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Rising stars in avian physiology: 2022

Edited by
Vincent M. Cassone and Massimiliano Petracci

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Rising stars in avian physiology: 2022

Topic editors

Vincent M. Cassone — University of Kentucky, United States

Massimiliano Petracci — University of Bologna, Italy

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Colin Guy Scanes,
University of Arkansas, United States

*CORRESPONDENCE
Massimiliano Petracci,
m.petracci@unibo.it

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Editorial: Rising stars in avian physiology: 2022

Massimiliano Petracci[1]* and Vincent M. Cassone[2]

[1]Department of Agricultural and Food Sciences, Alma Mater Studiorum—University of Bologna, Bologna, Italy, [2]Department of Biology, University of Kentucky, Lexington, KY, United States

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Editorial on the Research Topic
Rising stars in avian physiology: 2022

Recognizing the future leaders of Avian Physiology is fundamental to safeguarding tomorrow's driving force in innovation. This Research Topic is aimed to supporting scientists in the early stages of their careers across a wide range of disciplines by selecting and publishing their research output at the highest quality standards. In some sense, Avian Physiology is an amalgam of several fields and disciplines. This is reflected in the fact that veterinary oversight of animal research often distinguishes birds from poultry, birds that are used in agriculture. On one hand, avian physiologists who study poultry with the important objective of improving the health and profitability of poultry species are frequently called "poultry scientists". Poultry scientists have their own journals, such as Poultry Science, and their own societies, such as the Poultry Science Association and others. On the other hand, avian physiologists who study basic mechanisms in a variety of avian species, including poultry species, are often called "ornithologists". Ornithologists, like poultry scientists, have their own journals, such as Auk and Condor, as well as societies such as the International Ornithological Congress. And there are societies, such as the International Society for Avian Endocrinology, and journals, such as this journal, that address scientific issues from the breadth of Avian Physiology. This Research Topic reflects that breadth.

The progressive expansion in poultry production over the last several decades to meet growing demand for food has attracted much concern due to the adverse effects of the most challenging environmental stressor, heat stress on birds. For broiler chickens, their physiological response to thermal challenge varies according on several factors; among them, strains' genotype plays a key role. This is particularly true as the climate changes, especially in tropical and subtropical areas. In a couple of review articles, Brugaletta et al. and Teyssier et al. synthesize existing knowledge on the influence of heat stress on physiology, gut health, and live performance of chickens, with a special emphasis on nutritional strategies to be adopted for broiler chickens to mitigate the adverse effects of increasing temperature. Brugaletta et al. provide introductory knowledge on heat stress physiology to make good use of the nutritional themes covered by Teyssier et al. On the same topic, Malila et al. investigated the consequences of cyclic thermal stress in chickens

differing in growth rate and muscle development from different breeds on histological traits, gene expression related to adipose infiltration and inflammation in pectoral muscles. It was clearly confirmed that cyclic thermal challenge negatively affects live performances and breast yields, but infiltration of adipose tissue and inflammatory cells were reduced when fast-growing chickens were considered.

In the last few decades, growing demand for poultry meat has favoured the expansion of the use of fast-growing and high-breast-yield hybrids. This has enormously increased the pressure on pectoral muscular growth rate and mass in meat-type chickens and turkeys, indirectly increasing the occurrence of growth-related muscle abnormalities, such as white striping, an infiltration of fat into muscle, wooden breast, a condition where breast muscle is hard to the touch, and spaghetti meat, a condition where breast muscle is soft and has lost integrity. Tasoniero et al. investigated the onset of protein degradation processes occurring during post-mortem time and evolution of water distribution in pectoral muscles affected by spaghetti meat during refrigerated storage. The softer consistency and looseness of muscle integrity in breast fillets affected by spaghetti meat are not associated with greater proteolysis in live muscle and during post-mortem aging. On the other hand, spaghetti meat condition negatively affects water holding capacity due to abnormal water distribution caused by fibre myodegeneration. On the same topic, Soglia et al. tested the expression and distribution of muscle-specific proteins (vimentin and desmin) over the growing period in fast- and medium growing chickens to explore the existence of a relationship between the occurrence of muscle regeneration associated with the occurrence of growth-related muscle abnormalities and the growth profile of chicken genotype. The higher expression level of the desmin gene in fast-growing chickens supported its potential application as markers of the regenerative processes occurring in pectoralis major muscle affected by these muscle abnormalities. By using a risk analysis approach, Bordignon et al. investigated the role of production factors on the occurrence of the above-mentioned growth-related muscle abnormalities by using a large dataset collected in different trials conducted under varying experimental conditions. It was found that breast yield is a potential risk factor for white striping, while an elevated growth rate plays a major role in the induction of wooden breast and spaghetti meat which exhibited different probability levels when gender was considered.

During the last decade, there is a growing interest in the utilization of insects as a source of proteins in poultry diets. Biasiato et al. investigated the provision of insect larvae from Black soldier fly and yellow mealworm as potential environmental enrichment strategy to promote welfare in broiler chickens. It was evidenced that provision of insect larvae stimulated foraging behaviour, activity levels was increased and some overall behaviours potentially attributable to frustration were reduced, while plumage status, leg health and excreta corticosterone metabolites were not affected.

On the ornithological side, Hope et al. addressed a classical biological debate about the question of nature (genetics) *versus* nurture (environment). In birds, one key issue of the early developmental environment is the management of conditions (e.g., temperature, relative humidity, gas composition, lighting, *etc.*) during embryo development and the post-hatching period. In their study, Hope et al. focuses their attention on genetic background and post-hatching environmental conditions using zebra finches (*Taeniopygia guttata*) as their model. The results clearly showed that metabolic rate during embryo development and post-hatch is affected by parental inheritance, but pre- and post-hatch environmental conditions are of utmost importance. The latter can become particularly relevant considering climate change. How thermoregulatory ability is affected by early thermal environment and parental care will likely be shaped by natural selection as temperature continues to climb.

The melanocortin receptors (MCRs) are recognized to be gene family in the rhodopsin class of G protein-coupled receptors (MC1R-MC5R) which are only found in bony vertebrates. MCRs and their accessory proteins (MRAPs) are implicated in a large number of physiological processes such as pigmentation, lipolysis, adrenal steroidogenesis, and immunology. Indeed, Zhang et al. explored the relationship between MRAPs and sensitivity of cMC5R to natural chicken melanocortin peptides and studied how metabolism of liver is modulated in chickens. The main new finding is that ACTH likely plays a direct role in the regulation of glycolipid metabolism in the liver; this opens new research vistas to better explain the function of MC5R in multiple avian species.

West et al. evaluated the use of advanced behavioral tests to be used for deepening the knowledge on the foraging tactile specialization in Pekin ducks, *Anas playrhynchos domestica,* and Muscovy ducks, *Carina moschata domestica,* which were selected for their different profiles. It was found the females of both species have better predisposition toward learning strategies adopted in behavioral tests compared with males. As expected, differences were found between Pekin and Muscovy ducks in tactile ability to discriminate between hard and soft objects.

Finally, Smiley et al. provide a perspective on the inherent barriers with current research methods that lead to these biases in avian endocrinology in which studies on male birds predominate studies of female birds and experimental designs. This is a historical bias that continues to this day. For example, most studies of avian courtship focus of the generation of male birdsong without regard to female song and reaction to male advances. Considering that the rate-limiting steps in avian reproduction is the energy necessary to lay eggs and incubate them, both field and laboratory ornithologists must address

experimental designs that reflect female avian behavioural physiology and endocrinology.

Author contributions

MP wrote the first draft. VC added notes and revised the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Influences of Thermal Stress During Three Weeks Before Market Age on Histology and Expression of Genes Associated With Adipose Infiltration and Inflammation in Commercial Broilers, Native Chickens, and Crossbreeds

Yuwares Malila[1]*, *Pornnicha Sanpinit*[1], *Wilawan Thongda*[1,2], *Anuwat Jandamook*[3], *Yanee Srimarut*[1], *Yupin Phasuk*[3] and *Sajee Kunhareang*[3]

[1]National Center for Genetic Engineering and Biotechnology (BIOTEC), Thailand Science Park, Pathum Thani, Thailand, [2]Center of Excellence for Shrimp Molecular Biology and Biotechnology (CENTEX Shrimp), Faculty of Science, Mahidol University, Bangkok, Thailand, [3]Department of Animal Science, Faculty of Agriculture, Khon Kaen University, Khon Kaen, Thailand

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Edited by:
Vincent M. Cassone,
University of Kentucky, United States

Reviewed by:
Maurizio Mazzoni,
University of Bologna, Italy
Elizabeth Ruth Gilbert,
Virginia Tech, United States

***Correspondence:**
Yuwares Malila
yuwares.mal@biotec.or.th

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The objectives of this study were to examine the effects of cyclic thermal stress on histological characteristics of breast muscle and gene expression regarding adipose infiltration and inflammation in breast muscles collected from different breeds of chickens. The birds, from commercial broilers (CB, Ross 308, 3 weeks), native (NT, 100% Thai native Chee, 9 weeks), H75 (crossbred; 75% broiler and 25% NT, 5 weeks), and H50 (crossbred; 50% broiler and 50% NT, 7 weeks), were equally assigned into control or treatment groups. The control samples were reared under a constant temperature of 26 ± 1°C, while the treatment groups were exposed to 35 ± 1°C (6 h per day). After a 20-day thermal challenge, 12 male birds per treatment group were randomly collected for determination of live body weight, breast weight, numbers of growth-related myopathies, and breast meat chemical composition. Histological lesions were evaluated in the pectoralis major muscle immediately collected within 20 min postmortem based on hematoxylin and eosin staining. The results indicated that despite interaction between thermal stress and breed effects, thermal challenge significantly reduced feed intake, live body weight, and breast weight of the birds and increased moisture content in breast meat ($p < 0.05$). An interaction between the two main factors was found for protein content ($p < 0.05$) for which control CB showed less protein than the other groups. Heat stress decreased histological scores for adipose infiltration in CB ($p < 0.05$), but it did not significantly influence such scores in the other groups. CB received histological scores for adipose tissue at greater extent than those for the other groups. Differential absolute abundance of *CD36*, *FABP4*, *LITAF*, *PDGFRA*, *PLIN1*, *PPARG*, *POSTN*, *SCD1*, and *TGFB1* in the muscle samples well-agreed with the trend of histological scores, suggesting potential involvement of dysregulated fibro-adipogenic progenitors together with imbalanced lipid storage and utilization in the breast muscle. The

findings demonstrated that the cyclic thermal challenge restricted growth performance and breast mass of the birds, but such effects attenuated infiltration of adipose tissue and inflammatory cells in the CB breast muscle.

Keywords: chicken, droplet digital polymerase chain reaction, fat accumulation, immune cell infiltration, heat stress

INTRODUCTION

An increase in average environmental temperatures has urgently raised global attention as it inevitably impacts the well-being of lives on Earth. For chickens, their physiological response to thermal challenge depends on several factors, including breed, size, and age (Lu et al., 2007; Awad et al., 2020; Malila et al., 2021a). Commercial broilers, meat-type chickens, exhibit more susceptibility to thermal stress than their ancestors (Tabler et al., 2020) and other slow-growing strains (Washburn et al., 1980; Aengwanich, 2007; Malila et al., 2021a). This has been considered a negative consequence of an artificial breeding selection program focused on production performance and massive mass to meet high consumer demand. Development of thermoregulatory systems of broilers does not complement rapid growth of the birds (Havenstein et al., 2003). An ambient temperature above 30°C is considered sufficient to induce stress among the birds (Azad et al., 2010; Adu-Asiamah et al., 2021). The negative impacts of thermal challenge include diminished growth performance (Azad et al., 2010; Quinteiro-Filho et al., 2010; He et al., 2018; Aslam et al., 2021), increased mortality (Al-Fataftah and Abdelqader, 2014), compromised gut health, (Quinteiro-Filho et al., 2010; Awad et al., 2020), and deviated meat quality (Sandercock et al., 2001; Lu et al., 2017; Malila et al., 2021a). A recent meta-analysis performed by Andretta et al. (2021) highlighted that broilers with increasing age and weight become more sensitive to heat as their heat dissipation area becomes smaller. Their findings also indicated that heat stress negatively affects broilers over 21 days of age at a greater extent than the birds at an initial growing phase.

In regard to altered meat quality, increased fat along with decreased protein content was frequently reported in the meat yielded from chickens exposed to heat stress (Akşit et al., 2006; Lu et al., 2007; Zhang et al., 2012). The actual molecular etiology of the changes remained not fully comprehended. An increased level of serum corticosterone was observed in heat-stressed chickens and further linked with induced insulin resistance, stimulated lipid synthesis, and fat accumulation in abdominal, cervical, and thigh adipose tissues (Quinteiro-Filho et al., 2010; Xu et al., 2018). Serum corticosterone might also activate protein breakdown in the skeletal muscle of stressed animals (Scanes, 2016). Lu et al. (2019) reported widespread deposition of lipid droplets inside the liver of Arbor Acres broilers exposed to 14-day constant heat stress (32°C). They found that in the stressed animals, both apolipoprotein B gene and protein levels in the chicken liver and plasma corticosterone concentration remained unchanged, suggesting the disrupted transportation of the excessive triglycerides from the liver in the broilers exposed to chronic heat stress. Recently, Adu-Asiamah et al. (2021) analyzed histopathological characteristics of 56-day-old Chinese broiler chickens exposed to acute heat stress (35°C) for 8 h and reported the extent of tissue damage in the liver, spleen, breast, and leg muscles. In addition, experiments conducted using turkey' pectoralis major muscle satellite cells showed that temperature extremes altered the adipocyte-like properties of the satellite cells isolated from fast-growing and non-selected random-bred control lines (Clark et al., 2017).

The cyclic thermal stress, resembling the daily rise of temperature in the tropical region or in the summer of the temperate zone, exerted adverse impacts on growth performance of commercial broilers, although the impact was at a lesser extent than that of the constant heat challenge (Souza et al., 2016; Aslam et al., 2021). Under cyclic stress, commercial broilers are partly able to adjust to the stressed environment and have compensatory gain during the non-stressed period (Aengwanich, 2007; Andretta et al., 2021). Still, reduced growth performance and less meat yield, attributed to an exposure to cyclic heat stress, were consistently reported among previous studies (Quinteiro-Filho et al., 2010; Shao et al., 2019; El-Tarabany et al., 2021). Heat stress may exert a negative impact on meat quality through oxidative damages (Lu et al., 2017). This could impair the muscle regeneration process and lead to muscle abnormalities, including emerging myopathies known as white striping (WS) and wooden breast (WB), among the chickens exposed to thermal challenge (Aslam et al., 2021). Several studies indicated chemical changes, that is, reduced protein and increased fat content in chicken meat affected with those abnormalities (Kuttappan et al., 2012; Petracci et al., 2014; Malila et al., 2018; Thanatsang et al., 2020). Overall, the incidence could not only affect economic returns of broilers but also potentially dilute nutritional properties of chicken breast meat, which is generally recognized as an inexpensive source of food protein in several regions.

The objectives of this study were to examine the effects of cyclic thermal stress, mimicking the rise of temperature during the day in tropical regions, on histological characteristics of the breast (pectoralis major) muscle of chickens from different breeds. Absolute transcript abundance of associated genes was also evaluated to provide insights at molecular levels. The chickens included commercial broiler (CB, Ross 308), native (NT, 100% Thai native Chee), H75 (crossbred; 75% broiler and 25% NT), and H50 (crossbred; 50% broiler and 50% NT). We focused on the exposure of the challenge during the last 3 weeks before the specific market ages of each breed.

MATERIALS AND METHODS

Experimental Design, Animals, and Management

All chickens, including commercial broiler (CB, Ross 308), native (NT, 100% Thai native Chee), H75 (crossbred; 75% broiler and 25% NT), and H50 (crossbred; 50% broiler and 50% NT), were raised and maintained under an environmentally controlled

TABLE 1 | Chemical composition of standard commercial broiler diets.

Nutrient	Starting phase (1 day–21 days)	Growing phase (22 days to Market age)
Metabolizable energy (kcal/kg)	3,100	3,200
Moisture (%)	13	13
Crude protein (%)	21	20
Crude fiber (%)	5	5
Fat (%)	4	4

poultry facility of the Department of Animal Science, Faculty of Agriculture, Khon Kaen University (Khon Kaen, Thailand) following the standard practice for commercial broilers. The birds were housed in floor pens (1.3 m × 2.0 m) with rice hulls provided as bedding materials and received standard commercial broiler diets (**Table 1**). Feed and water were provided *ad libitum*. A routine vaccination program against coccidiosis, infectious bronchitis virus, and Newcastle disease was applied.

Three weeks prior to their specific market ages, the chickens of each strain were randomly divided into two groups: control and stressed treatment. The control (4 replications/group, 12–13 birds/replication, 5 birds/m^2) group was kept at a constant temperature of 26 ± 1°C, while the treatment groups (4 replications/group, 12–13 birds/replication, 5 birds/m^2) were exposed to 35 ± 1°C during 10:00 a.m. to 4:00 p.m., accounting for 6 h daily. The thermal challenge period was carried on for 20 days.

Upon completion of the thermal challenge, a total of 96 male chickens (n = 12 per group, 3 birds per pen replicate) were proceeded to the slaughtering process with 12-h fasting prior to the slaughter. The right side of the breast was immediately removed from the carcass and monitored for occurrences (presence or absence) of white striping (WS) and wooden breast (WB) abnormalities based on the criteria previously described by Malila et al. (2018) and Tijare et al. (2016), respectively. The evaluation was carried out by one fully trained staff to minimize the variation. The muscle samples were excised, oriented along the muscle fiber, from the cranial portion (approximately 1 cm deep from the ventral surface) of the muscle, and cut into 0.5 cm^3 cubes. Half of the muscle specimen was then snap-frozen in liquid nitrogen within 20 min postmortem and stored at −80°C until RNA isolation, whereas the other half was fixed within 10% buffered formalin fixative (pH 7.0) and stored at 4°C until histological evaluation.

The left side of the breast was collected, weighed, packed in a plastic bag, kept on ice during samples collection, and subsequently stored at refrigerated temperature until it reached 24 h postmortem. The samples were then ground and stored at −20°C until the analyses of chemical composition were performed.

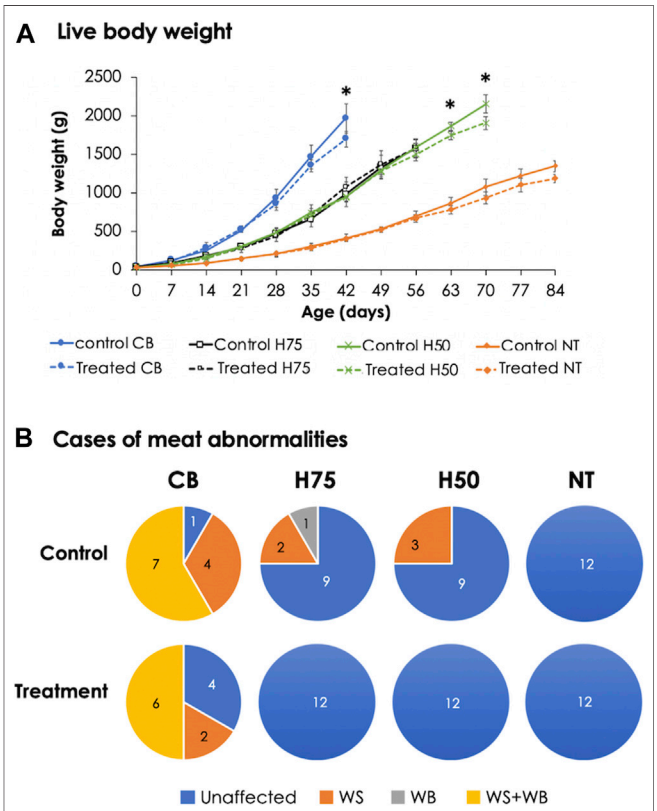

FIGURE 1 | Live body weight and cases of meat abnormalities in the chickens from different breeds. The birds were reared at a constant temperature (control, 26°C) or received heat challenge (treatment, 35°C, 6 h daily) for 20 days before reaching their market ages. **(A)** Markers and error bars depict average and standard deviation of live body weight (n = 12 per group), respectively. **(B)** Venn diagrams illustrate occurrence of growth-related myopathies, including white striping (WS) and wooden breast (WB), in breast muscles (n = 12 per group). CB: commercial broilers; H75: crossbreeds, 75% broiler background and 25% Thai native background; H50: crossbreeds, 50% broiler background and 50% Thai native background; NT: Thai native chicken.

Proximate Composition

Chemical compositions, including moisture, protein, fat, ash, and carbohydrate, of the samples were determined following the standard methods of AOAC (2016). In brief, moisture content was analyzed based on weight loss upon drying the samples at 105°C. Crude protein was determined following a Dumas combustion principle, and the conversion factor of 6.25 was used for calculation (AOAC 990.03). Crude fat was extracted from the breast samples with petroleum ether by using a Soxtherm (model SOX416, C. Gerhardt GmbH & Co. KG, Königswinter, Germany) following a Soxhlet method. Ash content was examined based on an incineration of the samples at 600°C. The analyses were performed in two technical replicates.

Histological Evaluation and Scoring

The muscle specimen stored in 10% buffered formalin was dehydrated in ethanol solutions with a serial concentration of 70, 80, 95% (repeated at this step three times), and 100%. The specimen was then embedded in paraffin and cross-sectioned into 5-μm sections. The tissue section slides were stained with hematoxylin and eosin (H&E staining). The slide section was examined under a bright-field microscope (Olympus, Tokyo, Japan) equipped with a digital camera (Olympus DP73 Microscope Digital Camera, Tokyo, Japan) and visualized using Olympus cellSens software (Olympus, Tokyo, Japan). The histopathological characteristics, including inflammation and adipose infiltration, were scored based on the criteria described by Prisco et al. (2021). In brief, inflammation was assessed based on the number of inflammatory cells as follows: score 0 = no infiltrated cells; score 1 = 5 to 25 cells per field; score 2 = 26 to 50 cells per field; and score 3 = more than 50 cells per field. As for adipose tissue infiltration: score 0 = no appearance of adipose tissue; score 1 = less than 10% of the skeletal muscle area was infiltrated by adipose tissue; score 2 = 10 to 20% infiltration; and score 3 = more than 20% infiltration. A total of ten fields at ×200 magnification (×20 for objective lens and ×10 for eyepiece lens) for each sample were selected for the evaluation. The scores for each muscle sample were an average score calculated from the ten fields.

Total RNA Isolation and cDNA Synthesis

Total RNA was isolated from the frozen breast muscle tissues using TriReagent (Molecular Research Center, Inc., Cincinnati, OH, United States) following the manufacturer's protocol. Contaminated DNA was removed by incubating the isolated total RNA with RNase-free DNase I (Thermo Fisher Scientific, Rockford, IL, United States), according to the company's instruction. The total RNA samples were then purified using a column-based ReliaPrep™ RNA clean-up and concentration system (Promega Corporation, Madison, WI, United States). The quantity and quality of total RNA were determined using a Nanodrop spectrophotometer (Thermo Fisher Scientific) and a fragment analyzer system (model 5300, Agilent), respectively. Only RNA samples exhibiting RNA integrity number greater than 7.0 were used for cDNA synthesis. Subsequently, total RNA (1.5 μg) was reverse-transcribed into cDNA using oligo(dT) as a primer and an ImProm-II™ reverse transcription system (Promega Corporation). The amount of the synthesized cDNA was determined using a Nanodrop spectrophotometer (Thermo Fisher Scientific).

Primers and Droplet Digital Polymerase Chain Reaction

The absolute expressions of 13 target genes (**Table 2**) associated with lipid metabolism and muscle injury were evaluated using an EVAGREEN droplet digital polymerase chain reaction (ddPCR) assay. The primers were designed using Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). To confirm primer specificity, PCR mixture, containing 1X EvaGreen supermix (Bio-Rad Laboratories, Inc., Hercules, CA, United States), 0.25 μM of each forward and reverse primer, and the cDNA template (**Table 2**), was prepared (**Table 2**) and performed using a thermocycler with conditions set as 95°C for 5 min; 40 cycles of 95°C for 30 s, 60°C for 1 min, 4°C for 5 min, and 90°C for 5 min. Primer specificity was then confirmed by the presence of a single band of amplicon products that corresponded to the correct molecular weight on a 2% agarose gel.

To perform ddPCR, the ddPCR reaction mixture consisting of 1X EvaGreen supermix (Bio-Rad Laboratories, Inc., Hercules, CA, United States), 0.25 μM of each forward and reverse primer, and the cDNA template (**Table 2**), was set up in a sterile microcentrifuge tube. The template was replaced by an equal volume of nuclease-free water for no template control. The ddPCR reaction mixture (20 μL) was then mixed with Evagreen generator oil (Bio-Rad Laboratories, Inc., Hercules, CA, United States) using a QX100™ droplet generator (Bio-Rad Laboratories, Inc.), according to the company's protocol. The generated droplets (40 μL) were subsequently transferred into a 96-well plate. The plate was then heat-sealed with an aluminum foil cover. The target genes in the samples were amplified in a thermocycler (model T100™, Bio-Rad Laboratories, Inc.) with a condition set as follows: 95°C for 5 min; 40 cycles at 95°C for 30 s, 60°C for 1 min, and 4°C for 5 min; and 90°C for 5 min. Afterward, the fluorescent signal intensity of the droplets was measured using a QX200™ droplet reader (Bio-Rad Laboratories, Inc.). The number of positive and negative droplets was automatically counted by QuantaSoft™ software (Bio-Rad Laboratories, Inc.), and transcript abundance was expressed as copies per 20-μL reaction.

Statistical Analysis

A statistical analysis was performed using the R package version 3.4.3. The significant level for all statistical analyses was set at $\alpha = 0.05$. The effects of thermal challenge and chicken breeds, as the main effects, were determined according to a 2 × 4 factorial analysis of variance (ANOVA). The mean differences were assessed using Duncan's new multiple range test. Prior to ANOVA analysis, the assumptions of normality and homogeneity of variance were examined using the Shapiro–Wilk normality test and Bartlett test, respectively. The data set that did not follow the assumptions was transformed using a function *varIdent* from the library nlme of the R package.

Pearson's correlation coefficient was calculated to define the correlation among histological scores and absolute expression of the tested genes. In this case, average histological scores from 10 observed microscopic fields and absolute transcript abundance (in the unit of copy number per 20 μL reaction) were included in the test. As for the relationship between growth-related myopathies and gene expression level, this categorical variable was labeled as 1 (presence) or 0 (absence) before the data were submitted to Spearman's rank correlation test.

RESULTS AND DISCUSSION

Feed Intake, Live Body Weight, Breast Weight, Chemical Composition, and Cases of Growth-Related Myopathies

Table 3 shows feed intake (during the 20-day thermal stress), live body weight, breast weight, and breast yield of the tested chickens

TABLE 2 | Primers.

NCBI accession number	Gene ID	Gene annotation	Sequence (5′ → 3′)	Amplicon length (bp)	Template amount (ng)	Annealing temperature (°C)
NM_206,991.1	*ADIPOQ*	Adiponectin	F: AGCAGAACCACTACGACAGC	166	10	60
—	—	—	R: ACGTTGTTCTCCTGG AACTGG	—	—	—
NM_001,030,731	*CD36*	CD36 molecule	F: TACCAGACCAGTAAGACC GTGAAGG	156	25	60
—	—	—	R: AATGTCTAGGACTCCAGC CAGTGT	—	—	—
NM_204,290.1	*FABP4*	Fatty acid–binding protein 4	F: TATGAAAGAGCTGGGTGTGG	168	10	60
—	—	—	R: GCTGTGGTCTCATCAAACTC	—	—	—
NM_204,267.1	*LITAF*	Lipopolysaccharide-induced tumor necrosis factor-alpha factor	F: ACTATCCTCACCCCTACC CTGTC R: TGTTGGCATAGGCTGTCCTG	95	25	60
XM_040,666,999.1	*LPIN1*	Lipin 1	F: CCTTTCACTGTAATGCTGGT	173	10	60
—	—	—	R: TGGAGTGGTATGGTCATCAG	—	—	—
NM_205,282.1	*LPL*	Lipoprotein lipase	F: AGGAGAAGAGGCAGCAATA	222	10	60
—	—	—	R: AAAGCCAGCAGCAGATAAG	—	—	—
NM_001,030,363.1	*MYF5*	Myogenic factor 5	F: TGAACCAAGCATTCGAGACC R: AGTAGTTCTCCACCTGTT CCCT	141	10	60
NM_204,749.2	*PDGFRA*	Platelet-derived growth factor receptor, alpha	F: AAGAGAGTGCCATTG AAACCG R: GCAGTTAGAAGGTGTCTG GGAT	155	10	60
NM_001,127,439.1	*PLIN1*	Perilipin 1	F: GCCAAGGAGAACGTGCT	142	10	60
—	—	—	R: TCACTCCCTGCTCATAGACC	—	—	—
NM_001,001,460.1	*PPARG*	Peroxisome proliferator activated	F: CCAGCGACATCGACCAGTTA	182	100	60
—	—	Receptor, gamma	R: TCCCATCCTTAAAGAGTTCA	—	—	—
NM_001,030,541.1	*POSTN*	Periostin	F: GCCTGGTGTGACAAACATCC	119	10	60
—	—	—	R: TGGTTGCCATGAGAT CAGGTT	—	—	—
NM_204,890.1	*SCD1*	Stearoyl-CoA desaturase 1	F: GGCTGACAAAGTGGTGATG	137	50	60
—	—	—	R: GGATGGCTGGAATGAAGA	—	—	—
NM_001,318,456.1	*TGFB1*	Transforming growth factor beta 1	F: GACGATGAGTGGCTC TCCTTC R: GTGCTTCTTGGCAATGCTCT	195	10	60

as affected by the thermal challenge. Focusing on the main effects, the heat condition reduced feed intake, live body weight, and breast weight ($p < 0.05$) but not breast yield ($p \geq 0.05$). Live body weight of the stressed CB, stressed H50, and stressed NT was approximately 13, 11, and 12%, respectively, less than their control counterparts. The current stress condition on reduced breast weight was more pronounced in CB as the breast weight was reduced to 28, 8, and 13% in stressed CB, stressed H50, and stressed NT, respectively, in comparison to their control counterparts. The reduced body weight and breast weight due to the thermal challenge in those breeds well agreed with that observed in previous studies (Sandercock et al., 2001; Duangjinda et al., 2017; Shao et al., 2019; Emami et al., 2021). Duangjinda et al. (2017) compared growth performance of commercial broilers and Thai indigenous chickens under either thermoneutral condition (26°C) or heat challenge (36–38°C, 6 h daily) for 3 weeks prior to market age and reported decreased growth performance for broilers but not in the indigenous birds. Shao et al. (2019) reported reduced body weight, average daily gain, and average feed intake in yellow-feathered broilers exposed to cyclic temperature of 35°C (8 h daily) for 7 days compared to the birds reared under thermal neutral condition (23°C). Using similar thermal challenge conditions (cyclic temperature of 35°C, 8 h daily vs. control condition of 23 °C), Emami et al. (2021) observed a significant decrease in body weight of broilers exposed to the stress during the age of 29–42 days. The decreased production performance has been shown to be associated with restricted feed intake upon exposure to thermal challenge (Souza et al., 2016). In this study, the difference in feed intake during the 20-day challenge was observed only between the control and stressed H50 ($p < 0.05$). The previous meta-analysis conducted by Andretta et al. (2021) found that under cyclic thermal stress, commercial broilers at the age of 21 days or older could sometime compensate their intake during the cooling period. The lack of thermal effects on growth and meat yield of H75 and NT suggested better heat tolerance and better adaptation of both strains over CB and H50 (Aengwanich, 2007).

The H50 birds exhibited the greatest live body weight ($p < 0.05$), followed by CB and H75, whereas NT showed the lowest ($p < 0.05$) body weight. In this regard, the different market age of each chicken strain must be emphasized. The CB, H75, H50, and

TABLE 3 | Live body weight at slaughter age, breast weight, and breast yield (%) of the chicken samples.

Sample		Feed intake[1] (g/bird/day)	Live body weight (g)	Breast weight (g)	Breast yield[2] (%)
CB (42d)[3]	Control	98.3 ± 11.6	1,962.5 ± 188.8	164.8 ± 43.6	8.3 ± 1.5
—	Treatment	85.6 ± 3.0	1,695.0 ± 106.3	119.3 ± 13.5	7.0 ± 0.4
H75 (56d)	Control	85.0 ± 3.8	1,575.0 ± 125.8	82.3 ± 19.1	5.2 ± 1.0
—	Treatment	88.2 ± 13.2	1,576.3 ± 115.9	88.8 ± 15.9	5.6 ± 0.8
H50 (70d)	Control	94.4 ± 8.6	2,150.0 ± 122.5	132.8 ± 15.7	6.2 ± 0.7
—	Treatment	79.8 ± 6.3	1,905.0 ± 85.4	121.5 ± 10.1	6.4 ± 0.4
NT (84d)	Control	64.4 ± 3.8	1,345.0 ± 68.1	60.5 ± 8.2	4.5 ± 0.4
—	Treatment	63.9 ± 8.2	1,185.0 ± 54.5	52.5 ± 4.4	4.4 ± 0.3
Main effects	—	—	—	—	—
Stress	Control	86.9 ± 15.05	1,758.1 ± 347.5	110.1 ± 48.1	6.8 ± 1.9
—	Treatment	78.8 ± 11.54	1,590.3 ± 283.2	95.5 ± 30.8	6.5 ± 1.2
Breed	CB	92.0^{a} ± 10.4	$1,828.8^{b}$ ± 201.4	142.0^{a} ± 38.5	8.4^{a} ± 1.4
—	H75	86.4^{a} ± 8.3	$1,575.6^{c}$ ± 112.0	85.5^{b} ± 16.7	6.1^{b} ± 1.0
—	H50	87.1^{a} ± 10.5	$2,027.5^{a}$ ± 163.4	127.1^{a} ± 13.6	6.9^{b} ± 0.6
—	NT	64.2^{b} ± 5.7	$1,265^{d}$ ± 102.8	56.5^{c} ± 7.5	5.0^{c} ± 0.4
p-value	—	—	—	—	—
Stress	—	0.03	0.0004	0.04	0.34
Breed	—	<0.0001	<0.0001	<0.0001	<0.0001
Interaction	—	0.12	0.11	0.09	0.15

Mean ± standard deviation (n = 12).

[1]Feed intake during the last 3 weeks before specific market age.

[2]Breast yield was expressed in percentage as breast weight relative to live body weight.

[3]Slaughter age (days) of each chicken strain.

[a, b, c, d] Significant differences due to different breeds (p < 0.05).

NT chickens were slaughtered at the age of 42, 56, 70, and 84 days, respectively, following their specific market ages. The superior yield of H50 over H75 might be mainly due to the later age of slaughter as both strains exhibited a similar growth rate (**Figure 1A**). Although the live body weight of H50 birds was greater than that of CB, the average weight of the breast portion collected from CB, particularly the control group, was greater than the others. The results well-agreed with those of previous studies comparing growth performance among the chickens with different growing rates and with the fact that modern broilers have been selected for their growth rate and pectoralis major yield (Zuidhof et al., 2014).

Concerning WS and WB cases (**Figure 1B**) of 12 control CB samples, only one breast sample exhibited unaffected characteristics, while four showed WS, and the other seven were classified as WS+WB. As for stressed CB, four unaffected, two WS, and six WS+WB breasts were observed. On the other hand, three of 12 birds from the control H75 and control H50 exhibited the abnormalities, whereas no WS or WB cases were observed among the NT chickens. It is worth mentioning that all WS-affected samples were classified as mild lesions (1–40 white lines clearly observed with line thickness < 1 mm), whereas the majority of the WB-affected breasts fell into the category of Grade 1 in which the breasts were hard mainly in the cranial region but flexible otherwise (Tijare et al., 2016). Of seven WS+WB breasts among the control CB samples, two samples were classified as moderate WB lesion (hardness throughout but slightly flexible in the middle) and one sample fell into severe WB severity (extreme hardness throughout the meat). The results strongly supported the influences of genetic selection for growth rate and meat yield on the development of abnormalities (Kuttappan et al., 2012; Pampouille et al., 2018; Lake et al., 2021). The lower number of cases for WS and WB abnormalities in the stressed CB group might be related with restricted growth among the stressed CB birds. This hypothesis was in agreement with previous reports of Kuttappan et al. (2013a) and Malila et al. (2018) that the high degree of WS and WB was significantly related with heavier birds and pectoral muscle yield. In accordance with our current findings, a recent study of Aslam et al. (2021) showed that Arbor Acres broilers received cycle heat stress (32°C, 12 h daily, 21–42 days) exhibited reduced body weight gain with less cases of WS in comparison to the birds raised under control condition (22–24°C).

Focusing on the chemical composition of the breast samples (**Table 4**), the cyclic heat stress significantly reduced the moisture content in breast samples ($p < 0.05$), whereas breed difference significantly influenced moisture, protein, fat, and ash content ($p < 0.05$). The interaction ($p < 0.05$) between the main effects (i.e., heat stress and breed) was observed for crude protein content. The control CB comprised lower protein than other samples ($p < 0.05$). Focusing on CB breeds, average values of moisture and fat content of the control CB were about 1.5 and 30% greater than those of their stressed counterparts. The results for CB breast were consistent with the characteristics of the breasts affected with growth-related myopathies (Soglia et al., 2016; Cai et al., 2018; Malila et al., 2018; Dalle Zotte et al., 2020; Maharjan et al., 2020; Thanatsang et al., 2020).

It is worth mentioning here that the temperature for control samples in this study was out of the optimum range (21–22°C) recommended for growing commercial broilers. However, as global ambient temperature continues to rise, it is difficult, particularly in tropical and subtropical regions, even for the houses equipped with an evaporative cooling facility, to maintain an optimum range during the day. The average

TABLE 4 | Chemical composition (g/100 g sample) of breast samples.

Sample		Moisture	Crude protein	Crude fat	Carbohydrate	Ash
CB	Control	75.87 ± 0.55	20.87^{d} ± 0.84	1.09 ± 0.19	1.01 ± 0.50	1.16 ± 0.12
—	Treatment	74.74 ± 0.22	22.51^{c} ± 0.27	0.76 ± 0.12	0.59 ± 0.27	1.40 ± 0.07
H75	Control	73.85 ± 0.29	23.03^{bc} ± 0.43	0.72 ± 0.30	0.70 ± 0.26	1.69 ± 0.16
—	Treatment	73.96 ± 1.03	23.05^{bc} ± 1.08	0.60 ± 0.17	0.82 ± 0.37	1.57 ± 0.30
H50	Control	74.12 ± 0.41	23.27^{abc} ± 0.34	0.63 ± 0.27	0.47 ± 0.49	1.51 ± 0.13
—	Treatment	73.25 ± 0.87	24.12^{a} ± 0.57	0.45 ± 0.13	0.45 ± 0.20	1.74 ± 0.20
NT	Control	73.31 ± 0.40	23.91^{ab} ± 0.60	0.28 ± 0.08	0.57 ± 0.21	1.94 ± 0.08
—	Treatment	73.39 ± 0.21	23.63^{ab} ± 0.34	0.42 ± 0.13	0.71 ± 0.32	1.85 ± 0.02
Main effects	—	—	—	—	—	—
Stress	Control	74.29 ± 1.06	22.77 ± 1.29	0.68 ± 0.36	0.69 ± 0.41	1.57 ± 0.32
—	Treatment	73.89 ± 0.87	23.33 ± 0.85	0.56 ± 0.18	0.64 ± 0.30	1.64 ± 0.24
Breed	CB	75.31^{a} ± 0.72	21.69^{b} ± 1.05	0.93^{a} ± 0.23	0.80 ± 0.43	1.28^{c} ± 0.16
—	H75	73.91^{b} ± 0.70	23.04^{a} ± 0.76	0.66^{b} ± 0.24	0.76 ± 0.30	1.63^{b} ± 0.23
—	H50	73.69^{b} ± 0.78	23.69^{a} ± 0.63	0.54^{bc} ± 0.22	0.46 ± 0.35	1.62^{b} ± 0.20
—	NT	73.35^{b} ± 0.30	23.77^{a} ± 0.48	0.35^{c} ± 0.12	0.64 ± 0.26	1.89^{a} ± 0.07
p-value	—	—	—	—	—	—
Stress	—	0.03	0.02	0.08	0.71	0.25
Breed	—	<0.0001	<0.0001	<0.0001	0.23	<0.0001
Interaction	—	0.08	0.02	0.10	0.38	0.07

*Mean ± standard deviation (*n = *12).*

*[a, b, c, d]Significant differences due to different treatment groups or different breeds (*p < *0.05).*

temperature of 24–26°C or even higher was frequently observed in the rearing houses (Tirawattanawanich et al., 2011). We observed that in some recent studies, the thermoneutral condition approximately at 24–26°C was also used (Sohail et al., 2012; Duangjinda et al., 2017; Xu et al., 2018).

Histological Findings

The examples of histological findings of the pectoralis major muscles of each sample group are illustrated in **Figure 2**. Based on histological scores (**Figure 3**), the control CB samples frequently received score 2 for inflammation, with less samples receiving score 0 (**Figure 3A**). Hence, the average score for inflammation (**Figure 3C**) of the control CB was greater ($p < 0.05$) than that of the samples from the other strains, but the average scores for such histological characteristics were not significantly different from that of the stressed CB ($p \geq 0.05$). The average scores for inflammation were least pronounced in the control NT, of which the average scores did not significantly differ from stressed NT and stressed H75.

Concerning adipose tissue infiltration (**Figure 3B**), the majority of the control CB received score 1, while the majority of the other groups, particularly NT samples, was scored 0 for adipose tissue infiltration. The average score (**Figure 3D**) was the most pronounced in control CB, followed by the stressed CB. The results indicated the significant inflammation and adipose infiltration in the pectoral muscle of the control CB at a greater extent in comparison to the other strains. The histopathological appearance was in correspondence with the abnormal histological characteristics of WS and WB consistently reported in commercial broilers (Kuttappan et al., 2013b; Russo et al., 2015; Sihvo et al., 2017; Prisco et al., 2021).

A previous study of Adu-Asiamah et al. (2021) demonstrated that local Chinese broilers exposed to an acute temperature rise from 30 to 35°C for 8 h at the age of 56 days exhibited infiltrations of fat tissues and inflammatory cells along with mild fibrosis and degenerated muscle fibers in the breast muscle. In this study, although the average histological scores of H75, H50, and NT followed the trend reported in the previous studies, no significant effects were observed, which again supported the ability of those strains to adapt under the current challenge. The discrepancy between our results and the findings of Adu-Asiamah et al. (2021) might be explained by the different thermal intensity between the studies. On the contrary, the CB chickens exposed to the thermal stress were affected with adipose infiltration at a lesser extent than control CB. The current results were in congruence with the report of Aslam et al. (2021) in which less incidence and severity of myodegeneration, inflammatory and lipid infiltration, and fibrosis were observed in the pectoralis major muscle collected from Arbor Acres Plus broilers exposed to cyclic heat stress compared to the birds reared under thermoneutral condition.

Absolute Gene Expression

To further elucidate the molecular events associated with histological characteristics, absolute expressions of 13 target genes were examined (**Figure 4**). Concerning the main effects (heat stress and breed), no significant effects of the cyclic heat stress on transcript abundance of the tested genes were detected ($p \geq 0.05$). The significant impacts of breed on the expression of *CD36*, *FABP4*, *LITAF*, *PDGFRA*, *PLIN1*, *PPARG*, *POSTN*, *SCD1*, and *TGFB1* were observed ($p < 0.05$). The interaction ($p < 0.05$) between the two main effects was observed for *TGFB1*. The absolute expression levels of those genes, except for *LITAF*, were positively correlated ($p < 0.05$) with histological scores and the occurrence of WS and WB abnormalities (**Table 5**). Positive correlation ($p < 0.05$) between *LITAF* abundance was detected with adipose tissue infiltration and WS incidence. In

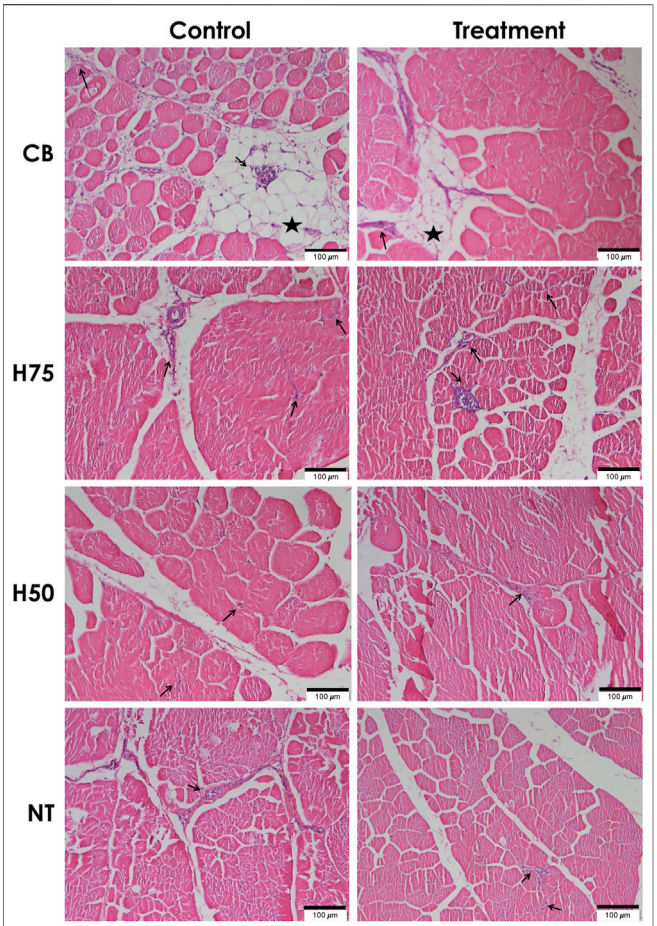

FIGURE 2 | Histological findings for hematoxylin and eosin (H&E)–stained pectoralis major muscle sections. Examples of microscopic images (scale bar of 100 µm) of H&E–stained pectoralis major muscles collected from different chicken breeds. The birds were reared at a constant temperature (control, 26°C) or received heat challenge (treatment, 35°C, 6 h daily) for 20 days before reaching their market ages. CB: commercial broilers; H75: crossbreeds, 75% broiler background and 25% Thai native background; H50: crossbreeds, 50% broiler background and 50% Thai native background; NT: Thai native chickens. Arrows and stars indicate accumulation of inflammatory cells and adipose tissue, respectively.

addition, the *LPL* expression level was found to be positively correlated ($p < 0.05$) with histological scores for adipose tissue infiltration and WS and WB abnormalities.

The proteins encoded by *ADIPOQ*, *CD36*, *FABP4*, *LPIN1*, *LPL*, *PLIN1*, *PPARG*, and *SCD1* play crucial roles in lipid metabolism. Adiponectin, encoded by *ADIPOQ*, regulates lipid metabolism by promoting transport of fatty acids into muscle cells and by activities and expression of several enzymes involved in β-oxidation. Adiponectin also promotes accumulation of triglycerides in adipocytes (Nguyen, 2020). Lipoprotein lipase, encoded by *LPL*, catalyzes the rate-limiting step in hydrolysis of plasma lipoprotein triglycerides to nonesterified fatty acids for further utilization of tissues, including re-esterification for storage within tissues (Mead et al., 2022). On the other hand, *LPIN1*-encoding enzyme participates in adipogenesis. *CD36* and *FABP4* encode long-chain fatty acid transport proteins, facilitating the uptake of free fatty acids across sarcolemma into skeletal muscle cells and mitochondria (Stahl et al., 2001). *PLIN1* encodes perilipin-1 which coats on lipid droplets and controls adipocyte triglyceride storage and lipolysis (Sun et al., 2019). *SCD1* encodes stearoyl-CoA desaturase, an integral membrane protein of the endoplasmic reticulum, which catalyzes the formation of monounsaturated fatty acids from saturated fatty acids (Heinemann and Ozols, 2003). In a previous study of Liu et al. (2019), pectoralis major muscle samples were collected from Jingxing-Huang female broilers, divided into two groups based on triglyceride (TG) content, and further submitted to RNA-seq. A transcriptome analysis between the two groups (i.e., high TG vs. low TG) revealed increased transcript abundance of *CD36*, *SCD1*, *PPARG*, *ADIPOQ*, and *LPL* in the high TG samples compared to the low TG ones. Those differentially expressed genes were mapped into the PPARγ signaling pathway, suggesting the crucial role of the PPARγ signaling pathway in lipid deposition in the chicken breast muscle with different TG content. Therefore, the differential expression of *CD36*, *FABP4*, *PLIN1*, *PPARG*, and *SCD1* identified in this study indicated the differences in lipid metabolism among the breeds upon exposure of the current cyclic thermal challenge. The altered lipid utilization could be attributed to the dysregulated PPARγ pathway in promoting adipogenesis and enhanced formation of lipid droplets through activity of *PLIN1* (Liu et al., 2019), leading to intramuscular fat deposition in the breasts.

It is worth noting that previous studies usually reported an increased fat content in the stressed broilers (Quinteiro-Filho et al., 2010; Xu et al., 2018; Lu et al., 2019). However, in this study, the trend was in the opposite direction, where stressed CB exhibited lower fat content along with decrease in absolute expression of *CD36* and *FABP4* than those of control CB. Although the actual reason of this discrepancy requires further investigation, it might be reasonable to hypothesize that the birds might be able to adapt to 20 days of cyclic thermal stress (Aengwanich, 2007). In addition, increased abundances of *CD36*, *PPARG*, *FABP4*, and *LPL* were previously found in the WB-affected broiler breast muscle (Abasht et al., 2016; Lake et al., 2019), while Marciano et al. (2021) reported increased *PLIN1* in the breast muscle of Cobb500 affected with WS condition. Papah and Abasht (2019) addressed significant increase in *CD36*, *PLIN1*, *FABP4*, and *LPL* abundance in the WB-affected pectoralis major muscle collected from 3-week-old Ross 708 broilers; however, in 7-week-old broilers, differential expressions of those genes between the affected and unaffected samples were not observed. Papah and Abasht (2019) hypothesized that as the breast muscle of 7-week-old broilers became larger, hypoxic conditions might be more profound and disrupt transcriptions of PPARγ and its targeted genes, shifting gene expression patterns.

It is widely accepted that an abnormal fat deposition in the skeletal muscle is associated with impaired muscle regeneration, especially in the breast muscle affected by WS. Upon muscle injury, satellite cells are activated and enter cell cycle for muscle regeneration. As myogenic factor 5 (Myf5), encoded by *MYF5*, play roles in activating quiescent satellite cells into proliferation, differential expression of *MYF5* (**Figure 4G**) among the current samples in correspondence with histological lesions was initially

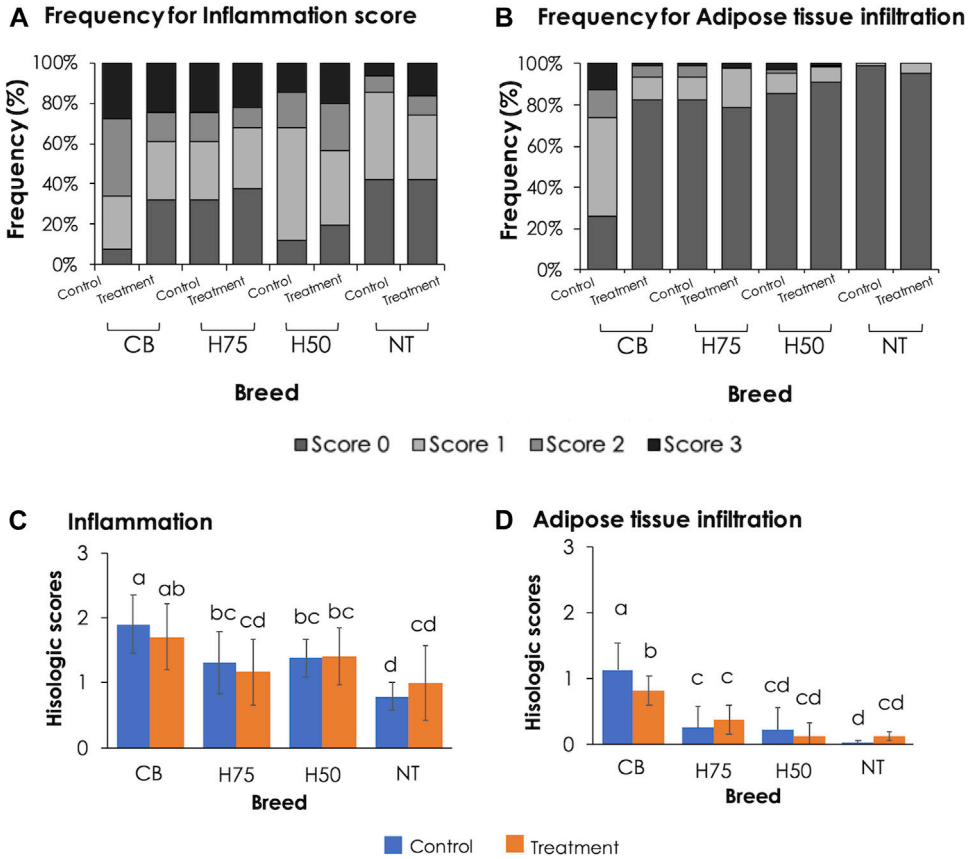

FIGURE 3 | Bar graphs indicating frequency and average histological lesion scores. **(A,B)** Frequency, expressed in percentage, of each histological score observed in pectoralis major muscles of each treatment group. **(C,D)** Average histological scores. Bars and error bars depict average and standard errors in the scores, respectively. CB: commercial broilers; H75: crossbreeds, 75% broiler background and 25% Thai native background; H50: crossbreeds, 50% broiler background and 50% Thai native background; NT: Thai native chickens. Different letters above bars denote statistical significance ($p < 0.05$).

anticipated. However, no significant changes of *MYF5* were found in this study ($p \geq 0.05$). Although further investigation remained to be elucidated to obtain full comprehension regarding the findings, our data agreed with the previous study of Praud et al. (2020) in which no differences in *MYF5* expression were addressed among slow-growing chickens and broilers exhibited either normal characteristics, WS, WB, or WS/WB abnormalities.

Apart from the adipogenic fate of satellite cells, intramuscular fat in the pectoralis major muscle may also be contributed by activities of fibro-adipogenic progenitors (FAPs). Recently identified by Uezumi et al. (2010), these multi-potent progenitors are localized in the interstitial area of the skeletal muscle and play important roles in muscle repair. During the early phase of muscle regeneration, FAPs are activated and differentiated into adipocytes and collagens to provide a transient support for satellite cell differentiation. FAPs are tightly regulated through TNF-α–induced apoptosis (Lemos et al., 2015). Under abnormal muscle regeneration, an excessive TGF-β could inhibit TNF-α–meditated FAP apoptosis (Pagano et al., 2019) along with imbalanced lipid storage and utilization (Lukjanenko et al., 2013), leading to fat deposition in the skeletal muscle. Overproduction of TGF-β has been observed in the injured skeletal muscle, and its expression is positively correlated with the differentiation fate of FAPs into adipogenic cells (Lukjanenko et al., 2013; Pagano et al., 2019) and fibrogenic cells (Davies et al., 2016; Song et al., 2017; Juban et al., 2018). In this study, absolute transcript abundances of *LITAF* (**Figure 4D**) and *TGFB1* (**Figure 4M**) in CB and H50 were greater ($p < 0.05$) than those of NT samples, while the expression levels of those genes in other samples were at intermediate levels. In agreement with the present study, upregulated *TGFB1* and *LITAF* in breast muscle of commercial broilers affected with growth-related myopathies were consistently addressed (Mutryn et al., 2015; Velleman and Clark, 2015; Marchesi et al., 2019; Praud et al., 2020; Prisco et al., 2021; Xing et al., 2021). The role of *LITAF*-encoded protein in regulating the transcription of TNF-α in inflammatory response was previously demonstrated by Hong et al. (2006). *PDGFRA* encodes a cell surface tyrosine kinase receptor for members of the platelet-derived growth factor family which is crucially required for normal development of several cells and organs, including connective tissue (Horikawa et al., 2016). Uezumi et al. (2010) demonstrated that the adipogenic fate of FAPs depended greatly on the muscle microenvironment, and only PDGFRA-positive FAP cells could undergo differentiation into adipocytes in the skeletal muscle. An increased *PDGFRA* abundance was also previously observed in the breast muscle of

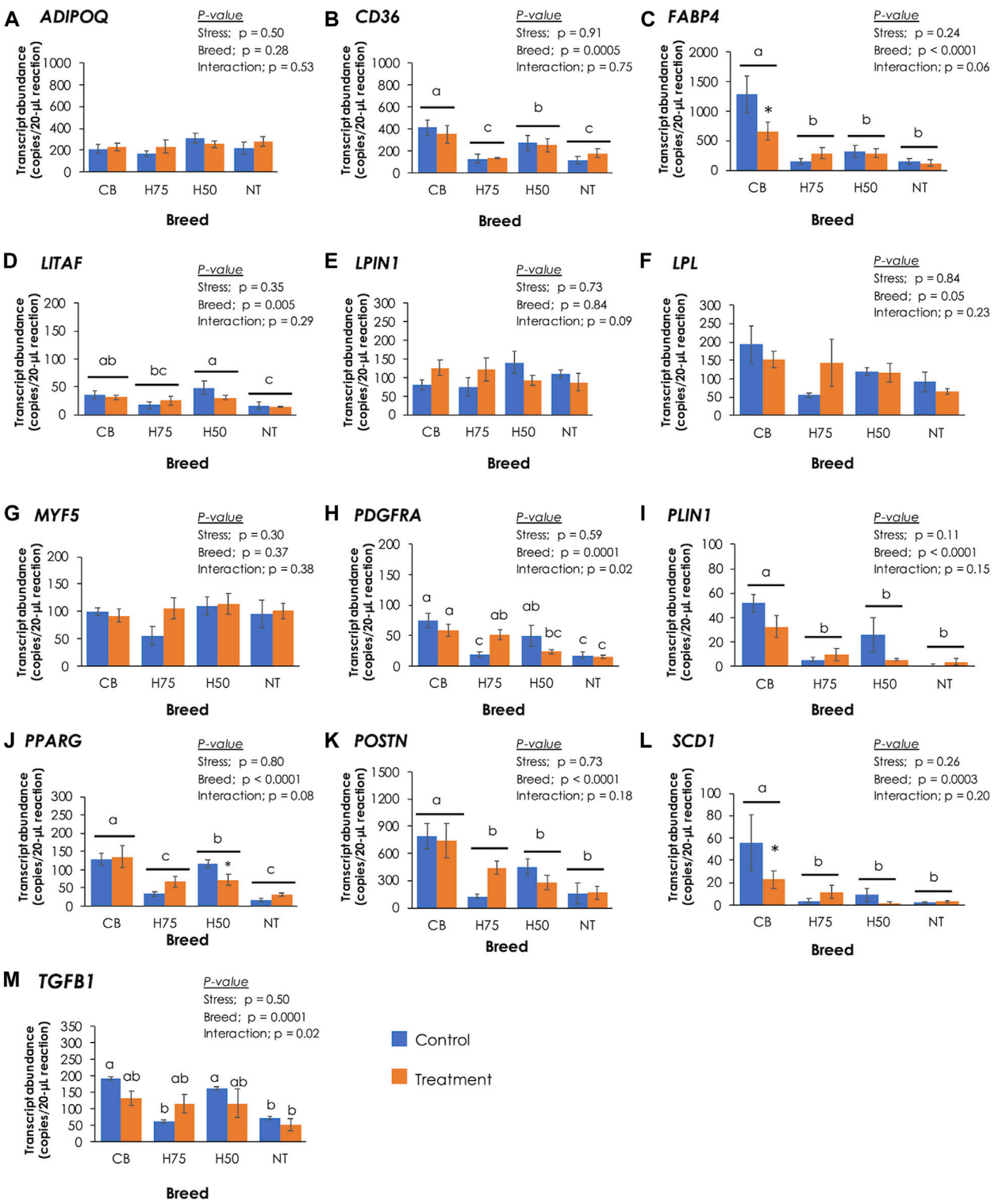

FIGURE 4 | Absolute transcript abundance of 13 target genes associated with lipid metabolisms and muscle injury in the pectoralis major muscle of chickens from different breeds. The birds were reared at a constant temperature (control, 26°C) or received heat challenge (treatment, 35°C, 6 h daily) for 20 days before reaching their market ages. Bars and error bars depict average and standard error in copies per 20-µL reaction, respectively. CB: commercial broilers; H75: crossbreeds, 75% broiler background and 25% Thai native background; H50: crossbreeds, 50% broiler background and 50% Thai native background; NT: Thai native chicken. Different letters above individual bars denote statistical significance among different treatment groups ($p < 0.05$). Different letters above horizontal lines indicate significance due to different breeds ($p < 0.05$). Asterisks indicate the reduced expression of particular genes in stressed samples compared to their control counterparts ($p < 0.05$).

TABLE 5 | Correlation coefficient between histological scores, white striping (WS) and wooden breast (WB) abnormalities, and absolute transcript abundance.

	Pearson's correlation coefficient		Spearman's rank correlation coefficient[1]	
	Inflammation	**Adipose tissue infiltration**	**WS**	**WB**
Inflammation	na	na	0.45*	0.36
Adipose tissue infiltration	na	na	0.46*	0.47**
WS	na	na	na	0.67***
WB	na	na	0.67***	na
ADIPOQ	–0.08	–0.12	0.03	0.01
CD36	0.43*	0.56**	0.66***	0.51**
FABP4	0.57**	0.76***	0.61**	0.51**
LITAF	0.29	0.37*	0.45*	0.31
LPIN1	–0.19	–0.17	0.14	0.03
LPL	0.27	0.40*	0.51*	0.48**
MYF5	–0.18	–0.03	0.37	0.30
PDGFRA	0.36*	0.73***	0.51**	0.47**
PLIN1	0.61**	0.81***	0.57**	0.47**
PPARG	0.49**	0.67***	0.52**	0.43*
POSTN	0.38*	0.66***	0.44*	0.48**
SCD1	0.51**	0.62**	0.54**	0.47**
TGFB1	0.47**	0.54**	0.54**	0.42*

*p < *0.05*, **p < *0.01*, ***p < *0.0001*, *na = not applicable.*

broilers affected with WB myopathy (Pampouille et al., 2019; Praud et al., 2020). The difference in the expression pattern of *PDGFRA* (**Figure 4H**) found in this study suggested a potential association between activities of FAPs and intensive histological lesions among the current chicken breast muscle samples.

Although fibrosis was not the main focus in this study, we observed an increased *POSTN* absolute abundance (**Figure 4K**) in significant association with severity of histological lesions (**Table 5**) in the current samples. *POSTN*-encoded protein, expressed in connective tissues rich in collagens, is recognized as a key player in regulation of organization of the extracellular matrix and shown to be induced by growth factors and cytokines, particularly TGF-β (González-González and Alonso, 2018). Our previous transcriptome analysis revealed an increased *POSTN* for approximately 2.5-fold in the WB-affected breast muscle of 7-week-old Ross 308 broilers compared to their normal counterparts (Malila et al., 2021b). The upregulated *POSTN* together with *TGFB1* identified in this study may also imply the fibronectin fate of FAPs potentially through the regulation of TGF-β in the control CB.

CONCLUSION

In summary, the results indicated that the current cyclic thermal condition significantly reduced live body weight and breast weight of the chickens ($p < 0.05$). As per chemical composition, stressed CB samples exhibited increased protein and ash ($p < 0.05$) content compared with their control counterparts. In addition, infiltration of inflammatory cells and adipose tissues was prevalent in control CB, and the histological scores correlated with the incidence of white striping. Differential absolute transcript abundances of the target genes in the breast muscle samples suggested potential involvement of dysregulated activities of FAPs together with perturbed lipid metabolisms in fat deposition in the CB breast muscle.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee at National Center for Genetic Engineering and Biotechnology (IACUC No. BT-Animal 13/2,564).

AUTHOR CONTRIBUTIONS

YM, YP, and SK conceived and designed the experiments. AJ, SK, and YP contributed to animal handling. YM, AJ, YS, PS, YP, and SK contributed to sample collection. PS and WT performed histological evaluation. PS performed total RNA isolation, cDNA synthesis, and ddPCR. YM conducted data analysis and drafted the manuscript with revisions provided by WT, YS, and SK. All authors read and approved the final manuscript.

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ORIGINAL RESEARCH
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Nature vs. Nurture: Disentangling the Influence of Inheritance, Incubation Temperature, and Post-Natal Care on Offspring Heart Rate and Metabolism in Zebra Finches

Sydney F. Hope[*†], *Louise Schmitt, Olivier Lourdais and Frédéric Angelier*[†]

Centre d'Etudes Biologiques de Chizé, CNRS—La Rochelle Université, Villiers en Bois, France

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Case Western Reserve University, United States

***Correspondence:**
Sydney F. Hope
sydney.hope@gmail.com

†ORCID ID:
Sydney F. Hope,
orcid.org/0000-0002-3711-8593
Frédéric Angelier,
orcid.org/0000-0003-2619-167X

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A historic debate in biology is the question of nature vs. nurture. Although it is now known that most traits are a product of both heredity ("nature") and the environment ("nurture"), these two driving forces of trait development are rarely examined together. In birds, one important aspect of the early developmental environment is egg incubation temperature. Small changes (<1°C) in incubation temperature can have large effects on a wide-array of offspring traits. One important trait is metabolism, because it is related to life-history traits and strategies, organismal performance, and energetic and behavioral strategies. Although it has been shown that embryonic and post-hatch metabolism are related to egg incubation temperature, little is known about how this may vary as a function of genetic differences or post-hatching environmental conditions. Here, we investigated this question in zebra finches (*Taeniopygia guttata*). We experimentally incubated eggs at two different temperatures: 37.5°C (control), which is optimal for this species and 36.3°C (low), which is suboptimal. We first measured embryonic heart rate as a proxy of embryonic metabolic rate. Then, at hatch, we cross-fostered nestlings to differentiate genetic and pre-hatching factors from post-hatching environmental conditions. When offspring were 30 days-old, we measured their resting metabolic rate (RMR; within the thermoneutral zone) and thermoregulatory metabolic rate (TMR; 12°C; birds must actively thermoregulate). We also measured RMR and TMR of all genetic and foster parents. We found that embryonic heart rate was greater in eggs incubated at the control temperature than those at the low temperature. Further, embryonic heart rate was positively related to genetic father RMR, suggesting that it is both heritable and affected by the pre-natal environment. In addition, we found that post-hatch metabolic rates were positively related to genetic parent metabolic rate, and interactively related to incubation temperature and foster mother metabolic rate. Altogether, this suggests that metabolism and the energetic cost of thermoregulation can be influenced by genetics, the pre-natal environment, and the post-natal environment. Our study sheds light on how environmental changes and parental care may affect avian physiology, as well as which traits may be susceptible to natural selection.

Keywords: incubation temperature, heritability, metabolic rate, thermoregulation, embryonic heart rate, cost of thermoregulation

1 INTRODUCTION

Whole-organism metabolism is a fundamental aspect animal physiology, and thus understanding the drivers of individual variation in metabolism is crucial (Burton et al., 2011; White and Kearney, 2013; Pettersen et al., 2018). Resting metabolic rate (RMR) represents an individual's minimum energy requirements for self-maintenance (Daan et al., 1990; Bryant, 1997), excluding physiological processes such as thermoregulation, digestion, and activity (McNab, 1997). RMR is important for understanding basal metabolic rate, and is also related to individual life history traits and strategies, performance, energetic strategies, behavior, reproductive success, and survival (Careau et al., 2008; Biro and Stamps, 2010; Williams et al., 2010; Careau and Garland, 2012; Rønning et al., 2016; Auer et al., 2018; Pettersen et al., 2018). While metabolic rate varies depending on current environmental conditions (Broggi et al., 2004; Norin and Metcalfe, 2019), there is evidence that, across taxa, individual differences in metabolic rate are repeatable (Nespolo and Franco, 2007; Careau et al., 2008; Broggi et al., 2009; Réveillon et al., 2019; Baškiera and Gvoždík, 2021; Dezetter et al., 2021) and heritable (reviewed in Pettersen et al., 2018). Further, conditions during early development and parental effects can also have lasting effects on individual metabolism (Burton et al., 2011). For example, in oviparous species, maternal hormone deposition to eggs can affect offspring post-hatch metabolic rate (Groothuis et al., 2005; Tobler et al., 2007; Nilsson et al., 2011). However, little is known about how different drivers (e.g., heritability and parental effects) may interact to influence metabolism (Burton et al., 2011; White and Kearney, 2013; Pettersen et al., 2018; McFarlane et al., 2021). Understanding the sources of inter-individual variation in metabolism will shed light on how environmental changes, parental care decisions, and natural selection can shape this important aspect of physiology.

In birds, some of the most important sources of variation in offspring physiology arise from parental care decisions. Aside from important maternal effects during egg-laying (e.g., nutrient/hormone transfer to eggs; (Groothuis et al., 2005; Tobler et al., 2007; Nilsson et al., 2011), two essential ways in which parents must ensure proper offspring development are through egg incubation and post-hatch nestling care. Incubation is necessary for eggs to hatch (Deeming and Ferguson, 1991), but energetically costly and time consuming for parents (Tinbergen and Williams, 2002; Nord and Williams, 2015). In turn, incubation investment varies among parents, due to factors such as ambient temperature, clutch size, parental experience, and individual quality (Aldrich and Raveling, 1983; Haftorn and Reinertsen, 1985; Conway and Martin, 2000; Ardia and Clotfelter, 2007; Coe et al., 2015; Amininasab et al., 2016; Hope et al., 2020; Williams et al., 2021). This causes incubation temperatures to vary both among and within nests (Boulton and Cassey, 2012; Coe et al., 2015; Hope et al., 2021). Importantly, small differences in temperature can have large effects on offspring physiology, such as metabolic rate, thermoregulation, glucocorticoid hormone levels, immune function, and telomere length (Nord and Nilsson, 2011; DuRant et al., 2013; Hepp et al., 2015; Wada et al., 2015; Stier et al., 2020; Hope et al., 2021). Similarly, in altricial species, parental food provisioning is essential for the proper growth and development of offspring. However, parents vary in their nestling provisioning rates due to factors such as food availability, the sex of the parent, parental experience, ambient temperature, brood size, and predation risk (Wright et al., 1998; Wiebe and Neufeld, 2003; Barba et al., 2009; Low et al., 2012; Ghalambor et al., 2013), with some evidence that provisioning behavior is repeatable and that some individuals are consistently "good" parents (Schwagmeyer and Mock, 2003). As with incubation temperature, differences in provisioning can affect offspring morphology and physiology. For example, food limitation during nestling development is related to low body masses, slow growth rates, altered glucocorticoid hormone levels, higher metabolic rates, and lower survival (Lepczyk and Karasov, 2000; Killpack and Karasov, 2012; Schmidt et al., 2012).

There is evidence that genetics, incubation temperature, and post-hatch parental care can influence both juvenile and adult avian metabolic rate. For example, avian RMR has been shown to be repeatable within individuals and heritable through adulthood (Bech et al., 1999; Rønning et al., 2005, 2007; Broggi et al., 2009; Nilsson et al., 2009). Further, studies have found that eggs incubated at lower temperatures have slower embryonic development and lower embryonic metabolic rates (DuRant et al., 2011; Stier et al., 2020) but, after hatching, produce offspring that have higher RMR early in life compared to those incubated at a warmer temperature (Nord and Nilsson, 2011; Wada et al., 2015). Moreover, environmental stressors during the post-hatch development, such as glucocorticoid exposure (Spencer and Verhulst, 2008; Dupont et al., 2019), food restriction (Moe et al., 2005; Criscuolo et al., 2008; Rønning et al., 2009; Careau et al., 2014), and sibling competition (Burness et al., 2000; Verhulst et al., 2006), can have long-lasting effects on offspring RMR. Additionally, another important aspect of metabolism is thermoregulatory metabolic rate (TMR), which is the metabolic rate organisms express under challenging thermal conditions, and represents the metabolic cost associated with thermoregulation (Broggi et al., 2004; Carleton and Rio, 2005; Nzama et al., 2010; DuRant et al., 2012; Dupont et al., 2019). Although less-often studied compared to RMR, there is also some evidence that avian TMR can be affected by incubation temperature and the post-hatch environment. For example, one study found that wood ducks (*Aix sponsa*) incubated at a lower temperature had higher TMR than those incubated at a warmer temperature (DuRant et al., 2012). Further, one study found that house sparrows (*Passer domesticus*) with increased glucocorticoid exposure during post-hatch development had lower TMR than control nestlings (Dupont et al., 2019). However, despite the evidence for the influence of genetics and pre- and post-hatch parental effects on both avian RMR and TMR, no study to date has investigated whether these different drivers may interact to affect metabolism.

In this study, we investigated whether genetics, incubation temperature, and/or post-hatch parental care interact to explain individual variation in avian RMR or TMR. To do this, we incubated zebra finch (*T. guttata*) eggs at two different temperatures: 37.5°C (control), which is optimal for this species and 36.3°C (low), which is suboptimal in this species,

as shown in other studies (Wada et al., 2015; Berntsen and Bech, 2016). During incubation, as a proxy of embryonic metabolic rate, we measured embryonic heart rate (Sheldon et al., 2018). Then, at hatch, we cross-fostered nestlings to decouple genetic and pre-hatching factors from post-hatching environmental conditions. Lastly, we measured the RMR and TMR of all offspring at Day 30 (i.e., nutritional independence), and of all parents after reproduction had ended. Our main hypothesis was that offspring metabolism is shaped through a combination of inheritance, incubation conditions, and post-natal care. We tested the following predictions:

1) Embryonic heart rate and offspring metabolic rate on Day 30 are positively related to the metabolic rate of genetic parents (i.e., metabolic rate is heritable; Rønning et al., 2007; Nilsson et al., 2009).
2) Lower incubation temperatures lead to slower embryonic heart rates (Rubin, 2019; Stier et al., 2020), but higher RMR (Nord and Nilsson, 2011; Wada et al., 2015) and TMR (DuRant et al., 2012) at Day 30.
3) We considered the relationship between foster parent and offspring metabolic rate to be representative of the overall influence of the post-hatch environment and predicted that offspring metabolic rate would also be related to the metabolic rate of foster parents.

Along with these predictions, we also tested for interactive effects among our incubation temperature treatment and parental metabolism, with the expectation that offspring metabolic rate and embryonic heart rate may have different relationships with parental metabolic rate, depending on the incubation temperature treatment.

2 MATERIALS AND METHODS

2.1 General Husbandry and Breeding

We used a breeding colony of zebra finches (*T. guttata*; $N = 20$ pairs; "parents") housed at the CEBC (CNRS) for this study. We first housed the 40 birds together in an indoor aviary for 10 days and we formed pairs based on mating behaviors that we observed (e.g., singing, proximity, etc.). We then housed pairs in cages (47.5 × 38 × 51 cm) with external nest boxes (12 × 13 × 16 cm). Ambient temperature was kept at a constant 22°C and the photoperiod was set to a 14:10 day:night cycle, for all aspects of the study, including pair formation, reproduction, and nestling rearing. We provided birds with ~10 g of alfalfa hay every day, and then ~1 g of coconut fiber once the hay completely covered the bottom of the nest box. We misted pairs with water once per day until their first egg was laid, to stimulate reproduction. We provided birds with *ad libitum* food (Versele-Laga Prestige Tropical Finches seed mix), water supplemented with vitamins, cuttlefish bone, and grit. We also gave birds ~2 g of chopped hard-boiled eggs (including the shells) every day from pair formation until nestling Day 30, along with endives and millet sprays once per week (Olson et al., 2014). All procedures in this study were approved by the national ethics committee for animal experimentation under file number APAFIS#23727-2020011311559318.

