Original Research Article

Restricted feeding regimens improve white striping associated muscular defects in broiler chickens

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A B S T R A C T

The current study investigated the effects of intermittent feeding (IF) and fasting strategies at different times post-hatch on muscle growth and white striping (WS) breast development. In the first trial, 32 one-day-old Arbor Acre broilers were fed ad libitum (AL) for 3 d post-hatch and then randomly allotted into 4 feeding strategies including AL, 1 h-IF group (1 h IF, 4 times feeding/d, 1 h each time), 1.5 h-IF (1.5 h IF, 4 times feeding/d, 1.5 h each time), and fasting (1 d acute fasting, 6 d free access to feed) groups and fed for 7 d. Although angiogenic genes including VEGFA, VEGFR1, and VEGFR2, and myogenic genes including MYOG and MYOD were upregulated (P < 0.05), the breast muscle satellite cell (SC) number and PAX7, MYF5 expression were decreased by the IF strategies (P < 0.05). One-day fasting at 6 d of age also upregulated angiogenic genes and MYOD expression (P < 0.05), downregulated MYF5 expression (P < 0.05), but did not change SC number (P > 0.05). In the second trial, 384 one-day-old birds were fed AL for 1 wk and then randomly allotted to the above 4 feeding strategies starting at 8 d of age until 42 d of age. Similarly, IF and fasting strategies upregulated the expression of angiogenic and myogenic genes (P < 0.05). Both 1 h-IF and 1.5 h-IF increased breast muscle SC number (P < 0.05). At slaughter, breast muscle fiber diameter of 1.5 h-IF was smaller but the SC number was larger than that of the birds fed AL (P < 0.05). The IF and fasting strategies prevented WS development, and reduced breast WS scores and triglyceride content (P < 0.05) without changing the body weight (P > 0.05). Fasting and 1 h-IF reduced the expression of adipogenic genes ZNF423 and PDGFRa (P < 0.05). Moreover, IF and fasting strategies reduced fibrosis in breast muscle and reduced skeletal muscle-specific E3 ubiquitin ligases (TRIM63 and MAFBx) (P < 0.05). Fasting significantly reduced CASPASE-3 in breast muscle (P < 0.05). In conclusion, IF starting in the first week decreases SC number. Compared to AL, IF or fasting promotes muscular angiogenesis, increases SC number, prevents muscle degeneration, and prevents the development of WS without impairing the growth performance of broiler chickens.

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1. Introduction

White striping (WS) and woody breast (WB) are 2 of the major myopathies impairing chicken meat quality and causing a high economic loss in the global chicken industry (Silvo et al., 2014). The modern broiler chickens have been genetically selected to achieve an extremely high growth rate which makes their muscle fibers capable to expand more than 5 times (Dalle Zotte et al., 2017; Havenstein et al., 2003). Although the high growth rate brings economic benefits, it is associated with a higher occurrence of WS and WB (Kuttappan et al., 2012a; Lorenzi et al., 2014). Muscle development undergoes 2 distinctive processes, hyperplasia and...
hypertrophy (Halevy, 2020; Relaix et al., 2005; Smith, 1963). Hyperplasia involves the mitotic division of satellite cells (SC) during embryogenesis and rapidly terminates at the early post–hatch stage. The rapid post–hatch growth of birds is achieved by muscular hypertrophy, during which SC fuse into multinucleated myofibers to enlarge the cross-sectional diameter of muscle fibers or repair the injured muscle fibers (Halevy, 2020; Wang and RUDNICKI, 2011). The number of SC and SC-mediated breast muscle regeneration decrease in birds with greater muscle hypertrophy (Daughtry et al., 2017). When muscle fiber repair fails, WS and WB develop because of ectopic fat deposition and fibrosis, respectively (Ayansola et al., 2021; Soglia et al., 2021).

The blood vessels are important for muscle growth and regeneration. Muscle hypertrophy leads to a compromised blood supply and hypoxia (Petracci et al., 2019). Moreover, angiogenesis and myogenesis are spatiotemporally associated processes, SC reciprocally interact with endothelial to support muscle regeneration (Christov et al., 2007). Satellite cells stimulate muscular angiogenesis (Rhoads et al., 2009) and recruit capillaries to establish a juxta-vascular niche that promotes their self-renewal and maintenance (Matsakas et al., 2013). To some extent, the blood vessels determine SC ability to proliferate (Yin et al., 2015). Higher vascular density increases SC numbers and prevents myoblast dyserythry (Matsakas et al., 2013; Verma et al., 2010). Of note, WS and WB muscles are characterized by blood vessel inflammation (Papah et al., 2017; Silivo et al., 2017) and lower vessel density (Silivo et al., 2018). The poor vascularity in WS and WB muscles cannot support SC re- generations during muscle repair processes (Christov et al., 2007; Luque et al., 1995; Rhoads et al., 2009). Thus, the functioning of SC and the blood vessels are critical for preventing the development of myopathies.

