≪Research Note≫

Differences in Breast Muscle Mitochondrial Respiratory Capacity, Reactive Oxygen Species Generation, and Complex Characteristics between 7-week-old Meat- and Laying-type Chickens

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The skeletal muscle growth rate is a major feature differentiating meat- and laying-type chickens. A large amount of ATP is required during skeletal muscle synthesis, in which mitochondrial energy production capacities play a significant role. Additionally, mitochondria may participate in muscle protein degradation via reactive oxygen species generation. To investigate the differences in mitochondrial energetic characteristics between chickens exhibiting different growth rates, this study evaluated respiratory capacities in response to different types of respiratory substrate, protein abundances, assembly of individual respiratory complexes (I-V) and supercomplexes, and reactive oxygen species generation rates. These characteristics were compared between mitochondria from the breast muscle (*M. pectoralis superficialis*) of seven-week-old meat- and laying-type male chickens. Blue native polyacrylamide gel electrophoresis analysis revealed that meat-type chickens exhibited a significantly lower protein abundance of complex III (cytochrome bc_1 complex), complex V (F_0F_1 ATP synthase), and total amount of super-complexes than did laying-type chickens. There were no differences between chicken types in the respiration rate of mitochondria incubated with either pyruvate/malate or succinate, each of which drives complex I- and complex II-linked respiration. Carnitine palmitoyltransferase-1-dependent and -independent respiration during ATP synthesis and carnitine palmitoyltransferase-2 enzymatic activity were significantly lower in meat-type chickens than in laying-type chickens. For mitochondria receiving pyruvate/malate plus succinate, the reactive oxygen species generation rate and its ratio to the oxygen consumed (the percentage of free radical leak) were also significantly lower in meat-type chickens than in laying-type chickens. These results suggested that the mitochondrial energetic capacities of the breast muscle of meat-type chickens could be lower than those of laying-type chickens at seven weeks of age. Furthermore, the lower reactive oxygen species generation rate in meat-type chickens might have implications for rapid muscle development, which is possibly related to their lower muscle protein degradation rates.

Key words: cardiolipin, carnitine palmitoyltransferase system, fatty acid oxidation, reactive oxygen species production, respiratory complex, respiratory supercomplex

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Introduction

Continual genetic breeding for the promotion of meat and egg production in chickens has led to a faster growth rate in meat-type chickens than in laying-type chickens. Skeletal muscles account for approximately 40% of a chicken's body mass; therefore, the muscle growth rate can have a great impact on the overall growth speed of chickens. It is well accepted that skeletal muscle mass is regulated by the balance between the rates of muscle protein synthesis and degradation, and it has been reported that their contribution may vary with age between meat- and laying-type chickens (Saunderson and Leslie, 1988).

In addition to muscle metabolism, energy balance determines animal body size. Meat-type chickens have been reported to exhibit a lower basal metabolic rate (Kuenzel and Kuenzel, 1977), and a higher mitochondrial coupling efficiency than laying-type chickens, possibly owing to the

lower expression of avian uncoupling protein (avUCP) (Toyomizu *et al.*, 2011). These findings allow us to hypothesize that mitochondrial function may participate in the skeletal muscle development of broiler chickens. Mitochondria produce a large amount of ATP by oxidative phosphorylation (OXPHOS), which is used for the maintenance of cell physiology as well as muscle development. Meanwhile, during electron transfer in OXPHOS, mitochondria generate reactive oxygen species (ROS), which cause oxidative damage to lipids, proteins, and nucleotides. It has been reported that low feed efficient broilers exhibited a higher level of oxidized proteins in their skeletal muscles than did high feed efficient broilers (Iqbal *et al.*, 2004). Moreover, muscle protein atrophy is induced by the overproduction of mitochondrial ROS (Li *et al.*, 2003, Furukawa *et al.*, 2015). From these findings, it can be suggested that mitochondria both inhibit and enhance muscle development.

Intracellular aerobic ATP production is a highly complex system consisting of *i*) the tricarboxylic acid (TCA) cycle; *ii*) fatty acid imported by the carnitine palmitoyl transferase (CPT) system and subsequent β-oxidation; and *iii*) electron transferring through respiratory complex I (NADH: ubiquinone oxidoreductase), complex II (succinate dehydrogenase), complex III (cytochrome bc_1 complex), complex IV (cytochrome c oxidase), and the chemiosmotic coupling to ATP synthesis in complex V (F_0F_1 ATP synthase). It is likely that the protein abundance in individual respiratory complexes may affect the energy production efficiency in mitochondria. Recent investigations have reported that the complexes co-assemble to form supercomplexes (SCs), as illustrated in Schon and Dencher (2009), which are able to enhance the electron transfer between complexes by reducing the diffusion distance for the mobile electron carriers ubiquinone and cytochrome c (Dudkina *et al.*, 2010). ROS are generated by electron leakage from the complexes, and it was reported that mitochondrial respiratory SC association limits ROS production from complex I (Maranzana *et al.*, 2013). Moreover, it has been reported that the SC assembly organizes the electron flux to optimize the use of available respiratory substrates (Lapuente-Brun *et al.*, 2013), and that SC disassociation reduces the mitochondrial respiration rate and intracellular ATP content (Ikeda *et al.*, 2013). These findings suggest that the SC formation level might play a role in the differences in muscle development between meat- and laying-type chickens, possibly via enhancing ATP-dependent respiration, as well as limiting ROS production in mitochondria.