2.2 Egg Incubation

We checked nest boxes daily at 10:00. Once an egg was found, we marked it with a unique ID using a small marker, weighed it, and placed it in an incubator (Brinsea© Ovation 28 Advance digital egg incubator) at one of two temperatures. We followed an incubation protocol similar to Wada et al. (2015). The "control" incubator was set at a constant 37.5°C (± 0.1 [SD]), which is likely optimal for zebra finches. The "low" incubator was set at a constant 36.3°C (± 0.1 [SD]), which is within the natural range of zebra finch incubation temperatures, but there is evidence that it produces suboptimal offspring phenotypes in this species (Wada et al., 2015). Both incubators were set at a humidity of 55%. We verified the temperature and humidity by placing iButton© (Hygrochron DS 1923, Maxim Integrated™) temperature loggers inside of each incubator. We randomly assigned the incubation treatment to the first laid egg of each breeding pair, and then systematically alternated among temperature treatments for each subsequent egg for the entire length experiment. Multiple clutches from each breeding pair were used in this study, to attain a sufficient sample size. During artificial incubation, we gave parents fake clay eggs to incubate so that they stayed in the breeding phase. One day before the predicted hatching date (day 13 for "control" eggs and day 14 for "low" eggs), we transferred eggs to a hatcher that was set at a temperature of 37.5°C and 67% humidity.

2.3 Embryonic Heart Rate

Embryonic heart rate is correlated with embryonic oxygen consumption (Du et al., 2010), and thus can be used as a proxy for energy expenditure during embryonic development. We measured embryonic heart rate by placing eggs in the Buddy digital egg monitor (Vetronic Services, Abbotskerswell, Devon, United Kingdom). We considered that a reading was reliable when the curve and heart rate outputs were relatively consistent for ~10 s (Sheldon et al., 2018). At each timepoint (see below), we took three repeated heart rate measures within 3 min of taking each egg (individually) out of the incubator and noted the time (seconds) that it took to take each measure. If any/all of the readings were unreliable (e.g., due to embryo movement; Sheldon et al., 2018), they were excluded from the analyses. All readings were taken in a room at a constant temperature of 22°C. If there was a consistent heart rate reading of "0", we candled eggs and determined if they were infertile or had died during development.

We measured heart rates of embryos after 11, 12 and 13 days for incubation for "control" eggs and after 12, 13 and 14 days of incubation for "low" eggs. These three measures are hereafter referred to as "readings 1, 2 and 3". We chose these days because the incubation period of "control" eggs is about 1 day shorter than that of and "low" eggs (**Table 1**) and, thus, we chose to investigate differences in heart rate among embryos at the same stage of development (i.e., "developmental age"), instead of after the same number of days (i.e., "calendar age"). We validated that our embryonic heart rate results were not driven by differences in eggshell temperature, and that they were not affected by our

TABLE 1 | Summary statistics of hatch success, incubation period, body mass, and metabolic rate.

Variable	Incubation temperature			
	Control (37.5°C)		Low (36.3°C)	
	Mean ± SE	N	Mean ± SE	N
Hatch success[a]	25.9%	135	23.9%	134
Incubation period (days)	13.8 ± 0.08	35	15.1 ± 0.06	32
Body mass day 0 (g)[b]	0.80 ± 0.02	33	0.83 ± 0.02	32
Body mass day 30 (g)	14.6 ± 0.39	20	14.4 ± 0.36	15
TMR (VO_2)	6.88 ± 0.16	20	6.93 ± 0.25	15
RMR (VO_2)	3.18 ± 0.08	20	3.06 ± 0.09	15

[a]*Excludes infertile and cracked eggs.*
[b]*Excludes two individuals that hatched but died in the incubator.*

choice of using "developmental age" instead of "calendar age" (see **Supplementary Appendix S1**). We placed eggs in the hatcher after the final heart rate readings.

2.4 Nestling Monitoring

We checked the hatcher for hatching multiple times each day and, at a minimum, once at 9:00 and once at 17:00. Once hatched, we weighed and marked nestlings by removing distinct patches of down feathers (Adam et al., 2014). Then, we cross-fostered nestlings. We gave parents up to two nestlings, which were never from the same incubation treatment, and nestlings were never more than 1 day apart in age. We housed nestlings with their foster parents until independence (i.e., Day 30), and then we conducted the metabolism measurements. Afterward, we housed independent offspring in sex-specific communal cages for use in future studies. We banded nestlings on Day 10 and determined their sex using plumage characteristics on Day 30.

2.5 Metabolism

We quantified energy metabolism in both parents and offspring by measuring oxygen consumption rates using multichannel open-circuit respirometry (Sable Systems Int., Las Vegas, NV, United States; Brischoux et al., 2017). We measured offspring when they were 32 ± 2.9 (range: 28–39) days-old, and we measured parents after they had finished reproduction [75 ± 26 days after their last foster nestling reached Day 30 (for those that successfully raised at least one nestling); 102 ± 20 days since their last laid egg (all birds)]. We measured metabolism of each bird twice: once at 32°C (RMR; within the thermoneutral zone; Calder, 1964) and once at 12°C (TMR; when birds must actively thermoregulate; Dupont et al., 2019), to determine whether incubation temperature might affect energy expenditure during a thermal challenge. Measurements at different temperatures were conducted on consecutive days and the order was randomized among incubation temperature treatments. We weighed birds at ~20:15 and began respirometry at ~20:30. Up to 7 birds were measured each night, and the system measured oxygen consumption of each bird for 10 min and systematically alternated among chambers, with 15 min of baseline reading (empty chamber) each time a full cycle was completed. Birds were removed and weighed again at ~8:30 the next morning. We did not analyze the first 3 h of data because this was the time when birds fasted (Wada et al., 2015). Oxygen flow was set at ~350 ml/min, and O_2 at 20.95%, which was recalibrated each night.

To calculate metabolic rate, we first chose the value of oxygen consumption for each 10 min run of each individual that was the lowest and most consistent, using the computer software ExpeData (Sable Systems). Then, we calculated metabolic rate using the Hoffman Equation for VO_2 (ml/h), and then corrected for body mass (ml/h/g). Lastly, we calculated the mean VO_2 of all runs of each individual to obtain the final VO_2 value (ml/h/g) for each individual.

2.6 Statistical Analyses

We conducted all statistical analyses using R v 3.5.1 (R Core Team, 2018). We reduced models using stepwise backwards elimination of non-significant terms ($p > 0.10$), starting with non-significant interactions. After eliminating the term with the highest p-value, we reran the model and continued this process until only significant ($p < 0.05$) or marginally significant ($0.05 < p < 0.10$) terms remained in the model. Incubation temperature was always treated as a categorical variable. We ensured that all models met the assumptions of normal and homoscedastic residuals by investigating histograms of residuals, normal quantile plots, and fitted vs. residuals plots. We verified that models met the assumption of non-multicollinearity by investigating the variance inflation factors (*vif*). Further, all continuous independent variables that were used in interactions were scaled and centered to reduce multicollinearity. We used the package *lme4* (Bates et al., 2015) for mixed effects models and *emmeans* (Lenth, 2018) for post-hoc tests, including slope comparisons for interactions. p-values were calculated using the *Anova* function using the *car* (Fox and Weisberg, 2011) package. R^2 values for mixed effects models were calculated using the *MuMln* package (Bartoń, 2018). Figures were created using the *plyr* (Wickham, 2011) and *ggplot2* (Wickham, 2016) packages. Two male parents died for reasons unrelated to the experiment before parental metabolic rates were measured, and thus their RMR, TMR, and ΔMR were not able to be included in the analyses. Neither of these males was a genetic father to any offspring that lived until Day 30 in this study, and only one of these males was a foster father to a single individual that lived until Day 30.

First, to determine whether embryonic heart rate was related to incubation temperature and/or parent metabolism, we built one linear mixed effect model with heart rate as the dependent variable. The independent variables were incubation temperature, reading (1, 2 or 3), the time it took to take the measurement (seconds), the RMR of both the genetic mother and father, along with all two-way interactions with incubation temperature. Parent TMR was not included in this model because 1) we predicted only that parental RMR would be related with embryonic metabolism, measured when embryos were at warm temperatures (i.e., incubation) and 2) parent TMR and RMR were correlated ($r = 0.42$; $p < 0.01$), and thus including them both in the model would increase multicollinearity. We also

included whether the egg hatched or not as an independent variable, and egg mass as a covariate. Egg ID was included as a random effect to control for repeated measures.

Second, to determine whether offspring metabolism was related to incubation temperature and the temperature at which the measurement was taken, we built one linear mixed effects model. Offspring metabolism (VO_2, ml/h/g) was the dependent variable, and it was log-transformed to meet model assumptions. The independent variables were incubation temperature, the temperature of the measurement (12°C or 32°C), and their interaction. Sex and age were also included as covariates, as well as the interaction between incubation temperature and sex. Individual ID, genetic parent ID, and foster parent ID were included as random effects to account for repeated measures within individuals and among siblings.

Next, we determined whether offspring metabolism was related to parental metabolism. First, we calculated heritability (h^2) as the slope of the regression between the mean value of genetic parent metabolism (either RMR or TMR) and offspring metabolism (Åkesson et al., 2008; Wray and Visscher, 2008). Then, to examine relationships among all parents (genetic and foster; separated by sex), and to test whether there was an interactive effect of incubation temperature and parental metabolism, we built three linear models. For all models, the dependent variable was offspring metabolism (VO_2, ml/h/g) and the independent variables were incubation temperature, the metabolism of the genetic mother, genetic father, foster mother, and foster father, along with all two-way interactions with incubation temperature. The difference among the three models was that the first included only RMR data (both parents and offspring), the second included only TMR data, and the third used the difference between TMR and RMR (i.e., additional amount of energy expended during thermoregulation; hereafter ΔMR) for all individuals.

Lastly, to determine whether embryonic heart rate and offspring metabolism (at Day 30) were correlated within individuals, we built one linear mixed effect model. The dependent variable was embryonic heart rate, and only individuals that lived until the metabolic measurement (~Day 30) were included in the model. The independent variables were incubation temperature, reading (1, 2 or 3), offspring RMR, and all two-way interactions with incubation temperature. Offspring TMR was not included in this model because 1) we predicted only that offspring RMR would be related with embryonic metabolism, measured when embryos were at warm temperatures (i.e., incubation) and 2) offspring TMR and RMR were correlated ($r = 0.73$; $p < 0.001$), and thus including them both in the model would increase multicollinearity. We also included sex and its interaction with incubation temperature in this analysis because, contrary to the first analysis, we had data on the sex of all individuals (i.e., only individuals that lived until Day 30 were included). The time it took to take the measurement (seconds) and egg mass were also included as covariates. Egg ID, genetic parent ID, and foster parent ID were included as random effects to control for repeated measures among siblings.

TABLE 2 | Full and reduced models investigating the relationship of embryonic heart rate with incubation temperature and parental metabolism.

	Embryonic heart rate (bpm)[a]	
	$N_{control}$ = 107 eggs; 839 readings	
	N_{low} = 117 eggs; 997 readings	
	Full model	
	R^2m = 0.43; R^2c = 0.70	
Term	**F**	***p***
Incubation temperature	**120.88**	**<0.0001**
Reading (1, 2, or 3)	**26.41**	**<0.0001**
Time until measurement (seconds)	**425.50**	**<0.0001**
Egg mass	**19.49**	**<0.0001**
Hatched (yes/no)	**4.18**	**0.042**
Mother RMR	**4.44**	**0.036**
Father RMR	0.86	0.36
Incubation X reading	**10.01**	**<0.0001**
Incubation X time	2.49	0.12
Incubation X Mother RMR	1.77	0.18
Incubation X Father RMR	0.42	0.52
	Reduced model	
	R^2m = 0.43; R^2c = 0.70	
Incubation temperature	**140.31**	**<0.0001**
Reading (1, 2, or 3)	**27.47**	**<0.0001**
Time until measurement (seconds)	**766.86**	**<0.0001**
Egg mass	**19.70**	**<0.0001**
Hatched (yes/no)	**3.91**	**0.049**
Father RMR	**13.55**	**0.0003**
Incubation X Reading	**9.48**	**<0.0001**

Bold values indicate statistical significance.
[a]*Egg ID was included as a random effect to control for repeated measures.*

3 RESULTS

3.1 Hatching Success, Incubation Period, and Body Mass

Summary statistics for hatching success, incubation period, and nestling body mass (Days 0 and 30) are reported in **Table 1**. There were no differences in hatching success, body mass at Day 0, or body mass at Day 30 between incubation temperature treatment groups (all $p > 0.25$; simple linear models). However, eggs incubated at the lower temperature had a longer incubation period than those incubated at the control temperature ($p < 0.001$).

3.2 Embryonic Heart Rate: Relationship With Incubation Temperature and Parental Metabolism

Embryonic heart rate was related to both incubation temperature and parent metabolic rate. We found that heart rate was greater in embryos from the control treatment compared to the low treatment ($p < 0.001$; **Table 2**; **Figure 1**), and that heart rate increased throughout the course of incubation (reading: $p < 0.001$; **Table 2**). There was an interactive effect of incubation

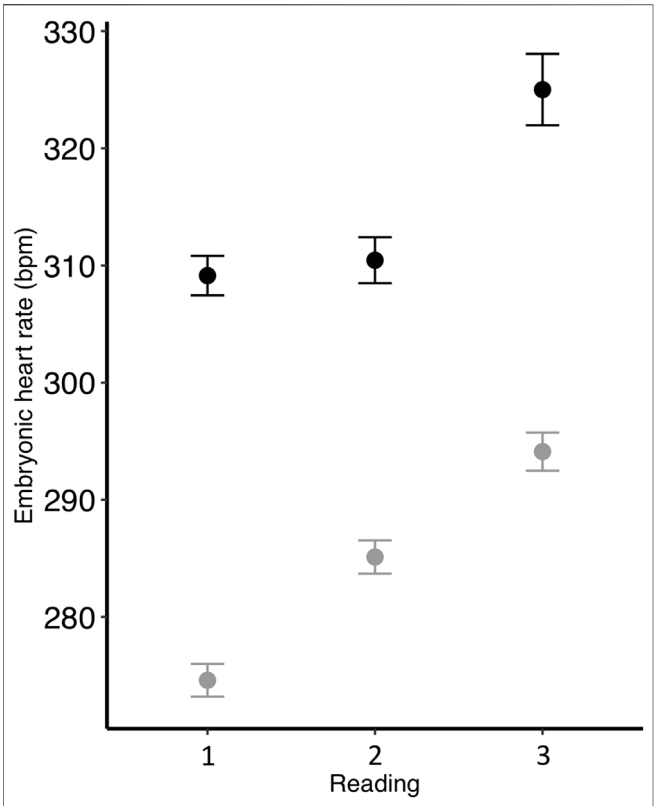

FIGURE 1 | Zebra finch embryonic heart rate (beats per minute; bpm) at three different time points during development and incubated at two different temperatures (black = control; gray = low). To correct for different developmental rates, readings were taken on control eggs after (1) 11, (2) 12, and (3) 13 days of incubation, while readings were taken on low eggs after (1) 12, (2) 13, and (3) 14 days. Mean ± SE are shown.

TABLE 3 | Full and reduced models investigating the effects of incubation temperature and temperature of measurement on offspring metabolism at Day 30.

	Offspring VO_2 (ml/h/g)[a,b]	
	$N_{control}$ = 20; N_{low} = 15	
	Full model	
	R^2m = 0.93; R^2c = 0.98	
Term	**F**	***p***
Incubation temperature	0.98	0.33
Temperature (12 or 32 °C)	**1847.1**	**<0.0001**
Sex	3.30	0.08*
Age	0.08	0.78
Incubation X Temperature	2.54	0.12
Incubation X Sex	1.55	0.22
	Reduced model R^2m = 0.92; R^2c = 0.98	
Temperature (12 or 32 °C)	**3380.8**	**<0.0001**

Bold values indicate statistical significance and asterisks (*) indicate marginal significance.

[a]*log-transformed to meet model assumptions.*

[b]*Parent (genetic and foster) and individual IDs were included as random effects in model.*

temperature and reading on heart rate ($p < 0.001$; **Table 2**), and post-hoc tests revealed significant differences for all pairwise comparisons (all $p < 0.001$), except between the heart rate of control embryos on days 11 and 12 ($p > 0.99$; **Figure 1**). Although embryonic heart rate was significantly related to mother RMR in the full model, it was not retained in the final model (**Table 2**; **Figure 2A**). However, embryonic heart rate was positively correlated with father RMR ($p = 0.0003$; **Table 2**; **Figure 2B**). There was also a relationship between whether or not the egg

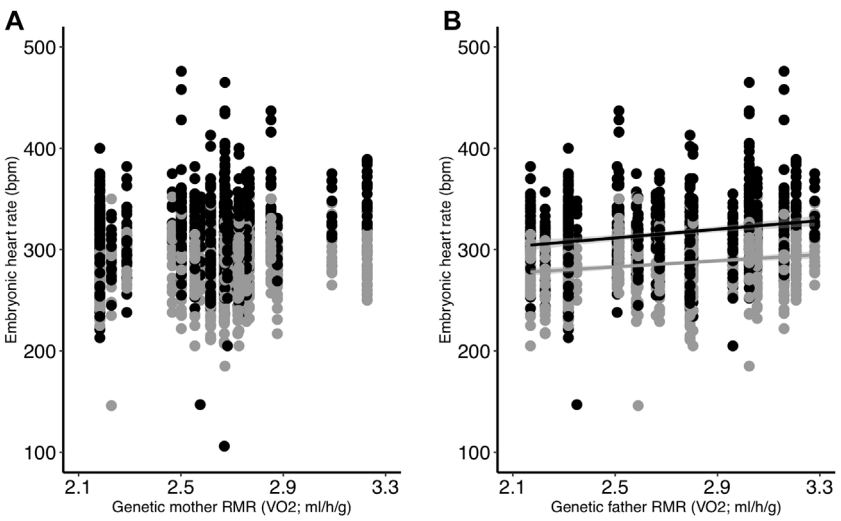

FIGURE 2 | The relationships between egg heart rate (bpm) and **(A)** genetic mother RMR and **(B)** genetic father RMR. Eggs were incubated at two different temperatures (black = control; gray = low). All egg heart rate measurements are shown. Regression lines are included only for significant relationships.

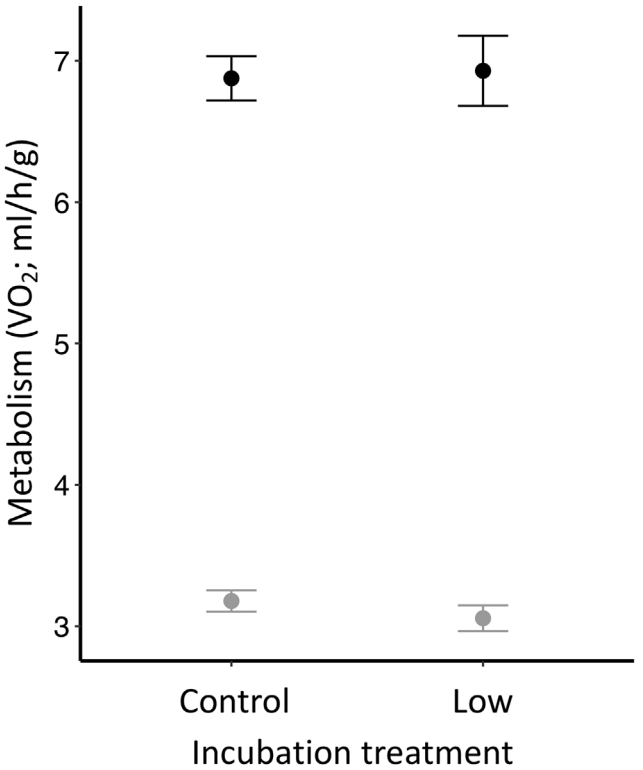

FIGURE 3 | Zebra finch metabolic rate (VO_2; ml/h/g) measured at two different temperatures (black = TMR: 12°C; gray = RMR: 32°C). Individuals were measured at Day 30 and had hatched from eggs incubated at two different temperatures (i.e., control or low).

hatched and its heart rate (p = 0.049; **Table 2**), where embryos with greater heart rates were more likely to hatch. Further, heart rate increased as egg mass increased (p < 0.001; **Table 2**), and heart rate decreased with the time that it took to take the measurement (p < 0.001; **Table 2**).

3.3 Offspring Metabolic Rate: Relationship With Incubation Temperature and Parental Metabolism

Although we found no differences in metabolic rate among offspring incubated at different temperatures or between sexes (**Table 3**), we found that offspring metabolism was related to the temperature at which the measurement was taken. As expected, metabolism was greater when the measurement was taken at 12°C (TMR) than at 32°C (RMR) (p < 0.0001; **Table 3**; **Figure 3**).

The regression of mean genetic parent RMR with offspring RMR revealed that RMR was significantly heritable [h^2 = 0.53 ± 0.22 (SE), p = 0.02]. When we examined the relationships of all parents (genetic and foster) as individual factors, we found that genetic mother RMR was positively related to offspring RMR (p = 0.014; **Table 4**; **Figure 4A**). However, offspring RMR was not related to any other parental RMR, and there were no interactive relationships with incubation temperature (**Table 4**; **Figure 4B–D**).

The regression of mean genetic parent TMR with offspring TMR revealed that the heritability of TMR was not statistically significant [h^2 = 0.38 ± 0.28 (SE), p = 0.19]. However, when examining parents separately (i.e., all genetic and foster parents as separate independent variables), we found a trend that genetic father TMR was positively related to offspring TMR (p = 0.093; **Table 4**). Further, there was an interactive effect of incubation temperature and foster mother TMR on offspring TMR (p = 0.033; **Table 4**), where the TMR of offspring from the control group was not related to foster mother TMR (slope estimate: 0.022; confidence interval: −0.32 to 0.37) while the TMR of offspring from the low group was negatively related to the TMR of their foster mother (slope estimate: −0.56; confidence interval: −0.95 to −0.16). There were no relationships between offspring TMR and their genetic mother or foster father (**Table 4**).

Similar to TMR, the heritability of ΔMR (i.e., TMR−RMR) was not statistically significant [h^2 = 0.18 ± 0.27 (SE), p = 0.51]. However, when examining parents separately, although there was no relationship between genetic mother ΔMR and offspring ΔMR (**Table 4**; **Figure 5A**), we found that there was a significant positive relationship between genetic father ΔMR and offspring ΔMR (p = 0.049; **Table 4**; **Figure 5B**). There was also an interactive effect of incubation temperature and foster mother ΔMR on offspring ΔMR (p = 0.025; **Table 4**; **Figure 5C**). This relationship mimicked that of TMR: the ΔMR of offspring from the control group was not related to foster mother ΔMR (slope estimate: −0.12; confidence interval: −0.34 to 0.11; **Figure 5C**) while the ΔMR of offspring from the low group was negatively related to the ΔMR of their foster mother (slope estimate: −0.55; confidence interval: −0.85 to −0.25; **Figure 5C**). There was no relationship between offspring ΔMR and that of their foster father (**Table 4**; **Figure 5D**).

3.4 Embryonic Heart Rate and Metabolic Rate: Relationship Within Individuals

Embryonic heart rate and offspring RMR were not correlated within individuals (**Table 5**). Further, there were no significant interactive effects of incubation temperature and RMR. The only terms that remained in the model (**Table 5**) were incubation temperature (p < 0.001), reading (p < 0.001), time to take the measurement (p < 0.001), and incubation temperature x reading (p < 0.001), as already found previously in **Section 3.2**.

4 DISCUSSION

Here, we manipulated the developmental environment of zebra finches to disentangle the impact of inheritance, incubation temperature, and post-hatch rearing conditions (i.e., cross-fostering) on the energy metabolism of embryos and offspring at nutritional independence (i.e., Day 30). We found that embryonic heart rate, a proxy of embryonic metabolism, was positively related to genetic father RMR and that embryos incubated at the higher incubation temperature had faster heart rates than those incubated at the lower temperature.

TABLE 4 | Full and reduced models investigating the relationship of offspring metabolism at Day 30 with interactions between incubation temperature and parental metabolism.

	Response variable: Offspring metabolism (ml/h/g)					
	$N_{control}$ = 20; N_{low} = 15 Includes RMR data		$N_{control}$ = 20; N_{low} = 15 Includes TMR data		$N_{control}$ = 20; N_{low} = 15 Includes difference between TMR and RMR (ΔMR)	
	Full models					
	Multiple R^2 = 0.42; Adjusted R^2 =0.20		Multiple R^2 = 0.32; Adjusted R^2 =0.06		Multiple R^2 = 0.45; Adjusted R^2 =0.25	
Term	**F**	**p**	**F**	**p**	**F**	**p**
Incubation temperature	0.006	0.94	0.079	0.78	0.89	0.35
Genetic mother metabolism	0.24	0.63	0.81	0.38	0.42	0.53
Foster mother metabolism	3.94	0.059*	0.034	0.85	0.50	0.49
Genetic father metabolism	0.11	0.74	0.57	0.46	0.58	0.45
Foster father metabolism	1.69	0.21	1.13	0.30	0.68	0.42
Incubation X Genetic mother	**4.31**	**0.049**	0.67	0.42	1.20	0.28
Incubation X Foster mother	0.87	0.36	3.92	0.059*	**4.42**	**0.046**
Incubation X Genetic father	2.82	0.11	0.14	0.72	1.35	0.26
Incubation X Foster father	3.32	0.081*	1.60	0.22	1.07	0.31
	Reduced models					
	Multiple R^2 = 0.17; Adjusted R^2 =0.14		Multiple R^2 = 0.25; Adjusted R^2 =0.15		Multiple R^2 = 0.38; Adjusted R^2 =0.30	
Incubation temperature	—	—	0.11	0.74	1.26	0.27
Genetic mother metabolism	**6.73**	**0.014**	—	—	—	—
Foster mother metabolism	—	—	0.017	0.90	1.10	0.30
Genetic father metabolism	—	—	3.01	0.093*	**4.20**	**0.049**
Incubation X Foster mother	—	—	**5.01**	**0.033**	**5.57**	**0.025**

Bold values indicate statistical significance and asterisks (*) indicate marginal significance.

Further, we found evidence that post-hatch offspring RMR is heritable and has a positive correlation with genetic mother RMR, although we found no relationship between offspring RMR and either foster parent RMR or incubation temperature. Lastly, we found that the metabolic cost of thermoregulation (i.e., TMR and ΔMR) had a lower heritability than RMR, but was positively related to genetic father TMR and ΔMR. Interestingly, foster mother TMR and ΔMR were negatively correlated with offspring TMR and ΔMR, respectively, but this relationship was only apparent when offspring were incubated at the lower temperature. This suggests that there are combined effects of the pre-natal environment and post-natal parental care on the metabolic cost of thermoregulation.

4.1 Effects of Incubation Temperature

As predicted, eggs that were incubated at the lower temperature had slower embryonic heart rates than those incubated at the higher temperature. Because embryonic heart rate should be an indicator of embryonic metabolism (Du et al., 2010; Sheldon et al., 2018), this suggests that low incubation temperatures lead to a lower embryonic metabolic rate. Our results agree with two other studies that have investigated the relationship between incubation temperature and embryonic heart rate (Rubin, 2019; Stier et al., 2020), and with one study that found that wood duck (*Aix sponsa*) eggs incubated at lower temperatures had lower daily embryonic oxygen consumption compared to those incubation at higher temperatures (DuRant et al., 2011). This lower energy expenditure during embryonic development should be related to slower developmental rates (Vedder et al., 2017; Sheldon and Griffith, 2018). Indeed, we found that eggs incubated at the lower temperature had a longer developmental duration (i.e., incubation period) than those incubated at the higher temperature (**Table 1**). Importantly, we still found a difference in embryonic heart rate between incubation temperatures when we corrected for differences in eggshell temperature and differences in developmental rate (i.e., developmental age vs. calendar age; see **Supplementary Material**). This suggests that incubation temperature alters physiology during development, more so than just a linear relationship with current temperature or developmental rate.

We predicted that offspring incubated at the low temperature would have greater post-hatch metabolic rates than those incubated at the control temperature because previous studies have found this effect of incubation temperature on both RMR (Nord and Nilsson, 2011; Wada et al., 2015) and TMR (DuRant et al., 2012) in birds. However, contrary to our predictions, offspring metabolic rate (RMR and TMR) was not affected by our incubation temperature treatments (control: 37.5°C; low: 36.3°C). This disagrees with some other avian studies. For example, Nord and Nilsson (2011) found that 14-day-old blue

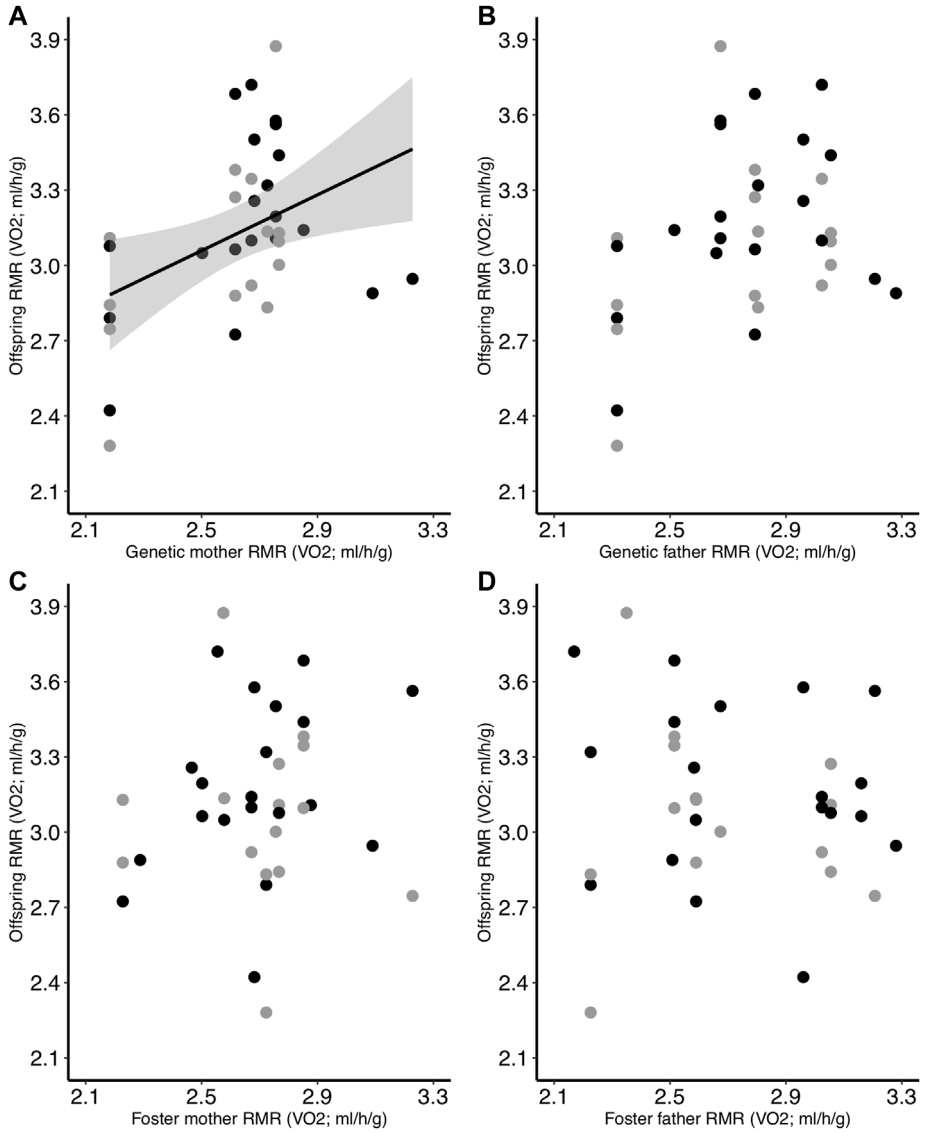

FIGURE 4 | Relationship between offspring RMR on Day 30 and the RMR of the **(A)** genetic mother, **(B)** genetic father, **(C)** foster mother, and **(D)** foster father. Offspring had been incubated at two different temperatures as eggs (black = control; gray = low). Regression lines are included only for significant relationships.

tits (*Cyanistes caeruleus*) incubated at a low temperature had higher RMR than those incubated at a warmer temperature and DuRant et al. (2012) found that 1-day-old wood ducks (*Aix sponsa*) incubated at a lower temperature had higher TMR than those incubated at a warmer temperature. To date, no study has investigated the effect of incubation temperature on zebra finch TMR, and two studies have examined the effects of incubation temperature on zebra finch RMR, with conflicting results. Supporting our findings, Berntsen and Bech (2021) found no difference in RMR among zebra finches that were incubated at 35.9°C and 37.9°C, measured at 15 and 45 days-old. In contrast, Wada et al. (2015) found that 25-day-old zebra finches incubated at 36.2°C had a higher RMR than those incubated at 37.4°C, although this effect was only found in females. In our study, we did not find an effect of sex on metabolic rate. In light of these conflicting results among species, it is possible that the effect of incubation temperature on metabolic rate is species-specific. For example, zebra finches may be a species that is more resistant to small changes in the embryonic thermal environment, and offspring develop similar physiological traits regardless of their developmental conditions. It is also possible that metabolic rate is more influenced by inheritance than by either the early developmental environment or sex, which could explain differences among zebra finch studies. Indeed, we found evidence that both RMR and TMR are heritable (see below). If our breeding parents displayed more genetic variation than those in the study of Wada et al. (2015), this could have masked any effects of incubation temperature and could explain the difference in results of our two studies. It should also be noted that there was relatively low hatching success in our study, although it was

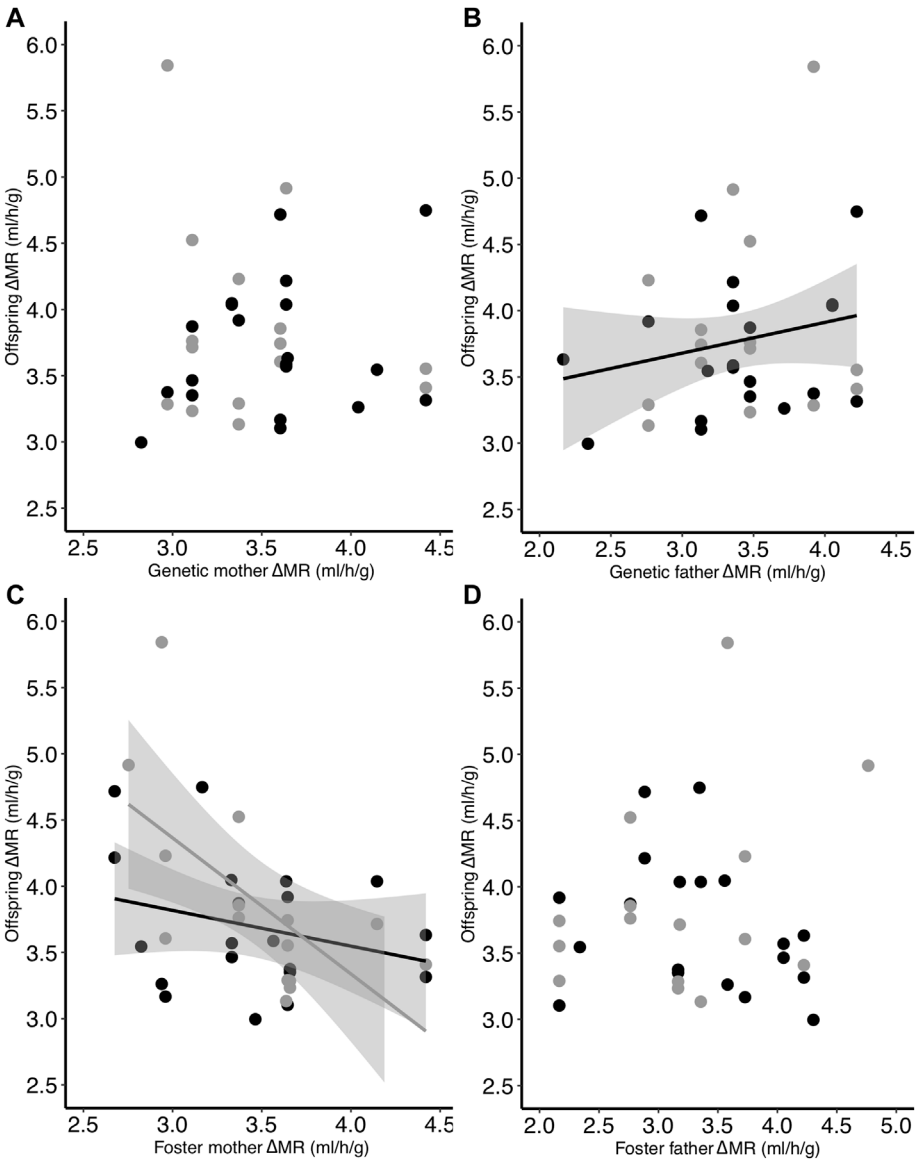

FIGURE 5 | Relationship between the difference in offspring TMR and RMR (ΔMR) on Day 30 and the ΔMR of the **(A)** genetic mother, **(B)** genetic father, **(C)** foster mother, and **(D)** foster father. Offspring had been incubated at two different temperatures as eggs (black = control; gray = low). Regression lines are included only for significant relationships.

within the range of hatching success found in other captive zebra finch studies (e.g., von Engelhardt et al., 2004; Von Engelhardt et al., 2006; Criscuolo et al., 2011; Winter et al., 2013). Nevertheless, we cannot exclude the possibility that there was a selective process during hatching, and that all offspring that succeeded to hatch had similar metabolic rates, regardless of incubation temperature.

4.2 Relationship With Genetic Parents

Embryonic heart rate was positively related to genetic father RMR, suggesting that there could be a genetic component to embryonic metabolism. Although there is evidence that embryonic heart rate and metabolism vary among different genetic lines in poultry (Druyan, 2010), and that there are significant among-clutch differences in embryonic heart rate in wild zebra finches (Sheldon et al., 2018), this is the first study to our knowledge that has explicitly investigated the relationship between parental and embryonic metabolism in birds. Although the correlation between genetic parent metabolism and embryonic heart rate could also be due to maternal effects during egg formation, such as egg yolk composition (Ho et al., 2011), the relationship between embryonic heart rate and genetic mother RMR was not significant. In contrast, because the relationship with genetic father RMR was statistically significant, this suggests that maternal effects may not be as important as genetics for determining embryonic metabolism in zebra finches.

TABLE 5 | Full and reduced models investigating the relationship between embryonic heart rate and Day 30 metabolism.

	Embryonic heart rate (bpm)[a]	
	$N_{control}$ = 20 eggs[b];163 readings	
	N_{low} = 15 eggs[b]; 132 readings	
	Full model	
	R^2m = 0.58; R^2c = 0.76	
Term	**F**	**p**
Incubation temperature	1.95	0.18
Reading (1, 2, or 3)	**83.26**	**<0.0001**
Time until measurement (seconds)	**155.24**	**<0.0001**
Egg mass	3.05	0.11
Offspring RMR	0.95	0.34
Sex	**5.84**	**0.03**
Incubation X Reading	**10.52**	**<0.0001**
Incubation X Offspring RMR	3.02	0.09*
Incubation X Sex	2.33	0.15
	Reduced model R^2m = 0.53; R^2c = 0.76	
Incubation temperature	**20.61**	**<0.0001**
Reading (1, 2, or 3)	**83.30**	**<0.0001**
Time until measurement (seconds)	**154.7**	**<0.0001**
Incubation X Reading	**10.60**	**<0.0001**

Bold values indicate statistical significance and asterisks (*) indicate marginal significance.

[a]*Parent (genetic and foster) and individual IDs were included as random effects in model.*

[b]*Only includes individuals that lived until Day 30 (metabolism measurement).*

As predicted, we found evidence that offspring RMR was highly heritable (h^2 = 0.53). This agrees with other studies that show that RMR is a heritable trait. For example, both Rønning et al. (2007) and Nilsson et al. (2009) measured RMR heritability by using restricted maximum likelihood to compare RMR among siblings and found evidence that RMR was heritable in zebra finches (h^2 = 0.25) and blue tits (h^2 = 0.59), respectively. Further, using methods similar to that of our study (i.e., parent-offspring regression), Bushuev et al. (2011) found evidence for heritability of RMR (h^2 = 0.43) in pied flycatchers (*Ficedula hypoleuca*). Further in line with our results, Bushuev et al. (2011) found that offspring RMR was correlated with genetic parent RMR, but not foster parent RMR. The relationship between genetic parent and offspring RMR that we found in this study could also be due to non-genetic maternal effects, such as hormone deposition to the egg. Indeed, in contrast to what we found for embryonic heart rate, when we examined the RMR of the genetic mother and genetic father as separate factors, we only found a relationship between offspring RMR and genetic mother RMR, and not genetic father RMR. This suggests that pre-incubation maternal effects may play a large role in determining post-hatch offspring RMR. For example, zebra finch eggs with higher testosterone concentrations produce nestlings and adults with higher RMR (Tobler et al., 2007; Nilsson et al., 2011). If the mothers with higher RMR in our study also deposited more testosterone into their eggs, this could partly explain the relationship that we found between parent and offspring RMR.

In comparison to RMR, the metabolic cost of thermoregulation (i.e., TMR and ΔMR) was less heritable. It may be expected that TMR would have a lower heritability than RMR because it may be more variable due to its dependence on the insulation capacity of plumage. For example, the body feathers of juvenile birds have different structural properties than those of adult birds (Butler et al., 2008), which could mask relationships between the TMR of parents and young offspring. However, although the h^2 of TMR (h^2 = 0.38) and ΔMR (h^2 = 0.18) were not statistically significant, the h^2 of TMR was still within the range of those found for RMR (see above). Further, offspring TMR tended to be positively related to genetic father TMR, and offspring ΔMR was positively related to genetic father ΔMR. This is the first evidence that we are aware of for the heritability of TMR or ΔMR, and suggests that the metabolic expenditure associated with thermoregulation could be shaped by natural selection. However, in contrast to RMR, we found little evidence for non-genetic maternal effects because, when genetic mother and genetic father were tested as separate factors, genetic mother TMR and ΔMR were not related to that of their offspring. Thus, any non-genetic maternal effect that may have influenced RMR either did not translate into differences in thermoregulatory capacity, or was masked by other driving factors (e.g., post-hatch environment; see below).

4.3 Relationship With Foster Parents

Although there were no relationships between foster parent and offspring RMR, we found that foster mother TMR and ΔMR were negatively related to that of their foster offspring, but only for offspring in the low treatment. This suggests that the impact of foster mother metabolism, and thus post-hatch parental care, is not on RMR, but rather on the ability of offspring to increase their metabolic rate when faced with a thermal energetic challenge. Thus, the ability of parents to increase their metabolic rate in response to an energetic challenge may be important for effective post-hatch parental care. Because most studies focus on RMR, our results call for future studies to focus more on TMR.

Specifically, we found that the more energy that foster mothers expended on thermoregulation, the less energy that their foster offspring expended on thermoregulation. It is possible that this relationship can be explained by differences in nestling provisioning. For example, foster mothers with a high metabolic rate should also have a high investment in parental care (Daan et al., 1990; Koteja, 2004; Sadowska et al., 2013), and provide a better developmental environment for their offspring (e.g., more food provisioning; Nilsson, 2002). In zebra finches, a greater food supply during nestling development, as opposed to food restriction, is related to lower offspring metabolic rate later in life (Criscuolo et al., 2008; Careau et al., 2014). Thus, this could explain the negative relationship that we found between foster mother and offspring TMR and ΔMR. In our study, offspring growth rate between Day 0 and Day 30 was not correlated with foster mother TMR (p = 0.9), and thus we did not find evidence to support the hypothesis that foster mother TMR is positively related to food provisioning and/or better parental care.

However, we did not measure parental care behavior (i.e., provisioning rates) in this study, and thus future studies are needed to determine whether there is a relationship between parental TMR and nestling provisioning rates, along with offspring growth rates and metabolism.

It is important to note that the relationships between foster mother and offspring TMR and ΔMR were only present in offspring incubated at the low temperature, and not the control temperature. This suggests that the impact of post-hatch care (e.g., nestling provisioning) is dependent on the quality of pre-natal care (i.e., incubation temperature). It is possible that offspring incubated at the control temperature are more resistant to differences in their post-hatch environment than those incubated at the low temperature, although little is known about how incubation temperature may influence trait plasticity or canalization. Future research is needed to investigate how different thermal environments shape avian thermoregulatory ability across generations, especially in the context of acclimation and adaptation in response to climate change (Nord and Giroud, 2020).

4.4 Relationship Between Heart Rate, Hatch Success, and Metabolic Rate Within Individuals

Embryonic heart rate is important because it can be used as a proxy for embryonic metabolic rate (Du et al., 2010), and can also provide insights into developmental rate, hatchling phenotype, and the effects of environmental stressors (reviewed in Sheldon et al., 2018). Contrary to what we expected, we did not find that embryonic heart rate was related to offspring RMR at Day 30. However, to our knowledge, this is the first study that has investigated whether there is a relationship between embryonic heart rate and offspring metabolism later in life. Our results suggest that individual metabolic rate can change throughout different stages of development, and that embryonic heart rate cannot be used to predict later-life metabolic rate in zebra finches. Indeed, studies that have found a positive relationship between heart rate and metabolic rate measured these two traits at the same developmental stage (i.e., embryonic; Du et al., 2010; Ide et al., 2017; Goodchild et al., 2020), and one study did not find a relationship between heart rate and metabolism even when measured at the same developmental stage (i.e., embryonic and hatching; Sartori et al., 2017). Similarly, one study on zebra finches found no relationship between embryonic heart rate and post-hatch growth rate or activity levels (Sheldon and Griffith, 2018). Thus, it appears that embryonic heart rate may not be able to be extrapolated to phenotypic differences later in life.

However, when we investigated an endpoint closer to embryonic development—hatch success—we did find a relationship with embryonic heart rate. Eggs that hatched had greater embryonic heart rates than those that did not hatch, suggesting that heart rate may be an indicator of embryo quality or hatching probability. Although heart rate has been used in other studies to predict hatching date or to confirm embryonic mortality (reviewed in Sheldon et al., 2018), this is the first study to our knowledge that has explicitly linked the magnitude of embryonic heart rate to hatching probability. Because all individuals that hatch also have high heart rates as embryos, this could create a selective process for a particular metabolic functioning. It is possible that this could mask potential effects of the incubation treatment or parental care on offspring metabolic rate, and could also explain why we did not find some of the relationships that we had predicted (e.g., effect of incubation temperature on RMR, relationship with foster parent RMR).

5 CONCLUSION

In this study, we show that avian metabolic rate throughout development, from the embryo to nutritional independence, is related to parental inheritance, the pre-hatch environment (i.e., incubation temperature), and post-hatch conditions (i.e., foster parent). Revealing how these different factors are related to RMR and TMR sheds light on how metabolism and the energetic cost of thermoregulation can be shaped by environmental changes, parental care decisions, and natural selection. Although most studies to date focus on RMR, our study reveals important relationships with TMR, which could be particularly important in the context of climate change for understanding how the early thermal environment and parental care affect thermoregulatory ability, and the possibility that thermoregulatory ability can be shaped by natural selection. More work is needed to determine if the differences in RMR and TMR that we found in this study have effects on short- or long-term offspring fitness.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the Autorisation de Projet Utilisant des Animaux à des Fins Scientifiques under file number APAFIS#23727-2020011311559318.

AUTHOR CONTRIBUTIONS

SH and FA contributed to the conception of the study. SH, OL and FA designed the study methods. SH and LS collected the data. SH performed the analyses and wrote the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2022.892154/full#supplementary-material

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BRIEF RESEARCH REPORT
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Quantitative Evaluation of Tactile Foraging Behavior in Pekin and Muscovy Ducks

*Aaron K. West, Emily M. Xu, Mitchell D. Nelson, Thomas R. Hart, Emelia M. Stricker, Alexandra G. Cones, Grace M. Martin, Kourtney Strickland, Devin L. Lambert, Lainey Burman, Bailey H. Zhu and Eve R. Schneider**

Biology Department, University of Kentucky, Lexington, KY, United States

Ducks have developed a variety of foraging strategies that utilize touch sensitive bills to match their ecological niche within wetlands. These techniques include diving, sieving, dabbling, and grazing. Ducks exhibiting tactile specialization in foraging outperform visual and non-tactile foraging ducks in behavioral experiments and have a higher percentage of light-touch mechanoreceptor neurons expressing Piezo2 in the trigeminal ganglia. Belonging to two different tribes of Anseriformes, the well-studied tactile specialist Pekin (Tribe Anatini: *Anas platyrhynchos domestica*) and lesser studied Muscovy (Tribe Cairinini: *Cairina moschata domestica*) ducks were tested on a series of experiments to assess these birds' functional tactile acuity. Both species of duck were able to separate out and consume edible items from increasing amounts of inedible plastiline clay distractors. They could also both be trained to associate a food reward with plastiline stimuli of differing size and shape using touch alone. However, only females of each species could learn to associate food reward with otherwise identical stimuli differing only in hardness. Pekin females performed significantly better than Muscovy females suggesting the anatomical specializations present in many Anatini may contribute to this type of tactile acuity. These findings have potential relevance in understanding the evolution of tactile ability and feeding ecology.

Keywords: Anseriformes, foraging behavior, *Cairina moschata*, tactile, duck bill, *Anas platyrhnchos*, touch

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Massimiliano Petracci, University of Bologna, Italy

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Michael Eichholz, Southern Illinois University Carbondale, United States
Andrew N. Iwaniuk, University of Lethbridge, Canada
Maude Baldwin, Max Planck Institute of Ornithology, Germany

****Correspondence:***
Eve R. Schneider
eves@uky.edu

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1 INTRODUCTION

Ducks are a group within the family Anatidae that display a diversity of specializations in feeding. The diets of these birds include an array of both plant matter (acorns, seeds, grasses, roots, leaves, etc.) and animal prey (mollusks, aquatic insects, eggs, fish, crabs, plankton, etc.) (Baldassarre et al., 2014). Ducks have developed a variety of foraging strategies to match their ecological niche. Several ducks dive for food, such as the lesser scaup (*Aythya affinis*) and ruddy duck (*Oxyura jamaicensis*) (Tome and Wrubleski, 1988), which sieve through underwater sediment, often in turbid conditions, in search of aquatic invertebrates. Others, such as the mallard, feed by straining for food at the surface or dipping their heads into the water to forage (behaviors known as "dabbling"). The technique of straining food items from water or mud is common in most ducks and is aided in part by specialized mouth morphology. Analysis of beak curvature in waterfowl shows a strong correlation between beak morphology and feeding ecology, with wider "duck-like" beaks being associated with filter feeding (Olsen, 2017). Foraging by ducks is commonly performed in conditions of poor visibility,

suggesting that tactile acuity in addition to beak morphology is critical for foraging (Mcneil et al., 1992). Recently, we examined anatomical and molecular signatures of tactile foraging ability across a range of species reported to have different foraging strategies. Trigeminal ganglia, which contain mechanosensitive neurons that innervate the bill, contain higher percentages of cells expressing the ion channel Piezo2 in tactile foraging species compared to species that use visual or non-specialized foraging methods (Schneider et al., 2014; Schneider et al., 2017). The mallard duck (*A. platyrhynchos*) and its domesticated descendent the Pekin duck (*A. platyrhynchos domestica*) are considered champion tactile foragers, with similarly high numbers of Piezo2-positive neurons in the trigeminal ganglia. Additionally, in a study examining late-stage duck embryos of seven species, the domestic duck had the fewest number of neurons expressing molecular markers consistent with a thermoreceptor/nociceptor phenotype, suggesting an evolutionary tradeoff between sensing light-touch and temperature (Schneider et al., 2019). Behaviorally, mallards are adept tactile foragers, capable of harvesting peas in wet sand while avoiding similar size inedible distractors (plastiline balls) (Zweers et al., 1977). They outperform non-tactile foraging wigeons (*Mareca penelope*) and visually foraging white-fronted geese (*Anser albifrons*) in filter-feeding tasks (Van Der Leeuw et al., 2003). Another study demonstrated that anesthetizing the bill significantly increases the time it takes for mallards to catch a tadpole in darkness (Avilova, 2017). However, most duck species dabble as ducklings regardless of their specialization in adulthood (Collias, 1958), and many grazing species dabble for insect larvae during breeding season (Drobney and Fredrickson, 1979). Thus, we wondered how closely linked tactile acuity is to adaptations in the anatomy of the trigeminal system.

To evaluate this, it is first necessary to develop behavioral tests disentangling ability from preference or other environmental factors. As our comparison species, we chose two domesticated ducks belonging to two distantly-related tribes: the well-known Pekin duck and the Muscovy duck (*C. moschata domestica*)— the only domestic duck not descended from mallard (Donkin, 1989), both of which have fully sequenced genomes. Muscovy ducks are members of the tribe Cairinini (perching ducks). Others in this tribe, such as the wood duck (*Aix sponsa*) and mandarin duck (*Aix galliculata*) display anatomical features consistent with a visual rather than tactile foraging strategy. For instance, wood ducks have large eyes, hooked bills, and small ratios of PrV (principal sensory nucleus of the trigeminal nerve) to the size of visual brain regions such as nucleus rotundus, or whole brain (Dubbeldam, 1990; Gutierrez-Ibanez et al., 2009). In a study by Olsen (2017) in which three-dimensional beak curvature was measured and compared across 43 waterfowl species, Muscovy ducks were shown to have a slightly more "goose-like" beak in comparison to the wide "duck-like" bill of mallards (Olsen, 2017). Muscovy ducks are considered generalists, often foraging by grazing. There are few studies of foraging ecology in the Muscovy duck and none of these studies directly assess tactile foraging ability (Baldassarre et al., 2014). Thus, we chose to compare Pekin and Muscovy ducks on a battery of behavioral tasks to assess tactile ability. Ducks were trained to forage in muddy water to eliminate the use of visual cues and assess their abilities to discriminate submerged food items from non-food items. Trials in clear water were used to assess possible reliance on visual cues by the species. To rule out the possibility of taste or olfactory cues playing a role in behavioral performance, two-alternative choice tasks were designed in which ducks were trained to associate food rewards with objects of different shape, size, and hardness. We predicted that in the tactile tasks, but not visual tasks, Pekin ducks would outperform Muscovy ducks.

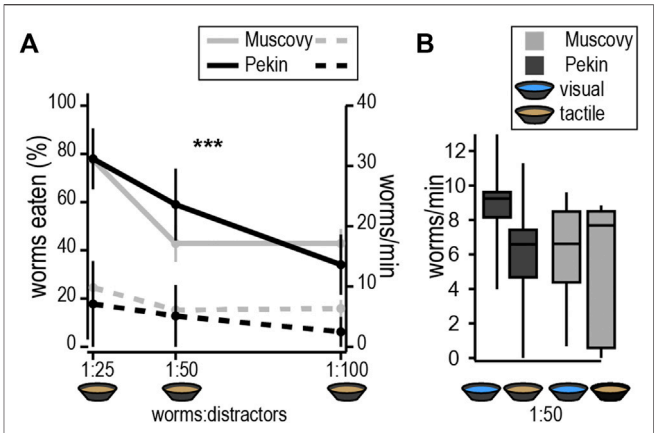

FIGURE 1 | Increasing difficulty of tactile foraging tasks. **(A)** Performance of target consumption (mealworms eaten, solid line, left y-axis) and non-target consumption (distractors eaten, dashed line, right y-axis) throughout tasks of increasing difficulty. Ratios of worms:distractors are calculated from weights in grams used in each task. Both Pekins and Muscovies consumed fewer worms as the ratio of distractors increased [$F(2,39) = 8.5$, $p < 0.0001$]. **(B)** Foraging-time corrected measurements (worms/min) showed no change as a function of the number of distractors. Conditions that allow ducks to see the worms did not improve task performance in either species.

2 RESULTS

Experiments were conducted to assess ducks' ability to pick out mealworms from among inedible distractors of a similar texture but slightly smaller diameter at various ratios of mealworms to distractors (**Figure 1A**). Both species ate less than 2% of the plastiline across all conditions (Muscovy = 0.78 ± 0.23 pieces, Pekin = 1.07 ± 0.55 pieces, n. s.). Contrary to our predictions, there was no significant difference between species in either the rate of consuming mealworms or the percentage of mealworms eaten [$F(2,24) = 3.94$, $F(1,22) = 0.005$, n. s.]. There was, however, a significant random effect of subjects in number, but not rate, of worms eaten (Wald test, $p < 0.05$), but this did not persist when corrected for the time spent foraging. As the ratio of distractors to mealworms increased, Pekins and Muscovies both consumed fewer worms [$F(2,39) = 8.5$, $p < 0.0001$, **Figure 1A**]. The only interspecies difference we observed was that Muscovies foraged for significantly less time than Pekins across all conditions [$F(1,27) = 8.74$, $p < 0.01$]. These data demonstrate that like the Pekin, Muscovy can separate edible items (worms) from inedible distractors of similar size and shape.

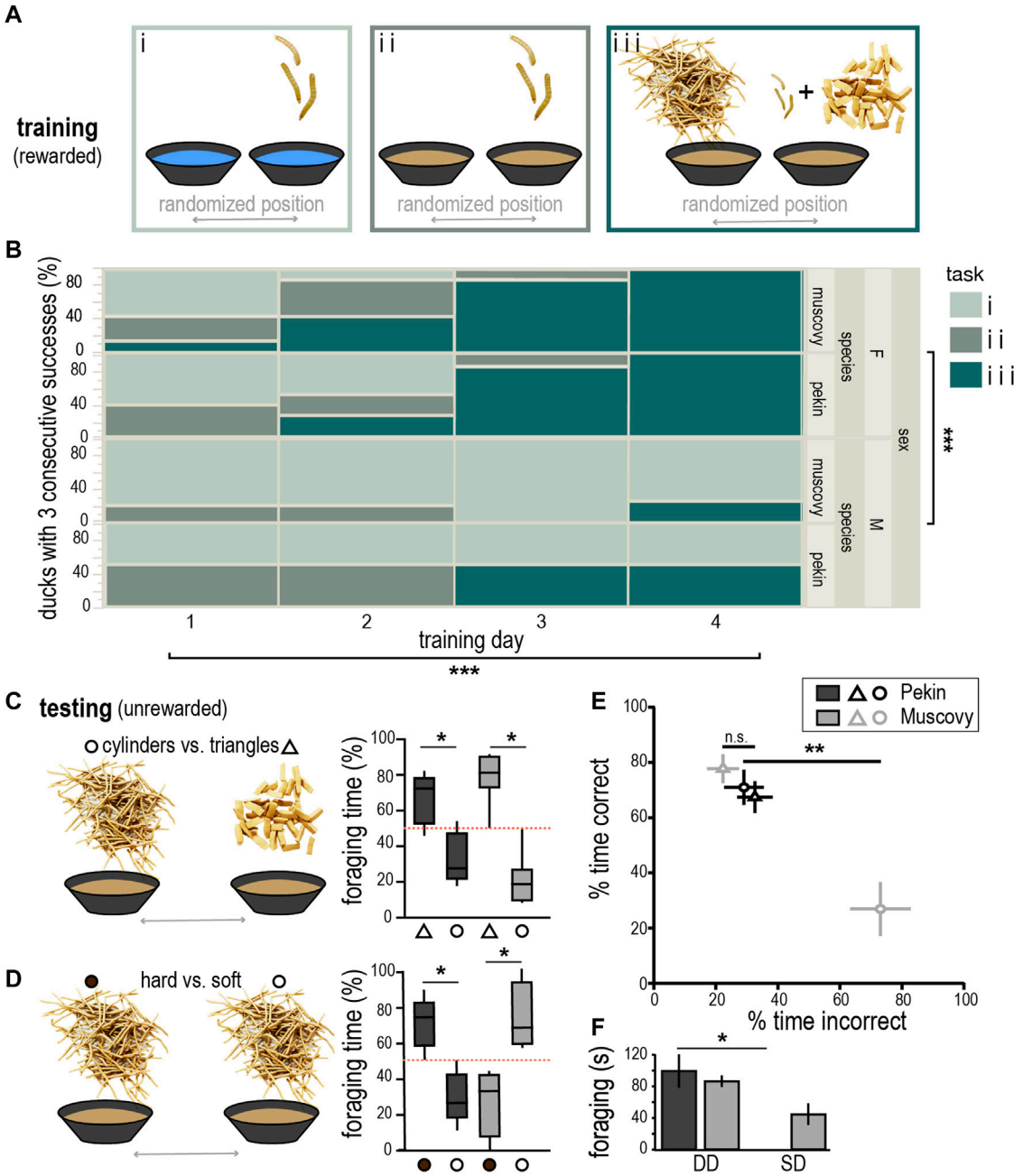

FIGURE 2 | Training and performance on tactile conditioning tasks. **(A)** Schematic of progressively more difficult tasks (i–iii) used to train ducks to learn shape discrimination (images of worms and plastiline not to scale with bowls, worms in i and ii = 2x scale from iii). **(B)** Progression of training on tasks i-iii indicating majority of ducks learned task iii after four training days [nominal logistic fit, Chi^2 (sex) = 16.7, df = 2; Chi^2 (training day) = 23.8, df = 6]. **(C)** Schematic of testing condition and box and whisker plot showing % foraging time in the correct bowl (triangle symbol) or incorrect bowl (circle), red dashed line indicates chance performance, whiskers indicate 10–90 percentile. **(D)** Schematic of hard (filled circles) vs. soft (open circles) testing condition, and box and whisker plot showing behavioral performance as in **(C)**. **(E)** Cross-species comparison of % time spent in the correct (hard) vs. incorrect (soft) bowls for Pekin (black) and Muscovy (grey). **(F)** Pekin ducks spent significantly more time doing deep dabbling (DD) than surface dabbling/gleaning (SD) on this task, whereas the Muscovy used both foraging strategies. Asterisk indicate *$p < 0.5$, **$p < 0.01$, ***$p < 0.0001$.

We then asked whether foraging performance of Muscovies would improve, compared to Pekins, under conditions that allowed ducks to use both touch and vision (clear water) (**Figure 1B**). Surprisingly, the number of worms eaten per minute was not significantly different between clear and muddy water for either species (Species x condition, $F(1,21) = 0.35$, $p = n.s.$), suggesting that adding vision did not improve performance.

To rule out taste and olfactory cues, ducks were trained to associate food reward with plastiline distractors in a two-choice foraging task (**Figure 2A**). Interestingly, significantly more females than males were able to learn this task after 3–4 days of training (Six females and one male for each species, nominal logistic fit, $Chi^2 = 16.7$, $p < 0.0001$, **Figure 2B**). Both species spent significantly more time exploring the shape associated with reward (triangles) than the unrewarded shape (cylinders), demonstrating they had learned to associate large plastiline triangles with reward (Pekin: $t = 4.29$, df = 10, $p < 0.05$, Muscovy: $t = 7.56$, df = 12, $p < 0.001$, **Figure 2C**). The fact they could make this association demonstrates that ducks could differentiate between objects of differing size and shape using touch. Additionally, there was no significant difference in the percentage of time the two species spent foraging in the correct bowl ($t = 1.3$, df = 11, $p = 0.21$). However, when restricting our analysis to the first minute of the task, Muscovy ducks performed significantly better than Pekin ($t = 2.7$, df = 11, $p < 0.05$).

Ducks successful on the previous task were tested on whether they could associate a food reward with objects differing only in hardness. Consistent with our predictions, only Pekins could successfully do the task on the unrewarded test trial, spending an average of 76% of the time foraging in the correct bowl ($t = 4.6$, df = 8, $p < 0.005$, **Figure 2D**), whereas Muscovies spent only an average of 27% of the time foraging in the correct bowl and spent significantly more time foraging in the incorrect bowl ($t = 3.3$, df = 6, $p < 0.05$). Thus, Pekins spent significantly more time in the correct bowl than Muscovies ($t = 3.9$, df = 7, $p < 0.01$, **Figure 2E**). This result suggests Pekin ducks have superior tactile discrimination ability that can be revealed by tasks of sufficient difficulty.

In addition to differing performance on this task, a closer examination revealed a significant difference in foraging behavior between the species (Kruskal-Wallis test, $p < 0.001$). Post-hoc comparison revealed that Pekins almost exclusively used a "deep dabbling" strategy when foraging on this task that involved fully submerging the bill and vigorously churning the water around them (Dunn's multiple comparisons test, multiplicity-adjusted $p < 0.05$), whereas Muscovy ducks shifted to a "surface dabbling" strategy, skimming the water's surface and producing very little agitation (**Figure 2F**).

3 DISCUSSION

We sought to evaluate tactile foraging performance in distantly related Pekin and Muscovy ducks. Our results demonstrate that both species can successfully discriminate between edible and inedible objects and associate objects of different size and shape with reward. This suggests both species have a reasonably high level of tactile acuity in the bill when conditions demand its use. In fact, individual differences on this task explained more of the variance in the number of worms eaten than interspecies differences. However, only Pekins were able to be conditioned using inedible objects of the same size and shape but different hardness. This result bears out and extends the prior studies on wild mallards and provides a novel point of comparison in the Muscovy duck, whose tactile foraging ability has not previously been studied. That Muscovy could not perform the hardest tactile conditioning task in our study is consistent with time-activity budget analysis of Muscovy ducks spending only 1.4% of their time foraging by dabbling or probing in soil (both considered tactile foraging methods) compared to 25% foraging by grazing or gleaning from the ground, which are presumably less tactile (Downs et al., 2017).

Interestingly, during training on the hard versus soft discrimination task, both species easily learned to associate hard plastiline distractors with reward. However, during testing when the reward was absent, Muscovies spent more time foraging in the bowl containing soft plastiline cylinders, which were never paired with reward on either conditioning task. This suggests they may have been using taste cues during training, and in absence of these cues they shifted to preferring the soft cylinders because of their similarity to natural food items such as mealworms or other insect larvae. Further, Muscovies behavior during foraging also shifted on this task, albeit to a less effective strategy. Indeed, the distinct performances of Pekin and Muscovy ducks on this task could be explained in the light of both tactile acuity and instinctual tendencies, both of which could also play roles in the foraging abilities of wild ducks in their natural setting. We observed domestic Pekins using a difference in hardness as an indirect indicator of the presence of food. In the wild such an ability may aid in prey detection, where the softness of substrates or the shape and softness of aquatic vegetation could serve as an indirect indicator that the duck is foraging in an environment suitable for specific prey, like amphipods or other aquatic invertebrates. Furthermore, females learned the conditioning tasks faster than males across both species. Because these studies were performed at the onset of sexual maturity, perhaps this is indicative of many female waterfowl's motivation to consume protein-rich insect larvae during the egg-laying period (Swanson et al., 1985). Further studies presenting distractors in a counterbalanced design across different life stages would be necessary to more fully understand the role tactile ability could play in seasonal and sex differences in foraging behavior.

Several neuroanatomical features may contribute to species differences in tactile discrimination between hard and soft objects. The mallard and Pekin duck have large PrVs (Gutierrez-Ibanez et al., 2009), and many neurons in the trigeminal ganglia express Piezo2, suggesting an expanded neural representation of tactile information from the bill. This has not been measured in the Muscovy duck; however, other members of tribe Cairinini (genus *Aix*) have small PrVs and very few mechanosensitive neurons in the trigeminal ganglia (Dubbeldam, 1990; Schneider et al., 2019). The bills of ducks contain numerous sensory corpuscles which can be found clustered in papillae in the maxillary and mandibular nails, making up a touch-sensitive region known as the bill tip organ. Filter-feeding ducks, such as the mallard, have significantly more corpuscle-containing papillae than species using other means of foraging (Avilova et al., 2018). Only a few studies exist examining Muscovy mouth morphology (Abdalla et al., 2018), and these do not quantify corpuscle density. Ducks also possess repeating comb-like ridges located along the edges of the mouth's interior, known as lamellae, which are traditionally believed to play a role in straining food items from water. Zweers et al. (1977) described a model for the straining process in mallards, likening the mouthparts to a suction pump (Zweers et al., 1977). The piston-like movement of the tongue draws water and food particles into the rostral end of the mouth, whereby the water is expelled past the lamellae. A comparison of

Pekin and Muscovy mouth morphology, including lamellae, could provide insights into how mouth morphology influences tactile ability.

Our tactile discrimination task assesses a distinct type of tactile acuity compared to previous work demonstrating Anatini could discriminate between particles of different sizes, which could be accomplished through size exclusion by lamellae. For instance, a study of mallard and shoveler ducks found that when the interlamellar space was increased by ablating a subset of lamellae filter feeding ducks performed worse on particle filtering tasks. In contrast, the dimensions of our plastiline distractors of varying hardness far exceed the interlamellar space, and are identical in size, and thus could not be differentiated by size exclusion using lamellae. Pekin ducks presumably benefit on our task from increased vibrotactile feedback consistent with a high density of Herbst and Grandry corpuscles in the bill, particularly in the bill tip organ (Berkhoudt, 1979). Ultimately, which neuroanatomical adaptations in tactile processing are most advantageous for tactile discrimination remains to be determined (Wylie et al., 2015). Since Pekin and Muscovy both have fully sequenced genomes they provide a compelling opportunity for further examination of the molecular/genetic basis of tactile acuity in waterfowl, as well as general principles of the evolution of tactile acuity.

Another intriguing area of future investigation is differences in foraging ability in domesticated and wild mallards and Muscovies. While we have demonstrated the Pekin duck as having higher tactile ability than Muscovy, mallards may have superior tactile ability to that of our meat-type Pekin ducks. Prior studies have found mallards foraging for peas among plastiline distractors in wet sand left no lamella marks on plastiline, raising the intriguing possibility that they can use remote touch, as has been observed in other water birds such as *Caladris canutus* (Piersma et al., 1998). In our studies, plastiline triangles appeared extensively chewed after testing, whereas in the hard versus soft tactile conditioning task there were few lamella markings on plastiline. Likewise, wild Muscovies may have differing abilities from domesticated Muscovies.

Though Anatini is a diverse group, the diets of many waterfowl overlap (Thomas, 1982). Our present study suggests that each species may have evolved to benefit from different feeding niches. If tactile sensitivity is better evolved in certain duck species, these species may be better suited to take advantage of the available food resources (Hitchcock et al., 2021). Characterizing tactile ability as well as the neural features that underpin behavior (e.g., pathways necessary for somatosensory development and specification, craniofacial development, and tactile response tuning of trigeminal and upstream neurons) across a wider range of species could provide a crucial lens to examine the evolution of foraging behavior. For instance, the lesser scaup (*A. affinis*), a diving duck, has the same percentage of mechanosensitive neurons in its trigeminal ganglia as the Pekin duck, but more of these neurons have rapid inactivation kinetics (Schneider et al., 2019). In contrast, the ruddy duck has relatively few mechanosensitive TG neurons but the largest PrV among waterfowl (Gutierrez-Ibanez et al., 2009). Using the behavioral methods outlined here to characterize foraging ability in other species of waterfowl, such as geese or diving ducks, with different foraging preferences or aptitudes to a standard domesticated mallard model could provide important insights into which adaptations most meaningfully contribute to the breadth of tactile abilities across species in Anseriformes.

4 MATERIALS AND METHODS

4.1 Animals and General Procedure

All procedures were approved by the University of Kentucky Institutional Animal Care and Use Committee under protocol # 2021-3810. Pekin duck eggs were obtained from Maple Leaf Farms (Cromwell, IA, United States) and hatched onsite. Day-old Muscovy ducklings were shipped from Freedom Ranger Hatcheries (Reinholds, PA, United States). Pekin and Muscovy ducks were raised separately in outdoor hanging wire aviaries for the first 4 months of life, then transferred for the next 4 months to a more secure metal frame outdoor aviary ($12' \times 11' \times 6.5'$) with concrete floors and large flake pine shavings as bedding. Food in hanging feeders (Mazuri starter pellets, then 19% protein pellets) and water in 15-gallon poultry drinkers were available *ad libitum.* Environmental enrichment included daily enrichment feeding (peas, mealworms, grain, or greens), large tubs of water for bathing and wading, and hanging shiny objects for pecking (compact disks, aluminum cans filled with mealworms). All training and testing experiments were done within a $30' \times 44.5' \times 31'$ cage placed within the aviaries.

4.2 Experiment #1: Increasing Ratio of Distractors to Targets

4.2.1 Training

To assess tactile foraging ability, we first trained Pekin and Muscovy ducks to forage in muddy water for mealworms hidden among inedible distractors of a similar shape and size made of plastiline clay (Roma Plastilina, Chavant Inc., Macungie, PA, United States). Mealworms (Josh's Frogs, Owasso MI, United States) were prepared a day in advance by drowning in water at 4°C to prevent floating or movement. Inedible distractors were prepared by extruding plastiline clay into 2 mm diameter × 18 mm cylinders. Muddy water was prepared from strained local mud and poured into a 4 L, 10.5 ″ diameter rubber feeding pan. As an initial training task, ducks were presented with muddy water containing 50 mealworms and 50 plastiline distractors and allowed to forage for 2 min (data not shown).

4.2.2 Testing

Tests for foraging ability were conducted with the same setup as training, but feeding bowls contained 20 mealworms and differing ratios of worms to distractors in the following order: 1:25, 1:100, 1:50 (i.e., 40, 80, 60 g plastiline; 4 g = approx. 50 pieces). After each experiment, mealworms were counted and remaining plastiline was dried and weighed. A camera (Raspberry Pi 4B/HQ camera or GoPro Fusion) was placed above the feeding area and used to record each duck's feeding behavior at 30–60 FPS. Additionally, the 1:50 test was repeated but with clear water to determine if introduction of visual information during foraging might aid the ducks in feeding. No additional

training was introduced for this visual test. Testing on the 1:50 conditions coincided with the event of sexual maturity/breeding season in both species, during which many ducks did not forage at all during testing. Thus, these conditions were tested twice and in cases where ducks foraged on both days results were averaged.

4.3 Experiment # 2: Operant Conditioning

4.3.1 Training

To rule out the use of gustatory or olfactory cues which may have been present in experiment #1, shaping behavior was used to train ducks on a two-alternative choice task of foraging between two bowls (**Figure 2A**). Training was done over 4 weeks, with a maximum of 3 days between training sessions (3–11 trials per task). Initially, ducks were given five mealworms in one of two bowls in randomized order in clear water (task i), then in muddy water (task ii). Worms were then placed in the container with 20 g (~50 pieces) of extruded isosceles triangles made from soft plastiline (Prima Plastilina) (5.6 mm × 7 mm side length × 18 mm long) in one bowl, while the other bowl contained 20 g (~120) soft plastiline cylinders (task iii). Ducks were given both bowls simultaneously. In other words, ducks learned to associate the triangles with the reward of locating worms while foraging during the training process. Ducks success on training trials (task i–iii) was quantified as the ability to actively forage for 5 s in the bowl containing the worms in under 20 s on three consecutive trials. Once ducks met this criterion on a given task they were moved on to the next task. To ensure ducks retained their learning, successful ducks were trained on Learning Task iii for at least 2 days. On the final 1–2 days of training on task iii the number of mealworms was reduced to three.

4.3.2 Testing: Size and Shape Discrimination

After ducks were taken through the series of training tasks associating the food reward (mealworms) with a certain plastiline stimulus (**Figure 2A**), ducks were tested once to assess if they correctly learned food association with plastiline triangles. On testing day, ducks were given one rewarded trial with the same setup as task iii of training: two bowls of muddy water were placed in front of each duck, one containing mealworms and triangles, the other containing cylinders and no worms; ducks were allowed to forage for 45 s. Immediately following this rewarded trial, ducks were given one unrewarded trial with the same setup but no worms were included with the triangles (**Figure 2C**). Testing videos were scored by two to three independent observers at least one to two of which were blind to condition and identity of individual ducks to measure time spent foraging in each bowl (dependent variable) by each duck during the 2-min trials.

4.3.3 Training and Testing: Hardness Discrimination

Ducks successful on the previous task were retrained for 3 days on a hardness discrimination task by pairing worms with hard plastiline cylinders (Roma plastiline) while the unrewarded bowl contained the same type of soft plastiline cylinders to increase similarity to the previous task. Ducks that met our criteria for success (foraging in the correct bowl in under 20 s on three consecutive trials) on this new task were then tested to determine which bowl they spent more time foraging in (dependent variable) for 2 min with no mealworms present (**Figure 2D**).