To improve growth performance, ad libitum (AL) feeding is preferred in the poultry industry, however, it has been reported to be a critical factor that exacerbates muscular defects in broiler chickens (Kuttappan et al., 2012b; Petracci and Velleman, 2021) because AL feeding leads to rapid muscle hypertrophy. Compared to AL, restricted (Meloche et al., 2018) or time-limited (Livingston et al., 2018) feeding regimes reduce the incidence of WS and WB. Intermittent feeding (IF) also alleviates stress and improves broiler welfare (Arrazola et al., 2019). As shown in rodent studies, caloric restriction increases blood vessel density and basal blood flow (Lynch et al., 1999). Time-restricted feeding improves vascular endothelial function (Azemi et al., 2022). Whether IF prevents WS and WB in broilers by improving blood vessel functions needs further investigation. The biggest concern of producers for IF is the potential reduction in growth performance. However, Fondevilla et al. (2020) reported that broilers restricted from eating for less than 6 h per day showed similar body weight gain compared with those birds fed ad libitum. Likewise, FARGLY et al. (2019) demonstrated that 4 h of IF did not decrease broiler growth performance when birds were fed from 1 d old till the end of a 6-wk experimental study. The prominent argument for these results is that broilers are likely to adapt quickly during restricted feeding regimens over a prolonged period, thereby attaining full compensatory growth performance (Fondevilla et al., 2020). Generally, the first week post–hatch is critical for muscle development, starvation or feed restriction during this period decreases SC proliferation (Halevy et al., 2000) and attenuates myogenesis (Velleman et al., 2014a). However, early study indicated that feed restriction at the second week post–hatch has a mild effect on muscle development of broilers (Velleman et al., 2014a). In this study, IF and fasting protocols were applied to birds starting from 3 or 8 d post–hatch. The muscular vasculature development, SC number, muscle growth, and health were analyzed to provide an alternative feeding regime that improves muscle health without impairing the growth performance of broiler chickens.

2. Materials and methods

2.1. Animal ethics

All experimental procedures followed the guidelines approved by the China Agricultural University Laboratory animal welfare and experimental ethical committee.

2.2. Experimental design, animal management, and sample collection

The research was conducted at the poultry unit, Zhuozhou research center, China Agricultural University. For the first trial, 32 one-day-old Abor acre male broilers were fed AL for 3 d post–hatch and then randomly allotted into 4 feeding strategies including AL, 1h-IF group, 1.5h-IF, and acute fasting groups and fed for 7 d. The 1h-IF and 1.5h-IF groups were intermittently fed 4 times daily during the following periods: 07:00 to 08:00, 13:00 to 13:30, 17:30 to 18:30, 22:30 to 24:00, and 07:00 to 08:30, 12:30 to 14:00, 17:30 to 19:00, 22:30 to 24:00 respectively. For the fasting group, birds fasted for 1 d after every 6 d of AL feeding. For this trial, birds fasted at 6 d of age. All birds were slaughtered at 10 d of age. Breast muscles were sampled and snap frozen in liquid nitrogen and stored at −80 °C for RNA extraction, or processed for histological analysis.

For the second trial, a total of 384 one-day-old Abor acre male broilers were fed AL for 7 d and then randomly divided into the 4 feeding groups mentioned above. Intermittent feeding was started from 8 d of age and the first fasting was at 13 d of age. Each feeding group had 8 replicates with each containing 12 birds. Feed intake and body weight were recorded weekly. On d 21 and 42, one bird from each cage/replicate was selected based on average body weight for sample collection. The selected birds were fasted overnight and slaughtered for sample collection, including blood, thymus, gizzard, glandular stomach, spleen, bursa of Fabricius, abdominal fat, and breast muscles. The samples were stored frozen in liquid nitrogen and stored at −80 °C for real-time PCR assay or fixed in 4% paraffin solution for histological analysis. In addition, on d 42, images of sampled broiler breast fillets were taken for further visual white striping scoring in accordance to Kuttappan et al. (2012b) with adjustments such that the grading scale adopted here represented normal (0 to 1), mild (2 to 3), moderate (3 to 4), and severe scores (5), respectively.

