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(Citation)
Animal Science Journal,88(11):1779-1787

(Issue Date)
2017-11

(Resource Type)
journal article

(Version)
Accepted Manuscript

The IGF-1/Akt/S6 pathway and expressions of glycolytic myosin heavy chain isoforms are upregulated in chicken skeletal muscle during the first week after hatching.

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Running title: Protein synthesis in skeletal muscle of newly hatched chicks

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Abstract

Skeletal muscle mass is an important trait in animal industry. We previously reported an age-dependent downregulation of the IGF-1/Akt/S6 pathway, major protein synthesis pathway, in chicken breast muscle after 1 week of age, despite a continuous increase of breast muscle weight. Myosin heavy chain (HC), a major protein in muscle fiber, has several isoforms depending on chicken skeletal muscle types. HC I (fast-twitch glycolytic type) is known to be expressed in adult chicken breast muscle. However, little is known about the changes in the expression levels of protein synthesis-related factors and HC isoforms in perihatching chicken muscle. In the present study, protein synthesis-related factors, such as IGF-1 mRNA levels, phosphorylation of Akt, and phosphorylated S6 content, increased in an age-dependent manner after post-hatch day (D) 0. The mRNA levels of HC I, III, and V (fast-twitch glycolytic type) dramatically increased after D0. The increase ratio of breast muscle weight was approximately 1,100% from D0 to D7. To our knowledge, these findings provide the first evidence that upregulation of protein synthesis pathway and transcription of fast twitch glycolytic HC isoforms play critical roles in the increase of chicken breast muscle weight during the first week after hatching.

Keywords: myosin heavy chain, perihatching chick, protein synthesis, skeletal muscle

1. Introduction

Broiler chickens have been genetically selected for growth rate and skeletal (especially breast) muscle weight (Arthur and Alber, 2003), resulting in larger number and diameter of muscle fiber than in layer chickens (Scheuermann et al., 2004). Since the total number of muscle fibers is set at hatching (Smith, 1963; Velleman, 2007), the posthatch growth of muscle is mainly attributable to muscle fiber enlargement (Velleman, 2007). For that to occur there must be an increase in protein synthesis (Velleman, 2007). Therefore, to clarify how the protein synthesis are regulated in posthatch skeletal muscle is important in poultry meat production industry.

The increase in muscle fiber size requires more nuclei for upregulated protein synthesis through the promotion of transcription and translation (Velleman, 2007). The additional nuclei are provided from satellite cells located underneath the basement membrane of the myofiber; satellite cells proliferate and then fuse with the existing myofibers (Mauro, 1961; Moss and LeBlond, 1971). Post-hatch day (D) 2 and 3 show the greatest number of satellite cells after which there is a subsequent decrease (Sobolewska et al., 2011), suggesting that the additional nuclei are actively provided to the existing myofibers and consequently, protein synthesis may be upregulated after D3 in chicken skeletal muscle.

Lines of evidence suggest that the IGF-1-induced phosphorylation of Akt and ribosomal protein S6 (S6) are critical events in protein synthesis in mammals (Glass, 2005; Sandri, 2008; Schiaffino *et al*., 2013; Shah *et al*., 2000). Phosphorylated S6 (p-S6) can be used as a marker of translational efficiency in muscle fibers (Goodman *et al*., 2012). Although IGF-1 is known to be synthesized in both the skeletal muscle and liver, previous studies using transgenic mice indicated that skeletal IGF-1 plays a major role in skeletal muscle growth (Sjögren et al., 1999; Yakar et al., 1999).

Previous studies in suckling rats (between 1 and 28 days postpartum) and chickens (between 1 and 6 weeks) showed that fractional protein synthesis rates decreased age-dependently (Davis et al., 1989; Kang et al., 1985), although skeletal muscle weight continuously increased. In addition, we previously reported that IGF-1 expression, phosphorylation rate of Akt, and pS6 content decreased coordinately in chicken breast muscle from 1 to 7 weeks of age (Saneyasu *et al*., 2016). These findings likely suggest the age-dependent downregulation of IGF-1/Akt/S6 pathway in chicken skeletal muscle during the first week after hatching. However, it remains to be elucidated how the IGF-1/Akt/S6 pathway is regulated in neonatal chicken skeletal muscle.

Myosin heavy chain (HC) is a major component of muscle fiber, and several isoforms have been identified in chickens (Rushbrook *et al*., 1997). The adult breast muscle (*pectoralis major*) consists of HC I only (Rushbrook *et al*., 1997, 1998). In contrast, several HC isoforms were shown to be expressed during the embryonic period; HC VI, VII, and IV were the major isoforms on embryonic day (E) 12, E14, and E19, respectively (Rushbrook *et al*., 1998). Furthermore, the post-hatch isoform HC V was reported to be expressed during D3 to D12 (Rushbrook *et al*., 1998). However, it remains unclear how the expression levels of HC isoforms (especially HC I, the major isoform in adults) change in chicken breast muscle during the perihatching period.

In an effort to understand the changes in protein synthesis and expression of HC isoforms in neonatal chicken muscle, we investigated the mRNA and protein levels of the protein synthesis-related factors and HC isoforms in the breast muscle of perihatch chickens. Our results indicate that IGF-1-stimulating protein synthesis pathway and transcription of HC I and V are markedly upregulated after hatching.

2. Materials and Methods

Animals and sampling

Commercial broiler (Ross308) eggs were purchased from a local hatchery (Ishii Poultry Farming Cooperative Association, Tokushima, Japan) and incubated at 37.5°C. After hatching, the chicks were given free access to water and a commercial starter diet (23.5% crude protein and 3,050 kcal/kg metabolizable energy; Nippon Formula Feed Mfg. Co. Ltd., Kanagawa, Japan). Six embryos or chicks of each sex were sampled at E18, E20, D0, D3, D5, and D7. D0 (post-hatch) chicks were euthanized before feeding. Body weights were measured, and embryos/chicks were euthanized by decapitation. The yolk sac and *pectoralis major* muscle were excised and measured. The dissected muscles were immediately frozen in liquid nitrogen and stored at -80°C until further analyses. The experimental procedures involving animals were approved by the Institutional Animal Care and Use Committee and carried out according to the Kobe University Animal Experimental Regulation.