4.4 Analysis

Data were analyzed using JMP Pro, Igor Pro and Graphpad Prism. All statistical tests were either two-tailed *t*-tests (t statistic reported) or linear mixed models (F statistic reported) unless otherwise noted. If data were normally distributed (Shapiro Wilk test), we performed two-tailed *t*-tests for pairwise comparisons or ANOVA/linear mixed model for multiple comparisons. When normality assumptions were violated the Kruskal-Wallis test was used. Linear mixed models were used in lieu of ANOVA to accommodate missing values from ducks that did not forage on individual test days, with subjects [species] included as a random intercept. Independent variables were number of worms eaten, worms eaten per minute, or time spent foraging in the correct bowl (on unrewarded test conditions). Dependent variables were species, sex, and test condition. One Pekin outlier was removed from 1:100 task who foraged for only 10 s (160 worms/min) to ensure residuals were normally distributed. Training data was coded as a nominal variable and analyzed using logistic regression in JMP Pro. Figures were made using Adobe Illustrator. Error bars on graphs represent S.E.M. unless otherwise noted.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the University of Kentucky Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

ES conceived the study. AW, EX, MN, AC, and ES designed the experiments. AW, EX, MN, AC, GM, KS, DL, LB, ES, and ES performed the experiments. AW, ES, GM, BZ, TH, LB, and DL analyzed the data. AW, TH, and ES wrote the manuscript. All authors approved the final content.

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ORIGINAL RESEARCH
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Biochemical and Physicochemical Changes in Spaghetti Meat During Refrigerated Storage of Chicken Breast

*Giulia Tasoniero, Hong Zhuang and Brian Bowker**

USDA, Agricultural Research Service, US National Poultry Research Center, Athens, GA, United States

This study investigated postmortem muscle protein degradation and myowater properties in broiler breasts afflicted with the Spaghetti Meat (SM) myopathy during 7 days of storage. Severe SM and unaffected (NORM) breast fillets were analyzed at days 0, 3, and 7 postmortem for TD-NMR myowater traits, myofibrillar protein profiles, calpain activity, free calcium, and desmin and troponin-T degradation patterns. Only at day 0, muscle histology, fiber size and sarcomere length were assessed on multiple fillet portions. In SM breasts, the intramyofibrillar water population exhibited longer relaxation times ($p = 0.0172$) and a lower proportion ($p = 0.0118$) compared to NORM. SM had a greater proportion of extramyofibrillar water ($p = 0.0080$) possessing a longer relaxation time ($p = 0.0001$). Overall, the SM myopathy had only a minor impact on the myofibrillar proteins profiles and did not affect either free calcium concentration, calpain activity, or the degradation of desmin and TnT, while storage time strongly affected all the traits measured. At microscopic level, muscle tissue from SM fillets exhibited the typical indicators of myodegeneration mostly in the superficial-cranial portion of the breast, while fiber size and sarcomere length were similar between the two muscle conditions irrespectively from the portion considered. The lack of overall significant interaction effects between muscle condition and storage period suggested that SM and NORM breast meat experience similar proteolytic and physical changes during the postmortem period.

Keywords: broiler chicken meat, spaghetti meat abnormality, postmortem proteolysis, calpain activity, myowater properties

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****Correspondence:***
Brian Bowker
brian.bowker@usda.gov

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INTRODUCTION

Spaghetti Meat (SM) is an emerging myopathy affecting broiler chicken *Pectoralis major* muscle, whose distinguishing macroscopic trait is an overall impaired muscle integrity and a stringy, soft consistency on the ventral-cranial portion of the muscle due to poor cohesion of muscle fibre bundles (Bilgili, 2016). Although the histological features of SM are similar to those of the White Striping and Wooden Breast myopathies, SM also exhibits a progressive rarefaction of the endomysium and perimysium coupled with loose, immature connective tissue deposition around thin and split fibers (Baldi et al., 2018). These observed alterations in the connective tissue are thought to contribute to the impaired muscle integrity of SM. As the visual acceptability of the breast fillet is compromised, processors are forced to use affected meat to manufacture further-processed products (Baldi et al., 2021). Initial studies on SM focused on understanding the impact of this myopathy on basic breast

meat quality and composition characteristics. The SM myopathy exerts a profound and negative impact on breast meat composition that leads to diminished functionality traits such as water-holding capacity and emulsifying properties (Baldi et al., 2018; Baldi et al., 2019; Baldi et al., 2021; Tasoniero et al., 2020). Despite having a greater pH, SM exhibits a greater proportion of extra-myofibrillar water and lower water-holding capacity compared to unaffected meat (Baldi et al., 2018; Baldi et al., 2021). Researchers observed that a diminished water-holding capacity in pork muscle is associated with decreased degradation of cytoskeletal muscle proteins both early postmortem and with increasing postmortem aging (Huff-Lonergan and Lonergan, 2005). Differently, in chicken muscle, the role of muscle protein degradation in determining muscle structural integrity and meat water-holding capacity has not been as clearly defined. In a recent study, it was suggested that a lower protein content in SM affected breasts could be related to a greater myofibrillar protein degradation accompanying the myofiber structural breakdown observed histologically (Tasoniero et al., 2020). However, little is known about the muscle protein fraction of SM or the potential impact of myofibrillar protein degradation on the muscle integrity and functional properties of SM. Thus, the purpose of this study was to investigate the effects of the SM myopathy on myofibrillar protein degradation, myowater properties, and muscle fiber characteristics. As postmortem aging is known to change some of these traits, indicators of postmortem proteolysis and myowater were measured at different postmortem times (0, 3, and 7 days postmortem). In addition, muscle histology was assessed at day 0 in order to confirm whether the histopathological lesions reflected the macroscopic assessment of the SM condition.

MATERIAL AND METHODS

Sample Collection and Preparation

During two trials, a total of 60 skinless boneless breast fillets—30 Spaghetti Meat (SM) affected and 30 unaffected (normal, NORM)—were collected at 3 h postmortem from a commercial broiler processing plant, transported to the US National Poultry Research Center (Athens, GA, United States), and subjected to 7 days of refrigerated storage. Selected SM samples exhibited a mushy and stringy consistency, due to muscle fiber bundle separation, especially in the ventral-cranial portion of the fillets (Bilgili, 2016; Baldi et al., 2018). Breasts exhibiting the white striping and/or woody breast conditions were excluded from this study. At 6–8 h post-mortem (day 0), all fillets were trimmed, weighed (NORM = 437 g, standard error = 10 vs. SM = 512 g, standard error = 10; $p < 0.0001$), and stored at 4 °C in vacuum sealed plastic bags. During each trial, analyses were carried out at 0, 3, and 7 days postmortem on five breasts (*P. Major*) per muscle condition per day. On each day of analysis, myowater properties were measured on intact fillets using time domain nuclear magnetic resonance (TD-NMR) analysis. All samples were then individually chopped in a food processor and free calcium concentration was determined in the fresh state. The remaining chopped meat was frozen in liquid nitrogen and individually stored at −80°C until myofibrillar protein, calpain, and western blot analyses were carried out. Samples for sarcomere length determination were collected only from samples processed at day 0, on knife-minced cubes of meat excised from the cranial portion of fillets (superficial and deep layers) and stored at −80°C until analysis. An additional set of five SM and five NORM fillets was collected for histological evaluation at day 0. At 24 h post-mortem, muscle samples were excised from the cranial (superficial and deep layers) and caudal portions of these fillets. Strips (approximately 0.5 cm wide, 0.5 heigh and 3–6 cm long) were cut parallel to the apparent muscle fibers, tied to a popsicle stick and frozen in isopentane chilled in liquid nitrogen, and stored at −80°C until histological analysis was performed.

Time Domain-Nuclear Magnetic Resonance Measurements

Myowater properties of intact fillets were assessed using transverse relaxation time (T_2) measurements collected with a LF 90II minispec NMR analyser (Bruker BioSpin, Rheinstetten, Germany). Transverse relaxation times (T_2) were acquired using the Carr-Purcell-Meiboom-Gill (CPMG) spin-echo sequence (Carr and Purcell, 1954; Meiboom et al., 1958) (τ = 1 ms; total number of acquired echoes = 200). To obtain the relaxation time and population percentage for each of the three water populations (T_{2B}, T_{21}, and T_{22}), the T_2 data were analyzed using distributed exponential fitting analysis carried out with Contin software (Bruker BioSpin, Rheinstetten, Germany). Plots of relaxation amplitudes for individual relaxation processes versus relaxation times revealed the presence of the three relaxation populations (no. points of relaxation spectrum = 500; relaxation spectrum = 0.1–1,000 ms). For each of the three water populations, relaxation times were calculated from the peak position and proportions of protons exhibiting those relaxation times were calculated from the corresponding area under each peak.

Free Calcium Concentration

To determine free calcium concentration, the procedures described by Pomponio and Ertbjerg (2012) and Soglia et al. (2018) were followed. Briefly, 25 g of chopped fresh breast meat were centrifuged at 18,000 × g for 30 min at 4°C and 5 ml of supernatant were collected. In order to provide a background ionic strength, 100 µl of 4 M KCl were added to the samples (ratio 50:1). A calibration curve was built each day of analysis, using solutions of 1,000, 100, 10, 1, and 0.1 ppm of $CaCl_2$ (with background ionic strength also adjusted at a ratio 50:1) as standards. Free calcium concentration was measured in duplicate at a constant temperature (22°C ± 1 °C) with a calcium ion selective electrode equipped with a reference electrode (Orion™ Calcium 9720BNWP Electrode, Thermo Fisher Scientific, Waltham, MA, United States). Sample output in mV was converted to free calcium ppm.

Protein Isolation and SDS-PAGE Analysis

Myofibrillar protein fractions were isolated from 2 g of minced meat according to the procedure of Pietrzak et al. (1997). A biuret

assay was performed to determine protein concentrations. Samples were then diluted to a protein concentration of 2 mg/ml in sample buffer (8 mol urea, 2 mol thiourea, 3% SDS (wt/vol), 75 mmol DTT, 25 mmol Tris-HCl (pH 6.8), 0.004% bromophenol blue) and denatured for 3 min in boiling water. Denatured protein samples (15 µg protein/lane) and a broad range molecular weight standard (5–250 kDa, Thermo Scientific PageRuler Broad Range Unstained Protein Ladders, Waltham, MA, United States) were loaded onto Novex precast 4%–20% tris-glycine polyacrylamide gels (Life Technologies Corp., Carlsbad, CA, United States) and ran at 4°C at a constant voltage. Gradient gels were utilized to allow for a broader range of proteins to be analyzed, and equal protein loads ensured that differences were because of actual variations in the protein profiles. Gels were then stained (Coomassie brilliant blue R-250) and destained. The densities of 13 myofibrillar protein bands were quantified using Alpha View software (v 3.4, ProteinSimple Inc., Santa Clara, CA, United States). The relative abundance of each individual protein band was expressed as a percentage of the total protein abundance of all bands within the lane.

Western Blot

Before performing western blots against desmin and troponin-T, electrophoretic separations were carried out as previously described with a protein load of 30 and 60 µg per lane for desmin and troponin-T, respectively. Myofibrillar proteins were transferred from SDS-PAGE gels to a 0.2 µm pore size PVDF membrane using a semi-dry blotting apparatus Trans-Blot® Turbo™ Transfer System (Bio Rad, Hercules, CA, United States). For desmin detection, membranes were soaked in blocking reagent (3% bovine serum albumin, BSA in TBS-T) for 1 h, washed in TBS-T (50 mM Tris, 150 mM NaCl, 0.5 g/L Tween-20; pH 7.5) for 5 min and soaked for 1 h on a shaker plate in a polyclonal rabbit anti-desmin primary antibody solution (Sigma-Aldrich Corp., St. Louis, MO, United States) diluted at 1:1,000 in TBS-T and 1% BSA. Membranes were then washed three times in TBS-T for 5 min each and soaked for 1 h in secondary goat anti-rabbit-IgG-AP antibody solution (Sigma-Aldrich Corp., St. Louis, MO, United States) diluted at 1:10,000 in TBS-T and washed again at the same conditions with TBS (50 mM Tris, 150 mM NaCl; pH 7.5). Membranes were then soaked in colorimetric solution (Opti-4CN diluent and substrate, Bio Rad, Hercules, CA, United States) until band development occurred. For troponin-T detection, dry membranes were re-wet with methanol, washed with phosphate buffered saline solution (PBS-T, 20 mM sodium phosphate, 150 mM NaCl, 0.1% v/v Tween-20, pH 7.4) for 5 min, and then soaked overnight in 5% blocking reagent solution (PBS-T + blocking reagent by Bio Rad, Hercules, CA, United States). Membranes were then washed five times with PBST for 10 min each and incubated for 2 h in a monoclonal mouse anti-troponin-T primary antibody solution (Sigma-Aldrich Corp., St. Louis, MO, United States) diluted at 1:1,000 in TBS-T and 1% BSA. Membranes were washed three times in PBS-T for 5 min each and then incubated with goat-anti-mouse-HRP secondary antibody diluted at 1:15,000 with PBS-T and 1% BSA. Membranes were washed three times in PBS-T for 10 min each. To amplify the reaction signal, membranes were incubated in amplification reagent (BAR, Bio-Rad Laboratories, Hercules, CA, United States) for 10 min, washed four times in 20% dimethylsulfoxide in PBS-T for 5 min each, washed two times with PBS-T for 5 min each, incubated in streptavidin-HRP diluted 1:3,000 in PBS-T and 1% BSA, and finally washed two times in PBS-T for 5 min each. For color development, membranes were incubated in Opti-4CN substrate (Bio-Rad Laboratories, Hercules, CA, United States) for 30 min under agitation. The density of immunoreactive bands was expressed as the relative abundance of each individual protein band within the lane.

Casein Zymography

Gels for casein zymography were cast with a 12% resolving gel (30% acrylamide/bis-acrylamide 29:1, 3 M Tris-HCL pH 8.8, 0.2% casein, 10% APS, 0.08% TEMED) and a 3.5% stacking gel (30% acrylamide/bis-acrylamide 29:1, 1 M Tris-HCL pH 6.8, 10% APS, 0.12% TEMED). 6 ml of extraction buffer (100 mM Tris; 5 mM EDTA; 10 mM Monothioglycerol; pH 8.0) were added to 1 g of muscle sample (in duplicate), and samples were homogenized three times at 13,500 rpm for 20 s and centrifuged at 15,000 × g for 30 min at 4°C. A biuret assay was performed and protein concentration was adjusted to 7 mg/ml using the extraction buffer. Thereafter, 75 µl of sample were mixed with 25 µl of sample buffer (300 mM Tris, 40% glycerol, 0.02% bromophenol blue, 100 mM DTT; pH 6.8) and gels were loaded with 15 µl sample/lane. Separation was carried out at increasing voltage (80–150 V) for 4 h at 4°C in a running buffer (25 mM Tris, 192 mM glycine and 1 mM EDTA, pH 8.3). Subsequently, gels were incubated in 100 ml of incubation buffer (50 mM Tris, 10 mM monothioglycerol, 4 mM CaCl2; pH 7.5) and shaken at room temperature for 1 h, changing the buffer three times. Gels were then washed overnight in stop buffer (20 mM Tris and 10 mM EDTA, pH 7.0) and stained with EZ Blue Gel Staining (Sigma-Aldrich, St. Louis, MO, United States) until the gel absorbed all the stain and white bands appeared. Native and autolyzed calpain activity was quantified by Alpha View software (v 3.4, ProteinSimple Inc., Santa Clara, CA, United States) and expressed as the relative abundance of each individual protein band within the lane.

Sarcomere Length

To prepare samples for sarcomere length determination, 14 ml of TX-rigor buffer (75 mM KCl, 10 mM imidazole, 2 mM $MgCl_2$, 2 mM EGTA, 1 mM NaN_3, 0.5% Triton X-100, pH 7.2) were added to 2 g of chopped muscle samples were then homogenized twice at 18,000 rpm for 5 s and centrifuged at 1,000 × g for 10 min at 4°C. The resulting pellet was then resuspended, homogenized, and centrifuged a second time. After centrifugation, the supernatant was discarded and the pellet was resuspended with 14 ml of rigor buffer (75 mM KCl, 10 mM imidazole, 2 mM MgCl2, 2 mM EGTA, 1 mM NaN3, pH 7.2), vortexed, and centrifuged. After discarding the supernatant, the pellet was resuspended in 28 ml of rigor buffer. 5 µl of the myofibril preparation was placed on a slide with 75 µl of slide fixative

(rigor buffer, 3% formaldehyde vol/vol). Slides were dried at 35°C for 10 min, rinsed with DI water, and sealed with a cover slip using 30 µl of mounting media (75 mM KCl, 10 mM Tris pH 8.5, 2 mM $MgCl_2$, 2 mM EGTA, 1 mM NaN_3, 1 mg/ml p-phenylenediamine, 75% glycerol vol/vol). Sarcomere length was assessed on 25–30 myofibrils per slide with a Carl Zeiss Axio Imager two light microscope using a 100X objective and Zen 2011 image processing software (Carl Zeiss AG, Oberkochen, Germany).

Histology

Frozen samples were cut (10 µm thickness) transversely to muscle fiber direction using a Leica CM3050S cryostat (Leica Biosystems, Nussloch, Germany), fixed in 10% buffered formalin, and used for hematoxylin-eosin (HE) staining according to the procedure described by Warren et al. (2020). Fiber size was measured in three randomly selected fields/portion/fillet on HE stained slides using Zen 2011 image-analysis software (Carl Zeiss AG, Oberkochen, Germany). The total number of fibers measured per fillet portion was 300–450 measurements for both NORM and SM samples.

Statistical Analysis

Statistical analysis was carried out using a mixed model (PROC MIXED) in SAS (Version 9.3, SAS Institute Inc., Cary, NC, United States). TD-NMR and free calcium content were evaluated with a two-way ANOVA mixed model considering muscle condition (M: NORM and SM), time (T: 0, 3, 7), and their interaction M × T as fixed effects and trial as a random effect. SDS-PAGE, casein zymography and Western Blot data were analyzed using the same two-way ANOVA mixed model, with the inclusion of gel among the random effects. A one-way ANOVA was used to evaluate the effect of the muscle condition on fiber size within fillet portion (considering sample ID and trial as random effects). Sarcomere length data were evaluated according to muscle condition, layer (superficial and deep), and their interaction (considering sample ID and trial as random effects). The individual fillet was considered as experimental unit for NMR, free calcium, SDS-PAGE, casein zymography and Western Blot analyses. The single fillet portion was considered as the experimental unit for histology and sarcomere length analyses. Means were separated using Bonferroni adjustments; $p < 0.05$ was assigned as significance level.

RESULTS AND DISCUSSION

Histological Analyses

Histological analysis of the breast samples confirmed that the SM fillets utilized in this study showed the typical histopathological muscle lesions observed in previous reports (Baldi et al., 2018; Baldi et al., 2019; Mazzoni et al., 2020). Differently from the normal tissue (**Figure 1A**), muscle tissue from SM fillets exhibited many of the typical indicators of myodegeneration associated with growth related breast myopathies, such as irregular fiber sizes, regenerative fibers, necrosis, and infiltration of inflammatory cells (**Figure 1B**). In agreement with previous literature (Baldi et al., 2018), the spatial differences regarding the distribution of the lesions within the fillets reflected the macroscopic assessment of the SM condition, as the SM histopathological traits were more abundant and severe in the superficial-cranial portion of the muscle compared to the deep-cranial and caudal parts.

In this study, average muscle fiber size was not statistically different between normal and SM samples either in the superficial (NORM = 3,201 μm^2, stdev = 1,592—vs. SM = 2,950 μm^2, stdev = 1,480; $p = 0.4542$), deep (NORM = 3,124 μm^2, stdev = 1,584—vs. SM = 2,861 μm^2, stdev = 1,320; $p = 0.5117$) or caudal portions (NORM = 2,438 μm^2, stdev = 1,337—vs. SM = 2,327 μm^2, stdev = 1,169; $p = 0.7002$). In previous studies on myopathies, however, fibers of White Striping and Wooden Breast -affected muscles exhibited larger cross-sectional areas compared to normal tissue (Dalle Zotte et al., 2017; Mazzoni et al., 2020). Interestingly, Mazzoni et al. (2020) found that SM muscles had both larger glycolytic fibers and smaller oxidative fibers than the normal counterparts at the same time; moreover, in that study, SM possessed a lower percentage of glycolytic fibers (−8.8%) and a higher percentage of oxidative fibers than the normal counterparts (+10.2%). The lack of significance in terms of fiber size between unaffected and abnormal tissues in the current study might be explained considering that both glycolytic and oxidative fibers were included in the measurement of the average size and metabolism type was not taken into account.

Myofibrillar Protein Profiles

To evaluate the effects of the SM myopathy and storage time on muscle protein composition, myofibrillar protein fractions were isolated, adjusted to equal concentrations and analysed using SDS-PAGE (**Figure 2**). The relative abundance of the 13 most prominent bands were quantified and shown in **Table 1**. The lack of significant interaction effects between muscle condition and postmortem time indicated that NORM and SM fillets exhibited a similar progression of protein degradation with postmortem storage time. Similarly, Soglia et al. (2018) reported that the myofibrillar protein degradation in Wooden Breast fillets globally occurred at the same rate as in normal fillets over a 7 day refrigerated storage period. The overall lack of significant muscle condition effects suggested that the SM myopathy had minimal impact on the myofibrillar protein profile of the breast muscle. Indeed, compared to normal fillets, SM samples only exhibited a difference in the relative abundance of a single protein band corresponding to 80 kDa (NORM = 1.00% vs. SM = 0.81%; $p = 0.0057$). Although the SM myopathy was found to reduce total muscle protein content in previous studies (Baldi et al., 2018; Baldi et al., 2019; Tasoniero et al., 2020), the electrophoretic data of the present study suggest that the myodegeneration associated with this myopathy only causes minor shifts in the myofibrillar protein profile, thereby corroborating the observations of our previous study (Tasoniero et al., 2020). Postmortem storage impacted the composition of the myofibrillar protein fraction. As the storage proceeded, the degradation of nebulin (d0 = 2.70%; d3 = 1.73%; d7 = 1.08%; $p = 0.0001$) and α-actinin (d0 = 2.42%; d3

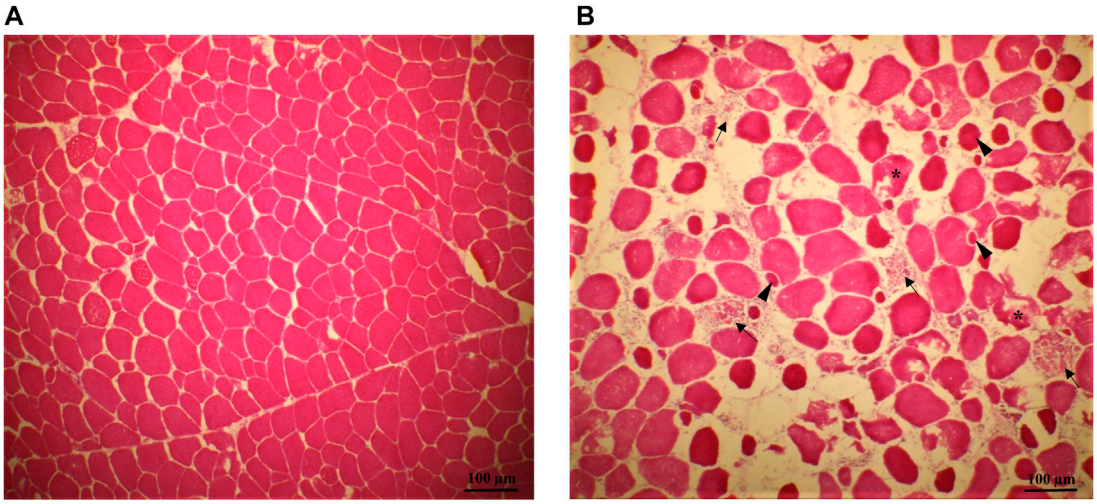


FIGURE 1 | Representative pictures of transversal histological sections from the superficial portion of normal [NORM—**(A)** and Spaghetti Meat fillets (SM—**(B)**]. Muscle tissue from SM fillets exhibited many of the typical indicators of myodegeneration associated with growth related breast myopathies: muscle fibers reduced in number, spaced apart and with irregular fiber sizes; regenerative fibers (arrowheads), necrosis (asterisks), infiltration of inflammatory cells and phagocytosis of the remainder of the myofibers (arrow).

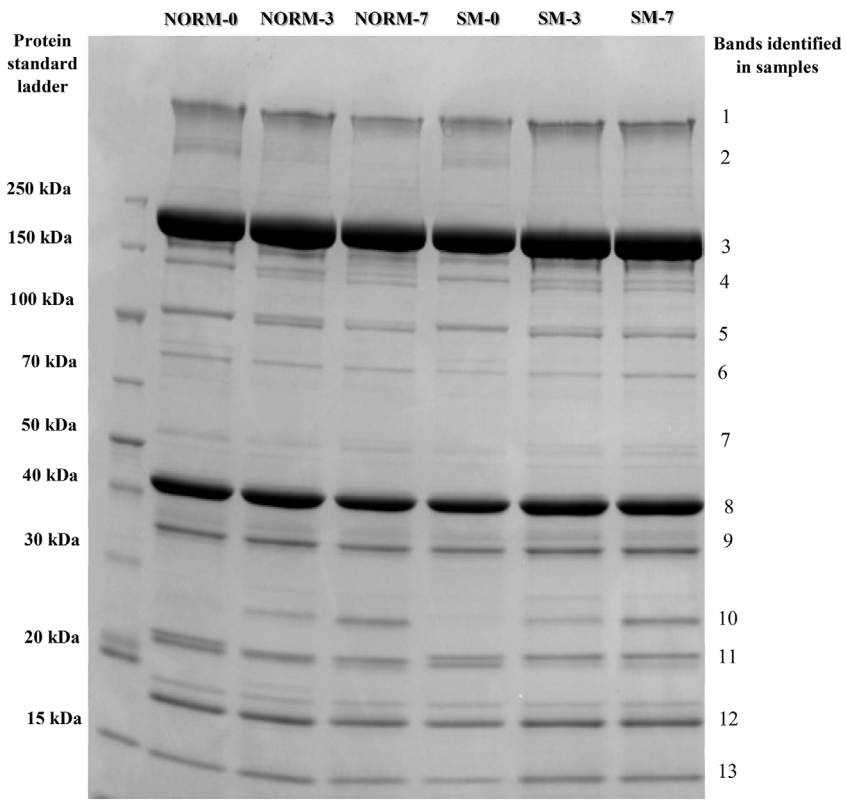


FIGURE 2 | Representative SDS-PAGE protein profiles of the myofibrillar fraction according to muscle condition (M—normal, NORM; Spaghetti Meat, SM) and postmortem time (T—0, 3, 7 days).

TABLE 1 | Relative abundance[1] of SDS-PAGE protein bands from the myofibrillar fraction of broiler breast fillets according to muscle condition (M—normal, NORM; Spaghetti Meat, SM) and postmortem time (T—0, 3, 7 days).

Bands	Approximate molecular size and identification	Muscle condition (M)		Time (T)			SE	*P*-value		
		NORM	SM	0	3	7		M × T	M	T
1	Titin	9.32	9.83	10.1	9.57	9.11	0.46	NS	NS	NS
2	Nebulin	1.85	1.83	2.70^{a}	1.73^{b}	1.08^{b}	0.34	NS	NS	0.0001
3	220 kDa, MyHC	39.8	40.6	38.7	40.4	41.5	2.0	NS	NS	NS
4	150 kDa, MyBPC	2.46	2.30	2.95	2.24	1.94	0.48	NS	NS	NS
5	105 kDa, α-actinin	2.16	2.03	2.42^{a}	2.14^{ab}	1.74^{b}	0.25	NS	NS	0.0053
6	80 kDa	1.00^{a}	0.81^{b}	0.91	0.94	0.87	0.15	NS	0.0057	NS
7	53 kDa, desmin	0.85	0.85	0.84	0.82	0.89	0.08	NS	NS	NS
8	42 kDa, actin	21.8	21.3	21.4	21.4	21.7	1.0	NS	NS	NS
9	38 kDa, TnT	5.04	5.01	5.08	5.04	4.95	0.49	NS	NS	NS
10	28–30 kDa	0.18	0.16	0.12^{b}	0.27^{a}	0.12^{b}	0.05	NS	NS	<0.0001
11	21 kDa, MLC-1	1.55	1.34	0.82^{b}	1.33^{b}	2.18^{a}	0.42	NS	NS	<0.0001
12	17 kDa, MLC-2	3.68	3.42	4.14^{a}	3.46^{b}	3.05^{b}	0.43	NS	NS	0.0005
13	15 kDa, MLC-3	1.51	1.75	1.45	1.68	1.76	0.30	NS	NS	NS

[1]Data expressed as individual protein band abundance as a percentage of total protein abundance in the entire lane. (MyHC, myosin heavy chain; MyBPC, myosin binding protein C; TnT, troponin T; MLC-1, myosin light chain 1; TnI, troponin I; TnC, troponin C; MLC-2, myosin light chain two; MLC-3, myosin light chain 3).
[ab]Means within the same row and effect followed by different superscripts differ $p < 0.05$.

TABLE 2 | Free calcium content and calpain activity of broiler breast meat according to muscle condition (M—normal, NORM; Spaghetti Meat, SM) and postmortem time (T—0, 3, 7 days).

Traits	Muscle condition (M)		Time (T)			SE	*P*-value		
	NORM	SM	0	3	7		M × T	M	T
Free Ca (μM)	48.0	44.4	43.6^{ab}	40.5^{b}	54.5^{a}	4.9	NS	NS	0.0392
Native μ/m calpain, %	48.8	49.5	70.1^{a}	48.0^{b}	29.4^{c}	4.6	0.0031§	NS	<0.0001
Autolyzed μ/m calpain, %	51.2	50.5	29.9^{c}	52.0^{b}	70.6^{a}	4.6	0.0031§	NS	<0.0001

[ab]Means within the same row and effect followed by different superscripts differ $p < 0.05$.
§Native and autolyzed μ/m calpain activity of broiler breast meat according to the interaction M × T shown in ***Figure 3***.

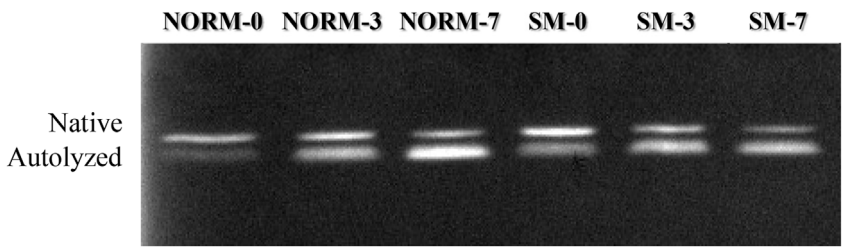
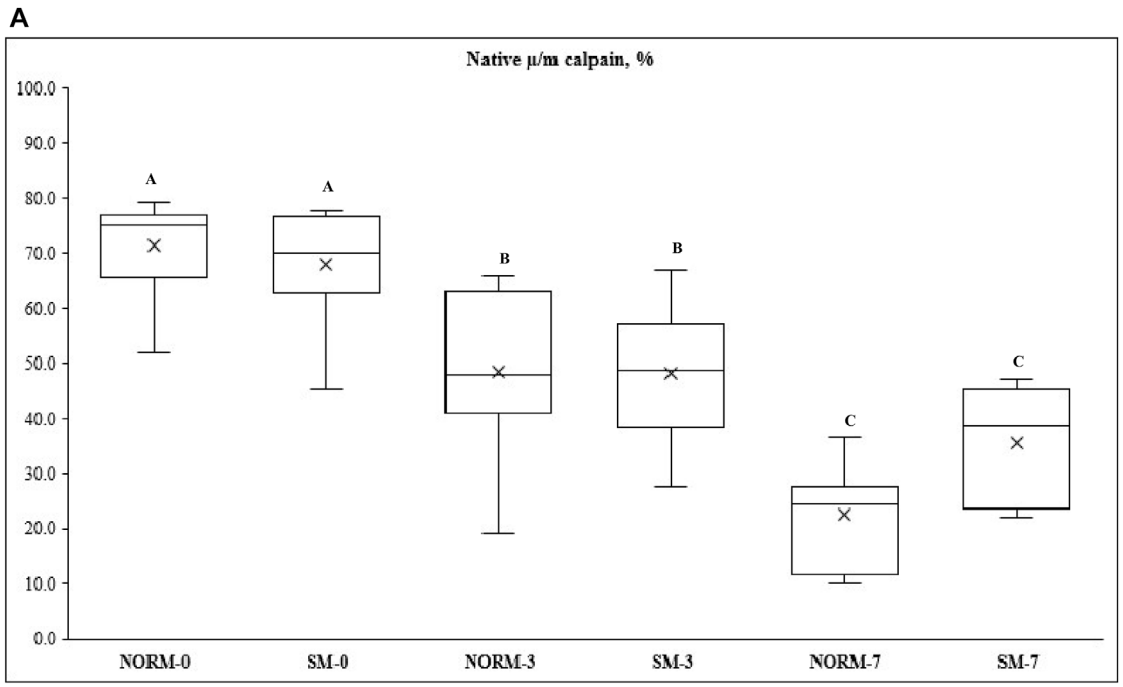


FIGURE 3 | Representative casein zymogram according to muscle condition (M—normal, NORM; Spaghetti Meat, SM) and postmortem time (T—0, 3, 7 days).

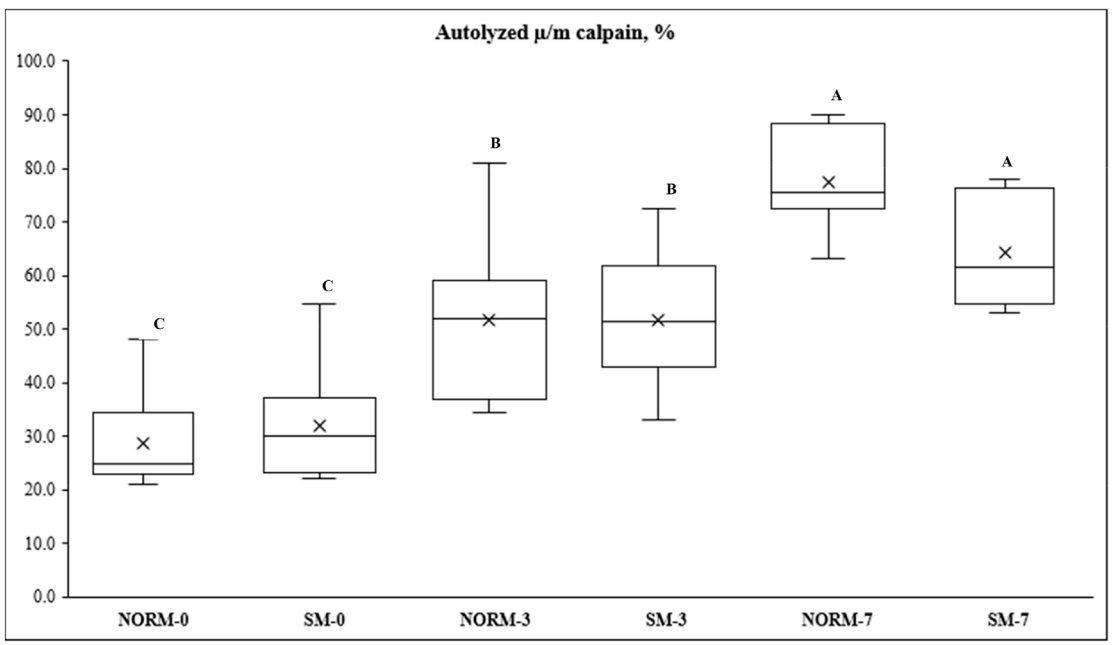
= 2.14%; d7 = 1.74%; $p = 0.0053$) occurs concurrently with the accumulation of a 21 kDa protein band (d0 = 0.82%; d3 = 1.33%; d7 = 2.18%; $p < 0.0001$) in both normal and SM samples.

Free Calcium and Calpain Activity

As SDS-PAGE analysis only provides a limited view of potential myofibrillar protein degradation, free calcium content and calpain activity were measured in this study as additional indicators and precursors of proteolysis (**Table 2**). The interaction M × T and the SM myopathy did not significantly impact free calcium concentration in the breast meat; however, postmortem storage time exerted a strong effect on calcium levels. From day 0 to 7, the concentration of free calcium increased (d0 = 43.6 μM; d3 = 40.5 μM; d7 = 54.5 μM; $p = 0.0392$). This trend may be ascribed to a gradual leakage of calcium ions from the sarcoplasmic reticulum into the sarcoplasm (Ushio et al., 1991) as well as to the proteolysis of calcium sequestering cytoskeletal proteins (Bond and Warner, 2007). Calpain activity in the tissue was assessed using casein zymography (**Figure 3**). The relative proportions of both the native and



A,B,C = different uppercase letters differ $P < 0.0001$



A,B,C = different uppercase letters differ $P < 0.0001$

FIGURE 4 | Native μ/m **(A)** and autolyzed μ/m **(B)** calpain activity of broiler breast meat according to the interaction M × T between muscle condition (M—normal, NORM; Spaghetti Meat, SM) and postmortem time (T—0, 3, 7 days).

autolyzed forms of μ/m calpain exhibited a significant muscle condition by storage time interaction effect ($p = 0.0031$, **Table 2**) and are shown in detail in **Figures 4A,B**. As it can be observed in the graphs, the proportions of the two forms were similar between NORM and SM fillets on days 0 and 3 postmortem, while the SM fillets tended to have a greater proportion of native μ/m calpain

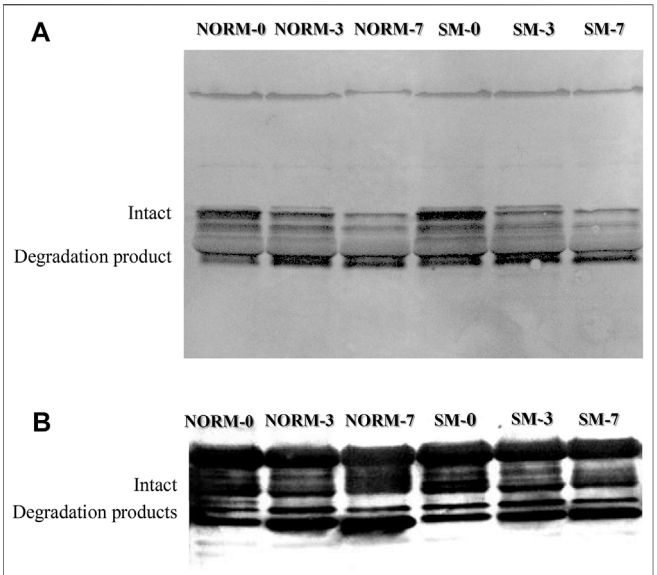


FIGURE 5 | Representative western blot for desmin **(A)** and troponin-T **(B)** according to muscle condition (normal, NORM; Spaghetti Meat, SM) and postmortem time (0, 3, 7 days).

(and consequently a lower proportion of its autolyzed form) at day 7 postmortem. In previous literature, similar to our current observations on SM, no significant differences were observed between Wooden Breast and unaffected samples in terms of native μ/m calpain activity and the accumulation of its autolyzed form with postmortem storage time (Soglia et al., 2018). With postmortem storage time, casein zymograms demonstrated a progressive decrease in the proportion of native μ/m calpain concomitant with the accumulation of its autolyzed form. These findings are consistent with previous studies describing the activity and autolysis over time of this calpain variant in avian muscles, where it has a crucial role for the proteolysis of cytoskeletal proteins during aging (Lee et al., 2007; Huang et al., 2019).

Desmin and Troponin-T Degradation

Desmin and troponin-T are often used as indicators of postmortem muscle protein degradation. In this study, western blot analysis was utilized to observe the relative abundance of desmin and troponin-T proteins (**Figures 5A,B**; respectively) and their degradation products in both NORM and SM fillets with postmortem storage (**Table 3**). Overall, the lack of significant interaction effect between muscle condition and storage time observed in the desmin and troponin-T blots (**Table 3**) suggested that the proteolytic processes taking place during the postmortem period are similar between SM and NORM breast meat. Due to its role in the structural organization of the sarcomere (Gallanti et al., 1992), it would have been reasonable to hypothesize that desmin accumulation may have occurred in muscle tissue during the fiber regeneration processes as a response mechanism to myodegeneration. Consistent with this hypothesis, Soglia et al. (2018) reported a larger amount of desmin in Wooden Breast samples compared to the normal ones at 10 h postmortem. Data from the current study, however, indicated that the relative abundance of intact desmin was similar between NORM and SM samples at all sampling times. This finding seems to corroborate the hypothesis that regeneration of fibres occurs in SM, but less intensely than Wooden Breast (Bilgili, 2016). Additionally, data from the current study suggest that the degree of desmin and troponin-T degradation was not affected by the occurrence of the SM condition. Consistent with the SDS-PAGE, free calcium, and calpain data, the western blot data suggest a progression of increasing myofibrillar protein degradation with postmortem aging in both NORM and SM fillets. According to Zhao et al. (2016), a strong correlation exists between μ/m calpain activity, desmin and troponin-T degradation, and the accumulation of a 28–32 kDa degradation product of troponin-T. Indeed, as a result of storage, the abundance of intact desmin decreased (d0 = 52.7%; d3 = 41.6%; d7 = 34.4%; p = 0.0001) and there was an accumulation of a 39-kDa degradation product (d0 = 47.3%; d3 = 58.4%; d7 = 65.7%; p = 0.0001). As for the degradation pattern of troponin-T, the bands ascribed to the intact form (42–40 kDa) exhibited a tendency to decrease over time (p = 0.0559), while protein bands ascribed to troponin-T degradation products tended to accumulate (p = 0.0559).

Myowater Properties

Myowater properties of the fillets as measured by Time Domain-NMR are presented in **Table 4**. Within the muscle, three water populations were identified by Time Domain nuclear magnetic resonance (NMR) transverse relaxation: water tightly bound to

TABLE 3 | Western Blot data for desmin and troponin-T of broiler breast meat according to muscle condition (M—normal, NORM; Spaghetti Meat, SM) and postmortem time (T—0, 3, 7 days).

Relative abundance, %	Muscle condition (M)		Time (T)			SE	*P*-value		
	NORM	SM	0	3	7		M × T	M	T
Desmin									
Intact, 52 kDa	43.8	41.9	52.7[a]	41.6[b]	34.4[b]	4.6	NS	NS	0.0001
Degradation product, 39 kDa	56.2	58.1	47.3[b]	58.4[a]	65.7[a]	4.7	NS	NS	0.0001
TnT									
Intact, 42–40 kDa	47.1	46.7	53.8	44.3	42.6	4.2	NS	NS	NS
Degradation products, 32–30 kDa	52.9	53.3	46.2	55.7	57.4	4.2	NS	NS	NS

[ab]*Means within the same row and effect followed by different superscripts differ* p < *0.05.*

TABLE 4 | Time Domain-Nuclear Magnetic Resonance traits of broiler breast meat according to muscle condition (M—normal, NORM; Spaghetti Meat, SM) and postmortem time (T—0, 3, 7 days).

NMR traits	Muscle condition (M)		Time (T)			SE	*P*-value		
	NORM	SM	0	3	7		M × T	M	T
T_{2B}, ms	0.69	0.71	0.70	0.72	0.69	0.01	NS	NS	NS
T_{21}, ms	45.9^{b}	46.9^{a}	47.1	45.9	46.2	0.6	NS	0.0172	NS
T_{22}, ms	180^{b}	200^{a}	227^{a}	181^{b}	162^{c}	6	NS	0.0001	<0.0001
P_{2B}, %	0.74^{a}	0.54^{b}	0.59^{b}	0.57^{b}	0.76^{a}	0.07	NS	0.0009	0.0134
P_{21}, %	83.8^{a}	80.7^{b}	78.7^{b}	83.8^{a}	84.1^{a}	2.1	NS	0.0118	0.0006
P_{22}, %	15.5^{b}	18.8^{a}	20.7^{a}	15.6^{b}	15.0^{b}	2.1	NS	0.0080	0.0005

^{ab}*Means within the same row and effect followed by different superscripts differ* $p < 0.05$.

macromolecules, water located within the myofibrillar matrix and free water held only by capillary forces. The latter population, or extramyofibrillar compartment, consists of the inter-myofibrillar spaces, as well as the spaces between muscle fibres and between fiber bundles. For each of the three compartments, the relaxation time (*T*) was measured as an indicator of water mobility. The relaxation times also provided information about population proportions (*P*), which indicated water molecule distribution. Consistent with previous literature (Baldi et al., 2018; Soglia et al., 2019; Wold and Løvland, 2020), the SM fillets possessed a lower percentage of bound water (P_{2B}; NORM = 0.74% vs. SM = 0.54%; p = 0.0009). The intramyofibrillar water population exhibited longer relaxation times in SM fillets (T_{21}; NORM = 45.9 ms vs. SM = 46.9 ms; p = 0.0172) and a lower proportion compared to NORM (P_{21}; NORM = 83.8% vs. SM = 80.7%; p = 0.0118). Concurrently, SM was characterized by a greater proportion of extramyofibrillar water (P_{22}; NORM = 15.5% vs. SM = 18.8%; p = 0.0080) possessing a longer relaxation time (T_{22}; NORM = 180 ms vs. SM = 200 ms; p = 0.0001). According to Bertram et al. (2002), a high correlation (r = 0.76) exists between P_{22} population and water-holding capacity in terms of drip loss, and the correlation results increased (r = 0.84) when T_{21} is included in the correlation analysis. Thus, altered intramyofibrillar water properties and a greater amount of free water detected by TD-NMR in SM fillets are consistent with a lower water holding capacity previously observed in affected meat (Tasoniero et al., 2020). Altered myowater distribution and mobility in SM samples are clearly ascribable to the histological lesions observed in the affected fillets: loss of normal tissue architecture, necrosis and lysis of fibers, immature collagen deposition coupled with a progressive rarefaction of endomysial and perimysial connective tissue that leads to muscle fibers detachment from each other and compromised fiber bundles cohesion (Baldi et al., 2018). Altered myowater properties in SM might also reflect a reduced protein content exhibited by affected fillets and potentially resulting from tissue degenerative processes (Tasoniero et al., 2020). Bertram et al. (2002) found that a high correlation (r = 0.84) exists between T_{21} and sarcomere length. The results of the current study are in accordance with this finding, as SM also exhibited longer sarcomeres than the normal counterparts (NORM = 1.71 μm vs. SM = 1.81 μm; SE = 0.03; p = 0.0231). In addition, the superficial layer tended to have longer sarcomeres than the deep layer (p = 0.0586) while the interaction effect was not significant. Currently, the structural and functional aspects of the contractile apparatus within the SM myopathy are unknown. Similar to Wooden Breast, it might be hypothesized that longer sarcomeres are ascribable to the loss of tissue architecture that could prevent muscle shortening (Tijare et al., 2016). Postmortem storage time exerted a strong effect on myowater properties. An increasing trend for both the P_{2B} (p = 0.0134) and P_{21} (p = 0.0006) water compartments coupled with a decrease of P_{22} (p = 0.0005) revealed that a redistribution of myowater occurred over time in both NORM and SM fillets as a consequence of the biochemical and structural changes occurring during the storage period. Specifically, greater relative intensities of the water bound to macromolecules and located within the myofibrillar matrix might be attributed to the cytoskeletal protein proteolysis occurring during meat aging, that contribute in myofibers swelling and thus increased water holding capacity (Melody et al., 2004).

CONCLUSION

The findings of the present study demonstrated that the SM myopathy only caused minor shifts in the muscle protein profile and revealed that the SM condition did not alter the initial levels of free calcium, calpain activity or the amounts of intact desmin and troponin-T. In addition, data from this study demonstrate that SM fillets do not have a greater degree of muscle protein degradation than normal fillets at 0, 3, or 7 d postmortem. These findings suggest that there is not enhanced proteolysis occurring either antemortem or early postmortem in SM that would be contributing to the soft consistency and diminished overall muscle integrity. Simultaneously, the TD-NMR data suggest that breast muscle tissue with the SM myopathy possess altered myowater properties compared to normal tissue, which is consistent with the lower WHC observed in SM fillets. Taken together, the findings in the current study on myowater properties and muscle protein degradation suggest that the differences in myowater properties observed between normal and SM fillets are not likely due to a more extensive breakdown of cytoskeletal muscle proteins in SM fillets. Further research is needed to understand more clearly how myopathy-induced changes to the physical and chemical properties of the muscle influence meat quality traits.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

GT, BB, and HZ planned the experiment. GT designed the study, performed the laboratory analyses, collected, and organized the data, performed the statistical analysis and wrote the first draft of the manuscript. GT, BB, and HZ interpreted the results and revised the manuscript.

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Factors Affecting Breast Myopathies in Broiler Chickens and Quality of Defective Meat: A Meta-Analysis

Francesco Bordignon[1], *Gerolamo Xiccato*[1], *Marija Boskovic Cabrol*[2], *Marco Birolo*[1] *and Angela Trocino*[3]*

[1]Department of Agronomy, Food, Natural Resources, Animal and Environment (DAFNAE), University of Padova, Padova, Italy, [2]Department of Food Hygiene and Technology, Faculty of Veterinary Medicine, University of Belgrade, Belgrade, Serbia, [3]Department of Comparative Biomedicine and Food Science (BCA), University of Padova, Padova, Italy

Fast-growing broiler chickens are subjected to breast myopathies such as white striping (WS), wooden breast (WB), and spaghetti meat (SM). Available studies about risk factors for myopathy occurrence often used flock data whereas a few reports evaluated chicken individual data. Thus, the present study aimed to elucidate the effect of growth and slaughter traits, besides sex and genotype on myopathy occurrence. Data were obtained from eight experimental trials, which used a total of 6,036 broiler chickens. Sex, genotype, daily weight gain, slaughter weight, and breast yield were evaluated as potential risk factors by logistic regression analyses. Then, the effects of myopathy and sex were evaluated on meat rheological traits (pH, colour, cooking losses and shear force). Based on a logistic regression, WS occurrence was associated with genotype, breast weight, and breast yield. Compared with chickens with intermediate breast weight and breast yield, higher odds of having WS were found in chickens with high breast weight (OR: 1.49) and yield (OR: 1.27), whereas lower odds were found in those with low breast weight (OR: 0.57) and yield (OR: 0.82). As for WB and SM, females had lower odds of having WB (OR: 0.55) and higher odds of showing SM (OR: 15.4) compared to males. In males, higher odds of having WB were found in chickens with a high daily weight gain (OR: 1.75) compared to those with an intermediate daily weight gain. In females, higher odds of having SM were associated to a high slaughter weight (OR: 2.10) while lower odds to a low slaughter weight (OR: 0.87). As for meat quality, only WB meat was clearly different for some technological and sensorial properties, which can play a major role also in meat processing. In conclusion, our analysis identified breast development as a potential risk factor for WS, while a high growth was the risk factor for WB and SM. A different probability of having WB or SM in females and male was found.

Keywords: white striping, wooden breast, spaghetti meat, risk factor, sex, breast yield, pH, cooking losses

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***Correspondence:**
Angela Trocino
angela.trocino@unipd.it

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1 INTRODUCTION

Among the most common animal food consumed worldwide due to its nutritional profile, as part of a well equilibrated (vegetable-rich) diet, poultry meat is linked to a lower risk of developing non-communicable diseases, including obesity, cardiovascular diseases, and type 2 diabetes mellitus (Marangoni et al., 2015). This issue supports the development of poultry production, one of the

fastest-growing agricultural subsectors, especially in developing areas where it contributes to food security and plays a major role in reducing global hunger (Mottet and Tempio, 2017) with an estimated production increase of 16% by 2029.

The expansion of the poultry production has been sustained by the extraordinary growth potential of chickens pushed in genetic selection programs. However, the increasing growth rate during a short life period (5–8 weeks) and the large body size of modern crossbred birds have resulted in cardiovascular diseases, different metabolic and musculoskeletal disorders, including the most recent emerging breast muscle abnormalities, such as white striping (WS), wooden breast (WB) and spaghetti meat (SM) (Baldi et al., 2021; Hartcher and Lum, 2019; Soglia et al., 2021; Che et al., 2022a, b). The WS breasts are characterized by the presence of visible white striations on the surface in the direction of muscle fiber, while a degenerative myopathy at the fiber level is present (Kuttappan et al., 2013; Radaelli et al., 2017). Sometimes, WS is also accompanied by the most recently discovered SM, though this latter can be found independently, as well (Che et al., 2022a). The WB muscles are characterized by pale and bulging hard areas and, at the morphological level, by polyphasic myodegeneration with regeneration and accumulation of interstitial connective tissue or fibrosis (Sihvo et al., 2014; Velleman and Clark, 2015). Finally, the few available data show that SM affects mainly the connective tissue within the perymisial compartments causing the formation of large intracellular spaces and consequently overall impaired integrity of the *Pectoralis major* (Soglia et al., 2021). In SM meat, muscle fibers can be easily separated resembling the long, thin, solid, and cylindrical appearance of spaghetti (Baldi et al., 2018).

Omics analyses have been used to define pathophysiological mechanisms of broiler myopathies with the main focus on WS and WB (Mutryn et al., 2015; Zambonelli et al., 2016; Kuttappan et al., 2017a; Boerboom et al., 2018; Soglia et al., 2020). These studies have suggested that a reduced vascularity and ischemia, with consequent hypoxia and lack of blood flow, lead to WB. Results of RNA-seq analysis support localized hypoxia, oxidative stress, increased intracellular calcium, and the possible presence of muscle fiber-type switching, as key features of WB (Mutryn et al., 2015). Furthermore, Zambonelli et al. (2016) found that breasts with WS and WB have an increased expression of genes associated with metabolic oxidative stress, inflammation, regeneration, glucose metabolism, lipidosis, fibrosis, and proteoglycan synthesis. Recently, Bordini et al. (2021) suggested that alterations in extracellular matrix composition could somehow activate the cascade of biological reactions that result in the growth-related myopathies onset, and hypothesized the involvement of Collagen IV alterations in activating the endoplasmic reticulum stress response.

From published data, it is not completely clear what factors influence the manifestation of a particular myopathy and neither what the initial trigger is (Bailey et al., 2020). Previous studies highlighted the importance of understanding the environmental and/or management factors that contribute greater than 65% of the variance in the incidence of WS and more than 90% of the variance of the incidence of WB (Bailey et al., 2015). This was stressed in recent analyses of risk factors for myopathies that collected data from different flocks in two Canadian slaughterhouses (Che et al., 2022b) which identified high environmental temperature during the grow-out period and absence of vaccination against coccidian as risk factors for increased probability of showing SM and WB, respectively.

Some studies suggest that there are biological characteristics in male broilers that make them more susceptible to WB (Brothers et al., 2019), while female broilers have a higher predisposition for SM (Pascual et al., 2020b). Other studies suggest that growth and carcass traits, in particular breast weight, are predominant factors for WS and WB occurrence (Aguirre et al., 2020; Santos et al., 2021). In addition, there is published information for the effects of feeding strategies, such as feed restriction (Trocino et al., 2015; Radaelli et al., 2017; Gratta et al., 2019) and time-limited feeding (Livingston et al., 2019), or the supplementation of sodium butyrate (Pascual et al., 2020b), guanidinoacetic acid (Córdova-Noboa et al., 2018a; Córdova-Noboa et al., 2018b), selenium (Cemin et al., 2018) and microalgae (Khan et al., 2021) on myopathies occurrence. Research has also focused on the effect of decreasing amino acid density to control growth and reduce the rates and degrees of myopathies (Cruz et al., 2017; Bodle et al., 2018; Meloche et al., 2018).

While quality of defective meat has been extensively compared within several studies, results among studies are often not consistent about changes in traits associated to *post mortem* metabolism and in rheological or sensorial properties of meat (i.e., pH, water retention, colour and hardness). Available data have been produced both using samples collected in controlled experimental trials and in commercial slaughterhouses from commercial flocks. Few studies investigated the correlation between individual growth traits and myopathy occurrence (Bailey et al., 2015; Aguirre et al., 2020; Santos et al., 2021). Also, according to our knowledge, only one study included SM in such analyses using flock data (Che et al., 2022b).

Thus, the present study aimed at elucidating the role of some major factors individually related to chickens (sex, growth and carcass traits) on the occurrence of myopathies, besides at characterizing rheological traits of defective meat, using a set of data collected from 2015 to 2021 within trials conducted in the controlled facilities of the University of Padova (Department of Agronomy, Food, Natural resources, Animals and Environment) using the same reference and lab methods under standardized conditions.

2 MATERIALS AND METHODS

2.1 Dataset

The present study used the data collected on 1,278 carcasses and 890 breasts obtained from eight trials performed at the University of Padova that used a total of 6,036 broiler chickens (**Table 1**). At commercial slaughtering the carcasses were used for scoring myopathy occurrence and the breasts were submitted to rheological analyses for meat quality (**Supplementary Tables S1, S2**).

The trials used three commercial genotypes selected for fast growth and high-breast yield: genotype B (Ross 308) was the

TABLE 1 | Summary of the main information about the eight experimental trials originating the dataset.

Trial	References	Main Experimental Factors	Sex	Genotype	Slaughtering Age, d	Recordings, n		
						Growth performance	Breast weight and myopathy occurrence	Meat quality traits
1	Trocino et al. (2015)	Early feed restriction from 13 to 21 days	Females and males	A, B	46	768	127	128
2	Gratta et al. (2019)	Early vs late feed restriction	Males	B, C	48	900	216	108
3	Not published yet	Light programs	Females and males	B, C	44	800	191	95
4	Pascual et al. (2020b)	Dietary supplementation with sodium butyrate	Females and males	B	45	768	192	96
5	Pascual et al. (2020a)	Dietary supplementation with yeast cell wall extracts	Males	B	44	576	120	72
6	Not published yet	Dietary supplementation with grape pomace extracts	Females	B	41	560	160	120
7	Pascual et al. (2022)	Dietary supplementation with grape pomace and chestnut extracts	Females and males	B	45	864	144	144
8	Huerta et al. (2022)	Dietary supplementation with grapeseed extracts	Females and males	B	42	800	128	128

Genotype A, Ross 708; Genotype B, Ross 308; Genotype C, Cobb 500.

mostly used (eight trials), genotype A (Ross 708) was used only in one trial, and genotype C (Cobb 500) in two trials (**Table 1**). Five trials used chickens of both sexes, half females and half males within trial; two trials used only males and one trial only females. Slaughtering age ranged from 41 to 42 days in two trials to 44–45 days in four trials, and 46–48 days in the remaining two trials. A variety of experimental factors was tested in the different trials which were directly addressed to control the occurrence of myopathies (e.g., feeding or management strategies aimed at reducing growth rate based on feed restriction; light management) or aimed at the use of different feed additives to improve gut health of broiler chickens with potential implication on myopathies occurrence (e.g., butyrate, yeast cell wall extracts, grape by-product extracts) (**Table 1**).

2.2 Growth Data Recordings

All trials were conducted at the Experimental Farm of the University of Padova (Legnaro, Padova, Italy) in a poultry house equipped with cooling system, forced ventilation, radiant heating, and controlled light systems. Broiler chickens were kept in wire-net pens with a concrete floor covered with wood shaving litter, each equipped with nipple drinkers and circular feeders for manual distribution of feed. Usually, a total of 24 h of light was provided during the first 2 days after the arrival of the chicks. Subsequently, the hours of light were progressively reduced until an 18L:6D photoperiod was achieved, which was then maintained from 12 to 13 days of age onward. Chicks were individually identified by a plastic band at the leg; they were always weighed on the day of their arrival and at the end of the trial for obtaining daily weight gain. Feed consumption was controlled on a pen level for which nor feed intake or feed conversion rate were used for the purposes of the present study.

2.3 Commercial Slaughtering and Carcass and Meat Quality Recordings

At the end of the trials, all chickens were slaughtered in a single commercial slaughterhouse, which was close to the experimental farm, therefore guaranteeing homogeneous slaughtering procedures and carcass preparation. Ready-to-cook carcasses were recovered after 2 h of refrigeration at 2°C and individually weighed to measure the slaughter dressing percentage. In all trials, a number of carcasses, previously selected on the basis of the final live weight as corresponding to the mean body weight within a pen, were subjected to gross examination between 8 and 24 h after slaughtering to evaluate the occurrence (presence or absence) in *pectoralis major* muscles of i) white striping (either moderate or severe) (Kuttappan et al., 2012a); ii) wooden breast (firm upon palpation, prominent ridge like bulge on caudal area of fillet, clear viscous fluid cover and/or petechial multifocal lesions on the fillet surface) (Sihvo et al., 2014); iii) spaghetti meat exhibiting an overall impaired integrity and tendency toward separation of the muscle fiber bundles especially within the cranial part of the fillet) (Baldi et al., 2018). Carcasses were stored at 2°C for 24 h after slaughtering and then dissected in the main cuts (breast, wings, thighs, and drumsticks). *Pectoralis major* muscles were separated from the breasts: the pH of the *p. major* muscles were measured in triplicates on their ventral side with a pH meter (Basic 20, Crison Instruments Sa, Carpi, Italy) equipped with a specific electrode (cat. 5232, Crison Instruments Sa, Carpi, Italy); the L*a*b* color indexes were measured in triplicate on the ventral side of the same muscles using a Minolta CM-508 C spectrophotometer (Minolta Corp., Ramsey, NJ, United States). After then, one meat portion (8 cm × 4 cm × 3 cm) was separated from the cranial side of the *p. major* muscle, parallel to the direction of the muscle fibers, and processed for the following analyses after storage under vacuum in plastic bags

at −18 °C. Thawing and cooking losses were measured in this cut (Petracci and Baéza 2011). Meat cuts (fresh or thawed depending on the trials) were individually placed in plastic bags and cooked in a water bath until an internal temperature of 80°C was achieved. After 40 min of cooling, another meat portion (4 cm × 2 cm × 1 cm) was separated from the cooked one to assess the maximum shear force using an LS5 dynamometer (Lloyd Instruments Ltd., Bognor Regis, United Kingdom) using the Allo-Kramer (10 blades) probe (load cell: 500 kg; distance between the blades: 5 mm; thickness: 2 mm; cutting speed: 250 mm/min) (Mudalal et al., 2015).

2.4 Statistical Analyses

Data were analyzed using SAS 9.4 (Statistical Analysis System, 2013). Descriptive statistics of growth and slaughter traits, myopathy occurrence and breast quality traits were obtained using PROC FREQ and PROC MEANS procedures.

To identify the risk factors related to myopathy occurrence, the effects of sex, genotype, growth traits (daily weight gain and slaughter weight) and slaughter traits (breast weight and breast yield) were evaluated by univariate and multivariate logistic regression analysis using the PROC LOGISTIC of SAS. Initially, variables were screened for multicollinearity (correlation coefficient $|r|<0.7$). A univariate analysis was performed for each independent variable and for each myopathy. Given the strong influence of sex on growth and slaughter traits of chickens, we firstly evaluated sex as a potential influencing factor on breast myopathy occurrence (**Supplementary Table S3**). Then, data were assigned to three classes of growth traits and slaughter traits with a similar number of data (33% of the available data set) within each sex, distinguishing between low, intermediate, and high growth traits or slaughter traits. Finally, we evaluated the effects of genotype and classes for daily growth traits or slaughter traits within each sex (**Supplementary Tables S4–S6**). Thereafter, independent variables that showed a $p < 0.05$ in the univariate analysis were included into a multivariate logistic regression analysis and the risk factors were identified through a forward stepwise selection based on $p < 0.05$. The regression coefficients were expressed as odds ratio (OR) with 95% confidence interval (CI). The evaluation of risk factors within each genotype was also performed and results are shown in **Supplementary Tables S7–S9**.

Individual data of pH, colour, cooking losses and shear force measured on *p. major* were submitted to ANOVA with myopathy, sex, and their interactions as the main effects and the trial as a random effect, using the PROC MIXED. Differences among means with $p < 0.05$ were assumed to be statistically significant. Differences among means with $0.05 < p < 0.10$ were regarded as approaching significance.

3 RESULTS

3.1 Descriptive Statistics of Growth, Carcass Data and Meat Quality Traits

Descriptive statistics for males and females and for the three genotypes are provided in **Tables 2** and **3**, respectively.

In the whole data set, individual daily weight gain of broiler chickens ranged from 53.5 g/d to 92.5 g/d, averaging at 70.7 g/d for a coefficient of variation (CV) at 11.2%. This growth corresponded to an average slaughter weight of 3,154 g (CV: 12.7%), a breast weight (with bone) of 912 g (CV: 13.4%) and a breast yield as a proportion of the carcass of 40.0% with a low variability (CV: 5%) (**Table 2**). While average daily weight gain, slaughter weight and breast weight were lower in females than in males, breast yield between the two sexes was rather similar (on average, 40.7% in females and 39.3% in males).

Breasts exhibiting WS (alone or in associations with other myopathies) averaged at 64.7% with minimum and maximum occurrence at 44.5% and 77.0%, respectively (**Table 2**). The occurrence of both WB and SM averaged at 11%–12% with a large variability (CV: 57.0% for WB and 108% for SM) which depended on differences between females and males. In fact, WB occurred more in males (average of trials 25.2% with a range from 5.09% to 55.6%) than in females (average of trials 12.1%, range 3.16%–17.5%), while SM showed an opposite trend (females: average of trials 36.7%, range 25.0%–48.6%; males: average 3.48%, range 1.56%–5.04%).

As for meat quality, the less variable traits in the whole data set and in the sub-sets per sex were final pH (CV: 2%–3%) and lightness (CV: 6%–7%), while meat water holding capacity (measured by means of thawing and cooking losses), texture (measured as shear force), and the colour indexes of redness (a*) and yellowness (b*) showed a large variability (**Table 2**). On the whole, no main differences in average values or in variability for meat quality traits were recorded between males and females.

3.2 Risk Factors for Breast Myopathy Occurrence

Potential risk factors influencing breast myopathy occurrence in broiler chickens were firstly identified based on a univariate logistic regression analysis and then included into the multivariate regression model. Based on the univariate analysis, potential influencing factors for WS occurrence were sex, genotype, daily weight gain, slaughter weight, breast weight, and breast yield (**Supplementary Tables S3, S4**). As for WB, potential influencing factors were sex, genotype, and daily weight gain (**Supplementary Table S5**), while sex, daily weight gain, and slaughter weight were identified as risk factors for SM (**Supplementary Table S6**).

Results from the multivariate logistic regression analysis of the factors selected for WS occurrence are reported in **Table 4**. Genotype, breast weight, and breast yield significantly influenced WS occurrence. Regarding the first factor, genotype C had higher odds of WS occurrence than genotype A (OR: 2.75; 95% CI: 1.37–5.52). As for breast traits, compared to intermediate-weight breasts (850–960 g), chickens with a high-weight breast (>960 g) showed higher odds of WS occurrence (OR: 1.49; 95% CI: 1.03–2.15), whereas those with a low-weight breast (<850 g) showed lower odds (OR: 0.57; 95% CI: 0.41–0.80). A similar trend was found for breast yield: compared to intermediate-yield breasts (39.0–40.7%), chickens with high-yield breasts showed higher odds of WS occurrence (OR: 1.27, 95% CI: 0.89–1.81) whereas those with low-yield breasts showed lower odds (OR: 0.82; CI: 0.58–1.15) (**Table 4**).

TABLE 2 | Descriptive statistics of growth traits, slaughter traits, myopathy occurrence and meat quality of *pectoralis major* in broiler chickens: data collected over eight experimental trials.

	All Chickens						Females						Males					
	No	Av	SD	Min	Max	CV	No	Av	SD	Min	Max	CV	No	Av	SD	Min	Max	CV
Growth and slaughter traits																		
Daily weight gain, g/d	1,278	70.7	7.94	53.3	92.5	11.2	549	66.1	6.01	53.3	85.9	9.09	729	74.3	7.43	57.4	92.5	10.0
Slaughter weight, g	1,278	3,154	401	2,186	4,260	12.7	549	2,873	309	2,186	3,887	10.8	729	3,368	323	2,561	4,260	9.59
Breast weight, g	930	912	122	560	1,258	13.4	453	835	83.1	560	1,112	9.95	477	986	107	721	1,258	10.9
Breast yield, %	930	40.0	2.05	32.9	46.4	5.13	453	40.7	1.88	32.9	45.1	4.62	477	39.3	1.97	32.9	46.4	5.01
Myopathies occurrence[a]																		
White Striping, %	8	64.7	11.6	44.5	77.0	17.9	6	60.0	12.5	37.1	70.5	20.8	7	68.6	12.2	52.3	83.3	17.8
Wooden breast, %	8	18.6	10.6	5.09	36.1	57.0	6	12.1	5.1	3.16	17.5	42.2	7	25.2	17.6	5.09	55.6	69.8
Spaghetti meat, %	8	13.0	14.1	5.00	41.9	108	6	36.7	10.6	25.0	48.6	28.9	7	3.48	1.50	1.56	5.04	43.1
Breast quality traits																		
pH	890	5.92	0.15	5.37	7.04	2.53	413	5.93	0.12	5.57	6.32	2.02	477	5.91	0.16	5.37	7.04	2.71
L*	890	47.2	3.11	38.3	55.5	6.59	413	47.5	2.78	41.2	55.5	5.85	477	47.0	3.37	38.3	54.8	7.17
a*	890	1.25	1.75	-2.50	5.85	140	413	1.34	1.63	-1.98	4.63	122	477	1.16	1.77	-2.50	5.85	153
b*	890	17.2	5.11	5.48	32.4	29.7	413	17.5	4.82	6.36	27.4	27.5	477	17.0	5.34	5.48	32.4	31.4
Thawing losses, %	818	9.75	3.07	1.04	26.7	31.5	339	10.1	3.00	3.33	19.9	29.7	334	9.44	3.12	1.04	26.7	33.1
Cooking losses, %	818	26.2	5.67	15.6	45.2	21.6	378	26.9	6.78	15.6	45.2	25.2	440	25.5	4.42	15.9	41.1	17.3
Shear force, kg/g	818	3.10	1.02	1.44	12.2	32.9	377	3.01	0.77	1.76	7.32	25.6	440	3.18	1.20	1.44	12.2	37.7

No, number of chickens; Av, Average; SD, standard deviation; Min, Minimum; Max, Maximum; CV, coefficient of variation.

[a]*Occurrence of myopathies within the trials. Not exclusive myopathy, i.e. white striping, wooden breast and/or spaghetti meat can be associated in the same breast.*

TABLE 3 | Descriptive statistics of growth traits, slaughter traits, myopathy occurrence and meat quality of *pectoralis major* in broiler chickens from three different genotypes: data collected over eight experimental trials.

	Genotype A						Genotype B						Genotype C					
	No	Av	SD	Min	Max	CV	No	Av	SD	Min	Max	CV	No	Av	SD	Min	Max	CV
Growth and slaughter traits																		
Daily weight gain, g/d	63	69.7	7.40	56.8	83.8	10.6	1,011	70.5	8.10	53.3	92.0	11.5	204	72.2	7.13	55.1	92.5	9.88
Slaughter weight, g	63	3,205	340	2,613	3,854	10.6	1,011	3,117	409	2,186	4,260	13.1	204	3,327	322	2,426	4,070	9.67
Breast weight, g	63	920	134	609	1,191	14.6	765	900	118	560	1,258	13.2	102	998	108	743	1,224	10.8
Breast yield, %	63	39.9	2.12	32.9	43.9	5.32	765	39.9	2.01	32.9	45.1	5.05	102	40.7	2.16	36.1	46.4	5.31
Myopathies occurrence[a]																		
White Striping, %	1	49.2	-	-	-	-	8	64.6	13.1	40.6	78.7	20.3	2	74.1	2.7	72.2	76.0	3.6
Wooden breast, %	1	14.2	-	-	-	-	8	18.7	10.4	6.32	36.1	55.7	2	5.00	1.80	3.70	6.25	36.2
Spaghetti meat, %	1	-	-	-	-	-	8	20.7	14.1	5.00	41.9	67.7	2	-	-	-	-	-
Breast quality traits							8											
pH	63	5.83	0.10	5.62	6.04	1.69	725	5.94	0.14	5.49	7.04	2.34	102	5.84	0.16	5.37	6.24	2.78
L*	63	46.1	2.27	41.3	52.3	4.91	725	47.6	3.08	39.1	55.5	6.46	102	44.8	2.56	38.3	51.1	5.71
a*	63	−0.97	0.59	−2.50	0.28	60.7	725	1.58	1.69	−1.6	5.85	107	102	0.27	0.66	−1.40	1.89	248
b*	63	13.1	1.97	9.01	19.0	15.0	725	17.8	5.40	5.48	32.4	30.3	102	16.0	2.14	10.4	20.7	13.4
Thawing losses, %	63	8.47	3.28	3.10	26.7	38.7	507	10.0	3.09	1.04	19.9	30.8	102	9.14	2.57	4.21	15.5	28.1
Cooking losses, %	63	23.8	3.28	18.6	35.3	13.8	653	26.4	6.08	15.6	45.2	23.0	102	25.8	3.30	21.2	36.3	12.8
Shear force, kg/g	63	2.99	1.05	1.92	6.69	35.2	653	3.19	1.06	1.44	12.2	33.3	102	2.60	0.48	1.64	4.54	18.4

No: number of chickens; Av: Average; SD: standard deviation; Min: Minimum; Max: Maximum; CV: coefficient of variation genotype A: Ross 708; genotype B: Ross 308; genotype C: Cobb 500.

[a]*Occurrence of myopathies within the trials. Not exclusive myopathy, i.e. white striping, wooden breast and/or spaghetti meat can be associated in the same breast.*

The representation of the significant effects of genotype within breast weight classes showed that the predicted probabilities of WS occurrence in chickens with low-weight breasts were 42.5% (95% CI: 30.1%–55.9%), 60.5% (95% CI: 54.8%–66.1%), and 72.0% (95% CI: 60.6%–81.1%) for genotype A, B, and C respectively (**Figure 1A**). Then, in chickens with intermediate-weight breasts, the probabilities were 48.3% (95% CI: 35.4%–61.4%), 66.0% (95% CI: 60.3%–71.3%), and 76.5% (95% CI: 66.1%–84.4%) for the same genotypes. Finally, in chickens with high-weight breasts, the probabilities were 54.8% (95% CI: 41.7%–67.3%), 71.6% (95% CI: 66.1%–76.5%), and 80.8% (95% CI: 72.0%–87.4%). As for breast yield (**Figure 1B**), the predicted probabilities of WS occurrence in chickens with low yields were 36.7% (95% CI: 25.3%–49.8%), 55.0% (95% CI: 49.3%–60.5%), and 61.6% (95% CI: 48.4%–73.3%) for genotype A, B and C, respectively; in chickens with intermediate yields the probabilities were 51.4% (95% CI: 38.0%–64.6%), 69.0% (95% CI: 63.6%–74.0%), and 74.6% (95% CI: 63.4%–83.2%), while in

TABLE 4 | Factors influencing white striping occurrence in broiler chickens extracted by forward selection in the multivariate logistic regression analysis.

Variable	Estimate	SE	Odds Ratio	95% CI		*p value*
				Lower	Upper	
Intercept	0.55	0.12	-	-	-	<0.001
Genotype						
A (Ref)	-	-	-	-	-	-
B	0.18	0.13	2.18	1.28	3.71	0.16
C	0.41	0.19	2.75	1.37	5.52	<0.05
Breast weight						
Intermediate: 850–960 g (Ref)	-	-	-	-	-	-
Low: <850 g	−0.50	0.10	0.57	0.41	0.80	<0.001
High: >960 g	0.45	0.11	1.49	1.03	2.15	<0.001
Breast yield						
Intermediate: 39.0–40.7% (Ref)	-	-	-	-	-	-
Low: <39.0% g	−0.21	0.10	0.82	0.58	1.15	<0.05
High: >40.7%	0.22	0.10	1.27	0.89	1.81	<0.05

SE, standard error; CI, confidence interval; Ref, reference. Genotype A, Ross 708; Genotype B, Ross 308; Genotype C, Cobb 500.

chickens with high yields the probabilities were 60.5% (95% CI: 47.0%–72.6%), 76.4% (95% CI: 70.8%–81.2%), and 81.0% (95% CI: 72.3%–87.4%).

Regarding the risk factors for WB occurrence, since the results of the univariate analysis showed that females had lower odds of having WB (OR: 0.55; 95% CI: 0.40–0.75) (**Supplementary Table S3**), the multivariate logistic regression analysis used only data from males (**Table 5**). In these latter chickens, WB occurrence was significantly influenced by genotype and daily weight gain. Compared to genotype A, genotype B showed higher odds (OR: 1.85; 95% CI: 0.61–5.58) and genotype C lower odds of having WB (OR: 0.35; 95% CI: 0.10–1.26). Males with a high daily weight gain (>78 g/d) showed higher odds of having WB (OR: 1.75; 95% CI: 1.12–2.73) compared with males with an intermediate daily weight gain (70.7–78.0 g/d), whereas no significant differences were found between males with intermediate and low daily weight gain (**Table 5**).