The chicks were raised in a controlled environment. The light was provided for 24 h from d 0 to 2 and then gradually reduced to 20 h at d 8, then gradually increased to 24 h from d 35 to the end. The room temperature was periodically adjusted to ensure optimum room temperature for brooding purposes. All the birds were fed standard commercial diets during the starter and grower phases of the experiment based on the National Research Council (NRC, 1994) recommendations for broiler chickens (Table 1).

2.3. RNA extraction and real-time quantitative PCR

According to the commercial protocol (Invitrogen Life Technologies, Carlsbad, CA, USA), the tissue samples stored at −80 °C were homogenized for total mRNA isolation in Trizol reagent. Subsequently, the mRNA concentration and quality were measured with a Nano-300 spectrophotometer. The total mRNA (approximately 1,000 ng) was reverse transcribed using the Beyotime biotechnology cDNA synthesis kit. Finally, the MyqQ2 real-time PCR machine was carried out using SYBR Green Supermix from Beyotime.
using GAPDH as the reference gene. The relative expressions of the targeted genes were calculated in fold changes to the AL group (Livak and Schmittgen, 2001), while the gene primer sequences targeted genes were calculated in fold changes to the AL group using the MS Excel frequency function, respectively. Specific primer sequences for quantitative real-time PCR are provided in Table 2.

### Table 1
Composition of experimental diets and nutrient contents (DM basis, %).1

| Item                  | Day 1 to 21 | Day 22 to 42 |
|-----------------------|-------------|--------------|
| Corn                  | 60.00       | 60.00        |
| Wheat offal           | 0.00        | 0.00         |
| Soybean meal          | 27.85       | 27.85        |
| Cottonseed meal       | 2.50        | 2.50         |
| Corn gluten meal      | 2.00        | 2.00         |
| Soybean oil           | 4.00        | 4.00         |
| Lignite (98.5%)       | 2.07        | 2.07         |
| Calcium hydrogen phosphate | 1.10    | 1.10         |
| Limestone             | 1.30        | 1.30         |
| Choline (50%)         | 0.10        | 0.10         |
| Vitamin premix2       | 0.05        | 0.05         |
| Mineral supplement3   | 0.20        | 0.20         |
| Salt                  | 0.30        | 0.30         |
| Phytase               | 0.02        | 0.02         |
| Antioxidant           | 0.02        | 0.02         |
| Total                 | 100         | 100          |
| Calculated nutrient content |          |              |
| Energy AME, Mcal/kg   | 3.04        | 3.04         |
| Crude protein, %      | 18.99       | 18.99        |
| Lysine, %             | 1.48        | 1.48         |
| Methionine, %         | 0.48        | 0.48         |
| Ca, %                 | 0.84        | 0.84         |
| Available phosphorus  | 0.39        | 0.33         |

1 The feeds were formulated based on the recommendation by the NRC (1994b).
2 Vitamin premix provided the following nutrients per kilogram of the diet: vitamin A, 9,500 IU; vitamin D3, 625 µg; vitamin E, 30 IU; vitamin K3, 2.65 mg; vitamin B1, 2 mg; vitamin B2, 2 mg; vitamin B6, 0.025 mg; biotin, 0.0325 mg; folic acid, 1.25 mg; panthenic acid, 12 mg; nicotinic acid, 50 mg.
3 Mineral premix provided the following nutrients per kilogram of the diet: Cu, 8 mg; Zn, 2.25 mg; Fe, 80 mg; Mn, 100 mg; I, 0.35 mg (KI).

