JPSA
Japan Poultry Science Association

http://www.jstage.jst.go.jp/browse/jpsa
doi:10.2141/jpsa.2024011

Sex differences in *COL1A1* Expression and Collagen Content in Skeletal Muscle of Mature and Juvenile Shamo Chickens

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Collagen content is an important parameter affecting meat consistency. Sex differences in collagen were therefore studied in mature and juvenile Shamo chickens. The pectoral (PT), lateral iliotibial (ITL), medial part of puboischiofemoral (PIF), and lateral part of gastrocnemius (GCL) muscles were weighed, and their *COL1A1* expression levels and total collagen content were analyzed. Body and muscle weights were significantly higher in males than in females of all ages. Muscle/body weight ratios were also higher in mature males than in females, but this difference was not observed in juveniles. In mature chickens, *COL1A1* expression was higher in the PIF and GCL muscles; this was not the case in juvenile chicken muscles. Sex differences in collagen content were observed only in the ITLs of mature chickens. A positive correlation between muscle weight and intramuscular collagen content was found for PT and GCL, but not for ITL and PIF, muscles. These results suggest that the sex difference in intramuscular collagen content only occurs in specific muscles and that *COL1A1* expression is not necessarily related to collagen content in mature chickens. Factors that determine the intramuscular collagen content likely differ by muscle type.

Key words: collagen, sex difference, Shamo chicken, skeletal muscle

J. Poult. Sci., 61: jpsa.2024011, 2024

Introduction

Toughness or tenderness is an important factor for consumers when choosing meats. Connective tissue, together with adipose tissue, are components that determine toughness. Collagen is the primary constituent of muscular connective tissue. It is present in the endomysium, perimysium, and epimysium of skeletal muscle. The shear force of broiler chicken breasts, perimysium width, and collagen fiber accumulation in the endomysium and perimysium all increase with growth[1–3]. This suggests that the toughness of chicken meat is related to increased collagen fibers in connective tissue.

Studies on the characteristics of intramuscular collagen content and fiber architecture have been conducted in several chicken breeds. It has also been shown that collagen content varies between muscles. The pectoral (PT) muscle has lower collagen content than the lateral iliotibial (ITL) muscle in adult Silky[4,5] and White Leghorn[4] chickens. In male broilers, PT collagen content shows little change with growth, but ITL shows the highest collagen content at 5 weeks and decreases thereafter[2]. Nutritional differences do not affect collagen content of PT[6], ITL, and puboischiofemoral (PIF) muscles[7] in male broilers. With respect to breed differences, ITL[8] and PT[3] muscles have different collagen content between layer- and meat-type cockerels: greater in latter than in the former in ITL until 10 weeks of age, but not consistent in PT over time.

There are few reports on sex differences in chicken muscle collagen content. In the PT muscle, it is lower in Silky hens than in cockerels but is not different between White Leghorn[4] and Italian local breeds[9]. The ITL muscle in hens has lower collagen content than Silky and White Leghorn cockerels. In broiler chickens, collagen content in the thigh muscle is higher in males than in females, but not in the breast muscle at 14 weeks of age[10]. Considering such differences, it may be useful to examine the difference in gene expression levels of collagen and related stimulating factors to clarify them, but few studies to date have done so.

Received: February 19, 2024, Accepted: April 9, 2024
Available online: April 26, 2024
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Table 1. **Primers used for real-time reverse transcriptional polymerase chain reaction**

Target gene	Accession No.	Primer forward	Primer reverse	Product length (bp)
COL1A1	XM_025144131.1	GAGCGACGGCTTCCAGTTTGAG	GTGTCGTGGTCCATGTAGGC	153
GAPDH	NM_204305.1	ACTGTCAAGGCTGAGAACGG	ACCTGCATCTGCCCATTTGA	99

The Shamo breed is used in cockfighting and for meat; it is cherished in Japan. Although it is rarely used commercially, it has been used as a parent or grandparent stock to produce branded high-quality meat for breed improvement in Japan for its large body size, firm flesh, and low fat content. Therefore, it would be beneficial to determine their intramuscular collagen properties to improve meat quality.

In the present study, we measured sex differences in mRNA expression levels of the alpha-1 type I collagen gene (*COL1A1*) and collagen content in the skeletal muscles of Shamo chickens, which may provide useful information for breed improvement when this chicken breed is selected as paternal or maternal stock.

