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Collagen Content and Collagen Fiber Architecture in the Skin of Shamo Chicken, a Japanese Game Fowl

Shotaro Nishimura[1], Sayaka Arai[2] and Yoshinao Z Hosaka[1]

[1] Faculty of Agriculture, Kyushu University, Fukuoka, Japan
[2] Graduate School of Bioresource and Bioenvironmental Sciences, Kyushu University, Fukuoka, Japan

Collagen content and collagen fiber architecture in the skin of Shamo chickens were compared between sexes and body parts. Cervical, thoracic, dorsal, femoral, and crural skin samples were collected and their collagen content was analyzed. Collagen fiber specimens were prepared for scanning electron microscopy using the cell maceration method with a NaOH solution. Sex differences in collagen content were only observed in the femoral skin of mature chickens, but not in 10-week-old chicks. The difference in collagen content between body parts was obvious; femoral and crural skin had higher collagen content than those of other parts in both sexes. Scanning electron microscopy indicated that the collagen fiber architecture was quite different between the superficial and deep layers in the dermis, with the former consisting of loosely tangled band-like collagen fibers, and the latter composed of thick and dense layers of collagen bundles in a parallel arrangement. The width of collagen fibers in the superficial layer of the dermis differed between sexes in the dorsal, femoral, and crural skin. From these results, it is likely that the difference in collagen content in the femoral skin is not due to sex hormones but other factors, such as mechanical stimulation in daily activity. Additionally, collagen fiber width in the superficial layer is likely related to the difference in collagen content between sexes and between body parts.

Key words: collagen, SEM, Shamo chicken, skin

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Introduction

A Japanese specific game fowl, termed “Shamo” in Japan, is a chicken breed that has been used for cockfights or meat production. Currently, cockfighting is prohibited by ordinances in some Japanese prefectures, and Shamo is not necessarily a popular commercial chicken breed. Instead, it has been widely used as a parent or grandparent stock to produce high-quality brand-named chicken meat, owing to its large body size, firmness, and low-fat meat.

Chicken meat is usually supplied with skin, and chicken skin is consumed together with meat or sometimes separately. The skin consists of the epidermis, dermis, and subcutaneous tissue. The epidermis is the outermost layer of the skin. Blood vessels, nerves, and lymph vessels are distributed in the dermis. The dermis is also a collagen-rich tissue, along with the bone and cartilage. Together with elastic fibers, collagen fibers maintain the structure of the skin and contribute to its strength and elasticity. In birds, the dermis is divided into two main layers: the superficial layer (*Stratum superficiale*) and deep layer (*Stratum profundum*).

Collagen is an important factor for skin strength. Collagen content in chicken skin differs among strains and between sexes in broilers[1]. Pines *et al*.[2] report that collagen content and collagen type I gene expression levels in the breast skin of broiler chicks increases with growth and are higher in males than in females in the early developmental stage. Regarding sex differences in skin collagen, Rhode Island Red cocks have higher collagen content than hens[3], but estradiol does not affect collagen content in the skin[3,4]. Rhode Island Red and White Leghorn cocks have similar skin collagen content[3]. In Shamo chickens, however, there is no information on the collagen content and morphological characteristics of collagen fibers in the skin, although a few investigations on the weight and histochemical properties of skeletal muscles have been conducted[5,6]. To improve chicken meat, understanding the characteristics or properties of Shamo chicken as an original stock for breeding is essential.

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Correspondence: Shotaro Nishimura, Department of Bioresource Sciences, Faculty of Agriculture, Kyushu University, 744 Motooka, Nishi-ku, Fukuoka 819-0395, Japan. (E-mail: shotaro@agr.kyushu-u.ac.jp)

In the present study, collagen content in the skin of several body parts of Shamo chickens was investigated. Data were compared between sexes, body parts, and between chicks and mature chickens. In addition, the collagen fiber architecture in the dermis was studied using scanning electron microscopy.

Materials and Methods

Ethics statement

All experiments were performed in accordance with the Guidelines for Proper Conduct of Animal Experiments published by the Science Council of Japan. All experimental protocols were approved by the Ethics Committee of Kyushu University (approval no. A20-028-0).

