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Characterization of Growth, Fat Deposition, and Lipid Metabolism-Related Gene Expression in Lean and Obese Meat-Type Chickens

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Excessive fat deposition adversely affects poultry production. In this study, we investigated growth, fat deposition, and hepatic mRNA expression of 13 lipid metabolism-related genes in three unique breeds of meat-type chickens with distinct breed origins and genetic relationships. One was Nagoya (NAG), a native Japanese breed, whereas the others were White Plymouth Rock (WPR) and White Cornish (WC), which have been used worldwide as the parental breeds of common broiler chickens. NAG chickens were phenotypically characterized by slow growth, lean body fat, and high gizzard and liver weights. In contrast, both WC and WPR chickens were characterized by rapid growth but high percentage of subcutaneous fat and abdominal fat weight, resulting from high feed intake. Among the three breeds, WC had the highest percentage of pectoral muscle weight, whereas WPR was the most obese. Among lipid metabolism-related genes, the expression of *PPARA*, *PPARG,* and *CD36* was mostly associated with obesity. These results provide basic information for quantitative trait locus (QTL) analysis related to growth and fat traits in an F_2 population of the lean NAG breed and the obese WPR breed of meat-type chickens in future.

Key words: fat, growth, lipid metabolism-related genes, Nagoya, White Cornish, White Plymouth Rock

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Introduction

Modern broiler chickens have been subjected to extensive artificial selection to obtain rapid growth and high feed conversion ratio, and they are now more than four times heavier than the broilers produced in 1957 at the shipping age of 56 d (Zuidhof *et al*., 2014). However, this has led to excessive subcutaneous and abdominal fat deposition in chickens, which has increased the incidence of cardiac/metabolic disorders and sudden mortality (Julian, 2005; Chen *et al*., 2017a, b). In addition, fat is a by-product with negligible commercial value and is usually discarded by the broiler industry.

In avian species, fatty acids are mainly synthesized in the liver and transported via the bloodstream as lipoproteins to target tissues for storage as triglycerides (Wang *et al*., 2017). Dietary nutrient composition, such as protein and fat content, can reduce fat deposition to a certain extent via changes in the expression of lipid metabolism-related genes (Wang *et al*., 2017). Nonetheless, genetic engineering may be the most effective method for decreasing fat deposition in chickens, as abdominal fat percentage has a high heritability of approximately 0.7 (Zerehdaran *et al*., 2004; Alnahhas *et al*., 2014). However, selection of a genetically modified new chicken line with combined characteristics of rapid growth and decreased fat deposition is challenging because of positive genetic correlation between the two traits (Zerehdaran *et al*., 2004; Wang *et al*., 2012). Certain studies have shown that hepatic lipid metabolism-related genes are differentially expressed in lean and fat chicken lines that have been divergently selected for fat deposition within a breed (Assaf *et al*., 2004; Bourneuf *et al*., 2006), and in broiler and layer chickens and their F_1 hybrids (Willson *et al*., 2018). However, studies on differential expression of such genes between genetically diverse breeds of meat-type chickens are limited. Identification of genes responsible for differences among breeds in terms of fat deposition is essential for genetic improvement in poultry production.

Nagoya (NAG) is a native Japanese breed, which yields high-quality meat and eggs. This breed was established in the Aichi Prefecture of Japan in 1912−1926, by removing the leg feathers from the Nagoya Cochin breed. Nagoya Cochin was established by crossing the Chinese Cochin breed with some native Japanese breeds in 1868−1912, although it is now extinct (Tsudzuki, 2003). On the contrary, White Plym-

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outh Rock (WPR) and White Cornish (WC) have been used worldwide as the parental breeds of common western broilers, which are F_1 hybrids of WPR females and WC males (Bell, 2002). NAG chickens are generally shipped to meat markets 20 weeks after hatching, whereas broiler chickens are normally shipped at 7–8 weeks because of rapid growth (Bell, 2002). However, the broilers undergo excessive fat deposition in the body. Comparative studies on growth and fat deposition in NAG, WPR, and WC for evaluating the potential of these breeds as genetic resources for the discovery of quantitative trait locus (QTL) are limited.

In this study, we characterized growth, fat deposition, and hepatic mRNA expression levels of lipid metabolism-related genes in NAG, WPR, and WC breeds of meat-type chickens with distinct origins and genetic relationships (Osman *et al*., 2006) for identifying possible functional candidate genes associated with trait differences, as well as breeds that can be used as parents for QTL identification. Owing to the recent increase in atmospheric temperature, maintenance of ambient conditions in chicken rooms/houses has turned out to be challenging, unlike regulating laboratory conditions for rearing experimental animals. Hence, we investigated the traits in summer and winter to determine whether trait responses change with room temperature between the two seasons.

Materials and Methods

Animals

Hatching eggs for the NAG (strain no. 87), WPR (strain no. 981), and WC (strain no. 60) breeds were purchased in two different seasons from the Hyogo station, National Livestock Breeding Center, Hyogo, Japan, and were hatched in May and December. Only five female chickens per breed were reared and dissected in July (referred to as the summer group) and January (the winter group), as females tend to accumulate more fat than males before egg-laying (Rondelli *et al*., 2003). In each breed, the chickens reared in the two seasons were obtained from two different parental populations. However, the genetic difference between the two populations was considered to be negligible because the two populations were derived from the same strain. In addition, it was likely that maternal and/or paternal effects on phenotypic traits varied between the two populations. These effects may be involved in the seasonal differences observed in the traits.

All chickens hatched were housed in a brooder until 25 d of age. Subsequently, they were moved to individual cages to collect phenotypic data (see below). All the chickens were provided with tap water and a commercial starter diet for broilers (23.0% crude protein and metabolized energy of $>$ 3,100 kcal/kg, Nosan Co., Yokohama, Japan) *ad libitium*. The brooding temperature was maintained at 32℃ till the hatching day and reduced gradually to 26℃ until 7 d of age. After that, the temperature was lowered by 2℃ weekly and maintained at approximately 18℃ till 35 d of age. The chickens were housed with 24 h light from hatching till 7 d of age, and thereafter at 14 h light (L): 10 h dark (D) condition. All chickens used in this study were handled in accordance with the regulations of the Animal Research Committee of Nagoya University.