Materials and methods

Ethics statement

All experiments were performed in accordance with the principles and specific guidelines presented in Guidelines for Animal Experiments by the Ethics Committee of Kyushu University (approval no. A20-028-0).

Chickens

Five male and four female Shamo chickens (29 weeks of age) and five male and five female juvenile chickens (10 weeks of age) were used. They were introduced as young chicks or fertilized eggs by the Fukuoka Agriculture and Forestry Research Center (Fukuoka, Japan) at the Poultry Breeding and Experiment Facility of the Faculty of Agriculture, Kyushu University, Japan. They were reared in a 2 × 1.5-m pen and fed commercial feed (Power chick ZK and Power Layer) purchased from JA Kitakyushu Kumiai Shiryo K.K. (Fukuoka, Japan) and water *ad libitum*. The floor was covered with wood chips or sawdust. After measuring body weight (BW), they were euthanized by exsanguination from the carotid artery under unconsciousness induced an intravenous injection of sodium pentobarbitone. While the dosage was set at 150 mg/kg BW, the required amount differed between individuals. PT, ITL, medial part of the PIF, and lateral part of the gastrocnemius (GCL) muscles were excised from the right side of each carcass and weighed after removing peripheral adipose tissue and tendons.

Real-time RT-PCR

Tissue fragments were excised from the centers of the muscles. Total RNA was isolated from them using ISOGEN II (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. Genomic DNA was removed using DNase I (Nippon Gene). Real-time RT-PCR was performed in duplicate using a One Step TB Green PrimeScript RT-PCR Kit II (TaKaRa Bio, Otsu, Japan). Primers were designed based on sequences from the National Center for Biotechnology Information (NCBI), listed in Table 1. PCR was performed using an Mx3000P QPCR system (Agilent Technologies, Inc., Santa Clara, CA, USA) under the following conditions: 42°C for 5 min, 95°C for 10 s; then 40 cycles of 95°C for 5 s and 60°C for 34 s; then 95°C for 15 s, 60°C for 1 min, and 95°C for 15 s as per the manufacturer's protocol. Data were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression.

Total collagen assays

Approximately 100 mg of tissue piece from each muscle was cut from the center of the muscle without epimysium, frozen in liquid nitrogen, and stored at −20°C. The Total Collagen Assay Kit (Quick-Zyme Biosciences B.V., Leiden, The Netherlands) was used following the manufacturer's protocol. Briefly, each sample was placed in 1 mL of 6 mol/L HCl and incubated for 20 h at 95°C. After cooling, samples were centrifuged for 10 min at 13,000 *g*. The supernatant was diluted 1.5 times with water. After adding assay buffer from the kit, samples were incubated for 20 min at room temperature (around 20 °C) and detection reagents were added. After mixing and incubation, absorbance was read at 570 nm using a Multiskan FC (Thermo Fisher Scientific Inc., Waltham, MA, USA). Data are indicated as μg collagen/mg tissue.

Statistical analyses

Sex differences in BW and muscle mass were analyzed using *t*-tests. The Smirnoff–Grubbs rejection test was used to exclude irregular data. Pearson's product-moment correlation coefficients were calculated between muscle weights and intramuscular collagen content. R software (https://www.r-project.org/) was used for all analyses. Data are shown as means ± standard error of the mean.

Results

Body and muscle weights

Body (BW) and muscle weights, as well as their ratios, for mature and juvenile Shamo chickens are shown in Table 2. For both ages, males had significantly higher body and muscle weights than females ($P < 0.01$). Cockerel BW was 1.5 times that of hens, but individual muscle weights in cockerels were 1.8- (PT) to 2.1- (GCL) fold greater than those of hens. On the other hand, juvenile male BW was 1.5 times that of females, as in mature chickens, but male muscle weights ranged from 1.4- (PT) to 1.6- (GCL) fold greater than those of females. Muscle/body weight ratios were significantly higher in cockerels than in hens for all four muscles ($P < 0.01$), but there were no significant sex differences observed for these muscles in juveniles.

