ABSTRACT

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POLYMORPHISMS WITH PHENOTYPIC
PRODUCTION TRAITS IN BROILER
CHICKENS

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This research investigated the association between SNPs and phenotypic production traits in fat and lean chicken broiler lines. In previous research, eleven SNPs in the promoter regions of four candidate genes were selected. In this study, significant associations were detected between AKR1B10 SNP1 and SDC1 SNP1 and fat yield. SDC1 SNP1 was significantly associated with fat weight. SOD3 SNP2 was associated with breast yield. Five sire-SNP interactions and one sex-SNP interaction were significant. There was a significant interaction between sex and SDC1 SNP3 on muscle-related factor. GPC3 SNP1 interacted with time period on body weight from week 1 to week 9. QTLs on chromosomes 1, 3 and 4 for body fat were refined by incorporating these SNPs into QTL analysis. These genetic markers may be of great value for marker-assisted selection (MAS) for chickens with less abdominal fat as well as genetic markers for body fat accumulation in humans.

ASSOCIATION OF SINGLE NUCLEOTIDE POLYMORPHISMS WITH
PHENOTYPIC PRODUCTION TRAITS IN BROILER CHICKENS

By

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Table of Contents

List of Tables

List of Figures

<u>Chapter 1: Introduction</u>

Broiler chickens are raised specifically for meat production. They are important sources of high quality protein. The growth rate and body weight of broiler chickens has been increased remarkably by decades of genetic selection (Havenstein et al. 2003). However, this selection is accompanied by excessive body fat accumulation (Deeb and Lamont 2002), which is a serious problem in the poultry industry, due to poor carcass quality and reduced feed efficiency, relative to leaner birds in the same population.

The domestic chicken is also widely used as a biological model (William R. A. Brown 2003), because it needs a short time to reach reproductive age, and the number of offspring is relatively large. The research of genetics for body fat in chickens might also be helpful in study of human obesity. Obesity is a serious health problem worldwide. In the United States, 34% of adults are considered to be overweight, and an additional 31% are obese (Hedley et al. 2004). Since obesity is associated with many other chronic diseases (M) (Muoio and Newgard 2006), it becomes one of the most common causes of death in the USA. About 280,000 adult deaths in the US every year are associated with obesity.

The aim of this study was to determine the association between single nucleotide polymorphisms (SNPs) and body composition in broiler chickens. This research focused on SNPs that have significant association with body fat accumulation, which could be used for further marker-assisted selection of chickens with less fat yield and can be helpful to research on obesity.

Chapter 2: Literature Review

Body Composition Regulation

Body composition, fat deposition, muscle accretion and body weight, are regulated by multiple factors, such as genetics, hormones and nutrition. The pituitary gland has profound effects on body composition. The anterior pituitary, which is the anterior lobe of the pituitary gland, is involved in the regulation of body growth, reproduction and stress responses by secreting different hormones to target organs. The anterior pituitary is functionally connected to hypothalamus by the median eminence and the hypothalamo-hypophyseal portal blood vessels. It is regulated by the hormones secreted by the hypothalamus and also by negative feedback from target organs. There are seven major hormones secreted by the anterior pituitary (Nakane 1970), adrenocorticotropic hormone, beta-endorphin, thyroid-stimulating hormone, follicle-stimulating hormone, luteinizing hormone, growth hormone and prolactin. At least three of them are involved in regulation of body composition: growth hormone, adrenocorticotropic hormone and thyroid-stimulating hormone.

Growth hormone (GH) is a single chain polypeptide hormone of 191 amino acids. It is synthesized and secreted by somatotrophs in the anterior pituitary gland. The production of growth hormone is mainly controlled by growth hormone-releasing hormone (GHRH) (Scanes and Harvey 1984), somatostatin (SS) and ghrelin. Growth hormone stimulates growth and is important for maintaining healthy body composition. It plays an important role in protein, lipid (Hall et al. 1987) and carbohydrate metabolism and indirectly stimulates growth. It exerts its effects mainly by binding to growth

4

hormone receptor (GHR) on target cells and stimulating insulin-like growth factor 1

(IGF-1) that are responsible for regulation of growth of many tissues. GH is associated

with decreased fat mass, increased muscle mass, increased bone density, increased skin

tone and texture and elevated energy levels. Growth hormone deficiency can result in

decreased muscle mass and increased body fat in adulthood.