The representation per genotype within classes of daily weight gain (low, intermediate, and high) showed that the predicted probabilities of WB occurrence in male chickens with low daily weight gain were 12.5% (95% CI: 4.76%–28.9%), 20.8% (95% CI: 15.4%–27.5%) and 4.76% (95% CI: 2.32%–9.51%) for genotype A, B, and C respectively (**Figure 1C**). In males with intermediate daily weight gain, the probabilities were 12.5% (95% CI: 4.87%–28.9%), 21.7% (95% CI: 16.4%–28.2%), and 5.01% (95% CI: 2.50%–9.77%) for the same genotypes A, B, and C, while in chickens with a high daily weight gain the probabilities were 18.7% (95% CI: 8.68%–43.8%), 32.7% (95% CI: 26.7%–39.4%), and 8.46% (95% CI: 4.31%–15.93%), respectively.

Regarding the risk factors for SM occurrence, since the results of the univariate analysis showed that females had significantly higher odds of having SM (OR: 15.4; 95% CI: 8.55–27.8) (**Supplementary Table S3**), the multivariate logistic regression analysis used only data from females (**Table 6**). In these latter, SM occurrence was significantly influenced only by slaughter weight. In fact, compared with females with an intermediate slaughter weight (2,720 g to 2,935 g), chickens with a high slaughter weight (>2,935 g) showed higher odds of having SM (OR: 2.10; 95% CI: 1.18–3.80), whereas those with a low slaughter weight (<2,720 g) showed lower odds (OR: 0.87; 95% CI: 0.53–1.42).

The results of the risk factor analysis within each genotype are reported in **Supplementary Tables S7–S9**. In male and female broiler chickens, WS occurrence was significantly influenced by breast weight in genotype B, and by breast weight and breast rate in genotype C (**Supplementary Table S7**). In males, WB was affected by breast rate in genotype B (**Supplementary Table S8**). In females, SM occurrence was influenced by slaughter weight both in genotype A and B (**Supplementary Table S9**).

3.3 Meat Quality

We firstly evaluated the effect of the presence of any myopathy (SM, WB, and WS alone or in combination) and the effect of sex on meat rheological traits (**Table 7**). Final pH, shear force, and thawing and cooking losses measured on *p. major* were significantly different in normal meat compared to defective meat, while colour (i.e. lightness, yellowness and redness indices) was not affected. In details, compared to normal meat, the pH of defective meat was significantly higher ($p < 0.01$), as it was for shear force ($p < 0.001$) and cooking losses ($p < 0.001$). Moreover, most traits were significantly different in females compared to males, except for the redness index and thawing losses. In details, breasts of males displayed higher pH, cooking losses, and lightness, while the yellowness index was higher in females (**Table 7**). No significant interaction between the presence of myopathy and the sex of chickens was measured.

To get deeper insight in the effect of the single myopathies on meat quality, data from female and male chickens affected by one myopathy per time were analysed separately because of the large differences in the occurrence of WB and SM in the two sexes, as discussed above. In females, cooking losses significantly increased from normal to WS to WB breasts, while these losses were similar between normal and SM breasts and between WS and SM breasts (**Figure 2**; $p < 0.001$). A similar trend was observed for shear force which significantly increased from normal to WS and WB breasts (2.88–3.18 and 3.58 kg/g; $p < 0.001$), whereas no difference was recorded between normal and SM breasts and between WS and SM breast ($p < 0.001$) (**Figure 2**). When data of males affected by a single myopathy were compared (**Figure 3**), cooking losses significantly increased from normal breasts to WS and, especially,

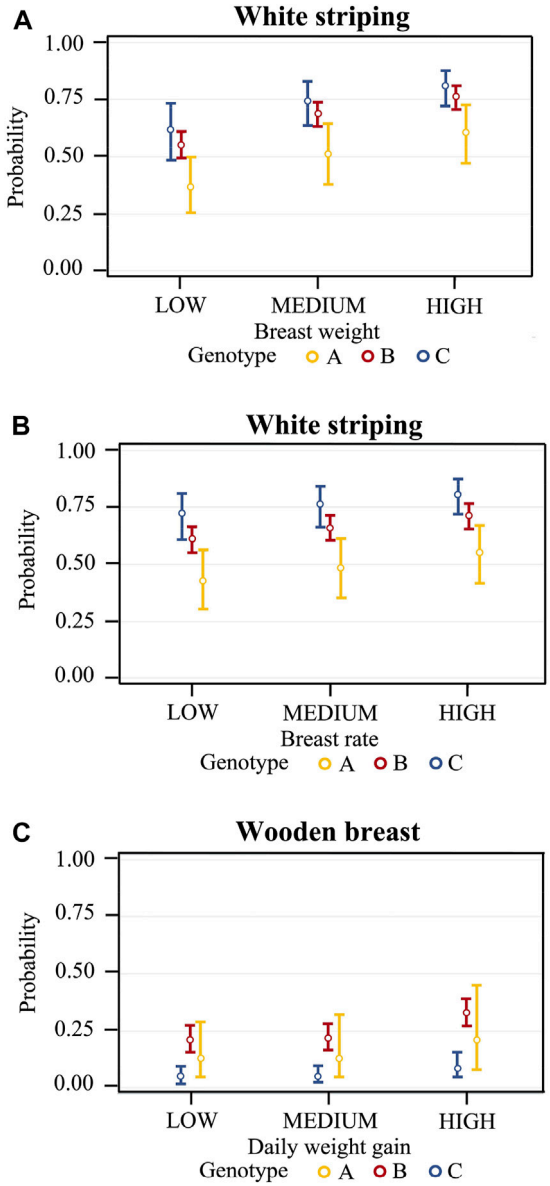

FIGURE 1 | Predicted probabilities of WS occurrence for breast weight class (LOW <850 g, MEDIUM 850–960 g, HIGH >960 g) in male and female broiler chickens **(A)**; predicted probabilities of WS occurrence for breast yield class LOW <39.0%, MEDIUM 39.0–40.7%, HIGH >40.7%) **(B)**; predicted probabilities of WB occurrence for daily weight gain class LOW <70.7 g/d, MEDIUM 70.7–78.0 g/d, HIGH >78.0 g/d) **(C)**. Predicted probabilities are given separately for the different genotypes (genotype A: Ross 708; genotype B: Ross 308; genotype C: Cobb 500) used in the experimental trials at the University of Padova.

WB ones (24.6% to 25.8% to 30.8%; $p < 0.001$) which corresponded to a higher shear force measured on WB compared to normal and WS breasts (5.34 kg/g vs. 3.03 and 3.29 kg/g; $p < 0.001$). Some trends approaching statistical significance were recorded for pH–which tended to be lower in normal meat compared to defective meat (5.90 vs 5.93–5.94 in normal vs WS and WB meat; $p = 0.10$)—and for redness index–which tended to be higher in normal and WS meat compared to WB meat (1.15 and 1.25 vs 0.93; $p = 0.11$).

Finally, when data of all animals were used (both females and males), categorizing breasts according to the presence of a single myopathy or concurrent myopathies (two or three at the same time) to confirm which was the most relevant condition affecting meat quality (**Figure 4**), significant differences were recorded for final pH, cooking losses, and shear force. As for pH, a lower value was recorded in normal breasts compared to those exhibiting WB alone or in combination with WS (5.90 vs 5.94; $p < 0.05$), while other defective meat showed intermediate values. As for cooking losses, lower values were recorded in normal and WS breasts (23.6% and 25.0%) compared to breasts exhibiting only WB (29.4%) or WB combined with WS (26.5%) and WS combined with SM (27.7%), or breasts exhibiting the three myopathies at the same time (29.5%), while intermediate losses were recorded in breasts affected by SM alone ($p < 0.001$). Finally, shear force was lower in normal meat compared to combined WS and WB breast (2.92 kg/g vs 3.41 kg/g) with the highest values in WB meat (4.30 kg/g) compared to all other types of meat ($p < 0.001$).

4 DISCUSSION

This study reviewed the rate of occurrence of different myopathies and was intended to clarify the role of some traits that can affect their occurrence based on data collected per single chicken under controlled conditions, besides to identify recurrent variations of meat quality according to the different myopathies.

Overall, the present study found the highest occurrence for WS myopathy (on average 64.7%) compared to WB and SM, which is consistent with literature data. Nevertheless, large differences among findings exist based on the ontogenetic and environmental factors tested in the different conditions. Kuttappan et al. (2017a) reported a high incidence of mild WS in 6-week-old broilers (78.4%), while the incidence of severe or very severe WS was higher at 9 weeks compared to 6 weeks (69% vs 14%), stipulating the adverse effect of older age and higher weight on the occurrence of severe myopathies. In an earlier study, Kuttappan et al. (2013) reported an incidence of 55.8% of breasts showing moderate and severe WS when birds were slaughtered between 59 and 63 days of age, whereas Petracci et al. (2013) observed only 12.0% of WS occurrence in birds with lower live weight, slaughtered earlier (45–54 days). Reports by Lorenzi et al. (2014) described a prevalence of 43.1% of WS fillets which reached 60.3% in broilers with higher slaughter weight. More recent reports (Petracci et al., 2019; Che et al., 2022b) showed high WS occurrence (>90%) which could be partly attributed to increased awareness of the issue and invested efforts to monitor and better understand the etiology and mechanisms leading to myopathies occurrence (Soglia et al., 2021).

Based on the results of the risk factor analysis, breast weight and breast yield significantly influenced WS occurrence whereas the multivariate model did not find a significant effect of the daily growth rate or the slaughter weight on the occurrence of this myopathy. Overall, our

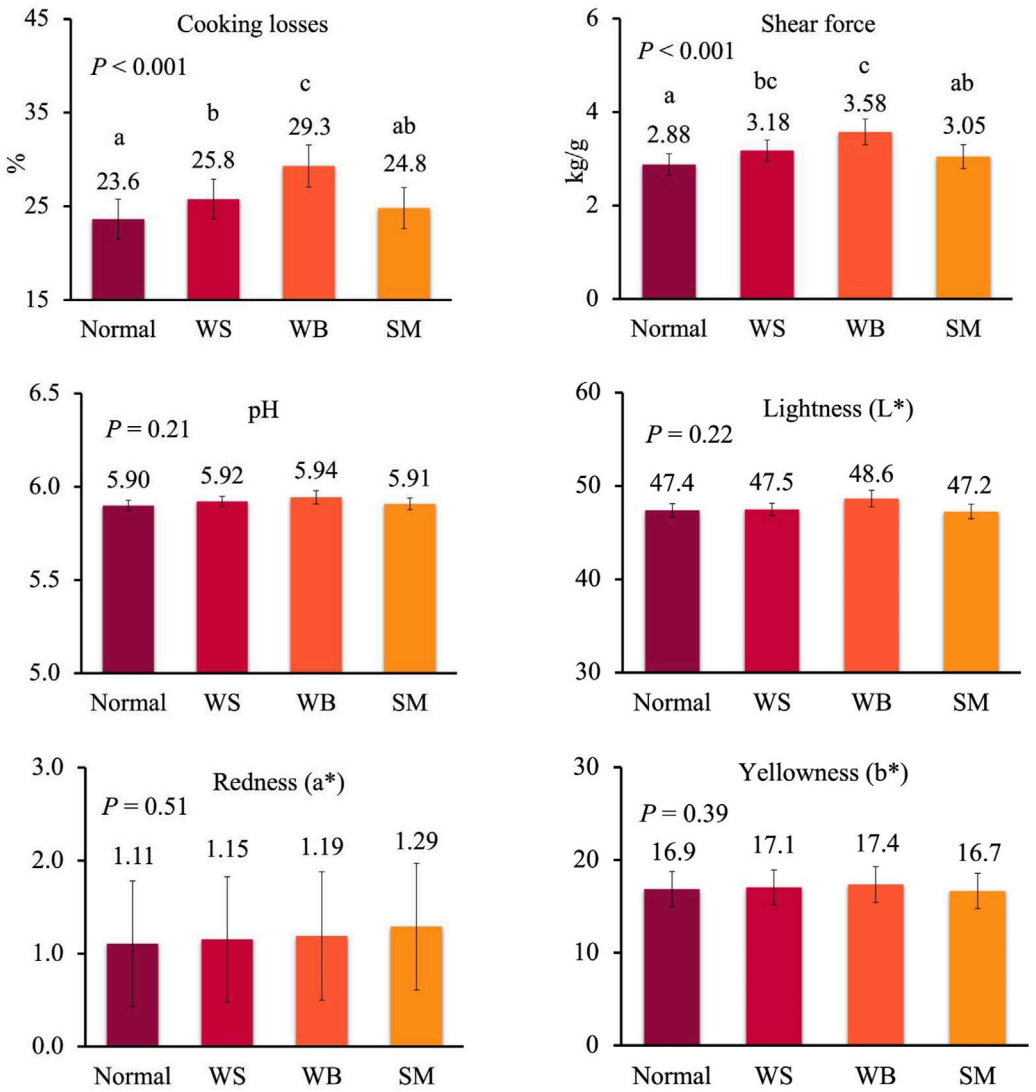

FIGURE 2 | Meat quality in normal breasts and in breasts affected by wooden breast (WB), white striping (WS) and spaghetti meat (SM) of female broiler chickens (samples with only one myopathy). Data are presented as LS means ± standard error. Different letters above bars indicate significant differences between LS means. [Number of chickens per group available in **Supplementary Table S2**].

results corroborate previous studies (Alnahhas et al., 2016; Aguirre et al., 2020) reporting the association between WS and fillet weight. Kuttappan et al. (2012a) already observed an association of increased fillet weight and yield with the WS severity degree. Based on the examination of 2,600 fillets from both sexes divided into different fillet-weight groups, Bowker et al. (2019) reported that WS scores slightly increased as fillet weight increased above 450 g. Our multivariate logistic regression model using data of both sexes also showed a significant effect of breast weight and yield on WS. In fact, in both sexes, the degree of the breast development as such and respect to the whole body is likely the challenging factor for the occurrence of white striping, whereas breast yields are rather similar in the two sexes as it was found in previous studies (Hussein et al., 2019; Santos et al., 2021).

As for the other myopathies, relating flock data of growth to individual data of myopathy, Che et al. (2022b) recently found that increased live weight is a risk factor for both WB and SM occurrence. Stated the high correlation between slaughter weight and daily weight gain in our data set (R^2: 0.86; data not reported in tables), this result is consistent with our observations. In fact, males with high daily weight gain had significantly higher odds of WB occurrence compared to those with low and intermediate daily weight gain. We also found that females with high slaughter weight had significantly higher odds of SM occurrence.

Our analyses clearly identified sex as a risk factor for WB or SM. On average, WB occurrence was 25.2% in males and 12.1% in females, while an opposite behaviour was observed for SM averaging at 36.7% in females and 3.48% in males. Consistently, Santos et al. (2021) found a higher WB occurrence in males than females (41.9%

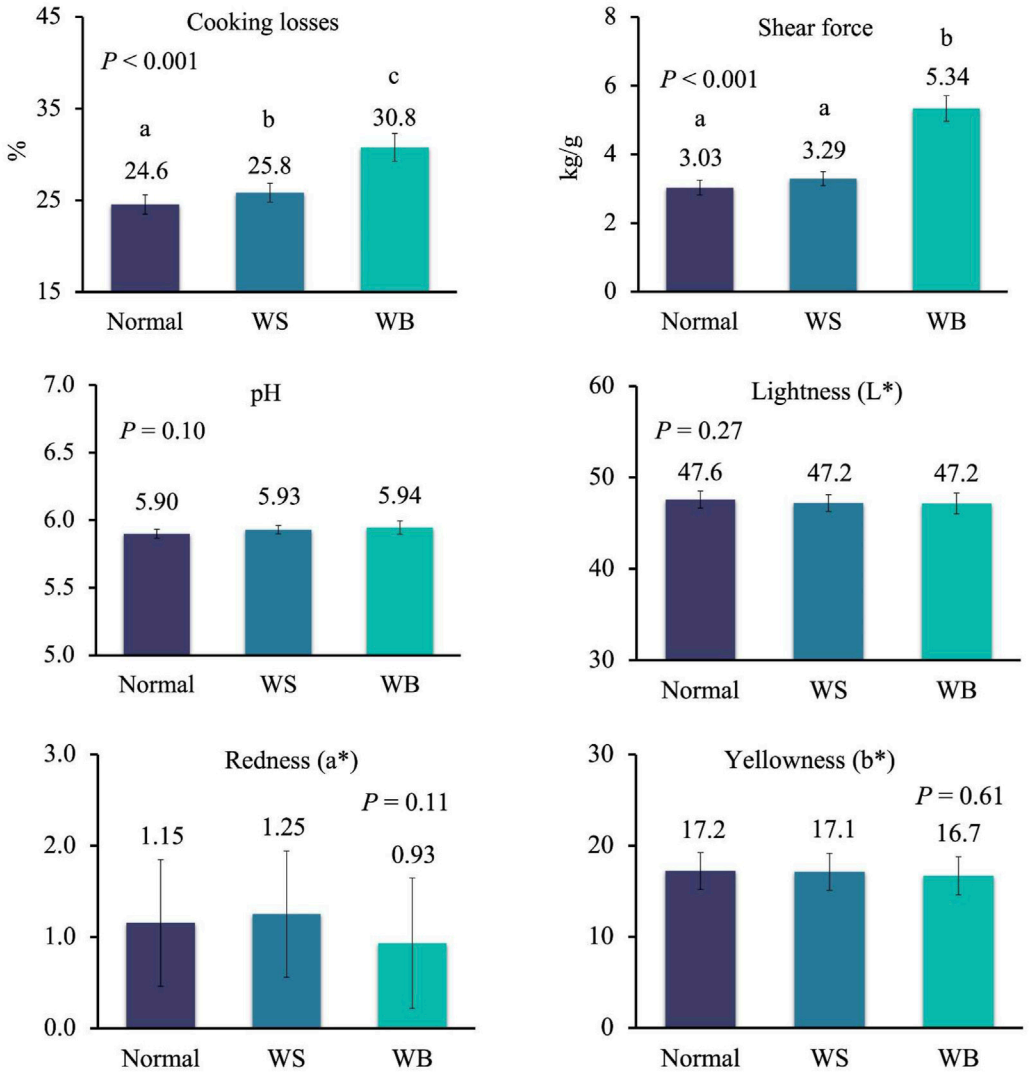

FIGURE 3 | Meat quality in normal breasts and in breasts affected by white striping (WS) and wooden breast (WB) of male broiler chickens (samples with only one myopathy). Data are presented as LS means ± standard error. Different letters above bars indicate significant differences between LS means [Number of chickens per group available in **Supplementary Table S2**].

vs 26.8%); Caldas-Cueva et al. (2021) found an approximately three times higher occurrence of moderate to severe WB myopathy in males compared to females; and Lake et al. (2021) reported a higher prevalence of WB (87% vs 71%) and WS (87% vs 73%) in males than females. As for SM, the only available data by Che et al. (2022b) report an average occurrence of 36.3% on a sample size of 9,250 chickens (average live weight at slaughter: 2.36 kg) collected in two slaughterhouses in Ontario (Canada) from flocks comprising only males, only females, and both sexes, whereas they did not identify sex as a risk factor.

Thus, based on the above results and available literature, we could argue that WS is likely associated to the general suffering of muscle fibers in the heaviest breasts in which muscle hypertrophy compromises the normal fiber metabolism regardless from sex. On the other hand, daily growth rate and slaughter weight have been identified as risk factors for WB and SM with higher odds of showing the myopathies in chickens with the highest growth performance. Possibly the different susceptibility to WB and SM of males and females depends on their different growth and metabolism (Kuttappan et al., 2013; Lee et al., 2017; Cui et al., 2021). Indeed, growth differences between sexes are the result of different hormones that influence metabolic processes (Varlamov et al., 2015), besides the impact of gut microbiota (Lee et al., 2017; Cui et al., 2021), that in turn can be predisposing factors for one or another myopathy. Cui et al. (2021) reported that the proteins involved in the process of transporting glucose and affecting the utilization rate of glycogen were higher in males than in female broilers, which would consequently impact body weight. Differences observed at the breast level suggested that increased expression of fat metabolism, oxidative stress response, antiangiogenesis, and connective tissue proliferation genes make male broilers more susceptible to WB compared to

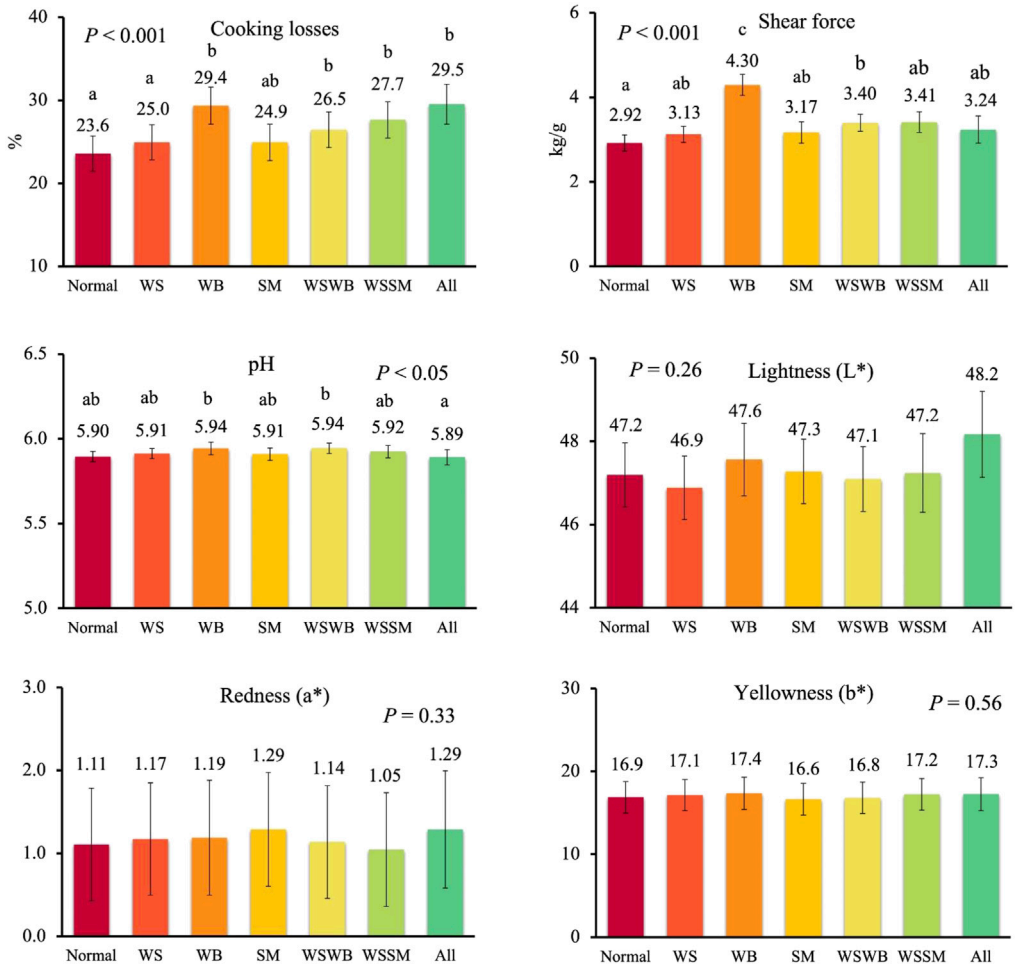

FIGURE 4 | Meat quality in normal breasts and in breasts affected by exclusively white striping (WS) or wooden breast (WB) or spaghetti meat (SM), or contemporarily all myopathies (All), or WS and SM, or WS and WB. Data are presented as LS means ± standard error. Different letters above bars indicate significant differences between LS means [Number of chickens per group available in **Supplementary Table S2**].

TABLE 5 | Factors influencing wooden breast occurrence in male broiler chickens and extracted by forward selection in the multivariate logistic regression analysis.

Variable	Estimate	SE	Odds Ratio	95% CI		*p value*
				Lower	Upper	
Intercept	–1.87	0.22	-	-	-	<0.001
Genotype						
A (Ref)	-	-	-	-	-	-
B	0.76	0.23	1.85	0.61	5.58	<0.001
C	–0.90	0.30	0.35	0.10	1.26	<0.01
Daily weight gain						
Intermediate: 70.7–78.0 g/d (Ref)	-	-	-	-	-	-
Low: <70.7 g/d	–0.22	0.15	0.95	0.58	1.56	0.12
High: >78.0 g/d	0.39	0.13	1.75	1.12	2.73	<0.01

SE, standard error; CI, confidence interval; Ref, reference. Genotype A, Ross 708; Genotype B, Ross 308; Genotype C, Cobb 500.

females (Brothers et al., 2019). Glycan biosynthesis and metabolism, lipid metabolism, and carbohydrate metabolism have been profiled in male broiler chickens as discriminating body weight groups (Lee et al., 2017). Finally, profiling of predictive metagenome functions from bacterial communities revealed that fructose, mannose, and galactose metabolisms

TABLE 6 | Factors influencing spaghetti meat occurrence in female broiler chickens and extracted by forward selection in the multivariate logistic regression analysis.

Variable	Estimate	SE	Odds Ratio	95% CI		*p* value
				Lower 95%	Upper 95%	
Intercept	–0.30	0.12	-	-	-	<0.05
Slaughter weight						
Intermediate: 2,720–2,935 g (Ref)	-	-	-	-	-	-
Low: <2,720 g	–0.34	0.16	0.87	0.53	1.42	<0.05
High: >2,935 g	0.54	0.18	2.10	1.18	3.80	<0.01

SE, standard error; CI, confidence interval; Ref, reference.

TABLE 7 | Effect of myopathy presence, sex and their interactions on breast quality traits.

	Myopathy (M)		Sex (S)		*p* value			RMSE
	Absent	Present	Male	Female	M	S	M×S	
Chickens, n	354	924	729	549	-	-	-	-
pH	5.90	5.92	5.93	5.89	<0.01	<0.001	0.50	0.12
Lightness (L*)	47.2	47.3	47.4	47.0	0.36	<0.05	0.15	2.33
Redness (a*)	1.38	1.45	1.32	1.45	0.15	0.45	0.94	0.64
Yellowness (b*)	17.9	18.0	17.8	18.1	0.94	<0.05	0.53	1.80
Thawing losses, %	9.32	9.70	9.49	9.70	0.11	0.87	0.90	2.58
Cooking losses, %	25.0	26.6	26.8	24.8	<0.001	<0.001	0.12	4.17
Total losses, %	33.9	36.1	35.9	34.1	<0.001	<0.001	0.56	4.77
Shear force, kg/g	2.92	3.26	3.18	3.00	<0.001	<0.05	0.94	0.90

(carbohydrate metabolism) and arachidonic acid metabolism (lipid metabolism) may be related to male broiler chickens, whereas progesterone-mediated oocyte (endocrine system), methane metabolism (energy metabolism), peptidoglycan biosynthesis (glycan biosynthesis and metabolism), glycerophospholipid metabolism, and lipid biosynthesis proteins (lipid metabolism) may be related to female broiler chickens (Lee et al., 2017). Nevertheless, relationships between metabolic patterns in the two sexes and myopathies susceptibility require further investigations especially for SM.

Conversely, as for relationships between meat quality and metabolic changes in muscle affected by myopathies, more information is already available even if differences in meat quality between normal and defective meat are not always consistent across studies. Based on our dataset, the myopathy that mostly affected meat quality was WB, with major changes for technological and sensorial traits, such as pH, cooking losses and shear force.

As for pH, some previous findings reported higher pH also in meat with WS (Mudalal et al., 2015), besides WB (Bowker and Zhuang, 2016; Baldi et al., 2020). Indeed, the severity of the myopathy can influence meat pH: greater values were previously found only in breasts affected with severe WS (Petracci et al., 2013) or severe WB (Campo et al., 2020). Moreover, consistently with our results, some studies showed a greater increase in pH value when WS and WB were simultaneously present (Tasoniero et al., 2016; Zambonelli et al., 2016; Kuttappan et al., 2017a). Finally, the pH of meat affected by SM has been found to be either higher (Baldi et al., 2018) or similar (Campo et al., 2020; Pascual et al., 2020b) to normal meat.

The greater ultimate pH of breasts affected by myopathies has been attributed to a reduced glycolytic potential (Berri et al., 2001; Berri et al., 2007) due to reduced carbohydrate metabolism (Kuttappan et al., 2017a, b). Proteomic analysis, comparing normal breasts and those with severe myopathy, revealed that in affected breast muscles there was a down-regulation of carbohydrate metabolic pathways related to reduced glycolysis, gluconeogenesis, tricarboxylic acid cycle, glycogen degradation, and pyruvate fermentation to lactate (Kuttappan et al., 2017b). Indeed, in heavier breast muscles, a lower storage of glycogen has been reported which has been related to their higher final pH (Le Bihan-Duval et al., 2008). In addition, the greater pH of WB samples have been attributed to lower buffering capacity coupled with reduced glycolytic potential resulting in decreased H+ accumulation (Baldi et al., 2020).

As for cooking losses, the compromised structural integrity of the muscle tissues compared to normal meat likely accounted for the higher values found in WB meat in our analyses. In particular, fiber degeneration and decreased myofibrillar proteins (Mudalal et al., 2015; Soglia et al., 2016; Tijare et al., 2016) can lead to greater mobility of water within the meat structure. In details, a great increase in proportion and mobility of extra-myofibrillar water fraction and a greater mobility of intra-myofibrillar water has been measured in WB and WS/WB meat compared to normal meat (Soglia et al., 2016). The relative abundance of the extra-myofibrillar water and the mobility of intra-myofibrillar water affect drip losses of WB meat, while the mobility of intra-myofibrillar water is crucial for cooking losses (Pang et al., 2020). Indeed, compared to normal meat, higher dripping and cooking losses have been reported also in SM meat (Soglia et al., 2016; Baldi et al., 2018; Pascual et al., 2021).

As for texture and tenderness, among the most important indicators of meat quality (Warner et al., 2022), results about changes due to myopathies are often inconsistent among studies, also as a consequence of differences in meat status (fresh vs frozen), cooking process (raw vs cooked), severity degree of myopathies,

sampled cuts, textural methods and, within the same method, measurement conditions (Soglia et al., 2017; Baldi et al., 2019; Pascual et al., 2021). For example, at low compression rates, the resistance is mainly due to the myofiber since the connective tissue has the property of expanding due to its elasticity without interfering with the muscle resistance, whereas at higher compression rates the connective tissue is an important part in the resistance of the muscle (Campo et al., 2000; Campo et al., 2020). Several authors (Mudalal et al., 2015; Dalgaard et al., 2018; Maxwell et al., 2018; Xing et al., 2020) did not find differences in texture between normal, WB, and WS + WB fillets. Other authors reported that differences between normal and WB meat found in raw samples disappeared after applying a thermal process (Soglia et al., 2017; Campo et al., 2020). On the other hand, in agreement with previous results (Tasoniero et al., 2016), across our dataset the highest force was required to cut cooked WB meat which has been attributed to the higher collagen content of WB fillets (Baldi et al., 2019).

Differently, regarding WS, since fat deposition tends to be higher in this meat (Velleman et al., 2010; Mudalal et al., 2015; Radaelli et al., 2017; Soglia et al., 2017), a comparable tenderness between WS and normal meat could be expected also as a results of the disruption of connective tissue cross-linkages in WS muscle fibers (Nishimura, 2010). On the other hand, in SM meat, reduced tenderness is attributed to the reduced collagen cross-linking degree of defective breasts (Baldi et al., 2019, 2021). In fact, Baldi et al. (2019) did not find differences in the texture between raw SM and normal samples, while Pascual et al. (2021) found that only the Meullenet–Owens razor blade test could distinguish between cooked normal and SM fillets.

Consistently with the present results, other studies reported that myopathies did not influence colour indices of breast meat (Zambonelli et al., 2016; Brambila et al., 2017). Nevertheless, within a single trial, Pascual et al. (2020b) found that SM and WB fillets had significantly higher lightness values than normal ones, while Petracci et al. (2013) and Tasoniero et al. (2016) did not find a difference in lightness between normal and defective meat. On the other hand, a higher yellowness (Mudalal et al., 2015) was found in defective compared to normal meat. Indeed, Campo et al. (2020) highlighted the effect of the severity degree where severe WB decreased lightness and increased redness and yellowness. The presence of WS resulted in redder meat, while more severe WS breasts showed higher lightness and yellowness; a moderate SM only increased yellowness (Pascual et al., 2021). Kuttappan et al. (2017a) also reported increased yellowness of fillets affected by severe WS, WB, and WS/WB compared with normal meat. Petracci et al. (2017) associated changes in colour of defective meat with increased fibrotic responses and reduced haem pigment levels.

Nevertheless, the impact on consumers' acceptance has been found to be relevant for all myopathies. In fact, raw WS meat resulted in lower acceptability and purchase intent compare to unaffected meat (de Carvalho et al., 2020) and consumer acceptance decreased along with the severity of WS (Kuttappan et al., 2012b). As for WB, neither traditional nor clean label marinades mask the undesirable eating characteristics of this defective meat and changes will be noticeable to consumers (Jarvis et al., 2020). Data about consumers' perception of SM meat have not been published, but the consumers' reaction is obvious and easily predictable.

Finally, as for the most relevant effects of sex on meat quality across all trials, the higher meat pH of males compared to females resulted in a higher water retention that is in turn responsible for a higher reflection of light and, thus, for the higher lightness index we measured in males (Campo et al., 2020). The higher pH of males could be related to their higher breast weights. In fact, a negative correlation between breast muscle weight and glycogen stored in the muscle, i.e., a positive correlation of breast weight with the final pH, has been reported (Le Bihan-Duval et al., 2008). While we measured a higher shear force for cutting meat of male chickens, other authors found a lower value than female chickens which was attributed to the smaller fiber diameter in males (Cygan-Szczegielniak and Bogucka, 2021).

In conclusion, among the growth and slaughter traits, our analysis identified breast development, measured as breast weight and breast yield at an individual level, as a potential risk factor for the prevailing myopathy, i.e., white striping. Conversely, a high growth, measured as daily weight gain and slaughter weight, was the risk factor for wooden breast and spaghetti meat. Along with the information available in literature, these results can pave the way for the evaluation of the etiopathogenic mechanisms that can trigger the onset of white striping on one side and wooden breasts or spaghetti meat on the other one. Additionally, we also found a different probability of having wooden breasts or spaghetti meat in females compared to males, which still deserves specific investigations. Finally, based on the results of the present analyses, only WB meat was clearly different for some technological and sensorial properties, which can play a role also in meat processing.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The data were obtained in eight trials approved by the Ethical Committee for Animal Experimentation (Organismo Preposto al Benessere Animale) of the University of Padova, Italy.

AUTHORS CONTRIBUTIONS

AT and GX contributed to the conception and design of the study and organized the database. FB performed the statistical analysis. FB, MBC, and AT wrote the first draft of the manuscript. FB, GX, MC, MBC, and AT contributed to manuscript revision, read, and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2022.933235/full#supplementary-material

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EDITED BY
Vincent M. Cassone,
University of Kentucky, United States

REVIEWED BY
Motoi Kikusato,
Tohoku University, Japan
Marcos Rostagno,
Phytobiotics North America,
United States

*CORRESPONDENCE
Federico Sirri,
federico.sirri@unibo.it

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A review of heat stress in chickens. Part I: Insights into physiology and gut health

Giorgio Brugaletta[1], Jean-Rémi Teyssier[2], Samuel J. Rochell[2], Sami Dridi[2] and Federico Sirri[1]*

[1]Department of Agricultural and Food Sciences, Alma Mater Studiorum—University of Bologna, Bologna, Italy, [2]Center of Excellence for Poultry Science, University of Arkansas, Fayetteville, AR, United States

Heat stress (HS) compromises the yield and quality of poultry products and endangers the sustainability of the poultry industry. Despite being homeothermic, chickens, especially fast-growing broiler lines, are particularly sensitive to HS due to the phylogenetic absence of sweat glands, along with the artificial selection-caused increase in metabolic rates and limited development of cardiovascular and respiratory systems. Clinical signs and consequences of HS are multifaceted and include alterations in behavior (e.g., lethargy, decreased feed intake, and panting), metabolism (e.g., catabolic state, fat accumulation, and reduced skeletal muscle accretion), general homeostasis (e.g., alkalosis, hormonal imbalance, immunodeficiency, inflammation, and oxidative stress), and gastrointestinal tract function (e.g., digestive and absorptive disorders, enteritis, paracellular barrier failure, and dysbiosis). Poultry scientists and companies have made great efforts to develop effective solutions to counteract the detrimental effects of HS on health and performance of chickens. Feeding and nutrition have been shown to play a key role in combating HS in chicken husbandry. Nutritional strategies that enhance protein and energy utilization as well as dietary interventions intended to restore intestinal eubiosis are of increasing interest because of the marked effects of HS on feed intake, nutrient metabolism, and gut health. Hence, the present review series, divided into Part I and Part II, seeks to synthesize information on the effects of HS on physiology, gut health, and performance of chickens, with emphasis on potential solutions adopted in broiler chicken nutrition to alleviate these effects. Part I provides introductory knowledge on HS physiology to make good use of the nutritional themes covered by Part II.

KEYWORDS

chicken, heat stress, physiology, metabolism, gut health

Abbreviations: AA, amino acids; AMP/HDP, antimicrobial/host defense peptide; BCO, bacterial chondronecrosis with osteomyelitis; CE, competitive exclusion; FASN, fatty acid synthase; GI(T), gastrointestinal (tract); HS, heat stress; IFN-γ interferon gamma; IGF-1, insulin-like growth factor 1; IL-1β, interleukin 1 beta; LPS, lipopolysaccharides; MAFbx, muscle atrophy F-box; MLCK, myosin light-chain kinase; NEFA, non-esterified fatty acids; PAMP, pathogen-associated molecular pattern; PI3K, phosphatidylinositol 3-kinase; RNS, reactive nitrogen species; ROS, reactive oxygen species; S6K, p70S6 kinase; IgA, immunoglobulin A; T_3, triiodothyronine; T_4, thyroxine; TEER, transepithelial electrical resistance; TJ, tight junction; TLR, Toll-like receptor; TNF-α, tumor necrosis factor alpha; ZO, zonula occludens.

Introduction

Heat stress (HS) affects performance, health, and welfare of commercially-reared birds (Renaudeau et al., 2012; Rostagno, 2020) and alters their meat (Song and King, 2015; Wang et al., 2017; Zaboli et al., 2019) and egg quality (Mack et al., 2013; Barrett et al., 2019), thereby endangering the sustainability of the poultry industry. This environmental stressor has commonly impacted poultry flocks raised in tropical and subtropical regions of the world. However, as a result of global warming, high environmental temperatures have become a large-scale issue that severely threatens poultry producers located in temperate areas as well (Renaudeau et al., 2012). Extreme heatwaves have already caused devastating events for the poultry industry, such as the sudden death of more than 700,000 birds in California (Nienaber and Hahn, 2007). St-Pierre et al. (2003) reported that the financial burden of HS amounts to $128–165 million per year for the US poultry industry alone. Although this figure still represents a general reference, recent estimates cannot be easily found in the literature.

In addition to causing evident changes in chicken behavior (Wang W. C. et al., 2018), HS negatively acts upon metabolism and general homeostasis (Rhoads et al., 2013) and impairs the functionality of the digestive system (Rostagno, 2020). Interestingly, several physiological and pathophysiological responses to HS are evolutionary conserved across different animal species (Lambert et al., 2002; Pearce et al., 2012; Koch et al., 2019; Kaufman et al., 2021), including humans (Snipe et al., 2018). For instance, the reduction in feed intake is one of the most common HS reactions because it is an effective way to limit the generation of metabolic heat due to digestion, absorption, and nutrient metabolism (Baumgard and Rhoads, 2013).

Given its tremendous relevance to the whole sector, poultry scientists and companies have been committed to developing reliable tools against HS. Engineering solutions and equipment intended to optimize the environmental control of poultry houses, along with management measures and genetic selection, can undoubtedly aid poultry producers in counteracting hot conditions (Lin et al., 2006b; Naga Raja Kumari and Narendra Nath, 2018; Saeed et al., 2019; Liang et al., 2020; Wasti et al., 2020; Goel, 2021; Nawaz et al., 2021; Vandana et al., 2021). Feeding strategies and dietary interventions can also help relieve HS effects on poultry (Gous and Morris, 2005; Lin et al., 2006b; Wasti et al., 2020; Vandana et al., 2021). For instance, increasing energy and nutrient density of the diet can counterbalance the decreased feed consumption of birds exposed to HS (Wasti et al., 2020). Moreover, researchers dealing with HS have been testing feed supplements aimed at promoting gastrointestinal (GI) health (Lian et al., 2020), which is a mainstay for the modern animal science and livestock industry (Kogut and Arsenault, 2016). Gut health is multifaceted and simultaneously influenced by composition and properties of the diet, digestion and absorption processes, integrity of the GI epithelium, plasticity and resilience of the GI immune system, and dynamics of the GI microbiota (Brugaletta et al., 2020). Pioneering studies conducted in the 1980s revealed that the GI microbiota is a main target of HS and that dietary supplementation of probiotic blends can attenuate HS in chickens (Suzuki et al., 1983, 1989). Probiotics have been shown to drive the formation of a desirable and protective GI microbiota (Baldwin et al., 2018), while properly reestablishing eubiosis following environmental stress, such as the exposure to elevated temperatures (Sugiharto et al., 2017). Along with probiotics, other GI microbiota modulators and potentially gut health-enhancing additives have been tested in poultry nutrition to promote eubiosis under HS conditions, such as prebiotics, synbiotics, postbiotics, phytochemicals, and amino acids, to name but a few (Lian et al., 2020; He et al., 2021).

Over the last years, HS-mediated alterations of physiology and gut health of chickens have received considerable attention. Therefore, this review series composed of Part I and Part II, was conceived to summarize relevant knowledge about these topics and examine some feeding and nutritional interventions that have been proposed to mitigate HS in broiler chickens. The present Part I discusses the effects of HS on physiology and gut health of chickens, while Part II (Teyssier et al., 2022a) provides an overview of potential solutions employed in broiler chicken nutrition to minimize the detrimental effects of HS.

Heat stress effects on physiology of chickens

Chickens are homeotherms that can keep their body temperature tightly regulated across a wide range of external temperatures. However, high environmental temperatures can overwhelm the thermoregulatory mechanisms, causing an imbalance between the amount of metabolic heat produced by chickens and their own capacity to dissipate body heat to the environment. This alteration results in an abnormal increase in body temperature (hyperthermia) and triggers HS (Renaudeau et al., 2012; Rostagno, 2020). In addition to being potentially lethal, HS has a broad-spectrum effect on behavior, physiology, gut health, welfare, and productive performance of chickens. It is worth pointing out that fast-growing and highly efficient broiler chickens (Havenstein et al., 1994, 2003b; Zuidhof et al., 2014; Tallentire et al., 2018), the outcome of about 70 years of genetic progress, are even less thermotolerant and more susceptible to HS than slow-growing lines due to extremely high metabolic rates and poorly developed cardiovascular and respiratory systems (Cahaner and Leenstra, 1992; Yunis and Cahaner, 1999; Havenstein et al., 2003a; Gous and Morris, 2005; Lu et al., 2007; Yahav, 2009; Xu et al., 2018). In this regard, Gogoi et al. (2021) recently proved that the physiological response to HS is more severe and heat tolerance is lower in heavy broilers than in lighter birds of the same line and age.

Heat stress and behavior

"Cooling behaviors" (Wang W. C. et al., 2018) are the most prominent HS-caused modifications in chicken behavior. As their name suggests, these abnormal behaviors are intended to cool the body down to restore normothermia. Chickens lack sweat glands, which would facilitate latent heat loss by evaporation of the perspiration, and have relatively limited unfeathered body surface areas to provide an effective loss of sensible heat through conduction, radiation, and convection (Nichelmann et al., 1986; Yunis and Cahaner, 1999; Renaudeau et al., 2012; Rostagno, 2020). As the ambient temperature rises, the thermal gradient between the body surface and the surrounding environment lessens while the dissipation of sensible heat becomes decreasingly effective. Therefore, chickens suffering from environment-induced hyperthermia increase their respiratory rate (thermal tachypnea/polypnea or panting) to maximize the loss of latent heat *via* evaporation of water from the respiratory tract (Jukes, 1971; Teeter et al., 1985). While sensible heat loss is restricted by the body-to-environment thermal gradient, relative humidity imposes a ceiling on water evaporation and, therefore, on latent heat dissipation (Renaudeau et al., 2012). Thus, elevated ambient temperature associated with high relative humidity considerably limit heat removal from the body and magnify the injurious effects of HS on chickens (Rajaei-Sharifabadi et al., 2017; Goel, 2021). Under persistent HS conditions, thermal polypnea turns into a slower and deeper panting phase, also called thermal hyperpnea (Hales, 1973; IUPS Thermal Commission, 1987; Renaudeau et al., 2012). Even though panting improves evaporative cooling through latent heat dissipation, it has some drawbacks for chickens (Marder and Arad, 1989). Dehydration, the most intuitive panting-related disadvantage, usually results in higher water requirement and consumption (Wang W. C. et al., 2018). Panting also increases CO_2 exhalation leading to hypocapnia and, eventually, to respiratory alkalosis, a disorder of the acid-base balance (Richards, 1970; Marder and Arad, 1989; Renaudeau et al., 2012; Beckford et al., 2020; Wasti et al., 2020). Alkalosis poses a risk to the egg industry because it reduces blood ionized calcium and therefore negatively affects eggshell mineralization (Odom et al., 1986). However, HS-induced respiratory alkalosis is a great threat to broiler growers as well (Teeter et al., 1985; Borges et al., 2007). Chickens subjected to HS frequently lift their wings (Wang W. C. et al., 2018) to expose body areas uncovered by feathers in an attempt to enhance the sensible heat flow toward the environment. Despite being fundamental to preserving or reestablishing euthermia, panting and raising wings are energy-intensive activities (Brackenbury and Avery, 1980; Dale and Fuller, 1980) which deplete the amount of calories that would be allocated to productive purposes (Yahav et al., 2004; Baumgard and Rhoads, 2013).

Chickens kept at high temperatures become lethargic, spending more time resting (e.g., squatting close to the ground) and less time feeding and walking. This unfavorably affects feed intake (Wang W. C. et al., 2018) and skeletal health (Hester et al., 2013). Limiting feed consumption is a highly conserved survival mechanism employed by animals to reduce the thermogenesis from digestive, absorptive, and nutrient utilization processes (Baumgard and Rhoads, 2013). Reduced performance of heat-stressed chickens have traditionally been attributed to reduced feed intake (Dale and Fuller, 1980; Teeter et al., 1985). However, pair-feeding models—adopted to minimize the confounding effects of dissimilar feed consumption between birds under thermoneutral conditions and their heat-stressed counterparts—revealed that up to 40% of body weight gain loss of broilers subjected to HS are unrelated to anorexia (Dale and Fuller, 1980; Geraert et al., 1996a; Ain Baziz et al., 1996; Lu et al., 2007; Zuo et al., 2015; Lu et al., 2018; Teyssier et al., 2022b). Readers are referred to Part II of this review series for more information on the effects of HS on feed intake (Teyssier et al., 2022a). Table 1 provides an overview of heat stress effects on chicken behavior.

Heat stress and lipid metabolism

Direct effects of HS upon physiology, other than reduced feed intake, remarkably contribute to impair chicken performance (Dale and Fuller, 1980; Geraert et al., 1996a; Renaudeau et al., 2012). Heat-stressed animals paradoxically show a restricted fat mobilization notwithstanding their negative energy balance and catabolic state (Baumgard and Rhoads, 2013). Indeed, not only chickens (Bobek et al., 1997; Lu et al., 2018), but also pigs (Pearce et al., 2013a; Victoria Sanz Fernandez et al., 2015), and dairy cattle (Rhoads et al., 2009) kept in warm environments show a progressive reduction in circulating non-esterified (free) fatty acids (NEFA)—a reliable indicator of lipid metabolism—suggesting a limited use of fat energy stores. Extensive research has also revealed that heat-stressed chickens deposit more visceral (abdominal), subcutaneous, and intramuscular fat (Kleiber and Dougherty, 1934; Kubena et al., 1972; Ain Baziz et al., 1996; Yunianto et al., 1997; He et al., 2015; Lu et al., 2018, 2019). A greater lipid retention at the peripheral body sites can further hinder the dissipation of sensible heat (Renaudeau et al., 2012), increasing the risk of severe hyperthermia. The hampered fat mobilization is a metabolic adaptation likely due to hyperinsulinemia triggered by HS, at least in pigs and cattle (Baumgard and Rhoads, 2013). In contrast to mammals, however, heat-stressed chickens do not usually show a spike in blood insulin levels (Geraert et al., 1996b; Tang et al., 2013; Belhadj Slimen et al., 2016), although Lu et al. (2019) reported an increase and a decrease in circulating insulin at 7 and 14 days of HS, respectively. Moreover, avian insulin lacks a powerful antilipolytic effect, while the importance of its

TABLE 1 Overview of heat stress effects on chicken behavior.

Class	Heat stress effect	Pros	Cons	References[a]
Behavior	↑ respiratory rate (thermal polypnea or panting) → thermal hyperpnea	↑ latent heat dissipation (evaporative heat loss through the respiratory tract)	Dehydration → higher water requirement and consumption ↑ CO_2 loss → hypocapnia → respiratory alkalosis (acid-base imbalance) → ↓ blood calcium for eggshell mineralization and ↓ growth performance ↑ energy expenditure to maintain euthermia → ↓ performance	**Richards (1970)**, **Jukes (1971)**, **Brackenbury and Avery (1980)**, **Dale and Fuller (1980)**, **Teeter et al. (1985)**, **Odom et al. (1986)**, **Marder and Arad (1989)**, **Yahav et al. (2004)**, **Borges et al. (2007)**, Renaudeau et al. (2012), Rhoads et al. (2013), **Wang W. C. et al. (2018)**, **Beckford et al. (2020)**, **Wasti et al. (2020)**
	Wing lifting	↑ exposition of unfeathered body surfaces → ↑ sensible heat loss	↑ energy expenditure to maintain euthermia → ↓ performance	**Dale and Fuller (1980)**, Baumgard and Rhoads (2013), **Wang W. C. et al. (2018)**
	Lethargy → ↓ feeding and walking	↓ metabolic heat from digestion, absorption, and nutrient utilization	↓ performance ↓ bone/skeletal health	**Dale and Fuller (1980)**, **Teeter et al. (1985)**, **Geraert et al. (1996a)**, Baumgard and Rhoads (2013), **Hester et al. (2013)**, **Wang W. C. et al. (2018)**

Upward arrow (↑), increase; downward arrow (↓), decrease; rightward arrow (→), consequence/degeneration.
[a]Include studies on non-avian species that have exhibited comparable heat stress effects and responses to those observed in chickens. Studies with the focus on chickens or poultry are highlighted in bold.

TABLE 2 Overview of heat stress effects on chicken lipid metabolism.

Class	Heat stress effect	Pros	Cons	References[a]
Lipid metabolism	↓ fat mobilization and ↑ hepatic lipogenesis → ↑ fat retention and deposition	—	↑ carcass adiposity ↓ sensible heat dissipation	**Kleiber and Dougherty (1934)**, **Kubena et al. (1972)**, **Ain Baziz et al. (1996)**, **Geraert et al. (1996a)**, **Yunianto et al. (1997)**, **Bobek et al. (1997)**, Rhoads et al. (2009), Renaudeau et al. (2012), Baumgard and Rhoads (2013), Pearce et al. (2013a), Victoria Sanz Fernandez et al. (2015), **He et al. (2015)**, **Flees et al. (2017)**, **Lu et al. (2018)**, **Lu et al. (2019)**

Upward arrow (↑), increase; downward arrow (↓), decrease; rightward arrow (→), consequence/degeneration.
[a]Include studies on non-avian species that have exhibited comparable heat stress effects and responses to those observed in chickens. Studies with the focus on chickens or poultry are highlighted in bold.

signaling cascades in the adipose tissue of chickens is still unclear and a matter of debate (Dupont et al., 2012, 2015). Therefore, several questions about the role of insulin in fat metabolism of chickens undergoing HS remain unanswered at present. It is worth noting, however, that the altered lipid metabolism is not limited to a reduced utilization of fat storages because heat-challenged chickens also show an overexpression of proteins involved in the hepatic *de novo* lipogenesis, along with fat accumulation in the liver (Flees et al., 2017; Lu et al., 2019). HS effects on chicken lipid metabolism are summarized in Table 2.

Heat stress and skeletal muscle protein metabolism

In addition to an increase in fat content, HS has been demonstrated to alter the carcass composition of broiler chickens by lowering the lean tissue proportion, especially the breast yield (Howlider and Rose, 1989; Geraert et al., 1996a; Ain Baziz et al., 1996; Temim et al., 2000; Zuo et al., 2015; Lu et al., 2018; Qaid and Al-Garadi, 2021; Zampiga et al., 2021). First molecular insights suggested that HS-mediated depression in muscle protein deposition is mostly attributable to a reduced protein synthesis rather than a more pronounced protein breakdown (Temim et al., 2000). Zuo et al. (2015) showed, however, that the cause for the diminished lean mass accretion can be muscle-specific, with the breast showing a decreased protein synthesis while the thigh an augmented protein degradation. They also associated the impaired protein synthesis with a lower expression of insulin-like growth factor 1 (IGF-1), phosphatidylinositol 3-kinase (PI3K), and p70S6 kinase (S6K) and the higher protein degradation with an upregulation of muscle atrophy F-box (MAFbx or atrogin-1). Ma et al. (2021) recently confirmed the modifications in S6K and MAFbx expression caused by HS. S6K is indispensable in controlling

TABLE 3 Overview of heat stress effects on skeletal muscle protein metabolism of chickens.

Class	Heat stress effect	Pros	Cons	References[a]
Protein metabolism	↓ protein synthesis and ↑ protein breakdown in skeletal muscles	Supply of glucogenic precursors to the liver	↓ lean tissue yield (especially breast yield)	**Howlider and Rose (1989)**, **Ain Baziz et al. (1996)**, **Geraert et al. (1996a)**, **Temim et al. (2000)**, **Boussaid-Om Ezzine et al. (2010)**, Rhoads et al. (2013), **Zuo et al. (2015)**, **Lu et al. (2018)**, **Ma et al. (2021)**, **Qaid and Al-Garadi (2021)**, **Zampiga et al. (2021)**

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[a]Include studies on non-avian species that have exhibited comparable heat stress effects and responses to those observed in chickens. Studies with the focus on chickens or poultry are highlighted in bold.

protein synthesis and muscle development in chickens (Bigot et al., 2003; Duchêne et al., 2008; Everaert et al., 2010). Interestingly, Boussaid-Om Ezzine et al. (2010) detected a limited response of the S6K signaling pathway to anabolic stimuli in heat-stressed broiler chickens. Lu et al. (2018) measured an increase in blood uric acid, urea, and proteinogenic amino acids (AA)—in spite of a marked decrease in feed intake and breast yield—along with a reduction in glucose and NEFA. Consequently, they postulated that heat-exposed chickens mobilize protein reservoirs of skeletal muscles, particularly the breast, to compensate for the inability to extract energy from stored fat. In this regard, plasmatic levels of creatine, 3-methylhistidine, and urea have been used as biomarkers to assess muscle protein breakdown induced by HS (Rhoads et al., 2013). The hypothesis formulated by Lu et al. (2018) has been supported by Ma et al. (2021) who found that HS reduces plasmatic glucogenic AA, increases AA uptake of the liver and its glucogenic potential, and enhances the activity of hepatic transaminases that deaminize AA to make them precursors for gluconeogenesis. Furthermore, Zampiga et al. (2021) observed that heat-stressed broilers exhibit a drop in blood glucogenic precursors and breast muscle free AA, despite a rise in circulating protein-building AA concomitant with a substantial feed intake reduction. Table 3 presents a summary of heat stress effects on skeletal muscle protein metabolism of chickens.

Heat stress and hormonal levels

Chickens subjected to HS share numerous hormonal variations with mammalian species. HS activates the hypothalamic-pituitary-adrenal axis, leading to a marked increase in circulating glucocorticoids, particularly corticosterone (Geraert et al., 1996b; Yunianto et al., 1997; Quinteiro-Filho et al., 2010, 2012; Rajaei-Sharifabadi et al., 2017; Lu et al., 2019; Beckford et al., 2020; Ma et al., 2021). In chickens, high levels of corticosterone have been reported to decrease growth potential, induce proteolysis and suppress protein synthesis in skeletal muscles, and increase fat deposition (Decuypere and Buyse, 1988; Dong et al., 2007; Yuan et al., 2008), all of which are typical HS consequences (Rhoads et al., 2013). It has been proposed that corticosterone impairs muscle protein metabolism by inducing the abovementioned changes in S6K and MAFbx expression (Ma et al., 2021), while also exerting a lipogenic effect by promoting the expression of fatty acid synthase (FASN) in hepatocytes and adipocytes (Gonzalez-Rivas et al., 2020). However, a recent investigation demonstrated that treating heat-stressed chicken myotubes with corticosterone does not intensify proteolysis and does not increase the expression of MAFbx compared to the HS treatment alone (Furukawa et al., 2021). The latter interesting results have been obtained *in vitro*, and therefore further research may be needed to elucidate the role of corticosterone in the altered protein metabolism of heat-stressed chickens.

Additionally, since hypercorticosteronemia is immunosuppressive (Quinteiro-Filho et al., 2010), heat-challenged chickens have a compromised immunocompetence and are more prone to infectious diseases (Renaudeau et al., 2012; Farag and Alagawany, 2018; Chauhan et al., 2021). In this regard, Hirakawa et al. (2020) detected serious immunological disorders in heat-stressed broilers, such as a decrease in immunoglobulin production against a prototype antigen as well as atrophy and dysfunction of primary and secondary lymphoid tissues accompanied by lymphocyte depression. The authors mentioned hypercorticosteronemia among the plausible reasons for these anomalies in the immune system. Corticosterone-related immune dysfunctions of chickens have thoroughly been described by Shini et al. (2010).

Reductions in hematic triiodothyronine (T_3) and thyroxine (T_4) have also been observed in laying hens (de Andrade et al., 1977; Bobek et al., 1997) and broiler chickens (Geraert et al., 1996b; Yunianto et al., 1997; Sohail et al., 2010; Rajaei-Sharifabadi et al., 2017; Beckford et al., 2020) undergoing HS. These variations, which might be caused by decreased size and activity of the thyroid (Huston and Carmon, 1962; Dale and Fuller, 1980; Yunianto et al., 1997), have also been measured in heat-stressed dairy cattle (Chen et al., 2018). It has commonly been assumed that the thyroid response to high environmental temperatures is an adaptive mechanism that allows animals to lower their basal metabolism and thermogenesis in order to prevent overheating (Renaudeau et al., 2012; Chen et al., 2018;

TABLE 4 Overview of heat stress effects on chicken hormonal levels.

Class	Heat stress effect	Pros	Cons	References[a]
Hormonal levels	Hypothalamic-pituitary-adrenal axis activation → ↑ circulating glucocorticoids (e.g., corticosterone)	—	↓ growth potential ↓ protein synthesis and ↑ protein breakdown in skeletal muscles → ↓ lean tissue yield ↑ fat deposition ↓ immunocompetence → ↑ infectious susceptibility and health care costs ↓ GI barrier	**Decuypere and Buyse (1988)**, **Geraert et al. (1996b)**, **Yunianto et al. (1997)**, **Dong et al. (2007)**, **Yuan et al. (2008)**, **Quinteiro-Filho et al. (2010)**, **Quinteiro-Filho et al. (2012)**, Renaudeau et al. (2012), Rhoads et al. (2013), **Rajaei-Sharifabadi et al. (2017)**, **Lu et al. (2019)**, **Beckford et al. (2020)**, Gonzalez-Rivas et al. (2020), **Hirakawa et al. (2020)**, **Wasti et al. (2020)**, Chauhan et al. (2021), **Ma et al. (2021)**
	Hypothyroid-like state	↓ basal metabolism and thermogenesis → ↓ metabolic heat generation	↓ growth potential ↑ carcass adiposity ↓ egg production and eggshell quality	**Huston and Carmon (1962)**, **de Andrade et al. (1977)**, **Dale and Fuller (1980)**, **Decuypere and Buyse (1988)**, **Geraert et al. (1996b)**, **Yunianto et al. (1997)**, **Sohail et al. (2010)**, Renaudeau et al. (2012), **McNabb and Darras (2015)**; **Rajaei-Sharifabadi et al. (2017)**, Chen et al. (2018), **Beckford et al. (2020)**, Gonzalez-Rivas et al. (2020)

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Gonzalez-Rivas et al., 2020). The hypothyroid-like condition can partly justify growth depression (McNabb and Darras, 2015), increased carcass adiposity (Decuypere and Buyse, 1988; Geraert et al., 1996b), and decreased egg production and shell quality (de Andrade et al., 1977) observed during HS. Table 4 briefly illustrates the effects of heat stress on chicken hormonal levels.

Heat stress effects on gut health of chickens

Gut health should be addressed in a holistic way (Oviedo-Rondón, 2019) by taking into consideration the major elements that synergistically affect it, namely the GI epithelium, the GI immune system, and the GI microbiota (Kogut et al., 2017). Being the largest body surface exposed to the environment, the gastrointestinal tract (GIT) is repeatedly threatened by a wide variety of harmful factors (Yegani and Korver, 2008), like noxious feed-derived compounds and pathogenic microorganisms. Applegate and Troche (2014) emphasized that the GIT accomplishes conflicting tasks, that is maximizing nutrient uptake while recognizing multiple antigenic stimuli and tolerating the resident microbiota. Hence, integrity and proper morpho-functionality of the GIT are of utmost importance in ensuring optimal health and productivity for chickens.

Heat stress and gastrointestinal epithelium

The GI epithelium, arranged in a single-cell layer, takes an active part in the integrated gut immune system, forming a barrier reinforced by tight junction (TJ) proteins, secreting mucus and antimicrobial/host defense peptides (AMP/HDP), and expressing pattern recognition receptors (PRR) that orchestrate the enteral immune response (Smith et al., 2014; Chen et al., 2015; Broom and Kogut, 2018a).

TJs, the uppermost component of the apical junctional complex, seal the interstice between adjoining columnar epithelial cells (Farquhar and Palade, 1963) and encompass transmembrane (claudins and occludin) and scaffolding/peripheral/plaque proteins (zonula occludens—ZO). Through their binding domains, ZO anchor to claudins and occludin on one side and to the perijunctional actomyosin ring on the other side, thereby making a bridge between transmembrane TJs and the cytoskeleton (Ulluwishewa et al., 2011). TJs are primarily responsible for controlling the paracellular pathway that, unlike the pump- and channel-dependent transcellular transports, allows a passive transepithelial diffusion *via* two main routes, known as the pore pathway and the leak pathway (Dokladny et al., 2016). The pore pathway relies on claudins and limits the passage of charged and large molecules (greater than 4 Å), while the leak pathway, governed by occludin and ZO, can be crossed by big solutes, including bacterial lipopolysaccharides (LPS) (Anderson and Van Itallie, 2009; Dokladny et al., 2016; France and Turner, 2017). Under HS, the cardiovascular system responds in another evolutionary-preserved adaptation whereby a large volume of blood is shunted from the splanchnic tissues to peripheral areas of the body to maximize the dissipation of sensible heat (Hales, 1973; Lambert, 2009). This occurs to the detriment of the GIT because the altered blood

TABLE 5 Overview of heat stress effects on the cardiovascular system of chickens.

Class	Heat stress effect	Pros	Cons	References[a]
Cardiovascular system	Peripheral vasodilatation and GIT vasoconstriction	↑ sensible heat dissipation	GIT hypoperfusion → ↓ nutrient supply to the GIT → ↓ GI barrier and functionality GIT hypoxia → oxidative stress → ↓ GI barrier and functionality	Hales (1973), Hall et al. (1999), **Koutsos and Arias (2006)**, Rao (2008), Lambert (2009), Baumgard and Rhoads (2013), Bischoff et al. (2014), **Rostagno (2020)**

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[a]Include studies on non-avian species that have exhibited comparable heat stress effects and responses to those observed in chickens. Studies with the focus on chickens or poultry are highlighted in bold.

pressure is mostly compensated by a sympathetically driven vasoconstriction of viscera (Table 5). The resulting hypoperfusion implicates a reduced supply of nutrients and oxygen to the GIT, which prompts deleterious effects on the intestinal mucosa (Lambert, 2009; Baumgard and Rhoads, 2013; Rostagno, 2020). In light of the remarkable energy and protein demands of the digestive system, a sub-optimal trophism of the GI epithelium negatively affects cell turnover and the maintenance of a robust intestinal barrier (Koutsos and Arias, 2006). On the other hand, the inadequate oxygenation leads to hypoxia, a condition that profoundly alters the cellular bioenergetic pathways and promotes the generation of reactive oxygen and nitrogen species (ROS and RNS, respectively) (Hall et al., 1999). Moreover, hyperthermia triggers ROS and RNS production per se (Hall et al., 2001) and impairs the enzymatic antioxidant systems (Farag and Alagawany, 2018), directly contributing to the establishment of a pro-oxidative scenario. Lin et al. (2006a) proved that elevated ambient temperatures provoke oxidative stress in chickens, while Tan et al. (2010) suggested that HS can depress the mitochondrial respiratory chain activity with consequent overproduction of ROS and oxidative injury. Worthy of mention is also the research on oxidative damage affecting the skeletal muscles, particularly the *Pectoralis major*, of heat-stressed broilers. Several authors demonstrated a rise in mitochondrial membrane potential, a high production of mitochondrial superoxide and ROS, and a considerable increase in malondialdehyde level (marker of lipid peroxidation) in breast muscles of broilers exposed to HS (Mujahid et al., 2006; Wang et al., 2009; Azad et al., 2010; Kikusato and Toyomizu, 2013). On the other hand, studies focused on the GIT have reported that oxidative stress destabilizes the TJ-regulated paracellular barrier and increases intestinal permeability (Rao, 2008; Bischoff et al., 2014). Myosin light-chain kinase (MLCK) is involved in this cascade of events because it regulates the circumferential contractions of the actomyosin ring and, indirectly, the TJ-controlled paracellular pathway (France and Turner, 2017). The actomyosin ring contractions can be triggered by several physiological and pathological stimuli. Oxidative stress has been shown to cause such contractions and, consequently, to affect the MLCK-regulated localization of ZO proteins and downregulate their expression, contributing to the deterioration of the paracellular barrier (González-Mariscal et al., 2011).

Along with oxidative stress, the aforementioned increment in corticosterone levels further weakens the intestinal barrier (Quinteiro-Filho et al., 2012). Transepithelial electrical resistance (TEER)—i.e., the epithelium resistance to ion passage—and mucosa-to-serosa flux of marker probes (e.g., fluorescein isothiocyanate-dextran) have commonly been used to evaluate the paracellular barrier stability and integrity (Shen et al., 2011; Bischoff et al., 2014; Awad et al., 2017; Ma et al., 2018; Gilani et al., 2021). A steady paracellular pathway shows high values of TEER and effectively obstructs the flux of markers, whereas low TEER and high marker passage indicate poor barrier functions. HS has been shown to considerably reduce TEER and significantly increase the migration of marker tracers across the intestinal epithelium in numerous animal models (Dokladny et al., 2016), pigs (Pearce et al., 2013b), and broiler chickens (Song et al., 2014; Tabler et al., 2020). These variations are indicators of a "leaky gut" that barely holds noxious luminal compounds (Shen et al., 2011; Awad et al., 2017; Ma et al., 2018; Ruff et al., 2020). Translocation of pathogen-associated molecular patterns (PAMP) from the intestinal lumen to the underlying lamina propria is a major consequence of an increased paracellular permeability. The gut contains a massive amount of PAMPs, mainly LPS of Gram-negative bacteria (Wassenaar and Zimmermann, 2018), which can bind to a class of PRRs known as Toll-like receptors (TLR). Intestinal TLRs are particularly differentiated in chickens (Keestra et al., 2013) and have been shown to play a pivotal role in maintaining gut homeostasis and evoking inflammatory responses in the case of infections or other insults, such as hypoxia and tissue injury (Gribar et al., 2008). These receptors are also involved in epithelial cell proliferation, wound healing, stability of TJs, and modulation of immunoglobulin A (IgA) production and AMP expression (Abreu, 2010; Iizuka and Konno, 2011). Furthermore, TLRs are rather non-responsive to the multitude of commensal microorganisms inhabiting the GIT, yet are constantly responsive to PAMPs and host indicators of cell damage (Harris et al., 2006; Kogut et al., 2017; Madsen and Park, 2017). The ability to distinguish between useful microbes and those undesirable—or that can become such, like

TABLE 6 Overview of heat stress effects on the GI epithelium of chickens.

Class	Heat stress effect	Pros	Cons	References[a]
GI epithelium	Altered GI epithelium morphology (microarchitecture) and enterocyte lifecycle	—	Digestive and absorptive dysfunctions → ↓ performance	Lambert (2009), **Song et al. (2014)**, Vaure and Liu (2014), Wang et al. (2015), **Wideman (2016)**, **Dokladny et al. (2016)**, **Alhenaky et al. (2017)**, Awad et al. (2017), France and Turner (2017), Ma et al. (2018), Wassenaar and Zimmermann (2018), **Ducatelle et al. (2018)**, **He et al. (2018a)**, **He et al. (2018b)**, Epstein and Yanovich (2019), **Nanto-Hara et al. (2020)**, **Ruff et al. (2020)**, **Tabler et al. (2020)**, **Liu et al. (2020)**, **Liu et al. (2022)**
	↑ paracellular permeability (↓ transepithelial electrical resistance and ↑ mucosa-to-serosa flux of markers) → "leaky gut"			
	↓ GI epithelium integrity		LPS/endotoxins leakage → ↑ pro-inflammatory cytokines → GI inflammation and ↓ GI barrier	
			↓ liver health and functionality Endotoxemia → systemic inflammation, multi-organ failure, and septic shock	
			"Bacterial translocation" → ↓ liver health and functionality bacterial chondronecrosis with osteomyelitis	

Upward arrow (↑), increase; downward arrow (↓), decrease; rightward arrow (→), consequence/degeneration.
[a]Include studies on non-avian species that have exhibited comparable heat stress effects and responses to those observed in chickens. Studies with the focus on chickens or poultry are highlighted in bold.

pathobionts (Round and Mazmanian, 2009)—is one of the most fascinating properties of the GI immune system (Mowat, 2018). At the basolateral membrane of the intestinal epithelium, LPS are recognized by the TLR4–MD-2 receptor complex (Shimazu et al., 1999; Abreu, 2010; Keestra et al., 2013) whose activation initiates an intracellular signaling cascade upregulating the expression of several pro-inflammatory cytokines (Vaure and Liu, 2014). The latter signaling molecules, also released by LPS-stimulated innate immune cells, foster a vicious cycle that deteriorates the intestinal barrier (Lambert, 2009). Tumor necrosis factor alpha (TNF-α), interleukin 1 beta (IL-1β), and interferon gamma (IFN-γ) have been reported to ruin the paracellular barrier, thereby increasing LPS leakage (Turner et al., 2014; Dokladny et al., 2016; Awad et al., 2017; Ma et al., 2018). Specifically, TNF-α has been shown to initiate the actomyosin ring contractions and, subsequently, to cause occludin internalization and TJ disassociation (Turner et al., 2014). Pro-inflammatory cytokines evoke a local inflammatory response aggravating the damages to the enteric mucosa. Quinteiro-Filho et al. (2010, 2012) reported that heat-stressed broilers manifest mild multifocal enteritis. Enteral inflammation has been shown to shorten the lifespan of enterocytes and cause crypt hyperplasia and villus atrophy (Smith et al., 2014). These alterations in intestinal epithelium morphology (microarchitecture), along with increased cell apoptosis and reduced cell proliferation, have recently been observed in broiler chickens exposed to HS (He et al., 2018a, 2018b; Liu et al., 2020, 2022; Nanto-Hara et al., 2020). In their pair-feeding study with broilers, Nanto-Hara et al. (2020) evidenced that intestinal morphological damage and increased intestinal permeability are direct consequences of HS rather than of feed intake reduction induced by HS itself. The resultant nutrient malabsorption and energy expenditure to sustain the GI immune response severely impact chicken performance and can be a predisposing factor for additional health problems (Broom and Kogut, 2018b).

In addition to initiating a local inflammation, luminal LPS can permeate the portal circulation whereby they reach and compromise the liver (Wang et al., 2015). Once exceeding the hepatic detoxification potential, LPS can diffuse throughout the bloodstream causing endotoxemia (Baumgard and Rhoads, 2013; Alhenaky et al., 2017; Epstein and Yanovich, 2019; Nanto-Hara et al., 2020). The resulting systemic inflammatory reactions force the organism to adjust nutrient partition and divert energy to support the immune system; this substantially depresses chicken performance (Broom and Kogut, 2018b; Ruff et al., 2020). At the worst, endotoxemia can lead to multi-organ failure and lethal septic shock (Wassenaar and Zimmermann, 2018).

According to Rostagno (2020), anomalies in the transcellular transport are another reason for intestinal permeability problems of chickens under HS. Indeed, a loss of epithelial integrity can degenerate into cell damage and consequent opening of TJ-independent pathways (France and Turner, 2017). Enteric bacteria can cross the altered and more permeable intestinal epithelium and, eventually, reach the liver or even migrate to other organs or tissues. For example, heat-stressed broilers showed a greater hepatic *Salmonella* invasion due to increased intestinal permeability (Alhenaky et al., 2017). This event, commonly called "bacterial translocation", can be prelude to extraintestinal issues, such as deteriorations of liver functionality and health (Ilan, 2012; Ducatelle et al., 2018) as well as bacterial chondronecrosis with osteomyelitis (BCO) (Wideman, 2016). HS effects on the GI epithelium of chickens are summed up in Table 6.

TABLE 7 Overview of heat stress effects on the GI microbiota of chickens.

Class	Heat stress effect	Pros	Cons	References[a]
GI microbiota	Perturbation of the GI ecosystem and microbial community stability → dysbiosis	—	Positive feedback loop among dysbiosis, GI barrier dysfunction, and GI inflammation → ↓ health and performance ↑ susceptibility to GI pathogen colonization → ↑ GI disorders (e.g., necrotic enteritis) → ↓ health and performance	**Suzuki et al. (1983)**, **Suzuki et al. (1989)**, **Lan et al. (2004)**, **Burkholder et al. (2008)**, **Soliman et al. (2009)**, **Song et al. (2014)**, Chen et al. (2018), **Tsiouris et al. (2018)**, **Wang X. J. et al. (2018)**, **Wang et al. (2020)**, **Ducatelle et al. (2018)**, **Shi et al. (2019)**, **Xing et al. (2019)**, **Zhu et al. (2019)**, **He et al.** (2019a), **He et al. (2019b)**, **He et al.** (2021), Le Sciellour et al. (2019), **Xiong et al. (2020)**, **Liu et al. (2020)**, **Liu et al. (2022)**

Upward arrow (↑), increase; downward arrow (↓), decrease; rightward arrow (→), consequence/degeneration.
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TABLE 8 Overview of heat stress effects on the inflammatory state of chickens.

Class	Heat stress effect	Pros	Cons	References[a]
Inflammatory state	Enteritis and systemic inflammation	Response to endotoxemia, microbial infection, and GI tissue injury	↑ energy expenditure to sustain the immune system → ↓ performance	**Quinteiro-Filho et al. (2010)**, **Quinteiro-Filho et al. (2012)**, Broom and Kogut (2018b), **Ruff et al. (2020)**

Upward arrow (↑), increase; downward arrow (↓), decrease; rightward arrow (→), consequence/degeneration.
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Heat stress and gastrointestinal microbiota

Microbiota and microbiome are similar-sounding words that are often used interchangeably. However, the microbiota represents a cluster of microorganisms residing in a specific environment (Marchesi and Ravel, 2015), such as an area of human or animal bodies (Clavijo and Flórez, 2018), while the microbiome unifies the metagenome of a microbiota (i.e., the collection of microbial genomes) and its surrounding environment (Marchesi and Ravel, 2015). The alimentary canal of chickens harbors an extremely complex microbial population that consists of bacteria, archea, protozoa, fungi, and viruses (Yeoman et al., 2012). The GI microbiota extends the genome of the host and substantially influences its physiology (Koutsos and Arias, 2006), almost acting as a supplementary—or "neglected" (Bocci, 1992)—organ. It is a widely held view that the GI microbiota is instrumental in programming and modulating both the gastroenteric (Iyer and Blumberg, 2018; Cheng et al., 2019) and systemic (Belkaid and Hand, 2014; Zheng et al., 2020) immune system of humans and animals, including poultry (Broom and Kogut, 2018c). This notion is supported by gnotobiotic models in which germ-free mice (Round and Mazmanian, 2009; Belkaid and Hand, 2014; Iyer and Blumberg, 2018) and chickens (Dibner et al., 2008) have been reported to suffer from severe developmental deficiencies and dysfunctions of the GI immunity. In addition to its immunogenic and immunoregulatory roles, the GI microbiota considerably influences growth, morphology, and function of the intestine in chickens (Dibner et al., 2008; Pan and Yu, 2014).