Chickens

Five male and five female Shamo chickens aged 24–29 weeks (five males and four females at 29 weeks old, and one female at 24 weeks) and the same number of 10-week-old chicks were used in this study. The chickens were introduced as fertilized eggs or chicks by the Fukuoka Agriculture and Forestry Research Center (Fukuoka, Japan) to the Poultry Breeding and Experiment Facility, Faculty of Agriculture, Kyushu University, Japan. After hatching, they were reared in a 2 × 1.5 m pen spread with wood chips or sawdust and fed a commercial feed mixture and water *ad libitum*. Because egg laying was confirmed in 25-week-old hens for the first time, the chickens were assumed to have reached maturity. Chickens were euthanized by exsanguination from the carotid artery under deep anesthesia with an intravenous injection of pentobarbital sodium salt (Nacalai Tesque, Inc., Kyoto, Japan). After plucking the feathers off, the cervical, thoracic, and dorsal skin was dissected, together with the femoral and crural skin from the lateral side.

Total collagen assay

Approximately 50 mg of skin pieces were excised to determine the collagen content. Samples were frozen in liquid nitrogen and stored at −20 °C until later analysis. A Total Collagen Assay Kit (Quick-Zyme Biosciences B.V., Leiden, The Netherlands) was used to analyze the collagen content in accordance with the manufacturer's protocol. Briefly, 1 mL of 6 mol/L HCl was added to each sample, followed by incubation at 95 °C for 20 h. After cooling the samples to room temperature, they were centrifuged at 13,000 × *g* for 10 min and the supernatant was collected. After the reaction of the supernatant with the kit reagent, the absorbance of the samples in a 96-well plate was measured at a wavelength of 570 nm using a Multiskan FC (Thermo Fisher Scientific Inc., Waltham, MA, USA).

Hematoxylin and eosin (HE) staining

Tissue specimens were mounted in the optimal cutting temperature (OCT) compound (TissueTek; Sakura Finetek Japan Co., Ltd., Tokyo, Japan) and frozen in liquid nitrogen. Frozen sections at 6 μm in thickness were cut using a cryostat (Leica CM1850; Leica Microsystems GmbH, Wetzlar, Germany), fixed in acetone for 5 min at 4 °C, and dried by airflow. HE staining was performed conventionally and the preparations were observed using a NIKON Eclipse E800 microscope equipped with the NIKON NIS-Elements D software, Ver 3.22.14 (Nikon Solutions Co., Ltd., Tokyo, Japan).

Scanning electron microscopy

To observe the three-dimensional arrangement of collagen fibers in the skin of mature chickens, the maceration method described by Ohtani[7] was applied with slight modification. After fixation with a 2% glutaraldehyde and 2% formaldehyde mixture in 0.1 M phosphate buffer (pH 7.4) for 16–17 days at room temperature, the tissue was rinsed with phosphate buffer and macerated in a 2N NaOH solution for 5 days. The tissue was then rinsed with ion-exchanged water for 3 days. The NaOH solution and water were replaced twice daily. The tissue was subsequently treated with 1% tannic acid for 2 h, followed by fixation with 1% osmium tetroxide for 2 h. The tissue was then rinsed with water, dehydrated using an ascending ethanol series, and displaced with 2-methyl-2-propanol. The tissue was freeze-dried using VFD-21S (Vacuum Device Inc., Ibaraki, Japan). The dried specimens were observed using a SU3500 scanning electron microscope (Hitachi High-Tech Corporation, Tokyo, Japan) installed at the Center for Advanced Instrumental and Educational Supports, Faculty of Agriculture, Kyushu University.

Morphometric analysis

Collagen fiber widths in the superficial layer of the dermis in the scanning electron micrographs were measured using the image analysis software, ImageJ (https://imagej.nih.gov/ij/index.html). Three pictures of the superficial layer of each bird were taken at 1,000× magnification. Twenty band-like fibers were randomly selected from each image, and their widths were measured. An average of 60 fibers in total was calculated for each bird.

