glycolytic fibres were most abundant in both muscles, but the *M. pectoralis* contained almost no oxidative fibres while 5–10% of the *M. gastrocnemius* fibres were oxidative ($P < 0.05$).

A tendency for interaction was observed for the *M. pectoralis* intramuscular fat content, which was higher in males than in females, except in the 39.0°C

Table 3. Muscle microstructure and intramuscular fat of M. gastrocnemius and M. pectoralis at hatch and 5 weeks post-hatch in female and male broiler chickens subjected to different incubations temperatures (36.5°C, 37.5°C, 38.5°C, 39.0°C) during embryonic day 4–7.

	Temperature (T)					Sex (S)			*p*-value	
	36.5°C	37.5°C	38.5°C	39.0°C	SEM	F	M	SEM	T	S
n[†]	12(11)	12	13(11)	12		25	21			
M. Gastrocnemius										
CSA day 35 [µm^2]	2681	2806	2627	2582	141.0	2674	2674	99.7	0.698	0.996
Fibre density d 35 [number/mm^2]	226	204	220	226	13.4	221	217	9.5	0.605	0.750
Intramuscular Fat [%]	0.58b	0.38b	0.91ab	4.19a	1.10	1.46	1.57	0.745	0.033	0.914
Intramuscular glycogen [µmol/g]	22.3b	28.9a	22.9b	21.4b	1.27	25.8	21.9	0.79	<0.01	<0.01
M. pectoralis										
CSA day 35 [µm^2]	3218	3278	3679	3385	158.0	3449	3331	112.0	0.152	0.448
Fibre density d 35 [number/mm^2]	150	153	132	159	7.7	143	153	5.4	0.059	0.207
Intramuscular Fat [%]	5.96	4.57	2.65	2.77	1.12	3.12	4.85	0.795	0.116	0.124
Intramuscular glycogen [µmol/g]	22.3b	27.4a	23.8ab	24.5ab	1.55	26.7	22.3	0.96	0.019	<0.01

CSA – cross-sectional area; [†]number in brackets = n at hatch, if different from 5 weeks; Trt – Treatment, S – Sex; *p*-values below 0.05 were considered statistically significant.
[a, b, c]LSM with different superscript are significantly different ($p < 0.05$).

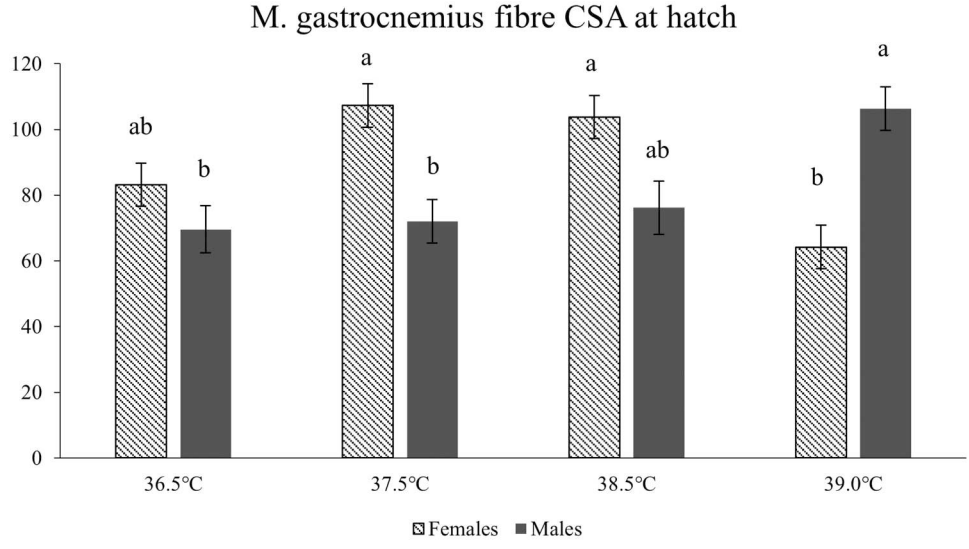

Figure 2. Cross-sectional areas µm^2 of *M. gastrocnemius* muscle fibres in newly hatched female and male broiler chickens subjected to different incubation temperatures on embryonic days 4–7. Animal numbers similar to Table 3, *n* at hatching. Different superscripts (a, b) indicate significant differences ($p < 0.05$).