For this purpose, the breast muscle (*M. pectoralis superficialis*), which presents a high growth rate in chickens, was used in this study to measure the rate of muscle growth per week. In addition, the differences in mitochondrial respiratory capacity, ROS generation, and complex characteristics between meat- and laying-type chickens at seven weeks of age were investigated, as differences in the rate of muscle growth exist between the types at 3, 7, and 10 weeks of age. Herein, we describe that the muscle mitochondria of meat-type chickens exhibited decreased fatty acid oxidation-supported respiration rate; protein abundance of complex III, complex V, and SCs; and mitochondrial ROS generation than did laying-type chickens.

Materials and Methods

Ethics Statement

The Animal Care and Use Committee of the Graduate School of Agricultural Science, Tohoku University, approved all procedures, and every effort was made to minimize the pain and discomfort of animals.

Animals and Experimental Design

One-day-old male meat-type (Ross) and laying-type (White Leghorn) chickens (*Gallus gallus domesticus*) were obtained from the domestic commercial hatcheries Matsumoto Poultry Farms & Hatcheries Co., Ltd., Miyagi and I-Hiyoko Co., Ltd., Niigata, respectively. They were housed in electronically heated batteries under continuous light for 14 days and provided *ad libitum* access to water and a standard diet of corn and soybean to avoid the effects of nutritionally different diets (Table 1). The body and breast muscle weights, which were excised after decapitation, were measured every week. The breast muscle gain:body weight gain ratio was calculated from the change in the average values from the preceding week (2, 6, and 9 weeks).

Isolation of Skeletal Muscle Mitochondria

Mitochondria were isolated from the breast muscles of seven-week-old chickens as previously described by Kikusato and Toyomizu (2015). The protein concentration of the isolated mitochondria was determined using bicinchoninic acid (BCA) assays (Sigma-Aldrich, St Louis, MO, USA), with bovine serum albumin (BSA) as the standard. All mitochondria were freshly prepared on the day of the ex-

Table 1. **Diet compositions**

Ingredients	(g/kg)
Corn	487
Soybean meal	402
Beef tallow	63.3
DL-Methionine	2
Lysine-HCl	0.4
Glucose	3.7
Choline chloride	0.7
Calcium carbonate	11.2
Dibasic calcium phosphate hydrate	19.3
Sodium chloride	3.5
Vitamin mixture [†]	1.0
Mineral mixture [††]	1.0

[†] Provided per kilogram of diet: retinol acetate, 3.4 mg; cholecalciferol, 0.1 mg; α-tocopherol acetate, 65 mg; thiamine hydrochloride, 2.5 mg; riboflavin, 6.5 mg; pyridoxine hydrochloride, 3.2 mg; calcium pantothenate, 18 mg; 2-methyl-1,4-naphthoquinone, 3.0 mg; folic acid, 1.5 mg; cyanocobalamin, 0.017 mg; and biotin, 0.2 mg. [††] Provided per kilogram of diet: $MnSO_4 \cdot 5H_2O$, 366.4 mg; $ZnSO_4$, 150 mg; $FeSO_4 \cdot 7H_2O$, 20 mg; $CuSO_4$, 20 mg, KI, 1.25 mg; Na_2SeO_3, 0.3 mg; $CoCl_2 \cdot 6H_2O$, 3.92 mg; and MoO_3, 0.6 mg.

periment, and a portion of mitochondria was stored at $-80℃$ for determination of the enzymatic activity and complex analyses.

Blue Native Polyacrylamide Gel Electrophoresis (BN-PAGE)

BN-PAGE was performed using the Novex® Native PAGE™ Bis-Tris Gel System (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. For this, the isolated skeletal muscle mitochondria (a total of 200 μg of protein) were treated with a sample buffer (50 mM Bis-Tris/HCl [pH 7.2], 50 mM NaCl, 10% [*w/v*] glycerol, and 0.001% [*w/v*] Ponceau S), containing either 1% (*w/v*) n-dodecyl-β-D-maltoside (DDM) or 1% (*w/v*) digitonin for solubilization of the individual respiratory complexes or respiratory SCs. After solubilization, samples were centrifuged at $20,000 \times g$ for 30 min at $4℃$, and then 0.25% Coomassie Brilliant Blue G-250 dye was added to the supernatant. The solubilized protein (12 μg of each) was loaded onto NativePAGE™ Novex™ 3−12% Bis-Tris Protein Gels (BN1001BOX; Life Technologies), with the Native Mark™ Unstained Protein Standard (LC0725; Life Technologies) used as a protein size marker. After electrophoresis, the gels were incubated in a fixing solution (40% [*v/v*] methanol and 10% [*v/v*] acetic acid) for 15 min at $18−23℃$, and then placed in 8% (*v/v*) acetic acid destaining solution overnight to visualize the protein bands. The banding patterns were scanned using an imaging system (VersaDoc™ MP 5000 system; Bio-Rad Laboratories, Hercules, CA, USA). The band intensity of each individual respiratory complex was densitometrically analyzed using Quantity One® software (Bio-Rad Laboratories, Hercules, CA, USA), with the intensity expressed relative to the total band intensity in the lane. The respiratory SC content was determined as the total area under the densitometric curves over 1236 kDa. The values of each complex and SCs are shown in arbitrary units.