Analysis of Growth Performance

Body weight was recorded weekly from the hatching day until 49 d of age. Feed intake was measured for one week before dissection. At 49–51 d, blood was sampled from the wing vein of each chicken without a coagulant, following which the chickens were slaughtered under anesthesia using isoflurane. Weights of the liver, gizzard, pectoral muscle, abdominal fat (sum of intraperitoneal fat and gizzard fat) and subcutaneous fat (around the neck) were recorded and expressed as a percentage of the organ weight to body weight at slaughter. Pieces of the organs and tissues, except for the gizzard, were frozen immediately in liquid nitrogen and stored at −80℃ until further analyses.

Sexing

The genomic DNAs of all birds were extracted from fresh blood cells obtained by washing blood with physiological saline, followed by centrifugation at 1,100×*g* for 5 min. Blood cells (1.0 μL) were digested with 2.0 μL 10 mg/mL proteinase K in 38 μL buffer solution (10 mM Tris-HCl, pH 8.0; 5 mM EDTA, pH 8.0; 0.5% Tween-20) for 1 h at 55℃. The reaction solution was centrifuged at 15,500×*g* for 10 min at room temperature, and 100 μL distilled water was added to 30 μL supernatant to prepare the DNA stock solution. The sexes of the birds were determined using polymerase chain reaction (PCR) amplification of the chromohelicase-DNA binding protein (*CHD*) gene on sex chromosomes, as described by Fridolfsson and Ellegren (1999). The PCR products were electrophoresed on 2.0% agarose gels, stained with ethidium bromide, and photographed under ultraviolet (UV) light.

Biochemical Assays

The coagulated blood was centrifugally separated into clot and serum. Serum triglyceride (TG) and total cholesterol (TC) levels were assayed using triglyceride E-test Wako (Wako Pure Chemical Industries Ltd., Osaka, Japan) and cholesterol E-test Wako (Wako Pure Chemical Industries Ltd.), respectively. Total lipids in the liver were extracted using Folch's method (Folch *et al*., 1957). A portion of this extract was dried and resuspended in isopropanol as described previously (Kodama *et al*., 2015). Liver TG and TC levels were determined using triglyceride E-test Wako and cholesterol E-test Wako, respectively, which are based on a glycerol-3-phosphate oxidase method. Absorbances at 600 nm for TC and TG levels were measured using a Sunrise absorbance microplate reader (Tecan Japan Co. Ltd., Kanagawa, Japan).

Histological Analysis

Liver and abdominal adipose tissues were fixed in 10% neutral-buffered formalin. After fixation, the tissues were dehydrated using ethanol, cleared with xylene, and embedded in paraffin. The paraffin-embedded tissues were sectioned at 6 μm thickness and stained with hematoxylin and eosin. The stained sections were examined using an Olympus BX51N-34 optical microscope (Olympus Co., Tokyo, Japan).

Table 1. **Primers used for quantitative reverse transcription PCR (qRT-PCR)**

Gene symbol	Forward primer (5′-3′)	Reverse primer (5′-3′)	Accession no.	Software[1]
FASN	AGAGGCTTTGAAGCTCGGAC	GGTGCCTGAATACTTGGGCT	NM_205155	Primer-BLAST
ME1	CCTCGAAGCCTTCATCCGTT	GCATCTTCAGGCCAGGTGTA	NM_204303	Primer-BLAST
SCD	ACCTTAGGGCTCAATGCCAC	TCCCGTGGGTTGATGTTCTG	NM_204890	Primer-BLAST
GPAT3	GGCGTGGCTCTCGTTGGTAT	CCACATGTAGGCCTCGGAGA	NM_001031145	Primer-BLAST
GPAM	TGGATGCTCTCTTCTCAAATGC	AATTATGCGATCGTAGGAGATTCC	XM_015288965	Primer Express
CD36	ACTGCGCTTCTTCTCCTCTGA	TCACGGTCTTACTGGTCTGGTAAA	NM_001030731	Primer Express
CPT1A	CTTGCCCTGCAGCTTGCT	AGGCCTCGTATGTCAAAGAAAATT	NM_001012898	Primer Express
CPT2	GCCTTCCCTCTTGGCTACCT	TCTCAGCAATGCCCACGTATC	NM_001031287	Primer Express
ACOX1	GATTTTTTGCAGGCGGGTATT	CACACGCTGGTTCACCTGAGT	NM_001006205	Primer Express
APOB	TGCAAATGTCCAAGGTGCAG	ACGCAGAGCATTGCTGAAAC	NM_001044633	Primer3Plus
apoVLDLII	GGTGCAATACAGGGCATTGG	GTCACGACGTTCTCTGTCAATGA	M25774	Primer Express
PPARA	CAAACCAACCATCCTGACGAT	GGAGGTCAGCCATTTTTTGGA	NM_001001464	Primer Express
PPARG	CACTGCAGGAACAGAACAAAGAA	TCCACAGAGCGAAACTGACATC	NM_001001460	Primer Express
18SrRNA	TCCCCTCCCGTTACTTGGAT	GCGCTCGTCGGCATGTA	AF173612	Primer Express

[1] The primers were designed using Primer Express Software for Real-Time PCR version 3.0.1 (Thermo Fisher Scientific Inc., Tokyo), Primer3 Plus version 2.4.2 (Untergasser *et al*., 2012), or Primer-BLAST (Ye *et al*., 2012).
FASN＝fatty acid synthase; *ME1*＝malic enzyme 1; *SCD*＝stearoyl-CoA desaturase; *GPAT3*＝glycerol-3-phosphate acyltransferase 3; *GPAM*＝glycerol-3-phosphate acyltransferase, mitochondrial; *CD36*＝CD36 molecule; *CPT1A*＝carnitine palmitoyltransferase 1A; *CPT2*＝carnitine palmitoyltransferase 2; *ACOX1*＝acyl-CoA oxidase 1; *APOB*＝apolipoprotein B; *apoVLDLII*＝very low-density apolipoprotein II; *PPARA*＝peroxisome proliferator-activated receptor alpha; *PPARG*＝peroxisome proliferator-activated receptor gamma; *18SrRNA*＝18S ribosomal RNA.