A myriad of host- and environment-related variables affects the GI microbiota (Kers et al., 2018). For instance, data from several studies indicate that high ambient temperatures can dramatically shape the GI microbiota. Specifically, it has been demonstrated that HS perturbs the GI microbiota in rats (Suzuki et al., 1983, 1989), poultry (Suzuki et al., 1983, 1989; Lan et al., 2004; Burkholder et al., 2008; Song et al., 2014; Wang X. J. et al., 2018; He et al., 2019b; Shi et al., 2019; Xing et al., 2019; Zhu et al., 2019; Liu et al., 2020; Wang et al., 2020; He et al., 2021; Liu et al., 2022), dairy cattle (Chen et al., 2018), and pigs (He et al., 2019a; Le Sciellour et al., 2019; Xiong et al., 2020), pushing it to dysbiosis. Dysbiosis (dysbacteriosis) is an alteration in the gut microbiota with an overgrowth of harmful microorganisms, or a depletion of beneficial bacteria, which can weaken the fragile equilibrium between the host and its GI microbiota (Walker, 2017; Ducatelle et al., 2018). A dysbiotic state is often associated with depression in nutrient digestion, loss of intestinal barrier function, and GI inflammation (Chen et al., 2015; Ducatelle et al., 2018), whereas eubiosis, referred to as a balanced microbial ecosystem (Iebba et al., 2016), can enhance health, productivity, and ability of chickens to withstand environmental stressors (Kogut, 2019). Although cutting-edge analytical techniques are currently available to study the GI microbiota (Borda-Molina et al., 2018), the modifications in structure, composition, and functions of the GI microbiota of heat-stressed chickens are still to be fully understood (He et al., 2021; Liu et al., 2022). However, changes in GI morphology,

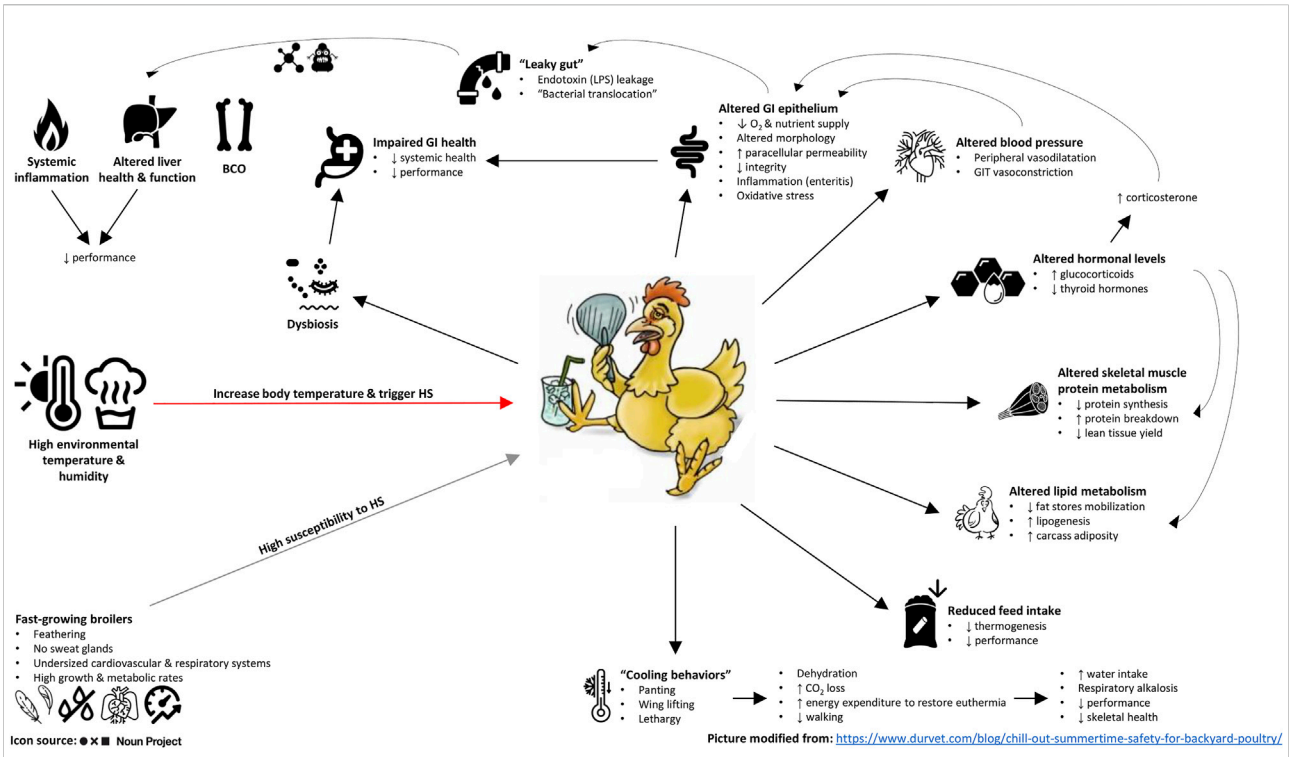

FIGURE 1
Summary chart of the main effects of heat stress on modern (broiler) chicken lines. LPS, lipopolysaccharides; BCO, bacterial chondronecrosis with osteomyelitis.

mucus quantity and composition, and attachment sites, coupled with an accumulation of poorly digested or even undigested feed components, are all plausible reasons for HS-caused dysbiosis.

The commensal microbiota is able to hinder the colonization and proliferation of allochthonous and pathogenic microorganisms in the GI ecosystem (Schneitz, 2005). This protective mechanism, conventionally termed competitive exclusion (CE) or "Nurmi concept", was firstly observed in newly hatched chicks acquiring resistance to *Salmonella* challenges if previously inoculated *per os* with a suspension of crop and intestinal contents collected from healthy adult chickens (Nurmi and Rantala, 1973). Chichlowski et al. (2007) specified that CE is a physical blockage of intestinal niches carried out by beneficial bacteria to the detriment of opportunistic pathogens. Desirable bacteria can also compete with pathogens for nutrients, and produce microbiostatic and microbicidal substances, such as organic acids and bacteriocins (Pan and Yu, 2014; Clavijo and Flórez, 2018). However, aberrant microbiotas of chickens subjected to HS have been related to an increased susceptibility to intestinal colonization of *Salmonella* Enteritidis (Burkholder et al., 2008; Soliman et al., 2009). Tsiouris et al. (2018) also demonstrated that HS can promote the expansion of *Clostridium perfringens* in the chicken intestine, becoming a predisposing factor for necrotic enteritis outbreak in flocks reared under hot conditions. *C. perfringens* can also release enterotoxins that, together with other harmful bacterial effectors, may impair TJs and gut barrier functions (Awad et al., 2017). Taken together, dysbiosis, intestinal barrier disorders, and mucosa inflammation are interconnected and fuel each other (Ducatelle et al., 2018), exacerbating the negative effects of HS on gut health, physiology, and performance of chickens (Tables 7, 8).

Conclusion

Nowadays poultry farmers must deal with HS at almost every latitude because climate change has made high temperatures a pressing issue no longer limited to hot countries. Consequently, it would be advisable to update the estimate of costs and economic losses caused by HS to realize its actual impact on the global poultry industry.

According to the literature reviewed here, HS provokes a wide range of deleterious effects on chickens, especially those belonging to modern high-performing lines (Figure 1). Firstly, HS negatively affects immunohomeostasis, hormonal equilibrium, and inflammatory and oxidative status. More studies on these physiologic alterations and their interconnections can help develop multitargeted solutions to help chickens combat HS more effectively. Secondly, HS promotes tissue catabolism and a substantial modification in protein and lipid metabolism. While there is evidence to assert that HS affects skeletal muscle accretion of chickens *via* both

protein synthesis inhibition and protein degradation stimulation, further investigations are needed to clarify the underlying causes of the blunted fat mobilization in heat-stressed chickens. Lastly, high temperatures can be deemed to be a "dysbiogenic stressor" that undermines gut functionality and disrupts the host-microbiota interrelationship. Reinforcing the intestinal barrier, restoring digestive and absorptive processes, rebalancing the GI microbiota, and lowering the GI inflammation and oxidative stress seem therefore essential to increase HS tolerance and resilience for chickens.

In conclusion, reversing the homeostatic and metabolic perturbations induced by HS and conferring enteral protection appear to be promising approaches to fight against this growing threat to the poultry industry sustainability.

Author contributions

GB and FS conceived this review series. GB wrote Part I of the series under the supervision of FS. J-RT, SR, SD, and FS revised this manuscript. All authors read and approved the submitted version of this manuscript.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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EDITED BY
Gale Strasburg,
Michigan State University, United States

REVIEWED BY
Paramjit S. Tappia,
I. H. Asper Clinical Research Institute, Canada
Markus Rodehutscord,
University of Hohenheim, Germany
Birger Svihus,
Norwegian University of Life Sciences, Norway

*CORRESPONDENCE
Jean-Rémi Teyssier,
jteyssie@uark.edu
Samuel J. Rochell,
rochell@uark.edu

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A review of heat stress in chickens. Part II: Insights into protein and energy utilization and feeding

Jean-Rémi Teyssier[1]*, Giorgio Brugaletta[2], Federico Sirri[2], Sami Dridi[1] and Samuel J. Rochell[1]*

[1]Center of Excellence for Poultry Science, University of Arkansas, Fayetteville, AR, United States, [2]Department of Agricultural and Food Sciences, Alma Mater Studiorum—University of Bologna, Bologna, Italy

With the growing global demand for animal protein and rising temperatures caused by climate change, heat stress (HS) is one of the main emerging environmental challenges for the poultry industry. Commercially-reared birds are particularly sensitive to hot temperatures, so adopting production systems that mitigate the adverse effects of HS on bird performance is essential and requires a holistic approach. Feeding and nutrition can play important roles in limiting the heat load on birds; therefore, this review aims to describe the effects of HS on feed intake (FI) and nutrient digestibility and to highlight feeding strategies and nutritional solutions to potentially mitigate some of the deleterious effects of HS on broiler chickens. The reduction of FI is one of the main behavioral changes induced by hot temperatures as birds attempt to limit heat production associated with the digestion, absorption, and metabolism of nutrients. Although the intensity and length of the heat period influences the type and magnitude of responses, reduced FI explains most of the performance degradation observed in HS broilers, while reduced nutrient digestibility appears to only explain a small proportion of impaired feed efficiency following HS. Targeted feeding strategies, including feed restriction and withdrawal, dual feeding, and wet feeding, have showed some promising results under hot temperatures, but these can be difficult to implement in intensive rearing systems. Concerning diet composition, feeding increased nutrient and energy diets can potentially compensate for decreased FI during HS. Indeed, high energy and high crude protein diets have both been shown to improve bird performance under HS conditions. Specifically, positive results may be obtained with increased added fat concentrations since lipids have a lower thermogenic effect compared to proteins and carbohydrates. Moreover, increased supplementation of some essential amino acids can help support increased amino acid requirements for maintenance functions caused by HS. Further

Abbreviation: AA, amino acid; AME, apparent metabolizable energy; AMEn, nitrogen-corrected apparent metabolizable energy; BCAA, branched-chain AA; BW, body weight; BWG, body weight gain; Cit, citrulline; CP, crude protein; DM, dry matter; dLys, digestible Lysine; FCR, feed conversion ratio; FI, feed intake; HP, heat production; HS, heat stress; ME, metabolizable energy; TSAA, total sulfur AA.

research to better characterize and advance these nutritional strategies will help establish economically viable solutions to enhance productivity, health, welfare, and meat quality of broilers facing HS.

KEYWORDS

heat stress, chicken, feed intake, digestibility, feeding strategies, amino acids, energy, broiler

Introduction

The poultry industry continues to play a critical role in meeting the growing demand for animal protein. The global production of chicken and turkey meat has doubled over the last 20 years, reaching 125.5 million tons in 2020 (FAO, 2022). This accounts for approximately 37% of global meat production, while poultry meat only represented 29% in the early 2000s. With the increasing global population projected to rise from 7.8 to 9.9 billion in 2050 (PRB, 2020) and better access to animal products in developing areas, it is predicted that animal-based food demand will grow by nearly 70% in the same timeline (Searchinger et al., 2019). Meanwhile, climate change represents one of the major concerns for livestock production in the coming decades. Some reports indicate that industrialized farming systems may lose 25% of their animal production, and this scenario may be worse for some tropical regions where extensive farming systems are more abundant (Nardone et al., 2010). Emerging estimates by the Intergovernmental Panel on Climate Change emphasized that global warming of more than 2°C will occur during the 21st century unless large reductions in CO_2 and other greenhouse gas emissions are acheived soon (IPCC, 2021). Also, the authors indicated that climate change is already and will continue increasing the frequency and intensity of extreme weather events like hot temperature waves. Therefore, the poultry industry needs to continue adopting technologies and practices that reduce its impact on the environment, but it should also adopt production systems that are resilient in the face of rising global temperatures.

Modern broiler chickens are particularly sensitive to hot temperatures due to their rapid growth rates resulting from genetic selection to enhance production efficiency, as well as from limitations in heat dissipation caused by feathering, an absence of sweat glands, and relatively high stocking densities in intensive commercial rearing facilities (Lara and Rostagno, 2013; Emami et al., 2020). Heat stress (HS) occurs when the amount of heat produced by an animal surpasses its capacity to dissipate the heat to the surrounding environment. When the environmental temperature rises above the thermoneutral zone, birds typically reduce their physical activity and feed intake (FI) to limit heat production (HP), as well as increase their panting and water consumption to favor heat loss by evaporation (Renaudeau et al., 2012). Indeed, elevated temperatues trigger important physiologic and metabolic changes as described in Part I of this review (Brugaletta et al., 2022), and chronic HS exposure results in significant losses in bird performance, negatively affects welfare, challenges food safety, and reduces the overall economic efficiency of poultry production (Lara and Rostagno, 2013; Pawar et al., 2016). Consequently, HS has been estimated to cause annual economic losses of $128 to $165 million for the United States poultry industry (St-Pierre et al., 2003), but these figures probably underestimate current and future losses due to the growth of the poultry industry over the last decade and the worsening of climate change predictions.

Mitigating the adverse effects caused by hot temperatures in poultry productions requires a holistic and multi-factorial approach. Housing (Oloyo, 2018), management practices (Saeed et al., 2019), genetic selection (Kumar et al., 2021), and feeding and nutrition (Syafwan et al., 2011; Fouad et al., 2016; Sugiharto et al., 2017; Wasti et al., 2020; Abdel-Moneim et al., 2021; Chowdhury et al., 2021) can all provide some benefit to birds under HS conditions and have been the topics of several recent global reviews (Lin et al., 2006b; Nawab et al., 2018; Goel et al., 2021; Vandana et al., 2021). This review aims to assess the effects of HS on FI and nutrient digestibility, as well as to evaluate different feeding strategies and nutritional solutions to mitigate some of the adverse effects of HS on poultry. Effects on broiler chickens will be emphasized, though research with other types of poultry will be discussed where relevant. Further, this review will focus on macro-nutritional solutions as carbohydrates, fat, and proteins are the main source of energy, and their oxidation results in HP (Costa-Pinto and Gantner, 2020), which needs to be limited under HS.

Impact of heat stress on feed intake regulation and nutrient digestibility

Nearly all studies that have investigated the effects of HS in poultry have observed reductions in FI of heat-stressed birds compared with those in thermoneutral conditions, including meta-analyses conducted in broilers (Liu et al., 2020) and laying hens (Mignon-Grasteau et al., 2015). This reduction of FI observed under HS conditions reduces endogenous HP associated with digestion, absorption, and metabolism of nutrients (Lara and Rostagno, 2013). However, the magnitude of the FI reduction depends on several parameters related to the characteristics of the HS model imposed on the birds, and this can complicate comparisons among studies. Temperature, length and cyclicality of the heat period, and age of the birds at the

beginning and the end of the HS period are all potential factors that can influence the intensity of the FI reduction. Many studies have used a constant HS model with high temperatures applied over a long period of time (Baziz et al., 1996; Geraert et al., 1996; Bonnet et al., 1997; Faria Filho et al., 2007). However, more recent studies have employed cyclic HS models combining higher temperatures during the day and lower temperatures during the night which may better simulate field conditions in temperate areas of the world (De Souza et al., 2016; Flees et al., 2017; Greene et al., 2021). When compared within the same experiment, cyclic HS decreased FI by 15% on average, while constant HS resulted in higher reductions ranging from 25% to 45% (De Souza et al., 2016; Awad et al., 2018; Teyssier et al., 2022). Therefore, cyclic HS resulted in a 1.5% reduction in FI per degree Celsius, while the values obtained under constant HS corroborate the expected response proposed by Baziz et al. (1996) of about a 3.5% reduction in FI per degree Celsius increase between 22°C and 35°C.

Interestingly, the reduction of growth observed under HS is greater than expected due to the reduced FI alone, leading to a lower feed efficiency (Renaudeau et al., 2012). The use of pair-feeding techniques, where birds under thermoneutral conditions are fed the same amount of feed consumed by heat-stressed birds, have shown that the reduction in growth due to decreased FI ranges between 60% and 99% (Geraert et al., 1996; Bonnet et al., 1997; Garriga et al., 2006; Lu et al., 2007; Zuo et al., 2015; De Souza et al., 2016; Zeferino et al., 2016; De Antonio et al., 2017; Emami et al., 2021; Ma et al., 2021; Teyssier et al., 2022). Therefore, the lower FI is the main factor explaining impaired performance of chickens observed under HS, with the remainder of the growth reduction attributable to impaired digestibility or physiological and metabolic changes that influence feed efficiency (Dale and Fuller, 1980; Geraert et al., 1996; Renaudeau et al., 2012).

Several studies have reported reduced dry matter (DM) digestibility in quails (Orhan et al., 2020) and laying hens (Kim et al., 2020) under HS conditions. In broilers, Bonnet et al. (1997) and De Souza et al. (2016) observed decreases of 1.6% and 3.9% in DM digestibility under constant HS. However, other studies have reported no DM digestibility losses due to HS (Faria Filho et al., 2007; Attia et al., 2016, 2017). At the nutrient level, even though no change in crude protein (CP) digestibility were observed by several authors (Faria Filho et al., 2007; Habashy et al., 2017b; Kim et al., 2020), numerous studies have reported decreases in CP or nitrogen digestibility ranging between 1.5% and 10% under hot temperatures (Zuprizal et al., 1993; Bonnet et al., 1997; Soleimani et al., 2010; Attia et al., 2016, 2017; De Souza et al., 2016; Orhan et al., 2020). The detrimental effect of HS has also been measured on amino acid (AA) digestibility. Wallis and Balnave (1984) observed a slight decrease in the digestibility for Thr, Ala, Met, Ile, and Leu, with greater impacts in male than in female birds. Standardized and apparent digestibility values of several AA (i.e., Arg, His, Thr, Val, Lys, Ile, Leu, Phe, Cys, Gly, Ser, Ala, Pro, and Tyr) were also reduced by approximately 5.5%, in the study of Soleimani et al. (2010). Regarding other nutrients, none of these studies observed an impact on crude fat digestibility, and only Kim et al. (2020) measured a reduction in NDF digestibility with laying hens.

Several mechanisms have been proposed to explain possible negative effects of HS on nutrient digestibility. Lower expression and activity of digestive enzymes, including trypsin, chymotrypsin, lipase, amylase, and maltase, have been observed in broilers reared under high temperatures (Hai et al., 2000; Song et al., 2018; Al-Zghoul et al., 2019). As described in Part I, oxidative stress induced by HS aggravates intestinal barrier disorders (Brugaletta et al., 2022), and hyperthermia has been associated with a reduction in upper gastrointestinal tract blood flow that can induce degradation of the intestinal mucosa (Song et al., 2014; Chegini et al., 2018). Following hot temperature exposure, the absorptive surface area of the small intestine is decreased due to a reduction in villi height, crypt depth (Song et al., 2018; He et al., 2019), and relative jejunal weight (Garriga et al., 2006). Heat stress also modulates the gene expression of several macronutrient transporters. Expression of glucose transporters SGLT1 and GLUT2 is downregulated when HS persists for several days (Sun et al., 2015; Habashy et al., 2017b; Al-Zghoul et al., 2019; Abdelli et al., 2021; Goel et al., 2021), whereas the expression of GLUT5 for the transport of fructose is increased (Habashy et al., 2017b). Despite the relatively greater decrease in AA digestibility compared to other macronutrients, several studies observed no influence of HS exposure on expression of AA transporters, including CAT1, y+LAT1, PePT1, and r-Bat (Sun et al., 2015; Habashy et al., 2017b; Al-Zghoul et al., 2019). On the other hand, Habashy et al. (2017a) measured a decrease in expression of several AA transporters (i.e., CAT1, LAT1, SNAT1, SNAT 2, SNAT 7, B0AT) after 12 days of HS. However, this reduction was not consistent with the slight increase in AA digestibility (+ 3%) observed in the same study.

Furthermore, even though HS does not seem to markedly affect fat digestibility, several studies have reported decreased intestinal expression of FABP and CD36 which are both involved in the uptake of fatty acids (Sun et al., 2015; Habashy et al., 2017b; Al-Zghoul et al., 2019), whereas the expression of FATP1 was increased under chronic HS (Habashy et al., 2017b).

While the regulation of nutrient transporter gene expression might be directly related to physiological adaptations to HS, it is important to consider that structural damages and the degradation of the epithelium induced by HS might be a potential factor indirectly causing the reduction of intestinal transporters (Habashy et al., 2017b). Overall, the slight decrease and inconsistent results regarding nutrient digestibility seem to indicate that reduced digestibility likely explains only a small proportion of reduced feed efficiency under HS conditions.

Feeding strategies

Lowering HP and improving heat dissipation are two ways to reduce the adverse effects of HS in poultry. While the reduction of HP is achievable by improving digestibility and by feeding the birds closer to their nutrient and energy requirements, an increased heat dissipation is possible by increasing the amount of water loss by evaporation (Syafwan et al., 2011). Several feeding strategies have been tested to attempt to mitigate the negative impact of hot temperatures through these means.

Feed restriction and withdrawal

Early studies focused on feed restriction before HS exposure, and its effects on HP and performance. In broiler breeders, feed restriction from 44 to 48 weeks before exposure to 4 days of elevated temperatures resulted in 23% decrease in HP compared with *ad libitum* fed birds. However, fed-restricted birds had a higher HP when adjusted for body weight (BW) differences and expressed per unit of metabolic body size ($BW^{0.75}$). The lower BW of fed-restricted birds was therefore responsible for the reduction in HP and not the feed restriction *per se* (MacLeod and Hocking, 1993). In broilers, no beneficial effect of a preventative feed restriction was measured on performance and carcass quality (Plavnik and Yahav, 1998), but more promising results were obtained when feed restriction was applied during the HS period. Abu-Dieyeh (2006) observed that feed restriction to 75% and 50% of the feed consumption of *ad-libitum* fed broilers reduced rectal temperature, mortality, and feed conversion ratio (FCR). However, feed restriction diminished the rate of BW gain (BWG) and delayed marketing age of the birds.

Similarly, feed withdrawal for at least 6 h during HS decreased the corporal temperature (Yalçin et al., 2001; Özkan et al., 2003; Lozano et al., 2006), mortality (Yalçin et al., 2001) and heterophil-to-lymphocyte ratio (Yalçin et al., 2003) of broilers, indicating a reduction of the adverse effects of HS. Nevertheless, effects on performance were not consistent throughout the studies, with some observing a growth improvement (Yalçin et al., 2001; Mohamed et al., 2019) and others reporting a growth degradation (Lozano et al., 2006) likely due to the timing and magnitude of feed restriction (Özkan et al., 2003). Therefore, a short feed withdrawal during the hottest period of the day appears to be the best strategy to minimize the negative effects of HS on growth and delayed market ages. Removing the feed a few hours before the HS period could also be beneficial to avoid the potential increased in HP induced by anticipatory feeding behavior observed in birds exposed to repeated intermittent fasting (Fondevila et al., 2020).

Dual feeding

Dual feeding is characterized by the distribution of two different diets, one more concentrated in protein and the other more concentrated in energy, that are provided either simultaneously for self-selection or in sequential order. Dietary proteins are known to have a higher thermogenic effect compared with carbohydrates (Geraert, 1991), and feeding high protein diets during the coolest period of the day has been hypothesized to improve the thermotolerance of birds. Sequential feeding of high energy and high protein diets decreased body temperature (De Basilio et al., 2001; Lozano et al., 2006) and mortality (De Basilio et al., 2001), but reduced or did not improve the growth of broilers. Syafwan et al. (2012) tested self-selection under hot temperatures by providing a high-protein diet (CP: 299 g/kg; ME: 2,780 kcal/kg) and a high-energy diet (CP: 150.7 g/kg; ME: 3,241 kcal/kg) and showed that choice-fed and control-fed birds with a standard diet (CP: 215 g/kg; ME: 2,895 kcal/kg) performed similarly, although the former had 14% lower protein intake and 6.4% higher energy intake. However, no data on carcass composition were reported, and a lower protein intake could reduce muscle deposition. While a dual-feeding approach might be feasible in tropical areas and less-intensive production systems, Iyasere et al. (2021) estimated that it is not suitable for most commercial production operations due to cost and logistical constraints.

Wet feeding

Water is the most important nutrient in broiler nutrition, and it plays an essential role for thermoregulation under hot temperatures. Heat stress increases water loss through the respiratory tract as birds pant to increase heat loss by evaporative cooling (Richards, 1970; Bruno et al., 2011). In the light of the importance of water for the nutrition and physiology of broilers, wet feeding attempts to maximize water intake and utilization. Several studies have investigated the effect of wet feeding, i.e., the use of high moisture diets, on poultry performance under thermoneutral conditions (Moritz et al., 2001; Shariatmadari and Forbes, 2005; Khoa, 2007) and during HS. In heat-stressed broilers, Kutlu (2001) measured increased BWG, DM intake, carcass weight, protein content, but also increased abdominal fat and lipid content per unit of carcass weight, and reduced DM conversion efficiency (DM intake/BWG), when feed was mixed with the same amount of water. Similarly, Awojobi et al. (2009) and Dei and Bumbie (2011) observed increased BWG with wet-fed birds (addition from 1 to 2 parts of water to 1 part of dry feed) reared in tropical conditions. In laying hens, Tadtiyanant et al. (1991) reported that wet feeding increased DM intake, but no beneficial effects were found on performance. In contrast to these results, egg production and egg weight were increased by wet feeding in Japanese quails (Okan et al., 1996a; 1996b). Despite somewhat positive impacts of wet feeding in poultry, its application in the field remains limited due to an increased risk of fungal growth and resulting mycotoxicosis in birds (Wasti et al., 2020).

Feed form (mash vs. crumble vs. pellets) and feed structure (particle size)

Three different forms of feed are generally used in the poultry industry: mash, crumble, and pellets. Under thermoneutral conditions, pelleted feed is known to increase FI and BWG and improve digestibility (Massuquetto et al., 2018; Massuquetto et al., 2019). During summer, increased feed efficiency and egg production of laying hens have been observed for pelleted diets compared with mash diets (Almirall et al., 1997). In broilers exposed to cyclic HS, Cardoso et al. (2022) measured increased FI (+ 10%), BWG (+ 8.3%), CP digestibility (+ 2.3%), and energy utilization (apparent metabolizable energy, AME and nitrogen-corrected apparent metabolizable energy, AMEn) when feeding a pelleted diet compared with a mash diet. However, pelleting did not improve FCR, livability, or the feed production cost to kg of bird produced ratio. Likewise, Hosseini and Afshar (2017a) observed beneficial effects of pelleting on performance and digestibility when comparing mash, crumbled and pelleted diets under similar cyclic HS conditions. These authors also reported improved carcass weight and yield in heat-stressed broilers fed pelleted diets. Comparable performance improvements were obtained by feeding pelleted diets under thermoneutral and HS conditions (Serrano et al., 2013), so it is likely that mechanisms responsible for the positive effects of pelleting under thermoneutrality can be applied to HS conditions. Pelleting feed has been shown to lower feed wastage (Gadzirayi et al., 2006) and increase feed consumption, while concomitantly reducing physical activity and HP (Skinner-Noble et al., 2005; Latshaw and Moritz, 2009). Furthermore, as observed under thermoneutral (Abdollahi et al., 2011; Serrano et al., 2013) and HS conditions (Hosseini and Afshar, 2017b), pelleted diets reduce the relative weight of the digestive tract compared with birds fed mash diets. The pelleting process can further reduce ingredient particle size, reducing the mechanical stimulation of the gizzard and could therefore lower the energy requirements for maintenance. It also could release some inaccessible nutrients and enhance energy utilization, which could explain the increase in abdominal fat observed by Hosseini and Afshar (2017a) with pelleted diets fed under cyclic HS. Other potential benefits of feeding pelleted feeds during HS shown by these authors include increased villus length and villus to crypt depth ratio in the jejunum (Hosseini and Afshar, 2017b) as well as decreased breast HSP70 mRNA expression, breast creatine kinase protein level, and heterophil-to-lymphocyte ratio (Hosseini and Afshar, 2017a). Collectively, these reports indicate that pelleting attenuates the harmful effects of high ambient temperature in broiler chickens.

Concerning particle size, the use of coarse particles (2,280 μm) of corn increased panting compared to finer particles (605 μm) in broilers fed a mash diet under natural HS conditions (Santos et al., 2019). Similar results were found in laying hens under a semiarid environment, where coarser corn particles increased rectal temperature, respiratory rate, and decreased eggshell quality (De Souza et al., 2015). However, while coarse particles may increase the thermal challenge, they are also known to increase FI and improve performance in broilers under thermoneutral conditions (Amerah et al., 2008; Naderinejad et al., 2016). Thus, more research on broiler performance would be required to fully understand the role of ingredient particle size during HS.

Dietary energy density and lipid supplementation

The marked decrease in FI and in turn, energy intake, caused by elevated temperatures negatively affects bird performance. The effect of HS on energy utilization of feedstuffs, which is usually represented as AME, is still not well defined. Indeed, responses probably depend on the parameters of the HS imposed and characteristics of the diet, as some studies observed an increase in AME due to hot temperatures (Keshavarz and Fuller, 1980; Geraert et al., 1992), some observed no difference between thermoneutral and HS conditions (Yamazaki and Zi-Yi, 1982; Faria Filho et al., 2007; De Souza et al., 2016), and some have reported a decrease in AME with HS birds (Bonnet et al., 1997). However, three studies using the comparative slaughter technique with broilers placed under thermoneutral and HS conditions from d 28 to 42 (Geraert et al., 1996), or d 21 to 42 (Faria Filho et al., 2007; De Souza et al., 2016), indicate a decrease in retained energy and increase in HP per unit of feed when birds are placed under hot temperatures. Similarly, a quadratic effect of the temperature on the energy requirement for maintenance functions was measured by Sakomura et al. (2005), with the lowest requirements estimated at 25.2°C: $ME_m = BW^{0.75}$ x (307.87 + 15.63 T + 0.31 T^2), with T being the temperature (°C) and $BW^{0.75}$ the metabolic body size. Therefore, the relative contribution of maintenance energy requirements to total energy requirements is partly increased by the lower growth of HS birds, but also directly impacted by the increased temperature, which results in a diminishing effect on feed efficiency.

To compensate for lower energy intake of birds during HS, it has become common for producers in hot climate areas to feed higher energy diets (Wasti et al., 2020). Early studies suggested that high dietary energy concentrations could improve bird performance under constant (Dale and Fuller, 1979) and cyclic HS (Dale and Fuller, 1980), but it should be noted that the CP content of the diets were adjusted to energy levels and thus higher in high energy diets. Nonetheless, more recent studies using isonitrogenous diets have confirmed previous observations and showed that an increase in dietary metabolizable energy (ME) between 100 and 200 kcal/kg for broilers improved BWG up to 17% and FCR up to 10% (Raju

et al., 2004; Ghazalah et al., 2008; Attia et al., 2011, 2018; Attia and Hassan, 2017) when reared under hot conditions. In addition, decreased skin and rectal temperatures have been observed in HS poultry fed diets with increased ME content (Al-Harthi et al., 2002; Attia et al., 2011). Increasing dietary ME content also improved ready-to-cook yield (Raju et al., 2004), although no improvement in carcass yield was observed by Ghazalah et al. (2008). However, both research groups reported an increased abdominal fat yield, thus the risk of increasing carcass yield from lipid and not protein deposition is a potential disadvantage of increasing dietary ME in HS broilers.

Increasing ME density in the diet is usually achieved by increasing the concentration of added lipid, and this strategy presents several potential advantages for HS birds. Feeding isocaloric diets with either higher proportions of carbohydrates or fat under HS conditions revealed that broilers had better performance when diets were supplemented with poultry fat, palm oil, or soybean oil compared to no fat supplementation (Zulkifli et al., 2006; Ghazalah et al., 2008). These observations are likely explained by the lower heat increment of fat oxidation compared with carbohydrates and proteins. Indeed, as measured by Fuller and Rendon (1977), high fat diets lead to lower heat increment than low-fat diets. Moreover, lipid inclusion improves nutrient digestion by slowing rate of passage (Mateos et al., 1982) and increasing the energy value of other nutrients (Aardsma et al., 2017). Lipid metabolism also generates more metabolic water than carbohydrate and protein catabolism, which can in turn be used for heat dissipation by evaporation (Barboza et al., 2009). Thus, as suggested by Ghazalah et al. (2008), a potential dietary recommendation for broilers exposed to hot temperatures could be to increase the ME level up to 3,300 kcal/kg, with lipid inclusion up to 5%, especially during the finishing period when birds are the most sensitive to high temperatures.

Although increasing dietary lipid additions has been shown to be a promising way to increase bird performance under HS conditions, less research has been conducted to compare the efficacy of different lipid sources. Zulkifli et al. (2006) did not observe a difference in BWG and FCR among broilers exposed to 34°C and supplemented either with 8% of palm oil or 8% of soybean oil. Abdominal fat and breast intramuscular fat deposition were also unaffected by the fat source. However, in broilers exposed to HS from 32 to 42 days post-hatch and fed isocaloric diets, improved FCR and BWG were observed when feeding diets with coconut oil or beef tallow than with diets containing olive or soybean oil (Seifi et al., 2020). The fatty acids within coconut oil and tallow are rich in saturated fatty acids and have chain lengths of mainly 12 and 16 carbons, respectively, while olive oil and soybean oil are rich in unsaturated fatty acids and have predominantly 18 carbon fatty acids. Short and medium chain fatty acids (SCFAs/MCFAs), containing up to 12 carbon atoms, are absorbed and metabolized more rapidly than longer chains, as they are transported to the portal vein as free fatty acid and do not require any transporter to get absorbed (Guillot et al., 1993), which could reduce the HP induced by digestion. Recent research also suggests saturated fatty acids, SCFAs, and MCFAs could have a beneficial impact on the mitochondrial metabolism and electron transport chain (Schönfeld and Wojtczak, 2016; Seifi et al., 2018, 2020; Hecker et al., 2021), which are known to be disrupted under HS condition (Akbarian et al., 2016).

Influence of dietary crude protein content

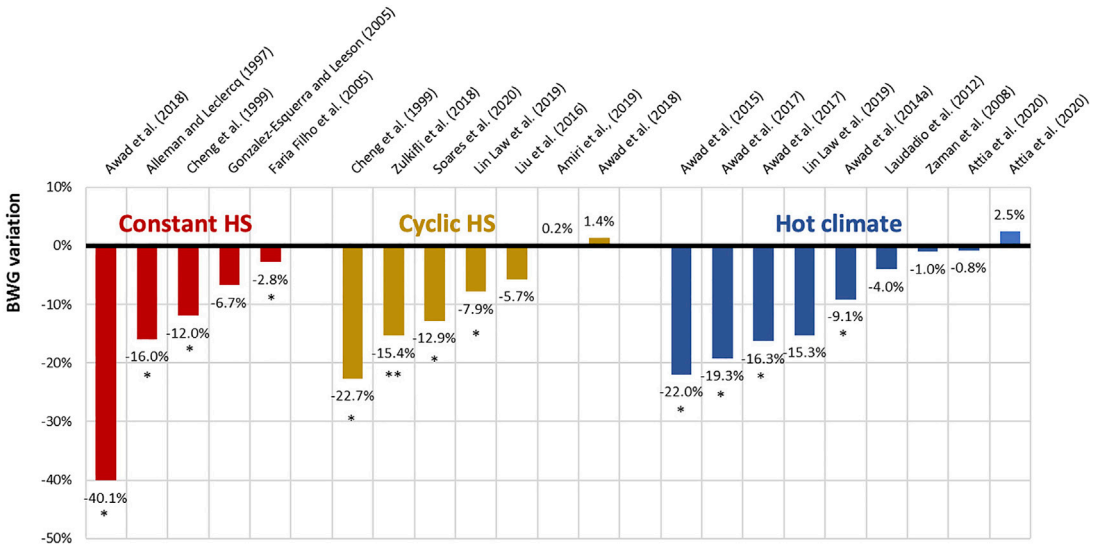
Proteins have a higher caloric increment than carbohydrates and fat (Musharaf and Latshaw, 1999) and therefore increase the diet-induced HP. When AA are metabolized for energy by birds, much of the HP is caused by deamination reactions and incorporation of N into uric acid (Smith et al., 1978; Swick et al., 2013). Therefore, optimizing dietary CP composition to better fit bird requirements decreases the heat produced during AA oxidation. So, in an effort to reduce the energy released during digestion, absorption, and metabolism of nutrients, dietary CP reductions have been proposed as a strategy to mitigate the harmful effects of HS in poultry (Furlan et al., 2004). Numerous studies in broilers have tested the effects of feeding a reduced CP diet versus a standard CP diet under constant HS (Alleman and Leclercq, 1997; Cheng et al., 1999; Faria Filho et al., 2005; Gonzalez-Esquerra and Leeson, 2005; Awad et al., 2018), cyclic HS (Cheng et al., 1999; Liu et al., 2016; Awad et al., 2018; Zulkifli et al., 2018; Amiri et al., 2019; Lin Law et al., 2019; Soares et al., 2020) and hot climates (Zaman et al., 2008; Laudadio et al., 2012; Awad et al., 2014, 2015, 2017; Lin Law et al., 2019; Attia et al., 2020). Table 1 summarizes 21 HS broiler trials comparing reduced CP diets (ranging from 143 to 190 g/kg CP) and standard CP diets (ranging from 183 to 223 g/kg CP), with both diets in each study formulated to meet or exceed a specific nutritional requirement, such as the Nutrient Requirements of Poultry (NRC, 1994) or breeder recommendations, or to contain similar AA profiles. Approximately half of these studies observed a significant reduction in performance when feeding broilers the reduced CP diet compared to the standard CP diet, while the other half did not observe dietary effects. The response variability can be partly explained by the range of low and standard CP levels, as well as the intensity and duration of the HS period, but it is important to note that feeding a low CP diet without degrading performance is still beneficial for reducing nitrogen excretion. Results for BWG, presented in Figure 1, indicate that regardless of the HS challenge type, reduced CP diets decreased BWG by 10.8% on average (ranging from a reduction of 40.1% to an improvement of 2.5%). Similar results were obtained with FCR, with an average increase of 6.9% (ranging from a decrease of 0.9% to an increase of 19.7%) when dietary CP was reduced (Figure 2). Some studies also reported a

TABLE 1 Summary of experimental conditions of broiler studies comparing reduced and standard CP diets under HS conditions.

Heat stress condition	Heat stress length	Average temperature (°C)	Standard CP (g/kg)	Reduced CP (g/kg)	Age start (d)	Age end (d)	Duration (d)	References
Constant HS	—	34	194	143	22	42	20	Awad et al. (2018)
	—	32	199	160	23	44	21	Alleman and Leclercq (1997)
	—	32.2	198	161	21	49	28	Cheng et al. (1999)
	—	31.4	200	180	21	42	21	Gonzalez-Esquerra and Leeson (2005)
	—	33	200	185	7	21	14	Faria Filho et al. (2005)
Cyclic HS	35°C for 8 h	29.4	198	161	21	49	28	Cheng et al. (1999)
	33°C for 6 h	25.5	190	162	22	35	13	Zulkifli et al. (2018)
	32°C for 8 h	26	200	160	22	42	20	Soares et al. (2020)
	34°C for 7 h	26.2	183	167	22	42	20	Lin Law et al. (2019)
	—	27.8	213	153	28	42	14	Liu et al. (2016)
	34°C for 8 h	NA	195	175	0	42	42	Amiri et al. (2019)
	34°C for 7 h	26.2	194	143	22	42	20	Awad et al. (2018)
Hot climate	—	At least 28.1	223	161	0	21	21	Awad et al. (2015)
	—	At least 28.1	223	162	0	21	21	Awad et al. (2017)
	—	At least 28.3	223 and 194	162 and 135	0	42	42	Awad et al. (2017)
	—	At least 28.5	216 and 187	176 and 156	0	35	35	Lin Law et al. (2019)
	—	At least 28.3	207	177	0	21	21	Awad et al. (2014)
	—	NA	205	185	14	42	28	Laudadio et al. (2012)
	—	NA	210	190	0	28	28	Zaman et al. (2008)
	—	34	190	155	28	49	21	Attia et al. (2020)
	—	35	186	152	30	45	15	Attia et al. (2020)

decreased FI with reduced CP diets (Cheng et al., 1999; Awad et al., 2014, 2015, 2017, 2018). In addition to a reduced CP diet, some researchers tested the effects of a higher CP diet, with CP levels above the standard recommendations. During HS, high CP diets resulted in a decrease (Cheng et al., 1999) or an increase in BWG (Faria Filho et al., 2005) and a decrease in FCR (Cheng et al., 1999; Faria Filho et al., 2005; Gonzalez-Esquerra and Leeson, 2005). However, other studies reported no effect of high versus standard CP diets (Zaman et al., 2008; Laudadio et al., 2012; Soares et al., 2020) and the increased diet cost associated with high CP diets could result in detrimental economical scenarios (Cardoso et al., 2022).

Feed-grade AA, which are included at higher levels in reduced CP diets to meet digestible AA requirements, allow to provide a balanced AA diet, and minimize the HP caused by AA oxidation, which is not possible to reach when relying on feed sources only. They also do not need enzymes for digestion and, as such, do not contribute to the digestion-related production of body heat (Morales et al., 2020). However, the performance degradations reported with reduced CP diets aligns with the lower HP observed in birds fed a high CP diet (220 g/kg) versus a low CP diet (160 g/kg) under cyclic HS conditions (Soares et al., 2020). Similar results have also been obtained under constant HS when comparing a high (230 g/kg), standard (200 g/kg), and low (170 g/kg) CP diets (Faria Filho et al., 2007). The lack of interaction between the CP level and environmental temperature reported by these authors is supported by studies conducted under thermoneutral conditions, where no difference (Noblet et al., 2003, 2007) or an increase (Swennen et al., 2004) in HP was measured with low CP diets, indicating that HS is not the cause *per se* of the higher HP with reduced CP diets. These results are surprising due to the higher caloric increment of proteins, but a possible explanation is that standard CP diets are usually formulated with a higher oil inclusion rate to reach the same amount of energy than reduced CP diets which generally have higher inclusion of corn (Soares et al., 2020). The extra-metabolic effect of dietary lipids, where the metabolizable energy value of the lipid exceeds its gross energy value (Aardsma et al., 2017), could compensate for the possible increase in heat increment derived from protein (Soares et al., 2020). Interestingly, reduced CP diets with AA deficiencies have also been associated with a greater plasma level of triiodothyronine (Carew et al., 1983, 1997; Buyse et al., 1992), which is known for its thermogenic effect (Collin et al., 2003).



$$BWG\ variation\ (\%) = \frac{BWG\ reduced\ CP\ diet - BWG\ standard\ CP\ diet}{BWG\ standard\ CP\ diet} \times 100$$

The presence of a * indicates a significant difference in BWG between the reduced and standard CP diet, while ** indicates a significant linear effect across several CP levels

FIGURE 1
Effect of reduced and standard CP diets on BWG of broilers exposed to different HS conditions.

Overall, simultaneously increasing dietary energy and CP could be a potentially beneficial strategy to limit the adverse effects of HS on broiler growth and feed efficiency. Indeed, improved performance has been demonstrated under HS conditions when broilers were fed both a high dietary ME and CP contents (Attia et al., 2006; Attia and Hassan, 2017). However, in a similar study in which broilers were exposed to thermoneutral temperatures or cyclic HS from day 19–42 and were fed with a dietary ME and CP content of 3,152 kcal/kg and 194.8 g/kg or 3,253 kcal/kg and 210.3 g/kg, respectively, no improvement in performance was observed with the higher nutrient and energy density diet in either environment. Consequently, an economic evaluation actually showed a decrease in overall profitability with the higher density diets (Cardoso et al., 2022).

The conflicting evidence of higher caloric increment of dietary protein and impaired performance of broilers fed reduced CP diets led Gonzalez-Esquerra and Leeson (2006) to conclude that no consensus has been reached on protein requirements of heat-stressed birds. More recent trials on reduced CP diets have shown no performance improvements or amelioration of HP reduction and do not support this dietary strategy under HS conditions. Nonetheless, when following the "ideal protein" concept, where all essential digestible AA are provided in balance (Baker and Chung, 1992), the supplementation of unbound feed-grade AA in reduced CP diets to satisfy the bird's requirements should result in similar performance as when feeding standard CP diets. Furthermore, most of the studies presented above based their requirements on NRC or breeder recommendations, albeit broiler's AA requirements under HS conditions still remain undefined. More importantly, although those studies met specific nutritional requirements for essential AA, some potentially limiting AA such as Arg, Thr, Ile, Leu, His, and Phe were not equally balanced between diets. Diets with AA imbalance can lead to adverse effects especially under HS condition as they normally increase HP (Sekiz et al., 1975). Also, the FI reduction triggered by HS reduces the amount of CP and AA ingested by the birds, potentially resulting in deficiency when compared with reduced dietary concentrations. Therefore, even if the inclusion level for all AA was formulated to meet or exceed a target nutritional requirement under thermoneutral conditions, the effective AA consumption may not have reached the bird's requirements for some AA under HS conditions.

Further research on dietary CP and its interaction with energy and AA content would be required to better characterize the biological response induced by those diet changes under HS conditions. This would allow for a better understanding on the utilization of those nutrients in poultry reared under hot temperature to ultimately facilitate better

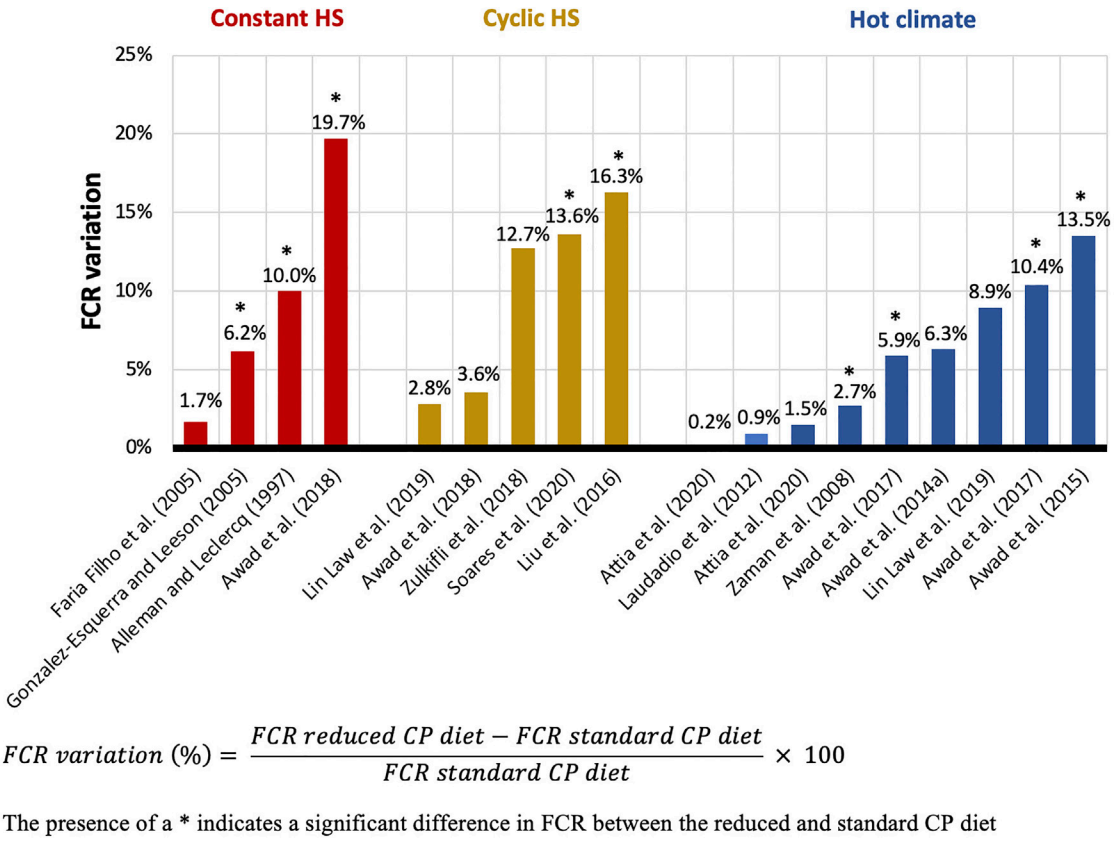


FIGURE 2
Effect of reduced and standard CP diets on FCR of broilers exposed to different HS conditions.

prediction of economic outcomes associated with nutritional dietary variation.

Supplementation of amino acids

Amino acid density

Altering dietary density of essential AA has been shown to have promising results in heat-stressed broilers. In broilers under hot temperatures, Maharjan et al. (2020) fed five levels of digestible Lys (dLys) from 80% to 120% of the recommended level with all other AA:dLys ratios held constant and observed quadratic responses in average daily gain and FCR up to the 120% dLys level, and no influence on FI. In contrast, the optimal average daily gain and FCR were closer to the 100% recommendation of dLys under thermoneutral conditions. This indicates a potential increase in overall AA requirements under HS, although the authors concluded that the requirement of AA/Mcal was not different in hot or thermoneutral environments, which was also observed by Hruby et al. (1995). Moreover, Alhotan et al. (2021) fed an AA density ranging from 80% to 110% of breeder recommendations to broilers exposed to cyclic HS. In contrast with the results reported by Maharjan et al. (2020), no interactions between environmental temperature and dietary AA density were observed on performance and processing data. However, linear effects of AA density indicated that BWG, feed efficiency, and breast muscle yield responded to increased AA density in both environments. Even though FCR was numerically improved by 10 points with the 110% AA diet relative to the 100% AA diet, this difference was not statistically significant and may indicate that higher AA levels were above the bird's requirements. In another trial, increasing the density of Met, Lys, and Thr in a reduced CP diet increased production performance of cyclically heat-stressed broilers over the ones obtained with standard CP diet and, in addition, improved intestinal health as indicated by changes in small intestinal morphology and increased mRNA expression of some tight junction proteins (Wang et al., 2022). Therefore, increasing AA density could be beneficial for broilers experiencing HS, especially when achieved with free AA to minimize diet-induced thermogenesis. However, further research is required to better characterize the true AA requirements of birds under HS conditions.

Individual amino acid supplementation

Methionine (Met) is the first limiting AA in avian species and is considered, along with cysteine (Cys), to meet total sulfur AA (TSAA) needs for the bird. Because of its importance in maintenance functions and muscle deposition that are greatly impacted during exposure to HS, defining Met requirements is an important step in optimizing poultry nutrition under HS conditions. Indeed, higher requirements of Met have been found in broilers under high temperatures compared to thermoneutral conditions (Silva Junior et al., 2006; Sahebi-Ala et al., 2021), but this does not appear to be the case in laying hens or pullets (Bunchasak and Silapasorn, 2005; Castro et al., 2019). Several physiological mechanisms have been proposed regarding the importance of Met under HS. First, Met supplementation has been shown to increase the antioxidant capacity of broilers (Del Vesco et al., 2015b; Gasparino et al., 2018; Liu et al., 2019; Santana et al., 2021). Under thermoneutral conditions, the production of reactive oxygen species and the antioxidant systems in chickens are balanced and can adapt to overcome normal challenge. Acute and chronic HS disturb this equilibrium due to an overproduction of reactive oxygen species, which ultimately surpasses the antioxidant capacity and leads to oxidative stress (Lin et al., 2000, 2006a; Azad et al., 2010; Akbarian et al., 2016). Furthermore, Met supplementation affected the inflammation-related gene expression in the liver of broilers placed under high temperature (Liu et al., 2019). Another potential benefit of Met supplementation under HS is its stimulatory effect on protein deposition and inhibition of protein breakdown as indicated by the increased expression of protein synthesis-related genes IGF1, GHR and PI3KR1 in the liver, and decreased expression of protein degradation-related genes atrogin1 and CTSL2 in the breast (Del Vesco et al., 2013; 2015a).

Beneficial effects of increasing the dietary amount of essential AA other than Met are not as well defined. Dietary levels of Lys, the second limiting AA in broiler chicken diets based on corn and soybean meal (Ishii et al., 2019), are closely associated with muscle protein deposition. However, the growth depression under HS does not seem to be ameliorated by supplementing broiler diets with Lys above the thermoneutral requirements (Mendes et al., 1997; Corzo et al., 2003; Attia et al., 2011). Interestingly, when Lys was supplemented in combination with Met in a reduced CP diet, broilers had similar performance and carcass characteristics to those fed a higher CP diet under hot climate conditions (Attia et al., 2020). In this study, additional treatments with supplementation of other essential AA besides Met and Lys did not ameliorate performance reductions caused by HS, emphasizing the potential importance of those two AA under HS conditions.

Threonine is almost invariably the third limiting AA in poultry diets (Kidd, 2000). In broilers, the earliest studies on Thr supplementation above the estimated requirements for birds under hot temperatures showed no or minimal benefits on performance (Dozier et al., 2000; Kidd et al., 2000; Ojano-Dirain and Waldroup, 2002; Shan et al., 2003), whereas more recent studies have shown some performance improvements (Debnath et al., 2019; Miah et al., 2022). In laying hens, increasing the supplementation of dietary Thr to 0.66% instead of 0.43% did not improve performance outcomes, but it decreased HSP70 in the ileum (Azzam et al., 2019) and increased SOD concentration in both serum and liver (Azzam et al., 2012), indicating potential antioxidant effects of Thr under HS condition.

Unlike mammals, poultry are highly dependent on dietary Arg supply because of less active *de novo* Arg synthesis pathways in birds (Klose et al., 1938; Tamir and Ratner, 1963; Castro and Kim, 2020). In broilers, the determination of Arg requirements under HS conditions have led to inconsistent results among different age periods. Over-supplementation was detrimental from 1 to 3 weeks of age (Chamruspollert et al., 2004), neutral from 3 to 6 weeks of age (Mendes et al., 1997), and beneficial from 6 to 8 weeks of age (Brake, 1998). Arg supplementation also improved FCR of Pekin ducks exposed to cyclic HS (Zhu et al., 2014) and enhanced several welfare indicators and decreased corticosterone plasma concentration in laying hens during the hot summer period (Bozakova et al., 2015). Furthermore, increasing dietary Arg improved performance, reproduction, antioxidant status, immunity, and maternal antibody transmission in quails (Kalvandi et al., 2022). The ability of Arg to reduce physiological stress is likely to be attributed to its antioxidative effects (Gupta et al., 2005). Arg is also the only nitrogen donor in the production of nitric oxide, which is involved in vasodilatation to potentially aid thermoregulation of heat-stressed birds (Uyanga et al., 2021). Interestingly, more focus is being placed on the potential beneficial effects of citrulline (Cit), a compound synthetized during Arg catabolism and the formation of nitric oxide. Recent studies have shown that Cit supplementation can effectively increase systemic Arg levels, even more than direct L-Arg supplementation (Morita et al., 2014; Agarwal et al., 2017). Cit concentration in blood has also been shown to be modulated by hot temperatures (Chowdhury et al., 2014; Chowdhury, 2019) and its supplementation may increase nitric oxide synthesis, provide an anti-inflammatory response, and enhance the central regulation of body temperature (Chowdhury et al., 2017; Uyanga et al., 2021, 2022).

Leu, Ile, and Val are three essential AA collectively known as branched-chain AA (BCAA). Their roles are diverse and include effects on performance, immunity, and intestinal health. They also serve as signaling molecules in the regulation of glucose, lipid, and protein synthesis (Kim et al., 2022). Kop-Bozbay et al. (2021) investigated the effect of increased BCAA density under HS conditions and did not observe any improvement in growth performance. These authors also tested various dietary Val

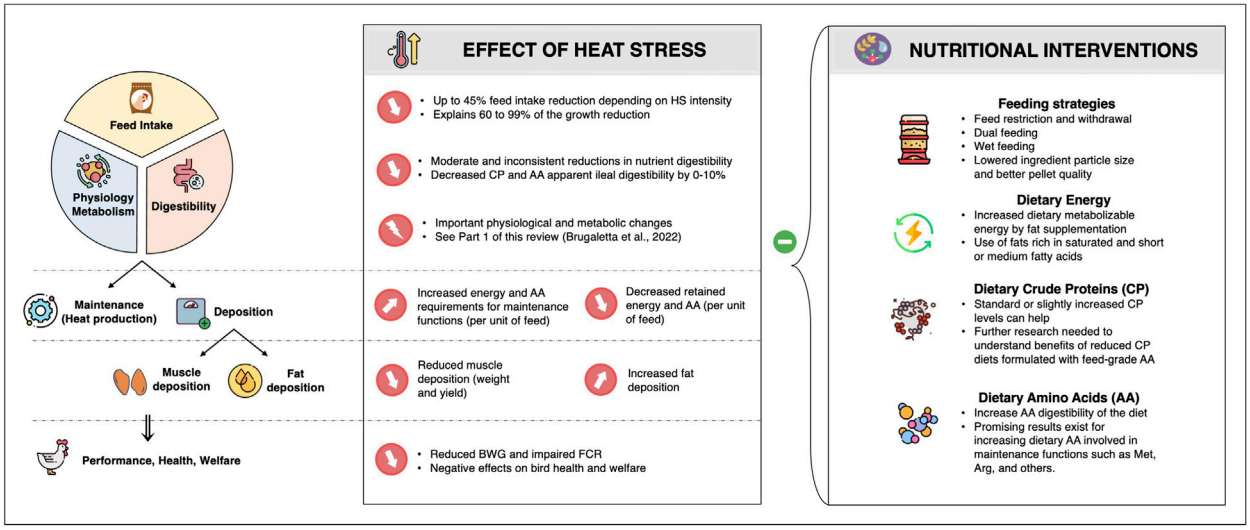


FIGURE 3
Conclusive scheme of the beneficial nutritional interventions on broilers exposed to heat stress conditions.

concentrations and did not observe effects on performance. However, high incorporation of Leu in those diets might have triggered the antagonist effect among BCAA (Ospina-Rojas et al., 2020). Interestingly, in ovo Leu injection improved BWG and thermotolerance of birds during subsequent exposure to HS (Han et al., 2017, 2019, 2020; Chowdhury et al., 2021). With the current increasing availability of feed-grade Val and Ile, further work is needed to define the potential for BCAA to combat HS in poultry.

Trp is an essential AA in poultry diets due to its need for protein synthesis, as well as serotonin and niacin production (Le Floc'h et al., 2011). Few studies have been published on the requirements of Trp under HS conditions, although high dietary concentrations did not improve performance in broilers (Tabiri et al., 2002; Shan et al., 2003; Badakhshan et al., 2021) or layers (Dong et al., 2012). However, Trp supplementation did decrease rectal temperature and abated corticosterone responses caused by HS in broilers (Badakhshan et al., 2021). Trp supplementation also increased eggshell quality and decreased SOD serum concentration in laying hens during HS (Dong et al., 2012). To our knowledge, no studies on the effect of dietary supplementation of less-limiting essential AA beyond Trp, such as His and Phe, have been conducted in poultry subjected to HS.

The remaining AA are non-essential AA and can be synthesized from other precursors. Besides altering essential AA needs, the reduced FI caused by HS limits the amount of nitrogen consumed by birds, which could potentially lead to a lack of sufficient nitrogen quantity for non-essential AA synthesis (Awad et al., 2014). Feeding low CP diets during hot temperatures could also worsen this nitrogen deficiency. Birds fed a diet with increased essential and non-essential AA concentrations under HS had a better performance than when a diet with only increased essential AA concentrations was fed (Awad et al., 2014, 2015). However, when comparing individual supplementation of several non-essential AA in low CP diets, only Gly improved broiler FCR under both normal and acute HS conditions (Awad et al., 2015, 2018). Recent research also suggests that Gly and Ser, which are normally evaluated together as Gly equivalents, are co-limiting or limiting before some BCAA in low CP diets under thermoneutral conditions (Chrystal et al., 2020; Maynard et al., 2022), which could make Gly equivalents important AA to consider during reduced FI caused by HS.

Therefore, for the essential AA, it seems possible that supplementation of Met and potentially Arg above current requirements could be beneficial under HS condition. However, further research is required to elucidate the effects of other essential AA, as well as Gly, non-essential AA, and overall nitrogen supply.

Conclusion

Adaptating to rising global temperatures while maintaining production efficiency is an important emerging challenge for the poultry industry. Under hot temperatures, birds reduce their FI to lower HP, and this is the main factor explaining the degradation of bird performance (Figure 3). Mitigation of those negative effects requires a holistic approach, and adjusting feeding practices and nutritional programs have a

critical role to play. Even though some feeding strategies are difficult to implement in the field, especially with intensive rearing systems, several practices discussed in this review have shown beneficial effects to reduce the heat load on poultry. Increasing dietary lipid concentration and maintaining a standard CP level are also recommended to compensate for the FI reduction and better fit the birds' requirements under elevated temperatures. Considering an increase in the density of some AA, like methionine and arginine, to meet the increased AA requirements for maintenance functions could also be advantageous. Therefore, further research is required to characterize nutrient partitioning and requirements of birds under HS conditions to ensure efficient and cost-effective solutions for the poultry industry.

Author contributions

JRT and SR conceived this review section (Part II) with input from GB and FS. JRT wrote Part II of the review under the supervision of SR. GB, FS, SD, and SR revised this manuscript. All authors read and approved the submitted version of this manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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EDITED BY
Vincent M. Cassone,
University of Kentucky, United States

REVIEWED BY
Andrew S. Cooke,
University of Lincoln, United Kingdom
Sabine G. Gebhardt-Henrich,
University of Bern, Switzerland
Xavier Averós,
Neiker Tecnalia, Spain

*CORRESPONDENCE
Ilaria Biasato,
ilaria.biasato@unito.it

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Welfare implications for broiler chickens reared in an insect larvae-enriched environment: Focus on bird behaviour, plumage status, leg health, and excreta corticosterone

Ilaria Biasato[1]*, Sara Bellezza Oddon[1], Giulia Chemello[2], Marta Gariglio[3], Edoardo Fiorilla[3], Sihem Dabbou[4], Miha Pipan[5], Dominik Dekleva[5], Elisabetta Macchi[3], Laura Gasco[1] and Achille Schiavone[3]

[1]Department of Agricultural, Forest and Food Sciences, University of Turin, Turin, Italy, [2]Department of Life and Environmental Sciences, Marche Polytechnic University, Ancona, Italy, [3]Department of Veterinary Sciences, University of Turin, Turin, Italy, [4]Center Agriculture Food Environment, University of Trento, Trento, Italy, [5]Entomics Biosystems, Cambridge, United Kingdom

The use of insect live larvae as environmental enrichment has recently been proposed in broiler chickens, but the concomitant administration of black soldier fly (BSF) and yellow mealworm (YM) has never been tested yet. Therefore, the present study aims to evaluate the effects of live BSF and YM larvae as environmental enrichments for broiler chickens by means of plumage status, behaviour, leg health, and excreta corticosterone metabolites (CM). A total of 180 4-day old male Ross 308 broiler chickens were randomly distributed in 3 experimental treatments (6 replicates/treatment, 10 birds/replicate) and fed for 35 days as follows: 1) control (C, commercial feed), 2) BSF: C + 5% of the expected daily feed intake [DFI] live BSF larvae and 3) YM: C + 5% of the expected DFI live YM larvae. Feathering, hock burn (HB) and footpad dermatitis (FPD) scores (end of the trial), as well as behavioural observations (beginning of the trial [T0] and every 11 days [T1, T2 and T3] during morning, larvae intake and afternoon) through video recordings, were assessed, and excreta samples collected to evaluate the CM. Feathering, HB and FPD scores, and excreta CM were unaffected by insect live larvae administration ($p > 0.05$). In the morning, the insect-fed birds displayed higher stretching, wing flapping, ground pecking (at T1 and T3), as well as lower preening (at T1 and T2), than the C group ($p < 0.05$). During the larvae intake, higher scratching, wing flapping and ground pecking, as well as lower stretching, preening and laying down, were observed in the insect-fed (scratching, stretching and laying down) or YM-fed (wing flapping, ground pecking and preening) groups than the C birds ($p < 0.05$). In the afternoon, insect live larvae administration increased wing flapping (YM) and laying down (BSF and YM), as well as decreased ground pecking (YM, $p < 0.05$). In conclusion, the administration of insect live larvae as environmental enrichment (especially YM) was capable of positively influencing the bird

welfare through the stimulation of foraging behaviour, increase in activity levels, and reduction in bird frustration, without affecting the plumage status, leg health, and excreta CM.

KEYWORDS

black soldier fly, broiler chickens, environmental enrichment, welfare, yellow mealworm

Introduction

Insects are nowadays recognized as excellent biofactories for their peculiar ability to valorise a wide spectrum of waste materials by nutrition upcycling, which allows obtaining edible high-quality micro- and macro-nutrients that can be incorporated in the animal feed chain (Gasco et al., 2020). The so-obtained insect larvae are, indeed, predominantly fractionated to obtain meals and oils, which can efficiently be utilized to replace the conventional protein and lipid sources in monogastric diets (Ravi et al., 2020). However, the scientific research recently carried on revealed that insect live larvae may also potentially reach an interesting market share in the form of environmental enrichments for either poultry (Pichova et al., 2016; Veldkamp and van Niekerk, 2019; Ipema et al., 2020; Star et al., 2020; Bellezza Oddon et al., 2021; Tahamtani et al., 2021) or pigs (Ipema et al., 2021a; Ipema et al., 2021b).

Environmental enrichment can be defined as a modification of the rearing environment of captive animals aimed at improving their biological functioning and stimulating their species-specific behaviours (Newberry, 1995). The enrichment strategies currently available for broiler chickens can be grouped in 2 main categories: 1) "point-source objects", which are enrichment objects/devices that are generally limited in size and whose use is often restricted to a single or a few locations in an animal enclosure; and 2) more complex enriched environments designed to meet the key behavioural needs of the animals within them (i.e., outdoor access) (Riber et al., 2018). Among the "point-source objects", the provision of food items to stimulate the bird foraging activity represents one of the most practical and effective enrichment techniques, as search for various types of food resources on the litter has been reported to increase foraging and movement in broiler chickens (Pichova et al., 2016; Ipema et al., 2020). Such increase in overall activity levels may have implications for the intensive farming, where the fast growth rates and the high body weights are the main cause of leg problems and lameness in broilers, thus, in turn, deeply limiting their ability to move (Reiter and Bessei, 2009). Furthermore, as fast-growing broilers spend between 60 and 80% of their time sitting (de Jong and Gunnink, 2018), contact dermatitis (i.e., hock burns, breast burns and foot pad dermatitis) may also frequently occur, as a consequence of continuing contact and pressure of the skin of the breast, hocks and feet against humid and soiled bedding (Ekstrand et al., 1998). The limited space and the absence of environmental stimuli of the commercial conditions can also impair broiler welfare by limiting the possibility to perform intrinsically motivated behaviours and diminishing activity levels, thus, in turn, furtherly increasing the occurrence of leg problems (Vasdal et al., 2019), and the susceptibility to abdominal dermatitis, plumage soiling and feet and hock dermatitis (Bruce et al., 1990; Opengart et al., 2018).

Black soldier fly (BSF) and yellow mealworm (YM) live larvae provision has recently been proposed as promising food environmental enrichment to promote welfare in broiler chickens, with increased activity and foraging behaviour (as a result of the search for larvae on the ground), and reduced occurrence of hock burns and lameness (as a result of the increased activity) being observed in the administered birds (Pichova et al., 2016; Ipema et al., 2020). Welfare assessment in broiler chickens is usually object of a multiperspective approach, as heterogeneous parameters (such as plumage status, hock burns and footpad dermatitis, lameness, behavioural patterns, and excreta corticosterone) are commonly evaluated (Weimer et al., 2018; Giersberg et al., 2021; Iannetti et al., 2021; Lourenço da Silva et al., 2021). Despite beneficial live insect larvae-related effects on bird behaviour and feathering scores having recently been highlighted in either turkeys (Veldkamp and van Niekerk, 2019) or laying hens (Star et al., 2020; Tahamtani et al., 2021), data about modulation of plumage status and excreta corticosterone in broiler chickens reared in live insect larvae-enriched environment are still missing. Furthermore, no studies assessing the effects of the concomitant administration of BSF and YM live larvae as environmental enrichments are currently available in poultry.

Therefore, the present study aims to investigate the effects of BSF and YM live larvae as environmental enrichments for broiler chickens, assessing the implications for bird welfare by means of behaviour, plumage status, leg health, and excreta corticosterone metabolites (CM).

Materials and methods

Birds and experimental design

The experimental design of the present study is reported in details by Bellezza Oddon et al. (2021), as the current research is part of the same project and was performed using the same birds. In order to provide a brief summary, a total of 180 4-day old male Ross

TABLE 1 Description of the broiler ethogram (frequency and duration behaviours) considered in the present study.

Frequency behaviour	Definition
Scratching	Scraping of the litter with the claws (Ipema et al., 2020)
Preening	Grooming of own feathers with beak (Ipema et al., 2020)
Trotting	Increasing walking step with head high and breast out (Veldkamp and van Niekerk, 2019)
Pecking pen mate	Pecking movements directed at the body or beak of a pen mate (Ipema et al., 2020)
Stretching	Stretching one wing together with the leg at the same side or both wings upward and forward (Martin et al., 2005)
Chasing	One hen chasing another, with fast running, no vocalisations, no hopping and no wing flapping (Sokołowicz et al., 2020)
Wing flapping	Number of wing beats, often while the bird is standing on the toes (Martin et al., 2005)
Shaking	Body/wing shake when the plumage is not in order (Martin et al., 2005)
Dust bathing	Sitting and performing: vertical wing-shaking, body shaking, litter pecking and/or scratching, bill raking, side and head rubbing (van Hierden et al., 2002)
Allopreening	Social preening (Kenny et al., 2017)
Duration behaviours	**Definition**
Walking	Taking one or more step (Webster and Hurnik, 1990)
Preening	Grooming of own feathers with beak (Ipema et al., 2020)
Standing still	Standing on the feet with extended legs (Webster and Hurnik, 1990)
Ground pecking	Pecking at the litter with the head in lower position than the rump (van Hierden et al., 2002)
Lying down	Sitting position (Webster and Hurnik, 1990)

308 broiler chickens were randomly allotted to 3 experimental treatments (6 replicate pens/treatment, 10 birds/treatment) as follows: 1) control (C), where a commercial feed only was provided (two feeding phases: starter [4–11 days] and grower-finisher [12–38 days]; ii), BSF, where the C diet was supplemented with 5% of the expected daily feed intake [DFI] of BSF live larvae (calculated on dry matter [DM]); and 3) YM, where the C diet was supplemented with 5% of the expected DFI of YM live larvae (DM). The starter commercial feed was characterized by 12.5 MJ/kg metabolizable energy (ME) and 224 g/kg crude protein (CP), while the grower feed contained 13.0 MJ/kg ME and 220 g/kg CP (Fa.ma.ar.co SPA, Cuneo, Italy). The pens were 1.20 m wide × 2.20 m long (bird density at the end of the growth: 10 kg/m^2). The daily amount of live larvae was distributed to all the pens in two plates at the same hour (11.00 a.m.) and 7 days/week for the whole trial (35 days). To avoid any potential bias, two plates with a known amount of control feed inside were also provided to the C animals to create the same interaction with the operators in all the treatments, and there was also a visual separation among the pens (Bellezza Oddon et al., 2021).

Feathering score

At the end of the experimental trial, all the birds were given feathering scores for back, breast, wing, under-wing and tail using scores of 1–5 for feather coverage as follows: score 1, minimal coverage (<25% coverage); score 2, 25%–50% coverage; score 3, 50%–75% coverage; score 4, >75% coverage; and score 5, complete coverage (Lai et al., 2010).

Behavioural observations

The behavioural observations were carried out using video recordings. A total of 3 pens/treatment were filmed for 5 min in the morning (9.00–9.05 a.m.), 5 min during the larvae intake (11.00–11.05 a.m.) and 5 min in the afternoon (6.00–6.05 p.m.) at the beginning of the trial (T0) and every 11 days until the end of the experiment (T1, T2 and T3). The recorded videos were analysed by the Behavioural Observation Research Interactive Software (BORIS, v 7.9.7) (Friard and Gamba, 2016). The considered behaviours were divided in two categories: the frequency (point event) and the duration (state event) behaviours (Table 1). The frequency behaviours were evaluated as the number of times that a specific behaviour occurred in the pen during the 5 min periods of observations. The duration behaviours were, instead, assessed as the percentage of the 5 min periods of observations that 4 identified subjects in the pen (named as alpha, beta, gamma and delta) spent performing a specific behaviour.

Feet and hock health assessment

The feet and hocks of the broiler chickens were examined at the end of the experimental trial in order to assess the incidence

and the severity of the footpad dermatitis (FPD) and the hock burns (HB). The FPD was scored as follows: 0 = no lesion, slight discoloration of the skin or healed lesion; 1 = mild lesion, superficial discoloration of the skin and hyperkeratosis; and 2 = severe lesion, affected epidermis, blood scabs, haemorrhages and severe swelling of the skin (Ekstrand et al., 1998). Differently, the HB were scored as follows: 0 = no lesion; 1 = superficial, attached (single) lesion or several single superficial or deep lesions ≤0.5 cm; 2 = deep lesion >0.5 cm to ≤1 cm or superficial lesion >0.5 cm; 3 = deep lesion >1.0 cm; 4 = whole hock extensively altered (Louton et al., 2020).

Excreta corticosterone analysis

At the beginning of the trial (T0) and every 11 days (after the video recordings of the administration of the insect live larvae) until the end of the experiment (T1, T2, and T3), all the birds from each pen were housed in wire-mesh cages (100 cm width × 50 cm length) for 120 min to collect fresh excreta samples. After collection, the excreta samples were pooled, immediately frozen at −20°C until corticosterone analysis, and processed according to Palme et al. (2013) and Costa et al. (2016). In particular, the excreta were freeze-dried and ground using a cutting mill (MLI 204; Bühler AG, Uzwil, Switzerland). A total of 0.25 g of the samples were placed into an extraction tube with 3 ml of ether and stored at −20°C for 1 h. After this time, the aliquots were mixed for 3 min through multivortex and the supernatant was recovered and transferred in a new tube. The tubes were then placed at 50°C for 14 h to obtain a dried extract. Lastly, excreta CM were analysed with a multi species enzyme immunoassay kit (Arbor Assay®, Ann Arbor, MI, United States) developed for serum, plasma, saliva, urine, extracted faecal samples, and tissue culture media. All of the analyses were performed in duplicate. The inter- and intra-assay coefficients of variation were less than 10% (7% and 9%, respectively). The sensitivity of the assay was 11.2 ng/g of excreta. All of the samples were analysed at multiple dilutions (1:4, 1:8, 1:16, and 1:32) and all the regression slopes were parallel to the standard curve (r^2 = 0.979).