### Table 2
A list of primer sequences for quantitative real-time PCR.

| Genes       | Primer ID   | Primer Sequence (5' → 3') |
|-------------|-------------|---------------------------|
| MYOD        | NM_204214.2 | F: CACGGAATCCACACATGGACCA |
|             |             | R: GACGCTGGGCTGAGGAAGAAAT |
| MYF5        | NM_204184.1 | F: AGTAGGAGGCATGCAAGACCG |
|             |             | R: GGAGGTGTCATCCTTCTTCCTA |
| MYOG        | NM_204184.1 | F: ACCAACACTCTGGCAAGACCA |
|             |             | R: TCCACATGAGACAGGACAGGAA |
| PAX7        | NM_205066.1 | F: TCAGTACGGCGGAGACAGGAGA |
|             |             | R: TGACGACCTGACCTGATTTTTC |
| VEGF        | NM_00110355.2 | F: CCGGAGATTGCTGAGTTGAAAGT |
|             |             | R: TGACGACCTGACCTGATTTTTC |
| HIF1A       | NM_204297.1 | F: AGTTCACTTGGAGCCCCAGCAG |
|             |             | R: AGGACACCAAAATTCCTCAAG |
| EGF         | NM_00100129.1 | F: CCTCTTTTGCTTTCTGAGCAGT |
|             |             | R: GGAATGGCTGAGGCGATTTTAC |
| FGF2        | NM_205433.2 | F: AGGGGCTTCCTTGGCAAGACG |
|             |             | R: CTCCTTGACATGATCCTGGC |
| VEGFR1      | NM_204252.1 | F: TTTCCTTTGGGGCCTCCCTCC |
|             |             | R: CTCTGGCTCTTGCTGAGAGG |
| VEGFR2      | NM_001004368 | F: CTATCCCCCAGGAAGAAGCAG |
|             |             | R: GATACGCGACCAGCCAGGACCA |
| FABP4       | NM_204290.1 | F: ATAGAGAGACGCTGGGCTGGGG |
|             |             | R: CTGCTCAGCTGGTCCACCTGGT |
| CEBPb       | NM_001031459.1 | F: TGGCGGACATCTGGAAAGAC |
|             |             | R: CTGCTATGCTGGAGAAGTCC |
| CEBP5       | NM_205253.2 | F: CAGACGCGGCGCCAGT |
|             |             | R: TCTGACAGCCGCTCACCTTC |
| ZNF423      | XM_025154318.1 | F: CCACAGCAGTCTCCAAAGTGT |
|             |             | R: CTCACCCGCCCACCTATGAT |
| PDGFRa      | NM_204749.2 | F: GGCGCTTATGAAACGAGGAGT |
|             |             | R: GCCACCTTGGTCTTCCG |
| PPARY       | XM_01529291.2 | F: AGGAGAACATTTTCCCCTGGT |
|             |             | R: CTCCATTTGAGCTCCTTGTC |
| GAPDH       | NM_204305.1 | F: TGACGCTGCAGAGGACACATA |
|             |             | R: GGCAGGATACGACCCAAACATC |

1 MYOD — myogenic differentiation; MYF5 — myogenic factor 5; MYOG — myogenin; PAX7 — paired box 7; VEGF — vascular endothelial growth factor A; HIF1A — hypoxia inducible factor 1 subunit alpha; EGF — epidermal growth factor; FGF2 — fibroblast growth factor 2; PAX7 — paired box 7; VEGFR1 — soluble vascular endothelial growth factor receptor-1; VEGFR2 — soluble vascular endothelial growth factor receptor-2; FABP4 — fatty acid binding protein 4; CEBP — peroxisome proliferator-activated receptor gamma; CEBPb — CCAAT/enhancer binding protein beta; ZNF423 — zinc finger protein 423; PDGFRa — platelet-derived growth factor receptor, alpha polypeptide; PPARY — peroxisome proliferator-activated receptor gamma; GAPDH — glyceraldehyde-3-phosphate dehydrogenase.

### 2.4. Histomorphology, immunohistochemistry, and immunofluorescence staining

The fixed breast muscles were subsequently embedded in paraffin wax, cut at 5 µm using a Leica microtome, then mounted on slides. Next, the mounted tissue slides were dewaxed, dehydrated, and stained with Hematoxylin and eosin (H&E) staining (histology composition and structure image) (He et al., 2019) or in Picrosirus red solution for collagen fiber evaluations (Sanden et al., 2021), then dehydrated, and cleared in xylene. Subsequently, the breast muscle images were quantified using Imagej software, to measure the fiber diameter sizes and their distribution percentages were quantified with MS Excel frequency function, respectively. Specifically, all muscle fibers were measured from 8 sectioned breast muscle samples per group. At least, we recorded above 1,500 muscle fiber counts for each sectioned sample to ensure a good representation of different muscle fiber diameters in the frequency percentage data.

For immunohistochemistry, fixed breast muscles were infiltrated with 30% sucrose for 8 h, then embedded with Tissue-Tek Optimal Cutting Temperature Compound and frozen in isopentane cooled in liquid nitrogen. Cryo-sections of 10-µm-thick were rinsed with PBS, then heated in citrate buffer (10 mmol/L citric acid, 0.05% Tween 20, pH 6.0) at 95 °C for 20 min, blocked with 5% goat serum in TBS containing 0.3% Triton X-100 for 2 h, then incubated with anti-CD31 (Cat No: ab119339, Abcam, USA) or anti-PAX7 (1:40, Developmental Studies Hybridoma Bank, Iowa City, IA, USA) antibodies overnight at 4 °C and then incubated with a goat anti-mouse antibody (Cat No: A5008, Abclonal, Wuhan, China) at room temperature for 1 h. Finally, sections were mounted in a fluoroshield mounting medium with DAPI (Cat No: C1002, Beyotime Biotechnology, China), then immediately viewed using an EVOS fluorescence microscope (ECHO model: RVL-100-G).