Measurement of Mitochondrial Respiratory Capacity

The mitochondrial O_2 consumption rate was measured using a Clark-type O_2-sensitive electrode (Rank Brothers, Bottisham, Cambridge, UK), as previously described by Kikusato and Toyomizu (2015). The mitochondria were incubated at $38℃$ in an assay medium (115 mM KCl, 10 mM KH_2PO_4, 3 mM HEPES [pH 7.2], 1 mM EGTA, 2 mM $MgCl_2$, and 0.3% [*w/v*] defatted BSA), which was assumed to contain atomic oxygen at 402 nmol/mL (Reynafarje *et al.*, 1985). To obtain complex I- or complex II-driven respiration rates, the mitochondria were stimulated using 10 mM pyruvate (Pyu), 2.5 mM malate (Mal) or 4 mM succinate (Suc), and 5.0 μM rotenone, which inhibit the reverse electron flow from ubiquinol. Mitochondria were incubated with 80 μM palmitoyl-CoA, 2.5 mM Mal in the presence of 2 mM carnitine or 80 μM palmitoyl-carnitine, and 2.5 mM Mal to measure the fatty acid oxidation-supported respiration depending on CPT1 (CPT1-dependent respiration) or independent CPT1 (CPT1-independent respiration). In the assays, Mal was added to promote the oxidation of acetyl-CoA, which was generated by β-oxidation. In each respiration measurement, the mitochondria were first incubated in the presence of 73 μM ADP to obtain coupled respiration, in which ADP

phosphorylation occurred at the maximal rate (state 3). After phosphorylation was completed, uncoupled respiration without ADP phosphorylation but with ATP retained in the mitochondria (state 4) was measured continuously. The measurement conditions are illustrated in Kikusato *et al.* (2015). The respiratory control ratio was calculated as the ratio of the state 3 and state 4 respiration rates to check the quality of mitochondrial preparations (Estabrook, 1967).

CPT1 and CPT2 Enzyme Activities

The mitochondrial CPT1 and CPT2 activities were measured as previously described in Zhu *et al.* (2013). Briefly, the mitochondria were incubated in an assay medium (20 mM HEPES [pH 7.4], 1 mM EGTA, 220 mM sucrose, 40 mM KCl, 100 μM 5,5′-Dithiobis (2-nitrobenzoic acid), 1.3 mg/mL BSA, and 40 μM palmitoyl-CoA) that was prewarmed to $25℃$, to which 1 mM carnitine was added to first measure the total CPT activity. The absorbance of the solution at 412 nm was monitored for 8 min using a spectrophotometer. The CPT2 activity was measured using the same reaction but in the presence of 10 μM malonyl-CoA, which inhibits CPT1 activity. The CPT1 activity was calculated by subtracting the CPT2 activity from the total CPT activity. The result was normalized according to the mitochondrial protein content.

Determination of Mitochondrial ROS Production and Glutathione Peroxidase Activity

Superoxide is a radical that is formed as primary ROS in mitochondria. As described by Kikusato and Toyomizu (2015), the ROS generation rate was determined as the H_2O_2 generation rate, which was enzymatically converted from superoxide and fluorometrically measured by the oxidation of 10-acetyl-3, 7-dihydroxyphenoxazine (Amplex® Red; Invitrogen, Carlsbad, CA, USA), in the presence of horseradish peroxidase (HRP). For this process, the mitochondria were incubated in an assay medium supplemented with either complex I- and complex II-linked substrates (10 mM Pyr/2.5 mM Mal/4 mM Suc) or 4 mM glycerol 3-phosphate (glycerol-3-P) in the absence of ADP. The superoxide released from the mitochondria was converted into H_2O_2 by 30 U/mL superoxide dismutase (S7571; Sigma-Aldrich, St Louis, MO, USA), which was then detected by 50 μM Amplex® Red coupled to the enzymatic reduction by 6 U/mL HRP. The rate of H_2O_2 production was spectrofluorimetrically determined by the change in fluorescence at excitation and emission wavelengths of 544 and 590 nm, respectively. The assay was carried out on a computer-controlled spectrofluorometer, with the appropriate correction for background noise and use of a standard curve generated with commercially available H_2O_2 solution (081-04215; Wako Pure Chemical Industries, Ltd., Tokyo, Japan).