Quantitative Reverse Transcription PCR (qRT-PCR)

Total hepatic RNA was isolated using TRIzol (Thermo Fisher Scientific Inc., Tokyo, Japan), and cDNA was synthesized from 1.0 μg total RNA using a PrimeScript RT reagent kit with genomic DNA eraser (Takara Bio Inc., Kusatsu, Japan) according to the manufacturer's instructions. Primers for qRT-PCR were designed using software programs, Primer Express Software for Real-Time PCR version 3.0.1 (Thermo Fisher Scientific Inc.), Primer3Plus version 2.4.2 (Untergasser *et al*., 2012), or Primer-BLAST (Ye *et al*., 2012). The primers used are listed in Table 1. qRT-PCR was performed in a reaction volume of 10 μL on a StepOne Plus real-time PCR system (Thermo Fisher Scientific) with SYBR Premix Ex Taq II (Tli RNaseH Plus) (Takara Bio Inc.). The following cDNA concentrations were used for qRT-PCR: 5.0 ng/μL for the very low-density apolipoprotein II (*apoVLDLII*) gene, 1.0 ng/μL for the peroxisome proliferator-activated receptor gamma (*PPARG*) gene, and 0.2 ng/μL for other genes. The thermal protocols for qRT-PCR included initial denaturation at 95℃ for 30 s, 40 cycles of denaturation at 95℃ for 5 s, annealing and extension at 60℃ for 30 s, and additional extension for 60 s. Melting curve fluorescence was measured every 0.3℃ from 60℃ to 95℃. *apoVLDLII* expression was determined using the $2^{-\Delta\Delta CT}$ method because of its low expression levels in the WC and WPR breeds. Quantitative relative standard curves with four serial dilution points of cDNA (20 ng, 4 ng, 0.8 ng, and 0.16 ng) were used to determine the expression levels of other genes. Dissociation curves, PCR amplification efficiencies, and R^2 values were determined to determine the precision of qRT-PCR (Pfaffl, 2004). All samples were analyzed in triplicate using the $2^{-\Delta\Delta CT}$ method and in duplicate using the relative standard curve method. The expression levels were normalized to that of the 18S ribosomal RNA (*18SrRNA*) gene.

Statistical Analysis

Statistical analysis was performed using the JMP Pro software version 13.2.0 (SAS Institute Japan Ltd., Tokyo, Japan). All data are shown as mean±standard error of the mean (SEM). Mean differences in phenotypic traits and gene expression levels among the breeds were compared using one-way analysis of variance (ANOVA), followed by Tukey's honestly significant difference (HSD) *post hoc* test. After Pearson's product-moment correlations for each pair of the phenotypic trait data were computed, principal component analysis was performed using the correlation matrix by the multivariate command of JMP Pro. Pearson's product-moment correlation coefficients were computed to measure relationships between phenotypic traits and gene expression levels. Two-way hierarchical clustering analysis was performed using Ward's aggregation method with the correlation matrix for the levels of liver TG and gene expression by the multivariate command of JMP Pro. Differences were considered statistically significant at $P<0.05$.

Results

Phenotypic Characterization

Figure 1a shows the growth curves for body weights of the NAG, WC, and WPR breeds in summer and winter. Both WC and WPR became significantly heavier than NAG from 7 d of age in summer and from the hatching day in winter. A significant difference in body weight between WC and WPR appeared from 14 d in summer and from 21 d in winter. Chickens had lower body weight in winter than in summer for all breeds, although the weight difference between seasons disappeared at 49 d of age. At that age, the body weights of WC and WPR were approximately 2.5-fold and 3-fold higher, respectively, than that of NAG (Table 2).