COL1A1 expression

From RT-PCR dissociation curves, a single amplicon peak was obtained for each primer pair (Fig. 1). Expression levels in the four skeletal muscles of mature and juvenile chickens are

Table 2. **Body weights, muscle weights, and muscle/body weight ratios in adult and young Shamo chickens**

	n	BW (g)	PT (g)	ITL (g)	PIF (g)	GCL (g)	PT/BW (%)	ITL/BW (%)	PIF/BW (%)	GCL/BW (%)
Cockerel	5	4117±52 ^{x}	202±6.9 ^{x}	61±1.6 ^{x}	13.6±0.46 ^{x}	32.5±0.66 ^{x}	4.91±0.121 ^{x}	1.48±0.038 ^{x}	0.331±0.0104 ^{x}	0.79±0.016 ^{x}
Hen	4	2708±153 ^{y}	107±8.1 ^{y}	31.9±0.56 ^{y}	6.8±0.24 ^{y}	16.0±0.74 ^{y}	4.0±0.25 ^{y}	1.19±0.060 ^{y}	0.25±0.013 ^{y}	0.59±0.039 ^{y}
Juvenile male	5	1623±43 ^{x}	66±1.6 ^{x}	11.0±0.42 ^{x}	4.4±0.17 ^{x}	8.1±0.19 ^{x}	4.05±0.081 ^{x}	0.68±0.027 ^{x}	0.268±0.0079 ^{x}	0.498±0.0054 ^{x}
Juvenile female	5	1098±42 ^{y}	47±2.5 ^{y}	7.6±0.75 ^{y}	3.0±0.15 ^{y}	5.0±0.31 ^{y}	4.27±0.076 ^{x}	0.69±0.046 ^{x}	0.269±0.0083 ^{x}	0.45±0.017 ^{x}

BW: body weight, PT: pectoral muscle, ITL: lateral iliotibial muscle, PIF: medial part of puboischiofemoral muscle, GCL: lateral part of gastrocnemius muscle.
Data are indicated as means ± standard error of the mean.
Cockerel and hen: 29 weeks old, juvenile male and female: 10 weeks old.
^{x,y}: Means with different letters are significantly different between sexes at the same age ($p < 0.01$).

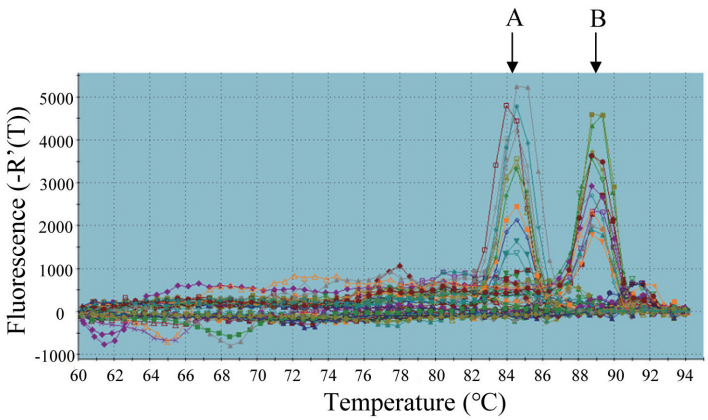

Fig. 1. **An representative dissociation curve from real-time RT-PCR performed on sixty-four samples from pectoral, lateral iliotibial, and gastrocnemius muscles, as well as blanks.** A single peak for *GAPDH* (A) or *COL1A1* (B) mRNA amplicons was observed for each primer pair.

shown in Figure 2. In mature chickens, expression in cockerels was higher in PIF ($P < 0.01$) and GCL ($P < 0.001$) muscles than those in hens, but not in the PT and ITL muscles. However, in juvenile chickens, we observed no significant differences in the expression levels between sexes for any of the four muscles.

Collagen content

Data for the four muscles are shown in Figure 3. In mature chickens, ITL collagen content was significantly higher in cockerels than in hens ($P < 0.05$); however, we observed no sex differences for the other three muscles (Fig. 3A). In contrast, no sex differences in collagen content were observed in juveniles (Fig. 3B). Because the sex difference was limited to a specific muscle in mature chickens, a correlation analysis was performed between muscle weight and intramuscular collagen content for all chickens in this study. These analyses are shown in Figure 4. For the PT and GCL, the positive correlation coefficients were 0.716 ($P < 0.001$) and 0.638 ($P < 0.01$), respectively. In contrast, no significant correlations were observed for the ITL and PIF: 0.265 ($P = 0.273$) and 0.202 ($P = 0.407$), respectively.