Statistical analyses

Data were analyzed using a two-way analysis of variance and significance was evaluated using the Tukey-Kramer test. Data are presented as means ± standard error of the mean (SEM). Irregular data were excluded based on the Smirnoff–Grubbs rejection test. Statistical analysis was performed using the programing language "R" (https://www.r-project.org/).

Results

Collagen content in the skin

The collagen content in the cervical, thoracic, dorsal, femoral, and crural skin of mature Shamo chickens is shown in Figure 1. When compared between sexes, a significant difference in collagen content was only observed in the femoral skin; collagen content was higher in cocks (24.6 ± 1.62 mg/g) than in hens (19.0 ± 0.24 mg/g) ($p < 0.01$). There were no significant differences observed in the skin of the other parts between sexes.

However, differences in collagen content among the skin parts were observed in both sexes. Collagen content was the lowest in the cervical skin of both cocks and hens. In cocks, femoral skin had the highest collagen content among the five parts, and it was significantly higher than in the cervical (8.6 ± 0.95 mg/g), thoracic (15.8 ± 0.99 mg/g), and dorsal (12.5 ± 0.46 mg/g) skin ($p < 0.0001$). Collagen content in the crural skin (22.8 ± 1.05 mg/g)

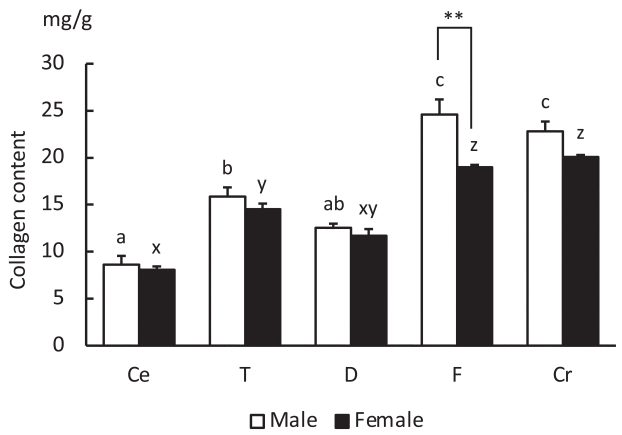

Fig. 1. **Collagen content in the cervical (Ce), thoracic (T), dorsal (D), femoral (F), and crural (Cr) skin in Shamo cocks and hens.** Data indicate the means ± standard error of the mean (SEM). N = 5. a, b, c: Means with different letters are significantly different among the skin of different body parts in cocks ($p < 0.0001$). x, y, z: Means with different letters are significantly different among the skin of different body parts in hens ($p < 0.001$). **: Significantly different between the sexes ($p < 0.01$).

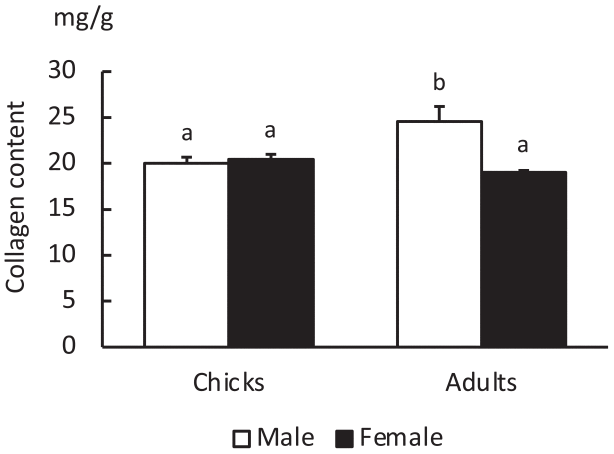

Fig. 2. **Collagen content in femoral skin of 10-week-old chicks and mature Shamo chickens.** Data indicate the means ± standard error of the mean (SEM). N = 5. a, b: Means with different letters are significantly different ($p < 0.05$).

