



Studies on the nutritional regulation of protein metabolism-related gene expression in chicken skeletal muscles

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Doctoral Dissertation

Studies on the nutritional regulation of protein metabolism-related gene expression in chicken skeletal muscles

(ニワトリ骨格筋におけるタンパク質代謝関連遺伝子発現の栄養学的制御に関する研究)

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Chapter 1

General Introduction

Chicken meat is an affordable, nutritious protein source that is gaining popularity worldwide, especially among budget-conscious consumers (Farrell, 2013; Mickiewicz, 2022). To meet the global challenges associated with food security, sustainability, and environmental change, strategies are needed to decrease nitrogen excretion and improve nitrogen retention in chicken meat production (Méda et al., 2011). Given that nitrogen excretion increases linearly with nitrogen intake, chicken rations with lower crude protein (CP) levels have been investigated (Liu et al., 2021). However, to further develop this nutritional strategy, the regulatory mechanisms of protein metabolism in chickens need to be elucidated in detail.

Genetic selection has had a profound impact on muscle growth rate and meat yield, leading to significant differences in the expressed genes in skeletal muscles of broiler chickens compared with those of layer chickens. For instance, the body weight of 21-day-old broilers was 4-fold higher than that of layer chickens (Willson et al., 2018). The breast muscle yield of broilers was approximately 1.5-fold higher than that of layers (Scheuermann et al., 2004). Moreover, broiler myoblasts proliferated faster under growth conditions and differentiated more rapidly into myotubes than layer myoblasts *in vitro* (Nihashi et al., 2019). Several muscle development and protein metabolism-related genes were differentially expressed between broiler and layer chicks, such as the ubiquitin-related protein degradation genes, showing lower expression levels in the pectoralis major (PM) muscle of broilers than in the PM of layers at 2 and 4 weeks of age (Zheng et al., 2009). The mRNA levels of insulin-like growth factor (IGF)-1 in the PM decreased by fasting in layer chicks, but not in broiler chicks, at 14 days of age (Saneyasu et al., 2015a). Therefore, broilers and layers have often been used as models for studying the regulatory mechanisms of skeletal muscle growth in

chickens.

The breast and thigh muscles, namely the PM and the biceps femoris (BF), constitute a large portion of the chicken's body and exhibit distinct properties (Lee et al., 2023; Zuidhof et al., 2014). Notably, the PM and BF muscles grow and respond differently to the variation of dietary essential amino acids (AAs) in broiler chickens. For example, the interaction effects of lysine and methionine were significant for the PM muscle, whereas the interaction effects of lysine and threonine were significant for the BF muscle (Mehri et al., 2016). Dietary supplementation of L-arginine suppressed protein synthesis in the PM but not in the BF of broiler chicks (Wang et al., 2022b). In addition, the protein metabolism-related genes in the PM and BF showed different responses to hormones (Dong et al., 2007; Wang et al., 2022a) and feeding status (Hu et al., 2016). Hence, the PM and BF of broilers and layers may be effective models for studying the regulatory mechanisms of protein metabolism in chickens.

Skeletal muscle mass increases when the overall rates of protein synthesis exceed the rates of protein degradation (Schiaffino et al., 2013). This process is regulated by circulating hormones such as IGF-1, insulin, and glucocorticoids (Glass, 2005; Millward, 2023; Schiaffino et al., 2013). Furthermore, evidence suggests that skeletal muscle-derived IGF-1 and IGF binding protein (IGFBP) play crucial roles in regulating muscle growth and regeneration in mammals. For example, Martín et al. (2021) proposed that skeletal muscle IGF-1 and IGFBP-3 are important for growth and muscle maintenance; the reduction of IGFBP-3 secretion using antisense oligonucleotides inhibits human myoblast differentiation (Foulstone et al., 2003), and the IGFBP-2 antibody inhibits C2C12 myoblast differentiation (Sharples et al., 2013). Silverman et al. (1995) proposed that skeletal muscle IGFBP-4 inhibits IGF-1 action on myogenesis. Additionally, antisense IGFBP-5 gene expression or knockdown of IGFBP-5 by siRNA in myoblasts influences myotube formation *in vitro* (James et al., 1996; Ren

et al., 2008). In chickens, Wang et al. (2019) found that the microRNA miR-34b-5p can repress the proliferation and promote the differentiation of chicken myoblasts by targeting IGFBP-2, and IGFBP-3 knockdown prevents myotube formation in chicken myoblasts (Guo et al., 2023). It is possible, therefore, that skeletal muscle IGF-related gene expression regulates muscle mass in chickens, similar to that in mammals.

Moreover, nutrients regulate the expression of IGF-1 and IGFBPs in skeletal muscles in vertebrates. For example, food deprivation decreased IGF-1 mRNA levels in the skeletal muscles of mice (Abe et al., 2019) and gopher rockfish (Bersin et al., 2023). High AA supplementation increased IGF-1 mRNA levels in rat L6 myotubes *in vitro* (Iresjö et al., 2022). In the skeletal muscles of fish during fasting, the expression levels of IGFBP-2, IGFBP-4, and IGFBP-5 are downregulated, whereas the expression of IGFBP-3 is upregulated (Safian et al., 2012). The addition of AAs to starved myotubes increased IGF-1 and IGFBP-5 mRNA levels in cultured muscle cells from Atlantic salmon (Bower and Johnston, 2010). In chicken, the mRNA levels of IGF-1 in skeletal muscles were decreased by fasting (Guernec et al., 2004) and increased by methionine supplementation (Wen et al., 2014). The mRNA levels of IGFBP-2 in the skeletal muscles of chickens were increased by dietary leucine (Zeitz et al., 2019) and by increasing the nutrient density (CP and energy) of the feed (Lu et al., 2009). Thus, the expression of IGF-1 and IGFBPs in skeletal muscles may be regulated by dietary nutrients and affect the growth of skeletal muscle mass in chickens.

The present study was conducted to characterize the gene expression of protein metabolism-related genes, including IGFBPs, in response to fasting and low-protein (LP) feeding in the skeletal muscles of broiler and layer chicks. Results suggest that skeletal muscle IGFBPs play physiological roles in response to fasting, but play minor roles in response to protein deficiency in chickens.

Chapter 2

Fasting Induces Gene Expression of Insulin-like Growth Factor-binding Proteins in Skeletal Muscles of Chicks

Introduction

Chickens are regarded as a model avian species in many aspects (Byers et al., 2017; Tesseraud et al., 2007; Vancamp and Darras, 2018). Furthermore, two major commercial chickens, broilers and layers, have been established due to long-term genetic selection and show differences in muscle development (Buzala and Janicki, 2016). For example, the breast muscle yield of broilers is approximately 1.5-fold higher than that of layer chicken (Scheuermann et al., 2004). Lee et al. (2023) have reported greater number and size of muscle bundles in the breast and leg muscles of broilers than in those of layer chickens. Therefore, comparing the responsiveness of broiler and layer chickens to dietary nutrients may provide valuable insights into the avian physiology of commercial chickens.

Breast and thigh muscles account for a large portion of the body mass of chickens (Zuidhof et al., 2014). The pectoralis major (PM; breast muscle) and biceps femoris (BF; largest skeletal muscle in the thigh) exhibit distinct muscle properties. For instance, dietary supplementation with L-arginine suppresses protein synthesis in the PM of broiler chicks, but not in the BF (Wang et al., 2022b). Another study has shown that satellite cells derived from the PM and BF of 5-week-old broiler chickens have different levels of responsiveness to serum mitogens (McFarland et al., 1997). Hu et al. (2016) have demonstrated a difference in insulin sensitivity between the PM and BF in 7-day-old broiler chicks. Thus, a comparison of the PM and BF in chickens may

provide new insights into the specificity of skeletal muscle physiology in chickens, which, in turn, will benefit the poultry industry.

Insulin-like growth factor-binding proteins (IGFBPs) function as carrier proteins for insulin-like growth factor-1 (IGF-1) in the bloodstream in vertebrates (Shimizu and Dickhoff, 2017). Most circulating IGF-1 binds IGFBPs that regulate IGF activity in mammals (Allard and Duan, 2018). In addition, *in vivo* and *in vitro* studies have shown that locally expressed IGFBPs affect skeletal muscle development in mammals. For instance, the IGFBP-2 antibody inhibits C2C12 myoblast differentiation (Sharples et al., 2013) and reduction of IGFBP-3 secretion using antisense oligonucleotides inhibits human myoblast differentiation (Foulstone et al., 2003). Positive effects of IGFBP-4 have also been reported during muscle hypertrophy (Awede et al., 2002), and knockdown of IGFBP-5 impairs myogenic differentiation and inhibits myotube formation in C2C12 cells (Ren et al., 2008). In chickens, the microRNA miR-34b-5p may repress proliferation and promote the differentiation of chicken myoblasts by targeting IGFBP-2 (Wang et al., 2019). IGFBP-3 knockdown prevents myotube formation in chicken myoblasts, suggesting that IGFBP-3 promotes the differentiation of chicken myoblasts into skeletal muscle (Guo et al., 2023). Moreover, transcriptional regulation of IGFBPs in skeletal muscles by feeding status has been reported in fish (Bersin et al., 2023; Bower et al., 2008; Safian et al., 2012; Strobel et al., 2020) and mammals (Chen and Arnqvist, 1994; Jagoe et al., 2002). These findings suggest that skeletal muscle IGFBPs play important physiological roles in vertebrate muscle growth.

In chickens, IGF-1 is the primary hormone that promotes skeletal muscle growth. For instance, continuous infusion of IGF-1 for 2 weeks significantly increases the growth rate of broiler chicks (Tomas et al., 1998) and treatment with IGF-1 promotes the proliferation of chicken embryonic myoblasts (Yu et al., 2015). Previous *in vitro* studies suggest that treatment with IGF-1 suppresses proteolytic pathways in chicken

myotubes (Nakashima et al., 2017; Nakashima and Ishida, 2018). Subcutaneously administered IGF-1 affects IGFBP-3 expression in the gastrocnemius of rats (López-Menduiña et al., 2012) and intraperitoneal administration of IGF-1 increases IGFBP-1 expression in the skeletal muscle of rockfish (Bersin et al., 2023). In addition, subcutaneous IGF-1 administration shows different effects on mRNA levels of muscle ring finger 1 between the gastrocnemius and soleus in rats (López-Menduiña et al., 2010) and on the proportion of Type I muscle fibers between the extensor digitorum longus and soleus in mice (Lynch et al., 2001). Therefore, it is possible that IGF-1 has different effects on IGFBP expression in different skeletal muscle types. Although the effects of IGF-1 on the gene expression of skeletal muscle IGFBPs have not been investigated in chickens, IGF-1 regulates IGFBP-2 gene expression in the ovarian granulosa cells of chicken follicles (Hu et al., 2024) and ovarian cells (Ahmadi and Ohkubo, 2022). Hence, IGF-1 may regulate the gene expression of IGFBPs in chicken skeletal muscles.