Sexing

The sex of chicks was determined as described by Fridolfsson and Ellegren (1999). Briefly, DNA was extracted from approximately 50 mg of *pectoralis major* muscle using Kaneka Easy DNA Extraction Kit for animal tissue (Kaneka Co., Osaka, Japan). The gene encoding chromo-helicase-DNA binding protein (CHD) was amplified using Kaneka High-Speed DNA polymerase (Kaneka Co., Osaka, Japan). PCR products were separated in 2% agarose gel including SYBR® safe Gel stain (Invitrogen, Carsbad, CA) and visualized in a transilluminator. Samples taken from males showed a single band (putatively 600 bp), whereas two different bands (putatively 450 bp and 600 bp) were

observed in those taken from females.

Real-time PCR analysis

Total RNA was extracted from the muscles using Sepazol-RNA I (Nacalai Tesque, Inc., Kyoto, Japan). First-strand cDNA was synthesized from total RNA using ReverTra Ace® qPCR RT Master Mix with gDNA Remover (Toyobo Co. Ltd, Osaka, Japan). mRNA levels were analyzed using Thermal Cycler Dice® Real Time System (Takara Bio Inc., Otsu, Japan) and SYBR Premix Ex Taq II (Tli RNaseH Plus; Takara Bio Inc., Otsu, Japan) according to the manufacturer's recommendations; sequences of the gene-specific primers used during the study are shown in Table 1. The expression levels of target genes were normalized to those of ribosomal protein S17 (RPS17).

Western blot analysis

Frozen muscles were homogenized by polytron in lysis buffer described previously by Duchêne *et al*. (2008). The homogenate were centrifuged at 17,900 g for 15 min at 4°C and the supernatants were stored at -80°C. Protein concentrations were determined by the method of Lowry *et al*. (1951). Muscle lysates were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting using the HorizeBlot (ATTO Co., Tokyo, Japan) according to the manufacturer’s recommendations. Bands were detected using Chemi-Lumi one Super (Nacalai Tesque, Inc., Kyoto, Japan), visualized with the Lumicube (Liponics Inc., Tokyo, Japan) and quantified using CS Analyzer (ATTO Co., Tokyo, Japan). Anti-Akt (#9272), anti-p-Akt (S473) (#9271), anti-S6 (#2217), anti-p-S6 (S240/244) (#5364), anti-α/β-tubulin (#2148), and horseradish peroxidase-conjugated anti-rabbit IgG (#7074) were purchased from Cell Signaling

Technology (Beverly, MA, USA). Band intensity of α/β-tubulin was used as a loading control.

Statistical analysis

Data were analyzed using the Tukey-Kramer method. All statistical analyses were performed using Excel 2013 (Microsoft, USA) with Statcel 3 add-in software (OMS, Tokyo, Japan).

3. Results

Figure 1 shows the changes in the weights of body without yolk sac, yolk sac, and *pectoralis major* muscle in male chicks. Body weight and weight of *pectoralis major* muscle increased in an age-dependent manner during the experimental period, whereas weight of yolk sac decreased in an age-dependent manner. A marked increase was observed in breast muscle weight from D3 to D5. Thus, we examined the changes in the expression levels of myogenic regulatory factors (MRFs), protein synthesis-related factors and HC isoforms in the breast muscle.

The MRF MyoD is expressed in the activated and proliferating satellite cells (Bentzinger *et al*., 2012; Legerlotz and Smith, 2008; Sobolewska *et al*., 2011), and activates the transcription of muscle-specific genes such as myogenin (Berkes et al., 2004) and HC (Beylkin *et al*., 2006; Wheeler *et al*., 1999). Myogenin is also categorized as a member of the MRFs and induces the fusion of myocytes (Nabeshima *et al*., 1993; Bentzinger *et al*., 2012). In the present study, MyoD mRNA levels increased in an age-dependent manner from E18 to D3 and maintained the highest level until D7 (Fig. 2A). In agreement with the results of previous studies (Halevy *et al*., 2004; Sobolewska *et al*., 2011),

myogenin expression peaked on D3 (Fig. 2B). These results likely suggest an increasing number of activated satellite cells (E18-D3) and their fusion with myofibers (at approximately D3).

The expression of breast muscle IGF-1 increased in an age-dependent manner after hatching (Fig. 3A). Phosphorylation of Akt and S6, and p-S6 contents increased in an age-dependent manner (Fig. 3C, D and E) in association with the changes in IGF-1 expression, although the total S6 contents, which is used as translational capacity (Goodman et al., 2012), did not change age-dependently (Fig. 3E). These results suggest that the IGF-1/Akt/S6 signaling pathway is upregulated in chicken breast muscle during the first week post-hatch.

Myostatin was reported to inhibit the IGF-1/Akt signaling pathway in mammals (Morissette *et al*., 2009; Gumucio and Mendias, 2013). In chicken, the myostatin mRNA level decreased in an age-dependent manner before hatching and gradually increased after hatching in a manner similar to the IGF-1 mRNA levels (Fig. 2B), indicating the possibility that IGF-1 regulates myostatin expression in the skeletal muscle of newly hatched chicks.

Figure 4 shows the mRNA levels of HC and troponin I (Tn I) isoforms. The expression levels of the embryonic/perihatch isoform HC IV and the Tn I type 1 (slow type) decreased significantly in chicken breast muscle after D0 and E20, respectively (Fig. 3C and E). In contrast, significant increases were observed in the mRNA levels of the post-hatch isoform HC V, adult isoform HC I, Tn I type 2 (fast type), and ratio of Tn I type 2 to type 1 (Fig. 3A, D, F, and G). Unexpectedly, HC III mRNA level peaked on D3 (Fig. 3B); previous studies report that this isoform does not appear in the developing and adult *pectoralis major* muscle (Rushbrook *et al*., 1997, 1998). To our knowledge, these findings

provide the first evidence that the expression of isoforms of HC and Tn I changes drastically in chicken breast muscle during the perihatching period.

The weights of body and tissue, and the levels of mRNA and proteins were examined in females as well as in males. They changed in females in a manner similar to those in males (Supplementary Figure S1, S2, S3, and S4).