Adrenocorticotropic hormone (ACTH or corticotropin) is a polypeptide tropic

hormone containing 39 amino acids. It is often introduced by the immune system in

response to biological stress, along with corticotrophin-releasing hormone. ACTH can be

regulated by corticotrophin-releasing hormone from the hypothalamus. ATCH plays an

important role in the hypothalamic-pituitary-adrenal (HPA) axis, which regulates

digestion, immune system, stress response and energy storage. ATCH can bind ACTH

receptors on adrenocortical cells. It stimulates the secretion of glucocorticoids from the

adrenal cortex (Chrousos et al. 2009), the transport of cholesterol into the mitochondria

and lipoprotein uptake into cortical cells.

Thyroid-stimulating hormone (TSH) is a glycoprotein composed of two subunits,

the alpha and beta sub-units. TSH is secreted by thyrotrope cells in the anterior pituitary

and regulates the thyroid gland. It is regulated by thyroid releasing hormone (TRH)

secreted by the hypothalamus and negative feedback of thyroid hormone levels (Harvey

and Baidwan 1990).   TSH is also involved in regulating body composition. It stimulates

the thyroid gland to synthesize and release thyroid hormones (Thorpe-Beeston et al.

1991) such as triiodothyronine (T3) and thyroxine (T4), which are involved in multiple

metabolic processes in the body that have influence on body growth, heart rate and body

temperature.

Body Fat Deposition

Adipose tissue, also know as body fat, is a type of connective tissue consisting chiefly of adipocytes (Weisberg et al. 2003). Adipose tissue is the major site for energy storage in the form of body fat (Kim et al. 2001). It also functions in heat insulation and mechanical protection. In addition, adipose tissue is also considered an endocrine organ (Kershaw and Flier 2004) by secreting proteins, known as adipokines, which have metabolic effects on other tissues and cells. There are two different forms of adipose tissue in mammals, white adipose tissue (WAT) and brown adipose tissue (BAT). White adipose tissue functions as an energy source. When energy is required, fatty acids are released for oxidation in other organs. Each white adipocytes contains a large single lipid droplet, a small amount of cytoplasm and a flattened nucleus located on the periphery. The distribution of WAT varies in different species. In mammals and birds, most fat is intra-abdominal and subcutaneous (Hausman et al. 2001). Brown adipose tissue stores fat and generates heat by burning the fat (Cannon and Nedergaard 2004). It has effects on thermogenesis. It utilizes electrochemical energy to generate heat by uncoupling the respiratory chain from ATP production. BAT plays an important role in hibernating animals and mammals in cold environments by maintaining body temperature. BAT contains a large volume of cytoplasm, round nuclei and a great amount of small droplets. BAT can be found around the neck and large blood vessels of the thorax. It is present mainly in newborn infants and hibernating mammals. In human newborn infants, BAT makes up to 5% of the body mass; this percentage decreases with age.

A certain amount of essential fat is necessary to maintain life and reproductive functions. Females tend to have a higher percentage of body fat than males (Clarys et al. 1999). In humans, the essential fat is 2-5% of the body weight in men and 10-13% in women. However, too much adipose tissue has an adverse effect on health.

In humans, obesity is an abnormal accumulation of excess body fat, which causes adverse metabolic effects on insulin resistance, blood pressure, blood cholesterol and triglycerides (Muoio and Newgard 2006). It is associated with many chronic diseases, including type II diabetes, hypertension, cardiovascular disease, osteoarthritis, gout, breathing problems, and stroke. It could even cause a higher risk for certain types of cancer, such as pancreatic cancer. The methods and criteria for the presence of obesity may vary. In humans, a key index for measuring body fat is body mass index (BMI), which is the ratio of body weight in kilograms to the square of height in meters. It helps to identify weight problems including underweight, overweight and obesity, since it provides an estimation of a healthy body weight according to a person's height (Hubbard 2000). The World Health Organization (WHO) defined a BMI of less than 18.5 kg/m^2 as underweight, 25-29.9 kg/m^2 as considered overweight, and greater than 30.0kg/m^2 as obese (Jezior et al. 2007). Over the past 20 years, the population of obese people increased dramatically. The most recent report from WHO shows that more than one billion adults in the world are considered to be overweight, while at least 300 million of those are obese. Twenty-two million children under the age of five are believed to be overweight, worldwide. Childhood obesity can also increase the risk of obesity in adulthood (Laitinen 1998). There are multiple factors contributing to the development of obesity, including genetics, hormones, diet, physical activity and environment. Obesity is

highly heritable. It is estimated that the genetic effect contributes about 80% of the inter-individual variation in BMI (Bell et al. 2005). Obesity genes could be recessive or dominant. To investigate the genetic basis of obesity, research has been conducted on candidate genes and genome-wide linkage.

Quantitative Trait Loci for Fatness in Chicken

Quantitative trait loci (QTL) are regions in the genome which contain genes involved in complex traits. QTLs are often found on different chromosomes. The use of QTL analysis allows in further understanding of the genetic architecture of a trait and the identification of candidate genes for the traits being measured.