The mitochondrial H_2O_2 production and O_2 consumption rates were measured in parallel under the same experimental conditions, which allowed the mitochondrial free radical leak (FRL) to be calculated. The FRL (expressed as a percentage) was calculated as described previously by Sanz *et al.* (2006) and Kikusato and Toyomizu (2013b). FRL provides a measure of the number of electrons that produce superoxide (and

subsequently H_2O_2) compared with the total number of electrons that pass through the respiratory chain. Two electrons are required to reduce 1 mol of O_2 to H_2O_2, whereas four electrons are transferred in the reduction of 1 mol of O_2 to H_2O. Therefore, the percentage of FRL is calculated as the rate of H_2O_2 production divided by twice the rate of O_2 consumption, and the result is multiplied by 100.

The mitochondrial glutathione peroxidase (GPx) activity was measured using a commercially available kit (No. 703102; Cayman Company, Ann Arbor, MI, USA), and the assays were performed according to the manufacturer's instructions. The values are expressed per mitochondrial protein content (mg).

Measurement of Muscle Cardiolipin Content

The cardiolipin content was measured as previously described by Barceló-Coblijn and Murphy (2008). For this process, the total lipids were extracted from the breast muscle according to Folch's method (Folch *et al*., 1957), and dissolved in a known volume of a mobile phase (n-hexane/2-propanol/water at a ratio of 56.7:37.8:5.5 [*v/v*]). An aliquot was then injected into the high-performance liquid chromatography system (LC-20AD; Shimadzu Co. Ltd., Kyoto, Japan), equipped with an Inertsil ODS-80A column ($4.6 \times$ 250 mm, 5 μm; GL Sciences Inc., Tokyo, Japan). The column was inserted into the column oven at 40℃. The eluent was monitored at 205 nm with a UV/Vis detector (SPD-20AD; Shimadzu Co. Ltd., Kyoto, Japan). To calculate the cardiolipin content, a standard curve was prepared using purified bovine heart cardiolipin (C0563; Sigma-Aldrich, St Louis, MO, USA).

Statistical Analysis

All data are presented as the mean±standard error (SE) of 4−6 individual mitochondrial preparations or tissue samples. Data were analyzed using Student's *t*-test, with *P*-values less than 0.05 considered statistically significant.

Results

Body Weight and Breast Muscle Weight

The body weight and breast muscle weights of 3-, 7-, and 10-week-old chickens are shown in Table 2. Meat-type chickens exhibited significantly higher body weights and breast muscle weights than did laying-type chickens, regardless of age. The breast muscle gain:body weight gain ratio per week was also significantly higher in meat-type than in laying-type chickens. The breast muscle gain:body weight gain ratio per week (2−3, 6−7, 9−10 weeks) was relatively higher in laying-type chickens at the age of seven weeks, wherein meat-type chickens exhibited higher breast growth rates (%) among the tested weeks.

BN-PAGE Analysis on Mitochondrial Respiratory Complexes, SC Protein Abundance, and Muscle Cardiolipin Content

The differences in the protein abundance of mitochondrial respiratory complex I (C-I), complex II (C-II), complex III (C-III), complex IV (C-IV), complex V (C-V), and SCs between the skeletal muscles of laying- and meat-type chickens were first evaluated. There were no differences in C-I, C-II, and C-IV between the two chicken types, while C-III and C-V were significantly lower in meat-type than in laying-type chickens (Fig. 1A). The total protein abundance of SCs was significantly lower in meat-type than in laying-type chickens (Fig. 1B).

The study further examined the muscle cardiolipin content, which is one of the inducers of SC formation (Mileykovskaya and Dowhan, 2014). Similar to the protein abundance of SCs, the muscle cardiolipin content was significantly lower in meat-type than in laying-type chickens (Fig. 1C).

Mitochondrial Respiratory Capacity and CPT Enzyme Activities

Next, we determined the mitochondrial respiratory capacity in response to the different substrates. There were no differences in complex I-driven (Fig. 2A) or complex II-

Table 2. **Body weight, breast muscle weight, and body weight gain:breast muscle weight gain ratio at 3, 7, and 10 weeks of age**

	Laying-type chicken	Meat-type chicken
Three weeks old		
Body weight (g)	211±9.9	868±25***
Breast muscle weight (g)	10.2±0.6	83.8±8.0***
Breast muscle gain/body weight gain (%)	6.2	11.2
Seven weeks old		
Body weight (g)	805±7.2	3534±42***
Breast muscle weight (g)	41.4±1.8	529±24***
Breast muscle gain/body weight gain (%)	5.2	20.6
Ten weeks old		
Body weight (g)	1149±45	5197±63***
Breast muscle weight (g)	63.9±1.2	875±17.3***
Breast muscle gain/body weight gain (%)	7.6	17.6