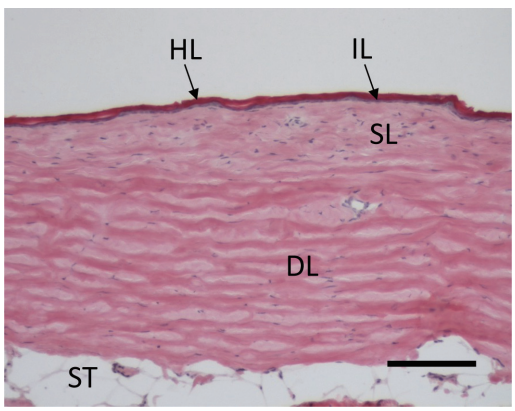

Fig. 3. **Representative photomicrograph of femoral skin in a Shamo cock. Hematoxylin and eosin staining.** DL: Deep layer of dermis, HL: horny layer of epidermis, IL: intermediate layer of epidermis, SL: Superficial layer of dermis, ST: subcutaneous tissue. Bar indicates 100 μm.

was significantly higher than in the cervical, thoracic, and dorsal skin ($p < 0.0001$) but was similar to that in the femoral skin. The collagen content in the thoracic skin was significantly higher than that in the cervical skin ($p < 0.0001$).

In hens, similar to cocks, collagen content in femoral (19.0 ± 0.24 mg/g) and crural (20.1 ± 0.20 mg/g) skin was higher than that in the cervical (8.1 ± 0.35 mg/g, $p < 0.0001$), thoracic (14.5 ± 0.59, $p < 0.05$), and dorsal (11.7 ± 0.70 mg/g, $p < 0.0001$) skin. The thoracic skin in hens also had significantly higher collagen content than that in the cervical skin ($p < 0.001$).

Since the sex difference in collagen content in mature chickens was only observed in the femoral skin, a comparison of collagen content between young and mature chickens was examined in the femoral skin. Figure 2 indicates the collagen content of the femoral skin of 10-week-old chicks and mature chickens. In chicks, collagen content was 20.0 ± 0.67 mg/g in males and 20.4 ± 0.56 mg/g in females, and a sex difference was not observed. When compared between chicks and mature chickens, collagen content in femoral skin was significantly higher in cocks than that in male chicks ($p < 0.05$) but was not different in females.

Histological properties

When the histological sections were observed, the Shamo skin had a very thin epidermis, thick dermis, and subcutaneous tissue with an adipose layer (Fig. 3). The epidermis is composed of a thin intermediate layer with only a few cell stacks and a thicker horny layer. A thick dermis was observed under the epidermis, and the superficial and deep layers were distinguishable. However, the distinction between the compact and loose layers and the subdivision of the deep layer was not obvious. The thickness of the dermis was not uniform. In the histological section, the superficial layer showed an amorphous histology, whereas the deep layer showed a striped pattern. The distribution of stromal cells (cell nuclei) was denser in the superficial layer than in the deep layer (Fig. 3). These histological properties of Shamo skin were common features in all parts of both sexes.

Scanning electron microscopy

In the observation of collagen fibers in the dermis of mature chickens using scanning electron microscopy, different collagen fiber constructions were observed between the superficial and deep layers (Fig. 4). In the superficial layer, band-like collagen fibers composed of fine collagen fibrils were loosely tangled. In

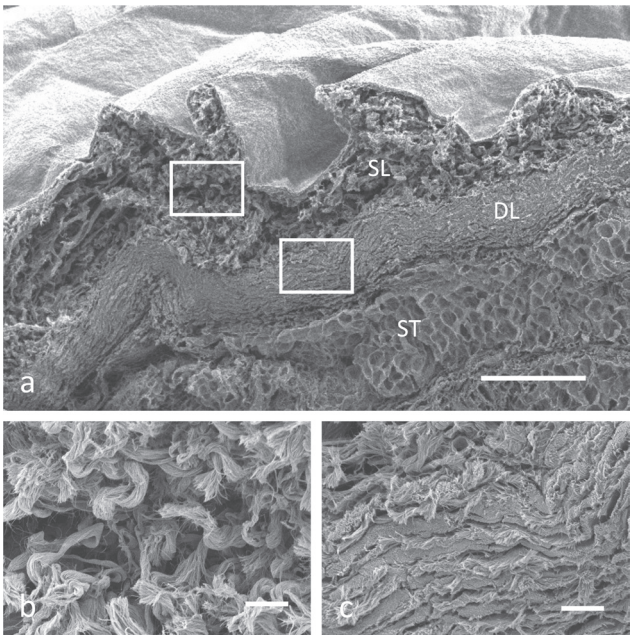

Fig. 4. **Scanning electron micrograph of crural skin in a hen after cell maceration treatment.** DL: Deep layer of dermis, SL: Superficial layer of dermis, ST: subcutaneous tissue. Pictures b and c are large magnifications of the left and right quadrangles in picture a, respectively. The bar in picture a indicates 300 µm. The bars in picture b and c indicate 30 µm.