### 2.5. Triglyceride assay

The triglyceride (TG) contents in the frozen breast samples were used as a marker to measure ectopic fat deposits in the chicken breast using the Folch extraction method (Folch et al., 1957). In summary, the breast muscle tissue samples (approximately 50 mg) were homogenized in 2:1 chloroform-methanol solution, centrifuged at 5,000 × g, 4 °C for 10 min, washed in 0.9% NaCl solution, then centrifuged again at low speed (400 × g) to separate into 2 phases: upper salt and methanol layer, while the lower phase contained the chloroform, respectively. The chloroform layer containing the lipid content was freeze-dried, resuspended in ACS-graded 2-propanol, followed by absorbance reading at 540 nm using a Dongou TG reagent kit (Cat No: A0-10017).
2.6. Protein expression using the Western blotting technique

The protein content in the breast muscle was quantified according to a Western blot protocol we previously described (Wang et al., 2021) with minor modifications. Briefly, the protein was digested using a lysis buffer containing radio immunoprecipitation assay, protease, and phosphatase inhibitors. The extracted protein lysate was separated by 10% SDS PAGE and electrophorosed onto the nitrocellulose membrane. This was followed by blocking in 5% Milkfat: TBST solution for an hour and subsequently incubated in primary antibodies at 4 °C overnight, including β-tubulin (Cat No: AF1216), NF-KB (AF5243), and CASPASE-3 (Cat No: AC030-1), purchased from Beyotime Biotechnology, while TRIM63 (Cat No: A3101), and FBXO32 (Cat No: A3193) were purchased from ABclonal Biotechnology. The membrane was washed with TBST buffer (137 mmol/L sodium chloride, 2.7 mmol/L potassium chloride, 50 mmol/L Tris–HCl, and 0.1% Tween 20) before being incubated with horseradish peroxidase-labeled secondary antibody (1:5,000, Shanghai Beyotime, Shanghai, China). The blotting bands were developed using a Super Enhanced Chemiluminescence (Shanghai Beyotime, Shanghai, China) and quantified using ImageJ software (NIH, USA).

2.7. Data analysis

The data were analyzed by one-way ANOVA tool of SPSS v20.0 software. Results are presented as means and standard error of the
mean. Duncan’s test compared the statistical differences among individual means at \( P < 0.05 \) as the significance level. The graphical representations were designed using GraphPad Prism 8.0.2 and MS Excel software packages.

3. Results

3.1. Intermittent feeding and fasting started at 3 d of age promoted angiogenesis and reduced satellite cell numbers in the broiler pectoralis major muscle

In the first trial, IF started at 3 d of age and one-day fasting was performed at 6 d of age. Compared to AL, 1h-IF upregulated angiogenic genes including \( VEGFA \) and \( VEGFR1 \), whereas the fasting treatment upregulated angiogenic genes including \( VEGFR2 \) and \( EGF \) (Fig. 1A, \( P < 0.05 \)). Consistently, all IF and fasting strategies increased vascular density in the pectoralis major muscle (Fig. 1B). Moreover, IF and fasting treatments upregulated the expression of \( MYOD \), a regulator of myocyte fusion (Ganassi et al., 2018) and the 1h-IF treatment upregulated the expression of another myogenic regulatory factor (MRF), \( MYOG \) (Fig. 1C, \( P < 0.05 \)). However, all IF and fasting strategies reduced the expressions of \( PAX7 \), the SC marker, and \( MYF5 \), another MRF (Hernandez–Hernandez et al., 2017) (Fig. 1D, \( P < 0.05 \)). Consistently, 1h-IF and 1.5h-IF reduced SC numbers in the broiler pectoralis major muscle (Fig. 1E, \( P < 0.05 \)).