Data on muscle weight and body weight are given as means±SE, *n*=6−8. ***P<0.001. Breast muscle gain:body weight gain ratio was calculated from the change in the average values from the preceding weeks (2, 6, and 9 weeks).
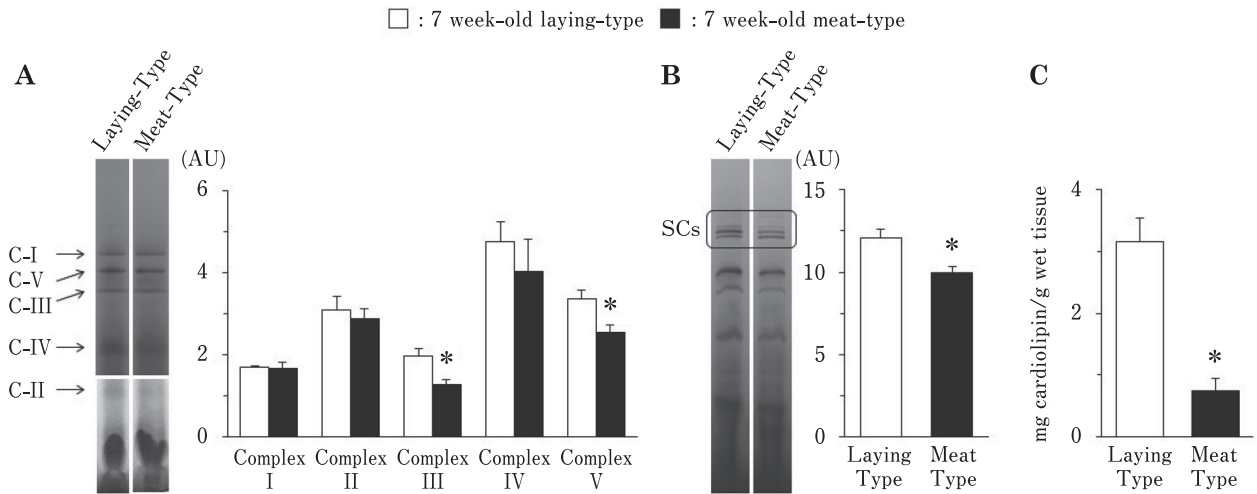
□ : 7 week-old laying-type ■ : 7 week-old meat-type

Fig. 1. **BN-PAGE analysis of the protein abundances of mitochondrial respiratory complex I (C-I), C-II, C-III, C-IV, and C-V (A) and supercomplexes (SCs) (B) in isolated breast muscle mitochondria and the muscle cardiolipin contents (C) of laying- and meat-type chickens at seven weeks of age.** Muscle mitochondria were solubilized with either n-dodecyl-β-D-maltoside (DDM) or digitonin to determine the individual complexes or SCs, respectively. Values are means$\pm$SEs (n=4–6). *P<0.05 compared to laying-type chickens.

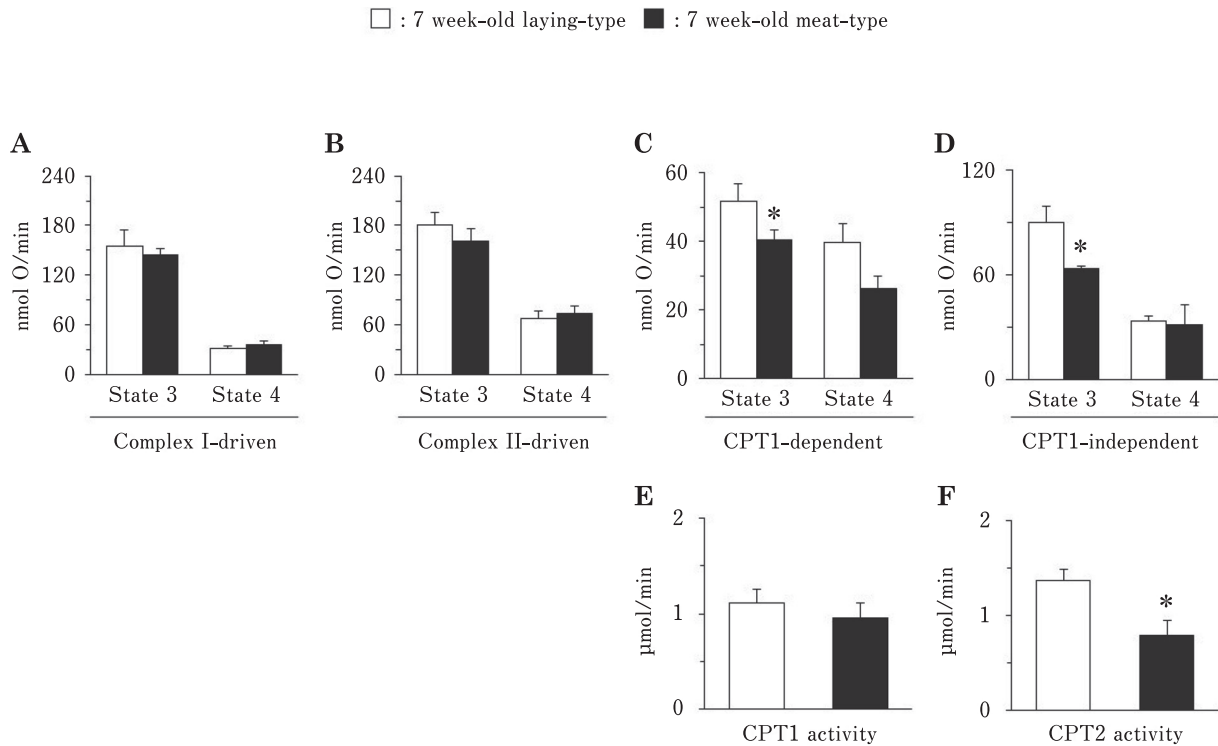□ : 7 week-old laying-type ■ : 7 week-old meat-type