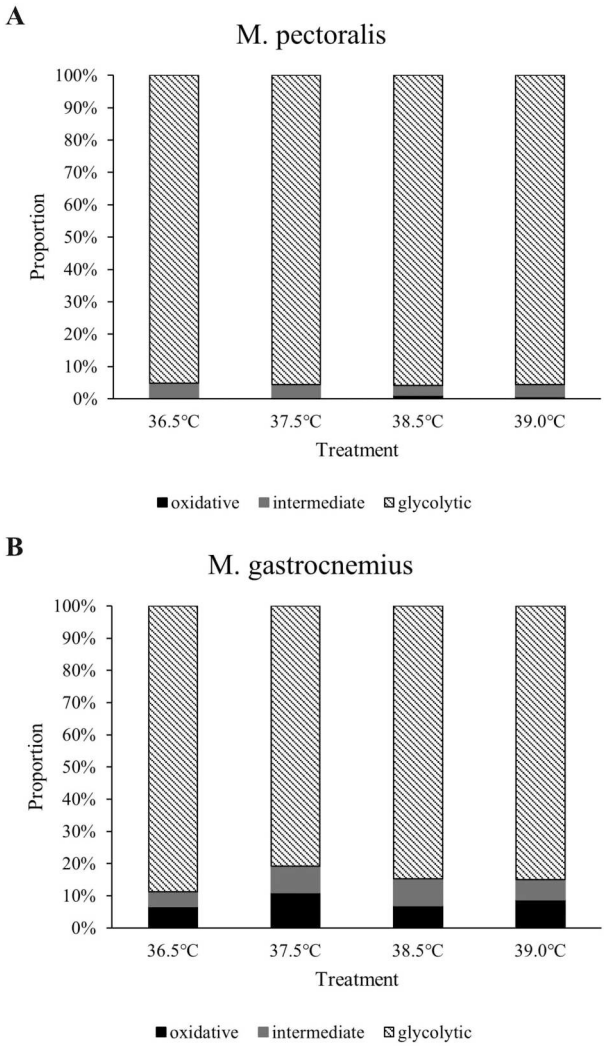

Figure 3. *M. pectoralis* and *M. gastrocnemius* fibre composition in 35-day-old female (A) and male (B) broiler chickens subjected to different incubation temperatures on embryonic days 4–7.

group ($P = 0.057$; not shown). The *M. gastrocnemius* fat percentage was larger in the 39.0°C group than in the other groups ($P = 0.033$). On average, the *M. pectoralis* contained more intramuscular fat than did the *M. gastrocnemius*. The glycogen contents of both muscles were significantly higher in females than in males (Table 3). The *M. gastrocnemius* glycogen content was significantly lower in all treatment groups than in the control group. The *M. pectoralis* glycogen content was significantly lower in the 36.5°C group than in the control group.

Gene expression

Differences in mean gene expression between the control and treatment groups are shown in Figure 4. Generally, the expression of the tested genes was low (as reflected by high Ct values) at hatching and 5 weeks.

At hatching, IGF-1 expression in the *M. pectoralis* was greater in the 36.5°C group than in the control group. The expression of the other genes in this muscle, and all genes in the *M. gastrocnemius*, was similar in the control and treatment groups. At 5 weeks of age, MyoG expression in the *M. pectoralis* was lesser in the 36.5°C group than in the control group. The other groups did not show changes in the gene expression. No differences in gene expression among the groups were found in the *M. gastrocnemius* at 5 weeks post-hatching.

Sex affected gene expression in the *M. pectoralis*; CS and PAX 7 expression was significantly greater in males than in females at hatching (Figure 5), and LDHA expression was significantly greater in females than in males at 5 weeks post-hatching ($P < 0.05$; data not shown). No significant effect of sex on gene expression in the *M. gastrocnemius* was observed at either timepoint.

Tibia characteristics

The tibia characteristics are summarised in Table 4. Interaction between sex and treatment was observed for the tibia weight; this weight did not differ significantly between males and females in the control group, but was significantly greater in males than in females in the three treatment groups (Figure 6). No parameter was affected by the treatment, but all parameters except the slope and distance to fracture were influenced significantly by sex. Males had heavier, longer and thicker tibiae than did females, with a significantly greater breaking force and more work performed (as represented by the AUC).

Gait scores

The prevalence of different gait scores is shown by group in Figure 7. Scores of 2 and 3, and 4 among males, were most prevalent in the 36.5°C group. The average gait score tended to be higher in this group than in the other groups ($P = 0.063$). No chicken had a gait score of 5. The average score was higher for males than for females ($P < 0.001$).

Discussion

This experiment was performed to investigate the effects of the incubation temperature on locomotor system development and performance in broiler chickens. The *M. pectoralis,* which is of economic importance, and the *M. gastrocnemius*, which has primarily functional relevance, were examined.