![Fig. 2](image-url) Intermittent feeding and fasting started at 8 d of age promoted angiogenesis and increased satellite cell numbers in broiler breast muscle. Intermittent feeding or fasting was applied on broiler chickens from 8 d of age, the birds were slaughtered and pectoralis major muscle was sampled at 21 d of age (n = 8). (A) \( VEGFA \), \( VEGFR1 \), \( VEGFR2 \), \( EGF \), \( HIF1A \), and \( FGF2 \) mRNA levels in the pectoralis major muscle. (B) Representative immunofluorescence images of pectoralis major muscle using an anti-CD31 antibody. (C) \( PAX7 \), \( MYF5 \), \( MYOD \), and \( MYOG \) mRNA levels in the pectoralis major muscle. (D) Representative immunofluorescence images of pectoralis major muscle using an anti-PAX7 antibody. (E) Quantification of \( PAX7 \)–satellite cells in pectoralis major muscle. AL = ad libitum; 1h-IF = 1 h intermittent feeding, 4 times feeding/d, 1 h each time; 1.5h-IF = 1.5 h intermittent feeding, 4 times feeding/d, 1.5 h each time; Fasting = 1 d acute fasting, 6 d free access to feed. Data are presented as means ± SEM. Means without a common letter differ significantly, \( P < 0.05 \). Scale bar = 100 μm. \( VEGFA \) = vascular endothelial growth factor A; \( VEGFR \) = soluble vascular endothelial growth factor receptor; \( EGF \) = epidermal growth factor; \( HIF1A \) = hypoxia inducible factor 1 subunit alpha; \( FGF2 \) = fibroblast growth factor 2; \( PAX7 \) = paired box 7; \( MYF5 \) = myogenic factor 5; \( MYOD \) = myogenic differentiation; \( MYOG \) = myogenin; CD31 = platelet and endothelial cell adhesion molecule 1; PAX7 = paired box protein Pax-7.
3.2. Effects of intermittent feeding and fasting starting at 8 d of age on vascular development, satellite cell numbers, and muscle growth of broiler pectoralis major muscle

In the second trial, IF was started at 8 d of age and weekly fasting was started at 13 d of age. On 21 d of age, IF upregulated VEGFR1, VEGFR2, and HIF1A (Fig. 2A, \( P < 0.05 \)), and all IF and fasting strategies increased vascular density in the pectoralis major muscle (Fig. 2B). Moreover, 1.5h-IF upregulated PAX7, MYF5, and MYOD (Fig. 2C, \( P < 0.05 \)). In addition, IF and fasting strategies increased SC numbers in breast muscles compared with that of the AL group (Fig. 2D and E, \( P < 0.05 \)). On 42 d of age, the breast muscle fiber diameters of the 1.5h-IF treated birds were smaller (Fig. 3A–C, \( P < 0.05 \)) and the SC number was larger than that of the AL (Fig. 3D and E, \( P < 0.05 \)).

3.3. Effects of intermittent feeding and fasting starting at 8 d of age on white striping development and ectopic fat accumulation in broiler breast muscle

The IF and fasting strategies applied in the second trial visually prevented the development of WS (Fig. 4A) and reduced WS scores (Fig. 4B, \( P < 0.05 \)). Consistently, both IF and fasting strategies reduced TG content in the broiler pectoralis major muscle (Fig. 4C, \( P < 0.05 \)). In addition, compared with AL, the expression of adipogenic genes ZNF423 and PDGFRα in the pectoralis major muscle of 1h-IF and fasting groups was lower (Fig. 4D, \( P < 0.05 \)).

3.4. Intermittent feeding and fasting alleviated muscle degradation and myofibrosis

Picrosirius red staining showed that both IF and fasting strategies alleviated collagen accretion compared to AL feeding (Fig. 5A and B, \( P < 0.05 \)). IF and fasting strategies reduced skeletal muscle-specific E3 ubiquitin ligases TRIM63 and MAFBX (Fig. 5C–E, \( P < 0.05 \)), which indicates lower muscle degeneration. Moreover, IF and fasting strategies reduced the content of apoptotic protein CASPASE-3 in the broiler pectoralis major muscle (Fig. 5C and F, \( P < 0.05 \)). We also analyzed the inflammatory protein NFκB but no difference was detected (Fig. 5C and G, \( P < 0.05 \)).

3.5. Intermittent feeding and fasting on growth performance of broiler chickens

Unsurprisingly, intermittent and fasting groups decreased the average daily feed intake consumed during wk 2 to 5 compared to AL group (Table 3, \( P < 0.05 \)). Despite that, none of the alternative feeding strategies impaired the growth (Table 4, \( P > 0.05 \)) or feed conversion ratio (Table 5, \( P > 0.05 \)) of birds compared to AL feeding. As depicted in Tables 4 and 5, it is apparent that despite the slowdown in the weight gain values between wk 5 and 6, the birds consistently increased in body weight till the end of the experiment (Table 4). Specifically, Table 5 shows that the 1.5h-IF birds also gained above 500 g during wk 5 to 6 compared with the AL group.

Foremost, no differences were observed in the weight of tissues or organs, including thymus, gizzard, glandular stomach, spleen,
and bursa of Fabricius at 21 or 42 d old (Tables 6 and 7, \( P > 0.05 \)). However, 1h-IF and fasting reduced abdominal fat of 21-d-old birds (Table 6, \( P < 0.05 \)). While the breast muscle mass of 1h-IF (157.85 g), 15h-IF (148.65 g), and fasting birds (148.41 g) were smaller numerically than that of the AL birds (167.15 g) on day 21, respectively, there were no differences on day 42 samples of the breast muscles (Table 7, \( P > 0.05 \)).