Fig. 2. **O$_2$ consumption rates in muscle mitochondria exhibiting complex I (C-I) -driven (Pyr/Mal) (A), C-II-driven (succinate [Suc] plus rotenone) (B), CPT1-dependent (palmitoyl-CoA/Mal plus carnitine) (C), and CPT1-independent (palmitoyl-carnitine/Mal) (D) respiration, as well as the enzymatic activities of CPT1 (E) and CPT2 (F), each of which were expressed per mitochondrial protein content (mg).** Values are means$\pm$SEs (n=4–6). *P<0.05 compared to laying-type chickens.

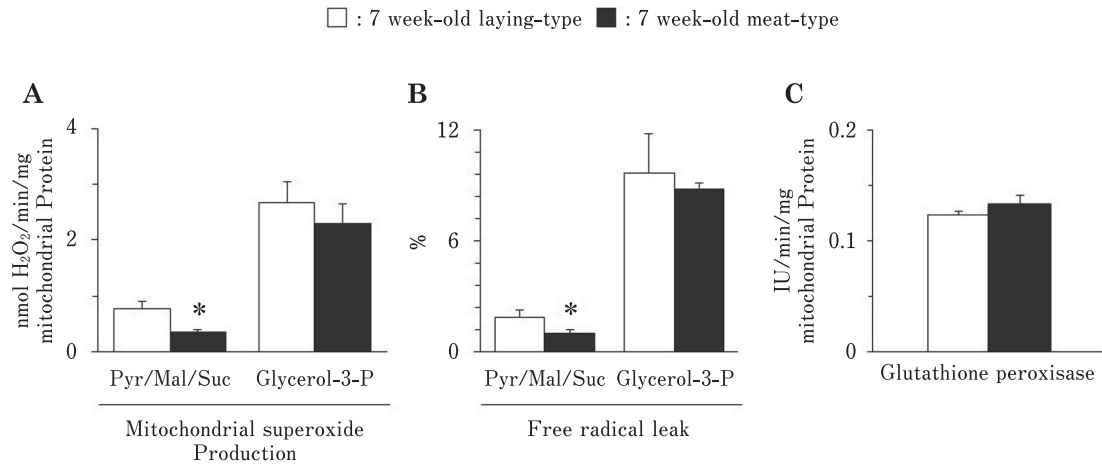□ : 7 week-old laying-type   ■ : 7 week-old meat-type

Fig. 3. **Superoxide generation rate (A) and free radical leak (B) in mitochondria receiving complex I (C-I)-linked and C-II-linked substrates (Pyr/Mal/Suc) and glycerol-3-phosphate as well as the enzymatic activity of GPx (C).** Values are means ± SEs ($n=4-6$). $*P < 0.05$ compared to laying-type chickens.

driven (Fig. 2B) respiration rates between laying- and meat-type chickens. In CPT1-dependent respiration, meat-type chickens showed a significantly lower state 3 respiration rate than did laying-type chickens; however, this difference was not observed in the state 4 respiration rate (Fig. 2C). In a similar manner, the CPT1-independent state 3 respiration rate was significantly lower in meat-type than in laying-type chickens, and no difference in state 4 respiration was observed between types. In these mitochondria, no difference in CPT1 activity was observed between the types, while the CPT2 activity was significantly lower in meat-type than in laying-type chickens (Fig. 2E, 2F).

Mitochondrial ROS Production, FRL, and GPx Activity

The mitochondrial superoxide production rates in response to complex I- and complex II-linked substrates (Pyu/Mal/Suc) and glycerol-3-P were evaluated. In muscle mitochondria receiving Pyr/Mal/Suc, meat-type chickens exhibited a significantly lower superoxide production rate than did laying-type chickens (Fig. 3A). There was no difference in superoxide production due to glycerol-3-P supplementation between the chicken types. Superoxide is formed by the one-electron reduction of an oxygen molecule. Radical generation could be increased with increasing respiration rates in the mitochondria because of the incomplete electron transferring system. FRL is the percentage of ROS generated per oxygen consumed and indicates incomplete electron transfer in the mitochondria. FRL values in the mitochondria receiving Pyr/Mal/Suc were significantly lower in meat-type than in laying-type chickens, but this decrease was not observed with glycerol-3-P supplementation (Fig. 3B). There was no difference in mitochondrial GPx activity between the chicken types (Fig. 3C).