4. Discussion

Early post-hatch fasting is shown to depress skeletal muscle growth in chickens, resulting in the lower body weight at marketing compared to those exposed to feed earlier (Gonzales *et al*., 2003; Halevy *et al*., 2000; Noy and Sklan, 1997). Previous studies in chickens and turkeys reported that mRNA/protein levels of myogenin, IGF-1, and p-Akt were lower in the breast muscle of unfed birds from D0 to D2 than in those of fed birds (Guernec *et al*., 2004; Halevy *et al.*, 2003). In the present study, the upregulations of myogenin expression and IGF-1/Akt/S6 pathway were observed after feeding. In addition, oral injection of carboxymethylcellulose to neonatal chick failed to increase the IGF-1 mRNA level in the breast muscle (unpublished data). All these findings suggest that initial intake of exogenous nutrients except fiber is one of the triggers to stimulate post-hatch growth via upregulation of the fusion of activated satellite cells and IGF-1/Akt/S6 pathway in the skeletal muscle of newly hatched chicks.

In contrast to the results of a previous study (Saneyasu et al., 2016), IGF-1/Akt/S6 pathway is upregulated in chicken breast muscle during the first week after hatching. The reason for the different pattern of changes in the basal level of IGF-1/Akt/S6 pathway before and after 1 week of age in chicken skeletal muscle remains unclear. However, our present and previous studies shows that the increase ratio of the *pectoralis major* muscle

weight is the highest during the first week after hatching: 80% (E18 to D0), 1,100% (D0 to D7), 495% (D7 to D14), 404% (D14 to D28), and 171% (D28 to D49) (Fig. 1, 3, and Saneyasu et al., 2016). It is therefore reasonable to consider that the IGF-1/Akt/S6 is upregulated in chicken skeletal muscle from hatching to 1 week of age.

Myostatin has been demonstrated to be a specific target of MyoD in *in vitro* study using C2C12 cells (Spiller *et al*., 2002). However, our data did not show any correlating changes in mRNA levels of MyoD and myostatin. Previously, an *in vitro* study on the differentiation of primary chicken myoblasts into the myotube showed that myostatin expression was enhanced by IGF-1 (Kurokawa *et al*., 2009). Our data shows that changes in mRNA levels of myostatin and IGF-1 were coordinated after D0, in agreement with previous study (Guernec *et al*. 2004). It is therefore likely that IGF-1 regulates myostatin transcription in chicken muscle post-hatch.

The postnatal development of muscle fiber type has been studied histochemically in chicken pectoral muscle (Ashmore and Doerr, 1971). At hatching, a majority of these fibers have many large diformazan granules. Neutral lipid droplets are large and numerous inside fibers that have higher mitochondrial density (Ashmore and Doerr, 1971). By 4 days of age, mitochondria and lipid-rich muscle fibers are clearly in a minority, these fibers being transformed rapidly into mitochondria-poor fibers in which histologically visible lipid is absent (Ashmore and Doerr, 1971). However, the mechanism underlying this histochemical change in the muscle fiber after hatching remains unclear. Previous studies reported that HC I, III, and V appeared to be associated with fast-twitch glycolytic fiber, and that HC IV appeared to be associated with fast-twitch-oxidative fiber (Barnard *et al*., 1982; Rushbrook *et al*., 1997, 1998). Therefore, we provide the first evidence on the drastic change in gene expressions of HC and Tn I

isoforms from oxidative to glycolytic type in the breast muscle of perihatching chicks, and suggest that this change may underlie the histochemical changes in the muscle fiber during the first week after hatching.

It remains unclear how expression of HC isoforms is regulated during the perihatching period in chicken muscle. The present results show a coordinated increase in the mRNA levels of IGF-1 and glycolytic type HC post-hatch and a preceding increase in MyoD mRNA level during perihatching period. *In vitro* studies using the C2C12 myotubes have shown that IGF-1 and MyoD activate the type IIB (fast-twitch glycolytic type) HC promotor (Shanely et al., 2009; Wheeler et al., 1999). Thus, our findings raise the possibility that IGF-1 and/or MyoD promote transcription of HC I, III, and V in the skeletal muscle of newly hatched chicks.

In conclusion, we show that protein synthesis pathway and transcription of fast-twitch glycolytic type HC are upregulated in an age-dependent manner in chicken breast muscle during the first week after hatching. These findings can provide new insights for further research on skeletal muscle growth in newly-hatched chicks, e.g., how protein synthesis can be enhanced in skeletal muscles after hatching, thereby contributing to improved industrial poultry meat productivity.

Acknowledgment

This work was supported by JSPS KAKENHI Grant Number 15K18770.

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Figure legends

Figure 1. Changes in the weights of body, yolk sac, and skeletal muscle in perinatal

male chicks. Data are expressed as mean ± SEM (n = 6). The Tukey-Kramer method was used to analyze differences. Groups with different letters were significantly different ($P < 0.05$).

Figure 2. Changes in mRNA levels of MyoD and myogenin in breast muscles of male chicks. Data are expressed as mean ± SEM (n = 6). The Tukey-Kramer method was used to analyze differences. Groups with different letters were significantly different ($P < 0.05$).

Figure 3. Changes in mRNA levels of IGF-1 and myostatin, phosphorylation of Akt and S6, and contents of phosphorylated and total S6 in breast muscles of male chicks. Data are expressed as mean ± SEM (n = 6). The Tukey-Kramer method was used to analyze differences. Groups with different letters were significantly different ($P < 0.05$).

Figure 4. Changes in mRNA levels of myosin heavy chain and troponin I isoforms in breast muscles of male chicks. Data are expressed as mean ± SEM (n = 6). The Tukey-Kramer method was used to analyze differences. Groups with different letters were significantly different ($P < 0.05$). HC, myosin heavy chain

孵化後 1 週間におけるニワトリ骨格筋の IGF-1/Akt/S6 経路及び解糖型ミオシン重鎖アイソフォームの遺伝子発現量は加齢依存的に上向き調節される

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骨格筋量は畜産業において重要な形質である。これまでに我々は、ニワトリのムネ肉重量は増加し続けるにもかかわらず、ムネ肉における IGF-1/Akt/S6 経路は 1 週齢以降加齢依存的に下向き調節されることを報告している。骨格筋線維中の主要なタンパク質であるミオシン重鎖（HC）には複数のアイソフォームが存在し、骨格筋の種類によって発現するアイソフォームが異なる。また、成鶏のムネ肉には HC I のみが発現していることが知られている。しかしながら、孵化前後のニワトリ骨格筋におけるタンパク質合成関連因子や HC アイソフォームの発現変動については殆ど知られていない。本研究では、タンパク質合成因子である、IGF-1 mRNA 量、Akt のリン酸化率、及びリン酸化 S6 タンパク質量は 0 日齢以降、加齢依存的に増加した。解糖型速筋線維型である HC I, III 及び V の mRNA 量は孵化後増加した。また、ムネ肉重量の増加率は 0 日齢から 7 日齢にかけて約 1,100%であった。これらの結果から、ニワトリのムネ肉では、タンパク質合成経路と解糖型速筋線維型 HC アイソフォームの転写は孵化後 1 週間において上向き調節されることが示唆された。