Principal component analysis was performed using all 12

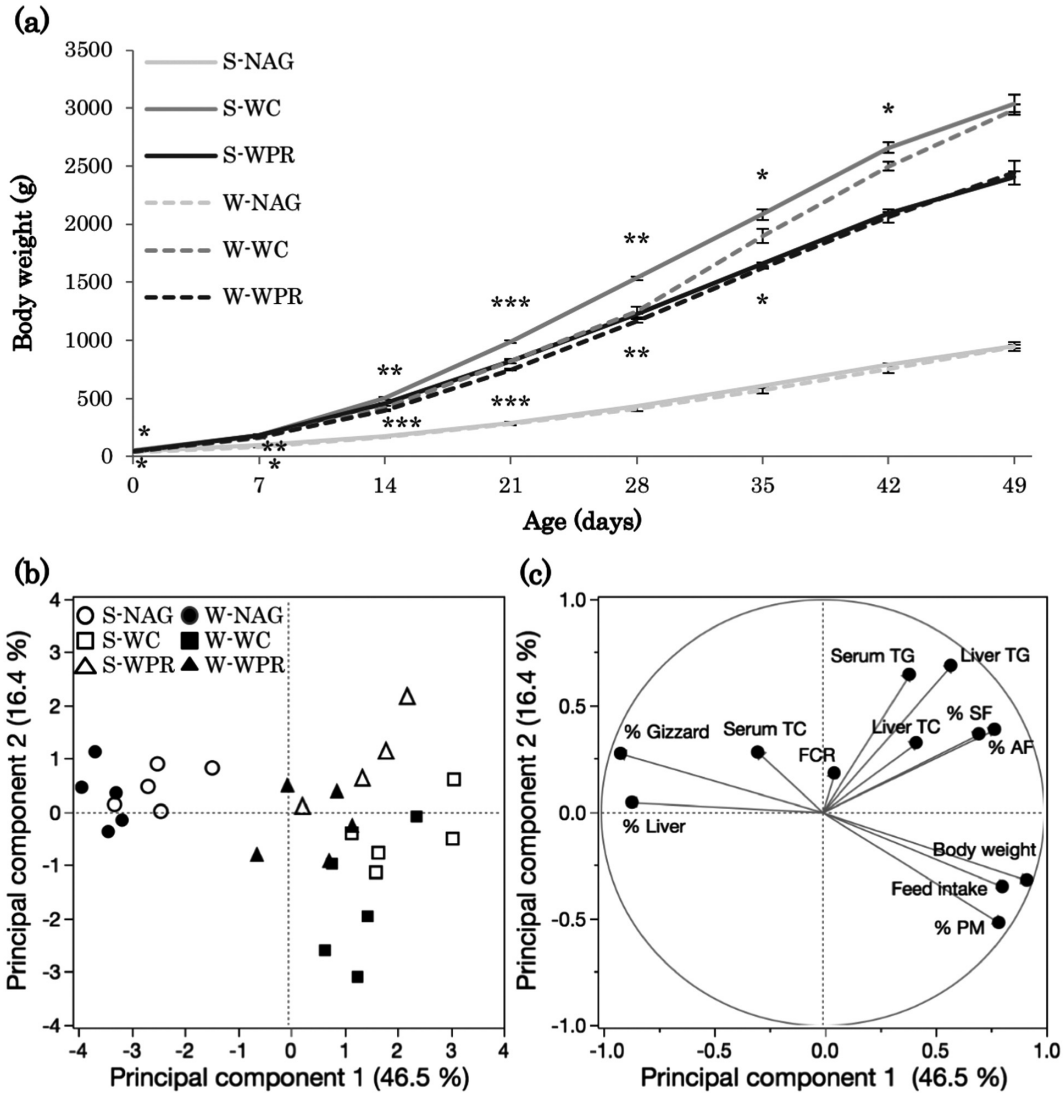

Fig. 1. **Growth curve of body weight (a), score plot (b), and factor loading plot (c) for principal component analysis of 12 phenotypic traits in Nagoya (NAG), White Cornish (WC), and White Plymouth Rock (WPR) chickens in summer (S-) and winter (W-).** (a) Each bar denotes the mean±SEM of five chickens. Significant differences between the two seasons in each breed are shown as * $P<0.05$, ** $P<0.01$, and *** $P<0.001$ (Student's *t*-test). (b) Detailed data for individual phenotypic traits are shown in Table 2. Each dot represents an individual. (c) The closer the loading value is to 1, higher is the effect of the principal component on the trait.

phenotypic traits shown in Table 2 to characterize the three breeds in perspective, rather than individually describe breed differences in each trait. Although the first four principal component axes explained 82.0% of the total trait variance, the first two principal component axes, explaining 62.9% of the total trait variance, were sufficient to characterize each of the breeds phenotypically (Fig. 1b). Based on the top seven factor loadings for traits mostly contributing to the first principal component, a group consisting of only NAG chickens was clearly distinguished from a group consisting of WC and WPR breeds in both seasons (Fig. 1b, c). Among the seven loadings, two were for percentages of gizzard and liver weight, with negative loading values of −0.91 and −0.86, respectively. This indicated that NAG chickens with slow growth had higher percentages of gizzard and liver weight than those of WC and WPR chickens with rapid growth (Fig. 1a and Table 2). The remaining five loadings were for body weight, feed intake, and percentages of pectoral muscle

Table 2. **Measurements of 12 phenotypic traits of NAG, WC, and WPR chickens**

Traits[1]		NAG		WC		WPR	
		Summer	Winter	Summer	Winter	Summer	Winter
Body weight (g)	Mean	950.55^{A}	946.42^{a}	3039.62^{B}	2986.58^{b}	2406.38^{C}	2442.88^{c}
at 49 d of age	SEM	15.96	36.94	75.82	46.30	47.37	100.78
Feed intake (g)	Mean	491.36^{A}	618.03^{a}	1204.14^{B}	1526.48^{b}	1070.26^{B}	1139.67^{b}
	SEM	15.65	20.39	73.58	76.00	67.01	162.3
Feed conversion ratio	Mean	3.09	3.25	3.25	3.33	3.41	3.61
	SEM	0.12	0.12	0.19	0.51	0.22	1.18
% Liver weight	Mmean	2.23^{A}	2.74^{a}	1.77^{B}	2.00^{b}	1.83^{B}	2.03^{b}
	SEM	0.08	0.04	0.07	0.08	0.09	0.13
% Subcutaneous fat	Mean	1.34	0.62^{a}	1.90	1.09^{b}	1.80	1.10^{b}
weight	SEM	0.17	0.06	0.15	0.11	0.20	0.07
% Abdominal fat	Mean	2.011^{A}	1.056^{a}	2.95^{A}	2.33^{b}	4.21^{B}	3.80^{c}
weight	SEM	0.17	0.13	0.22	0.49	0.38	0.24
% Pectoral muscle	Mean	10.30^{A}	9.64^{a}	19.58^{B}	19.59^{b}	14.27^{C}	13.92^{c}
weight	SEM	0.17	0.30	0.41	0.98	0.53	0.22
% Gizzard weight	Mean	1.40^{A}	1.54^{a}	0.52^{B}	0.60^{b}	0.74^{C}	0.83 ^{b}
	SEM	0.05	0.06	0.04	0.05	0.05	0.09
Serum TC (mg/dL)	Mean	109.82	112.86	111.79	81.07	105.00	88.22
	SEM	6.19	15.27	2.95	9.18	13.84	5.91
Serum TG (mg/dL)	Mean	45.68	44.40	50.82	48.68	68.81	46.11
	SEM	7.75	4.21	4.26	12.10	11.38	3.97
Liver TC (mg/g liver)	Mean	2.85^{A}	3.56	3.57^{AB}	3.39	3.68^{B}	3.75
	SEM	0.10	0.13	0.20	0.17	0.27	0.18
Liver TG (mg/g liver)	Mean	6.99^{A}	3.82	24.40^{AB}	8.85	55.53^{B}	7.29
	SEM	0.45	0.42	11.06	3.92	15.66	2.93

$^{A–C}$ Means with different superscript letters are significantly different between breeds in summer at $P<0.05$ (one-way ANOVA, followed by Tukey's HSD test).

$^{a–c}$ Means with different superscript letters are significantly different between breeds in winter at $P<0.05$ (one-way ANOVA, followed by Tukey's HSD test).

[1] Feed conversion ratio was calculated by dividing feed intake (g) by body weight gain (g) for one week before dissection. Each organ weight is expressed as the percentage of organ weight (g) to body weight at 49 d of age (g).