The present study was conducted to characterize the gene expression of IGFBPs in response to fasting in the skeletal muscles of chicks and to determine the influence of IGF-1 on chicken embryonic myotubes. Our findings suggested that several IGFBPs play physiological roles in chicken skeletal muscle growth.

Materials and Methods

Animals and tissue collection

This study was approved by the Institutional Animal Care and Use Committee (2021-11-02) and performed according to the Kobe University Animal Experimentation Regulations. One-day-old male layer (White leghorn) and broiler (Ross 308) chicks were purchased from local hatcheries (Japan Layer K. K., Gifu, Japan and Yamamoto

Co., Ltd., Kyoto, Japan, respectively). Chicks were reared in electrically heated battery cages. The temperature was kept at $31 \pm 2^{\circ}\text{C}$ during the first 7 days, and then reduced gradually according to age until reaching $25 \pm 2^{\circ}\text{C}$ at 21 days. Chicks were given free access to water and a commercial chick starter diet (NICHIIWA SANGYO Co., Ltd., Kobe, Japan) under a 23-h/1-h light/dark cycle—a light schedule commonly used in the poultry industry. The commercial chick starter diet met the nutritional requirements of both broiler and layer chicks.

Sixteen 21-one-day-old male layer or broiler chicks were weighed and allocated to two groups based on body weight (eight birds in each group). At 21 days of age, the chicks in the feeding group were euthanized by decapitation by a skilled person after 0 h of fasting. Chicks in the fasting group were euthanized by decapitation after 12 h of fasting. The central pieces of the PM and BF were excised and preserved in RNAlater® tissue storage reagent (Sigma-Aldrich Co., St. Louis, MO, USA). Muscles were stored at -80°C prior to total RNA extraction for gene expression analysis.

Myoblast culture and in vitro treatment

Transcriptional changes may not be induced by a short incubation time, whereas a long incubation time may induce not only a direct effect of IGF-1, but also a secondary effect of IGF-1-induced nutritional changes in primary cultures of chicken embryonic myotubes (CEM). There is evidence that IGF-1, insulin, and amino acids induce transcriptional changes in CEM after 3 h of incubation *in vitro* (Nakashima and Ishida, 2018). Therefore, a 3 h incubation time was chosen to examine the effect of IGF-1 on mRNA levels of IGF-related genes. Fertilized layer and broiler eggs were purchased from a commercial source and incubated at 37.5°C under 60%–70% relative humidity with rotation every 1 h until embryonic myoblasts were isolated from the excised breast or thigh muscles of 14-day-old embryos. After washing with phosphate-

buffered saline (PBS), muscle samples were minced using surgical scissors and digested with Hank's balanced salt solution (+) (Nacalai Tesque, Inc., Kyoto, Japan) containing 0.2% collagenase (Worthington Biochemical Corp., Lakewood, NJ, USA) for 20 min at 37 °C. Cells were collected by centrifugation and resuspended in a growth medium consisting of Dulbecco's modified Eagle's medium (DMEM, Nacalai Tesque) supplemented with 15% FetalClone III (GE Healthcare Bio-Sciences AB, Uppsala, Sweden), 1× non-essential amino acid solution (Nacalai Tesque), and 1× gentamicin/amphotericin solution (Life Technologies, Carlsbad, CA, USA). The cell suspension was filtered using a cell strainer to remove tissue debris and then transferred to an uncoated flask to allow fibroblast attachment. After 1 h, the unattached cells were transferred to another uncoated flask, and this procedure was repeated three times. The cell suspension containing the unattached cells was collected and centrifuged at 1200 × g for 5 min, and the resulting cell pellet was resuspended. Myoblasts were counted and seeded on to collagen I-coated 12-well plates (Iwaki, Tokyo, Japan) at a density of 1×10^5 cells/well. Myoblasts were incubated in the growth medium described above at 37 °C and 5% CO₂ in humidified air until myotube formation. On the 5th day, myotubes were treated with serum-free medium with or without 200 ng/mL recombinant human IGF-1 (Novus Biologicals, LLC, Centennial, CO, USA) for 3 h.

Real-time PCR

mRNA expression was measured using real-time reverse transcription polymerase chain reaction (RT-PCR) as previously described (Saneyasu et al., 2019a). Briefly, total RNA from the PM and BF muscles or CEM cultures after washing with PBS was extracted using Sepazol-RNA I (Nacalai Tesque). First strand cDNA was synthesized from total RNA using Rever Tra Ace® qPCR RT Master Mix with gDNA Remover (Toyobo Co. Ltd, Osaka, Japan). mRNA levels were quantified for each

primer using TB Green Premix Ex Taq II (Tli RNase H Plus; Takara Bio Inc., Otsu, Japan) according to the supplier's recommendations and based on a relative standard curve using the Thermo Scientific Piko Real Real-Time PCR System (Thermo Fisher Scientific Oy, Vantaa, Finland). Primer sequences are listed in Table 1. mRNA levels of the target genes were normalized to those of the ribosomal protein S17.

Data analysis

Data have been analyzed using the Student's *t*-test in Excel 2013 (Microsoft, Redmond, WA, USA). All differences were considered significant at $P < 0.05$.

Results

In the PM and BF of layer chicks (Fig. 1), mRNA levels of IGFBP-2 were significantly increased after 12 h of fasting, whereas IGFBP-3 mRNA levels significantly decreased. mRNA levels of IGFBP-1, -4, and -5 significantly increased with fasting in the BF, but not in the PM. In contrast, mRNA levels of IGF-1 significantly decreased with fasting in the PM, but not in the BF. mRNA levels of insulin-like growth factor-1 receptor (IGF-1R) significantly increased with fasting in both muscles.

In the PM and BF of boiler chicks (Fig. 2), fasting significantly decreased mRNA levels of IGFBP-3, similar to those in layer chicks. mRNA levels of IGFBP-2 slightly increased after fasting in the PM ($P = 0.060$) and BF ($P = 0.068$). Fasting significantly decreased IGFBP-5 mRNA levels in both muscles. Fasting significantly decreased IGFBP-4 and increased IGFBP-1 mRNA levels in the BF. Fasting significantly decreased IGF-1 mRNA levels in the PM. mRNA levels of IGF-1R significantly increased in both muscles with fasting. These results, combined with those

from layer chicks, suggested that fasting-induced changes in IGFBP-2 and -3 were conserved in chicken skeletal muscles.

Next, the effects of IGF-1 treatment on IGF-related gene expression in CEM cells were examined. Similar to previous studies (Nakashima et al., 2017; Nakashima and Ishida, 2018), mRNA levels of atrogin-1 significantly decreased with IGF-1 treatment in layer (Fig. 3) and broiler (Fig. 4) CEMs, suggesting that IGF-1 signaling induces gene expression under fasting conditions. mRNA levels of IGFBP-3 in the PM-derived CEMs significantly decreased with IGF-1 treatment (Fig. 3). However, most IGFBP genes were not affected by IGF-1. mRNA levels of IGF-1R in broiler BF-derived CEMs significantly decreased following IGF-1 treatment (Fig. 4). mRNA levels of IGF-1R in layer PM-derived CEMs slightly decreased with IGF-1 treatment (Fig. 3; $P = 0.053$).

Discussion

IGFBP-1 expression was significantly upregulated by fasting in the BF of layer and boiler chicks. There is evidence that mRNA levels of IGFBP-1 in the PM from 1 to 3 weeks of age are dramatically decreased compared to embryonic ages in both layer and broiler chicks (Guo et al., 2023). However, in the present study, mRNA levels of IGFBP-1 in layer and broiler BF muscles under fasting conditions were 3.79- and 3.89-fold higher than those under ad libitum feeding conditions. Although the autocrine and paracrine effects of IGFBP-1 on skeletal muscle development remain unclear, IGFBP-1 inhibits IGF-1-induced DNA synthesis and impairs cell growth in chicken embryonic fibroblasts (Liu et al., 1991). Burch et al. (1990) have reported that the addition of a 25 kDa amniotic IGFBP, later known as IGFBP-1, to the culture medium inhibits both basal and IGF-1-stimulated growth of cartilage *in vitro*. Thus, skeletal muscle IGFBP-1

may suppress the role of IGF-1 in the growth of chicken BF muscles under fasting conditions.

Fasting significantly upregulated IGFBP-2 expression in the PM and BF of layer chicks. In addition, IGFBP-2 mRNA levels in the PM and BF of broiler chicks slightly increased with fasting. Jia et al. (2018) have shown that skeletal muscle IGFBP-2 mRNA levels are indirectly related to the body weight of chicks and levels are higher in slow-growing chickens than those in fast-growing chickens. IGFBP-2 suppresses myotube formation by inhibiting the expression of myogenesis-related genes, such as myogenic differentiation 1 and myosin heavy chains, in cultured chicken primary myoblasts (Wang et al., 2019). Thus, IGFBP-2 may suppress skeletal muscle growth in chicks under fasting conditions.

Fasting significantly decreased mRNA levels of IGFBP-3 in the PM and BF of both chicken types. Safian et al. (2012) have reported that fasting increases IGFBP-3 mRNA levels in the skeletal muscles of flounder. These findings suggest that the role of IGFBP-3 in skeletal muscle is conserved between fish and birds. There is evidence that downregulation of IGFBP-3 in human (Foulstone et al., 2003) and chicken myoblasts (Guo et al., 2023) suppresses myoblast differentiation *in vitro*, suggesting that the role of IGFBP-3 in myoblasts is conserved between mammals and birds. These findings support the hypothesis that IGFBP-3 promotes muscle growth in birds under feeding conditions. Thus, the physiological role of skeletal muscle IGFBP-3 in chickens requires further clarification (e.g., the effects of IGFBP-3 knockdown on protein metabolism in CEMs).

The physiological roles of IGFBP-4 and -5 have not yet been elucidated in vertebrates. However, overexpression of IGFBP-4 in skeletal muscles is accompanied by increased IGF-1 gene expression and skeletal muscle hypertrophy in clenbuterol-treated rats (Awede et al., 2002). Ren et al., (2008) have reported that IGFBP-5

knockdown suppresses muscle cell differentiation in C2C12 cells. Additionally, mRNA levels of IGFBP-4 and -5 in breast muscles of layer and broiler chicks significantly decrease after hatching (Guo et al., 2023). These findings suggested that IGFBP-4 and -5 were involved in chicken muscle development at the embryonic stage. In the present study, fasting altered mRNA levels of IGFBP-4 and -5 in broiler and layer chicks and the responses differed between chick types. Further studies are required to clarify the role of IGFBPs in chicks after hatching.