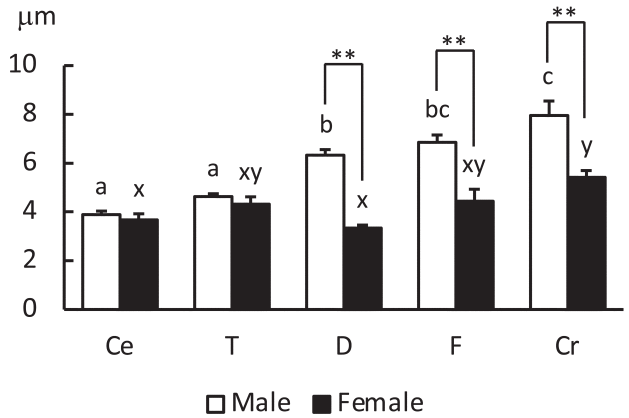

Fig. 5. **Collagen fiber width in the superficial layer of the cervical (Ce), thoracic (T), dorsal (D), femoral (F), and crural (Cr) skin in Shamo cocks and hens.** Data indicate the means ± standard error of the mean (SEM). There are five cocks and four hens. a, b, c: Means with different letters are significantly different among the skin of different body parts in cocks ($p < 0.05$). x, y: Means with different letters are significantly different among the skin of different body parts in hens ($p < 0.01$). **: Significantly different between the sexes ($p < 0.01$).

contrast, the deep layer was composed of thick and dense layers of collagen bundles in a parallel arrangement. The layers displayed parallel arrangements on the undulating skin surface. These structures of collagen fiber construction in the dermis were common features in all parts of both sexes.

Since the width of collagen fibers in the superficial layer of the dermis seemed to differ between sexes and between body parts, the width of 60 fibers in each bird was measured for comparison. Sex differences in the width of collagen fibers were not observed in the cervical and thoracic skin, but there were significant differences in collagen fiber width in the dorsal, femoral, and crural skin between sexes ($p < 0.01$) (Fig. 5). In cocks, the width of collagen fibers was significantly different between the body parts, in which the widths in the cervical (3.9 ± 0.14 µm) and thoracic (4.6 ± 0.13 µm) skin were smaller than those in the dorsal (6.3 ± 0.23 µm, $p < 0.05$), femoral (6.8 ± 0.31 µm, $p < 0.0001$), and crural (8.0 ± 0.59 µm, $p < 0.0001$) skin. In hens, the significant difference in the width of collagen fibers was only observed between cervical (3.7 ± 0.26 µm) and crural (5.4 ± 0.27 µm) skin ($p < 0.05$), and between dorsal (3.3 ± 0.12 µm) and crural skin ($p < 0.01$).

Discussion

In chicken meat production, increasing meat productivity and improving meat quality have been the focus to date, and the properties of chicken skin have not always been of interest, despite its large contribution as an edible part. Skin tearing has been one of the research themes in chicken skin because skin tearing injures the underlying skeletal muscles and reduces meat quality during the chicken meat production process[8]. Therefore, improving the quality of chicken skin is as important as improving the quality of chicken meat. The tensile strength of chicken skin increases with age in both male and female broilers, and fat may not be the only factor affecting their tensile strength[9]. Skin tearing is also affected by the chicken strain and diet owing to their significant interactions, and skin collagen is affected by diet in broiler chickens[1]. Pines *et al.*[2] report that the collagen content in the thoracic skin of broiler chicks increases with age without a significant age-by-sex interaction. These reports suggest that the amount of collagen affects the strength and toughness of chicken skin.