Statistical analysis

The statistical analysis was performed using IBM SPSS Statistics V28.0.0 software (IBM, Armonk, NY, United States). The pen was considered as the experimental unit for the plumage status, behaviour, and excreta CM analyses, while the bird was used for the assessment of the leg health. Shapiro-Wilk's test established normality or non-normality of distribution of both the data and the residuals. The feathering scores were analysed by fitting a generalized linear mixed model (GLMM) that allowed them to depend on linear predictors (diet, time, and their interaction) through a negative binomial response probability distribution with a nonlinear link function (log). The mean scores of each body area were included in the statistical model. A GLMM was also fit to allow the behaviour data to depend on the same linear predictors through a Poisson loglinear distribution (frequency behaviours) or a gamma probability distribution with a nonlinear (log) link function (duration behaviours). The total number of times that the specific frequency behaviours occurred in the pen, as well as the mean percentage of time that the 4 identified subjects of the pen spent performing the specific duration behaviours, were included in the corresponding statistical models. Frequency behaviours occurring less than 0.5 times on average per period of observation were excluded from the GLMM. The excreta CM were also analysed by fitting a GLMM that allowed them to depend on the same linear predictors through a gamma probability distribution with a nonlinear link function (log). The mean CM resulting from the duplicate analysis was included in the statistical model. The replicate was included as a random effect to account for repeated measurements on the same pen, and the interactions between the levels of the fixed factors were evaluated by means of pairwise contrasts. The HB and FPD scores were analysed by means of Kruskal-Wallis (post-hoc test: Dunn's Multiple Comparisons Test). The results were expressed as least square mean (plumage status, behaviour, and excreta CM) or mean (leg health) and standard error of the mean (SEM). p values ≤0.05 were considered statistically significant.

Results

Feathering score

The feathering scores of the broiler chickens of the current research are summarized in Table 2. The administration of both the BSF and the YM live larvae did not influence the feathering scores of the birds (p = 0.545). On the contrary, the feathering scores depended on the body area (p < 0.001). In particular, the back showed better scores when compared to the other body areas, with breast, under-wing and tail furtherly displaying greater scores than the wing (p < 0.001). No diet × body area interaction was also identified (p = 0.237).

Behaviour analysis

Frequency behaviours of the broiler chickens of the present study are summarized in Table 3 and Figures 1–3. In the morning, stretching and wing flapping were influenced by both the insect live larvae administration and the time (p < 0.001), but no diet × time interaction was identified (p = 0.686 and p = 0.220, respectively). In details, the insect-fed broiler chickens performed more stretching and wing flapping than the C group (p < 0.001), and, independently of diet, a

TABLE 2 Feathering score of the broiler chickens depending on diet, body area and their interaction.

	Diet (D)			Body area (B)					SEM		*p*-value			Wald test		
	C	BSF	YM	Back	Breast	Wing	Under-wing	Tail	D	B	D	B	D×B	D	B	D×B
Score, n	1.18	1.16	1.21	3.19^{a}	1.00^{b}	0.73^{c}	1.00^{b}	0.99^{b}	0.03	0.05	0.545	<0.001	0.237	1.214	854.780	8.010

C = control group; BSF = C diet + black soldier fly live larvae; YM = C diet + yellow mealworm live larvae. Means with superscript letters (a, b, c) denote significant differences ($p < 0.05$).

reduction (stretching) and an increase (wing flapping) of such behaviours was overall observed along the experimental trial ($p < 0.001$ and $p = 0.010$, respectively). The wing flapping frequency also abruptly decreased at T3 when compared to the other experimental times ($p = 0.010$). Preening depended on time only, with an increase being overall identified along the experimental trial, but an abrupt reduction at T3 ($p = 0.001$). On the contrary, no influence of insect live larvae administration or diet × time interaction were highlighted ($p = 0.102$ and $p = 0.110$, respectively). Allopreening, pecking pen mate and shaking behaviours did not depend on any of the considered variables (diet: $p = 0.549$, $p = 1.000$ and $p = 0.001$, respectively; time: $p = 0.549$, $p = 0.290$ and $p = 0.100$, respectively; diet × time: $p = 0.404$, $p = 1.000$ and $p = 1.000$, respectively). During the larvae intake, scratching and wing flapping behaviours were influenced by insect live larvae administration only ($p = 0.025$ and $p < 0.001$, respectively). In particular, the insect-fed broilers performed more scratching in comparison with the C birds ($p = 0.025$), while increased frequency in wing flapping was identified in the YM group only ($p < 0.001$). Differently, no influence of time ($p = 0.070$ or $p = 0.661$, respectively) or diet × time interaction ($p = 0.662$ and $p = 0.508$, respectively) were identified. Preening and stretching behaviours were influenced by either the insect live larvae administration or the time ($p < 0.001$). In particular, the insect-fed birds displayed less preening and stretching than the C broilers, with the YM group furtherly showing reduced stretching when compared to the BSF-fed birds ($p < 0.001$). Furthermore, independently of diet, preening and stretching frequencies progressively increased in the last 11 days of the experimental trial ($p < 0.001$). On the contrary, no diet × time interaction was highlighted ($p = 0.057$ and $p = 0.104$, respectively). Trotting and shaking behaviours depended on time only, with trotting frequency progressively decreasing in the last 11 days of the experimental trial ($p < 0.001$), and shaking displaying the opposite trend ($p < 0.001$). Differently, no influence of insect live larvae administration ($p = 0.098$ or $p = 0.687$, respectively) or diet × time interaction ($p = 1.000$ and $p = 0.492$, respectively) were identified. Allopreening and pecking pen mate behaviours did not depend on any of the considered variables (diet: $p = 0.624$ and $p = 0.105$, respectively; time: $p = 1.000$ and $p = 0.624$, respectively; diet × time: $p = 1.000$ and $p = 1.000$, respectively). In the afternoon, a diet × time interaction was observed for wing flapping only ($p < 0.001$). In details, the YM-fed broiler chickens performed more wing flapping than the other groups at T2 and T3 only ($p < 0.001$), while the C birds displayed higher wing flapping than the HI group at T1 ($p < 0.05$, Figure 3). On the contrary, preening, stretching and shaking behaviours depended on time only, with increasing frequencies being highlighted along the experimental trial ($p < 0.001$). On the contrary, no influence of insect live larvae administration ($p = 0.770$, $p = 0.302$ or $p = 0.378$, respectively) or diet × time interaction ($p = 0.127$, $p = 0.106$ and $p = 0.052$, respectively) were highlighted. Allopreening was not influenced by any of the considered variables (diet: $p = 1.000$; time: $p = 0.527$; diet × time: $p = 0.527$).

Duration behaviours of the broiler chickens of the current research are summarized in Table 4 and Figure 4–6. In the morning, a diet × time interaction was observed for both the ground pecking and the preening ($p < 0.001$ and $p = 0.006$, respectively). In particular, higher ground pecking was observed in the insect-fed broilers than the C group at T1 and T3 only ($p < 0.001$, Figure 4), whereas the C birds spent more time preening in comparison with the other groups or BSF group alone at T1 and T2, respectively ($p = 0.006$, Figure 4). Walking depended on either the insect live larvae administration or the time ($p = 0.001$ and $p < 0.001$, respectively). In details, the BSF birds spent more time walking when compared to the C group ($p < 0.001$), and, independently of diet, less walking was progressively observed along the experimental trial ($p < 0.001$). Differently, no diet × time interaction was identified ($p = 0.186$). Standing still and laying down behaviours were influenced by time only ($p < 0.001$ and $p = 0.045$, respectively). In particular, broiler chickens spent less time standing still along the experimental trial ($p < 0.001$), with an increase in laying down being also observed ($p < 0.05$). During the larvae intake, ground pecking and laying down depended on insect live larvae administration only ($p < 0.001$). In particular, the YM-fed birds displayed higher and lower, respectively, ground pecking and preening than the other groups, with either the BSF or the YM broilers spending less time laying down when compared to the C group ($p < 0.001$). On the contrary, no influence of time ($p = 0.703$ and $p = 0.190$, respectively) or diet × time interaction ($p = 0.118$ and $p = 0.141$, respectively) were highlighted. Preening was influenced by both the insect live larvae administration and the time ($p < 0.001$ and $p = 0.001$, respectively). In details, the YM-fed birds displayed lower preening than the other groups ($p < 0.001$), and,

TABLE 3 Frequency behaviours of the broiler chickens depending on diet, time and their interaction.

	Diet (D)			Time (T)				SEM		*p*-value			Wald test		
	C	BSF	YM	T0	T1	T2	T3	D	T	D	T	D×T	D	T	D×T
Morning															
Scratching, n	<0.5 times of occurrence														
Preening, n	9.72	8.35	9.96	5.45^{a}	8.05^{b}	26.70^{c}	6.44^{a}	9.34	0.88	0.102	0.001	0.110	4.980	13.913	4.342
Allopreening, n	0.00	0.00	0.00	0.00	0.00	0.00	0.48	0.00	0.03	0.549	0.549	0.404	1.200	1.200	1.810
Trotting, n	<0.5 times of occurrence														
Stretching, n	2.07^{a}	4.08^{b}	4.74^{b}	2.92^{a}	2.89^{ab}	3.31^{ab}	4.91^{b}	0.26	0.71	<0.001	<0.001	0.686	45.794	18.871	0.842
Pecking pen mate, n	0.00	0.00	0.00	0.00	0.00	0.67	0.00	0.00	0.17	1.000	0.290	1.000	0.000	2.412	0.000
Chasing, n	<0.5 times of occurrence														
Dust bathing, n	<0.5 times of occurrence														
Wing flapping, n	0.00^{a}	1.67^{b}	2.77^{c}	1.88^{b}	1.44^{b}	6.38^{a}	0.00^{c}	0.21	0.25	<0.001	0.010	0.220	136.671	9.294	3.030
Shaking, n	0.00	0.00	0.00	0.00	0.00	0.87	0.00	0.00	0.03	1.000	0.100	1.000	0.000	4.280	0.000
During larvae intake															
Scratching, n	0.33^{a}	2.28^{b}	2.52^{b}	1.20	1.21	1.06	1.49	0.27	0.41	0.025	0.070	0.662	7.416	9.787	0.825
Preening, n	13.05^{a}	3.59^{b}	4.74^{b}	4.00^{a}	3.85^{a}	7.32^{b}	7.89^{b}	1.16	0.98	<0.001	<0.001	0.057	75.693	206.003	5.716
Allopreening, n	0.00	0.00	0.00	0.40	0.42	0.00	0.00	0.00	0.07	0.624	1.000	1.000	0.240	0.000	0.000
Trotting, n	0.00	0.00	0.00	1.31^{a}	1.46^{a}	0.00^{b}	0.00^{b}	0.00	0.13	0.098	<0.001	1.000	4.645	39.095	0.000
Stretching, n	4.89^{a}	2.00^{b}	1.39^{c}	1.70^{a}	1.88^{a}	2.65^{b}	2.71^{b}	0.52	0.29	<0.001	<0.001	0.104	16.280	15.192	4.532
Pecking pen mate, n	0.00	0.00	0.53	0.00	0.00	0.00	0.00	0.07	0.00	0.105	0.624	1.000	4.950	0.786	0.000
Chasing, n	<0.5 times of occurrence														
Dust bathing, n	<0.5 times of occurrence														
Wing flapping, n	3.15^{a}	2.63^{a}	4.73^{b}	3.45	3.61	3.86	2.81	0.31	0.79	<0.001	0.661	0.508	82.131	0.829	1.356
Shaking, n	0.00	0.00	1.01	0.00^{a}	0.00^{a}	0.00^{a}	2.27^{b}	0.22	0.03	0.687	<0.001	0.492	0.752	84.592	0.472
Afternoon															
Scratching, n	<0.5 times of occurrence														
Preening, n	7.39	8.12	8.80	4.61^{a}	6.96^{b}	8.77^{c}	15.17^{d}	1.15	0.90	0.770	<0.001	0.127	0.522	143571.734	4.125
Allopreening, n	0.00	0.00	0.00	0.00	0.53	0.00	0.53	0.00	0.17	1.000	0.527	0.527			
Trotting, n	<0.5 times of occurrence														
Stretching, n	3.73	5.46	4.26	1.59^{a}	4.61^{b}	6.31^{b}	8.33^{c}	0.66	0.52	0.302	<0.001	0.106	1.891	49.443	5.231

(Continued on following page)

TABLE 3 (*Continued*) Frequency behaviours of the broiler chickens depending on diet, time and their interaction.

	Diet (D)			Time (T)				SEM		*p*-value			Wald test		
	C	BSF	YM	T0	T1	T2	T3	D	T	D	T	D×T	D	T	D×T
Pecking pen mate, n	<0.5 times of occurrence														
Chasing, n	<0.5 times of occurrence														
Dust bathing, n	<0.5 times of occurrence														
Wing flapping, n	0.00	0.00	1.25	1.30	1.52	0.00	1.19	0.09	0.31	0.309	0.888	0.001	2.346	0.237	14.554
Shaking, n	0.00	0.00	0.00	0.00^{a}	0.00^{a}	0.76^{b}	1.37^{c}	0.00	0.09	0.378	<0.001	0.052	1.947	20.694	5.975

C = control group; BSF = C diet + black soldier fly live larvae; YM = C diet + yellow mealworm live larvae. T0 = day 0; T1 = day 11; T2 = day 22; T3 = day 33. Means with superscript letters (a, b, c, d) denote significant differences ($p < 0.05$).

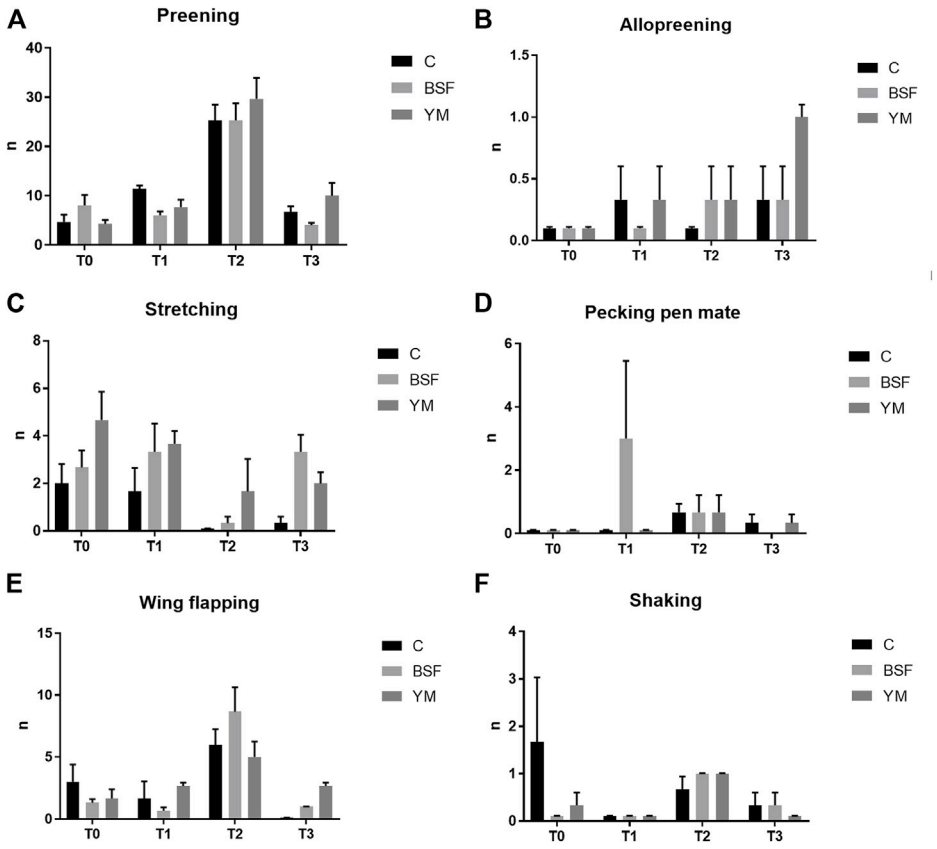

FIGURE 1
Frequency behaviours of the broiler chickens in the morning (diet*time interaction, $p > 0.05$). **(A)** Preening. **(B)** Allopreening. **(C)** Stretching. **(D)** Pecking pen mate. **(E)** Wing flapping. **(F)** Shaking. C = control group; BSF = C diet + black soldier fly live larvae; YM = C diet + yellow mealworm live larvae. T0 = day 0; T1 = day 11; T2 = day 22; T3 = day 33.

independently of diet, preening duration was reduced in the last 11 days of the experimental trial ($p = 0.001$). Differently, no diet×time interaction was identified ($p = 0.060$). On the contrary, no influence of insect live larvae administration or diet × time interaction were observed ($p = 0.208$ and $p = 0.077$, respectively). Standing still did not depend on any of the considered variables

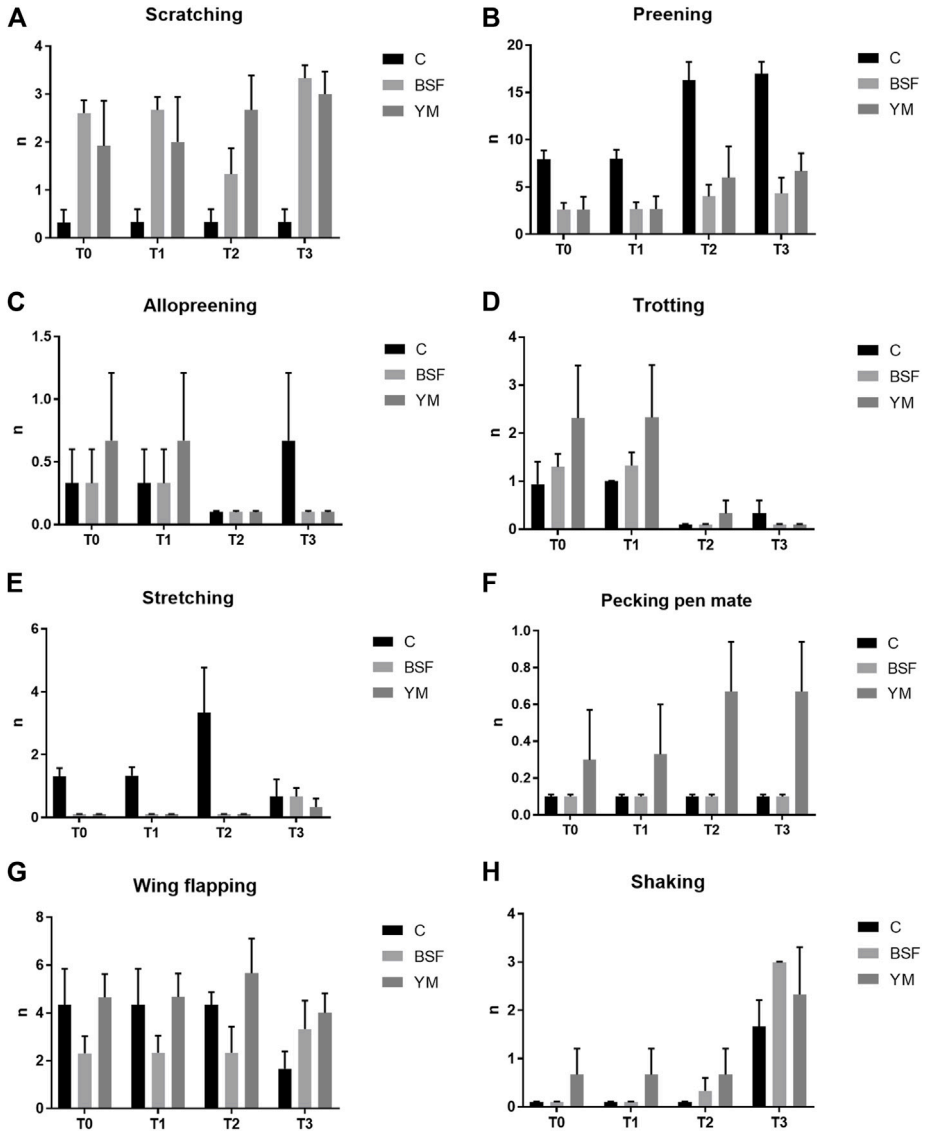

FIGURE 2
Frequency behaviours of the broiler chickens during the larvae intake (diet*time interaction, $p > 0.05$). **(A)** Scratching. **(B)** Preening. **(C)** Allopreening. **(D)** Trotting. **(E)** Stretching. **(F)** Pecking pen mate. **(G)** Wing flapping. **(H)** Shaking. C = control group; BSF = C diet + black soldier fly live larvae; YM = C diet + yellow mealworm live larvae. T0 = day 0; T1 = day 11; T2 = day 22; T3 = day 33.

(diet: $p = 0.218$; time: $p = 0.710$; diet × time: $p = 0.058$). In the afternoon, the insect-fed birds showed higher laying down in comparison with the C group at T3 only (diet × time interaction, $p < 0.001$; Figure 6). Ground pecking behaviour depended on insect live larvae administration, with the YM-fed broiler chickens spending less time ground pecking than the other groups ($p < 0.001$). On the contrary, no influence of time or diet × time interaction were highlighted ($p = 0.110$ and $p = 0.571$, respectively). Finally, walking, standing still and preening behaviours were influenced by time only ($p < 0.001$), with broiler chickens spending less time walking and standing still, as well as more time preening, along the experimental trial ($p < 0.001$). Differently, no influence of insect live larvae administration ($p = 0.678$, $p = 0.414$ and $p = 0.285$, respectively) or diet × time interaction ($p = 0.112$, $p = 0.215$ and $p = 0.116$, respectively) were observed.

Feet and hock health assessment

The administration of BSF and YM live larvae did not influence either the HB (H = 3.644; C: 0.37 ± 0.09; BSF: 0.73 ± 0.15; YM: 0.77 ± 0.17) or the FPD (H = 2.603; C: 0.60 ± 0.15; BSF: 0.60 ± 0.14; YM: 0.33 ± 0.11) scores ($p = 0.162$ and $p = 0.272$, respectively).

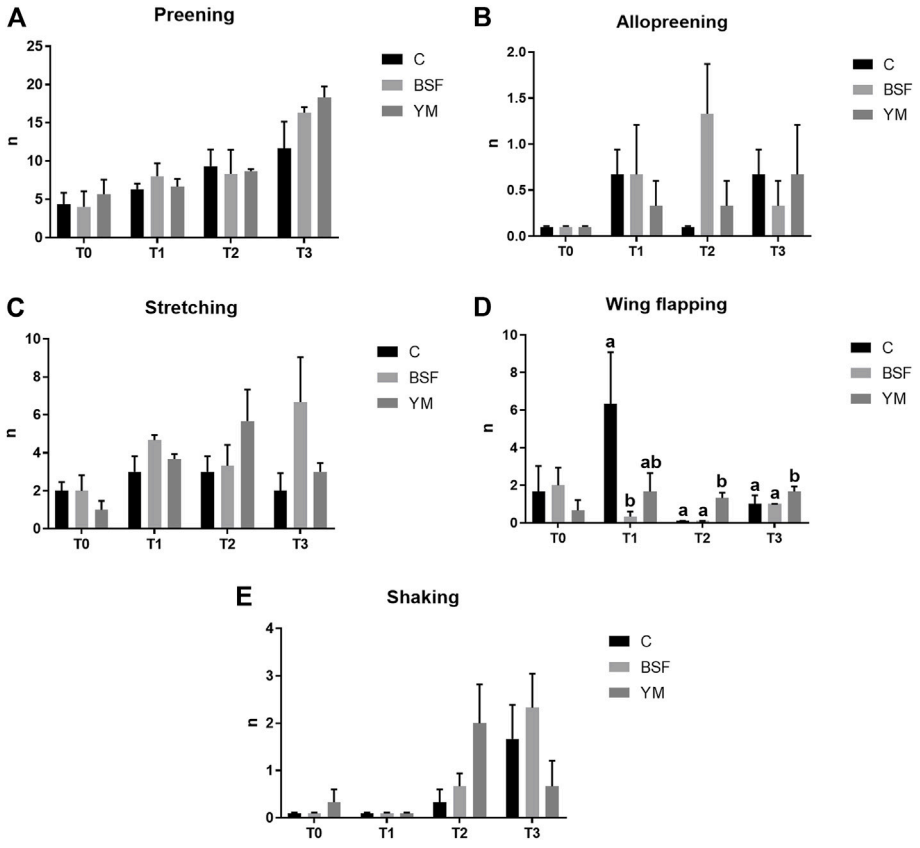

FIGURE 3
Frequency behaviours of the broiler chickens in the afternoon (diet*time interaction). **(A)** Preening. **(B)** Allopreening. **(C)** Stretching. **(D)** Wing flapping. **(E)** Shaking. Graph bars (representing least square means) with different superscript letters (a, b) indicate significant differences among the experimental treatments within the experimental times. C = control group; BSF = C diet + black soldier fly live larvae; YM = C diet + yellow mealworm live larvae. T0 = day 0; T1 = day 11; T2 = day 22; T3 = day 33.

Excreta corticosterone

The excreta CM of the broiler chickens of the present study are summarized in Table 5 and Figure 7. The administration of BSF and YM live larvae did not affect the excreta CM of the broiler chickens of the current research ($p = 0.684$). Similarly, no time-related effects or diet × time interactions were identified ($p = 0.288$ and $p = 0.369$, respectively).

Discussion

Feathering score

The administration of neither the BSF nor the YM live larvae was able to improve the feathering scores of the broiler chickens of the present study. Previous research highlighted a tendency towards improvement or a significant improvement in feather damage of BSF live larvae-fed turkey poults and laying hens, respectively (Veldkamp and van Niekerk, 2019; Star et al., 2020). Such improvement has been related to a reduction in the aggressive pecking directed at the back and tail base, as a consequence of the re-direction of this behaviour towards the floor and away from feathers (Veldkamp and van Niekerk, 2019). However, since the aggressive pecking displayed by the broilers of the current research was not influenced by the administration of either the BSF or the YM live larvae, it is reasonable that feather conditions were unaffected as well. Independently of the utilization of the insect larvae, the back and the wing of the birds showed the best and the worst feather coverage, respectively. Little information is currently available on the feathering scores of the different body parts in broiler chickens (Lai et al., 2010; Mahmoud et al., 2015; Sevim et al., 2022), with the totality of the body areas being not always assessed (Sevim et al., 2022), or the authors reporting a mean body score only (Mahmoud et al., 2015). Lai et al. (2010) previously identified similar feathering scores among the different body regions of broiler chickens, while a clear

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TABLE 4 Duration behaviours of the broiler chickens depending on diet, time and their interaction.

	Diet (D)			Time (T)				SEM		*p*-value			Wald test		
	C	BSF	YM	T0	T1	T2	T3	D	T	D	T	D×T	D	T	D×T
Morning															
Ground pecking, time %	2.59^{a}	7.12^{ab}	6.11^{b}	7.62^{a}	2.64^{c}	5.55^{b}	4.88^{b}	0.89	0.49	<0.001	<0.001	<0.001	101.932	366.984	235.8011
Walking, time %	4.74^{a}	5.99^{b}	3.95^{ab}	14.43^{a}	8.00^{b}	2.76^{b}	1.66^{c}	0.45	0.88	0.001	<0.001	0.186	14.706	128.630	3.362
Standing still, time %	23.52	19.91	19.89	41.98^{a}	27.22^{b}	8.28^{c}	20.71^{b}	2.67	3.21	0.573	<0.001	0.355	1.115	37.646	2.070
Laying down, time %	46.45	51.23	56.03	29.36^{a}	52.59^{ab}	73.21^{b}	60.24^{b}	3.49	6.56	0.055	0.045	0.107	5.793	6.184	16.710
Preening, time %	7.91^{a}	4.72^{b}	7.34^{b}	2.02^{a}	5.24^{b}	12.40^{d}	6.98^{c}	0.84	0.88	0.019	0.004	0.006	7.906	11.024	10.203
During larvae intake															
Ground pecking, time %	1.61^{ab}	1.66^{a}	2.52^{b}	2.10	2.06	2.14	2.52	0.85	0.58	<0.001	0.703	0.118	93.006	0.146	5.674
Walking, time %	3.29	4.24	4.78	5.58^{a}	5.50^{a}	5.64^{a}	2.92^{b}	0.63	0.24	0.208	<0.001	0.077	3.139	38.806	5.132
Standing still, time %	15.32	17.12	20.45	18.58	18.20	17.86	17.15	1.93	1.53	0.218	0.710	0.058	3.050	0.139	6.008
Laying down, time %	75.27^{a}	33.65^{b}	44.08^{b}	43.39	42.78	44.69	51.88	4.55	2.84	<0.001	0.190	0.141	251.827	1.714	3.918
Preening, time %	6.82^{a}	4.33^{a}	2.20^{b}	5.75^{a}	5.90^{a}	6.40^{a}	2.53^{b}	1.01	0.66	<0.001	0.001	0.060	140.920	12.020	5.640
Afternoon															
Ground pecking, time %	8.12^{a}	6.13^{a}	2.87^{b}	6.26	4.51	6.09	4.34	1.00	0.85	<0.001	0.110	0.571	19.931	4.421	1.120
Walking, time %	5.17	5.25	4.42	23.65^{a}	6.18^{b}	2.17^{c}	1.86^{d}	0.65	1.03	0.678	<0.001	0.112	0.778	18619.759	4.980
Standing still, time %	16.85	14.79	15.15	45.74^{a}	16.45^{b}	7.05^{c}	11.08^{b}	1.95	1.64	0.414	<0.001	0.215	1.761	1013.777	3.165
Laying down, time %	36.04^{a}	52.31^{b}	64.22^{b}	17.65^{a}	60.99^{b}	75.61^{b}	73.60^{b}	5.55	5.09	<0.001	<0.001	<0.001	370.193	44.580	486.225
Preening, time %	2.50	2.96	3.97	1.67^{a}	2.67^{b}	2.78^{b}	7.32^{c}	0.42	0.47	0.285	<0.001	0.116	2.510	11294.008	5.125

C = control group; BSF = C diet + black soldier fly live larvae; YM = C diet + yellow mealworm live larvae. T0 = day 0; T1 = day 11; T2 = day 22; T3 = day 33. Means with superscript letters (a, b, c, d) denote significant differences ($p < 0.05$).

separation between the back and the other body areas was herein outlined. The poor feather coverage of the breast can reasonably be attributed to the clear predominance of laying down behaviour in the whole behavioural time budget of the birds, while wing, under-wing and tail feather damage may be related to the progressively increase in preening frequency and duration along the experimental trial. Indeed, wing and tail—along with breast—represent the plumage areas receiving preferred attention from the birds during preening (Duncan and Wood-Gush, 1972). A significant role of the genetic selection—which aims at growth of meat and not feathers—cannot be excluded as well.

Behaviour analysis

The variations in the behavioural repertoire of the broiler chickens of the present study share several similarities between the morning and the moment of the larvae intake, while the afternoon was characterized by different behavioural patterns. During the morning and the larvae intake, birds receiving the insect live larvae spent more time ground pecking (with a statistical significance being detected at T1 and T3 only, as a consequence of the higher SEM of T2) and performing increased scratching behaviour when compared to the non-supplemented animals. This clear stimulation of a more natural behaviour such as foraging [characterized by ground pecking and/or scratching (Ipema et al., 2020)] has already been observed in turkey poults and broiler chickens administered with BSF live larvae (Veldkamp and van Niekerk, 2019; Ipema et al., 2020). Scattering food items on the litter (such insects) or using different bedding materials (sand, moss-peat, or oat husks) have previously been reported to stimulate foraging behaviour in broiler chickens (Arnould et al., 2004; Baxter and O'Connell, 2016; Pichova et al., 2016). However, similar environmental enrichments (such as whole wheat, wood shavings, rice hulls or straw pellets) are not capable of exerting an analogous effect (Bizeray et al., 2002; Shields et al., 2005; Toghyani et al., 2010; Jordan et al., 2011; Baxter and O'Connell, 2016; Pichova et al., 2016), thus suggesting that birds have a clear preference for certain types of substrates (Riber et al., 2018). Indeed, the motivational significance behind each food-based enrichment represents the main driver of the behavioural changes (Pichova et al., 2016), and the insect larvae—as alive, moving and part of the natural diet of birds—seem to be highly interesting for poultry (Bokkers

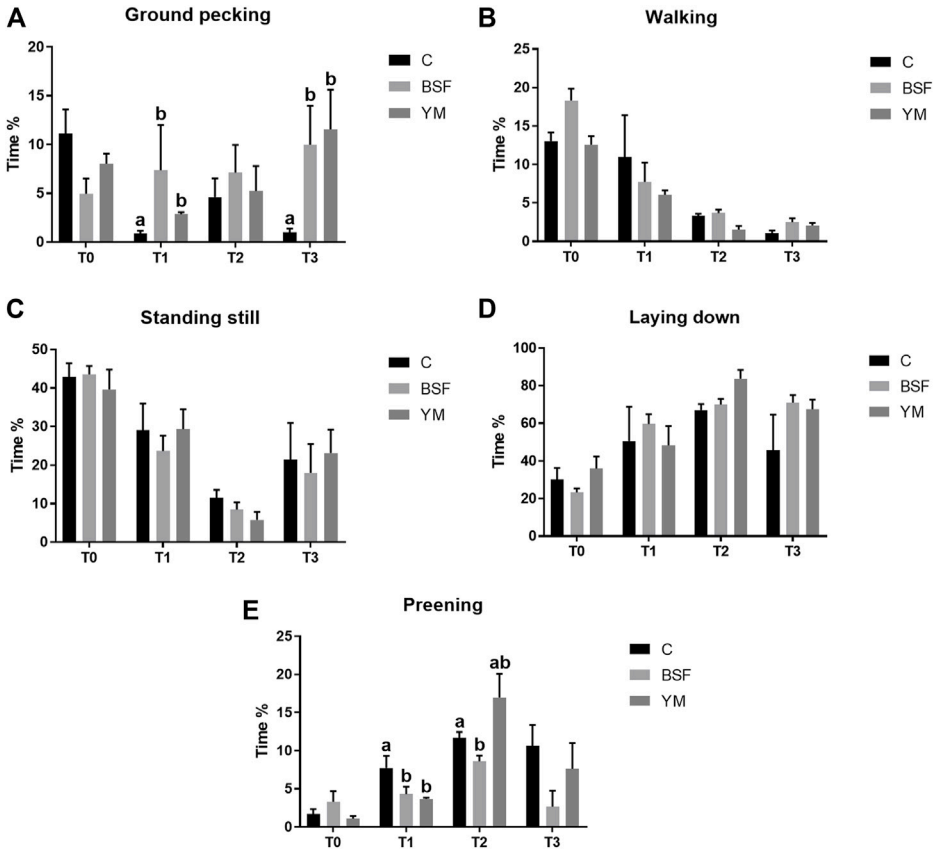

FIGURE 4
Duration behaviours of the broiler chickens in the morning (diet*time interaction). **(A)** Preening. **(B)** Allopreening. **(C)** Stretching. **(D)** Wing flapping. **(E)** Shaking. Graph bars (representing least square means) with different superscript letters (a, b) indicate significant differences among the experimental treatments within the experimental times. C = control group; BSF = C diet + black soldier fly live larvae; YM = C diet + yellow mealworm live larvae. T0 = day 0; T1 = day 11; T2 = day 22; T3 = day 33.

and Koene, 2002; Bruce et al., 2003; Ipema et al., 2020). The same motivational significance reasonably determined the increase in the activity levels of the insect-fed broiler chickens of the current research as well, as demonstrated by the increased frequency of stretching and wing flapping behaviours (the latter being mainly detected in the YM-fed birds), the increased time spent for walking and performing wing flapping, and the decreased time spent for laying down. An analogous scenario was also underlined in broilers and laying hens administered with BSF or YM live larvae as environmental enrichment (Pichova et al., 2016; Ipema et al., 2020; Star et al., 2020). It is, however, interesting to notice that the increase in stretching was observed in the morning only, while during the larvae intake such behaviour actually decreased. This may reasonably be related to the parallel increase in scratching and wing flapping behaviours. Another peculiar aspect to highlight is the reduced frequency (independently of time) and duration (mainly with BSF, as a consequence of the higher SEM of the YM group) preening displayed by the insect-fed birds of the present study. Preening, as it keeps plumages well-groomed by distributing lipid-rich oils from uropygial glands and removing parasites (Delius, 1988), could take a large time budget (~13%) out of the total behaviour repertoire of domestic fowl (Dawkins, 1989). However, overall time spent preening and number of preening bouts could give useful information about environment appropriateness for birds (Li et al., 2020). Indeed, absence of environmental stimuli (i.e., cages) stimulates the birds to spend more time preening (Delius, 1988) or to perform short-term and frequent preening (Duncan, 1998), as a sign of boredom and frustration. Therefore, the administration of insect live larvae may reduce such negative feelings in broilers. In the afternoon, birds receiving YM live larvae spent less time ground pecking than the other groups, whereas either the BSF- or the YM-fed broilers showed an increased duration of laying down behaviour (with a statistical significance being detected at T3 only, as a consequence of the higher SEM of T1 and T2). This may indicate that the need for foraging was fully rewarded during the morning and the larvae

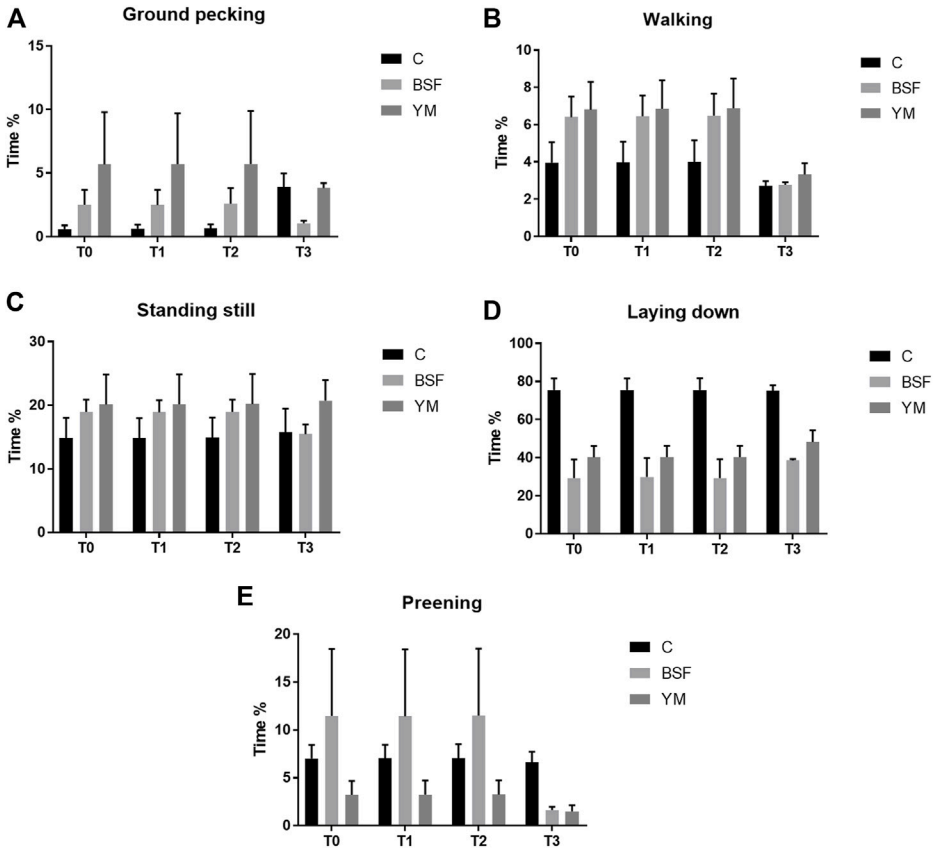

FIGURE 5
Duration behaviours of the broiler chickens during the larvae intake (diet*time interaction, $p > 0.05$). **(A)** Preening. **(B)** Allopreening. **(C)** Stretching. **(D)** Wing flapping. **(E)** Shaking. C = control group; BSF = C diet + black soldier fly live larvae; YM = C diet + yellow mealworm live larvae. T0 = day 0; T1 = day 11; T2 = day 22; T3 = day 33.

intake, and that the overall increased activity observed in the first part of the day predisposed the birds to rest in the afternoon. However, the wing flapping frequency remained higher in the YM-fed broiler chickens when compared to the other groups (with a statistical significance being detected in the last third of the experimental trial only, as a consequence of the higher SEM of T1).

Independently of the administration of the insect live larvae, the broiler chickens of the present study displayed less active behaviours (i.e., ground pecking, walking and standing still), as well as more passivity (i.e., laying down), with increasing age. This is in agreement with previous research on broilers (Bokkers and Koene, 2003; Castellini et al., 2016; Ipema et al., 2020; Jacobs et al., 2021), where the rapid increase in body weights leads to poor mobility and, in turn, inhibits their ability to express certain behaviours (Bokkers, 2004; Castellini et al., 2016). The overall increase in preening may similarly be attributed to frustration related to poor mobility (Bokkers and Koene, 2003). On the contrary, other active behaviours such as stretching, shaking and wing flapping increased with increasing age of birds. It is, however, important to underline that fast-growing broilers are motivated to perform the normal behavioural repertoire of chickens, even after 6 weeks of age and despite being hampered by the high body weights (Bokkers, 2004). Furthermore, as behaviours are performed in sitting position rather than in standing position with increasing age (Bokkers, 2004), it is reasonable to identify an increase in behaviours that birds can easily perform when laying down.

As a final aspect to consider, the use of YM live larvae yielded slightly more pronounced effects on bird behaviour (especially in terms of stimulation of foraging and increase in activity levels) than the BSF ones. Considering that the broiler chickens of the current research spent less time consuming the YM live larvae when compared to BSF (Bellezza Oddon et al., 2021), it is possible to speculate a bird preference towards the larvae of this insect

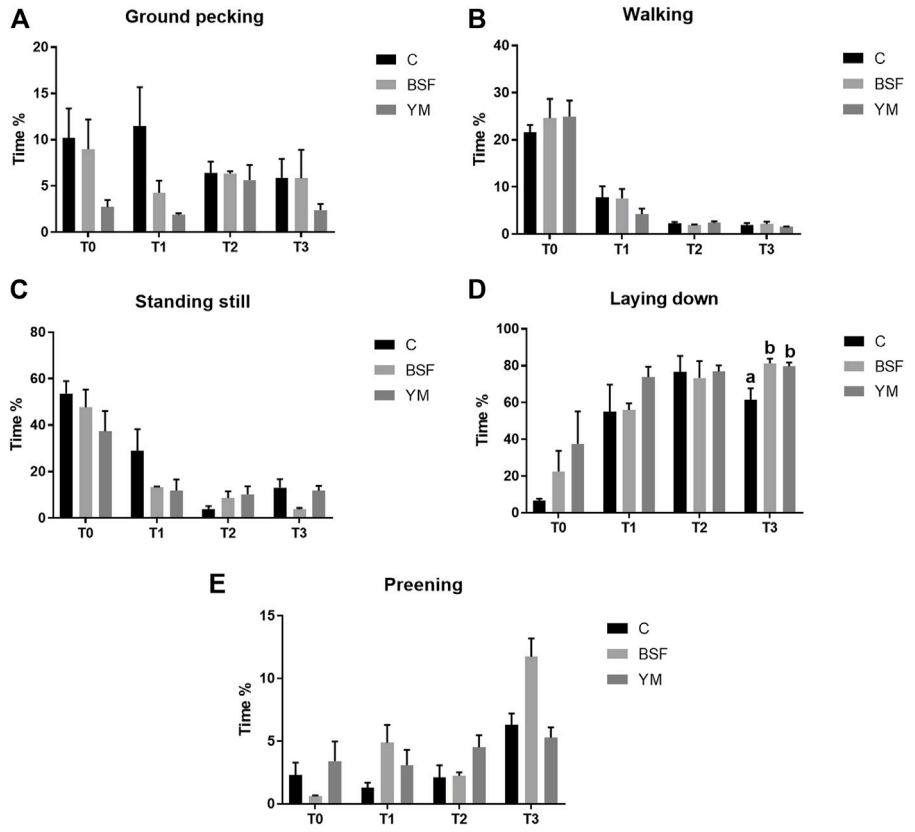

FIGURE 6
Duration behaviours of the broiler chickens in the afternoon (diet*time interaction). **(A)** Preening. **(B)** Allopreening. **(C)** Stretching. **(D)** Wing flapping. **(E)** Shaking. Graph bars (representing least square means) with different superscript letters (a, b) indicate significant differences among the experimental treatments within the experimental times. C = control group; BSF = C diet + black soldier fly live larvae; YM = C diet + yellow mealworm live larvae. T0 = day 0; T1 = day 11; T2 = day 22; T3 = day 33.

TABLE 5 Excreta CM of the broiler chickens depending on diet, time and their interaction.

	Diet (D)			Time (T)				SEM		*p*-value			Wald test		
	C	BSF	YM	T0	T1	T2	T3	D	T	D	T	D×T	D	T	D×T
CM, ng/g	2855.8	2955.6	3079.4	3210.3	2978.2	3024.4	2641.4	181.1	209.2	0.684	0.288	0.369	0.382	1.284	1.108

C = control group; BSF = C diet + black soldier fly live larvae; YM = C diet + yellow mealworm live larvae. T0 = day 0; T1 = day 11; T2 = day 22; T3 = day 33.

species. However, further studies are needed to confirm this hypothesis.

Feet and hock health assessment

Similarly to what was observed for the feathering scores, the HB and the FPD scores of the broiler chickens of the current research were not influenced by the administration of either the BSF or the YM live larvae. Ipema et al. (2020) highlighted that FPD occurrence was not affected by insect live larvae provision, whereas the larvae-administered birds displayed less HB when compared to the C birds. However, considering that FPD incidence has been reported to be influenced only in the first 3 weeks of age in turkey poults (Veldkamp and van Niekerk, 2019), it is reasonable that a single evaluation may not be enough

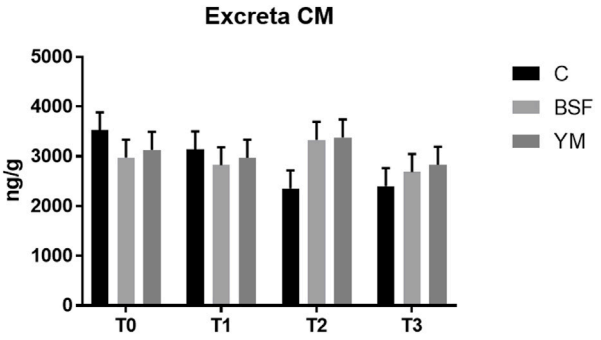

FIGURE 7
Excreta CM of the broiler chickens (diet*time interaction, $p > 0.05$). C = control group; BSF = C diet + black soldier fly live larvae; YM = C diet + yellow mealworm live larvae. T0 = day 0; T1 = day 11; T2 = day 22; T3 = day 33.

to observe potential differences in broilers as well. Furthermore, the identification of very low mean values for both the HB and the FPD scores of the C birds (less than 1) suggested the presence of an health status of the legs that was already good independently of insect live larvae administration, thus, in turn, making more challenging to improve it.

Excreta corticosterone

The excreta CM of the broiler chickens of the present study were not affected by the administration of both the BSF and the YM live larvae as well. The measurement of excreta CM is a well-recognized, non-invasive method to quantify the stress response in poultry, which offers a more convenient and less disruptive alternative to traditional measures that require bird restraint and blood sampling (Weimer et al., 2018), and does not interrupt the animal behaviour (Hirschenhauser et al., 2012). However, it is fundamental to underline that many factors (such as age, sex, diet, metabolic rate, social status, early life experience, diurnal and seasonal variations, and differences in the hormone metabolism of individuals) may influence the excreta CM (Alm et al., 2014). Therefore, despite the positive, insect-related modulation in the bird behaviour herein highlighted, such variability could have probably hidden the potential differences in the excreta CM.

Conclusion

In conclusion, the administration of BSF and YM live larvae as environmental enrichment for broiler chickens was capable of positively influencing the bird welfare through the stimulation of foraging behaviour, increase in activity levels, and reduction of behaviours potentially attributable to frustration, without affecting the plumage status, the leg health, and the excreta CM. As behavioural outcomes suggested some preference of the broilers for YM live larvae, further research to confirm this preference is recommended. Considering that the administration of insect live larvae in the intensive farming may potentially lead to different outcomes—as a consequence of the high rearing densities and competitiveness among birds—additional research testing such innovative environmental enrichment in the commercial setup are strongly recommended.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the corresponding author upon reasonable request.

Ethics statement

The animal study was reviewed and approved by the Bioethical Committee of the University of Turin (Italy).

Author contributions

AS, IB, SBO, and LG designed the study. IB, SBO, MG, EF, and SD carried out the rearing work. MP, and DD provided the insect live larvae. IB and SBO gave the feathering scores. GC and SBO analysed the behavioural video recordings. MG, EF, and SD collected the excreta samples. SBO, EF, and EM analysed the excreta corticosterone. IB and SBO performed the statistical analysis. IB wrote the first draft of the manuscript. All the authors contributed to the article and approved the submitted version.

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Conflict of interest

Authors MP and DD were employed by the company Entomics Biosystems.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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EDITED BY
Kent M. Reed,
University of Minnesota Twin Cities, United States

REVIEWED BY
Mert Colpan,
University of Arizona, United States
Daniel Hernandez-Patlan,
Facultad de Estudios Superiores Cuautitlán, Universidad Nacional Autonóma de México, Mexico

*CORRESPONDENCE
Martina Zappaterra,
martina.zappaterra2@unibo.it

†These authors have contributed equally to this work and share first authorship

‡These authors have contributed equally to this work and share last authorship

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The evolution of vimentin and desmin in *Pectoralis major* muscles of broiler chickens supports their essential role in muscle regeneration

Francesca Soglia[1†], Martina Bordini[1†], Maurizio Mazzoni[2], Martina Zappaterra[1*], Mattia Di Nunzio[3], Paolo Clavenzani[2], Roberta Davoli[1], Adele Meluzzi[1], Federico Sirri[1‡] and Massimiliano Petracci[1‡]

[1]Department of Agricultural and Food Sciences (DISTAL), Alma Mater Studiorum–University of Bologna, Bologna, Italy, [2]Department of Veterinary Medical Sciences, Alma Mater Studiorum–University of Bologna, Bologna, Italy, [3]Department of Food, Environmental and Nutritional Sciences (DeFENS), University of Milan, Milan, Italy

Vimentin (VIM) and desmin (DES) are muscle-specific proteins having crucial roles in maintaining the lateral organization and alignment of the sarcomeric structure during myofibrils' regeneration. The present experiment was designed to ascertain the evolution of VIM and DES in *Pectoralis major* muscles (PM) of fast-growing (FG) and medium-growing (MG) meat-type chickens both at the protein and gene levels. MG broilers were considered as a control group whereas the evolution of VIM and DES over the growth period was evaluated in FG by collecting samples at different developmental stages (7, 14, 21, 28, 35, and 42 days). After performing a preliminary classification of the samples based on their histological features, 5 PM/sampling time/genotype were selected for western blot, immunohistochemistry (IHC), and gene expression analyses. Overall, the findings obtained at the protein level mirrored those related to their encoding genes, although a potential time lag required to observe the consequences of gene expression was evident. The two- and 3-fold higher level of the VIM-based heterodimer observed in FG at d 21 and d 28 in comparison with MG of the same age might be ascribed to the beginning and progressive development of the regenerative processes. This hypothesis is supported by IHC highlighting the presence of fibers to co-expressing VIM and DES. In addition, gene expression analyses suggested that, unlike *VIM* common sequence, *VIM* long isoform may not be directly implicated in muscle regeneration. As for DES content, the fluctuating trends observed for both the native protein and its heterodimer in FG might be ascribed to its importance for maintaining the structural organization of the regenerating fibers. Furthermore, the higher expression level of the *DES* gene in FG in comparison with MG further supported its potential application as a marker of muscle fibers' regeneration. In conclusion, the findings of the present research seem to support the existence of a relationship between the occurrence of muscle regeneration and the growth rate of meat-type

chickens and corroborate the potential use of VIM and DES as molecular markers of these cellular processes.

KEYWORDS

broiler chicken, growth-rate, immunohistochemistry, western blot, gene expression

Introduction

Vimentin (VIM) and desmin (DES) belong to the family of type III intermediate filament proteins, specific components of the cytoskeletal network having a diameter that is intermediate between those of actin microfilaments and microtubules (Banwell, 2001). These proteins exert a pivotal role in maintaining the lateral organization and alignment of the myofibrils in developing and mature myotubes (Gard and Lazarides, 1980). In detail, although after their synthesis VIM and DES exhibit a diffused cytoplasmic distribution (Vater et al., 1994), upon myofibers' maturation they attain a sarcomeric pattern and are mainly located at the Z-disk (Bornemann and Schmalbruch, 1992; Vater et al., 1994; Vaittinen et al., 2001). In support of their strong interconnection and essential role in maintaining sarcomeres' integrity and functionality, VIM and DES were demonstrated to share a common structural organization, comprising a central α-helical rod domain–characterized by high homology in their amino acid sequences–flanked by amino- and carboxy-terminal domains (Tokuyasu et al., 1984; Cooper and Hausman, 2000). As for their dynamic expression, it is generally held that VIM can be transiently found in the early stages of myotubes differentiation, whereas DES content gradually increases to become the main intermediate filament protein in mature myofibers (Tokuyasu et al., 1984). In detail, since the newly synthesized DES filaments integrate into the pre-existing VIM ones and partially replace them, VIM and DES were found to co-assemble and co-distribute and, as a consequence, the initial distribution of DES mainly reflects that attained by VIM (Granger and Lazarides, 1979; Tokuyasu et al., 1984; Bornemann and Schmalbruch, 1992).

From the beginning of the XX century, several studies have been carried out to evaluate the intracellular organization and dynamic expression of VIM and DES and improve the knowledge concerning their interactions (Bornemann and Schmalbruch, 1992; Gallanti et al., 1992; Cheng et al., 2016). Later on, these aspects have been widely investigated both in humans affected by neuromuscular and myopathic disorders and in artificially induced animal models (Kottlors and Kirschner, 2010; Fröhlich et al., 2016; Agnetti et al., 2021) to ascertain their potential role in regenerating muscles. These studies allowed to shed light on their crucial role in maintaining muscle cytoarchitecture and recognized them as reliable markers for the regenerative processes taking place within the skeletal muscles (Tokuyasu et al., 1985; Gallanti et al., 1992).

A recent study was performed by our research group to evaluate the distribution and expression of VIM and DES in the *Pectoralis major* muscles (PM) belonging to fast-growing (FG) broilers having normal phenotype, as well as in those exhibiting the macroscopic features ascribable to the white striping, wooden breast, and spaghetti meat abnormalities to ascertain their potential involvement in the time-series of events leading to the progressive development of these conditions (Soglia et al., 2020). Indeed, the histological examinations carried out on PM affected by the abovementioned defects highlighted the occurrence of intense regenerative processes along with profound alterations of the connective tissue composing the perimysial septa (Soglia et al., 2019). Given the above and considering that growth-related abnormalities mainly manifest in broilers selected for growth performance parameters (e.g., growth rate, breast meat yield, etc.), the present experiment was designed to ascertain the evolution of VIM and DES in PM of modern chicken hybrids selected for meat production. In detail, the distribution of VIM and DES and the quantification of their expression, both at the protein and gene level, were evaluated in PM of chickens belonging to FG and medium-growing (MG) genotypes. In this context, MG broilers were considered as a control group in light of the allometric growth of their PM that should not imply the development of intense regenerative processes which are commonly observed in FG (Praud et al., 2021). In detail, to improve the current knowledge concerning the evolution of VIM and DES over the growth period of FG birds and assess the eventual differences which might be ascribed to the animals' growth rate, samples were collected at different developmental stages (i.e., 7, 14, 21, 28, 35, and 42 days of age).

Materials and methods

A total of 100 one-day-old male chicks, 70 FG and 30 MG both selected for meat production purposes, were allotted to an environmentally controlled poultry facility. The same commercial corn-wheat-soybean basal diet were provided to both genotypes according to a 3-phase feeding program: starter (0–14 days), grower (15–28 days) and finisher (29-end). Feed and water were administered *ad-libitum*. Stocking density (maximum 33 kg/m^2), birds' handling, raising, and processing conditions were defined according to the European legislation in force (European Union, 2007; European Union, 2009; European Union, 2010). The experiment was approved by the Ethical Committee of the Italian Ministry of Health (ID: 1194/2021).

As part of the experimental design, 10 FG and 5 MG birds were slaughtered when reaching different developmental stages (corresponding to 7, 14, 21, 28, 35, and 42 days) and samples were excised from the ventral surface of the PM (facing the skin), following the procedure described by Soglia et al. (2020). Briefly, samples collected for histology and immunohistochemical analyses were excised from the superficial section of the cranial portion of the PM, quickly frozen in isopentane (cooled with liquid nitrogen) and stored at -80°C. Further two samples were collected from the same position of each PM, quickly frozen in liquid nitrogen, and stored in a deep freezer (-80°C) until proteins and RNA extraction.

At each sampling time, PM were preliminary classified according to their macroscopic traits as unaffected muscles, exhibiting macroscopically normal appearance, or affected cases (i.e., showing features ascribable to the white striping and wooden breast abnormalities which are currently affecting the pectoral muscles of the FG hybrids) according to the criteria recently revised by Petracci et al. (2019). Then, before processing the samples for further analyses, a microscopic examination was performed to provide a more accurate and reliable classification of the samples not only based on their macroscopic traits but also considering their histological features (i.e., presence of necrotic fibers and inflammatory cells, increased deposition of connective tissue and fat). From each PM, 10 serial cross-sections (10 µm-thick) were cut on a cryostat microtome at −20°C, mounted on poly-L-lysine coated glass slides (Sigma-Aldrich, St. Louis, MO, United States) and stained with hematoxylin and eosin. Then, after evaluating their histological features, 5 PM/sampling time/genotype were selected and considered for further analyses.

Immunohistochemistry

Immunohistochemical analyses were performed following the procedure described in our previous study (Soglia et al., 2020) based on the avidin-biotin-peroxidase complex (ABC) method, with slight modifications. Briefly, for each PM, 10 serial cross-sections (10 µm thick) were cut on a cryostat microtome and mounted on poly-L-lysine coated glass slides (Sigma–Aldrich, St. Louis, MO). After rinsing in phosphate buffer saline, sections were incubated in 5% normal goat serum (for 30 min at room temperature, RT) to limit the eventual non-specific binding of the secondary antibodies. Then, sections were incubated (4°C in humid chamber for 24 h) with a monoclonal mouse anti-VIM and a polyclonal rabbit anti-DES (61013 and 10570, Progen Biotechnik GmbH, Heidelberg, Germany, respectively) antibodies both diluted 1: 1000. After washing, sections were incubated (RT for 1 h) with the biotin-conjugated goat anti-mouse IgG and biotin-conjugated goat anti-rabbit IgG secondary antibodies, both diluted 1:200 (Vector Laboratories, Burlingame, CA, USA), and then treated with ABC (Vector elite kit, Vector Laboratories, Burlingame, CA, USA). The immune reactions were then visualized through the 3,3′-diaminobenzidine (DAB) chromogen solution supplied by Vector (Vector DAB kit, Vector Laboratories, Burlingame, CA, USA). The sections were then washed in PBS and coverslipped with buffered glycerol, pH 8.6.

Western blot

Myofibrillar proteins were extracted following the procedure described by (Liu et al., 2014) with slight modifications. Briefly, 1 G of frozen PM was homogenized by Ultra-Turrax (IKA, Germany) (20 s at 13,500 rpm, in ice) in 20 ml of cold Rigor Buffer (75 mM KCl, 10 mM KH_2PO_4, 2 mM $MgCl_2$, 2 mM EGTA; pH 7.0) (Sigma–Aldrich, St. Louis, MO). The homogenate was centrifuged (10 min at 10,000 × g at 4°C) and the supernatant, containing the sarcoplasmic protein fraction, was discarded. The same procedure was repeated by adding 10 ml of cold Rigor Buffer, homogenizing (20 s at 13,500 rpm, in ice) and centrifuging the samples for 20 min under the same conditions previously adopted (10,000 × g at 4°C) and the resultant pellet, containing the myofibrillar proteins, was re-suspended by homogenization in 10 ml of cold Rigor Buffer. After being quantified (Bradford, 1976) by using bovine serum albumin as standard, the protein content of each extract was adjusted to 2.0 mg/ml and each sample was mixed 1:1 (v/v) with Sample Buffer (50 mM Tris-HCl, 8 M Urea, 2 M Thiourea, 75 mM DTT, 3% (v/v) SDS; pH 6.8) (Sigma–Aldrich, St. Louis, MO) (Fritz et al., 1989).

Myofibrillar proteins (10 µg) were loaded in 4–15% Mini-PROTEAN TGX Stain-Fee™ Gels (Bio-Rad Laboratories) and the electrophoretic separation was carried out with a Bio-Rad Mini Protean II electrophoresis apparatus at constant voltage (200 V) for 30 min. Gels were subsequently activated by UV exposure to produce a fluorescent signal resulting from the interaction of the tryptophan residues in the proteins polypeptide chains with the trihalo compounds incorporated into gel formulation (Gürtler et al., 2013), and protein fluorescence was acquired (to check the electrophoretic separation of the proteins) using a ChemiDoc™ MP Imaging System (Bio-Rad Laboratories) with the Image Lab software (version 5.2.1). Proteins were transferred onto a 0.2 µm nitrocellulose membrane using a Trans-Blot® Turbo™ Transfer System (Bio-Rad Laboratories) and incubated (45 min, at room temperature while shaking) with 15 ml TBST (Tris Buffered Saline with Tween® 20–20 mM Tris, 150 mM NaCl, 0.1% Tween 20; pH 7.4–7.6) with 5% skimmed milk powder. Membranes were probed (60 min, room temperature while shaking) with a monoclonal mouse anti-VIM (61013, Progen Biotechnik GmbH, Heidelberg, Germany) and a polyclonal rabbit anti-DES (10570, Progen Biotechnik GmbH, Heidelberg, Germany) antibodies diluted 1:4,000 and 1:6,000,

respectively. After washing, the membranes were incubated with secondary anti-mouse and anti-rabbit antibodies for 60 min (1: 15,000) (Merk Millipore, Burlington, Massachusetts, USA) and treated with Horseradish Peroxidase (HRP)-conjugated streptavidin (Merk Millipore, Burlington, Massachusetts, USA) for 20 min. Final detection was performed with enhanced chemiluminescence (Clarity™ Western ECL Substrate) Western Blotting detection kit (Bio-Rad Laboratories) and the images were acquired using the ChemiDoc™ MP Imaging System (Bio-Rad Laboratories). Densitometry differences were analyzed with the Image Lab software and normalized for the total fluorescent protein signal intensity (Valli et al., 2018). The results were expressed as %, considering 100% the intensity of the band assigned to VIM and DES in the PM belonging to the MG genotype sampled on d 7.

Gene expression

Total RNA was extracted from frozen PM muscles using TRIzol® Reagent (Invitrogen™, Thermo Fisher Scientific, Waltham, MA, United States), following the manufacturer's instructions. Quantification and purity of extracted RNA were tested by a ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, United States). Also, a visualization by agarose 1% was performed to check RNA integrity (Zappaterra et al., 2015). Then, iScript™ gDNA Clear cDNA Synthesis Kit (1725035, Bio-Rad Laboratories) was used for removing genomic DNA (gDNA) contamination from each sample and retrotranscribing 1 µg of total RNA to complementary DNA (cDNA), as recommended by the manufacturers. The expression levels of *Desmin* (*DES*) and two *Vimentin* sequences (*Vimentin* long isoform - *VIM long*; *Vimentin* common sequence - *VIM com*), as described in our previous study (Soglia et al., 2020), have been analyzed in the present paper. In particular, target and normalizing genes quantifications have been assessed by relative quantitative Real-Time polymerase chain reaction (qRT-PCR), using the standard curve method (Pflaff et al., 2004) on Rotor-Gene™ 6,000 (Corbett Life Science, Concorde, NSW, Australia). The qRT-PCRs were performed in a total volume of 10 µl using SsoAdvanced™ universal SYBR® Green Supermix (1725271, Bio-Rad Laboratories). Primer pairs were designed using Primer3Plus web online tool (Untergasser et al., 2012) and their complete information is reported in Supplementary Table S1. For each sample, every gene was quantified in triplicate to collect sample replications with coefficients of variation lower than 0.2. As regards the data normalization, genes coding for the ribosomal protein L4 (*RPL4*), ribosomal protein lateral stalk subunit P0 (*RPLP0*) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), which are reported as housekeeping in several studies (Velleman et al., 2014; Bagés et al., 2015; Powell et al., 2016; Zhang et al., 2018) were tested to identify the best couple of normalizing genes. In our study, *RPL4* and *GAPDH* were used as reference genes for the normalization of relative quantification of target genes, since the GeNorm algorithm (Vandesompele et al., 2002) evidenced them as the most stable genes. Amplification conditions of target and normalizing genes are reported in Supplementary Table S2.

Statistical analysis

Data were analyzed by using Statistica 10 (StatSoft Inc., 2014). In detail, within each sampling age (i.e., 7, 14, 21, 28, 35, and 42 days), the non-parametric Mann-Whitney U test was applied to assess the effect of the genotype (FG vs. MG) on the findings achieved for VIM and DES both at protein and gene level. For FG broilers, the one-way ANOVA option was used to evaluate the evolution of VIM and DES proteins as well as of their coding genes over their growth period. In addition, Spearman's correlations among the gene expression of *DES* and the two *VIM* sequences (*VIM long* and *VIM com*) were calculated per each sampling age and considering FG broilers on their whole. All statistical differences were considered significant at a level of $p \leq 0.05$.

Results

Histology

The results of the histological observations performed on PM of FG and MG chickens at different developmental stages (i.e., 7, 14, 21, 28, 35, and 42 days) are shown in Figure 1. A normal muscular architecture (Figure 1A,C), including fibers exhibiting polygonal profile and a normally structured connective tissue composing the endomysial and perimysial septa, was observed in MG during their whole growth period. As for FG, the histological features observed in the early stages of muscle development (i.e., 7, 14 and 21 days of age) were similar to those observed in MG whereas a prevalence of fibers having rounded profile along with a profoundly altered endomysial and perimysial connective tissue were found as the developmental stages proceeded (at d 28, 35, and 42). At these ages, several muscle fibers exhibited nuclear internalization, variable cross-sectional area (suggesting the contextual presence of degenerating and regenerating fibers), necrosis up to lysis, inflammatory cell infiltration, lipidosis, and fibrosis (Figures 1B,D).

Immunohistochemistry

Representative images of the pattern of immunoreactivity for VIM and DES in FG and MG chickens are shown in Figure 2. Overall, reactivity against VIM and DES was mainly observed in

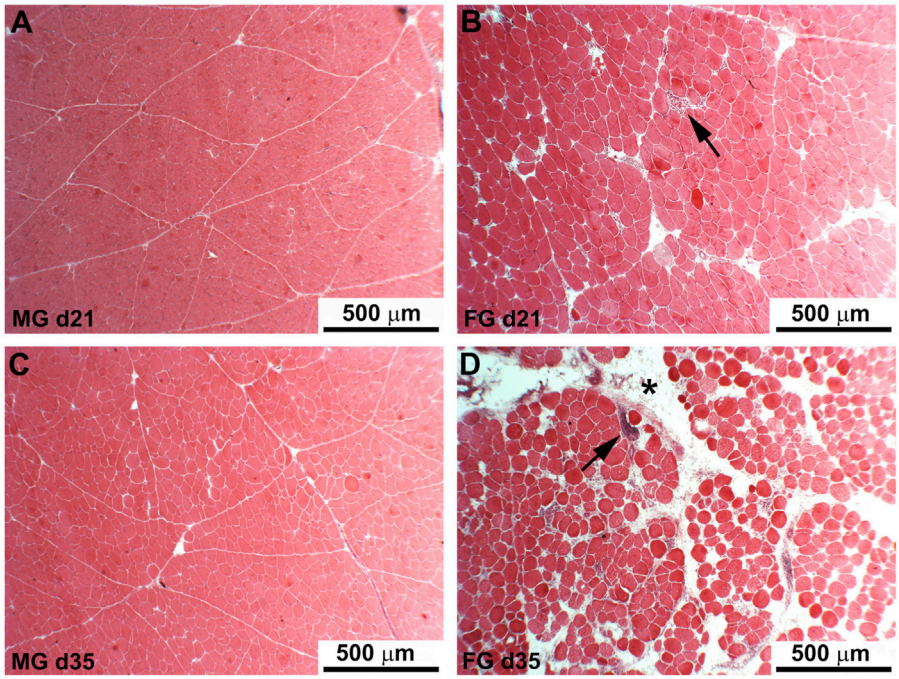

FIGURE 1
Hematoxylin and eosin staining of the *Pectoralis major* muscles of medium-growing chickens (21 and 35 days old, respectively) show fibers having a regular polygonal profile surrounded by normal endomysium and perimysium without any detectable damage or additional deposit of intermuscular adipose tissue **(A,C)**. In fast-growing chickens **(B,D)** many of the fibers exhibited rounded profile and variable diameter along with the presence of abundant adipose tissue infiltrated at endomysial and perimysial (D, asterisk) levels. Arrows indicate necrotic fibers surrounded by inflammatory infiltrates.

the intermyofibrillar network (peri and endomysial connective tissue), blood vessels and, in some cases, at the level of the fibers themselves. In detail, aside from the genotype, immunoreaction within the connective tissue was found particularly intense between 21 and 35 days of age. In addition, segments of the connective tissue composing the endo and perimysial septa as well as some fibers were found to co-express VIM and DES, whereas others exhibited a distinct reaction for VIM or DES (Figure 2A–F). As for their localization, VIM and DES were in some cases confined in the sub-sarcolemmal position while in others immunoreactivity was homogeneously distributed throughout the sarcoplasm (Figure 2). Regarding the effect of animals' growth, the greatest number of fibers immunoreactive to VIM and/or DES was observed at d 21, 28 and 35 (aside from the genotype), while a lower number of positive fibers was found earlier (i.e., d 7 and d 14) and at the end of the farming period (i.e., d 42).

Western blot

A representative image of the immunoblots obtained for VIM and DES is shown in Figure 3A,B (See also Supplementary Figure S3). Two distinct bands having, according to the marker, a molecular weight of 65 and 130 kDa were identified and ascribed to the native proteins (i.e., VIM and DES) and to their heterodimeric forms (i.e., VIM-VIM, DES-DES, VIM-DES).

The findings concerning the expression levels of VIM and DES in PM of FG and MG broilers at different developmental stages (7, 14, 21, 28, 35, and 42 days of age) are reported in Figures 4A,B and Figures 5A,B, respectively.

No significant differences among the genotypes were found in the relative concentration of the 65-kDa band with the only exception being the expression level of native VIM assessed at d 28 and d 35 of age (Figure 4A). Indeed, if compared with the results obtained in MG at d 28, a tendency ($p = 0.06$) of a higher VIM content was found in FG (25 vs. 50%) whereas its expression level was reduced to less than one half at d 35 (59 vs. 22%; $p < 0.01$).

As for the effect of the developmental stage, a progressive decline ($p < 0.001$) in the relative concentration of native VIM was observed within the PM of FG chickens as the age increased. In detail, if compared with the findings obtained at d 7, a significant reduction (-85%) in the relative concentration of native VIM was observed at d 42 (111 vs. 17%; $p < 0.001$).

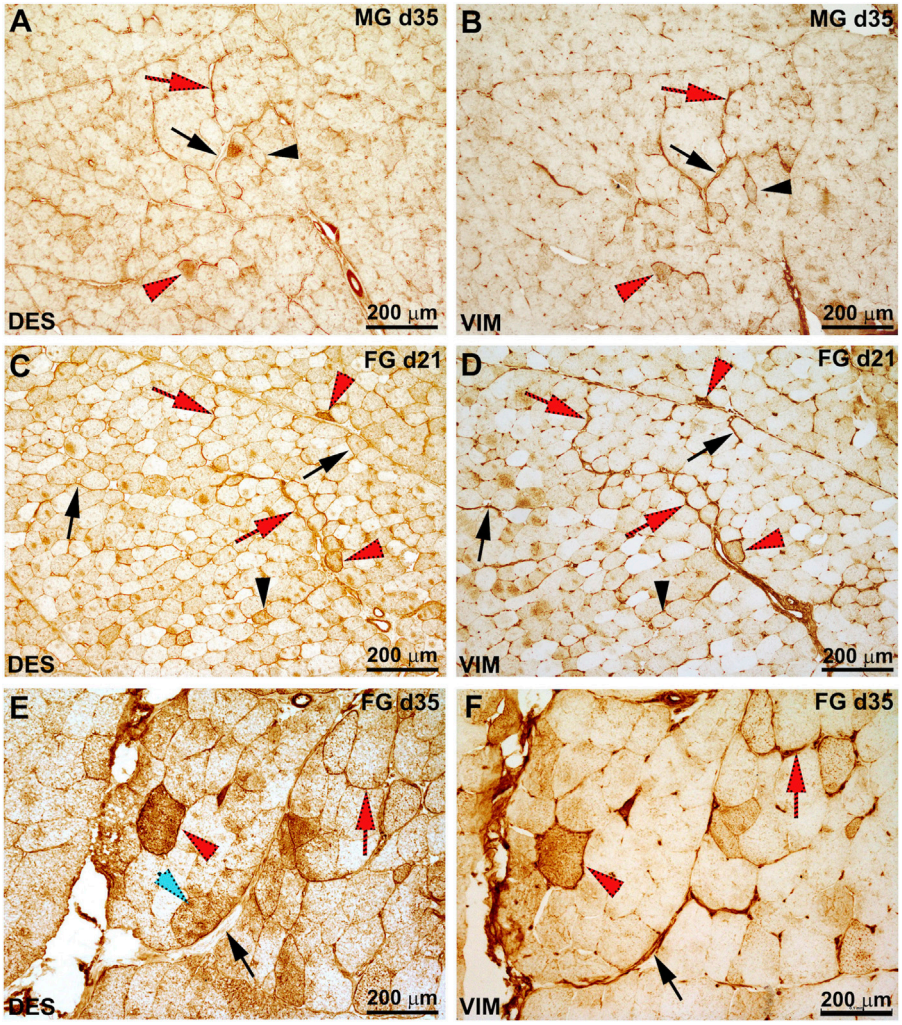

FIGURE 2
Representative images of the serial sections for desmin (DES) and vimentin (VIM) immunoreactivity in the medium-growing (MG) **(A, B)** and fast-growing (FG) **(C–F)** chickens at 21 and 35 days of age. The perimysial connective showed positivity against each target protein, while in other areas of the sections immunoreactivity against one protein rather that the other was evident. In detail, some tracts of the perimysial connective were found to co-express DES and VIM (red arrow) while in others only immunoreactivity for VIM was observed (black arrow). Similarly, some fibers were found to colocalize DES and VIM (red arrowhead) while others exhibit positivity only for VIM or DES (black arrowhead).

The findings concerning the relative concentration of the VIM heterodimeric form are shown in Figure 4B. No significant differences between FG and MG were found either at the beginning (i.e., d 7 and d 14) or at the end (d 42) of the rearing period. On the other hand, if compared with MG, two- and 3-fold higher ($p < 0.01$) expression levels of the 130-kDa band were found in FG at d 21 and 28, respectively. On the other hand, in agreement with the findings obtained for the native protein, a significantly higher ($p < 0.05$) relative concentration of the heterodimeric form was found at d 35 in MG in comparison with FG.

As for the effect ascribable to the developmental stage, the expression level of the 130-kDa band was found to sharply increase from d 14 to d 21, in which the highest values were found, followed by a progressive decline in the following ages.

The outcomes concerning the relative concentration of native DES and its heterodimeric form are reported in Figure 5A,B, respectively.

Native DES was found to be significantly higher within the PM belonging to FG if compared with MG at 7, 21, and 35 days of age (Figure 5A). On the other hand, no significant differences ascribable to the genotype were observed for the other developmental stages considered in the present study (i.e., 14, 28, 42 days).