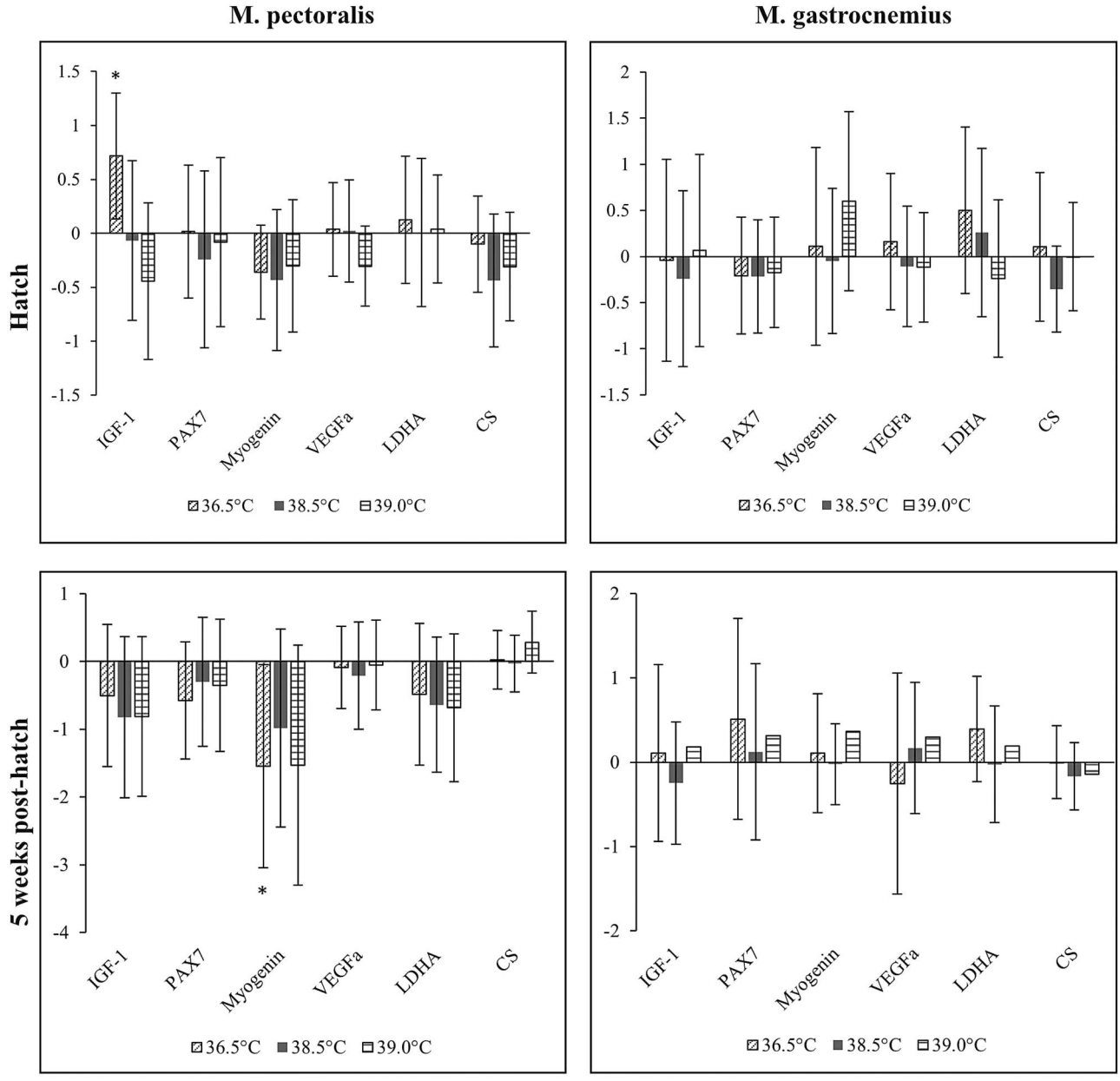

Figure 4. Gene expression in the *M. pectoralis* and *M. gastrocnemius* of newly hatched and 35-day-old female and male broiler chickens subjected to different incubation temperatures on embryonic days 4–7. The bars show differences in means of the $\log_2$ fold change between the control and treatment groups, with the error bars representing confidence intervals. *$P < 0.05$ vs. control.