Table 1. Primer sequences used for real-time PCR analysis

Gene name	Forward primer	Reverse primer	Accession number
MyoD	5’-gga atc acc aaa tga ccc aaa g-3’	5’-ggc agt cga ggc tgg aaa c-3’	NM_204214
Myogenin	5’-ggg aga agc gga ggc tga ag-3’	5’-cag agt gct gcg ttt cag agc c-3’	NM_204184
IGF-1	5’-gct gcc ggc cca gaa-3’	5’-acg aac tga aga gca tca acc a-3’	NM_001004384
Myostatin	5’-atg cag atc gcg gtt gat c-3’	5’-gcg ttc tct gtg ggc tga ct-3’	NM_001001461
HC I[#]	5’-aga gaa gat gtg ccg cac tct gga gga c-3’	5’-gta aat ccc tgc ttg cct cta gag-3’	
HC III[#]	5’-gcg gat gat caa tga cct caa tgc t-3’	5’-ctc ttg gcc ttt atc tcc tcc tct aaa tgc-3’	
HC IV[#]	5’-cct gga gaa gat tgc cgc aca ct-3’	5’-gag taa atg cct gtt tgc ctc ttg ata ac-3’	
HC V[#]	5’-acg gat gat caa tga tgt taa tgc t-3’	5’-agc aga ctg caa acc atg ggc t-3’	
Tn I type 1	5’-tga gcc aac tcc agg atc tgt-3’	5’-tct tcg tcc aca atc tca acc tt-3’	XM_004934839
Tn I type 2	5’-gtg ggc atg tct gct gat g-3’	5’-cat gtt gac ctt gtg ctt gga-3’	NM_205417
RPS17	5’-gcg ggt gat cat cga gaa gt-3’	5’-gcg ctt gtt ggt gtg aag t-3’	NM_204217

IGF-1, insulin like growth factor-1; HC, myosin heavy chain; RPS17, ribosomal protein S17; Tn I, troponin I. The primers of IGF-1, myostatin, RPS17 were used in previous studies (Saneyasu *et al*., 2015, 2016). [#], Refer to corresponding nucleotide sequences reported by Rushbrook *et al*. (1997, 1998).

Figure 1

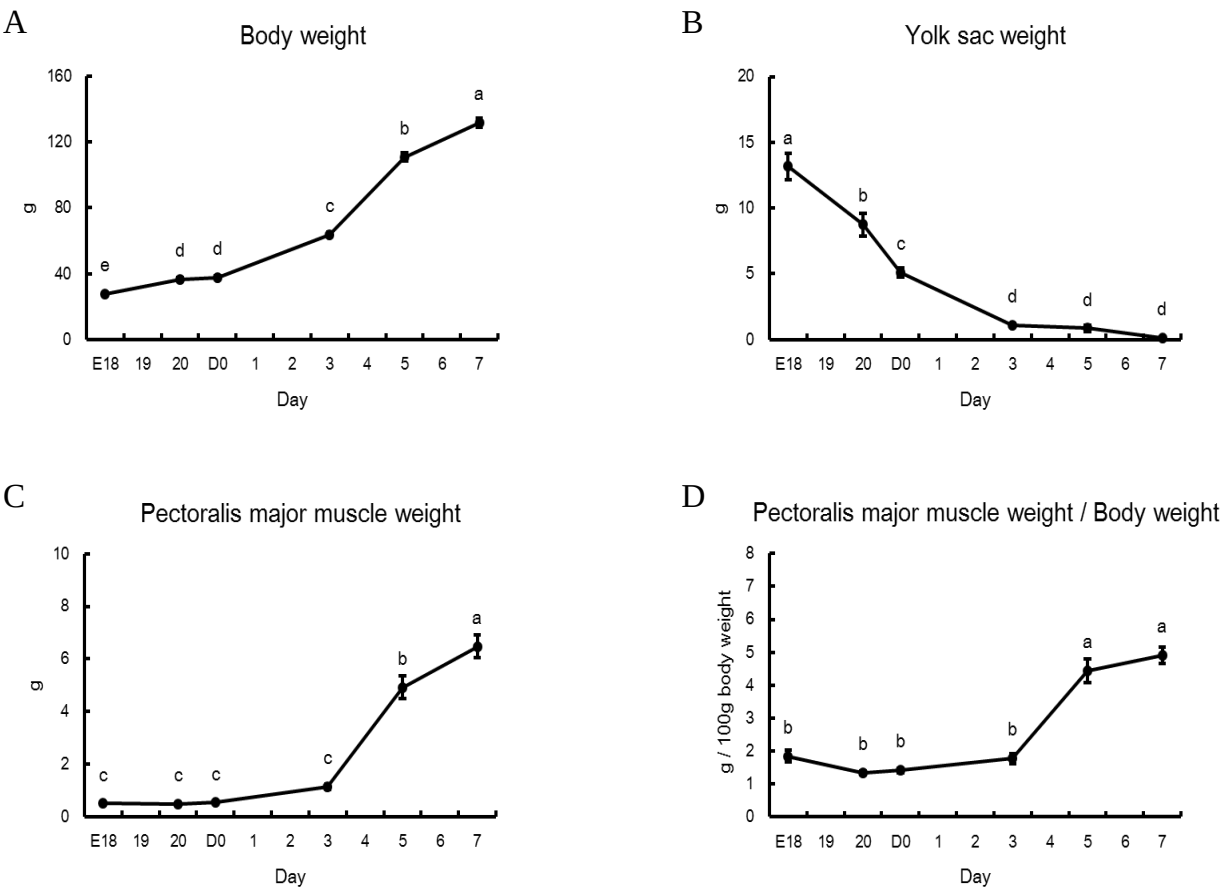

A
Body weight
160
120
80
40
0
g
e
d
d
c
b
a
E18 19 20 D0 1 2 3 4 5 6 7
Day
B
Yolk sac weight
20
15
10
5
0
g
a
b
c
d
d
d
E18 19 20 D0 1 2 3 4 5 6 7
Day
C
Pectoralis major muscle weight
10
8
6
4
2
0
g
c
c
c
c
b
a
E18 19 20 D0 1 2 3 4 5 6 7
Day
D
Pectoralis major muscle weight / Body weight
8
7
6
5
4
3
2
1
0
g / 100g body weight
b
b
b
b
a
a
E18 19 20 D0 1 2 3 4 5 6 7
Day