Regarding the collagen content in chicken skin, Bruce and Anastassiadis[3] report that hydroxyproline content is significantly higher in Rhode Island Red cocks than in hens. In the present study, sex differences in collagen content in mature Shamo chickens were not observed in the cervical, thoracic, dorsal, and crural skin, although the collagen content in the femoral skin was higher in cocks than in hens. This might be a breed-specific characteristic of Shamo chickens. Bruce and Anastassiadis[3] also report that estrogen administration to Rhode Island Red cocks does not affect hydroxyproline levels. In this study, even in the femoral skin, there was no significant difference in collagen content between the sexes of 10-week-old chicks. Our previous study indicates that the administration of estradiol in male chicks does

not affect the dermal thickness, *COL1A1* expression, or collagen content of the skin[4]. These results suggest that the sex difference in collagen content in the femoral skin of mature Shamo chickens is not an effect of sex hormones, such as estradiol, but due to other factors.

Other results in this study indicate that the collagen content differed between the skin of different body parts of Shamo chickens. In both sexes, leg skin had higher collagen content than that of the cervical, thoracic, and dorsal skin. The same tendency has been observed in our previous study using male Rhode Island Red chicks[4]. In addition, the thoracic skin had higher collagen content than that of the cervical skin. Because chickens live on the ground or floor exclusively, do not usually fly in the rearing environment, and game birds, such as Shamos, show a standing posture[6], it seems that their legs develop well through daily walking or running. Thus, the higher collagen content in leg skin than that in the skin of other body parts may be due to mechanical stimulation by daily activity. This presumption may also apply to the sex differences in collagen content observed only in the femoral skin.

Generally, the mammalian dermis is divided into two layers: the papillary layer (*Stratum papillare*) and reticular layer (*Stratum reticulare*). In dog skin, the papillary layer partly consists of reticular and elastic fibers and mostly of delicate collagen fibers arranged in different planes, although this layer is mostly absent[10]. The reticular layer of dog skin joins the papillary layer without any clear demarcation, and the density and size of the collagen fiber bundles increase towards the subcutaneous tissue[10]. Naffa *et al*.[11] have mentioned the differences in collagen fiber structure between deer and cow skin, in which deer have highly wavy collagen fibers and cows have straighter collagen fibers. In rat skin, loose and pleomorphic collagen fibers change into a parallel arrangement after prostaglandin E treatment[12]. As described earlier, the dermis of fowl consists of a superficial layer without a papillary structure and a deep layer, which is different from that of mammals. To the best of our knowledge, this is the first report of an electron microscopic observation of collagen fiber architecture in chicken dermis. Although Overton and Collins[13] have observed collagen fibers in embryonic chick skin using scanning electron microscopy, where the development of mesenchymal collagen fibers indicated a reticulated network. The present results showed that collagen fiber construction in the dermis was quite different between the superficial and deep layers, with the former composed of sparsely entangled collagen fiber strings and the latter a densely layered thick collagen sheet. These morphological features were also observed in histological sections stained with HE. The present study indicated that the cervical skin, which had thinner collagen fibers in the superficial layer, contained lower amounts of collagen than that of the leg skin, which had thicker collagen fibers in the superficial layer and higher collagen content. Although morphological differences in collagen architecture in the deep layer were difficult to determine among the skin of different body parts owing to their similar structure of thick collagen sheets arranged in parallel, the width of the collagen fibers in the superficial layer may be related to the collagen content. However, the significant differences in collagen fiber width in the superficial layer between the sexes did not correspond to similar amounts of collagen content in the dorsal and crural skin. Thus, it appears that the collagen content in these parts also reflects the amount of collagen in the deep layers.

In conclusion, this study indicated that collagen content in the Shamo skin was not different between sexes for all body parts, except the femoral skin of mature chickens, whose difference is likely due to mechanical stimulation by daily activity. We hypothesize that the difference in collagen content among the body parts is partly related to the width of collagen fibers in the superficial layer. These data may be useful for obtaining tearing-resistant skin in chicken breeding, using Shamo chickens as parent or grandparent stocks.

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Author Contributions

Shotaro Nishimura: supervision, histology, and draft writing; Sayaka Arai: collagen assay and SEM observation; Yoshinao Z Hosaka: draft review.