Muscle parameters

Our results indicate that the temperature of 38.5°C selectively hampered *M. gastrocnemius* development, leading to a lower weight of this muscle and a higher *M. pectoralis*/*M. gastrocnemius* ratio. In contrast, incubation temperatures higher and lower than 37.5°C tended to selectively support *M. gastrocnemius* growth in a previous study (Oksbjerg et al., 2019). Dalab and Ali (2019) also reported differing responses of breast and leg muscles to incubation temperature manipulation. The reasons for the difference in response between muscle groups have not been elucidated. We hypothesise that the difference in the muscle fibre composition confers different susceptibilities to outside influences during certain periods of embryogenesis. Whereas the *M. pectoralis* is composed mainly of type IIB fibres (Ono et al., 1993), leg muscles like the *M. gastrocnemius* contain more type I fibres (Williams & Dhoot, 1992). Type I fibres are formed from embryonic myoblasts during early embryogenesis, whereas type II fibres are formed from foetal myoblasts during mid-term and late embryogenesis (Crow & Stockdale, 1986). Temperature increases seem to decrease the

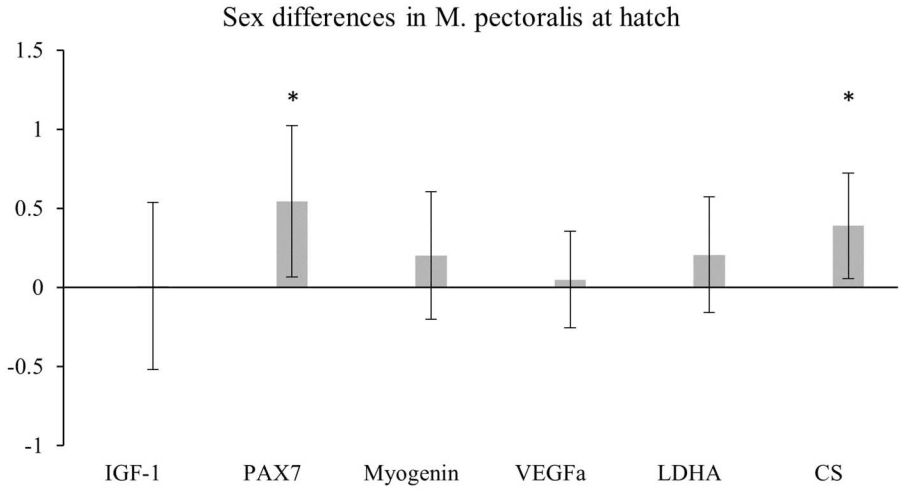

Figure 5. Gene expression in the *M. pectoralis* in newly hatched female and male broiler chickens subjected to different incubation temperatures (36.5°C, 37.5°C, 38.5°C and 39.0°C) on embryonic days 4–7. The bars show differences in means of the $\log_2$ fold change between females (set to 0) and males, with the error bars representing confidence intervals. *$P < 0.05$.

Table 4. Tibia measurements and breaking strength of 35-day-old female and male broiler chickens subjected to different incubation temperatures (36.5°C, 37.5°C, 38.5°C, 39.0°C) during embryonic day 4–7.

	Temperature (T)					Sex (S)			*p*-value	
	36.5°C	37.5°C	38.5°C	39.0°C	SEM	F	M	SEM	T	S
n	34	37	29	22		65	57			
Length [mm]	95	94.9	95.7	94.9	0.67	94.4	95.8	0.42	0.73	0.018
Diameter [mm]	7.37	7.80	7.53	7.24	0.198	7.14	7.84	0.126	0.094	<0.01
Peak Breaking Force [N]	360	347	339	328	13.5	317	370	8.5	0.298	<0.01
AUC [N*mm]	856	810	815	726	44.6	714	889	28.2	0.159	<0.01
Distance to Fracture [mm]	4.35	4.51	4.34	4.02	0.196	4.25	4.36	0.125	0.260	0.523
Slope [N/mm]	102.6	97.6	101.1	100.6	6.90	96.9	104.0	4.40	0.928	0.234

F – female; M – male; AUC – area under the curve; Trt – Treatment, S – Sex; *p*-values below 0.05 were considered statistically significant; values in a row without a common superscript differ significantly.