Figure 2

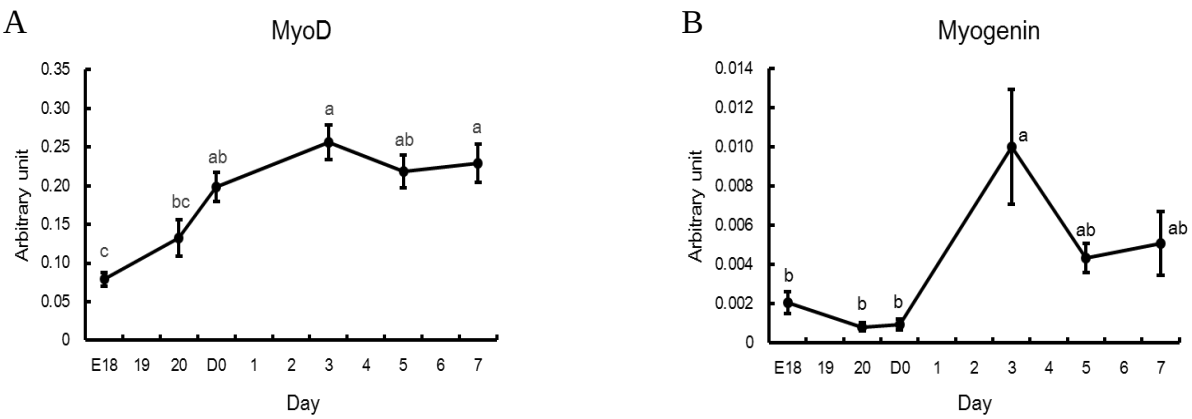

A
MyoD
Arbitrary unit
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0
c
bc
ab
a
ab
a
E18 19 20 D0 1 2 3 4 5 6 7
Day
B
Myogenin
Arbitrary unit
0.014
0.012
0.010
0.008
0.006
0.004
0.002
0
b
b
b
a
ab
ab
E18 19 20 D0 1 2 3 4 5 6 7
Day