Discussion

Chicken body weight is generally higher in males than in females in most breeds and ages[4,11–13]. We observed this sexual dimorphism in both mature and juvenile Shamo chickens. Weights of PT muscles and its ratio to carcass weight in Ross broiler chickens at 82 days of age were not different between sexes[13], different from our juvenile Shamo data. The relative mass of individual pelvic limb muscles in White Leghorn chickens[4,14] was greater in mature males than in females, consistent with the present results in Shamo chickens. In juvenile White Leghorns at 14–16 weeks of age, many muscles are similar in relative mass between sexes, but several pelvic limb muscles, such as the cranial iliotibial, lateral fibular, GCL, pelvic part of the lateral flexor muscle of the crus, and medial femorotibial muscle, are greater in females than in males[14], which is different in juvenile Shamo chickens in this study. In Ross broilers aged 8–10 weeks, the diameter of pectoral muscle fibers did not differ between sexes[15]. In this study, the weights of skeletal muscles and the weight ratios were larger in males than in females for the

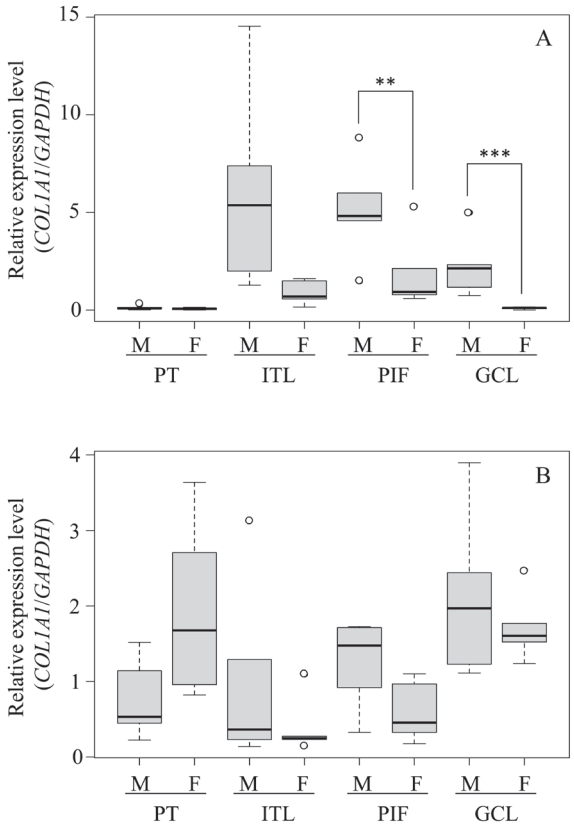

Fig. 2. **Relative expression levels of *COL1A1* in pectoral (PT), lateral iliotibial (ITL), medial part of puboischiofemoral (PIF), and lateral part of gastrocnemius (GCL) muscles in mature (A) and juvenile (B) Shamo chickens. Small circles denote outliers.**

M, male; F, female.

**Significantly different between sexes at the 0.01% level; ***Significantly different between sexes at the 0.001% level.

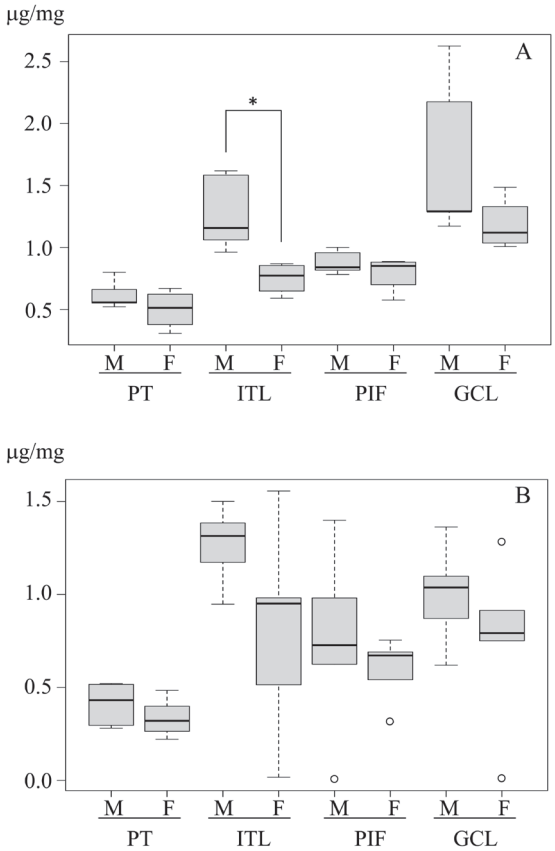

Fig. 3. **Intramuscular collagen content for the pectoral (PT), lateral iliotibial (ITL), medial part of puboischiofemoral (PIF), and lateral part of gastrocnemius (GCL) muscles in mature (A) and juvenile (B) Shamo chickens. Small circles denote outliers.**

M, male; F, female.