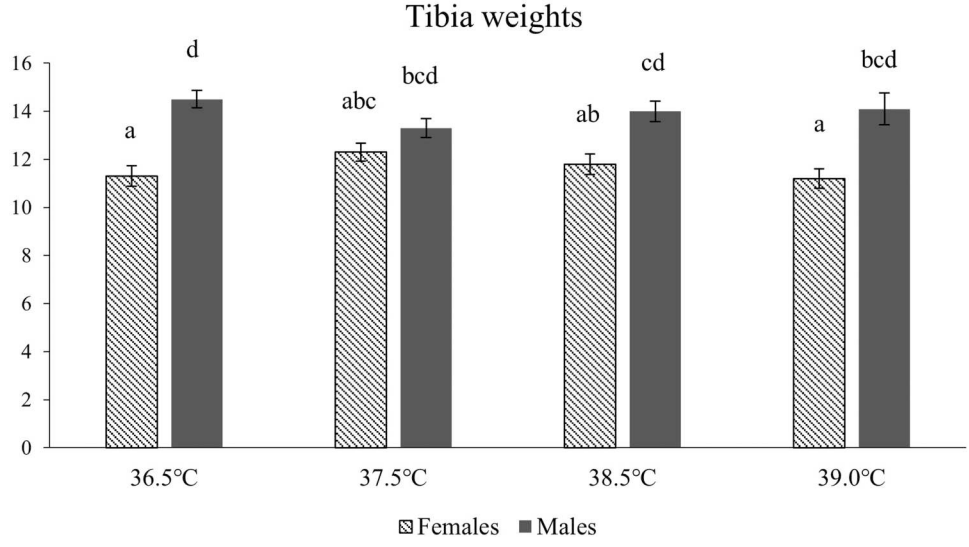

Figure 6. Tibia weights in 35-day-old female and male broilers subjected to different incubation temperatures on embryonic days 4–7. Numbers of animals are given in Table 4. Different superscripts (a, b, c, d) indicate significant differences ($P < 0.05$).

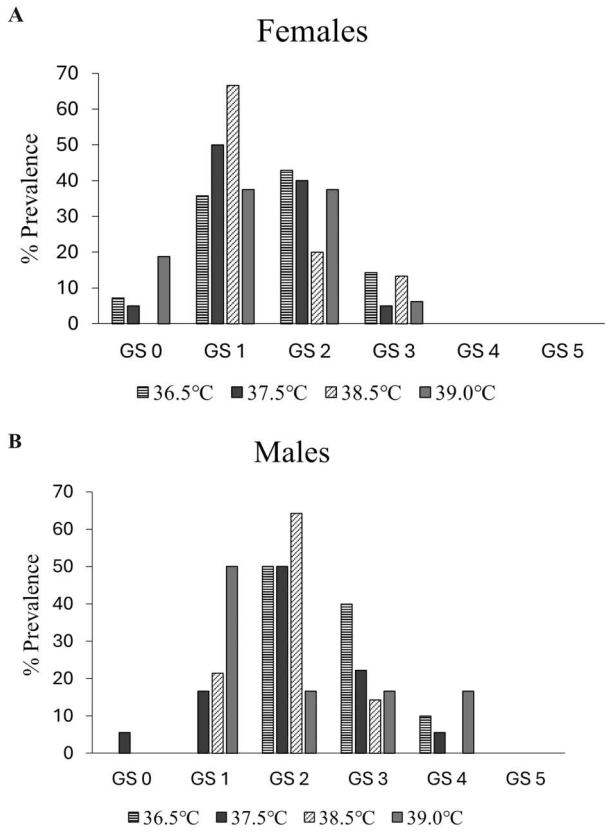

Figure 7. Distribution of gait scores (GSs) in 33-day-old female (A) and male (B) broiler chickens subjected to different incubation temperatures on embryonic days 4–7.

proliferation of type I fibres during early embryogenesis, leading to overall reduced *M. gastrocnemius* weights. Other studies have generally shown that incubation temperature increases stimulate muscle proliferation (Kettrukat et al., 2023), but they have focused mainly on the breast muscle and involved temperature manipulation mainly in later stages of incubation. More research on the differences in the responses of type I and type II muscle fibres to temperature manipulation is needed.

The histological analysis conducted in this study showed that muscle fibres are larger in females than in males at hatching. The temperature of 39.0°C inverted this condition, suggesting that it hampered muscle fibre growth in females and promoted it in males. This result may reflect the influence of this temperature on gene expression during incubation, leading to the lesser or delayed expression of growth, transcription and myogenic regulatory factors (Al-Musawi et al., 2012), or the slowing of chick metabolism, leading to the reduced uptake of nutrients and thus availability of limited resources for tissue growth (Dayan et al., 2020). On the other hand, more females than males hatched under this temperature treatment, suggesting that only the largest and most robust males, with the largest muscle fibres, survived. Sampling during the incubation period could have yielded more information on the physiological processes leading to the observation. In general, the fibre dimensions were in agreement with those reported previously (Al-Musawi et al., 2012; Yalcin et al., 2022).

After 5 weeks, the differences in muscle fibre size between sexes and among groups had disappeared, suggesting the occurrence of compensatory development in males. As the absolute muscle weights and body weight were greater in males than in females at 5 weeks of age, the smaller muscle fibres at hatching do not seem to be an indicator of inferior development or growth. Muscle growth in broiler chickens during the embryonic period has been shown to be the result of hyperplasia, rather than hypertrophy, meaning that the increase in the number of fibres contributes more to an increase in muscle mass (Liu et al., 2017). As the fibre number does not increase markedly after hatching, a larger number of muscle fibres at hatching is more supportive of muscle growth via hypertrophy than is a larger fibre size (Velleman, 2007), explaining the greater muscle weights of males at the end of the rearing period. In other words, chicks with the most muscle fibres at hatching have the greatest growth potential during the rearing period. Our finding that males had upregulated PAX7 expression in the *M. pectoralis* at hatching relative to females supports the hypothesis that they generate more fibres during embryogenesis and thus have greater muscle growth potential after hatching. Fibroblast growth factor 2 also promotes the formation of more muscle fibres before hatching (Velleman, 2007), but was not investigated in this experiment.