Figure 3

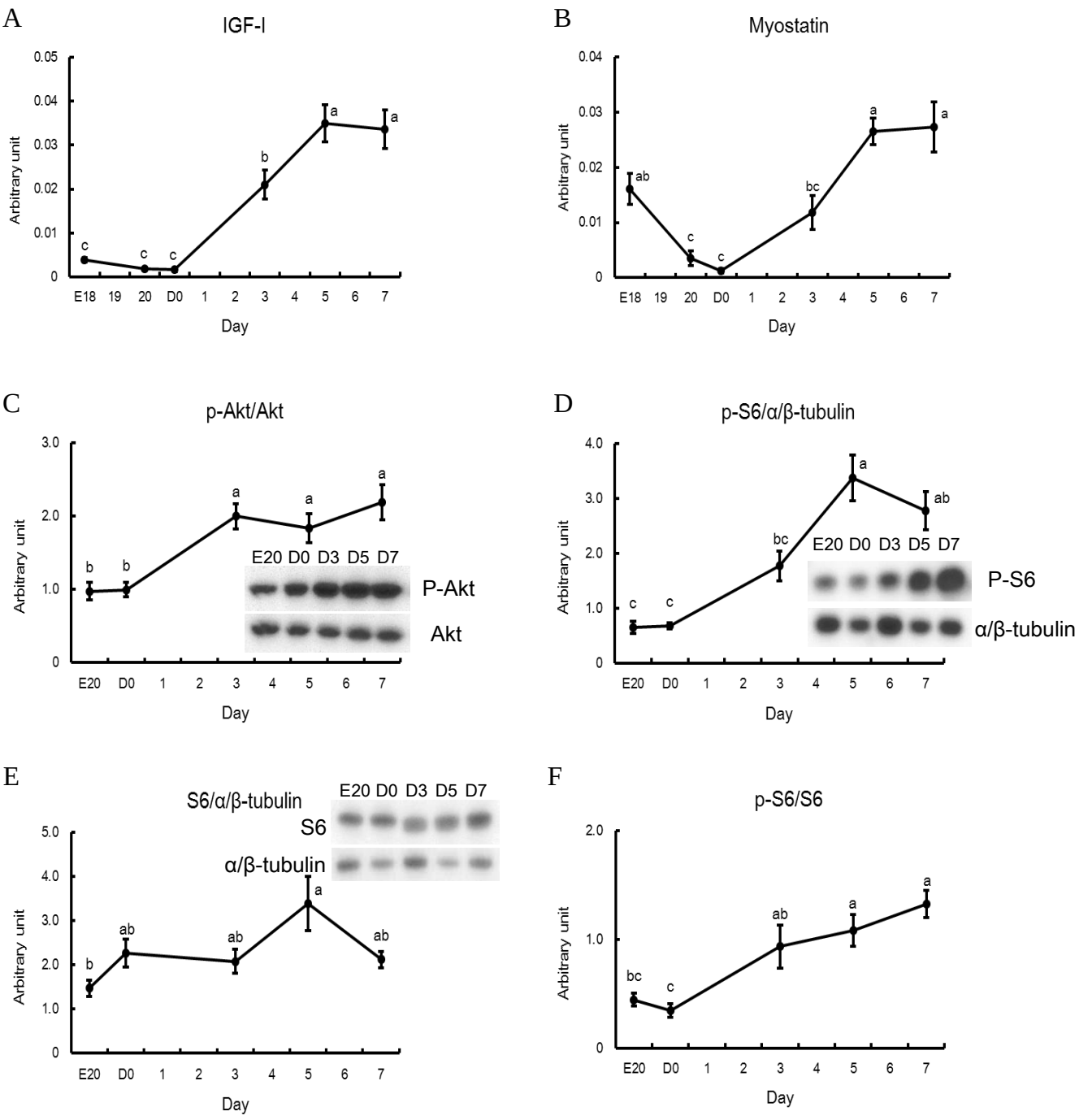
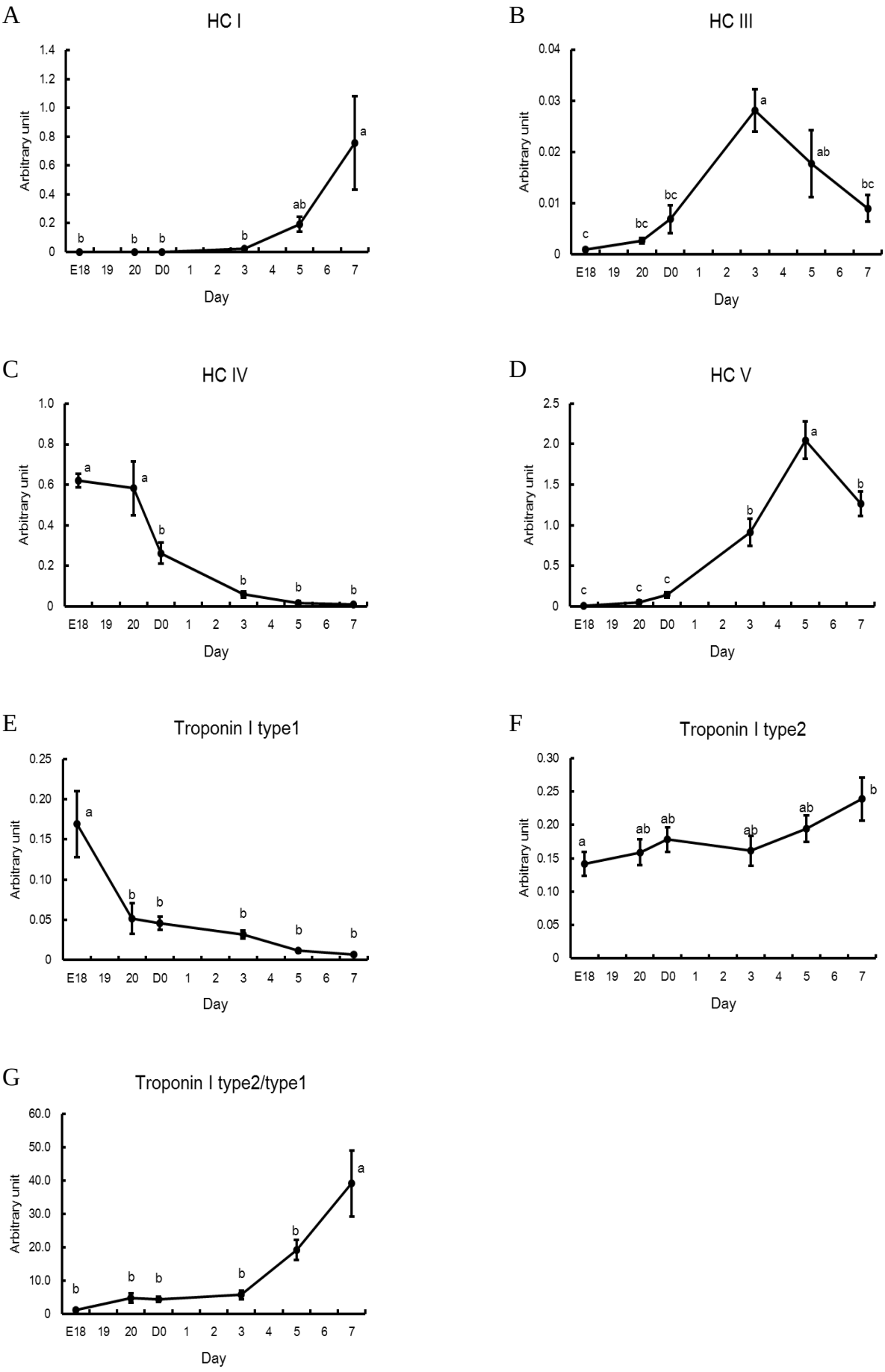
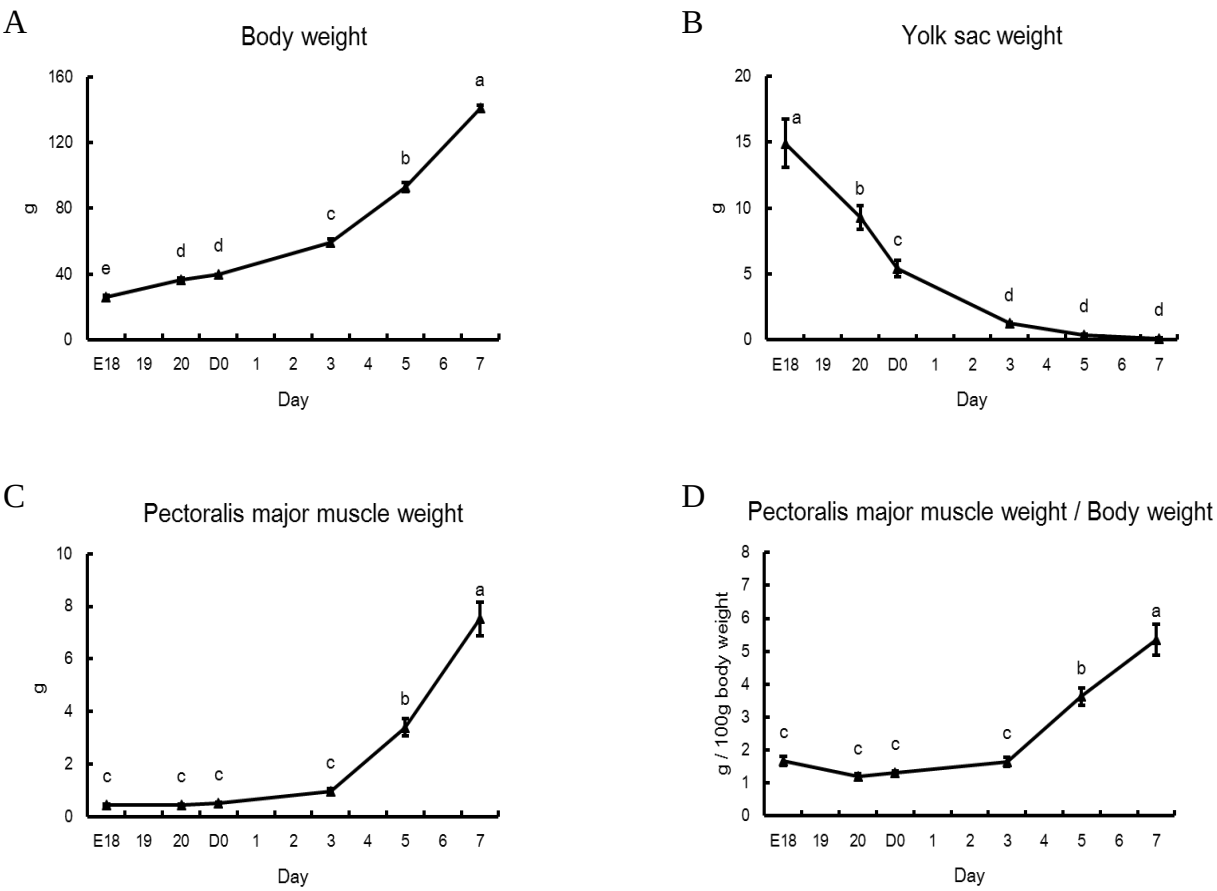