Fasting significantly affected the gene expression of many IGFBPs in chicken skeletal muscles. However, IGF-1 did not affect most IGFBPs in CEMs *in vitro* (Figs. 3 and 4). Only IGFBP-3 was observed in layer PM-derived CEMs, but mRNA levels of IGFBP-3 decreased with fasting in the PM and BF *in vivo*. Furthermore, the fasting decreased plasma IGF-1 concentration in chickens (Kita et al., 2002, 1996). Taken together, these findings suggested that chick skeletal muscle IGFBPs were transcriptionally regulated by feeding conditions in an IGF-1-independent manner. Intravascular administration of insulin suppresses fasting-induced IGFBP-2 expression in the liver and gizzard of 6-week-old layer chicks (Nagao et al., 2001). IGFBP-1 gene expression may be downregulated by insulin and upregulated by glucocorticoid in human osteoblasts (Conover et al., 1996) and rat hepatocytes (Orlowski et al., 1990; Villafuerte et al., 1992). Ernst and White (1996) have reported that insulin downregulates IGFBP-2 mRNA levels in C2C12 myotubes. Additionally, glucose inhibits IGFBP-1 secretion from Hep G2 cells (Cotterill et al., 1989). In chickens, fasting decreases plasma insulin (Beccavin et al., 2001; Saneyasu et al., 2015a) and glucose levels (Beccavin et al., 2001; Christensen et al., 2013; Saneyasu et al., 2015a, 2015b), and elevates plasma corticosterone and non-esterified fatty acid levels (Honda et al., 2022; Kadhim et al., 2019; Najafi et al., 2016; Wang et al., 2013). These findings suggest that changes in plasma hormone and nutrient levels affect IGFBP mRNA levels

in chicken skeletal muscle. Further studies are required to examine the effects of insulin, glucocorticoids, and/or other nutrient treatments on IGF-related gene expression in CEM cells.

mRNA levels of IGF-1R significantly increased with fasting in all muscles and chicks examined in this study. A mammalian study has proposed that the negative feedback circuit in the IGF-1 signaling pathway is activated by the suppression of IGF-1R expression in skeletal muscles (Huang et al., 2011). Plasma IGF-1 concentration decreases during fasting in chickens (Kita et al., 2002, 1996). In the present study, mRNA levels of IGF-1R significantly decreased with IGF-1 treatment in broiler BF-derived CEMs. mRNA levels of IGF-1R in layer PM-derived CEMs slightly decreased following IGF-1 treatment ($P = 0.053$). Although the effects were not consistent between CEMs, these findings suggest that the negative feedback circuit of IGF-1 signaling accompanied by downregulation of IGF-1R gene expression in skeletal muscles has been partly conserved between mammals and chickens.

Fasting decreased IGF-1 mRNA levels only in the PM of both chick types. IGF-1 is the primary hormone that promotes skeletal muscle growth in chicks. In chicks, IGF-1 mRNA levels in the PM are significantly higher than those in other skeletal muscles, including the BF (Shimamoto et al., 2016). Although the autocrine and paracrine effects of IGF-1 on skeletal muscle growth remain unclear, IGF-1 mRNA levels in the PM may be upregulated to maintain muscle mass under ad libitum feeding conditions. Further studies are required to fully elucidate the evolution of breast muscle development in chickens.

Because primer pairs differed between IGFBP genes, it was difficult to compare the abundance of each IGFBP mRNA in the PM and BF. In addition, there was no evidence of a comparison of mRNA levels between IGFBP genes in chicken skeletal muscles. However, Guo et al. (2023) have reported that the expression levels of all IGF

system members, including IGFBPs, sharply decreased after hatching in layer and broiler chickens, indicating that the IGF system plays a much greater role in the embryonic stage and early period after hatching. The findings in the present study suggest that skeletal muscle IGFBPs may also function in growing chickens, although the mRNA levels may be lower than those at embryonic age. Therefore, the physiological importance and major role of each IGFBP in the skeletal muscles of growing chickens needs to be investigated in future studies.

There is no evidence showing the circadian rhythm of IGFBP gene expression in mammals and birds, but significant daily rhythmic mRNA expression of IGF-1 and IGFBP-2 has been reported in goldfish skeletal muscle under a 12-h/12-h light/dark cycle (Bao et al., 2018). There is evidence that clock gene deficiencies in mice result in downregulation of IGF-1 production in skeletal muscles under a 12-h/12-h light/dark cycle (Chaudhari et al., 2017). However, in the present study, chicks were maintained under a 23-h/1-h light/dark cycle (23:00–24:00 dark) and fasted from 22:00 to 10:00. Therefore, the circadian rhythm would only have a small impact, if any, under these experimental conditions.

The present study showed that several IGFBP genes in chicken skeletal muscle were transcriptionally altered in response to feeding status. For example, mRNA levels of IGFBP-1 significantly increased in the BF, and mRNA levels of IGFBP-2 and -3 in the skeletal muscle of chicks appeared to be altered by fasting. Overall, these findings suggested that these IGFBPs play physiological roles in chicken skeletal muscle in response to feed intake.

Figure Legends

Fig. 1. Effects of fasting on the mRNA levels of insulin-like growth factor-related genes in the *pectoralis major* and *biceps femoris* in layer chicks. Data are the means $\pm$ S.E.M. of eight chicks in each group and are expressed as a percentage of the mean in the feeding group. *, and ** indicate statistical differences ($P < 0.05$, and $P < 0.01$, respectively) between the feeding and fasting group in each muscle.

Fig. 2. Effects of fasting on the mRNA levels of insulin-like growth factor-related genes in the *pectoralis major* and *biceps femoris* in broiler chicks. Data are the means $\pm$ S.E.M. of eight chicks in each group and are expressed as a percentage of the mean in the feeding group. †, *, and ** indicate statistical differences ($P < 0.10$, $P < 0.05$, and $P < 0.01$, respectively) between the feeding and fasting group in each muscle.

Fig. 3. Effects of insulin-like growth factor-1 on the mRNA levels of insulin-like growth factor-related genes in chicken embryonic myotubes derived from *pectoralis major* and *biceps femoris* of layer embryos. Data are the means $\pm$ S.E.M. of five to six wells in each group and are expressed as a percentage of the mean in the control group. †, *, and ** indicate statistical differences ($P < 0.10$, $P < 0.05$, and $P < 0.01$, respectively) between the control and treatment groups.

Fig. 4. Effects of insulin-like growth factor-1 on the mRNA levels of insulin-like growth factor-related genes in chicken embryonic myotubes derived from *pectoralis major* and *biceps femoris* of broiler embryos. Data are the means $\pm$ S.E.M. of six wells in each group and are expressed as a percentage of the mean in the control group. *, and ** indicate statistical differences ($P < 0.05$, and $P < 0.01$, respectively) between the control and treatment groups.

Table 1. Primer sequences for real-time PCR analysis

Gene		Nucleotide sequence	GenBank Accession Number
<i>IGFBP-1</i>	Sense	5'-GCC AGG ACA AAT CCA TCC CT-3'	NM_001001294
	Antisense	5'-GCT CCT TCT GAC AAG GTC CC-3'	
<i>IGFBP-2</i>	Sense	5'-AAT GGG CAG CGT GGA GAG T-3'	NM_205359
	Antisense	5'-CTG GAT CAC CTT CCC ATG GA-3'	
<i>IGFBP-3</i>	Sense	5'-ATC AGG CCA TCC CAA GCT T-3'	NM_001101034
	Antisense	5'-GAT GTG CTG TGG AGG CAA ATT-3'	
<i>IGFBP-4</i>	Sense	5'-GAG CAC CCC AAC AAC AGC TT-3'	NM_204353
	Antisense	5'-CCG TTG TTG ATG CGC TTT G-3'	
<i>IGFBP-5</i>	Sense	5'-CAA GGC CGA ACG GGA AT-3'	XM_422069
	Antisense	5'-TCC TCC GTC ATC TCC GAT GT-3'	
<i>IGF-1</i>	Sense	5'-GCT GCC GGC CCA GAA -3'	NM_001004384
	Antisense	5'-ACG AAC TGA AGA GCA TCA ACC A -3'	
<i>IGF1R</i>	Sense	5'- GGA GAA TTT CAT GGG TCT GAT TG-3'	NM_205032
	Antisense	5'- CAT GGG AAT GGC GAA TCT TC-3'	
<i>Atrogin-1</i>	Sense	5'-CAC CTT GGG AGA AGC CTT CAA-3'	NM_001030956
	Antisense	5'-CCG GGA GTC CAG GAT AGC A-3'	
<i>RPS17</i>	Sense	5'-GCG GGT GAT CAT CGA GAA GT-3'	NM_204217
	Antisense	5'-GCG CTT GTT GGT GTG GAA GT-3'	

IGFBP, insulin-like growth factor-binding protein; IGF-1, insulin-like growth factor-1; IGF1R, insulin-like growth factor-1 receptor; RPS, ribosomal protein