As for the evolution of the content of native DES at different developmental stages of the birds, a wavering trend was found: a

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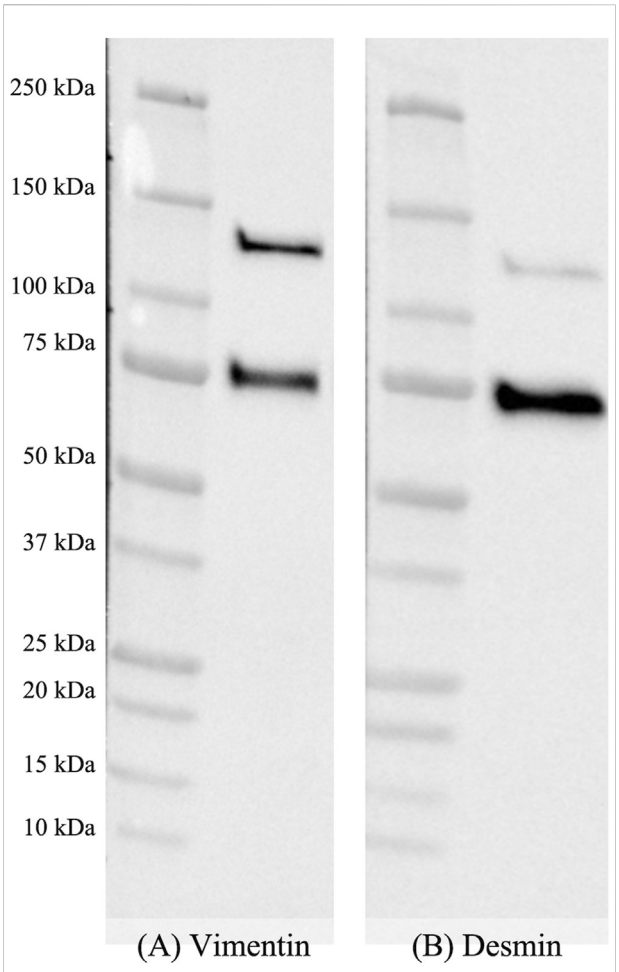

FIGURE 3
Representative image of nitrocellulose membranes incubated with monoclonal mouse anti-vimentin **(A)** and polyclonal rabbit anti-desmin **(B)** primary antibodies after performing the final detection with enhanced chemiluminescence.

remarkable increase in native DES observed at 7, 21, and 35 days was followed by a sharp decline in its content in the following ages (i.e., 14, 28, and 42 days). A similar trend was observed for the 130-kDa band (Figure 5B). In detail, if compared with MG, a remarkably higher content of the heterodimeric form was measured in FG at 7, 21, and 35 days (100 vs. 169%, 143 vs. 262%, and 37 vs. 185%; $p < 0.01$, respectively). No significant differences ascribable to the genotype were found at d 28 and d 42 whereas a significantly higher content of the heterodimer was observed at d 14 in MG in comparison with FG broilers of the same age (95 vs. 57%; $p < 0.01$).

As for the effect of the developmental stage, a significant decline in the relative concentration of the heterodimer was found at d 14 followed by a sharp increase in the concentration of this electrophoretic fragment (d 21). Then, the content of the 130-kDa band progressively declined as the age increased with the values assessed at the end of the rearing period being 4-fold lower than those measured at the beginning of the trial.

Gene expression

The normalized quantifications of the two *VIM* sequences (i.e., *VIM com* and *VIM long*) and the *DES* gene at each sampling time (7, 14, 21, 28, 35, and 42 days of age) are reported in Figure 6.

The *VIM* common sequence showed a normalized expression level higher in FG than in MG broilers at d 7 ($p < 0.05$) and d 35 ($p < 0.05$), and the difference was close to being significant at d 28 and d 42 ($p = 0.09$). No differences in the *VIM com* gene expression between FG and MG were found at d 14 and d 21 (Figure 6A). As for the *VIM long* (Figure 6B), the normalized expression level obtained using the primers specific for the *VIM* long-isoform showed significant differences between FG and MG at d 14 ($p < 0.05$), while a tendency was found at d 28 ($p = 0.06$). The normalized quantification of the *DES* gene is reported in Figure 6C. *DES* mRNA level was significantly higher expressed in FG than in MG broilers at d 28 ($p < 0.05$) and 42 ($p < 0.05$), whereas no differences were observed at d 7, d 14, d 21 and d 35.

As for the evolution of the normalized expression of the two *VIM* sequences in FG during the growth period (Table 1 and Figure 6A,B), transcription levels of *VIM com* progressively increased when passing from d 14 and d 21 to d 42. On the contrary, *VIM long* showed a progressive decline from d 14 to d 42. As for the effect of the developmental stage on the *DES* normalized expression in FG broilers, no statistical differences have been found (Table 1 and Figure 6C).

All the Spearman's correlation results obtained considering the normalized expression level of *VIM com*, *VIM long* and *DES* in FG broilers are reported in Table 2. Considering all the sampling times together, the normalized transcription levels of the two *VIM* sequences in FG broilers resulted to be negatively related to each other ($r = -0.58$; $p < 0.01$). Furthermore, Spearman's correlation analysis performed considering the sampling time separately showed a positive correlation between *VIM long* and *DES* in PM belonging to FG broilers at d 35 ($r = 0.90$; $p < 0.05$). On the other hand, the normalized quantification of *DES* in FG broilers resulted positively correlated to *VIM com* at d 42 ($r = 0.90$; $p < 0.05$).

Discussion

In the present study, the evolution of VIM and DES in the PM of FG and MG chickens has been assessed to improve the current knowledge concerning their evolution over the growth period of the birds and ascertain their eventual implication in the regenerative processes characterizing the pectoral muscles

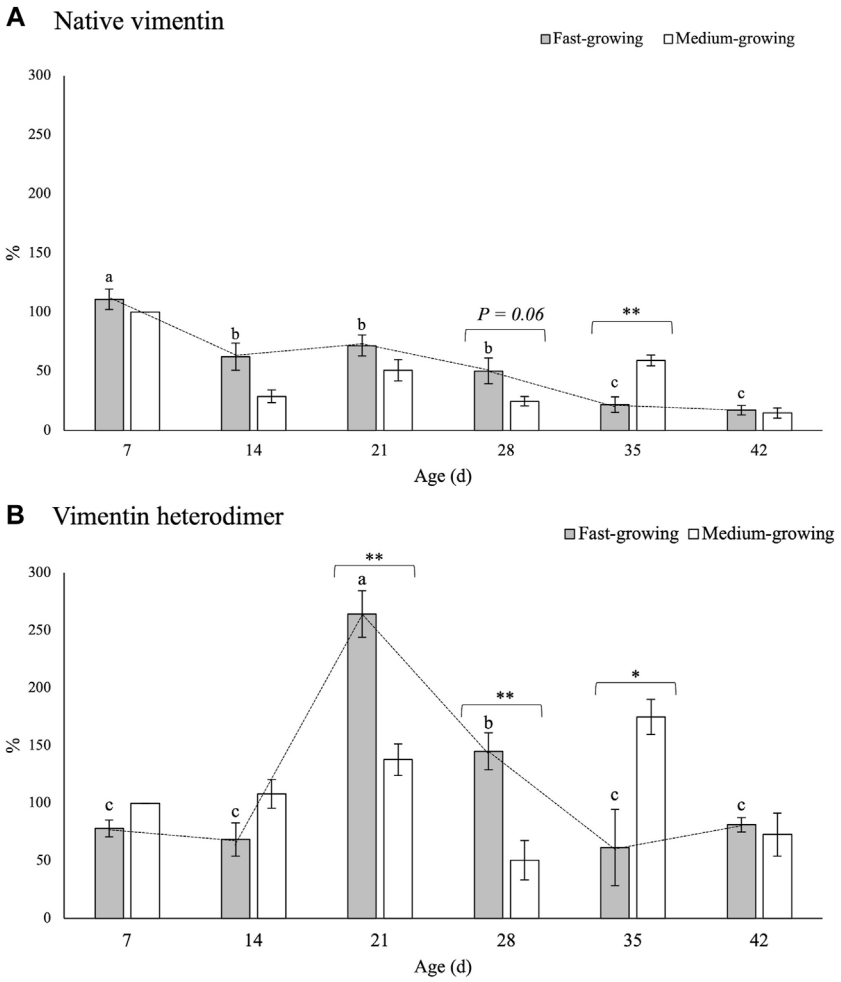

FIGURE 4
Relative concentrations of native vimentin **(A)** and of its heterodimeric form **(B)** in *Pectoralis major* muscles of broiler chickens belonging to fast- and medium-growing genotypes at different ages. The results are expressed as %, considering as 100% the intensity of the band in MG at the beginning of the rearing period (day 7–d 7). Error bars indicate standard error of mean. Within the same age (7, 14, 21, 28, 35, and 42 days) data were analyzed by using the non-parametric Mann-Whitney U test to investigate the effect of the genotype (FG vs. MG). * and ** = within the same age, mean values significantly differ between FG and MG for a p-value <0.05 and <0.01, respectively. On the other hand, the evolution of vimentin in FG over their growth period was assessed by One-way ANOVA. a-c = for FG, mean values followed by different letters significantly differ over the growth period ($p < 0.05$).

affected by growth-related abnormalities. The outcomes of the histological evaluations allowed us to obtain an accurate and precise classification of the PM. As expected, the PM of MG exhibited a normal muscular architecture in all the developmental stages considered in the present study whereas, although unaffected up to d 21, the FG ones displayed the microscopic lesions ascribable to white striping and/or wooden breast defects from d 28 onwards. These findings corroborated the evidence of an early onset of growth-related abnormalities in FG broilers, thus confirming that even those PM having a normal phenotype may exhibit microscopic features ascribable to muscular abnormalities (Sihvo et al., 2018; Mazzoni et al., 2020). In addition, the histological examinations seemed to support a strong association between the altered muscular architecture observed in FG broilers during growth and the breeding selection processes/plans which were implemented for their development.

As for the findings achieved through Western Blot analyses, the presence of two bands, having a molecular weight of 65 and 130 kDa, might be ascribed to native VIM or DES and to the development of either homo- or hetero-dimers (i.e., VIM-VIM, DES-DES, VIM-DES) resulting from their association at the Z-disk (Cooper and Hausman, 2000). This hypothesis is corroborated by previous studies demonstrating the existence of a VIM-DES heterodimer, consisting of face-to-face pairs of two different VIM and DES subunits, both in skeletal and smooth

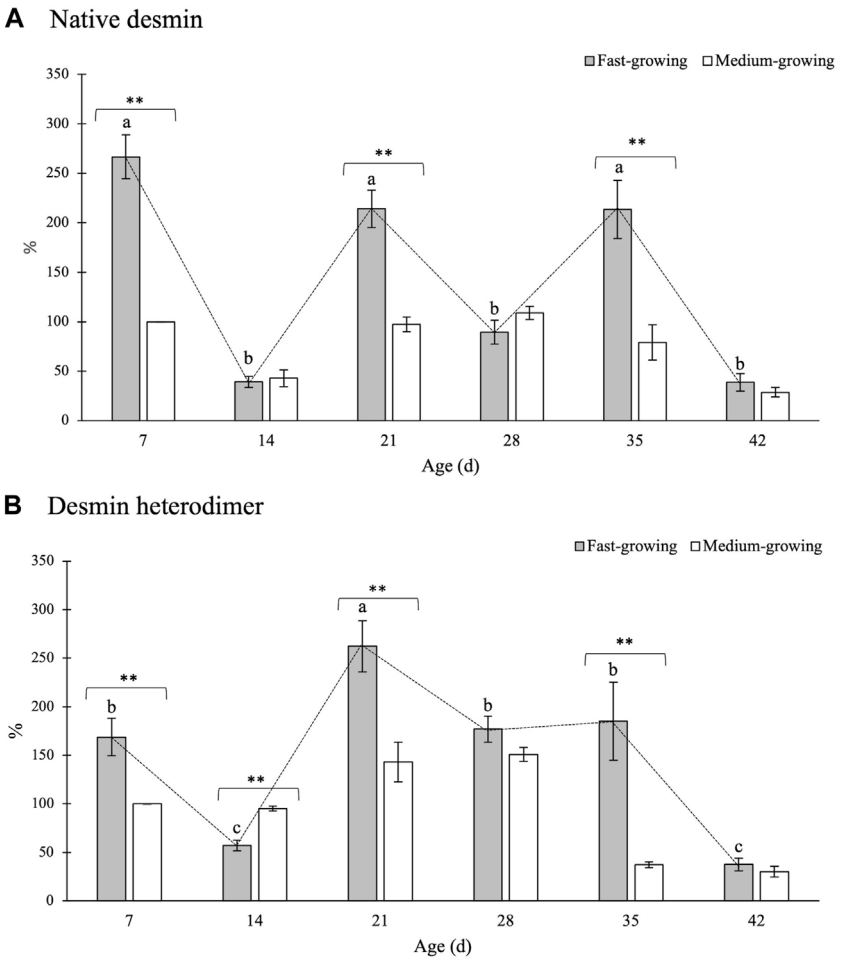

FIGURE 5
Relative concentrations of native desmin **(A)** and of its heterodimeric form **(B)** in *Pectoralis major* muscles of broiler chickens belonging to a fast- and medium-growing genotype at different ages. The results are expressed as %, considering as 100% the intensity of the band in MG at the beginning of the rearing period (day 7–d 7). Error bars indicate standard error of mean. Within the same age (7, 14, 21, 28, 35, and 42 days) data were analyzed by using the non-parametric Mann-Whitney U test to investigate the effect of the genotype (FG vs. MG). * and ** = within the same age, mean values significantly differ between FG and MG for a p-value <0.05 and <0.01, respectively. On the other hand, the evolution of desmin in FG over their growth period was assessed by One-way ANOVA. a-c = for FG, mean values followed by different letters significantly differ over the growth period ($p < 0.05$).

muscles as well as in cultured renal cells (Quinlan and Franke, 1982; Traub et al., 1993; Yang and Makita, 1996). Interestingly and consistently with what has been found by Traub et al. (1993), these two IF proteins seem to resist dissociation also using a high concentration of urea (e.g., 8 M urea) in the extraction buffer, thus suggesting a strong protein dimerization.

When considering the findings obtained for VIM protein, the differences related to the genotype might be presumably attributed to the higher growth rate of FG broilers. The two- and 3-fold higher level of the VIM-based heterodimer observed in FG at d 21 and d 28 in comparison with MG chickens of the same age might be ascribed to the beginning and progressive development of the regenerative processes taking place in these PM. Indeed, a sharp increase in VIM synthesis was already observed during the early stages of regeneration (Vater et al., 1994). Although at d 21 none of the PM belonging to FG exhibited the phenotypes and the macroscopic features associated with the white striping and/or wooden breast conditions, that did not exclude the eventual occurrence of occasional, up to intense, regenerative phenomena involving an augmented synthesis of VIM. Indeed, evident signs of muscle regeneration were demonstrated even within those PM exhibiting a normal visual appearance (Mazzoni et al., 2020). The histological observations further corroborated the results obtained by Western Blot analysis. Indeed, the first evidence of abnormalities affecting the fibers and the connective tissue

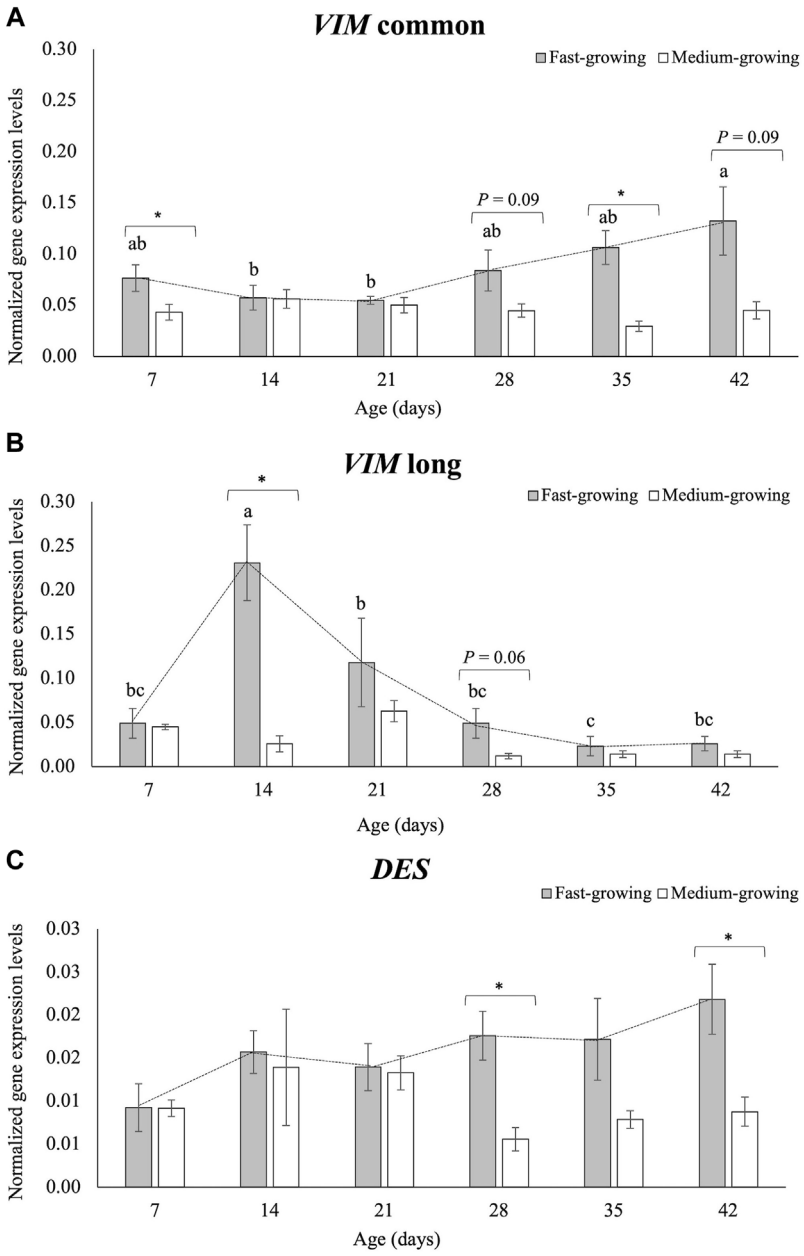

FIGURE 6
The normalized expression values of the *Vimentin* (*VIM*) common sequence **(A)**, *Vimentin* (*VIM*) long-isoform **(B)**, and *Desmin* (*DES*) gene **(C)** in *Pectoralis major* muscles of broilers belonging to a fast- and medium-growing genotype at different ages (7, 14, 21, 28, 35, and 42 days). For each panel **(A,B,C)**, the bar graphs show the mean values of the normalized expression level for each genotype (FG and MG) at each sampling time, and the error bars indicate the measured standard deviations. Significant differences between FG and MG at each sampling time were analyzed by using the non-parametric Mann-Whitney U. The significant *p*-values ($p \leq 0.05$; *) and trends towards significance ($p \leq 0.10$) are reported for the comparisons between FG and MG within each sampling age. On the other hand, the line graph evidences the evolution of the normalized expression level of each gene during the time in FG broilers. The evolution of the normalized gene expression of *VIM* common sequence, *VIM* long-isoform and *DES* gene in FG over their growth period was assessed by One-way ANOVA. a-c = for FG, mean values followed by different letters significantly differ over the growth period ($p \leq 0.05$).

were readily detected in FG at d 21. In agreement with that, the immunohistochemical analyses performed at this age revealed an increased expression of VIM both at the level of the endo and perimysial connective tissue as well as located within the muscle fibers in a sub-sarcolemmal and sarcoplasmic positions. Overall, these findings might support the hypothesis of the occurrence of

TABLE 1 One-way ANOVA results for the different developmental stages of fast-growing (FG) broilers. For each sampling time (d 7, d 14, d 21, d 28, d 35 and d 42), mean and standard deviation (SD) of the normalized gene expressions are reported.

FG

Gene	N	Mean	SD	Group effect	
				F Value	*P* (>F)
VIM long				6.792	0.001
d 7	5	0.049	0.017		
d 14	5	0.231	0.043		
d 21	5	0.118	0.050		
d 28	5	0.049	0.017		
d 35	5	0.023	0.011		
d 42	5	0.026	0.008		
VIM com				2.386	0.069
d 7	5	0.076	0.013		
d 14	5	0.057	0.012		
d 21	5	0.055	0.004		
d 28	5	0.084	0.020		
d 35	5	0.106	0.016		
d 42	5	0.132	0.033		
DES				1.541	0.214
d 7	5	0.009	0.003		
d 14	5	0.016	0.002		
d 21	5	0.014	0.003		
d 28	5	0.018	0.003		
d 35	5	0.017	0.005		
d 42	5	0.022	0.004		

TABLE 2 Spearman's correlations between the expression levels of *VIM* common sequence (*VIM com*), *VIM* long-isoform (*VIM long*) and *DES* genes in FG broilers considering all the samples together and each sampling time separately (d 7, d 14, d 21, d 28, d 35 and d 42). Significant correlations are reported in bold. *$p < 0.05$; **$p < 0.01$; n.s., not significant.

All	***VIM com***	***VIM long***	***DES***
VIM com	1	**-0.58****	n.s
VIM long	**-0.58****	1	n.s
DES	n.s	n.s	1
d 7	*VIM com*	*VIM long*	*DES*
VIM com	1	n.s	n.s
VIM long	n.s	1	n.s
DES	n.s	n.s	1
d 14	*VIM com*	*VIM long*	*DES*
VIM com	1	n.s	n.s
VIM long	n.s	1	n.s
DES	n.s	n.s	1
d 21	*VIM com*	*VIM long*	*DES*
VIM com	1	n.s	n.s
VIM long	n.s	1	n.s
DES	n.s	n.s	1
d 28	*VIM com*	*VIM long*	*DES*
VIM com	1	n.s	n.s
VIM long	n.s	1	n.s
DES	n.s	n.s	1
d 35	*VIM com*	*VIM long*	*DES*
VIM com	1	n.s	n.s
VIM long	n.s	1	**0.90***
DES	n.s	**0.90***	1
d 42	*VIM com*	*VIM long*	*DES*
VIM com	1	n.s	**0.90***
VIM long	n.s	1	n.s
DES	**0.90***	n.s	1

intense regenerative processes in an early stage within these PM which is in agreement with previous studies carried out on wooden breast-affected muscles (Papah et al., 2017; Sihvo et al., 2017; Griffin et al., 2018; Chen et al., 2019). As for the findings achieved at d 28, if compared with MG, the significantly higher VIM content (either in its native or heterodimeric forms) observed in FG broilers - which from this age exhibited the distinctive features associated with growth-related myopathies - might be due to the progression of the regenerative processes.

As for the effect of the FG birds' growth, overall, different trends were observed for the native protein and its heterodimeric form. In detail, the progressive reduction in native VIM observed as the developmental stage proceeded might be explained by considering previous studies, carried out at the end of the 1970s, demonstrating a higher VIM content in skeletal muscles belonging to young rather than old chickens (Granger and Lazarides, 1979; Gard and Lazarides, 1980). On the other hand, VIM heterodimeric form exhibited a bell-shaped evolution in which a sharp increase from d 7 and d 14 to d 21 (when it reaches its maximum expression) was then followed by a gradual decline thereafter. These outcomes perfectly matched with those obtained through IHC in which a higher and more intense immunoreactivity for VIM was observed in the inter-myofibrillar network at d 21 and d 35. This trend might be due to the progression of the regenerative processes within the PM requiring the synthesis of this protein. Indeed, VIM is transiently expressed in immature myotubes and, although it gradually disappears upon myoblasts fusion (Bennett et al., 1979; Granger and Lazarides, 1979; Bornemann and Schmalbruch, 1992; Gallanti et al., 1992), it can be detected in fibroblasts composing the connective tissue sheaths as well as in vascular endothelia (Čížková et al., 2009). In agreement with these observations, a 55% higher VIM content was found in wooden breast-affected PM if compared with their unaffected counterpart (Soglia et al., 2020). In this context, it is worth mentioning that VIM seems to be implicated in several cellular

processes occurring during muscle regeneration and that the available knowledge concerning their related pathway may help in understanding the mechanisms associated to muscle regeneration in FG broilers. The presence of degenerating/necrotic fibers, indeed, induces the activation of a satellite cell (SC)-mediated regeneration process whose first evidence at molecular and histological levels was observed in FG as early as two- and 3 weeks post-hatch, respectively (Papah et al., 2017, 2018; Sihvo et al., 2017). Intriguingly, this time course seems to perfectly match the findings achieved in the present study and support the hypothesis of a relevant role of VIM in muscle regeneration (Ostrowska-Podhorodecka et al., 2022) which, in its turn, contributes to explain the absence of differences at the protein level between FG and MG birds at the beginning of the growing period. This initial similarity may be partially due to the response time needed to induce the development of the first regenerative processes to counteract myofiber degeneration and necrosis. Indeed, VIM participates in many processes of crucial importance for tissue repair and regeneration (Cheng et al., 2016; Danielsson et al., 2018) and its expression is maximal during myotubes formation and SCs proliferation (Paulin et al., 2022). In this regard, the findings of the present study demonstrated that the occurrence of regenerative processes within the PM of FG birds can be detected at protein level (either by means of Western Blot or IHC analyses) as early as 3 weeks post-hatch when the content of the VIM-based heterodimer reaches its utmost level.

When considered on their whole, findings at protein level mirrored those related to gene expression. In detail, any change observed in the expression level of the *VIM* and *DES* genes seemed to be followed by a variation in protein synthesis in the subsequent sampling time, thus suggesting a potential time lag needed to observe the consequences of gene expression even at protein level.

As for gene expression, the two different primer pairs, previously used by Soglia et al. (2020), successfully amplified the *VIM* common sequence and the *VIM* long-isoform, thus validating that both *VIM* sequences are commonly expressed not only in FG but also in MG chickens. As previously reported, the *VIM* long-isoform differs from the common sequence by having a longer promoter and exon one regions (Soglia et al., 2020). Interestingly, in humans these regions of *VIM* gene were recently demonstrated to bind proteins playing important roles in the transcription regulation of other genes and proteins implicated in protein synthesis (e.g., the eukaryotic elongation factor-1 complex; eEF-1), cell migration and extracellular matrix remodeling (Al-Maghrebi et al., 2002; Pisani et al., 2016; Ostrowska-Podhorodecka et al., 2022). Therefore, dissimilarities between the two *VIM* sequences at the promoter level might suggest differences in regulating protein synthesis. In addition, it was also demonstrated that *VIM* exerts a role in contrasting cellular stress conditions (such as those induced by the accumulation of misfolded proteins) by interplaying with misfolded aggregates and interacting with proteins involved in the inflammatory response (Pattabiraman et al., 2020). Given the above and considering the numerous signs of stressful conditions (e.g., oxidative stress, inflammatory stress, and endoplasmic reticulum stress) observed in FG broilers, especially those affected by growth-related abnormalities (Abasht et al., 2016; Papah et al., 2018; Pampouille et al., 2019), a possible involvement of VIM in contrasting cellular stress may be hypothesized. According to this hypothesis, the remarkable increase in VIM heterodimeric form observed at d 21 might be attributed to muscle regeneration from one side and it might also represent a compensatory mechanism aimed at contrasting cellular stress in FG muscles. Moreover, the differences in *VIM com* observed in FG during the growth period and between FG and MG broilers at d 28, d 35 and d 42 suggest that this isoform may belong to one or more transcripts having a role not only in muscle regeneration but also in contrasting cellular stressful events. The role of VIM protein in protecting mitochondria during oxidative stress has been demonstrated in cultured cells, and mutations causing aminoacidic substitution in specific VIM sites or isoforms lacking some specific sequences in the N-terminal were found to cause a loss of VIM protective ability on mitochondria (Matveeva et al., 2010). In agreement with that, different variants of *VIM* transcripts were demonstrated to have diverse functions (Danielsson et al., 2018). Considering the opposite trend of the two *VIM* sequences as the growth proceeded, different roles between *VIM com* and *VIM long* during the animal growth could be hypothesized. In detail, a potential involvement of *VIM com* in the development of growth-related abnormalities in FG might be hypothesized whereas *VIM long* might not have substantial roles in the mechanisms underlying broilers' abnormalities.

As mentioned above, the *VIM* gene may regulate extracellular matrix remodeling through post-transcriptional regulatory pathways concerning collagen synthesis (Ostrowska-Podhorodecka et al., 2022) and fibroblast proliferation (Danielsson et al., 2018). Differences in the expression level of *VIM* between FG and MG birds have been detected since the early stages of growth. In this respect, considering the important role of VIM in SCs proliferation (Vater et al., 1994), the high level of *VIM com* observed in FG at d 7 might be partially related to the growth rates of these genotypes and to SCs' activity, which reaches its maximum level during the first week post-hatch (Halevy et al., 2000; Daughtry et al., 2017; Velleman and Song, 2017; Velleman et al., 2019). Moreover, since *VIM* was found to play a key role in protecting stem cells during proliferation (Pattabiraman et al., 2020), its potential involvement in counteracting the development of cellular stress during SCs proliferation and muscle regeneration could be hypothesized. To support this hypothesis, it is worth mentioning that selection for increased growth rate and muscle mass accretion resulted in a hypertrophic

growth of the fibers and concurrently favored the development of thicker PM, which are however perfused by an insufficient circulatory supply (Sihvo et al., 2018; Pampouille et al., 2019). Under these circumstances, the SCs function and muscle repair mechanism may be hindered (Velleman, 2019) thus contributing to explain the severe histological lesions observed in FG broilers from d 35 to d 42. Overall, these findings further corroborate the hypothesis of a strong association between SCs proliferation (which is necessarily required for muscle fiber regeneration) and the expression and subsequent synthesis of VIM (Vater et al., 1994). In addition, VIM was found to exert a profound effect on fibroblasts' proliferation thus leading to collagen production from one side and TGF-β secretion from the other (Velleman, 2019). The expression of this last transforming growth factor may be further exacerbated under hypoxic conditions, frequently occurring in FG (Abasht et al., 2016; Malila et al., 2019; Pampouille et al., 2019; Lake and Abasht, 2020) and, in its turn, regulates decorin expression during muscle growth, which is essential for collagen fibril formation and crosslinking. In addition, along with decorin, TGF-β is involved in the modulation of muscle fibrosis by means of regulating connective tissue growth factors (Velleman et al., 2019). Overall, VIM was found to have the ability to tip the balance from the regenerative process to the fibrotic repair (Walker et al., 2018). In this regard, given the above and considering the upregulation of the genes encoding for decorin and TGF-β previously observed in wooden breast-affected muscles (Velleman and Clark, 2015), it seems reasonable to hypothesize a regulatory role of VIM in this whole process, with special regard to the *VIM com* sequence at the gene level. On the other hand, the progressive decline in the amount of *VIM long* sequence and VIM protein observed in FG broilers respectively from d 14 and d 21 onward might be explained by considering that VIM synthesis gradually decreases with myotubes maturation (Bornemann and Schmalbruch, 1992). This trend was observed both in FG and MG with the last exhibiting an increase in VIM protein at d 35 (even exceeding that assessed in FG), likely ascribable to their growth-rate which may have slowed down the onset of muscle degeneration and subsequent regeneration. The regeneration processes likely ceased or being impaired, became largely ineffective during the developmental stages corresponding to the achievement of market weight (i.e., d 35 and d 42) so that no further variations in VIM content were observed in FG broilers. Accordingly, the progressive decline of the *VIM* long-isoform seemed to be associated with the physiologic role of this protein aimed at maintaining cell architecture, adhesion and migration (Danielsson et al., 2018). Overall, results obtained for VIM protein agreed with those of the *VIM long* gene in which a progressive decline of its normalized quantification was observed in FG and no differences were found between the two genotypes at d 35 and d 42. On the contrary, considering the significant differences in the normalized expression level of *VIM com* observed between FG and MG at d 35, along with the tendency toward significance at d 28 and d 42, the common sequence of the *VIM* gene may be considered a reliable marker of the regenerative processes in FG broilers, at least from d 35 onwards. On the whole, the present results seemed to support the hypothesis that different *VIM* transcripts may determine the synthesis of proteins having diverse biological roles, as previously described in humans (Zhou et al., 2010), and that *VIM long* may not be directly related to muscle regeneration, as opposed to what hypothesized for *VIM com*.

The outcomes of the investigations carried out to quantify native DES and its heterodimeric form exhibited analogous and fluctuating trends in which an increased synthesis of this protein is then followed by a sharp decline in its content in the subsequent sampling age. These findings perfectly matched with the events related to VIM thus confirming their sequential and differential synthesis during the regeneration processes taking place within the PM. Indeed, once synthesized, DES integrates into the pre-existing VIM filaments and, as a consequence of their progressive decline, ultimately results in the development of a DES-dominated network (Cary and Klymkowsky, 1994). Accordingly, a co-expression of VIM and DES was observed by means of IHC both within the connective tissue composing the endomysial and perimysial spaces and in the fibers. Indeed, some fibers were found to be immunoreactive to both VIM and DES (thus demonstrating their co-expression) whereas others exhibited a distinct reaction for one protein rather than the other.

Although there is still an ongoing debate, since its biochemical identification at the end of 1970s, different functions have been ascribed to DES. Among the others, DES was demonstrated to exert a primary role in maintaining sarcomeres' alignment (Morgan, 1990) and, in light of being one of the first muscle-specific genes expressed during development, it was also hypothesized to play a biological role in muscle development (Rudnicki et al., 1993). Indeed, desmin-null mice showed impaired myoblast fusion and myotube formation (Hnia et al., 2015) along with altered nuclear and mitochondrial shape/positioning (Paulin and Li, 2004; Capetanaki et al., 2007). Not surprisingly, DES was also found to regulate a broad spectrum of cellular processes by serving as a platform for the integration of signals between the outside and the inside of some organelles, such as the nucleus and mitochondria (Clemen et al., 2013; Hnia et al., 2015). Given the above, the distribution and expression of DES were investigated in muscular disorders (e.g., Duchenne Muscular Dystrophy) affecting humans and other species (Gallanti et al., 1992; Fröhlich et al., 2016), and it was considered a reliable marker of muscle regeneration under pathological conditions. Thus, the fluctuating trends observed for both native DES and its heterodimeric form might be due to the degenerative and subsequent regenerative processes occurring in PM of FG chickens. Muscle degeneration, implying fibers' necrosis and loss of myofibrils, likely resulted in DES degradation followed by an increased synthesis of this protein to support the structural

organization of the regenerating fibers. In this regard, it is worth mentioning the lack of correspondence between *DES* gene and its encoded protein at d 42 when a 2-fold higher expression of *DES* gene was found in FG compared with MG, whilst no differences were found at the protein level. The higher transcriptional level of *DES* observed in FG at d 42 could be likely due to an up-regulation aimed at synthesizing the degraded DES protein and thus re-establishing the structural organization of the regenerating fibers. In addition, the discrepancy in the protein content may be imputable to a potential time lag needed for the translation of the DES protein thus determining a delay in the observation of the consequences of the gene expression at the protein level. In addition, although not significant, the increasing trend observed for *DES* gene in FG over the growth period was consistent with the differential synthesis of this protein, which progressively integrates and replaces the pre-existing VIM filaments (Cary and Klymkowsky, 1994). Indeed, the opposite general trends of *VIM long* and *DES* observed in FG from d 7 to d 42 perfectly overlapped with the evidence that *VIM* expression is downregulated as muscle regeneration proceeds and/or during myotubes' maturation whereas *DES* level increases (Hnia et al., 2015).

Regarding the role of DES in muscle regeneration processes, Soglia et al. (2020) evidenced an increased abundance both at the gene and protein level of DES in chickens affected by growth-related abnormalities, thus proposing its level of expression as a marker for the regenerative phenomena taking place in PM muscles belonging to FG broilers. In this regard, the higher level of *DES* in FG compared with their MG counterpart observed in the present research corroborated the statement that this gene can be effectively exploited as a marker of muscle fibers regeneration in broiler chickens, especially from d 28 onwards. In addition, it is worth mentioning the potential role of DES in regulating mitochondrial morphology and bioenergetic capacity (Kuznetsov et al., 2020) along with the evidence that its defective anchorage and/or spacing profoundly affect these features (Knowles et al., 2002). The abovementioned traits mirrored those observed at ultrastructural level by Sihvo et al. (2018) in the early phase of wooden breast (in 22-days-old broilers), when mitochondrial swelling, vacuolation, and loss of cristae were apparent and associated with endoplasmic reticulum stress (Papah et al., 2018; Sihvo et al., 2018). Therefore, considering the presence of DES aggregates observed within the fibers through IHC, a synthesis and subsequent accumulation of an altered DES protein (Delort et al., 2019) might be hypothesized to occur also in FG broilers.

Our hypothesis concerning the essential role of VIM and DES in the regeneration processes occurring in FG chickens might be further supported by the findings of the correlations existing at gene level. Indeed, the positive correlation between *VIM com* and *DES* found in FG at d 42 may corroborate the hypothesis that *VIM com* could play a role in counteracting stressful conditions (probably exacerbated by an altered DES deposition) occurring in PM. These results agreed with those reported in our previous study performed on broiler chickens (Soglia et al., 2020). Besides, Vater et al. (1994) demonstrated that *VIM* may up-regulate the expression of *DES* at later stages of muscle regeneration, thus contributing to explain their positive correlation at the latest stages of PM development in broiler chickens prior to slaughter (i.e., d 42). Noteworthy, at d 35, *DES* was positively correlated with the *VIM* long-isoform: since the normalized expression of *VIM long* decreased during the growth period, a tendency to a decreased expression of *DES* might be hypothesized, which is then mirrored by the decreased protein amount identified at d 42.

In conclusion, the present study represented the first attempt to investigate the expression and distribution of VIM and DES over the growth period (from 7 to 42 days of age) in FG and MG broiler chickens and confirmed their potential use as markers of the regenerative processes occurring in skeletal muscle. In detail, the findings of the present research seem to support the existence of a relationship between the occurrence of muscle regeneration and the growth rate of the meat-type chickens with the FG hybrids being more susceptible to this phenomenon (as well as to the occurrence of growth-related muscle defects). This evidence may be of a relevant importance when considering that VIM and DES could be potentially exploited as molecular markers to identify breeders bearing/prone to develop the growth-related abnormalities and exclude them from the breeding practices, thus improving the economic and environmental sustainability of the system. In addition, this study allowed to ascertain the key function of VIM in coordinating the sequence of events occurring during muscle regeneration and shed light on the biological roles of DES in preserving the stability of the sarcomeric structure as well as its involvement in several cellular processes. In this context, considering the similarities existing between broilers' abnormalities and other disorders affecting humans (such as desminopathies as well as vimentin-related myopathies) *Gallus* may be potentially proposed as a spontaneous alternative animal model for studying the pathogenesis of these conditions which currently requires the use of artificially induced laboratory animals.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was reviewed and approved by Ethical Committee of the Italian Ministry of Health (ID: 1194/2021).

Author contributions

All the Authors have made a substantial contribution to the work and approved it for the publication. MB, FS, MZ, and FS

have conceptualized the study. FS, MB, MD, MM. performed the analyses. All the Authors have made the interpretation of data. FS and MB drafted the manuscript, and MP, FS, RD and AM supervised it. All the Authors have substantively revised the work.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

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EDITED BY
Massimiliano Petracci,
University of Bologna, Italy

REVIEWED BY
Gregoy Y. Bedecarrats,
University of Guelph, Canada
Marta C. Romano,
Instituto Politécnico Nacional de México (CINVESTAV), Mexico

*CORRESPONDENCE
Kristina O. Smiley,
ksmiley@umass.edu

†These authors have contributed equally to this work and share first authorship

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Beyond a biased binary: A perspective on the misconceptions, challenges, and implications of studying females in avian behavioral endocrinology

Kristina O. Smiley[1,2*†], Sara E. Lipshutz[3†], Abigail A. Kimmitt[4], M. Susan DeVries[5], Kristal E. Cain[6], Elizabeth M. George[7] and Kristen. M. Covino[8]

[1]Centre for Neuroendocrinology and Department of Anatomy, School of Biomedical Sciences, University of Otago, Dunedin, New Zealand, [2]Center for Neuroendocrine Studies and Department of Psychological and Brain Sciences, University of Massachusetts-Amherst, Amherst, MA, United States, [3]Biology Department, Loyola University Chicago, Chicago, IL, United States, [4]Department of Ecology and Evolutionary Biology, University of Michigan, Ann Arbor, MI, United States, [5]Department of Biological Sciences, University of Wisconsin-Whitewater, Whitewater, WI, United States, [6]School of Biological Sciences, University of Auckland, Auckland, New Zealand, [7]Biology Department, Texas A&M University, College Station, TX, United States, [8]Biology Department, Loyola Marymount University, Los Angeles, CA, United States

For decades, avian endocrinology has been informed by male perspectives and male-focused research, leaving significant gaps in our understanding of female birds. Male birds have been favored as research subjects because their reproductive behaviors are considered more conspicuous and their reproductive physiology is presumably less complex than female birds. However, female birds should not be ignored, as female reproductive behavior and physiology are essential for the propagation of all avian species. Endocrine research in female birds has made much progress in the last 20 years, but a substantial disparity in knowledge between male and female endocrinology persists. In this perspective piece, we provide examples of why ornithology has neglected female endocrinology, and we propose considerations for field and laboratory techniques to facilitate future studies. We highlight recent advances that showcase the importance of female avian endocrinology, and we challenge historic applications of an oversimplified, male-biased lens. We further provide examples of species for which avian behavior differs from the stereotypically described behaviors of male and female birds, warning investigators of the pitfalls in approaching endocrinology with a binary bias. We hope this piece will inspire investigators to engage in more comprehensive studies with female birds, to close the knowledge gap between the sexes, and to look beyond the binary when drawing conclusions about what is 'male' versus 'female' biology.

KEYWORDS

testosterone, progesterone, prolactin, challenge hypothesis, sex hormones, female birds, sex differences, estradiol

Introduction

From Berthold's first experiment in the 19th century documenting that the testes are necessary for the expression of male sex characteristics in chickens (Berthold and Quiring, 1944), the field of avian endocrinology has been dominated by male-focused research. Although females have been well-studied in some domesticated species such as poultry, the study of natural biological variation in other female birds has received considerably less attention in the fields of behavioral endocrinology and evolutionary biology (Hrdy, 1986; Cotton et al., 2006; Shansky, 2019; Shansky and Murphy, 2021), particularly in studies examining mate quality and reproductive strategies. This sex bias in the literature was demonstrated in a systematic review, which found that 84% of avian physiology, ecology, and reproduction studies between 2003 and 2011 involved male birds, whereas only 58% involved female birds (Caro, 2012). A 10-years follow up

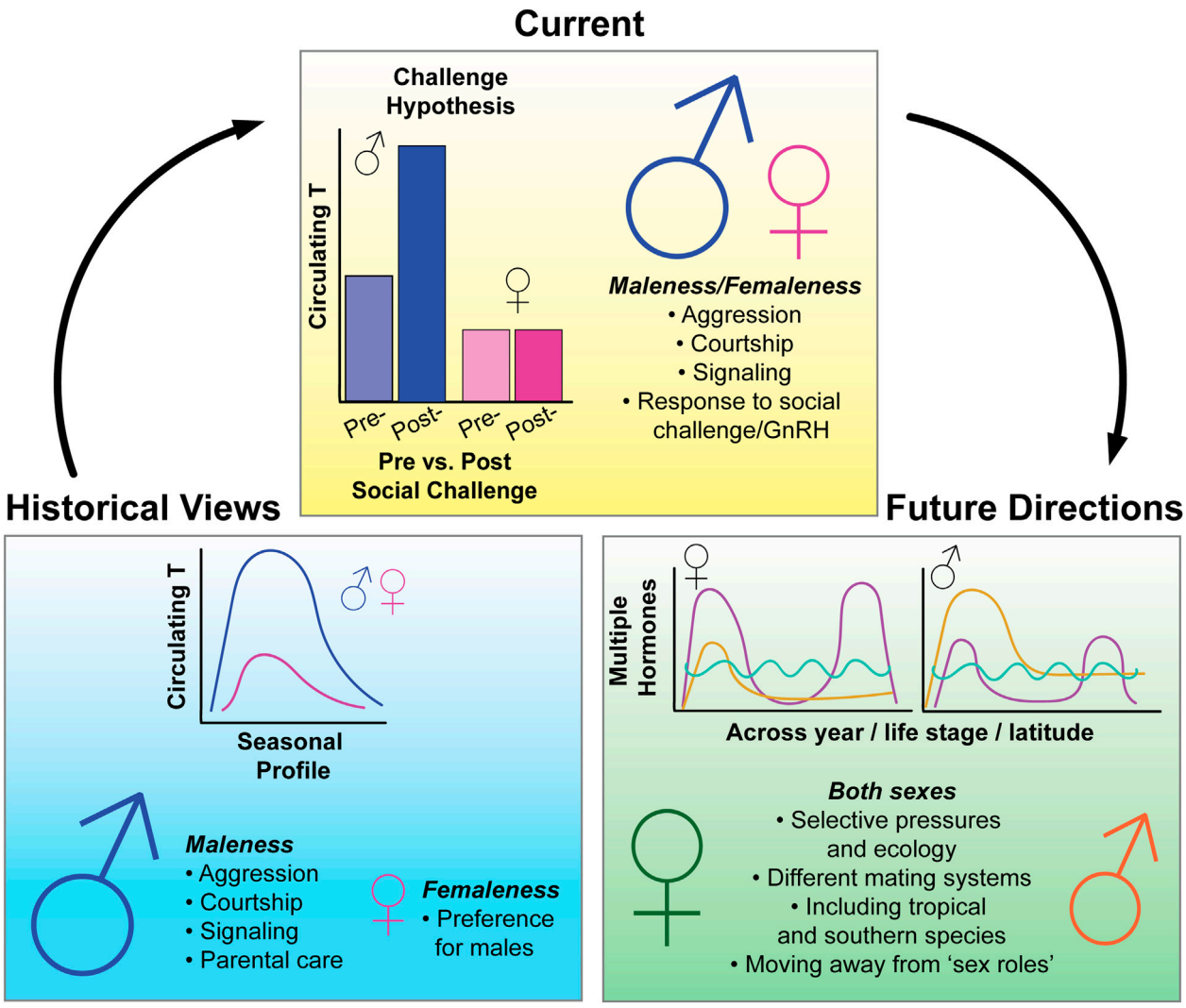

FIGURE 1
Summary figure of historical, current, and future state of research in avian behavioral neuroendocrinology. Historically, the field of avian endocrinology has focused on seasonal variation of testosterone (T) and its effects on "male traits", with little emphasis on how hormones may be affecting similar traits in females. Recent research, which has taken a more female-directed approach, has overturned some previously held dogmas of the field, such as the Challenge Hypothesis (Wingfield 1990)—which seeks to explain variation in T levels across the breeding season. The inclusion of more female-perspectives in avian research has also demonstrated that similar hormones can have similar effects in both females and males and previously thought "male traits" such as dominance, aggression, and bright coloration, also persist in females. In the future, we hope to see more emphasis on female-research, leading to more even study of the sexes on a range of hormone-mediated traits and behaviors. However, to develop more inclusive research programs, we must put increased emphasis on studying and understanding the unique selective pressures and ecology of both sexes, conduct research that focuses on the spectrum of variation for all individuals, and become aware of potential sources of sex bias in our research methodology and design. Note that figures schematics are for illustrative purposes.

study found that this discrepancy persists at similar rates in the current literature (Kimmitt, 2020). In addition to being understudied, female variation in key life history traits is typically examined in the context of how such variation affects males, rather than an interesting and important subject itself (Alonzo and Warner, 2000; Rosenthal and Ryan, 2022). Such male-biased perspectives have misled research on female birds for decades, causing major misconceptions in the field of behavioral endocrinology.

Although female avian endocrinology has received increased attention in the past 20 years, male-dominated research programs and male-biased study designs persist (Figure 1). In this perspective piece, we address inherent barriers with current research methods that lead to these biases. In the second part of this piece, we highlight recent advances that showcase the importance of studying female avian endocrinology and challenge historic applications of an oversimplified, male-biased lens. Lastly, we offer solutions to address and overcome male bias in future research and increase female inclusivity. Our perspective shares important insights with many recent publications on the inclusion of female animals in biology (Shansky, 2019; Orr et al., 2020; Rosvall et al., 2020) including birds (Ball and Ketterson, 2008; Caro, 2012; Kimmitt, 2020). This is by no means an exhaustive list of topics for which significant improvements can be made. Rather, we consider this a starting point for investigators interested in designing female-inclusive research.

Barriers to our understanding of avian endocrinology in females

Here, we highlight specific examples of logistical, technical, and cultural biases that have impeded our understanding of hormones in female birds.

Capture techniques

One commonly used explanation for persistent sex bias in avian endocrinology is that males are more conspicuous or aggressive than females, and therefore easier to capture and study in the field (Caro, 2012; Kimmitt, 2020). However, as evidenced by recent research efforts studying free-living females (e.g., Cain and Ketterson, 2012; DeVries et al., 2015; Covino et al., 2018; George and Rosvall, 2018; Kimmitt et al., 2019; Lipshutz et al., 2021), a better understanding of the natural history of female birds can make sampling less difficult. For example, in cavity-nesting species, females can be more easily captured in artificial nest boxes compared to traditional mist netting techniques (George and Rosvall, 2018; Bentz et al., 2021). In open cup nesting species, females can be easier to capture prior to egg laying (Kimmitt et al., 2019; Needham et al., 2019), as they might be more active or aggressive in the early breeding season (Cristol and Johnsen, 1994; Sandell and Smith, 1997; Reichard et al., 2018). Females in open cup nesting species can also be captured in peak breeding season if researchers devote time to find nests and use the appropriate sex as a lure, as females are often more aggressive towards other females (Cain and Ketterson, 2012; DeVries and Jawor, 2013; Graham et al., 2017). Overall, females are not necessarily "more difficult" to capture, but their capture likelihood may be limited when using methods developed for studying males.

Seasonal bias

Research efforts in avian biology are biased toward reproductive seasons (Marra et al., 2015), when capture likelihood may be most likely to differ by sex. During non-breeding seasons, including migration, females and males may be equally likely to be captured, making this a prime opportunity to ask questions about both sexes (e.g., Covino et al., 2018; Covino et al., 2015; Covino et al., 2017; DeVries et al., 2011). Distinguishing between females and males in sexually amorphic species can be difficult when breeding anatomical features (e.g., brood patch, cloacal protuberance) are absent, but this can be resolved with inexpensive molecular sexing techniques (Fridolfsson and Ellegren, 1999). Monomorphic species also provide a natural control for sex-related biases in sampling and/or data (e.g., behavioral assays).

Tropical vs. temperate bias

Most studies in behavioral endocrinology focus on temperate-breeding migrants, with the implied assumption that these species are models for birds more generally. However, the vast majority of avian species are year-round tropical residents (Hau et al., 2008). Drastic differences in environmental and life-history traits between temperate and tropical species likely have considerable effects on hormone-behavior relationships for both sexes (Levin and Wingfield, 1992; Hau et al., 2004). Despite the dearth of research on tropical species, the available evidence undermines traditional hypotheses, especially as related to the role of testosterone (hereafter, "T") in aggression and song (Peters et al., 2001; Moore et al., 2004; York et al., 2016). For example, in tropical and southern hemisphere species, females defend territories (Levin, 1996; Hall, 2004; Tobias et al., 2011; Cain and Langmore, 2016), sing in the context of territorial defense (Illes, 2015; Riebel et al., 2019; Loo and Cain, 2021), and are brightly colored (Dale et al., 2015; Price, 2015), all traits which are

more traditionally associated with T and male-male competition in the northern hemisphere (Hau and Goymann, 2015).

Captive vs. wild studies

Captive experiments, where environmental conditions can be manipulated, are important for testing hypotheses in avian endocrinology. However, females of many species do not readily enter reproductive conditions in captivity, whereas males do (Rosvall et al., 2020). Females of domesticated species like canaries (*Serinus canaria*) and zebra finches (*Taeniopygia guttata*) readily breed in captivity and are models for endocrinology (e.g., Adkins-Regan, 1999; Williams and Martyniuk, 2000; Hurley et al., 2008; Smiley and Adkins-Regan, 2016; Madison et al., 2020), but domesticated species may not best represent how physiological traits evolve in the wild. Some research groups have successfully studied reproduction in captive females by hand-rearing chicks (Baptista and Petrinovich, 1986) or providing spacious aviaries (Caro et al., 2007; Perfito et al., 2015; Lindner et al., 2021). Both solutions are time-consuming and expensive, however, which may limit sample size and feasibility. Expanding the number of species for which females can be studied in captivity requires funding sources to support these efforts and costs.

Measuring hormones

The ability to measure hormones from blood in free-living birds gave birth to the subdiscipline of "field endocrinology" (Wingfield et al., 2020). However, there are several logistical barriers to quantifying hormone concentrations in female birds. For instance, female T concentrations may be lower than the detection limits of enzyme linked immunosorbent assays (ELISA) designed to measure higher "male-typical" T levels. Additional hormones can be difficult to measure in both sexes, due to limitations of current ELISA kit sensitivity and sample volumes (e.g., estradiol) or a lack of reliable, commercially available ELISA kits that work for birds (e.g., prolactin, IGF1, LH, and FSH). Liquid Chromatography/Mass Spectrometry (LC/MS) is a promising method for quantifying multiple hormone concentrations from small volumes of blood or tissue (Jalabert et al., 2021; Munley et al., 2022). However, LC/MS has drawbacks, as it requires expensive equipment and rigorous calibration. Alternative methods of non-invasive sampling (e.g., fecal or feather) could also increase sample sizes for measuring hormone variation in both sexes (Chávez-Zichinelli et al., 2010; Chávez-Zichinelli et al., 2014). Although these sampling methods are not equivalent to plasma sampling, they reveal integrated hormone profiles on longer timescales (Bortolotti et al., 2008), which may be beneficial depending on the research question. We are optimistic that further advancements will support the quantification of hormone levels in female birds.

"Sex roles"—flipped and reversed

Substantial emphasis has been placed on conventional "sex roles" for female and male animals, in line with the Darwin-Bateman paradigm of sexual selection (Janicke et al., 2016; Gonzalez-Voyer et al., 2022). This binary framing reinforces what is expected for sex-specific courtship, competition, parental care, and their endocrinological correlates, thereby limiting how we examine and interpret variation in natural history (Ah-King and Ahnesjö, 2013). We also highlight the problematic framing of birds as "sex-role reversed", in which females compete for multiple male mates (i.e., social polyandry), and males conduct the majority of parental care (Emlen and Oring, 1977). In these avian systems, the degree of sexual dimorphism in average T secretion varies, depending on whether males are conducting parental care or seeking courtship (Lipshutz and Rosvall, 2020). Similarly, in cooperatively or group breeding species, sex differences in hormones are often considerably less than differences between breeders and non-breeders, though female levels are rarely reported (Pikus et al., 2018). We argue that sex (i.e., the default framing of "sexual dimorphism") is not necessarily the main predictor of variation in avian endocrinology. We envision a future framework for which phenotypic traits like behavior, morphology, and hormone levels can be viewed across a spectrum, rather than categorically by sex.

Misleading terminology

Many misconceptions about the endocrinology of females stem from misleading terminology. The language we use can create and reinforce bias, which constrains our understanding of hormones and behaviors. For example, the pervasive labeling of T and its metabolites as "male hormones" with "masculinizing effects," and estrogen and progesterone as "female hormones" with "feminizing effects" (e.g., 68, a widely used undergraduate textbook) presents a false binary that these hormones only have sex-specific functions, when in reality these hormones are functionally important in both sexes. Whereas titers of T may be higher in males than in females, T's functional capabilities are similar in both sexes (Staub and De Beer, 1997). Furthermore, hormonal values exist along a continuous spectrum, and a binary approach that emphasizes differences between groups might

ignore important similarities and within-group variation (Williams, 2008). In some cases, hormonal variation between the sexes can be seen as overlapping bell curves (Muck and Goymann, 2011; Goymann and Wingfield, 2014), and variation within sex categories is greater than variation between them. Mislabeling hormones as the "male hormone" or "maternal hormone" disregards their broader regulatory functions and can lead us to overlook their importance in all individuals.

Advances that highlight the importance of studying female avian endocrinology

Below, we highlight some specific examples of how studying hormones in female birds has broadened our understanding of avian endocrinology, by facilitating the testing of old and new hypotheses.

Testosterone is more than a "male hormone"

Our understanding of T's role in female behavior is in its infancy (Rosenthal and Ryan, 2022), despite decades of study on T in male birds (Wingfield et al., 1990; Goymann et al., 2019). Recent work reveals T-behavior relationships, which have been classically described as "male", also exist in females. For example, research in female songbirds has demonstrated that both T and aggression are elevated early in the breeding season (Cain and Ketterson, 2012; George and Rosvall, 2018), and that territorial aggression positively correlates with circulating T levels (Lipshutz and Rosvall, 2021). Meanwhile, many purported effects of T on classically "male" behavior are instead mediated by the "female" hormone estradiol. For example, in certain brain regions T is converted to estradiol, and both hormones activate either the androgen or estrogen receptor to promote singing behavior (Frankl-Vilches and Gahr, 2018) and aggression (Ubuka and Tsutsui, 2014).

Even as researchers began to recognize the biological relevance of T in females, it has nevertheless been studied within a male-typical framework. For example, the Challenge Hypothesis established an important framework for answering questions concerning T's role in modulating male social behavior (Wingfield et al., 1990). Yet, as males of more species were assessed, conflicting results emerged (Wingfield et al., 2019) and recent modification suggests that the presence of females, rather than male competitors, explains variation in male T levels within the breeding season (Goymann et al., 2019). In female birds, assessment of the Challenge Hypothesis suggests that T elevations do not accompany acute social challenges in most species examined thus far (Rosenthal and Ryan, 2022). However, T's influence on aggressive behavior cannot entirely be ruled out (Cain and Ketterson, 2012; Lipshutz and Rosvall, 2021; George et al., 2022). To build a conceptual framework that works for T-behavior relationships in females, future work should account for the unique selective pressures relevant to their life history.

Prolactin is more than a "maternal hormone"

Whereas androcentric terminology has led our field to overlook important aspects of female biology, the same can be said for using gynocentric terminology and male biology. One such example is the anterior pituitary hormone prolactin, which is most well-known for its regulation of mammalian lactation and maternal behavior. There is little research focused on prolactin in males—a rare female bias in research! However, biparental care is widespread in birds, with both males and females participating in egg incubation and/or chick provisioning in over 80% of avian species (Cockburn, 2006). In some species, including ring doves and pigeons, both females and males produce and regurgitate "crop milk," a nutrient-rich substance secreted from the crop sac organ to feed young (Lehrman, 1955; Buntin et al., 1991). Though most other birds do not produce crop milk, in virtually all avian species studied to date, prolactin levels increase just before hatching in all individuals (both sexes) that provide parental care (Smiley, 2019). In both male and female zebra finches, the rise in prolactin before hatching is required for parenting behaviors (Smiley and Adkins-Regan, 2018). Inter- and intra-specific differences in paternal investment have also been linked with prolactin levels in male songbirds (Van Roo et al., 2003; Badyaev and Duckworth, 2005). Together, these experiments demonstrate that prolactin plays a similar role in both female and male birds, and is far from solely a "maternal hormone."

Females are active participants in courtship behaviors

Female courtship behavior is often interpreted from a "male perspective" or is neglected altogether. For instance, breeding territory quality and resource access has been well-studied in males. In contrast, much less attention is given to which females mate with these males, how females acquire high-quality resources, and whether females choose mates based on male traits or territory characteristics (Hasegawa et al., 2012; Cain and Rosvall, 2014). In avian endocrinology, studies of courtship often center on T's role in regulating elaborate male traits (Riters et al., 2011), while little is known about the endocrine regulation of mating signal perception in females. Although courtship is generally thought to be male-driven, hormones such as progesterone and gonadotropin-releasing hormone stimulate copulation solicitation and other courtship behaviors, which are an active female mating signal (Maney et al., 1997; Smiley

et al., 2012). Given that courtship is a critical component of both male and female reproductive success, we encourage studies that highlight the bidirectionality of behavioral and physiological mechanisms.

Female birds sing

Bird song is a classic subject in behavioral endocrinology, and the link between T and singing is well-established in males. The bulk of song research has focused on a highly derived clade of northern hemisphere migrants that are seasonally territorial (Riebel et al., 2019; Rose et al., 2022), but songbirds evolved in the southern hemisphere, where birds often sing year-round (Rose et al., 2022; Theuerkauf et al., 2022). Thus, patterns in this group may not be generalizable (Gahr, 2014; Ball, 2016). Further, our understanding of hormones and song is primarily based on males, or to a lesser extent experimentally manipulated females in species without female song (Riebel et al., 2019; Rose et al., 2022; Catchpole, Slater, Song). However, female song is widespread and ancestral (Ball, 2016), often functioning in an analogous manner to male song—acquiring and defending critical reproductive resources (Langmore, 1998; Cain et al., 2015; Hall et al., 2015; Odom et al., 2014). Neuroanatomical comparisons of sex differences in the song control system have found that HVC (used as a proper name) and RA (robust nucleus of the arcopallium) volumes are larger in males, even in species for which females sing similarly, or more often (Ball, Balthazart). However, androgen receptor distribution appears similar in the song control nuclei of females for which both sexes sing (Gahr, 2014). Much remains to be studied on the role of hormones in regulating female song development, neuroendocrine processes, and performance (Riebel et al., 2019; Rose et al., 2022; Rouse, 2022).

Where do we go from here?

Here, we propose and reflect on next steps for a more inclusive field of avian endocrinology.

Males are not the baseline

Research on males has been retrofitted to females instead of coming from first principles. We argue that by grounding research in the natural history of female birds, we can make better predictions about hormones-behavior relationships. This requires countering the biased ideas that females do not compete, are always maternal, and are relatively interchangeable (i.e., do not exhibit functionally important variation). In particular, more work is needed to identify the specific selection pressures that shape female behavior and physiology (Buchanan and Fanson, 2014). We encourage studies that evaluate whether a hormone has the same function in both males and females, or whether it has sex-specific effects, and under which developmental, physiological, social, and ecological circumstances we expect to find these similarities and differences. Changing our perspective and assumptions alters the questions we ask and how we test them, and helps us avoid the errors that are too often engrained in experimental design. This will facilitate progress on developing a deeper and more integrative understanding of how phylogeny, ecology, and physiology interact to shape female behavior, and by extension, population persistence and dynamics. We offer a set of questions to ask next time a research paper is being critically evaluated or better yet, while a study is being designed, to increase awareness of potential sex biases (dBox 1).

More and different data

A collective goal in biology is to establish patterns and determine the mechanisms driving those patterns. Reviews and meta-analyses are key for evaluating the predictive strength of these hypotheses. However, such work requires empirical data on a broad array of taxa, locations, and life-histories. With each new study on a different aspect of the behavioral endocrinology, we have found new patterns or nuances to previously well-supported patterns, and we are still lacking in general theories for many aspects of female avian endocrinology. This is particularly evident as we have moved away from the original model species—namely migratory sparrows, zebra finches, and canaries - and towards species with different life-histories and ecologies, such as tropical, non-migratory, cooperative breeders, polyandrous females, etc. As research expands to other species in other parts of the world, these gaps are slowly filling in.

Measuring the complete system of signal and reception

Studying circulating hormones alone cannot provide a complete understanding of a bird's underlying physiology, as this approach ignores other crucial components of endocrine signaling systems, such as receptors, enzymes, and carrier proteins (Hau, 2007; Ketterson et al., 2009). Focusing on only circulating hormones can lead to a binary understanding of these endocrine systems. As an example, we might conclude that sex differences in circulating levels of T have some functional importance across species, but this singular focus on T signal ignores the many other components of the androgen signaling system, including tissue sensitivity to the signal, and the rate of conversion to other hormones (Rosvall, 2013; Lipshutz et al., 2019; Schuppe et al., 2020). Therefore, progress in our understanding of the hormonal phenotypic continuum must include a more comprehensive study of these endocrine axes, in both females and males.

DBOX 1 Beyond Male-Centered Research

The **Bechdel test** is a tool for examining representation of women in entertainment, asking whether women-identifying characters are represented and whether they talk to each other about anything other than a man-identifying character (roughly half of movies fail the test). We argue a similar test should be applied studies of avian endocrinology and beyond, as females and other sex/gender minorities are largely understudied. Next time you read a manuscript, ask yourself the following questions:

1) Do the authors discuss the potential effects of sex (or gender in human-centered research) on variation in the trait of interest?
2) Do the authors report sample sizes of each sex and are the sexes equally represented?
2a) If the research is centered on one sex, do the authors discuss previous findings in the other sex (es)?
3) Do the authors include sex as a fixed effect in their statistical models?
Bonus: Does the research challenge pre-existing sex-related biases?

Revising our language and perpetuating correct terminology

One challenge to building a more inclusive avian endocrinology is that many biased ideas are heavily entrenched in the minds of the general, well-educated public. Outdated concepts are perpetuated by inaccurate textbooks (Raven, 2020) and popular media which use misleading headlines to generate clicks, when the reality is much less sensational. We encourage researchers to avoid terms like "masculinization" and instead use terms like "androgenization" or "increased T", to help us move away from the false binary of "male" versus "female" hormones. We hope this shift in terminology will also help address misinterpretations and misuses of our research by the public, fellow scientists, and legislators alike.

Increasing diversity in scientists

We all bring biases to our research, but through self-awareness of positionality, greater inclusion of diverse backgrounds, and explicit reckoning with barriers and biases, we can minimize these blind spots (Kamath et al., 2022). As the number of scientists of underrepresented genders increases in our community, so do the number of studies that include different perspectives, make different assumptions, and examine questions from a new angle (Baran, 2018; Haines et al., 2020; Tang-Martínez, 2020). Increasing diversity among scientists is an important antidote to the issues we have addressed here.

dBox 1: To combat potential sex-bias research, we propose a set of questions to ask next time a research paper is being critically evaluated. These same questions can be applied when designing new studies. Discussing these concepts with trainees and other colleagues can increase awareness and can encourage scientists to consider the 'female-perspective' in future research efforts.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

Author contributions

KC conceived of the general idea for the manuscript. All authors contributed to the framework, focus, and drafting of the manuscript. KS and SL organized manuscript sections, directed editing, and finalized the manuscript. KS, KC, and AK made the figures. All authors approved the final version of the manuscript and agreed to be accountable for the content of the work.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Vincent M. Cassone,
University of Kentucky, United States

REVIEWED BY
Alexandra Gouveia,
University of Porto, Portugal
Anderson O. L. Wong,
The University of Hong Kong, Hong Kong SAR, China

*CORRESPONDENCE
Jiannan Zhang,
biozhangjn@gmail.com
Yajun Wang,
cdwyjhk@163.com

†These authors have contributed equally to this work

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Characterization of the chicken melanocortin 5 receptor and its potential role in regulating hepatic glucolipid metabolism

Xiao Zhang†, Jiancheng Su†, Tianjiao Huang, Xinglong Wang, Chenlei Wu, Jing Li, Juan Li, Jiannan Zhang* and Yajun Wang*

Key Laboratory of Bio-resources and Eco-environment of Ministry of Education, Animal Disease Prevention and Food Safety Key Laboratory of Sichuan Province, College of Life Sciences, Sichuan University, Chengdu, China

Melanocortin receptors (MC1R-MC5R) and their accessory proteins (MRAPs) are involved in a variety of physiological processes, including pigmentation, lipolysis, adrenal steroidogenesis, and immunology. However, the physiological roles of MC5R are rarely characterized in vertebrates, particularly in birds. In this work, we cloned the full-length cDNA of chicken MC5R and identified its core promoter region. Functional studies revealed that cMC5R was more sensitive to ACTH/α-MSH than β-MSH/γ-MSH, and was coupled to the cAMP/PKA signaling pathway. We demonstrated that MRAP2 decreased MC5R sensitivity to α-MSH, whereas MRAP1 did not have a similar effect, and that both MRAPs significantly reduced MC5R expression on the cell membrane surface. Transcriptome and qPCR data showed that both MRAP1 and MC5R were highly expressed in chicken liver. Additionally, we observed that ACTH might increase hepatic glucose production and decrease lipogenesis in primary hepatocytes, and dose-dependently downregulated the expression levels of *ELOVL6* and *THRSPA* genes. These findings indicated that ACTH may act directly on hepatocytes to regulate glucolipid metabolism, which will help to understand the function of MC5R in avian.

KEYWORDS

MC5R, MRAP1, functional analysis, tissue expression, THRSPA, ELOVL6

Introduction

Melanocortin receptors (MCRs) are a set of five G-protein coupled receptors (MC1R-MC5R) that may be divided into two groups depending on ligand selectivity for α-melanocyte-stimulating hormone (α-MSH) and adrenocorticotropin (ACTH) in bony vertebrates (Cone, 2006). The MC2R, also known as the adrenocorticotropic hormone receptor (ACTHR), can only be activated by ACTH when melanocortin receptor accessory protein 1 (MRAP1) is present, while the other receptors (MC1R, MC3R, MC4R, and MC5R) can be activated by α-MSH or ACTH in the presence or absence of MRAP1/MRAP2 (Chan et al., 2009; Rouault

et al., 2017b; Yang and Harmon, 2017). As a single transmembrane protein that forms a homodimer, MRAPs may interact with and regulate the trafficking and signaling of all MCRs (Chan et al., 2009). In numerous vertebrates, MCRs interact with MRAPs and play a role in pigmentation, lipolysis, adrenal steroidogenesis, energy homeostasis, immunology, and cardiovascular function (Cooray and Clark, 2011; Dores et al., 2016; Scanes and Pierzchala-Koziec, 2021; Ji et al., 2022).

In the chicken genome, there are five paralogous MCRs and two MRAPs (Ling et al., 2004; Barlock et al., 2014; Zhang et al., 2017; Thomas et al., 2018). In recent years, the pharmacological characterization and physiological activity of chicken MCRs (cMCRs) have been widely explored (Scanes and Pierzchala-Koziec, 2021). It has been shown that chicken MC1R binds to the radioligand or responds to α-MSH/ACTH by promoting intracellular cAMP accumulation (Ling et al., 2003; Ling et al., 2004; Mundy, 2005). MC1R of dark-feathered chickens with a Glu to Lys mutation at position 92 showed constitutive activity, which was associated with feather color in chickens (Ling et al., 2003). When chicken MC2R was co-expressed with MRAP1 in CHO cells, the ligand selectivity properties of the receptor were identified, demonstrating that cMC2R can be activated by $ACTH_{1\text{-}24}$, but not by NDP-MSH (Barlock et al., 2014). Further research revealed that cMC2R was potently activated by chicken or human ACTH only in the presence of MRAP1 (Zhang et al., 2017; Thomas et al., 2018). The major melanocortin receptor expressed in the adrenal gland, MC2R, was thought to be a key regulator of the hypothalamus-pituitary-adrenal (HPA) axis in stress adaption (Dores and Garcia, 2015; Thomas et al., 2018). Chicken MC3R and MC4R were predominantly expressed in the hypothalamus, which were activated by both α-MSH and ACTH. Unlike cMC2R, their constitutive activity and ligand sensitivity were affected by both MRAP1 and MRAP2 (Zhang et al., 2017; Thomas et al., 2018). Single nucleotide polymorphisms (SNPs) in MC4R have been reported to be associated with the differences in body weight and egg production in chickens (Karim and Aggag, 2018; Kubota et al., 2019).

In addition, previous studies on MC5R in chickens have offered some information. Ling et al. investigated the ability of chicken MC5R to couple with the intracellular messenger cAMP and observed that the EC50 values with different ligands (human α-MSH and $ACTH_{1\text{-}24}$) were equivalent (Ling et al., 2004). Furthermore, recent studies have shown that MRAP1 supplementation improved chicken MC5R sensitivity to human $ACTH_{1\text{-}24}$, and demonstrated that the KKRRP motif of $ACTH_{1\text{–}24}$ was needed for complete activation of cMC5R when co-expressed with MRAP1 in cultured CHO cells (Thomas et al., 2018; Dores et al., 2020). However, chicken MRAP2 had no influence on cMC5R sensitivity to human $ACTH_{1\text{–}24}$ (Thomas et al., 2018). Another study indicated the F254A mutation in chicken MC5R displayed a significant increase in basal activity and significantly reduced the reactivity to α-MSH/NDP-α-MSH. They also found that the MC5R mutants D119A and D204A were completely unresponsive to three agonists (α-MSH, NDP-α-MSH and SHU9119), suggesting that the acidic amino acids D119 and D204 in cMC5R played an essential role in intracellular signal transduction (Min et al., 2019).

However, some key information, including the physiological role of MC5R in chickens, remains uncertain. Although human melanocortin peptides have been used to characterize chicken melanocortin receptors in previous studies. α-MSH is identical at all positions in chicken and human, but Arg15 and Ile20 in chicken ACTH differ from Lys15 and Val20 in human ACTH. Arg15 is the first amino acid of the KKRRP motif, which may play a key role in the activation of cMC5R (Dores et al., 2020). We hypothesized that the mutations in the ligand ACTH might yield different results in functional studies. In addition, MC5R has been detected to be expressed in the liver, adrenal gland, kidney, fat, and lung of chickens (Takeuchi and Takahashi, 1998; Yabuuchi et al., 2010; Thomas et al., 2018; Min et al., 2019), but its physiological functions in these tissues remain unknown. The high level of MC5R expression in chicken liver increases the possibility that the liver is an important target tissue for melanocortin (Thomas et al., 2018; Min et al., 2019). In the present study, we evaluated the effects of the accessory protein MRAPs on the sensitivity of cMC5R to natural chicken melanocortin peptides and investigated the role of ACTH on avian liver metabolism.

Materials and methods

Chemicals, primers, peptides, and antibodies

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, United States) and the restriction enzymes were obtained from TaKaRa (Dalian, China). Chicken (c-)$ACTH_{1\text{–}39}$, α-MSH (acetyl-α-MSH), β-MSH, and γ-MSH were synthesized by GL Biochem Ltd (Shanghai, China). The synthesized peptides have a purity of above 95% (as determined by HPLC) and their structures have been confirmed by mass spectrometry. H89 (371,963), MDL (444,200), forskolin (344,273) and 8-Br-cAMP (203,800) were purchased from Calbiochem (Merck KGaA, Darmstadt, Germany). Antibodies for total CREB (48H2) rabbit mAb (1:1,000, #9197), phosphorylated CREB (Ser133) (87G3) rabbit mAb (1:1,000, #9198) and anti-rabbit IgG, HRP-linked antibody (1:5,000, #7074) were purchased from Cell Signaling Technology (CST, Bevely, MA). All primers used in this study were synthesized by Beijing Genome Institute (BGI, China) and listed in Supplementary Table 1.

Animals

Adult chickens and chicks of the Lohmann Layer strain were purchased from local commercial companies. All animal

experiments were carried out in accordance with the Guidelines for Experimental Animals issued by the Ministry of Science and Technology of the People's Republic of China. All animal experimental protocols used in this study were approved by the Animal Ethics Committee of College of Life Sciences, Sichuan University.

RNA extraction, RT-PCR, and quantitative real-time PCR assays

Three female and three male adult chickens were sacrificed and tissues including the cerebellum, midbrain, cerebrum, hindbrain, hypothalamus, anterior pituitary, kidney, liver, lung, muscle, skin, testis, adrenal gland, abdominal fat, heart, spleen, and ovary were immediately collected. Fresh tissues were frozen in liquid nitrogen and then placed in a refrigerator at −80°C until use. RNAzol reagent (Molecular Research Center, Cincinnati, OH) was used to extract total RNA, and all operations were performed in accordance with the manufacturer's instructions. The total RNA obtained was resuspended in H_2O treated with diethylpyrocarbonate (DEPC). These RNA samples were then reverse transcribed by Moloney murine leukemia virus (MMLV) reverse transcriptase (Takara, Dalian, China). In brief, oligodeoxythymide (0.5 μg) and total RNA (2 μg) were mixed in a total volume of 5 μl, incubated at 70°C for 10 min, and cooled at 4°C for 2 min. Then, the buffer containing 0.5 mM each of deoxynucleotide triphosphate and 100 U MMLV reverse transcriptase were added into the reaction mix, for a total volume of 10 μl. The reverse transcription (RT) reaction was performed at 42°C for 90 min.

cDNA samples were subjected to quantitative real-time PCR assay of chicken MC5R mRNA levels in different chicken tissues, as described in our previous study (Fang et al., 2021; Zhang et al., 2021). Quantitative real-time PCR (qPCR) was performed on the CFX96 Real-time PCR Detection System (Bio-Rad, Hercules, CA). Briefly, the reaction system contained 1 μl of EvaGreen (Biotium Inc., Hay-ward, CA), 1 μl of cDNA, 1 × PCR buffer, 0.2 mM each dNTP, 0.2 mM each primer, 0.5 U Taq DNA polymerase (TaKaRa) and RNase-free H_2O to a final volume of 20 μl. The PCR profile consisted of 40 cycles of 94°C for 3 min, followed by 94°C for 15 s, 60°C for 15 s, and 72°C for 20 s. To assess the specificity of PCR amplification, melting curve analysis and agarose gel electrophoresis were performed at the end of the PCR reaction to confirm that a specific PCR band was produced. In addition, the identity of PCR products for all genes was confirmed by sequencing.