TC=total cholesterol; TG=triglyceride; $n=5$ per season per breed.

weight, abdominal fat weight, and subcutaneous fat weight, which had positive loading values of 0.92, 0.81, 0.80, 0.78, and 0.71, respectively (Fig. 1c). Hence, the WC/WPR group was characterized by higher body weight, feed intake, and fat deposition than NAG (Table 2). Two vectors of factor loadings for body weight and feed intake were positioned closely with similar directions and values, indicating that extreme breed differences in growth (Fig. 1a) appeared to be caused by more than 2-fold differences in feed intake among the three breeds (Table 2). Interestingly, despite the extreme breed differences in feed intake, feed conversion ratios were not significantly different among the breeds (Table 2).

The top three factor loadings for liver TG levels, serum TG levels, and the percentage of pectoral muscle weight, the values of which were 0.69, 0.65, and −0.52, respectively, contributed to the second principal component. Based on these loadings, the obese WC/WPR group was further subdivided into two groups of WC and WPR, with some chickens slightly overlapping between the groups (Fig. 1b, c). WPR was characterized by high levels of liver and serum TGs. WC was uniquely characterized by the highest percentage of pectoral muscle weight among the three breeds, reaching nearly 20% of the body weight (Table 2).

Principal component anaysis comprehensively revealed seasonal differences in the 12 traits within each of the three breeds. All summer groups in the breeds shifted right or diagonally upper right on the component score plot shown in Fig. 1b, indicating that chickens in the summer group became more obese than chickens in the winter group.

Figure 2 shows the hematoxylin-eosin-stained micrographs for the livers and abdominal fat tissues of representative chickens of the three breeds. Large numbers of lipid droplets were observed in some WC chickens and all WPR chickens in summer, whereas no lipid droplets were observed in NAG (Fig. 2a). Regardless of the season or breed, small lipid droplets were usually observed in chickens with liver TG level of approximately 15 mg/g liver or higher. Large and obvious lipid droplets were observed in chickens with liver TG level of 25 mg/g liver or higher. The size of the lipid droplets appeared to increase in a concentration-dependent manner. One of five WC chickens and all WPR chickens with liver TG levels $>$ 25 mg/g displayed a clear tendency of developing fatty liver. Similar to NAG, no lipid droplets were observed in WC livers with TG levels $<$ 15 mg/g (Table

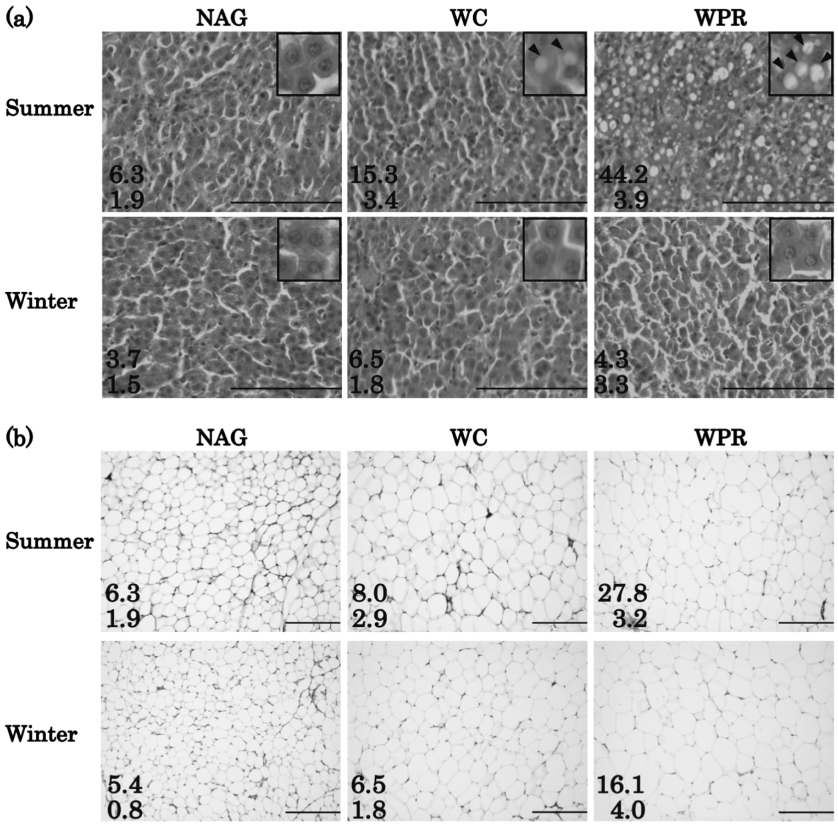

Fig. 2. **Hematoxylin-eosin-stained micrographs of the liver (a) and abdominal fat (b) of NAG, WC, and WPR chickens in summer and winter.** Insets show enlarged views of the liver sections with or without lipid droplets (arrowheads). Upper and lower numbers in the bottom left corner indicate the liver TG level (mg/g liver) and percentage abdominal fat weight of the individual examined, respectively. Scale bars=100 μm.

2 and Fig. 2a). Larger adipocytes were observed in abdominal fat of WC and WPR than in the abdominal fat of NAG in both seasons (Fig. 2b).

Gene Expression

Figure 3 shows how the 13 genes examined in this study are involved in hepatic lipid metabolism of chickens. Figure 4a shows hepatic mRNA expression levels of the 13 genes involved in fatty acid synthesis, TG synthesis, fatty acid oxidation, TG transport, and transcription factors in the three breeds of chickens reared in two seasons. In summer, the expression levels of *FASN*, *APOB*, *CD36*, *ACOX1*, *PPARA,* and *PPARG* were up-regulated in WC and WPR compared to those in NAG. Among the up-regulated genes, the expression levels of *CD36*, *ACOX1*, *APOB, PPARA,* and *PPARG* were down-regulated in winter in WC and WPR. A similar pattern was observed for *GPAM*, *CPT1A,* and *CPT2*. In contrast, *apoVLDLII* expression was down-regulated in the fatty livers of WC and WPR compared to that in NAG in summer. In winter, no significant differences in the expression levels of the 13 genes, with the exception of *PPARG,* were observed among the breeds.