Fig. 1

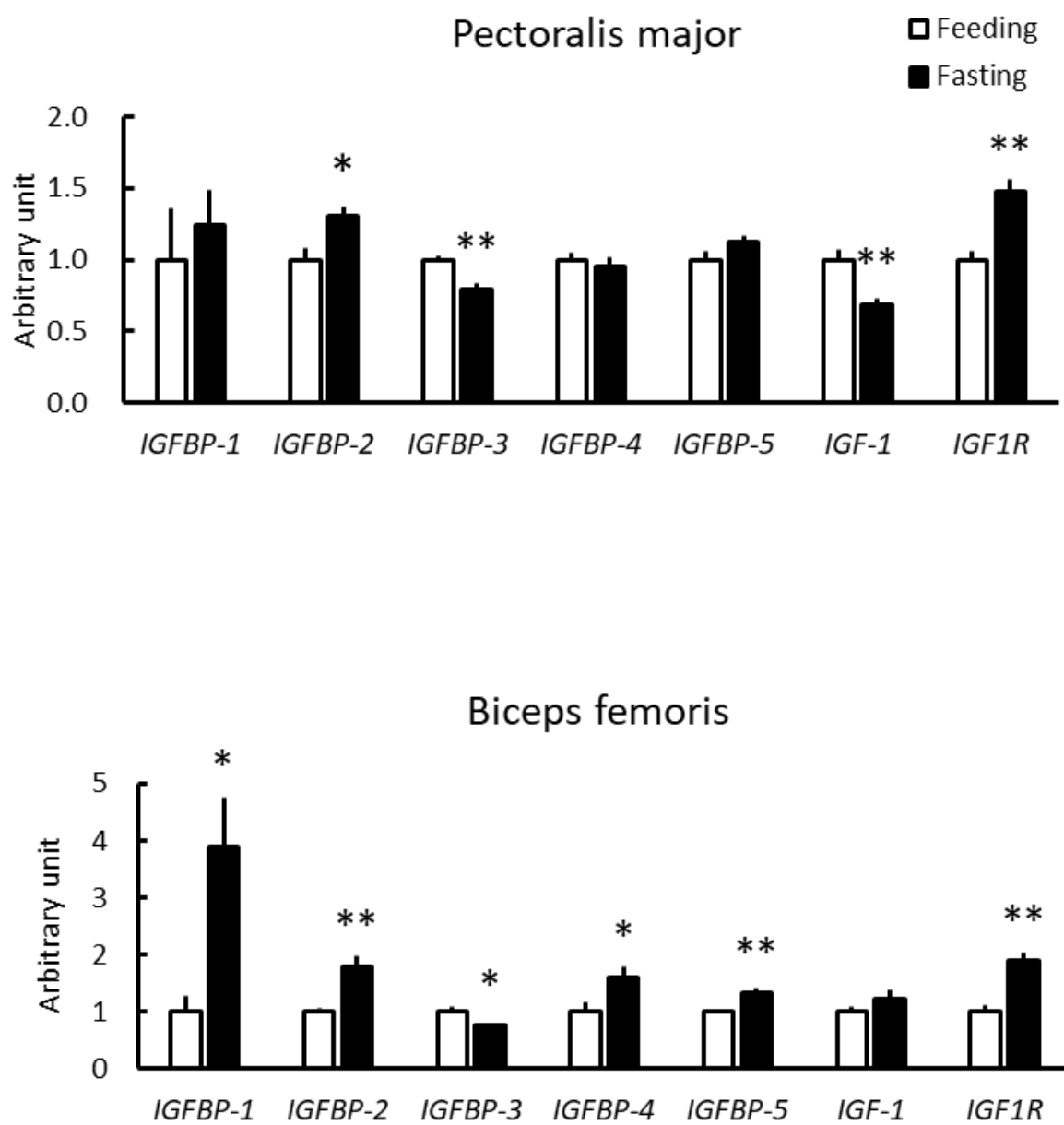


Fig. 2

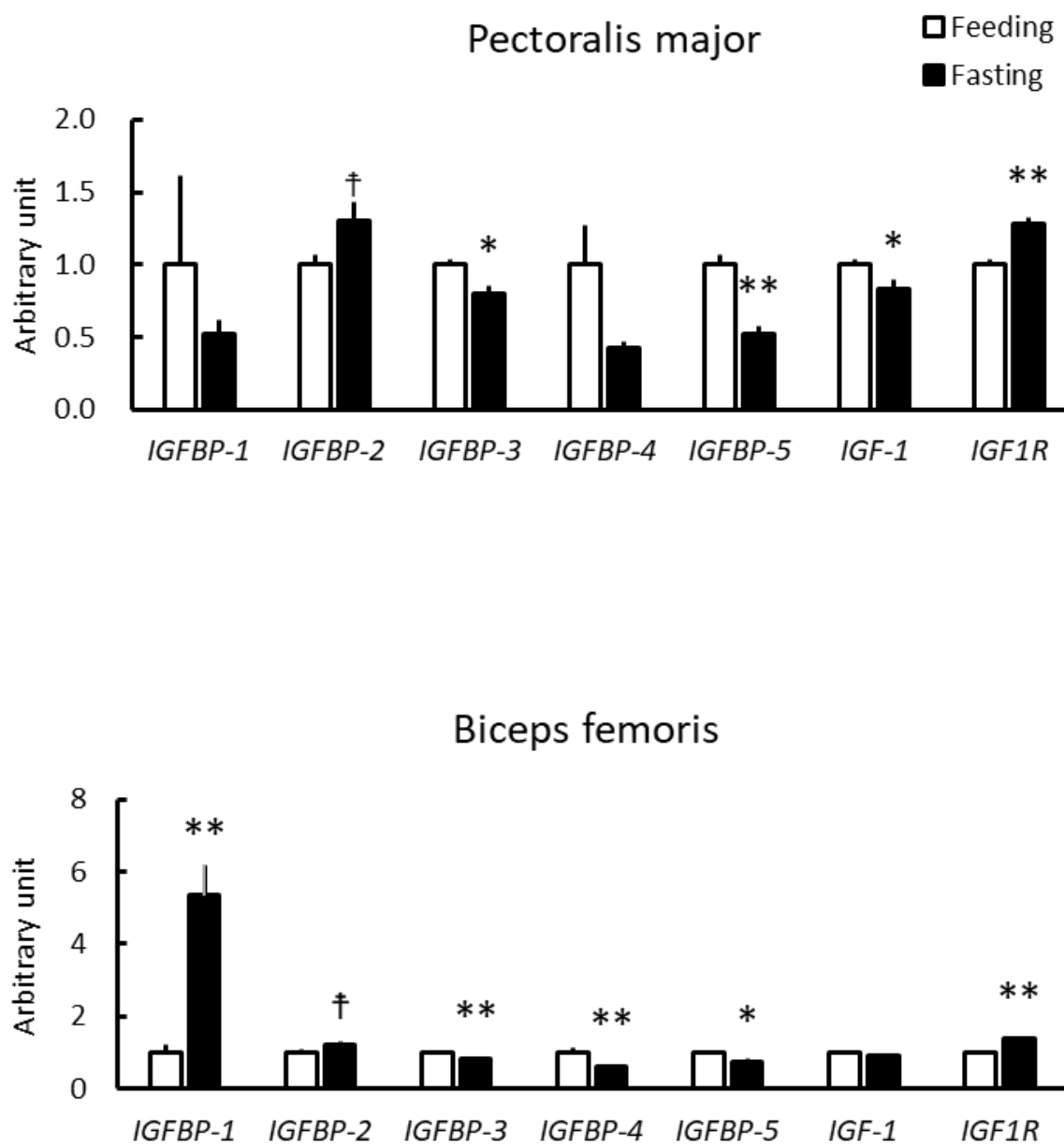


Fig. 3

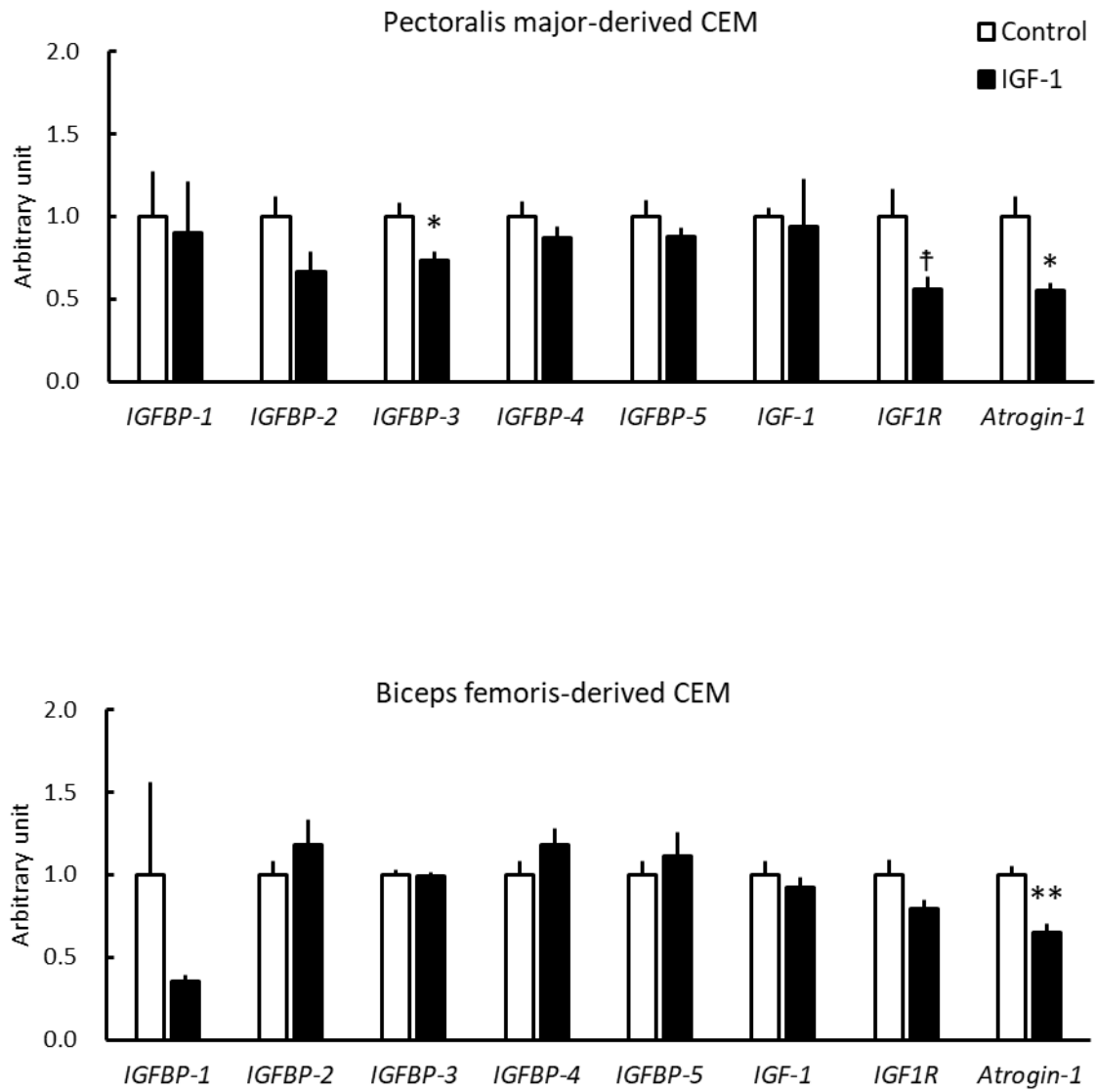
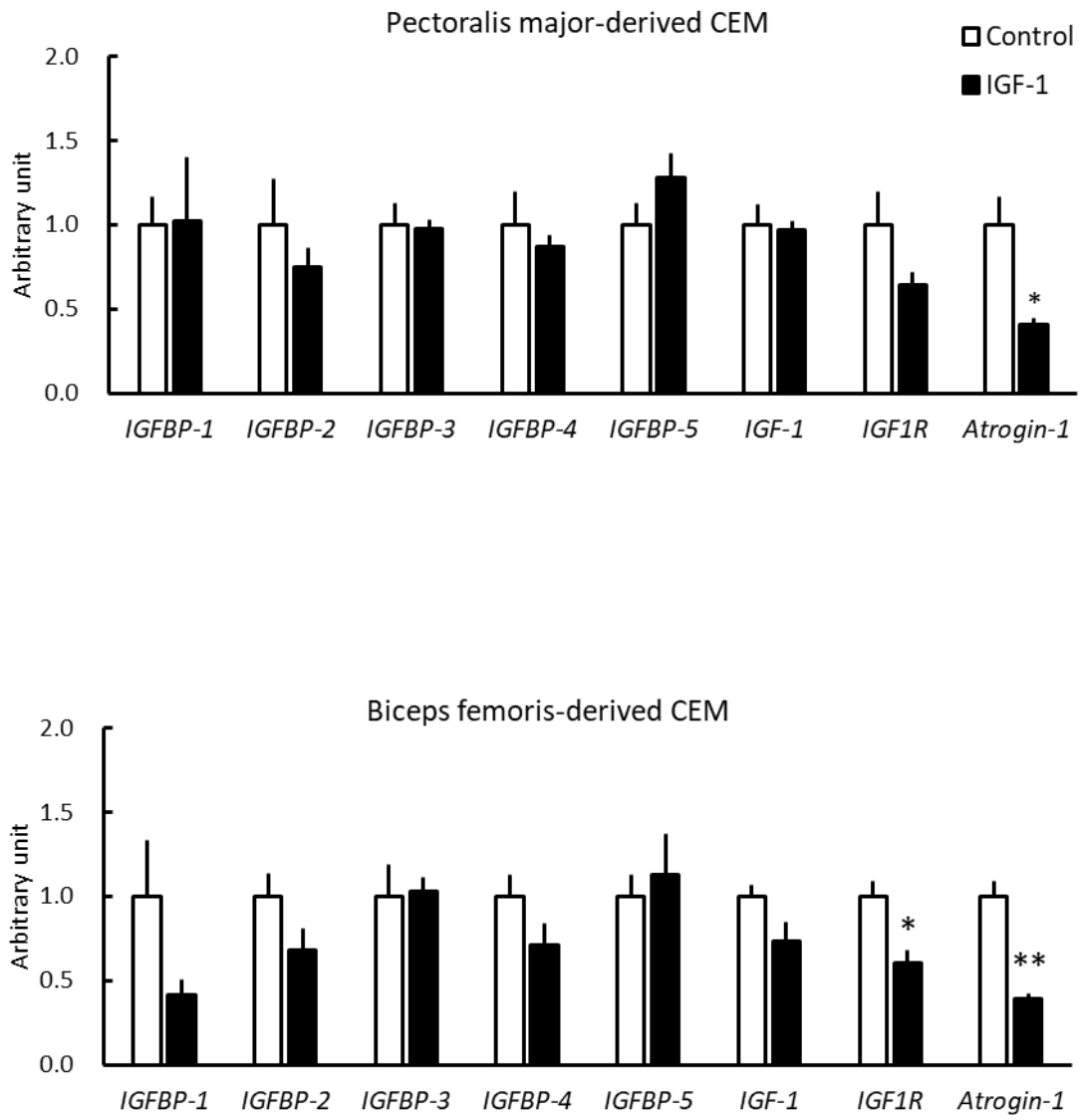