Whereas the myofiber dimensions do not differ significantly between male and female broilers under standard rearing conditions, differences among muscles are pronounced (Tejeda et al., 2019). In our experiment, the *M. pectoralis* generally had larger fibres and a lower fibre density than did the *M. gastrocnemius* at 5 weeks. This difference is the result of genetic selection for breast muscle growth, which favours the development of large fibres (Aberle & Stewart, 1983). The differential regulation of satellite cells has been suggested to contribute to the increased growth of muscles, especially the *M. pectoralis* (Zheng et al., 2009). Furthermore, the proportions of fibre types play a role; glycolytic fibres have larger diameters than do oxidative fibres, and the *M. pectoralis* is made up almost exclusively of these fibres (Verdiglione & Cassandro, 2013), as confirmed in the present study. In general, the *M. pectoralis* fibre dimensions in the present study are within reported ranges (Werner et al., 2010; Krischek et al., 2018). The *M. gastrocnemius* was found to have a mixed fibre

composition in this study, in agreement with previous findings (Williams & Dhoot, 1992), but the proportion of glycolytic fibres was larger in the present study because the samples were taken from the pale part of the muscle, whereas Williams and Dhoot (1992) sampled the red part. Moreover, the progress made in selective breeding since 1992, which has likely impacted the *M. gastrocnemius* fibre composition, must be taken into account. Changes in the week-2 incubation temperature have been found to alter embryonic breast and leg muscle metabolism (Krischek et al., 2016), which could be related to the fibre composition. Effects at slaughter age, especially in the breast muscle, are less clear (Krischek et al., 2018). Fast – and slow-twitch fibres are formed during the first week, whereas only fast-twitch fibres are formed in later periods (Miller & Stockdale, 1987). The incubation temperature's promotion or hampering of the proliferation and differentiation of muscle fibres during these time windows would alter the muscle fibre composition. As we observed no difference in this composition among temperature treatments, the influence of temperature does not seem to be sufficiently strong to have such an effect. This is supported by the near lack of difference in gene expression among temperature treatments.

Intramuscular fat deposition in the *M. gastrocnemius* of broiler embryos has been studied using Sudan Black staining, a method similar to Oil Red O staining (Al-Musawi et al., 2012). However, data on chickens at slaughter age are lacking. Al-Musawi et al. (2012) reported an increase in such deposition in 18-day-old embryos incubated at a temperature of 38.5°C on EDs 4–7. In this study, incubation at 39.0°C, but not 38.5°C, during the same period increased intramuscular fat deposition, generally supporting previous findings that higher temperatures increase the lipid content in muscles. Higher temperatures in week 1 of incubation have been proposed to promote adipocyte differentiation, resulting in higher intramuscular fat contents (Al-Musawi et al., 2012). Increased abdominal fat deposition has been observed in chicks exposed to chronic heat stress after hatching (El-Tarabany et al., 2021; Li et al., 2024). The same process could happen in muscle tissue, with pre-hatching heat stress (39.0°C treatment) affecting lipid metabolism up to slaughter age. The increase in lipid accumulation in heat-stressed chickens may be associated with an increase in the corticosterone level, which increases fat synthesis via the liver X receptor-α pathway (Lu et al., 2019). Corticosteroids such as dexamethasone have also experimentally increased lipid accumulation in tissues such as muscle (Wang et al., 2010). A higher intramuscular fat content in meat, especially the chicken breast muscle, is regarded as desirable, but increased fat deposition in muscle tissue has also been found in association with myopathies such as wooden breast and white striping, possibly due to the replacement of degenerated fibres with adipose tissue (Soglia et al., 2016). In the present study, we observed wooden breast in chickens from all groups, especially in the heaviest chickens, but did not specifically document it. In addition, some samples showed more connective tissue between muscle fibres on histological analysis. Oviedo-Rondon et al. (2020) proposed that the incubation temperature, intramuscular fat content and myopathy development were associated, but only for week 3 of incubation, as intramuscular fat accumulation begins on ED 17 (Liu et al., 2017). Such associations should be investigated for early temperature manipulation in future studies, e.g. by incorporating a scoring for wooden breast.