Relationship between Obesity and Gene Expression

Table 3 shows the correlations between 12 phenotypic traits and the expression levels of 13 lipid metabolism-related genes. The expression levels of *CD36*, *CPT1A*, *ACOX1*, *PPARA,* and *PPARG* correlated positively with the TG level of the liver, which is the site of lipogenesis, i.e., the conversion of glucose to TG. *CD36*, *APOB*, *PPARA,* and *PPARG* expression levels correlated positively with the percentages of subcutaneous and abdominal fat weights. Thus, *CD36*, *PPARA,* and *PPARG* correlated maximally with obesity traits. Interestingly, *FASN* and *ME1* expression levels correlated positively with feed intake.

We performed two-way hierarchical clustering analysis to comprehensibly understand the associations between liver TG levels and the expression levels of the 13 lipid metabolism-related genes. As shown in Figure 4b, individuals clustered clearly into two chicken groups, A and B. Group A consisted of chickens of WC and WPR breeds reared in winter and four NAG chickens with low liver TG levels in summer. Group B consisted of WC/WPR chickens in summer and one WPR chicken and two NAG chickens in winter. In contrast,

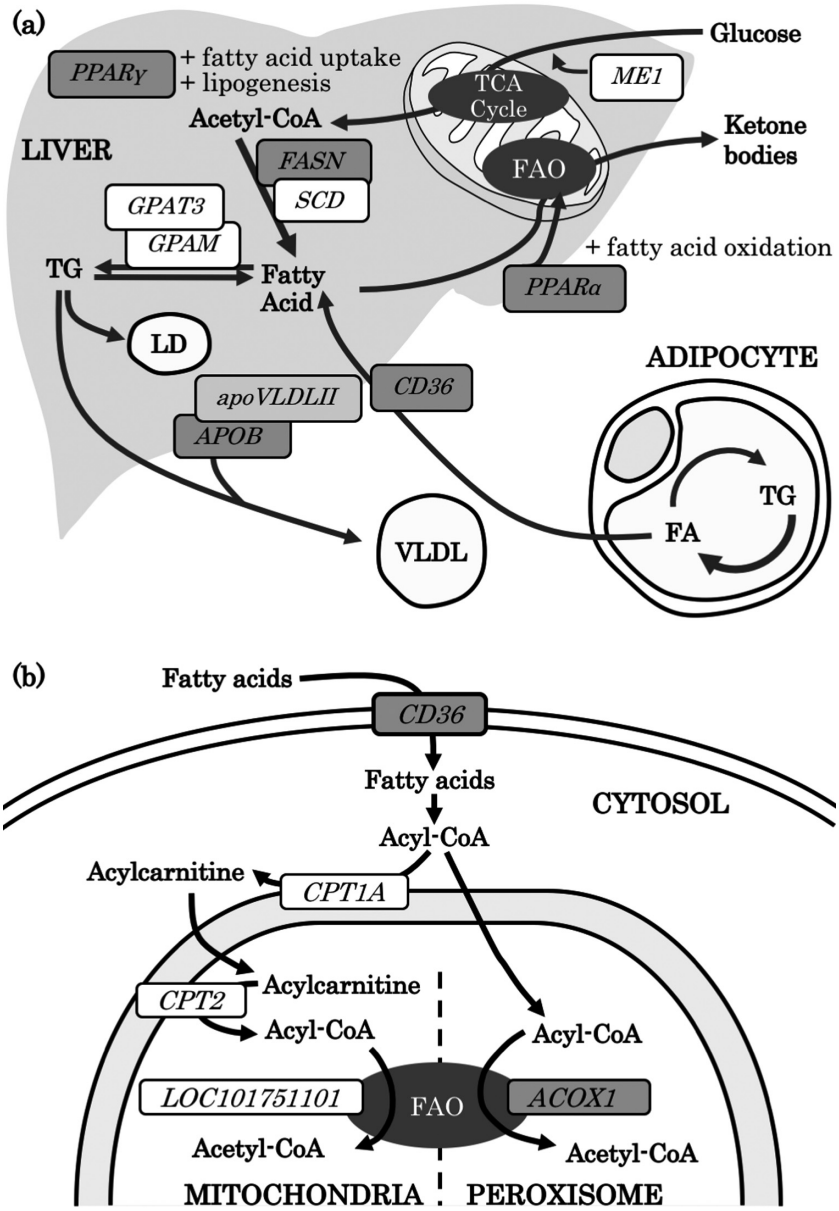

Fig. 3. **Overview of pathways for hepatic lipid metabolism (a) and fatty acid oxidation (b).** Genes involved in these pathways are shown in boxes based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways (gga00071, gga00561, gga00564, gga00620, gga01040, gga01100, gga01212, map03320, and map04979). See the footnote to Table 1 for the complete names of the genes. Genes in red and blue boxes are up-regulated and down-regulated, respectively, in WC and WPR chickens compared to NAG chickens in summer (see Fig. 4a). *PPARA* expression stimulates fatty acid oxidation. Similar to mammals, PPARγ is involved in fatty acid uptake and lipogenesis in chicken liver.

the liver TG level and gene expression levels were clustered into two groups, C and D. Group C consisted of liver TG level, *APOB,* and eight genes in total for triglyceride synthesis (*GPAT3* and *GPAM*), fatty acid oxidation (*CD36*, *CPT1A*, *CPT2* and *ACOX1*), and transcription factors (*PPARA* and *PPARG*). Group D consisted of *apoVLDLII* and three genes for fatty acid synthesis (*FASN*, *SCD* and *ME1*). In chickens of Group A, the expression levels of genes belonging to Group C were low, whereas they were high in chickens of Group B. These results, therefore, indicated that,