Fig. 4



Chapter 3

Effects of Amino Acid Supplementation to a Low-Protein Diet on the Growth Performance and Protein Metabolism-related Factors in Broiler Chicks

Introduction

Using a low-protein (LP) diet may alleviate the environmental impact of chicken meat production by reducing nitrogen excretion and ammonia emissions (Greenhalgh et al., 2020; Méda et al., 2011). Supplementation with synthetic free amino acids (AAs) ameliorates the low growth performance of broiler chicks fed an LP diet (Ospina-Rojas et al., 2014; Selle et al., 2020). For example, Belloir et al. have reported that supplementation with AAs reduces the dietary crude protein (CP) content by at least 17% in growing-finishing male broilers without impairing growth performance or meat quality (Belloir et al., 2017). Thus, formulating chicken rations with lower CP levels and supplementing with the required AAs would enable environmentally friendly and economical poultry production. However, the mechanisms underlying the restorative effects of dietary AAs on the growth performance of LP diet-fed broiler chicks have not yet been clarified.

Skeletal muscle mass is regulated by the balance between protein synthesis and degradation (Maruyama et al., 1978; Masiero et al., 2009) and is governed by anabolic hormones, such as insulin-like growth factor (IGF)-1, and catabolic hormones, such as glucocorticoids (Glass, 2005; Millward, 2023; Schiaffino et al., 2013). For example, IGF-1 treatment promotes hypertrophy of C2C12 myotubes (Rommel et al., 2001) and proliferation of chick embryonic myoblasts (Yu et al., 2015). IGF-1 treatment significantly decreases the expression of atrogin-1, a regulatory enzyme of the ubiquitin-proteasome system, and increases intracellular protein levels in chicken

embryonic myotubes (Nakashima et al., 2017). IGF-1 also significantly decreases mRNA levels of microtubule-associated protein 1 light chain 3 isoform B (LC3B), an autophagy-related protein, in chicken embryonic myotubes (Nakashima and Ishida, 2018). The synthetic glucocorticoid dexamethasone significantly upregulates atrogin-1 mRNA levels in the *pectoralis major* (PM) muscles of chicks when administered intraperitoneally, as well as in chick embryonic myotubes *in vitro* (Nakashima et al., 2016). Moreover, an LP diet may induce alterations in adrenocortical function in chicks and increase plasma corticosterone concentrations (Carsia et al., 1988). However, AAs protect against or alleviate dexamethasone-induced skeletal muscle atrophy in mammals (Lee et al., 2022; Shimizu et al., 2011). In addition to circulating hormones, locally expressed IGF-1 may be involved in protein metabolism in chicken skeletal muscles. For example, the PM of heavy broiler chicks expresses high mRNA levels of IGF-1 and pro-myogenic genes, and low mRNA levels of anti-myogenic genes (Xiao et al., 2017). We suggest that IGF-1 in thigh muscles contributes to protein synthesis via the protein kinase B (Akt)/ribosomal protein 6 (S6) pathway in chicks (Saneyasu et al., 2019b). Dietary methionine supplementation increases IGF-1 protein and mRNA levels in breast muscle and the relative weight of whole breast muscle in broilers with low hatching weights (Wen et al., 2014). Therefore, circulating hormones and locally expressed IGF-1 may be involved in the impaired effects of a LP diet and the ameliorative effect of AA supplementation to an LP diet on the growth performance of broiler chicks.

Insulin-like growth factor binding proteins (IGFBPs) are produced in the liver and skeletal muscles (List et al., 2014; Vaccaro et al., 2022). Hepatic IGFBPs are released in the bloodstream and function as carrier proteins of serum IGF-1 in mammals and chickens (McMurtry et al., 1997). Supplementation with dietary valine and isoleucine significantly decreases IGFBP-1 mRNA levels in the liver of LP diet-fed pigs (Habibi et al., 2022). Jousse et al. have reported that AA limitation significantly induces

IGFBP-1 expression in mammalian hepatoma cells (Jousse et al., 1998). Saxena et al. have found that an LP diet significantly increases IGFBP-2 mRNA levels in the liver of broiler chicks (Saxena et al., 2020). These findings suggest that dietary protein influences the function of IGF-1 by regulating hepatic and blood IGFBPs. Although the effect of dietary protein levels on IGFBP mRNA levels in skeletal muscle has not been investigated in chickens, mRNA levels of IGFBP-2 in breast muscle are increased by dietary leucine supplementation above recommended levels in 10-day-old broiler chicks (Zeitz et al., 2019). In broiler chicks with high muscularity, IGFBP-2 mRNA levels are high in the PM (Vaccaro et al., 2022). Therefore, IGFBPs expressed in liver and skeletal muscles may be involved in the effects of AA supplementation on the growth performance of LP diet-fed broiler chickens.

In the present study, the effects of an LP diet and AA supplementation to the LP diet on various protein metabolism-related factors in the liver, skeletal muscle, and blood of broiler chicks were investigated. These findings suggested that transcriptional changes in proteolytic genes in skeletal muscles might be related to the adverse effects of an LP diet in broiler chicks.

Materials and methods

Animals, experimental design, and diets

This study was approved by the Institutional Animal Care and Use Committee and performed according to the Kobe University Animal Experimentation Regulations (2021-11-02). One-day-old Ross 308 male broiler chicks were purchased from a local hatchery (Yamamoto Co., Ltd.) and reared together using a commercial starter feed (22.0% CP and 3,050 kcal/kg metabolizable energy; Nichiwa Sangyo Co., Ltd.). Chicks were reared in battery cages with free access to feed and water under a 23 h/1 h

light/dark cycle (23:00–24:00 dark), and the temperature was kept at 31 ± 2 °C during the first 7 days and then reduced gradually until reaching 25 ± 2 °C at 21 days of age. At 8 days of age, chicks were allocated based on body weight (BW) into three experimental groups of four replicates (12 chicks per group, 3 chicks per cage) and fed experimental diets from 10 days of age. Chicks in the control group received corn-soybean-based mash diets formulated to meet the nutrient recommendations of the breed (Aviagen, 2019) for the grower (10–24 days of age) and finisher (25–35 days of age) phases (Tables 1 and 2). AA content of each diet was calculated using data described in the Standard Tables of Feed Composition in Japan (“Standard tables of feed composition in Japan. National agricultural research council organization,” 2009). In a preliminary experiment, the detrimental effects of a 20% reduced protein diet were not ameliorated by AA supplementation. Therefore, a 15% reduced protein diet was used in this study. Either an isocaloric LP diet (LP, 85% CP) or LP supplemented with AAs (LP+AA) to meet only the essential AA (EAA) requirements were given in the other two groups. Feed intake (FI) of the replicates was measured every day and individual chick BW was measured every week.

Sampling and analysis of plasma biochemical parameters

At the end of the experiment and under carbon dioxide anesthesia, blood was collected from the carotid arteries of chicks and chicks were euthanized by decapitation. Plasma was separated immediately by centrifugation at $1,910 \times g$ for 10 min at 4 °C and then stored at -80 °C until analysis. Plasma corticosterone and uric acid concentrations were measured using commercial kits (Corticosterone ELISA Kit, AssayPro LLC, MO, USA; Uric Acid C-Test, Code: 437-17301, FUJIFILM Wako Pure Chemicals Co., Osaka, Japan). The weights of the breasts, legs, and livers were recorded. Then, the center pieces of the PM, BF, and liver were excised and immediately frozen in liquid

nitrogen and stored at -80°C for real-time polymerase chain reaction (PCR) analyses.