The targets of the gene expression analysis performed in the present study are involved in muscle proliferation and development (IGF-1, PAX7 and MyoG) and muscle metabolism (CS and LDHA). Expression in the *M. pectoralis* changed only in response to the 36.5°C treatment, with the stimulation of the muscle's proliferation at hatching and reduced proliferation at 5 weeks compared with the control. However, as the expression of only one gene was altered at each timepoint (IGF-1 at hatching and MyoG at 5 weeks), we cannot definitively conclude that the treatment clearly stimulated or suppressed proliferation. Surprisingly, no change in gene expression in the *M. gastrocnemius* was observed. As gene expression and muscle proliferation are dynamic processes, we suspect that the sampling timepoints were too late to capture the changes in gene expression that ultimately led to differences in muscle weights. The increased expression of genes related to muscle proliferation after temperature manipulation has been well documented in newly hatched and 5-week-old chickens (Al-Zghoul & El-Bahr, 2019; Yalcin et al., 2022), but data on the *M. gastrocnemius* have been obtained only for embryos (Al-Musawi et al., 2012). Due to the development of type I fibres during early incubation, earlier sampling during the embryonic period would more likely to show such changes. The greater expression of PAX7 and MyoG in the *M. pectoralis* in males than in females at hatching reflects a sex difference in the timing of muscle development or a longer proliferation period in males relative to females. It also supports males' greater growth potential muscle weights at slaughter age. Greater LDHA expression at slaughter age has been linked to wooden breast (Young & Rasmussen, 2020), and supports the hypothesis that myopathy develops in the heaviest chickens based on the larger intramuscular fat

percentages in males than in females. Oviedo-Rondon et al. (2020) hypothesized that thermal stress, especially close to hatching, could increase the incidence and severity of myopathies in chickens, but mentioned no relationship to early temperature manipulation.

The lower glycogen contents observed in the muscles of females relative to those of males and in the treatment groups relative to the control group in this study reflect differences and changes in muscle metabolism and fibre composition. The metabolism of male chickens is more adapted to glucose utilisation, whereas that of females is more adapted to lipid utilisation (López et al., 2011; Cui et al., 2021). This difference has been related to a difference in the cecal microbiota composition; lipid metabolism correlates positively with the relative abundance of *Ruminococcaceae* and *Enterococcus* in females, whereas glycan metabolism correlates positively with the relative abundance of *Bacteroides*, *Megamonas* and *Lactobacillus* in males (Cui et al., 2021). Similar effects on the cecal microbiota could have been induced by early temperature manipulation; this possibility has not been studied, but data from chickens raised under thermoneutral vs. heat stress conditions suggest that temperature influences the cecal microbiota composition and metabolic pathways (Campos et al., 2023). Our observation of metabolic changes after temperature manipulation is supported by the finding of lower glycogen contents in the liver after an incubation temperature increase on EDs 16–18 (Willemsen et al., 2010). Further investigation involving the analysis of glucose and lipid metabolism – related gene expression, enzyme activity and gut microbiota composition is warranted. The discovery of new ways to influence the energy metabolism of broiler chickens is relevant to the improvement of feed efficiency and sustainability in chicken meat production.

Tibia characteristics

The influence of the early incubation temperature on bone development in broilers has been investigated by examining bone dimensions and strength (Oksbjerg et al., 2019) and rickets, a feed-induced abnormality (Shim & Pesti, 2011), with no notable difference observed among different temperatures. The application of an increased temperature in the second week of incubation has been found to promote bone strength and mineralisation (Guz et al., 2020), and to increase the tibial and tarsal lengths in layer embryos (Hammond et al., 2007). In this study, we investigated the tibia strength and dimensions due to their associations with the walking ability (Toscano et al., 2013).

Most bone parameters were unaffected by the treatments applied in this study, consistent with the findings of Oksbjerg et al. (2019). The temperature manipulation seems to have increased the sex difference in the tibia weight, with males having heavier tibias, relative to the control. This finding suggests that male bone development was supported while female bone development was hampered. Bone development starts with cartilage formation on EDs 5 and 6 (Bellairs & Osmund, 2005), although most bone growth happens in the last week of incubation. Mineralisation proceeds faster in females than in males (Rose et al., 1996). Due to sex-specific changes in gene expression or nutrient uptake, the temperature manipulation applied in the first days of incubation in this study could have affected cartilage formation differently in males and females. The treatment also could have delayed bone mineralisation in females, leading to the increased sex difference relative to the control. However, mineralisation was not analysed in this study. The analysis of gene expression related to bone development during broiler chicken incubation and rearing could help to elucidate sex-specific differences in bone responses to temperature manipulation.