Fatness is controlled by multiple factors including environment and genes. Genes involved in metabolism, energy balance and behavior contribute to the regulation of body fat accumulation and fat distribution in animals. It has been reported that abdominal fat weight in broiler chickens has a 0.50 to 0.80 heritability (Chambers 1990), which indicates a strong genetic basis for fatness (Chambers 1990; Griffin et al. 1991; Le Bihan-Duval et al. 1998; Rance et al. 2002). Experimental strains for leaner broiler chickens have been developed by the Institut National de la Recherche Agronomique (INRA) in France. These fat and lean broiler chicken lines were selected according to the difference in the proportion of abdomonial fat to live weight at 9 weeks of age (Leclercq 1980).

The measurement of body fat content is expensive and laborious. Therefore, successful identification of genetic markers for fatness allows the use of marker-assisted selection (MAS) (Jean-Marcel Ribaut 1998) to select chickens with low fat yield in breeding operations. Although many functional genes have been identified, a large number of genes in the chicken genome have not been investigated, since the genetic basis of fat deposition is not completely known. QTL mapping does not require any

previous knowledge of the underlying genes. It uses genetically divergent strains and the

established linkage map that covers the genome. The chromosomal regions that are

detected as a QTL can be further investigated for functional genes.

Experimental Populations:

Most of the QTL studies in chickens so far were based on F_2 populations generated by crossing extreme lines (Jennen et al. 2004). F_2 populations obtained from crossing between less extreme lines could also be used for QTL analysis for growth and fatness traits. One of the experimental populations used by Jennen et al. (2004) and Van Kaam et al. (1998, 1999), is an active breeding population. The experimental design was a three-generation full-sib-half-sib design, which contains parents (generation 1), full-sib offspring (generation 2) and half-sib grand-offspring (generation 3). Sewalem and Ikeobi did their experiment on an F_2 chicken population established by crossing a commercial broiler sire line and a White Leghorn egg laying line (Ikeobi et al. 2002; Sewalem et al. 2002). The broiler sire line had been selected for higher breast muscle yield and rapid growth rate. The population used by Atzmon and collaborators was established by Arbor Acres Farm poultry breeding company (Atzmon et al. 2006). Grandsires were produced by crossing male-line L-03 and female-line L-14. It was backcrossed with granddames of female-line L14. High growth (HG) and low growth (LG) broiler chicken lines created by the INRA in France were used by Javad et al (Javad Nadaf 2007). Those two lines were selected according to body weight (BW) at 8 and 36 weeks of age for more than 20 generations, resulting in divergence in growth rate. They also show large differences in body weight and abdominal fat content. The F_2 population was established by an HG× LG cross. The study of X. Liu et al. (2007) was based on the Northeast Agricultural University Resource Population (NEAURP). The sire had been selected for increased abdominal fat. It was crossed with Baier layer dams, which is a Chinese native breed. The

F_2 population was produced by an intercross of F_1 birds. Two commercial White Leghorn lines, which show different susceptibility to Marek's Disease, were used by Yonash et al (Yonash et al. 1999). Chickens were crossed to generate F_2 birds. Thus, most QTL studies conducted in chickens have used an F_2 generation resulting from the crossing of diverse lines.

Quantitative Trait Loci Mapping Methods:

In QTL analyses, there are three QTL genotypes (QQ, Qq and qq) at each position. The probability of an F_2 offspring being of each of the genotypes can be calculated. A linear model can be applied including additive (a) and dominance effects (d) of a QTL at each given position by the least square method. The additive effects are estimated by half the difference between two homozygotes; the dominance effects are the difference between heterozygotes and the mean of homozygotes. The statistical model could include fixed effects such as sex, sire, family, hatch and their interactions. The significance of the interactions between sex ×QTL, sire ×QTL, family ×QTL, hatch ×QTL could also be tested. Other traits like carcass weight may also be included as a covariate (Ikeobi et al. 2002).

Interval mapping (IM), proposed by Lander and Botstein is the most popular approach for QTL mapping (Lander and Botstein 1989). This method considers one QTL at a time. It constructs one putative QTL for likelihood ratio test at every position by using one marker interval at a time. Interval mapping can be biased when there are multiple QTL located in the same linkage group. Composite interval mapping (CIM) is an improvement on IM. It is based on applying multiple regression analysis in IM to fix the problems for multiple QTL (Jansen 1993; Zeng 1994). CIM uses other makers as covariates to reduce the residual variance when testing for the putative QTL in an interval. A more powerful method, multiple interval mapping (MIM) was developed by Chen-Hung Kao (Kao et al. 1999). This method can construct multiple putative QTL by

using multiple marker intervals simultaneously. MIM allows analysis of epistatic QTL and estimation of the individual genotypic value as well as the heritability of the trait. Desired genotypes can be selected in marker-assisted selection based on the MIM result.