Real-time PCR

Real-time PCR analysis was performed as previously described (Fujita et al., 2018, 2017; Saneyasu et al., 2019a). Total RNA was extracted from tissues using Sepazol-RNA I Super G (Nacalai Tesque, Inc., Kyoto, Japan). First-strand cDNA was synthesized from total RNA using Rever Tra Ace® qPCR RT Master Mix with gDNA Remover (Toyobo Co. Ltd, Osaka, Japan). mRNA levels were quantified for each primer using TB Green Premix Ex Taq II (Tli RNaseH Plus; Takara Bio Inc., Otsu, Japan) according to the supplier's recommendations based on a relative standard curve using the Thermo Scientific Piko Real Real-Time PCR System (Thermo Fisher Scientific Oy, Vantaa, Finland). Primer sequences for IGF-1, IGF1R (Fujita et al., 2017), IGFBP-1–3 (Fujita et al., 2018), atrogen-1, muscle RING-finger protein-1 (MuRF-1) (Saneyasu et al., 2019a), myoblast determination protein 1 (MyoD), myogenin (MyoG), and ribosomal protein S17 (RPS17) (Saneyasu et al., 2017) may be found in previous studies. The following primer sequences were used for LC3B (NM_001031461) and glucocorticoid receptor (GCR, NM_001037826): LC3B sense, 5'-TCC GAG ATC AGC ATC CAA CT-3', LC3B antisense, 5'-CAC CAT GCT GTG TCC GTT C-3'; GCR sense, 5'-CAT GAA CCT CGA AGC TCG CAA G-3,' and GCR antisense, 5'-ACC TCC AGC AGT GAC ACC AG-3'. mRNA levels of target genes were normalized to those of RPS17.

Data analysis

All data were statistically analyzed using the Tukey–Kramer method ($P < 0.05$) and Stat View 5.0 software (SAS Institute Inc.).

Results

The initial body weight of chicks was as follows: control, 205 ± 1.7 g; LP, 205 ± 0.7 g, and LP+AA, 208 ± 1.6 g. As shown in Table 3, BW gain (BWG) significantly decreased and the feed conversion ratio (FCR) significantly increased in the LP group throughout the experimental period compared to those in the control group. A significant decrease in BWG and an increase in FCR were observed in the LP+AA group in the first week (10–17 days of age) compared to those in the control group. Total feed intake was significantly higher in the LP+AA group than that in the control group.

As shown in Table 4, weights of the body, breast muscle, and legs significantly decreased in the LP group. There was no significant difference in liver weight and plasma corticosterone levels between groups, whereas plasma uric acid concentrations were significantly lower in the LP+AA group than those of the other groups.

To identify the mechanisms underlying the effects of the LP diet and AA supplementation, myogenesis- and proteolysis-related gene expression in the PM and BF were analyzed. mRNA levels of LC3B were significantly increased in the PM of the LP group, whereas mRNA levels of atrogen-1, MuRF-1, and MyoD were significantly increased in the BF (Fig. 1).

The expression levels of IGF-1 and its related genes in the liver, PM, and BF are shown in Fig. 2. Only IGFBP-2 mRNA levels in the BF were significantly higher in the LP group than that in the other groups. Therefore, hepatic and skeletal muscle IGF-1-related genes may not be involved in the significant differences in growth and skeletal muscle mass between groups, at least under the present experimental conditions.

Discussion

In this study, EAA supplementation in a 15% reduced CP diet from 10 to 35 days of age restored the decreases in growth performance and meat production observed in the LP group. Chrystal et al. have reported that a 14% reduced CP diet supplemented with EAA maintains the BWG and does not influence FI or FCR in broiler chicks between 14 and 35 days of age (Chrystal et al., 2020). Supplementation of EAA to 10% reduced CP diets in broilers from 11 to 35 days of age (Van Harn et al., 2019) or 21 to 35 days of age (Belloir et al., 2017) results in the recovery of growth performance and meat production. Thus, the current experimental conditions appeared to be comparable to those of previous studies and were desirable for clarifying the mechanisms underlying the effects of an LP diet and EAA supplementation in broiler chicks.

In this study, the LP diet significantly decreased BWG without any significant changes in FI compared to that of the control diet. In addition, changes in skeletal muscle weight and proteolytic gene expression in the LP group might suggest the induction of skeletal muscle proteolysis. Therefore, it is possible that reduced protein intake, and not energy intake, is one of the causes of impaired growth and loss of muscle mass in the LP group.

As shown in the LP+AA group, BWG and the FCR recovered with AA supplementation, although this group showed the highest feed consumption. Notably, the effects of AA supplementation on FI in the LP diet differed depending on experimental conditions. For example, a 15.5% reduced CP diet supplemented with EAAs increases FI without affecting BWG in broiler chicks from 1 to 17 days of age (Kriseldi et al., 2018). A 14% reduced CP diet supplemented with EAAs does not affect the BWG or FI of broiler chicks from 14 to 35 days of age (Chrystal et al., 2020). Moreover, the BW and FI of broiler chicks are not influenced by feeding a 17% reduced

CP diet supplemented with EAAs from 10 to 28 days of age (Namroud et al., 2010). In the present study, BWG decreased and the FCR increased during the first week (10–17 days of age) in the LP+AA group; however, these changes were not detected during the second and third weeks (17–24 and 24–31 days of age). Subsequently, FI increased in the LP+AA group during the remaining four days (31–35 days of age). These results suggested that changes in metabolism occurred during the experimental period in the LP+AA group and influenced growth performance during each growing period.

In the LP group, mRNA levels of LC3B were significantly higher in the PM, whereas those of atrogin-1 and MuRF-1 were significantly higher in the BF. These changes were accompanied by decreased muscle weight. However, no significant difference in these mRNA levels was detected between the control and LP+AA groups. There is evidence that AAs regulate proteolytic systems and preserve skeletal muscle mass in chickens, as well as in mammals. For example, oral administration of leucine or isoleucine suppresses myofibrillar proteolysis *in vivo* and *in vitro* by downregulating the ubiquitin-proteasome pathway in chick skeletal muscle (Nakashima et al., 2005). L-arginine treatment suppresses protein levels of atrogin-1 and MuRF-1 and increases muscle fiber diameter in primary chicken myoblast cultures (R. Wang et al., 2022). In cultured chick myotubes, non-essential AA supplementation decreases mRNA levels of LC3B and atrogin-1 (Nakashima and Ishida, 2018). These findings suggested that skeletal muscle proteolysis might be induced in the LP group and that dietary AAs attenuated the changes in both PM and BF in the LP+AA group.

Protein malnutrition increases plasma corticosterone levels in rats (Jacobson et al., 1997) and chickens (Carsia and Weber, 2000). Glucocorticoids regulate protein metabolism in chickens (Wang et al., 2015). The GCR is an intracellular receptor that conveys glucocorticoid signals and is implicated in the induction of catabolic responses in skeletal muscles of mammals (Kuo et al., 2013; Zhao et al., 2009). Therefore, plasma

corticosterone levels and skeletal muscle expression of GCR were compared between groups. However, in the present study, no significant differences were observed in plasma corticosterone or GCR mRNA levels in the PM or BF between groups. Considering that metabolic changes might have occurred during the feeding period in the LP+AA group, the corticosterone-induced proteolytic pathway might be temporarily upregulated in the first week of the feeding period. Further studies are required to clarify the effects of an LP diet over a short period in broiler chickens.

In the LP group, MyoD mRNA levels were significantly higher in the BF. MyoD and MyoG play important roles in the regeneration of adult skeletal muscle and remodeling of damaged fibers to restore fiber integrity and function (Dumont et al., 2015). Ishido et al. have proposed that high expression of MyoD in myonuclei and satellite cells in skeletal muscle under neurotrophs contributes to protection against apoptotic cell death and enhances myoblast proliferation in rats (Ishido et al., 2004). Therefore, the increased gene expression of MyoD in the BF of the LP group might be an adaptive mechanism that accelerates muscle regeneration.

In this study, the PM, but not the BF, showed significantly higher levels of LC3B mRNA, suggesting that the autophagic lysosomal system was induced in the PM, but not in the BF. The PM is mainly composed of glycolytic muscle fibers (Barnard et al., 1982; Roy et al., 2006), whereas the BF, the largest skeletal muscle in the thigh, is composed of both glycolytic and oxidative muscle fibers (Zhao et al., 2012). A comparison of autophagy (GFP-LC3 localization) between different muscle types reveals that autophagic rates in glycolytic muscles are more upregulated than those in oxidative muscle after 24 h starvation in mice (Mizushima et al., 2004). Thus, the different responses in LC3B gene expression might be attributed to different fiber types.

In the present study, the LP diet resulted in different changes in mRNA levels of proteolytic genes, such as atrogin-1, MuRF-1, and LC3B, between the PM and BF groups (Fig. 1). However, the reasons for these different responses remain unclear. Site-specific responses to nutrients and hormones have been reported in chicken skeletal muscles. For example, the first exogenous nutrient significantly suppresses mRNA levels of atrogin-1 in the *sartorius*, but not in the PM of newly hatched chicks (Ijiri et al., 2017). Intraperitoneal injection of clenbuterol significantly suppresses mRNA levels of atrogin-1 in the *sartorius*, but not in the PM, in neonatal chicks (Ijiri et al., 2014). The capacity for protein synthesis, estimated from the RNA: protein ratio, is significantly suppressed in the BF, but not in the PM, in broiler chickens by pair-feeding (Saneyasu et al., 2015b). Therefore, it is likely that chicks maintain protein homeostasis by transcriptionally regulating protein metabolism-related genes in skeletal muscle in a site-specific manner.

IGF-1 produced in the liver plays an important role in chicken growth (Scanes, 2009). IGF-1 in the thigh muscles may contribute to protein synthesis via the Akt/S6 pathway in chicks (Saneyasu et al., 2019b). An extremely deficient LP diet (CP 12.15%) suppresses growth and decreases plasma IGF-1 concentrations in broiler chicks compared to those fed a high protein diet (CP 21.58%) (Tesseraud et al., 2003). A reduction in soybean protein from 20% to 5% in the diet significantly suppresses growth and decreases plasma IGF-1 concentrations in layer chicks (Kita et al., 2002). However, in the present study, no significant difference was observed in IGF-1 mRNA levels in the liver, PM, or BF between groups. These results suggested that neither circulating nor skeletal muscle IGF-1 was involved in the changes in growth or skeletal muscle mass induced by LP or EAA-supplemented LP diets, at least under the present experimental conditions.