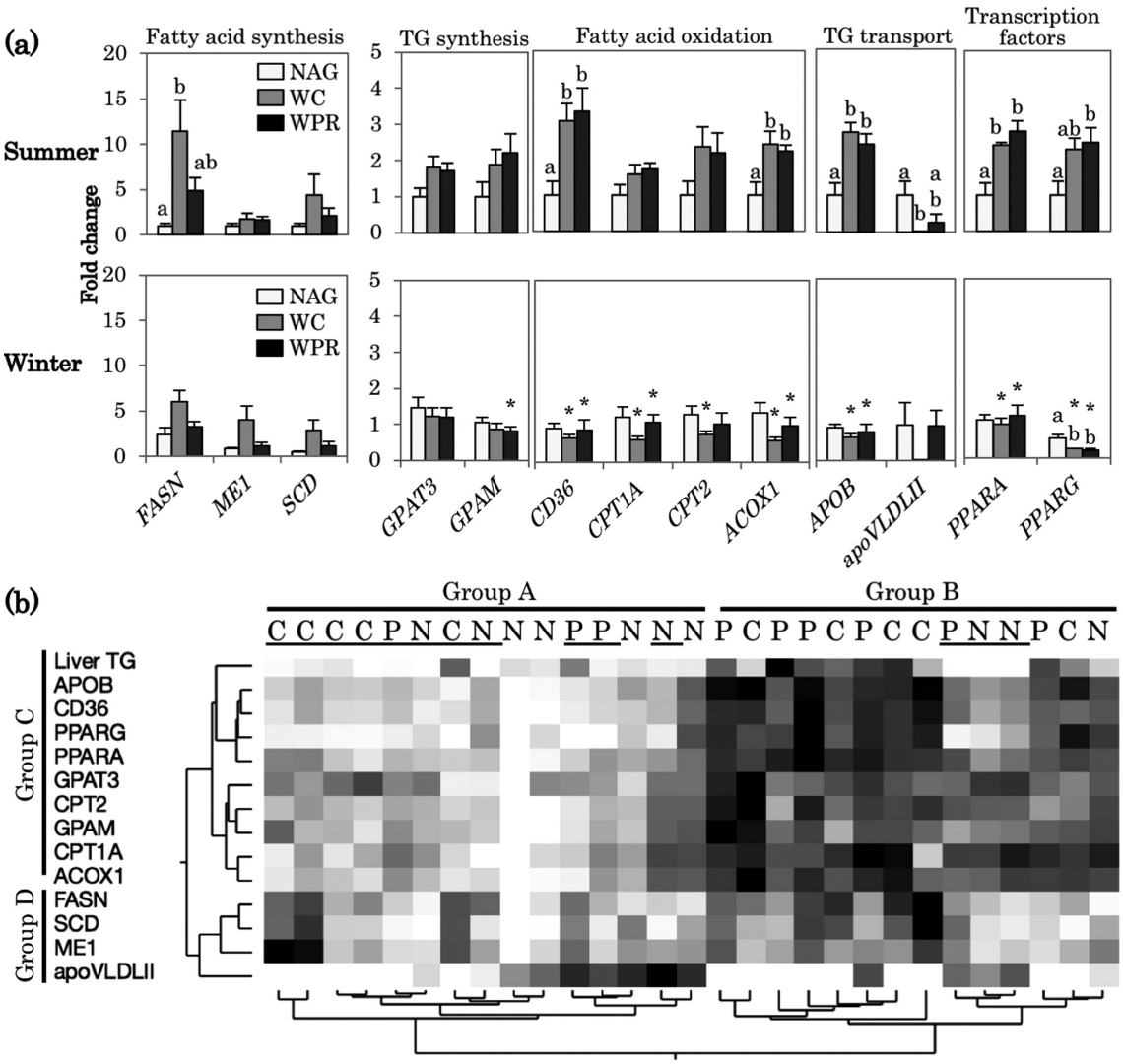

Fig. 4. **Changes in hepatic mRNA expression levels of 13 lipid metabolism-related genes (a), and a heat map showing two-way hierarchical clustering analysis of the gene expression levels and liver triglyceride (TG) contents in NAG, WC, and WPR chickens in summer and winter.** (a) The expression levels of genes for fatty acid synthesis (*FASN*, *ME1,* and *SCD*), triglyceride synthesis (*GPAT3* and *GPAM*), fatty acid oxidation (*CD36*, *CPT1A*, *CPT2,* and *ACOX1*), triglyceride transport (*APOB* and *apoVLDLII*), and transcription factors (*PPARA* and *PPARG*) were determined using qRT-PCR. The expression levels were normalized to *18SrRNA* levels. Each bar indicates the mean fold change±SEM of five chickens relative to the expression level of NAG chickens in summer. Different letters above each bar show significant differences between breeds in each season at $P<0.05$ (one-way ANOVA, followed by Tukey's HSD test). Asterisks indicate significant seasonal differences in each breed at $P<0.05$ (Student's *t*-test). (b) Dark and light cells indicate higher and lower levels, respectively, of liver TG and gene expression than the corresponding mean values. A cell indicates data pertaining to an individual. The underlined and not underlined letters (N, C, and P) show individuals reared in winter and summer, respectively. N＝NAG; C＝WC; P＝WPR.

regardless of the breed, the expression levels of Group C genes were generally low in chickens with low TG levels but high in chickens with high TG levels.

Discussion

Significant seasonal effects were observed on most of the traits examined in this study. It is well known that birds housed at high room temperature do not require energy to

Table 3. **Pearson's product-moment correlation coefficients between 12 phenotypic traits and hepatic expression levels of 13 lipid metabolism-related genes**

	FASN	*ME1*	*SCD*	*GPAT3*	*GPAM*	*CD36*	*CPT1A*
Body weight	0.55**	0.38*	0.42*	0.19	0.19	0.33	0.08
Feed intake	0.40*	0.45*	0.29	0.06	0.05	0.09	−0.08
Feed conversion ratio	0.08	0.45*	0.09	0.18	0.22	0.07	−0.09
% Liver weight	−0.39*	−0.17	−0.35	−0.14	−0.27	−0.49**	−0.27
% Subcutaneous fat weight	0.25	0.02	0.22	0.05	0.34	0.46*	0.30
% Abdominal fat weight	0.21	0.07	0.19	−0.06	0.19	0.39*	0.24
% Pectoral muscle weight	0.55	0.33	0.40*	0.28	0.16	0.32	0.07
% Gizzard weight	−0.54**	−0.35	−0.40*	−0.18	−0.30	−0.39*	−0.11
Liver TC	0.18	0.09	0.04	0.13	−0.05	0.06	0.23
Liver TG	0.19	−0.07	0.06	0.10	0.24	0.41*	0.38*
Serum TC	0.13	−0.04	0.09	0.26	0.40	0.32	0.29
Serum TG	0.11	0.02	0.11	−0.05	0.02	0.20	0.19