Bone strength was unaffected by the incubation temperature in this study, which is to be expected as the bone diameter and weight were not affected. The tendency of higher and lower incubation temperatures to affect the slope of the bone breaking curve that was reported by Oksbjerg et al. (2019) was not reproduced in this study. Manipulation at a later stage of embryogenesis seems to more effectively increase bone strength (Guz et al., 2020).

Almost all bone strength values were affected by sex in this study, which is expected due to males' larger overall size and bones. Studies of the influence of the incubation temperature on bone development until slaughter age in male and female chickens are scarce. Oviedo-Rondon et al. (2009) reported a significantly higher prevalence of leg health problems (crooked toes and valgus deformity) in male than in female 41-day-old broilers, underlining the importance of sex as a factor. Measured parameters, such as the bone breaking force, should be considered together with welfare assessments, such as the gait score, to better evaluate bone functional aspects. In this study, the measure of bone strength (greater in males) did not reflect leg health, as males had higher gait scores than females, indicating inferior leg health.

Performance parameters

The total and yolk-free body weights did not differ among groups at hatching in this study. The yolk-free body weight was measured to exclude the residual yolk, a sign of underdevelopment (Meijerhof, 2009). In

previous studies, the overall body weights of chicks subjected to lower incubation temperatures were greater than those of controls, whereas the yolk-free body weights were lower than those of controls (Joseph et al., 2006; Ipek et al., 2014), indicating that the residual yolk sacs had concealed inferior growth. This was not the case in our experiment.

As early as 10 days after hatching, the body weights of males were greater than those of females in this study. In a recent review, England et al. (2023) showed that differences in broiler body weight, growth and carcass characteristics are associated with sex-specific differences in feed intake, social dominance, nutrient requirements, hormone production, the gut microbiota and gene expression. For example, males in mixed flocks have higher feed intake and tend to exclude females from feeders. The authors noted that evidence exists for sex differences in the gut microbiota and nutrient transporter expression, but that more research on these factors is needed.

The application of lower temperatures during several incubation periods has been shown to slow embryonic and post-hatching growth (Joseph et al., 2006; Ipek et al., 2014; Janisch et al., 2015), consistent with the present results for days 1–10 post-hatching. Lower incubation temperatures slow chicks' metabolism and nutrient uptake, resulting in higher residual yolk weights, and lower body weights at hatching and during rearing (Leksrisompong et al., 2007; van der Pol et al., 2014). Interestingly, the slower growth of the 36.5°C group in this study did not affect the final body weight. Similar observations have been made in chickens subjected to early feed restriction, which induces compensatory growth due to increased feed consumption in the later rearing period, leading to the similarity of these chickens' weight and feed efficiency at slaughter to those of controls (Zhan et al., 2007; Butzen et al., 2013). The initial slowing of growth with temperature manipulation could support efforts to make commercial broilers more resilient to pathogens and skeletal abnormalities without reducing their performance. Unlike feed restriction, the reduction of the incubation temperature does not compromise chickens' welfare by inducing hunger, stress, aggression and abnormal behaviour (Jong & Guémené, 2011). However, the number of animals included in the present study, and especially those subjected to higher temperatures, was small; this topic should be investigated further in larger field studies.

The present finding that the temperature of 38.5°C has no negative effect on growth is supported by previous findings that the application of this temperature in the first (Krischek et al., 2013) or second (Janisch et al., 2015) week of incubation results in no body weight change.

Gait

The higher average gait score in males than in females in this study is likely a result of males' greater body weight, as reported previously (Oviedo-Rondon et al., 2009; Oksbjerg et al., 2019). Studies conducted with larger samples, especially for higher temperature treatments, are needed to draw definitive conclusions on differences among groups. The tendency for the 36.5°C group to have a higher average gait score than the other groups may also be related to the body weight, as this group was heaviest at slaughter age. The results of the bone strength analysis do not indicate that any treatment resulted in the development of unfavourable mechanical properties.

Conclusion

This study provides a comprehensive view of the influence of the early incubation temperature on *M. pectoralis* and *M. gastrocnemius* development in broiler chickens, assessed at hatching and 5 weeks of age. Incubation temperature changes may serve as a tool to influence muscle energy metabolism, with beneficial effects on broiler chicken welfare and meat quality. Furthermore, due to its growth-slowing effect through day 10 post-hatching, the temperature of 36.5°C could be used to ameliorate problems related to fast growth without compromising broiler chickens' feed efficiency and body weight at slaughter age. Such effects are desirable for alternative production systems and have been incorporated in legislation in some countries, including the Netherlands. In conclusion, the manipulation of the incubation temperature is a cost-efficient means of influencing broiler chicken growth and muscle development, and can be fine-tuned to support more sustainable production.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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