In the LP group, IGFBP-2 mRNA levels were higher in the BF. Kita et al. have reported that a 5% soybean protein diet significantly increases gizzard IGFBP-2 mRNA levels and significantly decreases brain IGFBP-2 mRNA levels compared to that of a 20% soybean diet in layer chicks (Kita et al., 2002). Therefore, transcriptional regulation of IGFBPs is likely tissue-dependent in chickens. Wang et al. have reported that IGFBP-2 knockdown significantly increases MyoD mRNA levels in chicken myoblasts; however, in the present study, both IGFBP-2 and MyoD mRNA levels are increased in the BF of the LP group (Wang et al., 2019). Thus, the upregulation of IGFBP-2 expression in the LP group might not play an important role in the suppression of skeletal muscle growth.

In chickens, ammonia produced by AA catabolism is excreted in the form of uric acid. The LP+AA group showed a significant decrease in plasma uric acid concentration compared to those of the control and LP groups, although the plasma uric acid concentration did not significantly change in the LP group. Feeding diets with 11%–13% reduced protein from 1 to 48 days of age do not influence the plasma uric acid concentrations or decrease BW in broilers (Hernández et al., 2012). Feeding diets with 23%–27% reduced dietary protein from 1 to 21 days of age decreases the BWG and blood uric acid concentration in broilers (Awad et al., 2015). Hence, diets with more than 23% reduced CP might influence both the BWG and plasma uric acid levels in broiler chicks. Abou-Elkhair et al. have reported that 5%–10% reduced dietary CP supplemented with EAAs from 1 to 35 days of age decreases serum uric acid levels and improves growth in broiler chicks compared to that broiler chicks fed the normal diet (Abou-Elkhair et al., 2020). These results and the current findings suggest that the addition of EAAs might suppress AA catabolism without affecting BWG in broiler chicks.

In this study, corn and soybean meals were the major feed ingredients. Further studies are required to examine the effects of other feed ingredient-based LP diets and LP+AA diets in broiler chicks. For example, wheat, sorghum, distillers, dried grains with soluble corn gluten meal, and rapeseed meal may all be used as feed ingredients. However, soybean meal is the major dietary protein source in animal feed throughout the world (Kim et al., 2019). Therefore, these findings provide essential information that contribute to future research.

In summary, the effects of a 15% reduced LP diet, with or without EAA supplementation, were examined on growth performance and protein metabolism-related factors in skeletal muscle of broiler chicks. These findings suggested that changes in mRNA levels of proteolytic genes in skeletal muscle might be related to the impaired effects of the LP diet in broiler chicks.

Figure Legends

Fig. 1. Effects of amino acid supplementation to a low-protein diet on mRNA levels of protein metabolism-related factors in skeletal muscle of broiler chicks.

Data are expressed as means $\pm$ the standard error of the mean (SEM) of four replicates in each group. Groups with different letters are significantly different ($P < 0.05$). LP, low-protein; LP+AA, low-protein+amino acids; MuRF-1, muscle RING-finger protein-1; LC3B, microtubule-associated protein 1 light chain 3 isoform B; GCR, glucocorticoid receptor; MyoD, myoblast determination protein 1; MyoG, myogenin.

Fig. 2. Effects of amino acid supplementation to a low-protein diet on mRNA levels of insulin-like growth factor 1 (IGF-1) and IGF-1 related genes in the liver, *pectoralis major*, and *biceps femoris* muscles of broiler chicks. Data are expressed as means $\pm$ the standard error of the mean (SEM) of four replicates in each group. Groups with different letters are significantly different ($P < 0.05$). LP, low-protein; LP+AA, low-protein+amino acids; IGF-1, insulin-like growth factor 1; IGF1R, insulin-like growth factor 1 receptor; IGFBP, insulin-like growth factor binding protein.

Table 1. Ingredients and nutrient compositions of the experimental diets

Ingredient (%)	10–24 days			25–35 days		
	Control	LP	LP+AA	Control	LP	LP+AA
Yellow corn, ground	55.01	65.19	66.14	58.92	68.54	69.35
Soybean meal (CP 45%)	33.7	25.05	22.65	28.82	20.7	18.55
Fish meal (60%)	3	3	3	3	3	3
Soybean oil	4	2.6	2.9	5	3.6	3.9
Calcium carbonate	0.85	0.85	0.85	0.85	0.85	0.85
Dicalcium Phosphate	1	1.1	1.1	1	1.1	1.1
Sodium chloride	0.3	0.3	0.3	0.3	0.3	0.3
Vitamin and mineral mix ¹	1.91	1.91	1.91	1.91	1.91	1.91
DL-Methionine	0.23	-	0.35	0.2	-	0.32
L-Lysine, HCL	-	-	0.27	-	-	0.27
L-Threonine	-	-	0.13	-	-	0.1
L-Valine	-	-	0.11	-	-	0.09
L-Isoleucine	-	-	0.15	-	-	0.14
L-Arginine	-	-	0.14	-	-	0.12
Total	100	100	100	100	100	100
Calculated composition						
CP (% in diet)	21.5	18.3	18.3	19.5	16.6	16.6
CP (% of requirement ²)	100	85	85	100	85	85
ME (Kcal/kg diet)	3110	3110	3110	3211	3205	3207
ME (% of requirement ²)	100	100	100	100	100	100

LP, low-protein diet; LP+AA, amino acid supplemented low-protein diet.

1 Supplied the following per kg diet: vitamin A, 47,200 IU; vitamin D3, 11,800 IU; vitamin E, 71 mg; vitamin K3, 11.6 mg; vitamin B1, 5 mg; vitamin B2, 15.8 mg; vitamin B12, 0.02 mg; pantothenic acid, 23.8 mg; niacin, 35 mg; pyridoxine, 7.8 mg; biotin, 0.17 mg; folic acid, 2.7 mg; choline, 550 mg; copper, 11 mg; zinc, 110 mg; iron, 112 mg; manganese, 112 mg; selenium, 0.10 mg; iodine, 1.1 mg; cobalt, 0.11.

2 Ross Broiler Nutrition Specifications [29]

Table 2. Calculated composition of essential amino acids in the experimental diets

Nutrient	10–24 days			25–35 days		
	Control	LP	LP+AA	Control	LP	LP+AA
Percentage in diet						
Methionine	0.60	0.34	0.67	0.55	0.31	0.61
Methionine + Cystine	1.00	0.68	0.99	0.91	0.63	0.92
Lysine	1.41	1.16	1.29	1.26	1.02	1.17
Threonine	0.94	0.80	0.88	0.85	0.73	0.78
Valine	1.11	0.95	1.01	1.01	0.87	0.91
Arginine	1.60	1.33	1.38	1.44	1.18	1.23
Isoleucine	0.97	0.81	0.90	0.87	0.72	0.81
Leucine	2.11	1.87	1.78	1.95	1.73	1.65
Tryptophan	0.29	0.24	0.23	0.26	0.21	0.20
Percentage of requirement ¹						
Methionine	118	66	130	117	67	131
Methionine + Cystine	101	69	100	100	69	101
Lysine	109	90	100	109	88	101
Threonine	107	91	100	110	93	100
Valine	111	95	101	113	96	101
Arginine	117	97	101	118	97	100
Isoleucine	109	91	102	108	89	100
Leucine	148	132	126	154	136	130
Tryptophan	139	115	107	138	113	105

¹ Ross Broiler Nutrition Specifications [29]

Table 3. Growth performance of broiler chicks from 10 to 35 days of age

Parameter		Days of age				Total
		10-17	17-24	24-31	31-35	
Weight gain (g)	Control	351.1±10.5 ^a	464.8±20.1 ^a	580.1±40.2 ^a	295.6±36.2	1691.5±68 ^a
	LP	269.1±6.6 ^c	364.8±7.3 ^b	443.8±4.7 ^b	272.9±2.6	1350.6±17.5 ^b
	LP +AA	311.4±4.5 ^b	460.3±8.6 ^a	578±13.2 ^a	337.1±15.3	1686.8±29 ^a
Feed intake (g)	Control	433.7±9.4	640.1±18 ^{ab}	916.6±35.1	509.5±39 ^b	2499.8±62.4 ^b
	LP	402.2±16.4	602.3±24.9 ^b	951.6±19.6	578.6±15.3 ^{ab}	2534.8±55.3 ^{ab}
	LP +AA	429.2±7.8	675.9±6.3 ^a	983.4±21.8	613.8±13.6 ^a	2702.2±27.3 ^a
FCR (g/g)	Control	1.24±0.01 ^b	1.38±0.05 ^b	1.6±0.14 ^b	1.76±0.11 ^b	1.48±0.05 ^b
	LP	1.5±0.05 ^a	1.65±0.04 ^a	2.14±0.03 ^a	2.12±0.06 ^a	1.88±0.02 ^a
	LP +AA	1.38±0.03 ^a	1.47±0.02 ^b	1.7±0.03 ^b	1.83±0.06 ^{ab}	1.6±0.02 ^b

Values are mean ± SEM for four replicates in each group. Groups with different superscripts in the same column are significantly different ($P < 0.05$).

Table 4. Body and tissue weights and blood parameters of 35-day-old broiler chicks

	Control	LP	LP+AA
Final body weight (g)	1896.4±68.4 ^a	1555.2±18.1 ^b	1894.9±30.4 ^a
Breast muscles weight (g)	261.0±14.2 ^a	180.3±3.5 ^b	266.2±6.5 ^a
Legs weight (g)	389.8±11 ^a	315.8±6.2 ^b	390.0±7.7 ^a
Liver weight (g)	34.1±1.3	33.8±0.4	38.6±1.8
Plasma Uric acid (mg/dL)	4.1±0.2 ^a	4.1±0.2 ^a	2.8±0.3 ^b
Plasma Corticosterone (ng/mL)	3.15±0.21	3.23±0.19	3.19±0.34

Values are mean ± SEM for four replicates in each group. Groups with different superscripts in the same row are significantly different ($P < 0.05$).