Conflicts of Interest

The authors declare no conflict of interest.

References

[1] Granot I, Pines M, Plavnik I, Wax E, Hurwitz S and Bartov I. Skin tearing in broilers in relation to skin collagen: effect of sex, strain, and diet. Poult Sci, **70**: 1928–1935. 1991. https://doi.org/10.3382/ps.0701928, PMID:1780263

[2] Pines M, Schickler M, Hurwitz S and Yamauchi M. Developmental changes in skin collagen biosynthesis pathway in posthatch male and female chickens. Poult Sci, **75**: 484–490. 1996. https://doi.org/10.3382/ps.0750484, PMID:8786937

[3] Bruce KR and Anastassiadis PA. Connective tissue constituents of the fowl. Effects of exogenous estrogen. Poult Sci, **56**: 1073–1085. 1977. https://doi.org/10.3382/ps.0561073, PMID:605071

[4] Nishimura S, Arai S, Ohtani M, Shimomura Y and Tabata S. Effects of estradiol on expression of estrogen receptor and collagen mRNAs in chick skin. J Poult Sci, **59**: 162–167. 2022. https://doi.org/10.2141/jpsa.0210093, PMID:35528379

[5] Iwamoto H, Katoh F, Gotoh T, Nishimura S, Ono Y, Nishio Y, Murakami E and Nishio T. Effects of parent Shamo cocks on the histochemical properties of *M. iliotibialis lateralis* and *M. supracoracoideus* on their crossbred broilers. Br Poult Sci, **39**: 589–595. 1998. https://doi.org/10.1080/00071669888449, PMID:9925310

[6] Endo H, Tsunekawa N, Kudo K, Oshida T, Motokawa M, Sonoe M, Wanghongsa S, Tirawattanawanich C, Phimphachanhvongsod V, Sasaki T, Yonezawa T and Akishinonomiya F. Comparative morphological study of skeletal muscle weight

among the red jungle fowl (*Gallus gallus*) and various fowl breeds (*Gallus domesticus*). J Exp Zoolog B Mol Dev Evol, **338**: 542–551. 2022. https://doi.org/10.1002/jez.b.23111, PMID:34826346

[7] Ohtani O. Three-dimensional organization of the connective tissue fibers of the human pancreas: a scanning electron microscopic study of NaOH treated-tissues. Arch Histol Jpn, **50**: 557–566. 1987. https://doi.org/10.1679/aohc.50.557, PMID:3326543

[8] Salim HM, Lee HR, Jo C, Lee SK and Lee BD. Effect of dietary zinc proteinate supplementation on growth performance, and skin and meat quality of male and female broiler chicks. Br Poult Sci, **53**: 116–124. 2012. https://doi.org/10.1080/00071668.2012.658757, PMID:22404812

[9] Weinberg ZG, Angel S and Navrot C. The effect of sex, age, and feed on tensile strength of broiler skin. Poult Sci, **65**: 903–906. 1986. https://doi.org/10.3382/ps.0650903

[10] Schwarz R, Le Roux JM, Schaller R and Neurand K. Micromorphology of the skin (epidermis, dermis, subcutis) of the dog. Onderstepoort J Vet Res, **46**: 105–109. 1979. http://hdl.handle.net/2263/53794 PMID:551356

[11] Naffa R, Maidment C, Ahn M, Ingham B, Hinkley S and Norris G. Molecular and structural insights into skin collagen reveals several factors that influence its architecture. Int J Biol Macromol, **128**: 509–520. 2019. https://doi.org/10.1016/j.ijbiomac.2019.01.151, PMID:30703425

[12] Lupulescu AP. Cytologic and metabolic effects of prostaglandins on rat skin. J Invest Dermatol, **68**: 138–145. 1977. https://doi.org/10.1111/1523-1747.ep12492460, PMID:839087

[13] Overton J and Collins J. Scanning electron microscopic visualization of collagen fibers in embryonic chick skin. Dev Biol, **48**: 80–90. 1976. https://doi.org/10.1016/0012-1606(76)90047-6, PMID:1245263