Cloning the full-length cDNA and promoter regions of chicken MC5R

To construct the expression plasmid of MC5R for functional assay, several specific primers (Supplementary Table S1) were designed according to the chicken MC5R sequence (KF670718.1) in GenBank, and PCR was performed using these primers and liver-derived cDNA as a template to obtain the coding region sequence of cMC5R. The amplified PCR products were cloned into the pcDNA3.1 (+) vector (Invitrogen, Carlsbad, CA) and sequenced by Tsingke Company (Beijing, China).

To determine the complete gene structure of chicken MC5R, gene-specific primers (Supplementary Table S1) were used to amplify the 5′-untranslated region (5′-UTR) and 3′-UTR of chicken MC5R from the adult chicken liver using the SMART-RACE cDNA amplification Kit (Clontech, Palo Alto, CA). The amplified PCR products were cloned into the pTA2 vector (TOYOBO) and sequenced by the Tsingke Company (Beijing, China). The sequences of 5′-UTR and 3′-UTR of the chicken MC5R gene were then compared to the chicken genome database (www.ensembl.org/gallus_gallus). Finally, the full-length cDNA of MC5R was determined based on the sequences of 5′- and 3′-cDNA ends with an overlapping region, which has been deposited in GenBank with an accession number OP259502.

To determine the chicken MC5R promoter region, specific primers (Supplementary Table S1) were generated to amplify 5′-flanking regions of different lengths from the genomic DNA template. These PCR products were cloned into the pGL3-Basic vector (Promega, Madison, WI) and sequenced. In this study, the transcription start site of MC5R exon 1 was designed as "+1", and the first nucleotide upstream of the transcription start site was designed as "−1". Finally, a series of promoter-luciferase reporter constructs for cMC5R (P1: −1827/+171-Luc, P2: −828/+171-Luc, P3: −343/+171-Luc, P4: −66/+171-Luc) were obtained.

Functional analysis of chicken MC5R, MRAP1 and MRAP2

The expression plasmids encoding MC5R, MRAP1 and MRAP2 were established by cloning their entire open reading frame (ORF) into the pcDNA3.1 (+) expression vector (Invitrogen). Chinese hamster ovary (CHO) cells transiently expressing MC5R were treated with chicken $ACTH_{1-39}$/α-MSH/β-MSH/γ-MSH (10^{-12}–10^{-6} M, 6 h), and the receptor-activated cAMP signaling pathway was then monitored using the pGL3-CRE-luciferase reporter system according to our previously established methods (Zhang et al., 2017; Zhang et al., 2020).

In brief, CHO cells were cultured on a six-well plate (Nunc, Roskilde, Denmark) and grown for 24 h before transfection. The cells were then transfected with a mixture containing 700 ng pGL3-CRE-Luciferase reporter construct, 200 ng of receptor expression plasmid (or empty pcDNA3.1 vector as a negative control), 20 ng of pRL-TK construct (containing a Renilla luciferase gene, used as an internal control), and 2 μl jetPRIME transfection reagent (Polyplus Transfection, Illkirch,

France) in 200 μL buffer. Twenty-four hours later, CHO cells were sub-cultured into a 96-well plate at 37°C for an additional 24 h before treatment. After removal of the medium from the 96-well plate, the cells were treated with 100 μl ligand-containing medium (or ligand-free medium) for 6 h. Finally, CHO cells were lysed with 1 × passive lysis buffer for luciferase assay (Promega) and the luciferase activity of the cell lysate was measured by a Multimode Microplate Reader (TriStar LB941, EG&G Berthold, Germany) according to the manufacturer's instruction.

To test whether cMRAP1 and cMRAP2 can alter the pharmacological properties of cMC5R, CHO cells co-expressing MC5R and cMRAP1/cMRAP2 were treated with chicken $ACTH_{1-39}$/α-MSH, and the relative potential of these two peptides to activate the receptor was also determined using the pGL3-CRE-luciferase reporter system. At the same time, H89 (a PKA inhibitor, 10 μM) and MDL12330 A (an AC inhibitor, 5 μM) were used to further determine the signal pathways activated by $ACTH_{1-39}$/α-MSH.

Western blot

To investigate whether the activation of cMC5R can enhance CREB phosphorylation, 100 ng of cMC5R expression plasmid, or an empty pcDNA3.1 (+) vector, was transfected into CHO cells cultured in a 24-well plate (Nunc) using jetPRIME transfection reagent (Polyplus Transfection). After 24 h transfection, ACTH (10 nM) or α-MSH (10 nM) was added to treat the cells for 10 min. Then the whole-cell lysates were used to examine the level of phosphorylated CREB using western blot. The level of total CREB protein was also examined and used as internal controls in each experiment. The phosphorylated CREB (p-CREB) and total CREB levels were quantified by densitometric analysis with ImageJ software. Relative optical densities of p-CREB normalized by CREB levels compared to the densities in the absence of ACTH (or α-MSH) are shown as relative p-CREB/CREB expression (fold changes).

Detection of cell surface expression of MC5R by Nano-Glo HiBiT detection system

To quantify the cell surface expression of cMC5R, the Nano-Glo® HiBiT Extracellular and Lytic Detection System purchased from Promega Corporation (Promega) were used according to our previously established methods (Zhang et al., 2020). Nano-Glo® HiBiT Extracellular Detection System can quantify HiBiT-tagged MC5R expressed on the cell membrane, while the Lytic Detection System can determine the total HiBiT-tagged MC5R levels in cultured cells.

In brief, CHO cells cultured in a 96-well plate were transfected with HiBiT-tagged receptor (HiBiT-MC5R) expression plasmid (or co-transfected with HiBiT-MC5R and MRAP1 (or MRAP2) plasmids) and incubated for an additional 24 h. To quantify the cell surface expression of HiBiT-MC5R, 40 μl Nano-Glo® HiBiT Extracellular Reagent, in which LgBiT protein can bind to HiBiT-MC5R expressed on the cell surface and generate luminescence, was added, and the luminescence values were measured by a Multimode Microplate Reader (TriStar LB 941, EG&G Berthold, Germany). To quantify the total expression levels of HiBiT-MC5R in cells, Nano-Glo® HiBiT Lytic Reagent was added to lyse cells, and the luminescence values were also measured according to the manufacturer's instruction. Finally, the relative cell-surface expression level of MC5R (HiBiT-MC5R signal on the cell membrane) was first normalized by the total HiBiT-MC5R signals in cells, and then expressed as the percentage to the control group.

Tissue expression of chicken *MC5R*, *MRAP1*, and *MRAP2*

To examine the mRNA abundance of chicken *MC5R*, *MRAP1* and *MRAP2* in different domestic chicken tissues, we used a large-scale RNA-Seq dataset representing all the major organ systems from adult Lohmann White domestic chickens (Zhang et al., 2022). An open-access chicken tissue gene expression atlas (TGEA) (https://chickenatlas.avianscu.com/) is presented based on the expression of 224 samples across 38 well-defined chicken tissues. It allows us to view and download the expression profile of chicken *MC5R*, *MRAP1* and *MRAP2* across tissues. Expression levels were estimated in transcript per million (TPM) units. The raw data that support the findings of this RNA-Seq dataset have been deposited into CNGB Sequence Archive (CNSA) of China National GeneBank DataBase (CNGBdb) with accession number CNP0003404.

To examine the expression levels of chicken *MC5R* and *MRAP1* mRNA at different development stages, RNA-Seq data were downloaded from the SRA database (Accessions: PRJEB26695, University of Heidelberg). This dataset covers the development of liver organs from day 10 post-conception to day 155 post-hatch. The quantification of reads was performed with Salmon v1.4.0 (Patro et al., 2017) against the NCBI GRCg7b database (https://www.ncbi.nlm.nih.gov/assembly/GCF_016699485.2/). The transcripts per million (TPM) values were used to estimate the abundance of *MC5R* and *MRAP1* mRNA transcripts.

Identification of the promoter regions of cMC5R

The promoter activity of these constructs was detected in DF1 cells, which is a continuous cell line of chicken embryo fibroblasts, by the dual-luciferase reporter assay (Promega,

Madison, WI), as described in our previous study (He et al., 2016; Gao et al., 2017). In brief, DF-1 cells were cultured in a 48-well plate at a density of 1 × 10^5 cells per well before transfection. After 24 h incubation, a mixture containing 100 ng of promoter construct, 5 ng of pRL-TK construct and 0.5 μl of JetPRIME (Polyplus-transfection, France) was prepared in 200 μl of buffer and transfected following the manufacturer's instructions. 24 h later, the medium was removed and 100 μl 1 × passive lysis buffer (Promega) was added to each well. Luciferase activities of 15 μl of cellular lysates were measured using DLR assay kit (Promega). Luciferase activity of promoter-luciferase construct in DF-1 cells was normalized to *Renilla* luciferase activity derived from the pRL-TK vector (Promega). Then, the luciferase activities in each treatment group were expressed as fold change as compared with the control group. The cells transfected with the empty pGL3-Basic vector was used as an internal control group.

Evaluation of the effect of $ACTH_{1\text{-}39}$ on primary cultured chicken hepatocytes

To evaluate the effect of $ACTH_{1\text{-}39}$ on chicken liver, the chicken hepatocytes were prepared and maintained as a monolayer culture. Briefly, one-month-old male chicken hepatocytes were isolated by perfusion of a liver with Ca^{2+}-free KRB buffer to remove blood cells, and being digested with 0.25% collagenase-I for 10 min. Hepatocytes were obtained following filtering and washing using M199 medium. Then the hepatocytes were plated in a Corning CellBIND 48-well microplate (Corning, NY, cat. no.3338) with M199 medium containing 100 U/ml penicillin and 100 mg/ml streptomycin (Gibco), 5 μg/ml bovine insulin (Sigma), and 5% FBS at 37°C in a 5% CO_2 atmosphere. To adhere to the wall, the cells were incubated on the 48-well plate for 4 h.

Glucose production from primary cultured chicken hepatocytes was measured as previously described (Collins et al., 2006). Briefly, cells were washed three times with warm phosphate-buffered saline (PBS) to remove glucose, followed by treatment with 100 nM (or different concentration gradients) chicken $ATCH_{1\text{-}39}$ for 30 min or 60 min in glucose-free medium containing gluconeogenic substrates (20 mM sodium lactate and 2 mM sodium pyruvate). Glucose concentration was determined with a glucose assay kit (Applygen, Beijing, China, catalog no. E1011). Briefly, the 5 μl medium was added into 195 μl reagent and incubated for 20 min at 37°C. Absorbance in 550 nm was measured in a standard glucose solution.

For lipid quantification by Oil Red O staining, after treatment with $ACTH_{1\text{-}39}$ for 24 h, primary cultured chicken hepatocytes were washed with PBS and then fixed with 4% paraformaldehyde for 30 min. After PBS washing, the Oil Red O working solution was added to stain for 30 min. The stained lipids were then visualized by light microscopy after washing in PBS. To quantify the lipid content, the Oil Red O stained in the cells was extracted with isopropanol by measuring the OD value at 540 nm.

For measurement of triglyceride content, after treated with $ACTH_{1\text{-}39}$ for 24 h, hepatocytes were harvested and triglyceride (TG) content was analyzed using a commercial triglyceride content assay kit (Applygen, Beijing, China, catalog no. E1013). Results were normalized to the protein content of each sample, as determined using a BCA assay kit (Beyotime Institute of Biotechnology, China, catalog no. P0010).

For quantitative real-time PCR, primary cultured chicken hepatocytes were treated with various concentrations of $ACTH_{1\text{-}39}$ (or other composition) at 37°C for 6 h. The relative amount of mRNA was calculated using the comparative Ct method. Chicken β-actin gene was used as the reference gene. Amplification of specific transcripts was confirmed by analyzing the melting curve profile performed at the end of each run and by determining the size of the PCR products using agarose electrophoresis and ethidium bromide staining.

Data analysis

All statistical analysis was performed using GraphPad Prism 9 (Graph Pad Software Inc., San Diego, CA). The dose-response curves were constructed using nonlinear regression models. Student's test was used to compare two groups, for more than two groups one-way ANOVA was performed followed by Dunnett's test. To validate our results, all *in-vitro* experiments were repeated three times, and representative data are reported.

Results

Characterization of the chicken *MC5R* full-length cDNA

According to the predicted cDNA sequences of *MC5R* deposited in GenBank (KF670718.1), we amplified and cloned the cDNAs of *MC5R* from chicken liver tissue. The chicken full-length *MC5R* gene contains two exons (GenBank accession no. OP259502), and its 5′-untranslated region (5′-UTR) and 3′-UTR is 253 bp and 601 bp in length, respectively. It is predicted to encode a G protein-coupled receptor of 325 amino acids (Figure 1).

Amino acid sequence alignment of chicken MC5R with their corresponding orthologs in other vertebrate species was shown in Figure 1A. According to multiple sequence alignment, the deduced amino acid sequence of MC5R is similar to the sequences of other known MC5Rs with a high degree of amino acid sequence identity to that of human (78%), mouse (79%), duck (98%) and zebrafinch (97%). The C-terminal and the seven transmembrane domains (TM1-TM7) of these MC5R

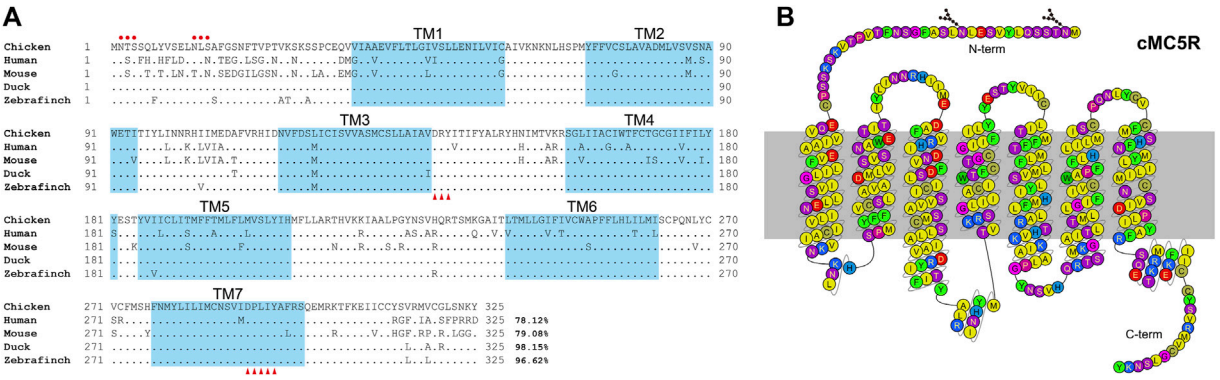

FIGURE 1
(A) Alignment of mature MC5R amino acid sequences among five species. NCBI accession numbers of these sequences are OP259502 (chicken), NP_005904.1 (human), NP_038624.3 (mouse), XP_005018248.1 (duck) and XP_004175266.1 (zebrafinch). The TM1-7 (seven transmembrane domains) are shaded in blue. The red dots indicate the predicted N-glycosylation sites. The conserved DRY motif and DPXXY motif were marked with red triangles. "." represents identical amino acid, and "–" indicates a gap in the sequence alignment. **(B)** Snake-like diagram of the chicken MC5R receptor. Residues are colored based on their chemical properties. N-term represents the N terminal sequence, C-term represents the C terminal sequence.

sequences are more similar than the N-terminal. Further analysis of MC5R sequences revealed several structural features, including two putative N-glycosylation sites (NXS/T, where X stands for any amino acid except proline), an Asp-Arg-Tyr (DRY) motif at the bottom of TM3, and a highly conserved Asp-Pro-X-X-Tyr (DPXXY) motif at the TM7 (Figure 1B).

Functional characterization of chicken MC5R

To investigate the functionality of chicken MC5R under stimulation with synthetic chicken melanocortin peptides (α-, β-, γ-MSH and $ACTH_{1-39}$) (Figure 2A), the pGL3-CRE-luciferase reporter system was used to monitor receptor-stimulated intracellular cAMP/PKA signaling pathway (Zhang et al., 2017). All four chicken melanocortin peptides were shown to potently activate cMC5R in CHO cells (Figure 2B). Among the four ligands, ACTH and α-MSH had the highest potency to activate cMC5R, with similar EC50 values (6.12 ± 1.18 nM and 4.23 ± 0.83 nM). The EC50 values for β-MSH and γ-MSH upon activation of cMC5R were 38.7 ± 6.51 nM and 144.4 ± 15.41 nM, respectively, which were approximately 10-fold and 30-fold lower than ACTH/α-MSH. In addition, the control plasmid pcDNA3.1 expressed in CHO cells did not respond to treatment with chicken melanocortin peptides (Figure 2C).

We also investigated the effect of cMC5R activation on CREB phosphorylation. Western blotting was used to show that CREB phosphorylation (43 kDa) was significantly increased when CHO cells expressing cMC5R were stimulated with 10 nM ACTH and 10 nM α-MSH for 10 min (Figure 2D). To further confirm the functional coupling of cMC5R to the intracellular cAMP/PKA signaling pathway, inhibitors targeting the intracellular cAMP/PKA signaling pathway were used to test whether they might inhibit the receptor-activated signaling pathway. H89 (a PKA inhibitor, 10 μM) and MDL12330 A (an AC inhibitor, 5 μM) could significantly inhibit ACTH-stimulated (Figure 2E) and α-MSH-stimulated (Figure 2F) luciferase activity in CHO cells expressing cMC5R, confirming the functional coupling of cMC5R to the AC/cAMP/PKA signaling pathways.

Interaction of chicken MRAP1 and MRAP2 with MC5R

To investigate the influences of chicken MRAPs on the responsiveness of cMC5R to α-MSH and ACTH, CHO cells co-expressing cMC5R and cMRAPs were treated with $ACTH_{1-39}$ and α-MSH, and the receptor activation was monitored by pGL3-CRE luciferase reporter system. Following stimulation with chicken $ACTH_{1-39}$, the EC50 value for MC5R expressed alone was 5.91 ± 1.18 nM, whereas the EC50 values for MC5R co-expressed with cMRAP1 or cMRAP2 were 2.12 ± 0.37 nM and 3.19 ± 1.09 nM, respectively (Figure 3A). It indicated that either MRAP1 or MRAP2 had no effect on the sensitivity of chicken MC5R for ACTH. Following stimulation with α-MSH, the EC50 value for MC5R expressed alone was 4.23 ± 0.83 nM, whereas the EC50 values for MC5R co-expressed with cMRAP1 or cMRAP2 were 7.85 ± 1.58 nM or 44.2 ± 10.21 nM, respectively (Figure 3B). Notably, co-expression of MRAP2 significantly reduced the sensitivity of MC5R to α-MSH (Table 1).

To test whether MRAP1 and MRAP2 could alter the trafficking of MC5R, we measured the surface expression of chicken MC5R in the absence or presence of cMRAPs using Nano-Glo HiBiT

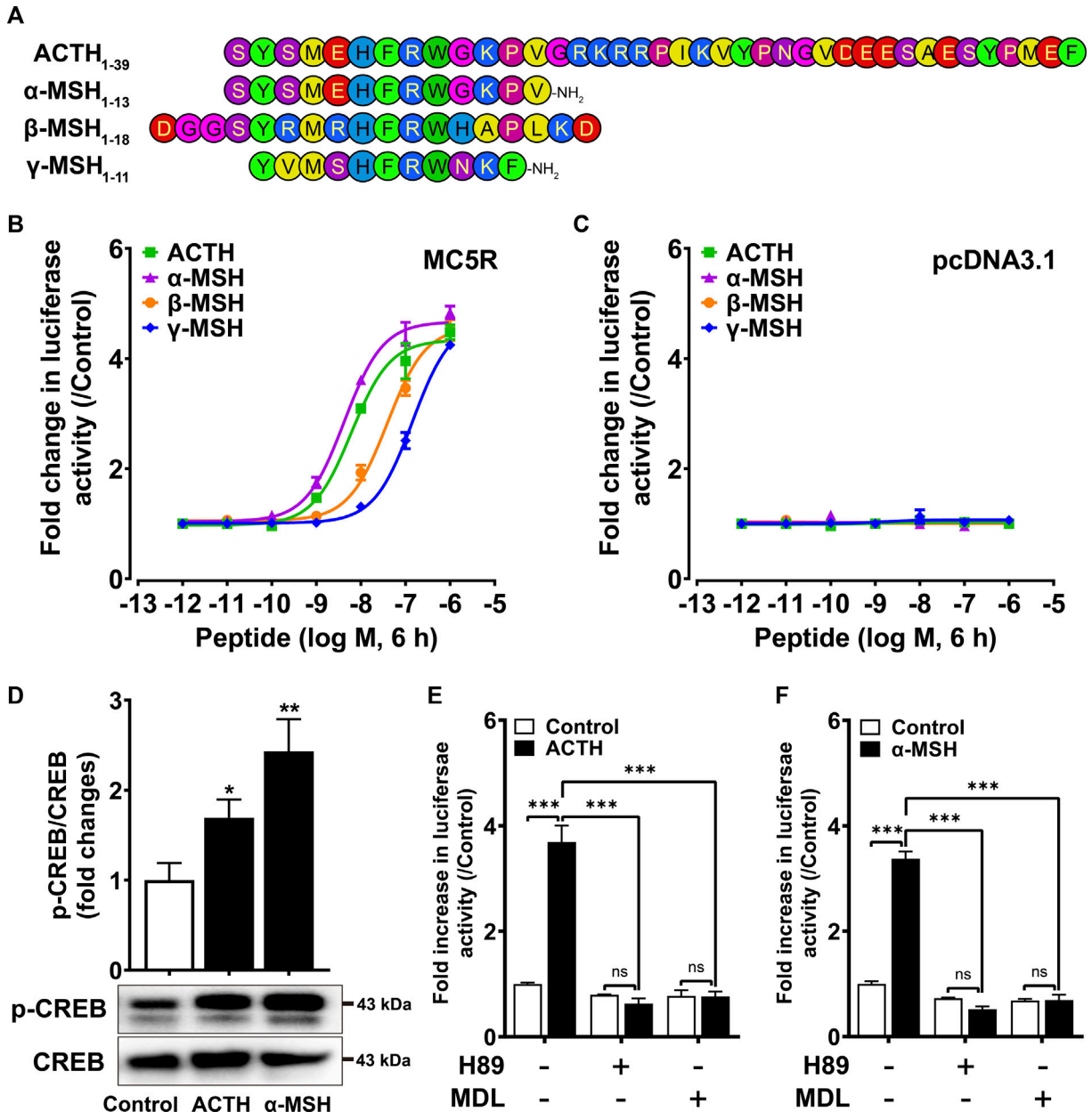

FIGURE 2
(A) Amino acid sequences of chicken ACTH, a-MSH (acetyl-a-MSH with an amidated C-terminus), β-MSH and γ-MSH (acetyl-γ-MSH with an amidated C-terminus) used in this study. **(B)** Effects of ACTH, a-MSH, β-MSH and γ-MSH in activating chicken MC5R expressed in Chinese hamster ovary (CHO) cells, as monitored by the pGL3-CRE-luciferase reporter system. Data are shown as the mean ± SEM of three replicates (N = 3) and are representative of three independent experiments. **(C)** Effects of ACTH, a-MSH, β-MSH and γ-MSH in activating negative control pcDNA3.1 expressed in CHO cells. Data are shown as the mean ± SEM of three replicates (N = 3) and are representative of three independent experiments. **(D)** Both ACTH and a-MSH treatment (10 nM, 10 min) could enhance CREB phosphorylation levels of CHO cells expressing cMC5R. The phosphorylated CREB (p-CREB) levels were quantified by densitometric analysis, normalized by that of cellular total CREB, and expressed as fold difference compared to the control (0 min). Data points represent the mean ± SEM of three independent experiments performed in triplicate. The representative set of Western blots is shown at the bottom. *, $p < 0.05$, **, $p < 0.01$ vs. control. **(E–F)** Effects of H89 (10 μM) and MDL (5 μM) on ACTH (10 nM, 6 h) **(E)** or a-MSH (10 nM, 6 h) **(F)** induced luciferase activities of CHO cells expressing cMC5R, monitored by pGL3-CRE-luciferase reporter system. H89 or MDL was added 0.5 h before treatment. Each figure shows one representative experiment repeated three times. ***, $p < 0.001$, ns, non-significant.

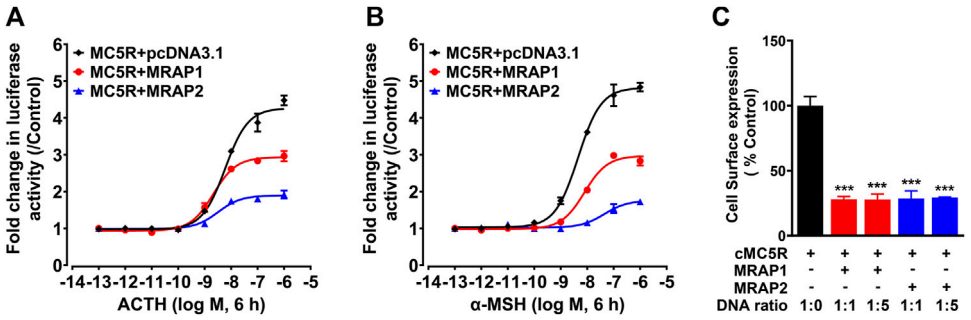

FIGURE 3
(A) Effect of ACTH in activating chicken MC5R expressed in CHO cells co-transfected with cMRAP1 or cMRAP2 expression plasmid, as monitored by the pGL3-CRE-luciferase reporter system. **(B)** Effect of a-MSH in activating chicken MC5R expressed in CHO cells co-transfected with cMRAP1 or cMRAP2 expression plasmid, as monitored by the pGL3-CRE-luciferase reporter system. Data are shown as the mean ± SEM of four replicates ($N = 4$) and are representative of three independent experiments. **(C)** Surface expressions of chicken MC5R in CHO cells transfected with MC5R and MRAP1 (or MRAP2) at the indicated ratio were measured by the HiBiT-tagging extracellular detection system (Promega) and the total expression levels of HiBiT-MC5R measured by HiBiT lytic assay were used as an internal control to normalize transfection efficiencies. Each figure shows one representative experiment repeated three times. ***, $p < 0.001$.

TABLE 1 EC_{50} values of chicken $ACTH_{1-39}$ and α-MSH in activating cAMP/PKA signaling pathways in CHO cells expressing chicken MC5R.

EC_{50} (nM)	MC5R	MC5R + MRAP1	MC5R + MRAP2
$ACTH_{1-39}$	5.91 ± 1.18	2.12 ± 0.37	3.19 ± 1.09
α-MSH	4.23 ± 0.83	7.85 ± 1.58	44.20 ± 10.21[a]

Results were expressed as the mean ± SEM, of at least three independent experiments.
[a]Significantly different from the parameter of MC5R, $p < 0.01$.

Detection System (Figure 3C). Compared with CHO cells transfected with MC5R only (1:0), the expression of chicken MC5R on the cell surface was significantly reduced to about 25% in the presence of MRAP1 or MRAP2 at a progressive ratio (1:1 and 1:5). This finding clearly indicated that MRAP1 and MRAP2 inhibit the cell surface expression of chicken MC5R.

Tissue expression of chicken *MC5R*, *MRAP1*, and *MRAP2*

To examine the tissue distribution of *MC5R*, *MRAP1* and *MRAP2* in adult chickens, we analyzed the expression in 36 chicken tissues with reference to the RNA-seq data previously obtained in our lab. We found that *MC5R* was widely expressed in various tissues, including liver, lung, adrenal gland, anterior pituitary, abdominal fat, visceral fat, hypothalamus, and kidney (Figure 4A). The highest level of *MC5R* transcript was detected in the liver. *MRAP1* was abundantly expressed in the adrenal gland and liver as illustrated in Figure 4B. RNA-seq results showed that *MRAP2* was also highly expressed in the cerebellum, hypothalamus, hindbrain, cerebrum, midbrain, adrenal gland, retina, and anterior pituitary, weakly expressed in the thymus gland, spinal cord, testis, and fat, and with almost undetectable expression in the liver (Figure 4C).

Using quantitative reverse transcription PCR (RT-qPCR), we re-examined the mRNA expression of *MC5R* in adult chicken tissues, including the cerebellum, midbrain, cerebrum, hindbrain, hypothalamus, anterior pituitary, kidney, liver, lung, muscle, skin, testis, adrenal gland, abdominal fat, heart, spleen, and ovary. In agreement with the RNA-Seq data, *MC5R* transcript had the significantly highest abundance in the liver, which was about 2.3-fold and 3.5-fold higher than those detected in the adrenal gland and lung (Figure 4D). Transcripts of *MC5R* have also been detected weakly in other tissues, including anterior pituitary, skin, muscle, kidney, testis, fat, ovary, and some brain regions. In addition, the expression of the other four MCRs were also examined *via* the RNA-Seq data atlas (Figure 4E), and only *MC5R* could be detected in adult chicken liver tissue, while the TPM values of the other MCRs were almost zero.

Since *MC5R* is highly expressed in the liver, we examined the mRNA expression of *MC5R* in liver at different developmental stages, including at embryonic day 7 (E7), embryonic day 15 (E15), day 1 after hatch (D1), day 7 after hatch (D7), day 35 after hatch (D35) and day 300 after hatch chicken (D300). As shown in Figure 5A, expression of *MC5R* gradually increased from day 7 of embryonic development and reached its peak significantly on day 1 after hatch. During the post-hatch growth period, *MC5R* expression was downregulated significantly.

In addition, we analyzed the expression trends of *MC5R* and *MRAP1* genes in the red junglefowl (*Gallus gallus*) at different developmental periods using transcriptome data from the NCBI

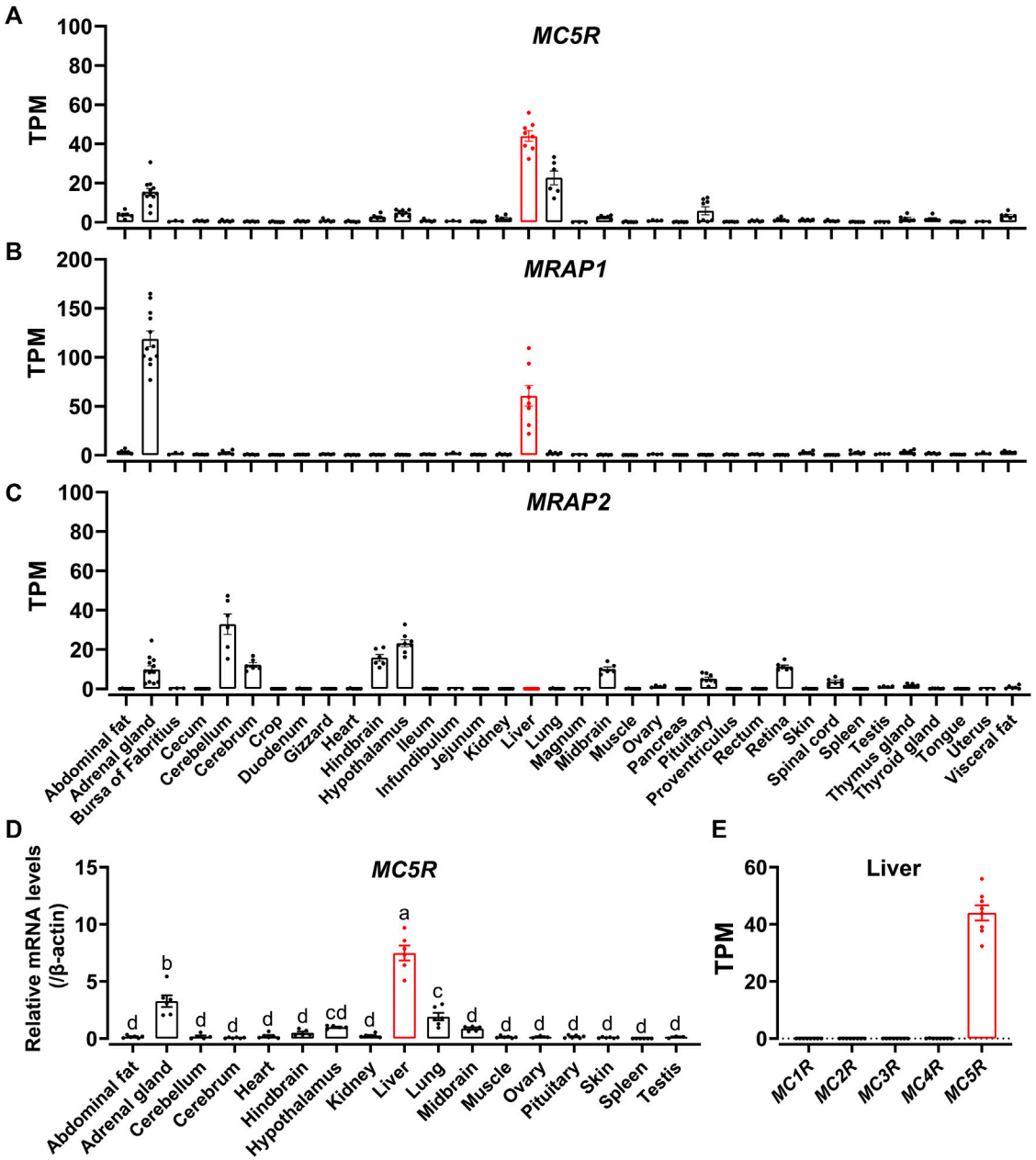

FIGURE 4
(A–C) RNA-seq data analysis showed the expression of *MC5R* **(A)**, *MRAP1* **(B)**, and *MRAP2* **(C)** in adult Lohmann Layer strain chicken tissues. Each dot represents an individual. The transcripts per million (TPM) values were used to estimate the abundance of mRNA transcripts. **(D)** Quantitative real-time PCR assay of *MC5R* mRNA levels in Lohmann Layer strain chicken tissues, including the cerebellum, midbrain, cerebrum, hindbrain, hypothalamus, anterior pituitary, kidney, liver, lung, muscle, skin, testis, abdominal fat, heart, spleen, and ovary. The mRNA levels of target genes were normalized to that of β-actin and expressed as the fold difference compared with that of the midbrain. Each data point represents the mean ± SEM of six adult chickens ($N = 6$, three males and three females, one-year-old), except for that of ovary and testis, which represent the mean ± SEM of three adult chickens ($N = 3$). Different superscripts **(A–D)** among the different developmental stages are significantly different ($p < 0.05$) by one-way ANOVA test, followed by Dunn's multiple comparison test. **(E)** The mRNA expression of five MCRs in the Lohmann Layer liver tissue using transcriptome data from our gene expression atlas (https://chickenatlas.avianscu.com/). The transcripts per million (TPM) values were used to estimate the abundance of mRNA transcripts.

public database (Accessions: PRJEB26695). In high agreement with the quantitative PCR results in Lohmann Layer strain, *MC5R* was expressed at the highest level in chicken liver at day 0 after hatch, and *MC5R* expression gradually decreased after

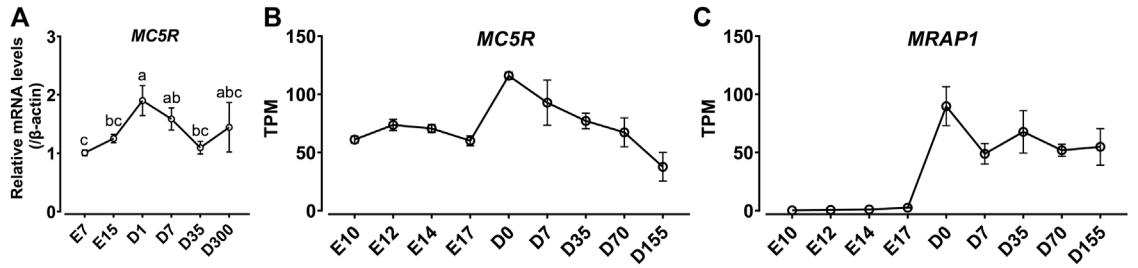

FIGURE 5
(A) Quantitative real-time PCR assay of *MC5R* mRNA levels in Lohmann Layer strain chicken tissues at different development stages, including embryonic day 7 (E7), embryonic day 15 (E15), day 1 after hatch (D1), day 7 after hatch (D7), day 35 after hatch (D35) and day 300 after hatch chicken (D300). The mRNA levels of target genes were normalized to that of β-actin and expressed as the fold difference compared with that of embryonic day 7 (E7). Each data point represents the mean ± SEM of six adult chickens (N = 6, three males and three females). Different superscripts (a–c) among the different developmental stages are significantly different ($p < 0.05$) by one-way ANOVA test, followed by Dunn's multiple comparison test. **(B–C)** The mRNA expression of *MC5R* **(B)** and *MRAP1* **(C)** genes in the red junglefowl (*Gallus gallus*) at different developmental periods using transcriptome data from the NCBI public database (Accessions: PRJEB26695). This dataset covers the development of liver organ from day 10 post-conception to day 155 post-hatch. The transcripts per million (TPM) values were used to estimate the abundance of mRNA transcripts.

hatch (Figure 5B). Unexpectedly, *MRAP1* expression was barely detectable in chicken embryos, while *MRAP1* had the highest expression level at day 0 after hatch and then maintained a stable expression level in the chicken liver (Figure 5C).

Analysis of the chicken *MC5R* promoter region

To identify the promoter region of chicken *MC5R*, we constructed several promoter-luciferase constructs containing the 5′ flanking region of *MC5R* with different lengths and tested their promoter activities in cultured DF1 chicken embryonic fibroblasts. A 1998 bp PCR fragment containing 171 bp of *MC5R* cDNA sequence (exon 1) and 1827 bp of 5′-flanking sequence was first obtained by genomic PCR. To determine the core promoter region of *MC5R*, four promoter-luciferase reporter constructs of chicken *MC5R* were generated by cloning the 1.9 kb fragment or its truncated fragment into pGL3-basic vector.

As shown in Figure 6A, the 5′-flanking regions of *MC5R* from -1827 to +171 (P1) exhibited promoter activity in DF1 cells. When the 5′-end of P1 was truncated (P2: −828/+171), the activity of luciferase in DF1 cells was greatly increased. Interestingly, continued truncation of the 5′-end fragments of P2 (P3: −343/+171 and P4: −66/+171) reduced the promoter activity. We also noted that although the length of P3 was 514 bp, it had a 32-fold increase in luciferase activity compared to the control vector, indicating that this region (-343 to +171) retains the essential minimum activity. Using the online software AnimalTFDB (v3.0) (Hu et al., 2019), the putative binding sites for many transcription factors, including CREB1, SRF, CTCF, AR, FOXA3, FOXA1, PPARX:RXRA and CEBPB were predicted to exist within or near the promoter region P2 (Figure 6B).

To clarify whether the cAMP/PKA/CREB pathway stimulates *MC5R* transcription or not, we examined the effect of forskolin (an adenylyl cyclase activator), 8-Br-cAMP (a cell-permeable cAMP analog, PKA activator) and glucagon (induce cAMP elevation in a dose-dependent way) on the expression level of *MC5R* in primary chicken hepatocytes. Treatment of hepatocytes with forskolin significantly increased *MC5R* mRNA abundance (Figure 6C). Incubating hepatocytes with 8-Br-cAMP also stimulated a dose-dependent increase in *MC5R* mRNA abundance (Figure 6D). Incubating chicken hepatocyte cultures with glucagon (10–100 nM) stimulated a 3.5-fold increase in *MC5R* mRNA abundance after 6 h of treatment (Figure 6E). These results demonstrated that glucagon increased hepatic *MC5R* mRNA abundance and provided evidence that the PKA branch of the cAMP pathway may play a role in mediating this effect.

Effect of ACTH on glucolipid metabolism in primary hepatocytes

Both *MC5R* and *MRAP1* were highly expressed in chicken liver after hatch, which indicated that the liver might be another target organ for ACTH, in addition to the adrenal gland, when the chicken HPA axis is active. We then evaluated the influence of $cACTH_{1\text{-}39}$ on the glucose production and lipid contents of primary hepatocytes to explore the biological roles of ACTH mediated by MC5R and MRAP1 in the chicken liver. As shown in Figures 7A,B, $cACTH_{1\text{-}39}$ significantly increased the level of glucose production in a dose (≥1 nM)- and time (≥0.5 h)-dependent manner in primary hepatocytes. When compared with the control, ACTH prominently decreased triglyceride (TG) contents, oil red O staining showed that lipid contents

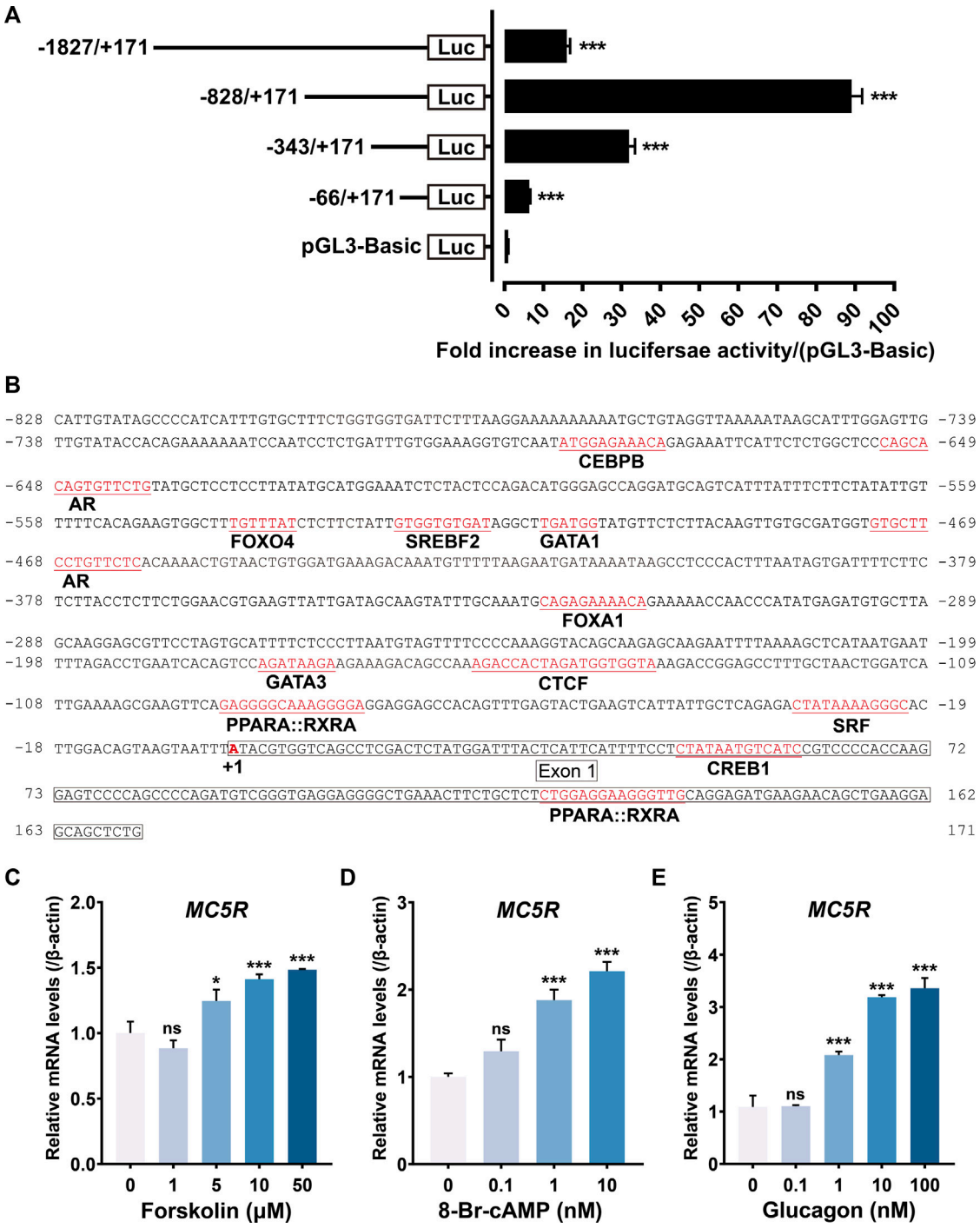

FIGURE 6
(A) Detection of the promoter activities of the 5′-flanking regions of chicken *MC5R* gene in cultured DF-1 cells. Various stretches of the 5′-flanking regions of chicken *MC5R* were cloned into a pGL3-Basic vector for the generation of four promoter-luciferase constructs (P1: −1827/+171-Luc, P2: −828/+171-Luc, P3: −343/+171-Luc, P4: −66/+171-Luc). Their promoter activities were determined by the Dual-Luciferase Reporter (DLR) assay. All experiments were performed in triplicate and represent at least three independent biological repeats. Data shown represent mean ± SEM. ***, $p < 0.001$ vs. promoter-less pGL3-Basic vector. **(B)** Partial sequence (-828/+171) of the chicken *MC5R* promoter region. The predicted binding sites for transcriptional factors, such as CREB1, SRF, CTCF, AR, FOXA3, FOXA1, and CEBPB were shaded. The transcriptional start site 'A' was marked with red and designated as '+1'. The sequences of exon 1 was boxed. **(C–E)** Effects of forskolin **(C)**, 8-Br-cAMP **(D)** and chicken glucagon **(E)** with different concentrations on *MC5R* gene expression in cultured chicken primary hepatocytes examined by RT-qPCR. Each data point represents means ± SEM of four replicates ($N = 4$). *, $p < 0.05$, ***, $p < 0.001$, ns, non-significant vs. respective control (without peptide treatment).

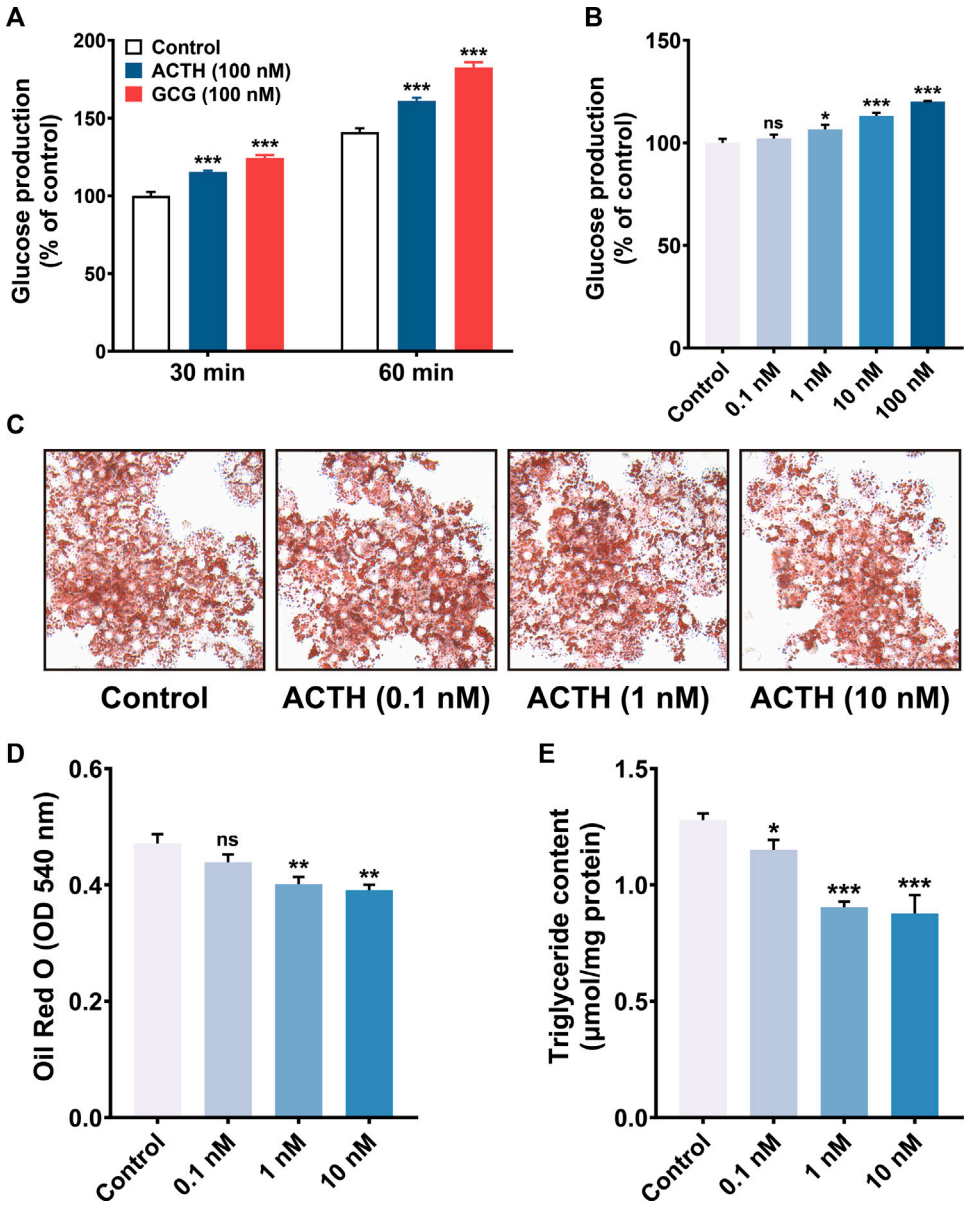

FIGURE 7
(A) ACTH and glucagon (GCG) induction of gluconeogenesis in primary cultured chicken hepatocytes. Primary cultured chicken hepatocytes were incubated with 100 nM of ACTH and GCG for various times (30 and 60 min), glucose production was then measured. Values represent means ± SEM of four independent experiments with triplicate dishes. ***, $p < 0.001$ vs. control. **(B)** ACTH induction of gluconeogenesis in primary cultured chicken hepatocytes. Primary cultured chicken hepatocytes were incubated with different concentrations (0.1–100 nM) of ACTH for 60 min. Values represent means ± SEM of four independent experiments with triplicate dishes. *, $p < 0.05$, ***, $p < 0.001$, ns, non-significant. **(C–D)** Oil red O staining (magnification: 10×40) and quantification analysis ($N = 6$), red circle drops mean fat droplets. The Oil Red O stained in the cells was extracted with isopropanol by measuring the OD value at 540 nm **, $p < 0.01$, ns, non-significant vs. control. **(E)** The effects of ACTH on triglyceride content in primary chicken hepatocytes. *, $p < 0.05$, ***, $p < 0.001$ vs. control.

were lower in the ACTH-treat group in a dose-dependent manner (Figures 7C–E).

In addition, ACTH dose-dependently decreased *ELOVL6* (Figure 8A) and *THRSPA* (Figure 8D) mRNA expression, instead of *ACACA*, *FASN* and *SCD* (Figures 8G–I), which were all related to lipogenesis. The expression level of *ELOVL6* and *THRSPA* in primary hepatocytes was not affected by Forskolin (adenylyl cyclase activator) (Figures 8B,E) and 8-Br-cAMP (PKA activator) in a dose-dependent way (Figures 8C,F). These results demonstrated that ACTH may relieve triglyceride deposition by inhibiting *de novo* lipogenesis.

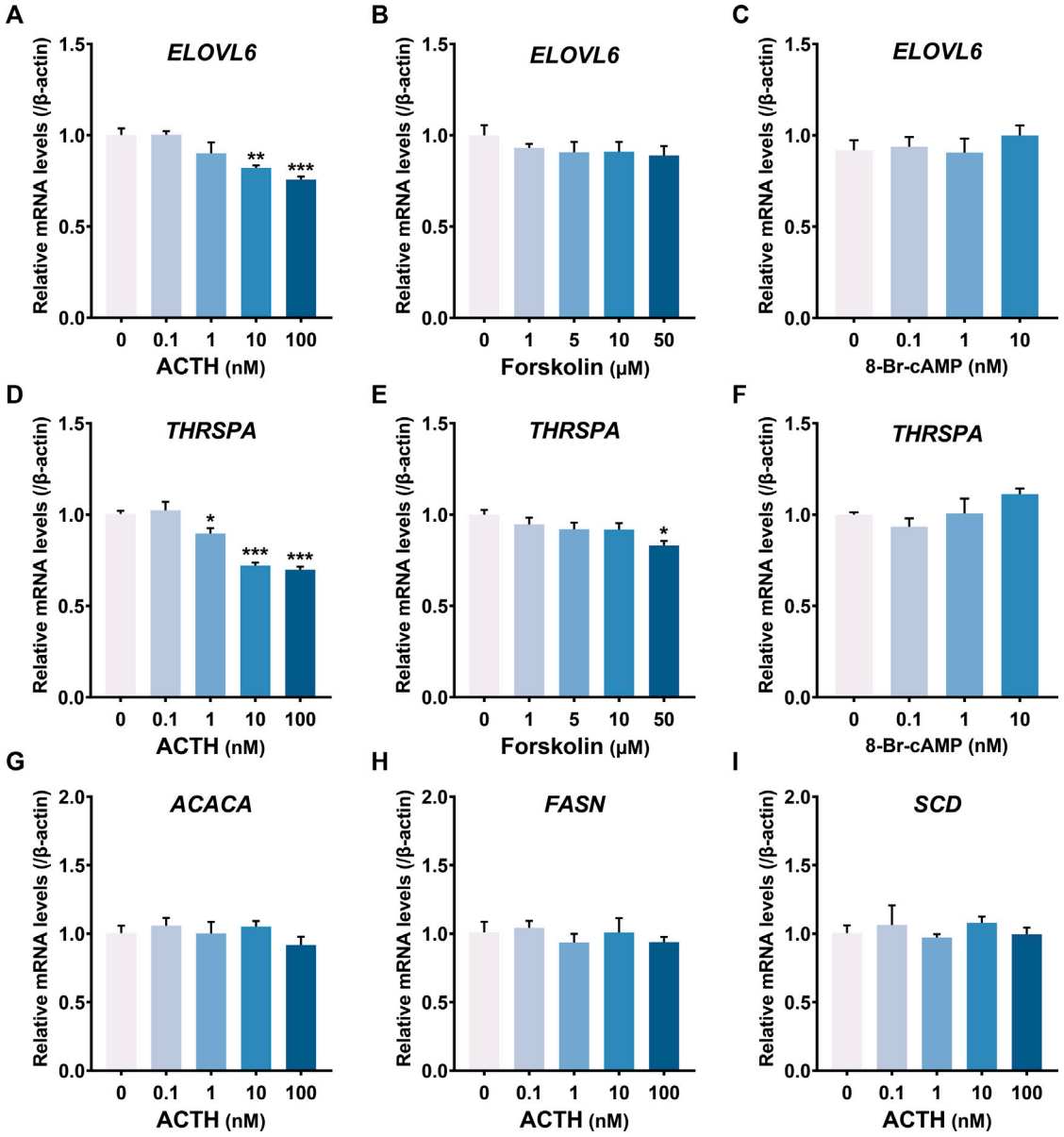

FIGURE 8
(A–F) Effects of chicken $ACTH_{1-39}$, forskolin and 8-Br-cAMP with different concentrations on gene expression, including *ELOVL6* **(A–C)** and *THRSPA* **(D–F)**, in cultured chicken primary hepatocytes examined by RT-qPCR. **(G–I)** Effects of chicken $ACTH_{1-39}$ with different concentrations (0.1–100 nM) on gene expression, including *ACACA* **(G)**, *FASN* **(H)** and *SCD* **(I)**, in cultured chicken primary hepatocytes. Each data point represents means ± SEM of four replicates ($N = 4$). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. respective control (without peptide treatment)..

Discussion

In this study, *MC5R* cDNAs were cloned from the chicken liver. The current work provided evidence for the promoter activity of MC5R in chickens and confirmed that MC5R was sensitive to multiple endogenous melanocortin ligands in chickens. The effects of chicken MRAP1 and MRAP2 on MC5R signaling and trafficking were also discussed. Based on transcriptomic and qPCR data, MC5R was highly expressed in the liver along with MRAP1 in post-hatch chickens. Then we found that chicken $ACTH_{1-39}$ enhanced glucose production and decreased triglyceride contents in primary hepatocytes, dose-dependently. Gene expression studies revealed that *ELOVL6* and *THRSPA* were down-regulated, suggesting ACTH supplementation could suppress *de novo* lipogenesis. These results indicating that ACTH may play a direct role in hepatic

metabolism. Previously, little information was available on the physiological functions of chicken MC5R, and our findings provided new evidence to explore the physiological roles of MC5R in avian.

Here, from the chicken brain, we cloned the full-length cDNA sequence of MC5R. Similar to cMC2R, the cMC5R transcript has an upstream non-coding exon and a coding exon with a long 3′-UTR. Both the MC2R and MC5R genes in chickens contain two exons, adding to the evidence that the MC2R and MC5R genes evolved from a common ancestor (Klovins et al., 2004; Dores, 2013). Sequence alignment showed that MC5Rs in different species all consisted of 325 amino acids with sequence similarity higher than 78% and were highly conserved between chicken and other birds or with human. The transmembrane regions of chicken MC5R were highly conserved to those of other vertebrates, but the N-terminal region differed considerably. This is consistent with earlier findings, which indicated that the MCR's binding domain is likely to be present inside TM1, TM2, TM3, TM6, and TM7 (Schioth, 2001).

The luciferase reporter system was then employed to investigate the signal pathway of MC5R coupling. In response to α-MSH or ACTH, we demonstrated that the chicken MC5R can functionally couple to the Gs-linked cAMP/PKA signaling pathway, which is consistent with previous findings in human (Fathi et al., 1995), mice (Haskell-Luevano et al., 2001), blunt snout bream (Liao et al., 2019), zebrafish (Zhu et al., 2019), fugu (Klovins et al., 2004), sea bass (Sanchez et al., 2009), whale shark (Hoglin et al., 2020), and gar (Wolverton et al., 2019). Human peptides have previously been widely employed in the investigation of chicken MC5R (Ling et al., 2004; Thomas et al., 2018; Min et al., 2019; Dores et al., 2020). α-MSH is conserved in all positions between chicken and human, but the substitution of R15K and I20 V occurred in the KKRRPV motif of $hACTH_{1-24}$. It was concluded that there were no statistical differences in the EC50 values when stimulated with either $hACTH_{1-24}$ or $srACTH_{1-24}$ (R15 and I20) (Dores et al., 2020). The synthesized chicken $ACTH_{1-39}$, α-MSH (acetyl-α-MSH), β-MSH and γ-MSH were used in our study to confirm the pharmacological impact of ligands acting on the MC5R. Remarkably, our results showed that the ability of melanocortin to activate adenylate cyclase is ranked in the following order: $ACTH_{1-39}$ ≈ α-MSH > β-MSH > γ-MSH. The ability of $hACTH_{1-39}$ and its shortened form, $hACTH_{1-24}$, to activate human and chicken MC5R has been shown to be no different in previous studies (Schioth et al., 1997; Ling et al., 2004). Our results were consistent with an early study that showed human $ACTH_{1-24}$ and α-MSH had equal potency for chicken MC5R (Ling et al., 2004). The intracellular response of cMC5R to β-MSH and γ-MSH was first investigated in chicken. In contrast to $ACTH_{1-39}$/α-MSH, the EC50 values of β-MSH and γ-MSH were approximately 10-fold and 30-fold lower, which is consistent with previous studies on human MC5R (Fathi et al., 1995) and blunt snout bream MC5R (Liao et al., 2019).

Melanocortin receptor accessory proteins (MRAP1 and MRAP2) were involved in the regulation of trafficking and signaling of vertebrate melanocortin receptors (Chan et al., 2009; Asai et al., 2013; Ramachandrappa et al., 2013; Zhang et al., 2017). As revealed in Figure 3, chicken MRAP1/MRAP2 did not affect the sensitivity of cMC5R to $ACTH_{1-39}$, while MRAP2, instead of MRAP1, significantly decreased the sensitivity of cMC5R to α-MSH. At the same time, we observed that co-expression of MRAP1/MRAP2 with MC5R could reduce the plateau phase of the sigmoid curve, which is nicely correlated to the drop in membrane MC5R detected with MRAP1/MRAP2 co-expression. Using the Nano-Glo HiBit detection system, which is a novel method that allows quantitative and sensitive measurement of total and cell surface receptor expression (Rouault et al., 2017a; Reyes-Alcaraz et al., 2018), we further verified that both MRPA1 and MRAP2 could significantly decrease cell surface expression of MC5R compared to control, even at a 1:1 ratio.

Our results were consistent with previous results. In human, MRAPs were thought to down-regulate the cell surface expression of MC5R *in vitro*, and MRAP2 reduced the efficacy of hMC5R to NDP-MSH (Chan et al., 2009). It was reported that mouse MRAP1 had a negative effect on the surface expression of the MC5R receptor (Sebag and Hinkle, 2009). In zebrafish, MRAP2a decreased the surface expression and efficacy of MC5Ra/MC5Rb, but not MRAP2b, in the same manner as mouse MRAP2 did (Zhu et al., 2019). In ricefield eel (*Monopterus albus*), two isoforms of MRAP2 (maMRAP2X1 and maMRAP2X2) significantly decreased efficacy of maMC5R (Liu et al., 2021). In gar (*Lepisosteus oculatus*), MRAP2 and MRAP1 did not affect the sensitivity of MC5R, but increased the trafficking of MC5R to the plasma membrane (Wolverton et al., 2019).

However, our findings are in sharp contrast to the previous observations on chicken MC5R, which showed that chicken cMRAP1 significantly increased the sensitivity of cMC5R to $hACTH_{1-24}$ (Thomas et al., 2018). Their further study confirmed this result and suggested that when cMC5R was co-expressed with cMRAP1, the KKRRP motif of $hACTH_{1-24}$ is necessary to increase the sensitivity of MC5R to $hACTH_{1-24}$ nearly 1,000 fold (Dores et al., 2020). Since the R15K and I20 V substitutions in chicken and human ACTH occur in/near the KKRRP motif (Figure 2A), it might lead to different regulatory effects of MRAP1 on MC5R in chicken, although more in-depth studies are still needed to verify this. Furthermore, recent reports have shown that co-expression of MC5R orthologs with MRAP1 in whale sharks, elephant sharks, stingrays, and rainbow trout could increase sensitivity to $ACTH_{1-24}$ (Dores et al., 2018; Barney et al., 2019; Dores et al., 2020; Hoglin et al., 2022), implying that MRAP1's effect on MC5R sensitivity may be species specific.

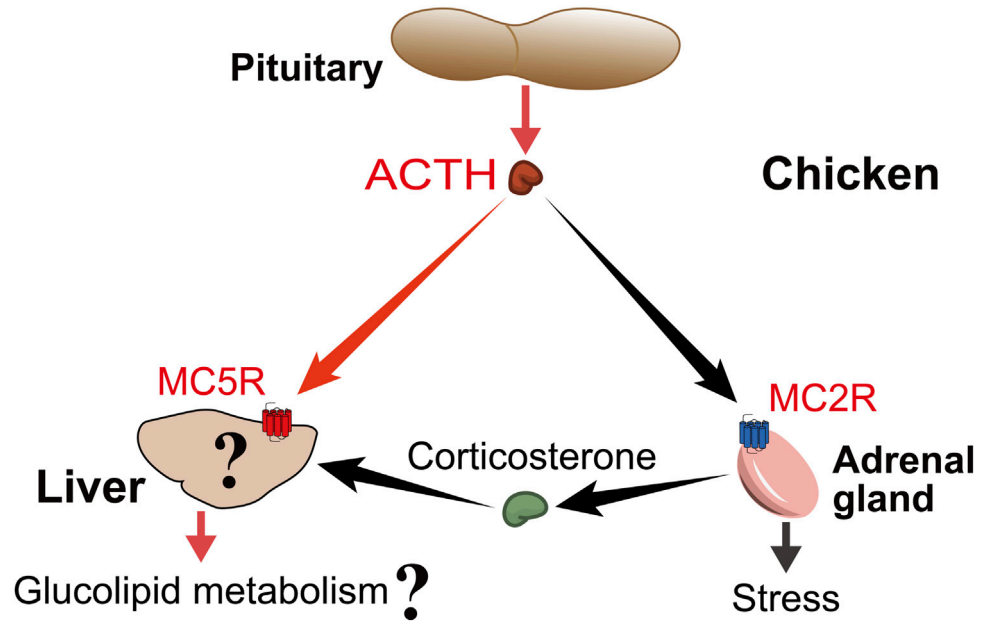

FIGURE 9
Proposed model for ACTH actions on chicken liver. Pituitary-derived ACTH hormone can act not only on MC2R, which is highly expressed in adrenal tissue, to participate in the stress, but also on MC5R, which is highly expressed in liver tissue, to participate in the regulation of glucolipid metabolism. At the same time, the liver is also the main target organ of ACTH-induced corticosterone, so it is worthwhile to investigate the difference between the direct effect of ACTH and the effect of glucocorticoids.

In previous work (Zhang et al., 2017), we focused on the expression patterns of *MC3R*, *MC4R*, *MRAP1* and *MRAP2* among the chicken melanocortin system. RNA-Seq and RT-qPCR were used in this work to assess the expression pattern of *MC5R* in Lohmann Layer strain. As illustrated in Figure 4, the highest levels of chicken *MC5R* mRNA were detected in the liver, lung, and adrenal gland. *MRAP2* mRNA was detected mainly in the chicken brain, retina, pituitary, and adrenal gland. The expression of *MRAP1* was limited to the adrenal gland and liver. Our finding was consistent with earlier findings in chicken (Ren et al., 2017; Zhang et al., 2017; Thomas et al., 2018), mouse (Asai et al., 2013) and zebrafish (Sebag et al., 2013). In addition, it also demonstrated that only *MC5R* could exist in liver tissue, which is supported by the RNA-Seq data atlas (Zhang et al., 2022). *MC5R* and *MRAP1* could also be detected in the liver at different developmental stages, especially with the highest expression levels simultaneously after hatch, suggesting that both may have potential physiological effects on the liver metabolism of newborn chicks.

In this study, we found that chicken *MC5R* expression is likely controlled by a functional promoter near exon 1 (within -828 to +171), which contains putative binding sites for many transcriptional factors (such as CREB1, FOXO4, AR, and FOXA1) and displays a strong promoter activity in DF-1 cells. Notably, the promoter activity of P2 (-828/+171) was the highest, 89-fold higher than that of the control. Like its mammalian counterparts, numerous putative transcription factor binding sites were found in the upstream of the *MC5R* transcription start site. According to reports, Foxo4 may bind to the *MC5R* promotor and inhibit *MC5R* transcription in mouse adipocytes (Liu et al., 2017). In the chicken *MC5R* promoter region, a putative FOXO4 binding site was discovered, suggesting that the transcription factor FOXO4 may control *MC5R* transcription. We also found a potential CREB-binding site in the proximal promoter region of the *MC5R* promoter, demonstrating that CREB may be involved in the transcriptional control of *MC5R*. Glucagon has been shown to enhance the intracellular cAMP accumulation and activate CREB (Herzig et al., 2001; Noriega et al., 2011), and the glucagon receptor (GCGR) was highly expressed in the liver of chickens (Wang et al., 2008). In this work, we found that forskolin, 8-Br-cAMP and glucagon enhance the expression of *MC5R* in cultured hepatocytes in a dose-dependent manner. These findings provided support for a role of cAMP/PKA pathway in mediating the increase of *MC5R* mRNA abundance. The present findings established a foundation for further investigation of the regulatory mechanism governing *MC5R* expression in chicken liver tissues. However, further studies are needed to determine the extent that ACTH (or other MSHs) interact with other hormones, such as the fasting-related hormone glucagon, influence liver tissue metabolism.

The high expression of both *MRAP1* and *MC5R* in the liver suggested that the melanocortin ligands (ACTH and other MSHs) may regulate essential physiological functions in this organ. Due to the avian pituitary lacking the intermediate lobe, it secretes relatively little α-MSH, hence $ACTH_{1-39}$ is considered as the primary circulating melanocortin peptide released from pituitary in chickens (Hayashi et al., 1991; Takahashi and Mizusawa, 2013). The ACTH in the plasma was about 10 pg/ml (~2.2 pmol/l) in 4–6 days chicks determined by a two-site sequential chemiluminescent immunometric assay (Gaston et al., 2017). The concentration of plasma ACTH was about 12 pg/ml in hatched chicks (Okur et al., 2022) and 7–18 pg/ml in three-week-old male chicks from our recent study (Liu et al., 2022). There is little information on the circulating concentrations of chicken α-MSH, which is also known to be present in systemic circulation for regulating pigmentation (Ling et al., 2003) or lipolytic activity (Shipp et al., 2017) in different chicken tissues. The concentration of plasma α-MSH was about 3.1 ng/ml (1.86 nmol/l) in 4-day-old chicks (Shipp et al., 2017). It is unclear if circulating α-MSH is three orders of magnitude higher than circulating ACTH or if this is an artifact of limited investigations. Therefore, we first investigated the effects of ACTH on hepatocytes here. In future research, we intend to reveal the concentration and effects of α-MSH in chickens.

Early studies have tested the effect of continuous ACTH administration in 7-day chickens (Puvadolpirod and Thaxton, 2000a; Puvadolpirod and Thaxton, 2000b; Puvadolpirod and Thaxton, 2000c; Puvadolpirod and Thaxton, 2000d; Thaxton and Puvadolpirod, 2000). Continuous ACTH administration through mini-osmotic pumps offered an ideal model for examining chicken stress, and it was shown that ACTH induced not only an increase in plasma glucose and relative liver weight in this model, but also a fast rise in plasma corticosterone (Puvadolpirod and Thaxton, 2000a). A series of effects of ACTH in the liver were attributed to the release of corticosterone in the adrenal cortex. Corticosterone could mobilize glucose reserves and promote gluconeogenesis *via* fatty acid and protein degradation (Lin et al., 2004). The high expression of MC5R in the liver raises new questions as to whether ACTH can act directly on MC5R/MRAP1 complex to involve in glycolipid metabolism in the liver.

The present study provided evidence that chicken ACTH can act on primary hepatocytes to increase glucose production and decrease triglyceride contents, while downregulating several genes associated with lipogenesis. Importantly, this result was obtained with physiological concentrations of ACTH. MC5R was implicated in metabolic regulation as it regulated α-MSH signaling in skeletal muscle, which derived glucose disposal and thermogenesis (Enriori et al., 2016). In addition, central activation of MCRs has also been shown to play a role in skeletal muscle glucose uptake in mammals (Nogueiras et al., 2007; Toda et al., 2009). Here, this interesting finding led us to hypothesize that ACTH may directly act on MC5R/MRAP1 complex in chicken hepatocytes to regulate glucose and lipid metabolism, which needs further verification (Figure 9).

The phenomenon of lower *ELOVL6* and *THRSPA* mRNA, instead of *ACACA*, *FASN* and *SCD* mRNA, in the ACTH group has also attracted our attention. All these genes played an important role in hepatic *de novo* lipogenesis (Nematbakhsh et al., 2021). The elongation of very long chain fatty acids protein 6 (ELOVL6) is responsible for the final step in endogenous saturated fatty acid synthesis and involves in *de novo* lipogenesis (Shimano, 2012). The regulation of ELOVL6 expression is important for altering the hepatic lipid composition in response to alterations in dietary and hormonal status (Wang et al., 2006). Thyroid hormone-responsive Spot 14 protein *α* (THRSPA), as a primary lipogenic transcription factor, has emerged as the highest-expressed hepatic gene supporting enhanced lipogenesis and thermogenesis in newly hatched chicks (Resnyk et al., 2013; Cogburn et al., 2018). THRSPA appeared to be a key transcriptional regulator of the switch in metabolism from ectothermy to endothermy (Cogburn et al., 2018). The chicken *THRSPA* gene variations were significantly associated with fat deposition and plasma lipid profiles such as TC and LDL (Hirwa et al., 2010). We demonstrated that ACTH regulates lipogenesis by influencing the expression of *ELOVL6* and *THRSPA* in chicken liver.

Unexpectedly, we found that both forskolin and 8-Br-cAMP did not affect the expression of *ELOVL6* and *THRSPA* genes. In addition to the cAMP pathway *via* Gαs, Activated MC5R can be coupled to the Ca^{2+} pathway *via* Gαq in mammals (Hoogduijn et al., 2002). Moreover, MC5R also can activate some cAMP- and Ca^{2+}-independent pathways. For example, MC5R triggers the PI3K-ERK1/2 pathway, which can further mediate downstream pathways in fatty acid re-esterification, cellular proliferation, and immune responses (Rodrigues et al., 2009; Rodrigues et al., 2013; Xu et al., 2022). Then, further investigation will be required to elucidate the exact mechanisms and pathways by which ACTH affects the expression or activity of other transcription factors or downstream metabolic genes in chicken.

In conclusion, we cloned the full-length sequence of chicken *MC5R* gene and characterized its promoter activity in DF1 chicken embryonic fibroblasts. The functional assay demonstrated that MC5R exhibited higher sensitivity to chicken ACTH/α-MSH compared to β-MSH/γ-MSH. It also showed that both MRAP1 and MRAP2 inhibited the trafficking of chicken MC5R to the plasma membrane, and that only MRAP2 significantly reduced the sensitivity of MC5R to stimulation by α-MSH. *MC5R* and *MRAP1* mRNA were co-expressed in the liver of post-hatch chickens. We found that ACTH may increase glucose production, decrease triglyceride content, and dose-dependently downregulate the expression levels of *ELOVL6* and *THRSPA* genes in hepatocytes, suggesting that ACTH has a unique endocrine role in regulating hepatic glucolipid metabolism. However,

further studies are needed to characterize and confirm the critical role of the ACTH-MC5R/MRAP1 axis in regulating glucose and lipid metabolism in chicken liver.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: Nucleotide sequence of chicken MC5R is available in the GenBank databases under the accession number OP259502. The raw data that support the tissue gene expression atlas (TGEA) (https://chickenatlas.avianscu.com/) from adult Lohmann White domestic chickens have been deposited into CNGB Sequence Archive (CNSA) of China National GeneBank DataBase (CNGBdb) with accession number CNP0003404. RNA-Seq data publicly available used to evaluate the expression level of *MC5R* and *MRAP1* at different development stages was downloaded from NCBI under accession code PRJEB26695.

Ethics statement

The animal study was reviewed and approved by the Animal Ethics Committee of College of Life Sciences, Sichuan University.

Author contributions

XZ: Investigation, Data analysis, Writing-original draft, and editing. JS: Data analysis, Writing-review, and editing. TH: Conceptualization, Investigation. XW: Investigation. CW: Investigation and Data analysis. JiL: Investigation and Data analysis. JuL: Resources and Writing-review and editing. JZ: Investigation, Data analysis, Writing-original draft, and editing. YW: Resources and Writing-review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2022.917712/full#supplementary-material

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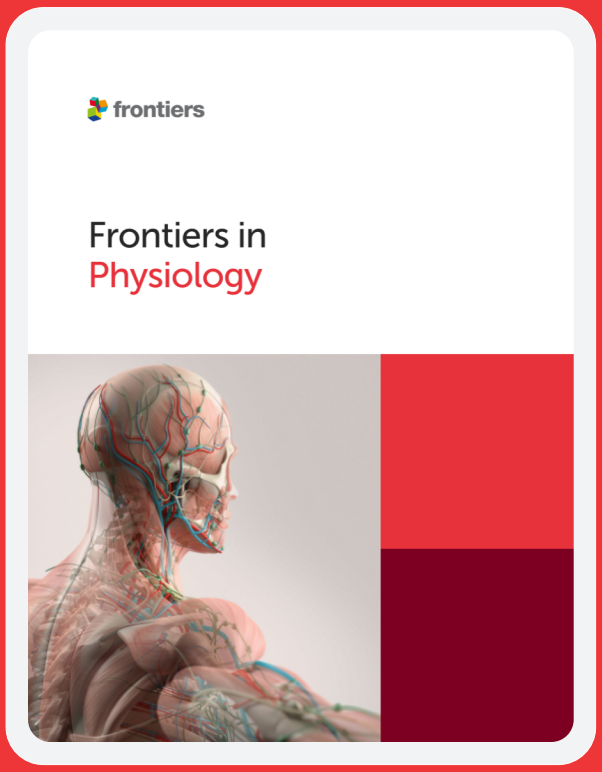

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