4. Discussion

The occurrence of myopathies is positively associated with the growth rate or body size of broiler chickens (Kuttappan et al., 2012a, 2013). Post-hatch skeletal muscle growth is achieved through muscle fiber hypertrophy, a process that SC fuse into existing muscle fibers (Velleman et al., 2014a; White et al., 2010). Satellite cells have the highest mitotic activity immediately after hatch (Velleman et al., 2014a). Early post-hatch starvation decreases SC proliferation (Haley et al., 2000). We observed that restrictions on the timing of access to feed in the first week post-hatch decreased the SC number but IF or fasting started on the second week post-hatch increased the SC number. Whether the increase in SC number is because of enhanced proliferation or retarded muscle growth is unknown. Although the IF and Fasting started on the second week post-hatch did not reduce growth performance, the muscle fiber size of the IF or fasted birds were smaller than that of the control. Because for most animals the number of muscle fibers is fixed before birth (Albrecht et al., 2006; Du et al., 2010), we do not expect more muscle fibers in the IF or fasted birds. Nevertheless, the slower increase in muscle fiber size may have contributed to WS prevention.

The enlargement of muscle fibers reduces the vascular density within the muscular region (Joiner et al., 2014). The compromised blood supply, hypoxia, and oxidative damage that occur during muscle hypertrophy are major causes of muscle degeneration (Petracci et al., 2019). Whole transcriptome analysis also revealed that insufficient capillary blood supply triggered hypoxia facilitates tissue damage and WS incidence (Marchesi et al., 2019). Thus, blood vessel development is crucial to the function and health of high-speed growing muscle. Intermittent fasting is well known to benefit human health. As reported, intermittent fasting promotes angiogenesis in different tissues (Katare et al., 2009; Kim et al., 2017; Mattson and Wan, 2005). In the current study, IF and fasting also upregulated angiogenic genes including VEGF, VEGFR1, VEGFR2, EGF, and HIF1A, and increased capillary blood vessel density in broiler breast muscle. Other than alleviating hypoxia, the increased blood vessels may have also contributed to the maintenance of SC number and its myogenic potential, promoting muscle fiber regeneration and preventing the occurrence of WS. It is interesting that, although both IF and fasting strategies started on the first and the second week post-hatch promoted angiogenesis in broiler breast muscle, the SC numbers were decreased by these alternative feeding regimes applied earlier but increased by the same regimes applied at the later stage. This indicates that nutrient supply played a major role in SC proliferation in the first week post-hatch. For birds applied to IF or fasting on the second week post-hatch, no matter whether it is because of enhanced proliferation or retarded muscle growth, the increased SC reserve can support the compensatory growth in the later stage (Haley et al., 2000) and contribute for muscle fiber fixation or maintenance together with the improved blood vessel system.

Some studies showed that altered nutrition induces transdifferentiation of SC into adipocytes (Powell et al., 2014; Velleman et al., 2014b). Velleman showed that 20% feed restriction in the first week post-hatch increased the expression of PPARγ and C/EBPδ in broiler breast muscle (Velleman et al., 2014b). In the current study, 1h-IF and fasting started on the second week post-hatch reduced the expression of ZNF423 and PDGFRα, which is consistent with the reduced TG content and WS scores. Moreover, 1h-IF started on the second week post-hatch significantly reduced the abdominal fat of 21-d-old birds, which could improve the economic benefit.

In conclusion, IF and fasting strategies in the first week post-hatch reduce SC number and may impair broiler growth; IF and fasting strategies starting in the second week post-hatch
Fig. 5. Intermittent feeding and fasting alleviated muscle degradation and myofibrosis. Intermittent feeding or fasting was applied on broiler chickens from 8 d of age, the birds were slaughtered and pectoralis major muscle was sampled at 42 d of age (n = 8). (A) Representative images of Picro-Sirius red stained pectoralis major muscle sections. (B) Quantification of collagen area in pectoralis major muscle. (C) Immunoblotting bands of NFκB, MAFBX, TRIM63, and β-tubulin of pectoralis major muscle lysate. (D-G) Quantifications of band intensity of NFκB, MAFBX, TRIM63, and CASPASE-3 normalized to β-tubulin. AL = ad libitum; 1h-IF = 1 h intermittent feeding, 4 times feeding/d, 1 h each time; 1.5h-IF = 1.5 h intermittent feeding, 4 times feeding/d, 1.5 h each time; Fasting = 1 d acute fasting, 6 d free access to feed. Data are presented as means ± SEM. Means without a common letter differ significantly, P < 0.05. Scale bar = 200 μm. NFκB = nuclear factor kappa-B; MAFBX = Muscle Atrophy F-box protein; TRIM63 = tripartite motif-containing protein 63; CASPASE-3 = caspase 3.