Fig. 1

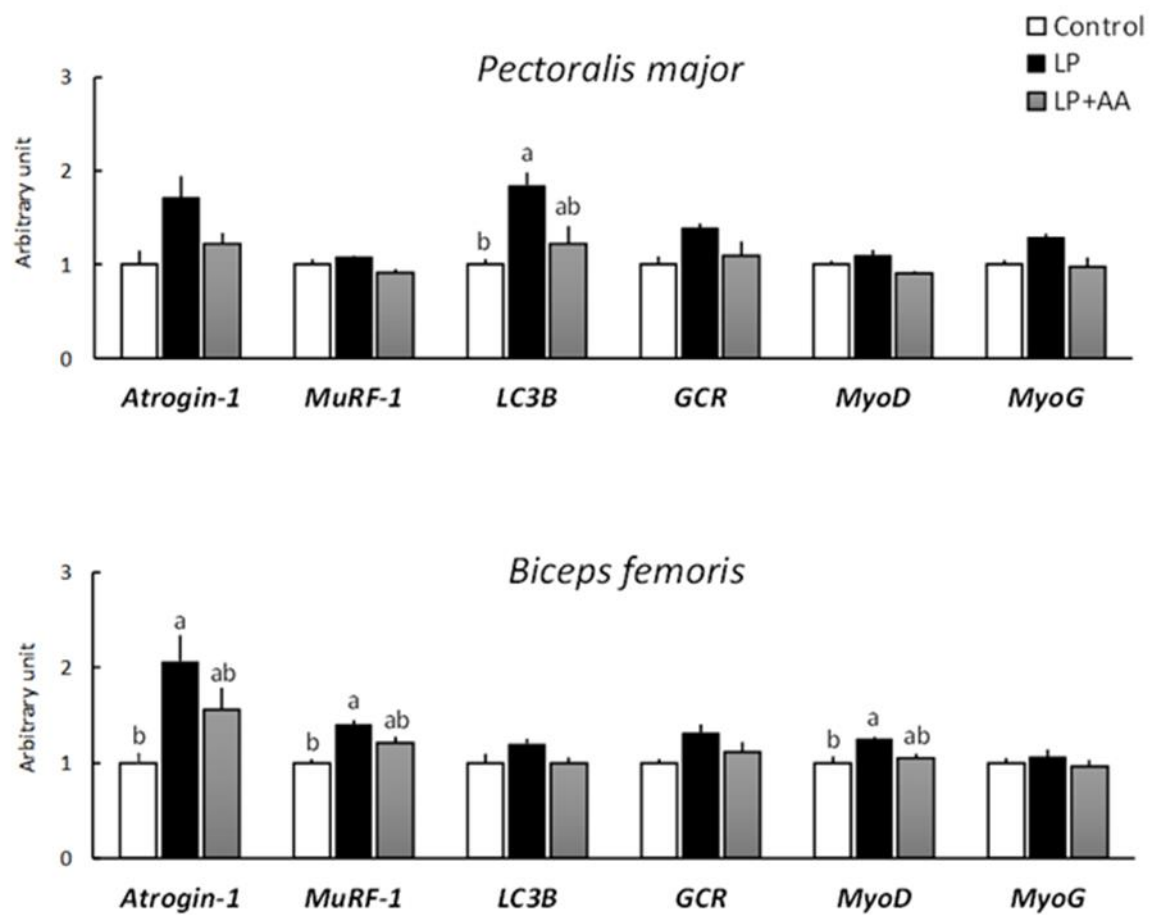
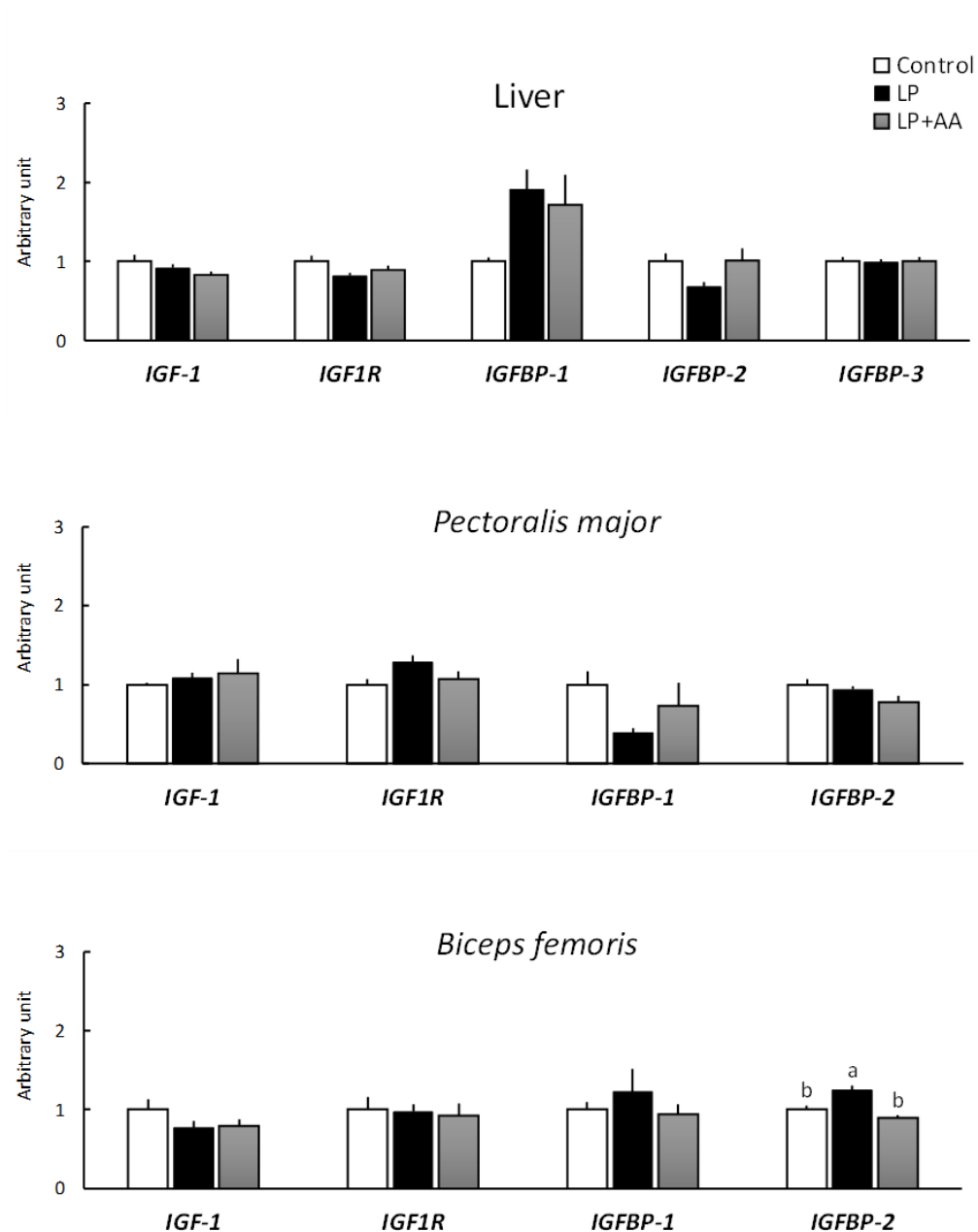


Fig. 2



Summary

Chicken meat is an affordable, nutritious protein source that is gaining popularity worldwide, especially among budget-conscious consumers. To meet the global challenges associated with food security, sustainability, and environmental change, strategies are needed to decrease nitrogen excretion and improve nitrogen retention in chicken meat production. However, to further develop this nutritional strategy, the regulatory mechanisms of protein metabolism in chickens need to be elucidated in detail.

Skeletal muscle mass increases when the overall rates of protein synthesis exceed the rates of protein degradation. This process is regulated by circulating hormones such as IGF-1, insulin, and glucocorticoids. Furthermore, evidence suggests that skeletal muscle-derived IGF-1 and IGF binding protein (IGFBP) play crucial roles in regulating muscle growth and regeneration in mammals.

In the present study, the expression of protein metabolism-related genes including IGF-related genes in the skeletal muscles of broiler and layer chicks was investigated, particularly in response to fasting and an LP diet.

In Chapter 1, the background was introduced, emphasizing the need to clarify the physiological roles of skeletal muscle IGFBPs under fasting and LP diet conditions in chickens.

In Chapter 2, the effects of fasting on the expression of IGFBP genes in the pectoralis major (PM) and biceps femoris (BF) muscles of broiler and layer chicks were examined. Twelve hours of fasting significantly increased the mRNA levels of IGFBP-1 in the BF of both chick types. The mRNA levels of IGFBP-2 were significantly increased in both the PM and BF of layer chicks, but only slightly increased in the muscles of broiler chicks. Fasting significantly decreased the mRNA levels of IGFBP-3

in both the PM and BF of both chick types. The mRNA levels of IGFBP-4 and -5, however, did not show consistent responses in both the PM and BF of layer and broiler chicks. Next, the effects of IGF-1 treatment on IGFBP genes were examined in primary CEM cultures derived from broiler or layer muscles. The mRNA levels of most IGFBP genes were not influenced by IGF-1 in the CEMs, suggesting that skeletal muscle IGFBPs are transcriptionally regulated in an IGF-1-independent manner in chickens. Overall, these findings suggest that the physiological roles of IGFBP-1, -2, and -3 are conserved between the two types of chickens and the two types of skeletal muscles.

In Chapter 3, the effects of a 15% low protein (LP) diet with or without amino acid (AA) supplementation on the growth performance of broiler chicks from 10 to 35 days of age and the underlying mechanism for loss of skeletal muscle mass were investigated. Thirty-six male broiler chicks were allocated into three experimental groups based on body weight: control, LP, and essential AA-supplemented LP (LP+AA). The body weight gain, feed conversion ratio, and weights of the breast muscles and legs significantly decreased only in the LP group at the end of the feeding period. Plasma uric acid levels were significantly lower in the LP+AA group than in the other groups. In the LP group, the mRNA levels of LC3B were significantly higher in the PM, whereas those of atrogin-1, MURF-1, MyoD, and IGFBP-2 were significantly higher in the BF, compared with those in the control group. There were no significant differences in IGF-1 mRNA levels in the liver or skeletal muscle between groups. These findings suggested that supplementation with essential AAs ameliorated the impaired effects of an LP diet on growth performance in broiler chicks and that the transcriptional changes in IGFBP-2, atrogin-1, and MyoD in the BF and LC3B in the PM are related to the impaired effects of the LP diet.

In conclusion, this work showed that dietary nutrient availability significantly influences transcriptional changes in IGFBP-1, -2, and -3 in the skeletal muscles of

chickens. Dietary protein availability and AA supplementation influenced IGFBP-2, atrogin-1, and MyoD gene expression only in the BF and LC3B gene expression only in the PM of broiler chicks. These findings may contribute to the development of feeding strategies that harness nutrigenomic approaches to improve chicken growth, optimize feed utilization, and reduce nitrogen waste, ultimately promoting more efficient and sustainable poultry production.

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