A
IGF-I
Arbitrary unit
Day
B
Myostatin
Arbitrary unit
Day
C
p-Akt/Akt
Arbitrary unit
E20 D0 D3 D5 D7
P-Akt
Akt
Day
D
p-S6/α/β-tubulin
Arbitrary unit
E20 D0 D3 D5 D7
P-S6
α/β-tubulin
Day
E
S6/α/β-tubulin
E20 D0 D3 D5 D7
S6
α/β-tubulin
Arbitrary unit
Day
F
p-S6/S6
Arbitrary unit
Day

Figure 4


A
HC I
Arbitrary unit
Day
B
HC III
C
HC IV
D
HC V
E
Troponin I type1
F
Troponin I type2
G
Troponin I type2/type1
E18 19 20 D0 1 2 3 4 5 6 7

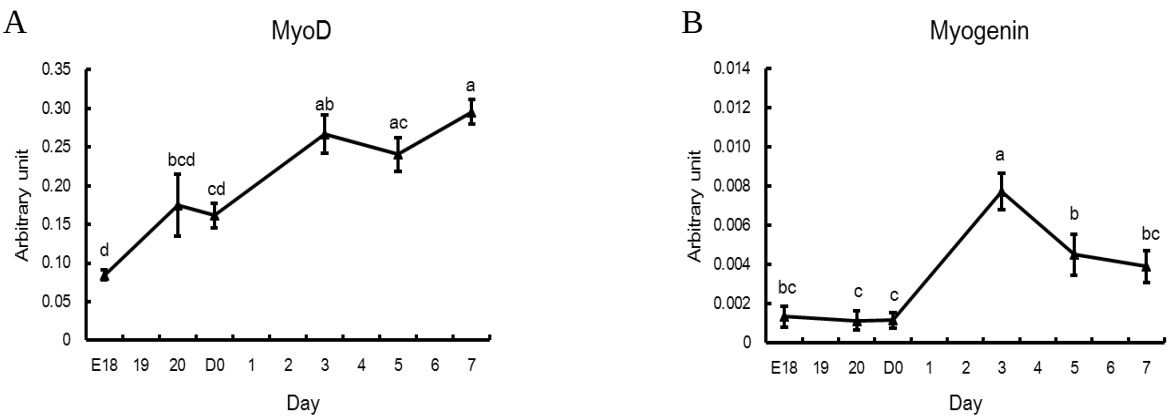
Supplementary Figure S1



Supplementary Figure 1. Changes in the weight of body, yolk sac, and skeletal muscle in perinatal female chicks.

Data are expressed as mean ± SEM (n = 6). The Tukey-Kramer method was used to analyze differences. Groups with different letters were significantly different ($P < 0.05$).