Table 3

| Time  | AL     | 1h-IF  | 1.5h-IF | Fasting | SEM  | P-value |
|-------|--------|--------|---------|---------|------|---------|
| Wk 1  | 20.32  | 19.68  | 20.12   | 20.19   | 0.26 | 0.843   |
| Wk 2  | 52.66a | 45.22b | 45.14b  | 51.49a  | 0.82 | <0.001  |
| Wk 3  | 79.35a | 74.19b | 75.04b  | 73.34b  | 0.78 | 0.028   |
| Wk 4  | 124.75a| 112.56b| 111.04b | 110.18b | 1.46 | <0.001  |
| Wk 5  | 158.70b| 149.63b| 140.02b | 138.68b | 1.89 | 0.001   |
| Wk 6  | 159.15b| 146.33  | 152.19  | 150.14  | 2.27 | 0.231   |

AL = ad libitum; 1h-IF = 1 h intermittent feeding, 4 times feeding/d, 1 h each time; 1.5h-IF = 1.5 h intermittent feeding, 4 times feeding/d, 1.5 h each time; Fasting = 1 d acute fasting, 6 d free access to feed. Within a row, means without a common letter differ significantly (P < 0.05, n = 8, mean ± SEM).

Table 4

| Time  | AL     | 1h-IF  | 1.5h-IF | Fasting | SEM  | P-value |
|-------|--------|--------|---------|---------|------|---------|
| Initial | 41.80  | 41.32  | 41.76   | 41.05   | 0.16 | 0.343   |
| Wk 1  | 114.91  | 116.13 | 115.18  | 112.99  | 0.67 | 0.451   |
| Wk 2  | 252.30  | 227.86 | 231.38  | 250.17  | 4.01 | 0.051   |
| Wk 3  | 335.82  | 306.56 | 299.98  | 288.57  | 7.92 | 0.186   |
| Wk 4  | 614.66  | 590.07 | 604.31  | 542.24  | 12.37 | 0.193 |
| Wk 5  | 678.10  | 648.52 | 616.51  | 598.29  | 12.40 | 0.107 |
| Wk 6  | 570.13  | 492.18 | 516.41  | 477.91  | 13.99 | 0.091 |

AL = ad libitum; 1h-IF = 1 h intermittent feeding, 4 times feeding/d, 1 h each time; 1.5h-IF = 1.5 h intermittent feeding, 4 times feeding/d, 1.5 h each time; Fasting = 1 d acute fasting, 6 d free access to feed.
Intermittent feeding and fasting effects on carcass and organs of broiler chickens at 21 d of age (g/kg BW).

| Items            | AL   | 1h-IF | 1.5h-IF | Fasting | SEM  | P-value |
|------------------|------|-------|---------|---------|------|---------|
| Thymus           | 1.2  | 1.13  | 1.18    | 1.12    | 0.05 | 0.938   |
| Gizzard          | 15.91| 15.63 | 14.61   | 16.07   | 0.28 | 0.266   |
| Glandular stomach| 5.07 | 4.36  | 4.67    | 4.87    | 0.13 | 0.219   |
| Abdominal fat    | 10.10| 7.08  | 10.47   | 7.64    | 0.49 | 0.017   |
| Spleen           | 1.7  | 1.4   | 1.49    | 1.23    | 0.08 | 0.17    |
| Bursa of Fabricus| 1.1  | 1.15  | 1.13    | 0.99    | 0.05 | 0.747   |
| Breast muscle    | 167.15| 157.85| 148.65  | 148.41  | 3.15 | 0.132   |

Table 6

| Items            | AL   | 1h-IF | 1.5h-IF | Fasting | SEM  | P-value |
|------------------|------|-------|---------|---------|------|---------|
| Thymus           | 1.27 | 1.48  | 1.36    | 1.37    | 0.06 | 0.643   |
| Gizzard          | 1.06 | 1.24  | 1.21    | 1.2     | 0.05 | 0.453   |
| Abdominal fat    | 10.27| 11.04 | 9.99    | 9.44    | 0.25 | 0.152   |
| Glandular stomach| 14.07| 12.73| 10.38   | 11.52   | 0.6  | 0.145   |
| Spleen           | 0.45 | 0.49  | 0.48    | 0.42    | 0.02 | 0.726   |
| Breast muscle    | 212.15| 195.23| 212.12  | 195.05  | 4.04 | 0.241   |

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