*Significantly different between sexes at the 0.05% level.

four muscles and in both mature and juvenile chickens, but intramuscular collagen contents were not different between sexes, except for mature ITL muscle. This indicates that the weight or size of the muscles is not directly related to the intramuscular collagen content.

Many studies have reported sex differences and the effects of sex hormones on intramuscular collagen content in domestic animals. For example, collagen content in collagen-rich muscles such as *M. triceps brachii caput lateralis* and *M. pectoralis profundus* in cattle is higher in bulls than in steers between 12 and 24 months, but not different in *M. longissimus thoracis*, *M. semimembranosus*, and *M. semitendinosus*, indicating a higher ratio of collagen to sarcoplasmic and contractile protein growth in bulls than in steers[16]. In contrast, Destefanis et al.[17] reported that *M. longissimus thoracis et lumborum* in bulls had a higher hydroxyproline content than that in steers, indicating the lack of anabolic effects of testosterone on intramuscular collagen synthesis in steers. In pigs, the total collagen concentration in the semitendinosus muscle is greater in boars than in gilts, although that in the supraspinatus muscle is lower in boars than in gilts, and there is no difference in other muscles, such as the longissimus muscle, serratus ventralis muscle, and lateral head of the triceps brachii muscle[18]. There is no significant difference in the intramuscular collagen of the longissimus muscle between barrows and gilts[19–22]. In summary, in some but not all, skeletal muscles collagen was more abundant in males than in females, suggesting an effect of sex hormones on intramuscular collagen synthesis. In chickens, the total collagen in the PT muscle was not different between the sexes in Ross[13] and White Leghorns[4], but that of the ITL muscle in White Leghorns is higher in cockerels than in hens. The sex differences in Shamo chickens we observed were only in the adult ITL muscle and not in the PT, PIF, and GCL muscles of juveniles. Faria et al.[23] reported that total and soluble collagen content in the breast and thigh muscles