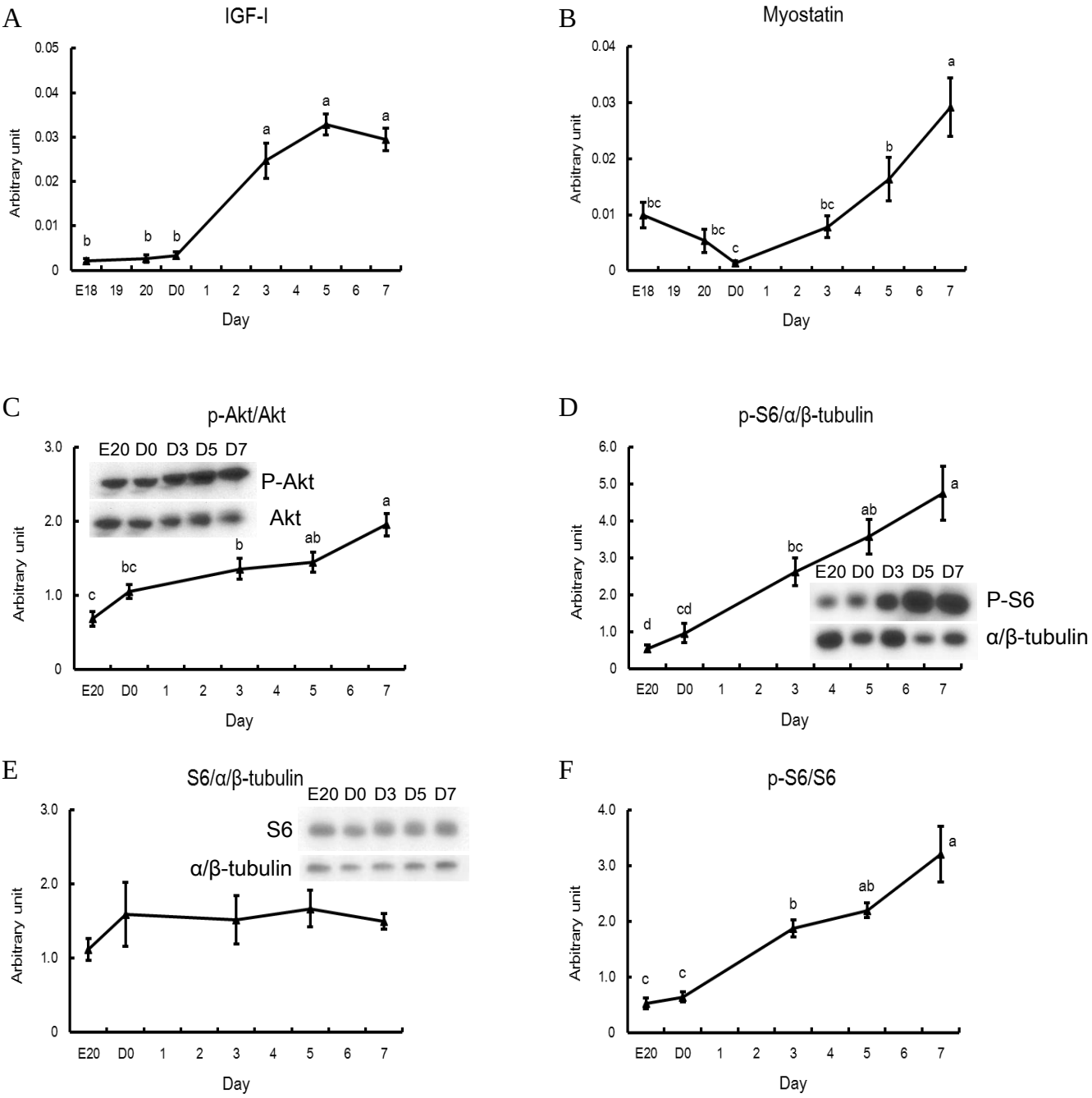
Supplementary Figure S2



Supplementary Figure 2. Changes in mRNA levels of MyoD and myogenin in breast muscles of female chicks.

Data are expressed as mean ± SEM (n = 6). The Tukey-Kramer method was used to analyze differences. Groups with different letters were significantly different ($P < 0.05$).

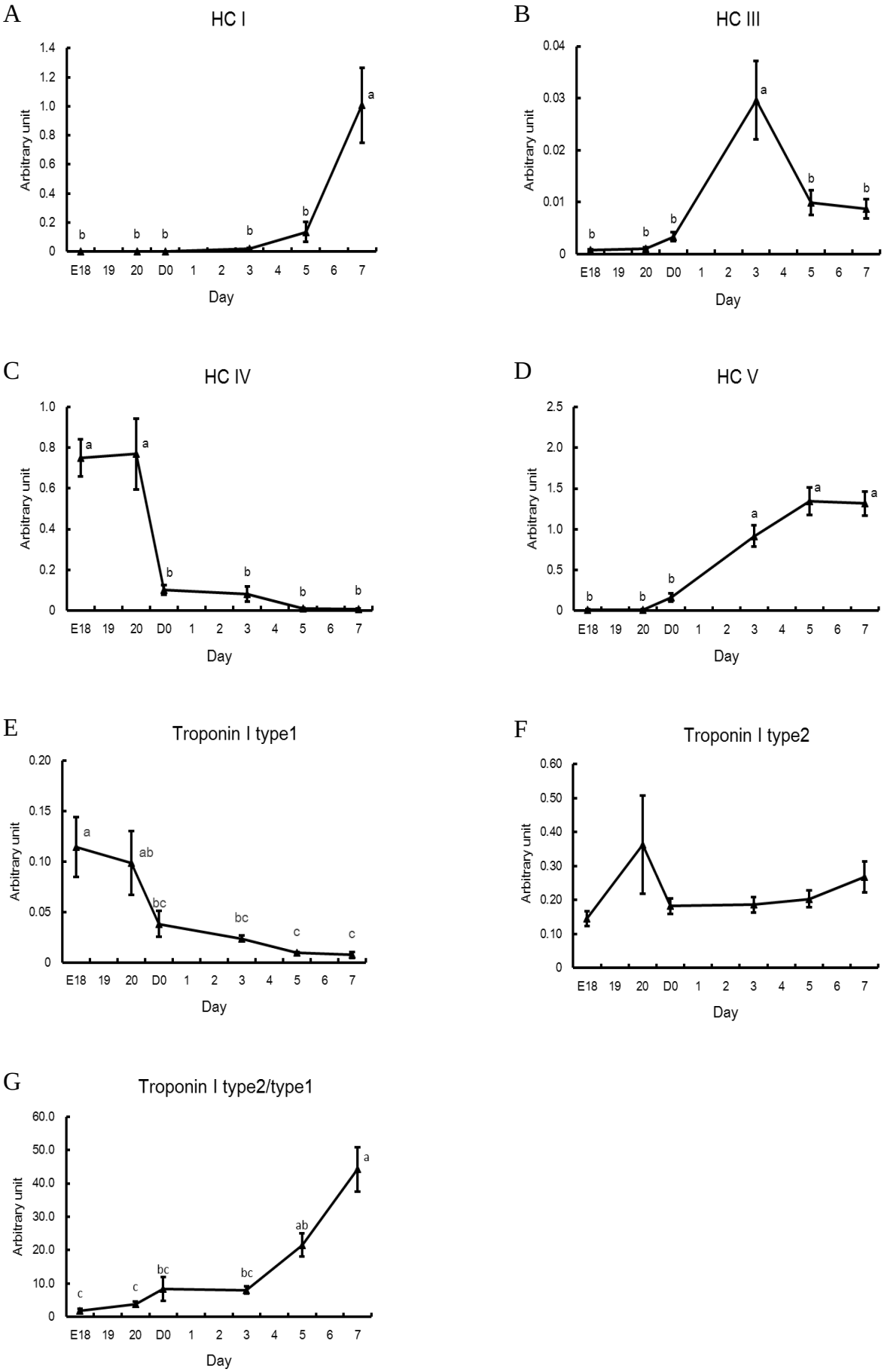
Supplementary Figure S3



Supplementary Figure 3. Changes in IGF-1 mRNA, phosphorylation of Akt, and phosphorylated S6 content in breast muscles of female chicks.

Data are expressed as mean ± SEM (n = 6). The Tukey-Kramer method was used to analyze differences. Groups with different letters were significantly different ($P < 0.05$).

Supplementary Figure S4



Supplementary Figure 4. Changes in mRNA levels of myosin heavy chain isoforms in breast muscles of chicks. Data are expressed as mean ± SEM (n = 6). The Tukey-Kramer method was used to analyze differences. Groups with different letters were significantly different ($P < 0.05$). HC, myosin heavy chain.