Discussion

Several studies have used transgenic manipulation techniques for cultured cells to clarify the role of SCs in intracellular bioenergetics and metabolism (Acín-Pérez *et al*., 2008; Ikeda *et al*., 2013; Lapuente-Burn *et al*., 2013). Recently, investigations focusing on the physiological role of SC formation are gradually increasing. The formation levels of SCs vary with age and long-term exercise patterns in mammals (Lombardi *et al*., 2009, Greggio *et al*., 2017). However, there are no comparative investigations on the protein abundance of mitochondrial SCs in avian skeletal muscles. One of the aims of this investigation was to compare mitochondrial respiratory capacities between chicken types that exhibit different muscle growth rates. We hypothesized that in terms of increased energy production, the muscle mitochondria of meat-type chickens would exhibit a higher abundance of protein complexes than would laying-type chickens. However, this study obtained the opposite results, with meat-type chickens exhibiting lower C-III, C-V, and total SC levels than laying-type chickens (Fig. 1). A similar investigation was previously conducted using broilers with different feed efficiencies within a single genetic line, and found that the mitochondrial proteins that were components of C-III and C-IV were lower in high feed efficiency broiler chickens than in low feed efficiency broiler chickens (Iqbal *et al*., 2004). The results suggest that the protein abundance of mitochondrial complexes or its components might be negatively correlated with the body size of chickens.

This study found that meat-type chickens exhibited lower CPT1-dependent and CPT1-independent state 3 respiration rates than did laying-type chickens (Fig. 2C, 2D). One can assume that this lower respiration rate in meat-type chickens may be due to the lower CPT2 enzymatic activity (Fig. 2F).

It has been reported that fast-growing chickens exhibited lower mRNA expression levels of fatty acid degradation-related genes (including CPT2) in the breast muscle than did slow-growing chickens at the neonatal stage (Lin *et al.*, 2017). In our investigation using younger (3-week-old) chickens, the fatty acid oxidation-driven respiration rate was also lower in meat-type than in laying-type chickens (Supplemental Fig. 1C, 1D). From the results, it is conceivable that the breast muscle of fast-growing chickens may have a lower fatty acid oxidation capacity than slow-growing chickens. Moreover, a study using recombinant human CPT2 protein reported that the enzymatic activity was stimulated in the presence of cardiolipin (Motlagh Scholle *et al.*, 2018). We found that meat-type chickens exhibited a lower muscle cardiolipin content than did laying-type chickens (Fig. 1C). The results suggest that decreased CPT2 activity might be attributed to the lower cardiolipin content in meat-type chickens.

Meanwhile, it is also necessary to consider the differences in the respiratory complex protein abundance between types to determine the respiratory properties. Fatty acids are incorporated into the mitochondrial matrix through the CPT system and subsequently undergo β-oxidation, from which NADH and $FADH_2$ are produced. Acyl-CoA is first metabolized by mitochondrial acyl-CoA dehydrogenase to produce $FADH_2$ in the matrix, which then reduces coenzyme Q through the activity of electron transferring flavoprotein: ubiquinone oxidoreductase and subsequently donates the electrons to C-III (Tahara *et al.*, 2009). The systemic pathway indicates that C-III is one of the most important factors in reducing the equivalents from β-oxidation. Therefore, it could be suggested that the decrease in fatty acid oxidation-supported respiration rate in meat-type chickens might be associated with the decrease in C-III protein abundance. However, this idea may not be widely applicable as there was no difference in C-I-driven and C-II-driven respiration rates between meat- and laying-type chickens (Fig. 2A, 2B). This study found no differences in the protein abundance of C-I and C-II between the chicken types, which might be associated with the respiratory outputs. Considering that C-III and C-V are essential factors for the respiratory pathways initiated by C-I and C-II, the lower protein abundance of C-III and C-V in meat-type chickens should have reflected the results of the C-I- and C-II-driven respiration rates in the chicken types. For this discrepancy, we also need to focus on the constituents of SCs formed in each chicken type but not the formation levels in mitochondria. One might assume that SCs in the muscle mitochondria of laying-type chickens contain a higher proportion of factors that relate to fatty acid oxidation. However, the BN-PAGE analysis did not address the constituent analyses, such as 2D-electrophoresis and subsequent immunoblotting, because of the non-reliable antibodies for mitochondrial proteins. The protein electrophoresis analysis did not determine the coenzyme Q and cytochrome *c* contents, each of which are essential factors of oxidative phosphorylation. Moreover, the additional concentration of TCA metabolites used for evaluating the respiration rate may be due to the physiological levels in the muscle tissues of each chicken type. Thus, further investigations are required to verify the relationship between the protein abundance of respiratory complexes and the muscle mitochondria respiratory capacities in meat- and laying-type chickens.