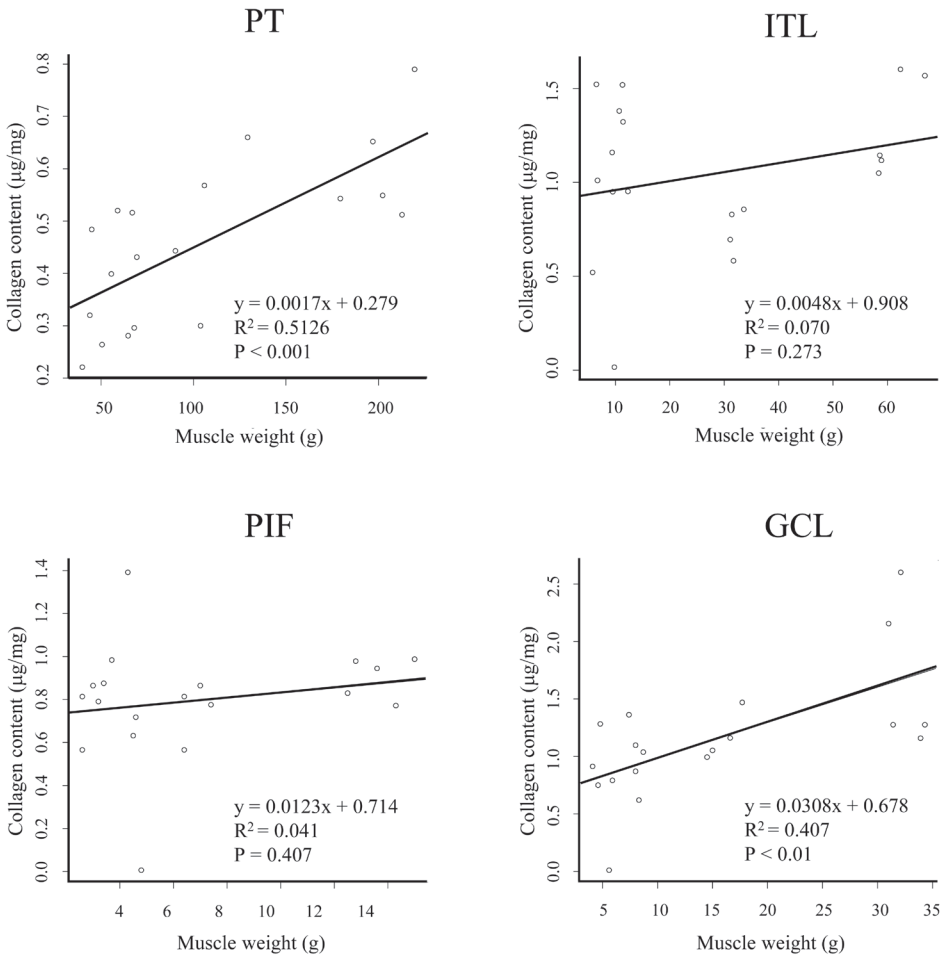

Fig. 4. **Regression graphs for muscle weight and collagen content in pectoral (PT), lateral iliotibial (ITL), medial puboischiofemoral (PIF), and lateral gastrocnemius (GCL) muscles from all Shamo chickens analyzed.**

of broilers aged 65–95 days did not differ between sexes or ages. These results suggest that sex hormones do not necessarily affect collagen synthesis in chicken muscle.

Type I is the most abundant collagen type and is composed of two α1(I) chains and one α2(I)-chain triple helix[24], encoded by the *COL1A1* and *COL1A2* genes. Although we also attempted to analyze the expression of *COL3A1*, encoding a type III collagen α1(III) chain, we could not obtain an appropriate primer set. This may be because the sequence in the NCBI database was merely predicted for *Gallus gallus*. Therefore we analyzed only *COL1A1* expression.

In juvenile Shamo chickens, *COL1A1* expression levels did not differ between sexes in any of the four muscles we analyzed; nor did intramuscular collagen content. Therefore, it is likely that the gene expression level of *COL1A1* during this growth stage is directly related to collagen content. However, *COL1A1* expression levels in the PIF and GCL muscles of mature Shamo chickens were significantly higher in cockerels than in hens, independent of intramuscular collagen content. Hosper et al.[25] compared *COL1A1* expression and type I collagen deposition between TGF-β1-stimulated epithelial-to-mesenchymal transition (EMT) epithelial cells and TGF-β1-induced myofibroblasts. They found that myofibroblasts had markedly lower *COL1A1* expression, with much higher collagen I deposition, than EMT epithelial cells. Collectively, it seems that *COL1A1* expression is not necessarily, or at least not directly, related to total collagen; alternatively, the difference in the gene expression levels was not sufficiently large to affect total collagen content in mature chickens.

Sex differences in collagen content in Shamo chickens were not universal among the muscles, which did not correlate with sex differences in muscle weights. Therefore, we analyzed correlations between muscle weight and collagen content with age and sex to detect another possible determinant of intramuscular

collagen content. Significant correlations were found for the PT and GCL, but not for the ITL and PIF, muscles. Thus, intramuscular collagen content is not necessarily related to muscle weight in all muscles; as a muscle grows heavier, intramuscular collagen accumulates.

In this study, a sex difference in intramuscular collagen content was observed only in the ITL muscle of mature chickens, but not in the PT, PIF, or GCl muscles. Our findings suggest that when Shamo chickens are used as an original stock for breed improvement for meat production from the point of view of intramuscular collagen content, both is fine as a paternal or maternal stock. Considering the present results, it is thought that the factors that determine intramuscular collagen content in chickens differ depending on muscle type.

Acknowledgments

We thank the Fukuoka Agriculture and Forestry Research Center (Fukuoka, Japan) for the kind provision of chickens.

Author Contributions

Nishimura S, supervision and draft writing; Ohtani M, bird rearing, sample collection, collagen analysis, and real-time PCR; Kabunda GM, collagen analysis and real-time PCR; Arai S, bird rearing and sample collection; Nishimura H, real-time PCR; Hosaka YZ, draft review. All authors have approved of the manuscript as submitted.

Declaration of AI and AI-assisted Technologies

TRINKA software (https://www.trinka.ai/) was used for English proofreading and iThenticate (https://www.ithenticate.com/) was used to check for plagiarism using AI and AI-assisted technologies.

Conflicts of Interest

The authors declare no conflicts of interest.

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