Table 3. **Pearson's product-moment correlation coefficients between 12 phenotypic traits and hepatic expression levels of 13 lipid metabolism-related genes (continued)**

	CPT2	*ACOX1*	*APOB*	*apoVLDLII*	*PPARA*	*PPARG*
Body weight	0.20	0.22	0.35	−0.45*	0.39*	0.24
Feed intake	−0.00	0.02	0.11	−0.35	0.23	0.04
Feed conversion ratio	0.10	0.01	0.12	−0.05	0.17	0.07
% Liver weight	−0.30	−0.36	−0.51**	0.28	−0.55**	−0.47*
% Subcutaneous fat weight	0.25	0.44*	0.60***	−0.24	0.60***	0.59***
% Abdominal fat weight	0.16	0.28	0.35	−0.17	0.54**	0.37*
% Pectoral muscle weight	0.23	0.24	0.37	−0.41*	0.28	0.24
% Gizzard weight	−0.24	−0.26	−0.42*	0.43*	−0.43*	−0.3480
Liver TC	−0.0621	0.17	0.10	−0.01	0.28	0.04
Liver TG	0.13	0.39*	0.36	−0.18	0.59***	0.48**
Serum TC	0.40*	0.36	0.33	0.05	0.28	0.34
Serum TG	−0.04	0.15	0.15	−0.22	0.29	0.26

See the footnote to Table 1 for the full names of the genes. *=$P<0.05$; **=$P<0.01$; ***=$P<0.001$; NS=not significantly different from zero.

maintain their body temperature, and hence, excessive energy intake is stored in the body as ectopic fat, which is used at low room temperature to maintain high body temperature of approximately 41℃ (Weaver Jr, 2002). In our study, room temperature might have increased in summer and decreased in winter compared to the thermoneutral zone of 18–24℃, at which adult chickens can maintain their thermal balance (Weaver Jr, 2002). Hence, we speculated that the chickens had become fatter in summer than in winter, leading to elevation in liver TG levels in obese chickens of WC and WPR reared in summer.

Our results revealed that, among the 13 lipid metabolism-related genes examined, *CD36*, *CPT1A*, *ACOX1*, *PPARA,* and *PPARG* were significantly up-regulated in fat chickens of WC and WPR, and that these genes correlated positively with the liver TG level. Furthermore, our hierarchical clustering analysis showed that *PPARA* was classified into the same gene expression group (Group C) as that of genes for fatty acid oxidation (*CD36*, *CPT1A*, *CPT2,* and *ACOX1*). Fatty acid oxidation, a catabolic process that breaks down fatty acids and produces energy in fasting mammals, is regulated by the transcription factor PPARα, which results in decreased fat accumulation (Evans *et al*., 2004; Karagianni and Talianidis, 2015), as *PPARA* expression correlated significantly with *FAT*, *CPT1A*, *CPT2,* and *ACOX1* expression in the present study. Although *Ppara* null mice show impaired fatty acid oxidation, resulting in fatty liver (Reddy and Sambasiva Rao, 2006), the development of obesity has been reported to be accompanied by increased expression of *Ppara* in mouse models of obesity (Memon *et al*., 2000). Thus, our results strongly suggest that chickens have gene regulatory mechanisms for fatty acid oxidation that are similar to those in mammals.

Our results showed that *PPARG* expression was up-regulated in the livers of obese chickens. It is well known that PPARγ, an important transcription factor for fat accumulation, is expressed mainly in adipose tissues and induces adipose differentiation in both rodents (Schoonjans *et al*., 1996) and chickens (Meng *et al*., 2005; Wang *et al*., 2008). Recently, PPARγ has been reported to be involved in hepatic lipid metabolism in mammals (Evans *et al*., 2004; Karagianni and Talianidis, 2015). Memon *et al*. (2000) observed that the expression of *CD36*, a downstream target of PPARγ, was induced in the liver but not in adipose tissue, after treatment

of obese mice exhibiting high hepatic levels of PPARγ with a PPARγ agonist. Similar to rodents, the up-regulated *PPARG* expression in chickens might have activated *CD36* expression, leading to fatty acid uptake.

Our results indicated that feed intake correlated positively with *FASN* and *ME1* expression, genes involved in fatty acid synthesis. Previous reports indicated that in broiler chickens, feed restriction and nutritional factors alter the expression of many genes involved in lipid metabolism (Richards *et al*., 2003; Wang *et al*., 2017). Thus, the differences in expression of *FASN* and *ME1* observed among NAG, WC, and WPR chickens is possibly caused by the breed difference in feed intake.

In the present study, only *apoVLDLII* expression was up-regulated in lean NAG chickens, whereas it was down-regulated in obese breed chickens. *apoVLDLII* expression is well known to be dependent on estrogen in chickens (Berkowitz *et al*., 1993). The estrogen effect might not have been significant in our study because we used only 49-d-old prelaying female chickens. However, we will confirm the difference in expression using male chickens in future.

In conclusion, NAG chickens, a native Japanese breed, were phenotypically characterized by slow growth with lean body fat but high percentages of gizzard and liver weights. In contrast, the WC and WPR chickens, which have been used worldwide as the parental breeds of common broiler chickens, were characterized by rapid growth and high percentage of pectoral muscle weight, but elevated subcutaneous and abdominal fat deposition due to increase in feed intake. Among the 13 lipid metabolism-related genes, the expression levels of *PPARA*, *PPARG,* and *CD36* were mostly associated with obesity phenotypes. However, whether these three genes are *bona fide* functional candidates responsible for the difference in fat deposition between lean NAG chickens and obese WC/WPR chickens remains unclear in the absence of further transcriptional analysis using a segregating population. Our results provide basic information for future QTL analysis of growth and fat traits in an F_2 population obtained from an intercross between the lean NAG breed and the obese WPR breed of meat-type chickens. QTL analysis can identify chromosomal regions associated with the trait differences between NAG and WPR breeds. These chromosomal regions can be used to develop a new rapidly growing chicken line with low fat deposition, using marker-associated selection with genetic markers flanking these regions. Further identification of causal genes underlying the trait differences will allow us to perform direct gene-associated selection. *PPARA*, *PPARG,* and *CD36* identified in the present study might be involved with the causal genes.

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