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 \textit{PPARA}, \textit{PPARG}, and \textit{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\textsubscript{2} population of the lean NAG breed and the obese WPR breed of meat-type chickens in future.

\textbf{Keywords}: fat, growth, lipid metabolism-related genes, Nagoya, White Cornish, White Plymouth Rock
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; Chen et al., 2017b). 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 F1 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 Plymouth Rock (WPR) and White Cornish (WC) have been used worldwide as the parental breeds of common western broilers, which are F₁ 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 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 libitum*. The brooding temperature was maintained at 32°C till the hatching day and reduced gradually to 26°C until 7 d of age. After that, the temperature was lowered by 2°C weekly and maintained at approximately 18°C 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°C 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°C. 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 chromo-helicase-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).

**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 StepOnePlus 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°C for 30 s, 40 cycles of denaturation at 95°C for 5 s, annealing and extension at 60°C for 30 s, and additional extension for 60 s. Melting curve fluorescence was measured every 0.3°C from 60°C to 95°C. 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 R² 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 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 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 analysis 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 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 comprehensively 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, 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, 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 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°C (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°C, 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 F2 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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**Explanation of Figures**

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.

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.

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). PPARα expression stimulates fatty acid oxidation. Similar to mammals, PPARγ is involved in fatty acid uptake and lipogenesis in chicken liver.
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, CPTIA, CPT2, and ACOXI), 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.
| Gene symbol | Forward primer (5′-3′) | Reverse primer (5′-3′) | Accession no. | Software |
|-------------|------------------------|------------------------|---------------|----------|
| FASN        | AGAGGCTTTGAAAGCTCGGAC  | GGTGCTGAATACTTGGGCT    | NM_205155     | Primer-BLAST |
| ME1         | CCTCGAAGCCTTCATCCGTT   | GCATCTTCAGGCCAGGTGTA   | NM_204303     | Primer-BLAST |
| SCD         | ACCTTAGGGCTCAATGCCAC   | TCCCGTGGGTTGATGTTCTG   | NM_204890     | Primer-BLAST |
| GPAT3       | GGCGTGCTTCCTGTTGGTAT   | CCACATGTAGGCCTCGGAGA   | NM_001031145  | Primer-BLAST |
| GPAM        | TGGATGCTCTCTCTTCAATGC  | AATTATGGGATCGAGAATTCC  | XM_015288965  | Primer Express |
| CD36        | ACTCGCCTTCTCTCTCTCTGA  | TCACGGCTTTACTGCTCGTA   | NM_001030731  | Primer Express |
| CPT1A       | CTTGCCCCGTACGGTCTGCT   | AGGCCCTGATACATAAGAATT  | NM_001012898  | Primer Express |
| CPT2        | GCCTTCCCTCTTGCTACCT    | TCTCAGCAATGCCAGTATC    | NM_001031287  | Primer Express |
| ACOX1       | GATTTTTTGCAGGGGGTATT   | CACACGCTGCTGACTCTGA    | NM_001006205  | Primer Express |
| APOB        | TGCAATGTCCAGGGGGTCA    | ACGCAGAGCATGGCTGAAGAC  | NM_001044633  | Primer3Plus |
| apoVLDLII   | GGTGCAATACAGGGGATACGG  | GTCACGACGTCTCTGCAATGA  | M25774        | Primer Express |
The primers were designed using Primer Express Software for Real-Time PCR version 3.0.1 (Thermo Fisher Scientific Inc., Tokyo), Primer3Plus 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.
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      |
|                           | 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 weight | Mean 1.34      | 0.62 a         | 1.90           | 1.09 b         | 1.80           | 1.10 b         |
|                           | SEM 0.17       | 0.06           | 0.15           | 0.11           | 0.20           | 0.07           |
| % Abdominal fat weight    | Mean 2.011 A   | 1.056 a        | 2.95 A         | 2.33 b         | 4.21 B         | 3.80 c         |
|                           | SEM 0.17       | 0.13           | 0.22           | 0.49           | 0.38           | 0.24           |
| % Pectoral muscle weight  | Mean 10.30 A   | 9.64 a         | 19.58 B        | 19.59 b        | 14.27 C        | 13.92 c        |
|                           | 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          |
|                | Mean      | SEM  | Mean    | SEM  | Mean  | SEM  | Mean  | SEM  |
|----------------|-----------|------|---------|------|-------|------|-------|------|
| Liver TC (mg/g liver) |           |      |         |      |       |      |       |      |
| SEM            | 7.75      | 4.21 | 4.26    | 12.10| 11.38 | 3.97 |       |      |
| Mean           | 2.85^A    | 3.56 | 3.57^AB | 3.39 | 3.68^B| 3.75 |       |      |
| Liver TG (mg/g liver) |           |      |         |      |       |      |       |      |
| SEM            | 0.10      | 0.13 | 0.20    | 0.17 | 0.27  | 0.18 |       |      |
| 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.
Table 3. Pearson’s product-moment correlation coefficients between 12 phenotypic traits and hepatic expression levels of 13 lipid metabolism-related genes