One of the features differentiating between the muscle mass of meat- and laying-type chickens is the muscle proteolysis rate. It has been reported that the mRNA expression levels of proteasome C2 subunit and atrogin-1 were lower in meat-type than in laying-type chickens (Nakashima *et al.*, 2009). A previous study using broiler chickens with different feed efficiencies reported that low feed efficiency chickens showed a higher level of oxidized proteins than did high feed efficiency chickens, from which proteolytic activity might be induced (Iqbal *et al.*, 2004). Considering the evidence and the finding that the atrogene mRNA level was induced by superoxide generated from mitochondria in chicken muscle cells (Furukawa *et al.*, 2015), it can be hypothesized that mitochondrial ROS generation may be lower in meat-type than in laying-type chickens. As expected, this study demonstrated that meat-type chickens exhibited a lower superoxide generation rate and FRL value in response to Pyr/Mal/Suc (Fig. 3A, 3B). It has been proposed that mitochondrial SC formation can attenuate ROS production via the improvement of the electron transfer process (Maranzana *et al.*, 2013). In contrast, the results of this study suggest that the SC formation level might not be associated with the ROS production rate in chicken muscle mitochondria. Moreover, it can be assumed that the larger SC formations in laying-type chickens is a compensatory response to suppress ROS generation in the mitochondria.

Superoxide needs to be released into the intermembrane space to act as a transcriptional-related factor that upregulates proteolytic genes, as the radicals cannot move across the membrane because of low water-solubility; however, it is permeable to the outer membrane. Superoxide is mainly formed at C-I and C-III, and the superoxide generated from C-III is released into both sides of the inner membrane in mitochondria. Glycerol-3-P is metabolized by mitochondrial glycerol-3-P dehydrogenase, wherein the superoxide generated is also released into the intermembrane space (Mráček *et al.*, 2013). However, this study found no difference in the superoxide generation rates between the chicken types. Therefore, it can be suggested that the lower C-III protein abundance may contribute to the superoxide production rate and possibly affect the proteolytic gene expression in meat-type chickens.

This study showed that the muscle cardiolipin content was lower in meat-type than in laying-type chickens (Fig. 1C). Considering that both chicken types were fed the same diet during the study, it is likely that the SC protein abundance in the chicken muscle mitochondria may be metabolically regulated. This study found that meat-type chickens exhibited lower fatty acid oxidation-supported respiration due to decreased CPT2 activity, and similar metabolic features were also found in the mitochondria of broiler skeletal

muscles, which consist entirely of oxidative muscle fibers (type I) (Hakamata et al., 2018). This finding allows us to suggest that muscle fiber compositions in the breast muscle might induce different energetic properties between chicken types: However, the muscle of meat-type and laying-type chickens have the same compositions: 100% fast-glycolytic muscle fiber (type IIB) (Ueda et al., 2005, Hakamata et al., 2018). Meanwhile, previous studies have shown that the diameter of muscle fibers with a lower oxidative capacity is smaller than that of fibers with a higher capacity (van der Laarse et al., 1989, van Wessel et al., 2010). Moreover, a co-transcription factor, peroxisome proliferator-activated receptor gamma coactivator 1-α (PGC-1α), is a marker that differentiates muscle fiber type and mitochondrial contents, and we previously found that the mRNA expression of this marker tended to be higher in laying-type than in meat-type chickens (Kikusato et al., 2013a). Therefore, it could be assumed that muscle fiber morphology and mitochondrial contents are also associated with differences in the metabolic characteristics of meat- and laying-type chickens.

Our previous investigation showed that the C-II-driven respiration rate was higher in the meat-type than in the laying-type chickens when the muscle mitochondria were isolated from birds aged 17 to 21 days (Toyomizu et al., 2011). However, in this study, meat-type chickens at 3, 7, and 10 weeks of age exhibited lower respiratory capacities (including C-II-driven respiration) than did laying-type chickens at the same ages (Supplemental Fig. 1). The reason for this discrepancy between the previous and current investigations remains unclear. Differences in the nutritional value of the diets used in the studies and/or genetic selection over the past decade might be involved in this discrepancy. This study also found that mitochondrial ROS production was significantly lower in meat-type than in laying-type chickens at 3 and 10 weeks of age (Fig. 3C, Supplemental Fig. 2). Based on these results, the difference in breast muscle development between the chicken types may be attributable to the rate of mitochondrial ROS generation rather than the respiratory capacity.

This study revealed differences in the mitochondrial respiratory capacity and complex characteristics (including SCs) between meat- and laying-type chickens at seven weeks of age. This suggests that the rate of mitochondrial ROS generation plays an important role in the development of the breast muscle at this age. Further investigations are required to explore the role of mitochondrial functions (including SC formation) in the control of skeletal muscle growth and mass, in terms of protein electrophoretic analysis and chicken age.

Acknowledgments

Conflict of Interest

The authors declare no conflict of interest.

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