| Phenotypic Trait                  | FASN  | ME1   | SCD   | GPAT3 | GPAM  | CD36  | CPT1A | CPT2  | ACOX1 | APOB  | apoVLDLII | PPARA | PPARG |
|----------------------------------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-----------|-------|-------|
| Body weight                      | 0.55**| 0.38* | 0.42* | 0.19  | 0.19  | 0.33  | 0.08  | 0.20  | 0.22  | 0.35  | -0.45*     | 0.39* | 0.24  |
| Feed intake                      | 0.40* | 0.45* | 0.29  | 0.06  | 0.05  | 0.09  | -0.08 | -0.00 | 0.02  | 0.11  | -0.35      | 0.23  | 0.04  |
| Feed conversion ratio            | 0.08  | 0.45* | 0.09  | 0.18  | 0.22  | 0.07  | -0.09 | 0.10  | 0.01  | 0.12  | -0.05      | 0.17  | 0.07  |
| % Liver weight                   | -0.39*| -0.17 | -0.35 | -0.14 | -0.27 | -0.49**| -0.27 | -0.30 | -0.36 | -0.51**| 0.28       | -0.55**| -0.47*|
| % Subcutaneous fat weight        | 0.25  | 0.02  | 0.22  | 0.05  | 0.34  | 0.46* | 0.30  | 0.25  | 0.44* | 0.60***| -0.24      | 0.60***| 0.59***|
| % Abdominal fat weight           | 0.21  | 0.07  | 0.19  | -0.06 | 0.19  | 0.39* | 0.24  | 0.16  | 0.28  | 0.35  | -0.17      | 0.54**| 0.37* |
| % Pectoral muscle weight         | 0.55  | 0.33  | 0.40* | 0.28  | 0.16  | 0.32  | 0.07  | 0.23  | 0.24  | 0.37  | -0.41*     | 0.28  | 0.24  |
| % Gizzard weight                 | -0.54**| -0.35 | -0.40*| -0.18 | -0.30 | -0.39*| -0.11 | -0.24 | -0.26 | -0.42*| 0.43*      | -0.43*| -0.3480|
| Liver TC                         | 0.18  | 0.09  | 0.04  | 0.13  | -0.05 | 0.06  | 0.23  | -0.0621| 0.17  | 0.10  | -0.01      | 0.28  | 0.04  |
| Liver TG                         | 0.19  | -0.07 | 0.06  | 0.10  | 0.24  | 0.41* | 0.38* | 0.13  | 0.39* | 0.36  | -0.18      | 0.59***| 0.48**|
| Serum TC                         | 0.13  | -0.04 | 0.09  | 0.26  | 0.40  | 0.32  | 0.29  | 0.40* | 0.36  | 0.33  | 0.05       | 0.28  | 0.34  |
| Serum TG | 0.11 | 0.02 | 0.11 | -0.05 | 0.02 | 0.20 | 0.19 | -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.
Fig. 1

(a) Body weight (g) vs. Age (days)

(b) Principal component 2 vs. Principal component 1

(c) PCA biplot showing correlations between different parameters.
Fig. 2

(a) NAG WC WPR

Summer

|   | NAG | WC | WPR |
|---|-----|----|-----|
|   | 6.3 | 15.3 | 44.2 |
|   | 1.9 | 3.4  | 3.9  |

Winter

|   | NAG | WC | WPR |
|---|-----|----|-----|
|   | 3.7 | 6.5 | 4.3  |
|   | 1.5 | 1.8 | 3.3  |

(b) NAG WC WPR

Summer

|   | NAG | WC | WPR |
|---|-----|----|-----|
|   | 6.3 | 8.0 | 27.8 |
|   | 1.9 | 2.9 | 3.2  |

Winter

|   | NAG | WC | WPR |
|---|-----|----|-----|
|   | 5.4 | 6.5 | 16.1 |
|   | 0.8 | 1.8 | 4.0  |
Fig. 3

(a) LIVER
- PPARγ
- fatty acid uptake + lipogenesis
- Acetyl-CoA
- TCA Cycle
- ME1
- Ketone bodies
- fatty acid oxidation
- GPAT3
- GPAM
- FASN
- SCD
- LD
- apoVLDLII
- APOB
- CD36
- VLDL
- FA
- TG

(b) CYTOSOL
- Fatty acids
- CD36
- Acyl-CoA
- Acylcarnitine
- CPT1A
- CPT2
- LOC101751101
- ACOX1
- MITOCHONDRIA / PEROXISOME
Fig. 4

(a) Fatty acid synthesis

(b) Liver TG

Group A

Group B

Group C

Group D

Liver TG

APOB

CD36

PPARG

PPARA

GPAT3

CPT2

GPM

CPT1A

ACOX1

FASN

SCD

ME1

apoVLDLII