Many good models and packages for statistical QTL analysis of genomic data are available. Examples include: JMP software produced by SAS Institute, Cary, NC (Liu et al. 2007), MapQTL (Van Ooijen 1996) and MultiQTL (Mester et al. 2004) for multiple QTL, R/qtl (Sen and Churchill 2001) for QTL diagnostics, IM/EM for multiple imputation and QTL Express (Seaton et al. 2002) for mapping quantitative trait loci in simple and complex pedigrees.

Quantitative Trait Loci Mapping Results:

QTL mapping was reported for abdominal fat and some other production traits in chickens. Selection for abdominal fat is accompanied by corresponding changes in other fat depots.

Van Kaam et al. (1999) scanned the whole genome in chickens for QTL affecting growth, feed efficiency and carcass traits using 420 makers on 27 autosomal linkage groups. They found four QTL that exceeded the significance thresholds. Quantitative trait loci were found for feed intake between 23 and 48 days on Chromosome 1 at 235 cM, linkage group WAU26 at 16 cM and at 147 cM on Chromosome 4. The QTL located on Chromosome 1 at 235 cM is the most significant one; it explains 4% of the variance in feed intake between 23 and 48 d. On Chromosome 2, at 41 cM, a QTL for feed intake adjusted for body weight (BW) was detected. On Chromosome 2 at 345 and 369 cM, there was another QTL detected for meat color.

Tatsuda & Fujinaka (2001) performed a QTL analysis on chicken growth traits using 78 microsatellite makers on 14 linkage groups. They found two QTLs for body weight at 13 and 16 weeks on Chromosome 1 at 220 cM and Chromosome 2 at 60 cM. They also found a QTL for the ratio of abdominal fat deposition to live body weight (abdominal fat %) at 16 weeks of age on Chromosome 7 at 38 cM.

Ikeobi et al. (2002) completed a study of QTL mapping on abdominal fat weight, skin fat weight and fat distribution by using 102 microsatellite markers in 27 linkage

15

groups. The authors detected QTLs for abdominal fat weight on Chromosome 3 at 40 cM, Chromosome 5 at 51 cM, Chromosome 7 at 41 cM, Chromosome 15 at 0 cM and Chromosome 28 at 17 cM. They also found QTL for abdominal fat weight adjusted for carcass weight on Chromosomes 1 (126 cM), 5 (50 cM), 7 (39 cM) and 28 (17 cM), QTLs for subcutaneous fat on Chromosome 3 (170 cM), 7 (78 cM), and 13 (35 cM), QTL for skin fatness on Chromosome 3 (166 cM) and Chromosome 28 (0 cM). QLTs for fat distribution (skin fat weight adjusted for abdominal fat weight) are located on Chromosome 5 (51 cM), 7 (36 cM) and 15 (0 cM). The QTL effects ranged from 3.0 to 5.24% of the total phenotypic variances.

Sewalem et al. (2002) conducted QTL mapping using 101 microsatellite markers for body weight at 3, 6 and 9 weeks of age in a broiler-layer cross(Sewalem et al. 2002). QTLs located on Chromosomes 1, 2, 4, 7, and 8 were significant for body weight at two ages. A QLT for body weight at all three ages was identified on Chromosome 13. The QTLs explained 0.2 to 1.0% of the phenotypic variance.

Jennen et al. (2004) also completed QTL mapping for fatness in broiler chickens. They used 410 markers on 25 chromosomes and found two genome-wise significant QTL. One was for percentage abdominal fat at 10 weeks. It was located on Chromosome 1 at 241 cM. The other was for body weight at 10 weeks on Chromosome 13 at 9 cM. They also found multiple suggestive QTL on Chromosome 1, 2, 4, 13, and 18.

Lagarrigue et al. (2006) found 6 QTL for abdominal fat weight adjusted for live body weight on Chromosome 1 (449 cM), 3 (84 cM and 121 cM), 5 (68 cM and 150 cM), and 7 (32 cM). These QTL effects ranged from 0.9 to 1.3% of the phenotypic variation.

Nones et al.(2006) conducted QTL mapping for carcass traits on chicken chromosome 1, which is the largest chromosome. 24 microsatellite markers on Chromosome 1 were selected to be genotyped in the study. The QTL for body weight, feed intake, carcass weight, drum weight, thigh weight and abdominal fat weight were detected in their study. Those regions are consistent with the results from other populations. They also found QTL for weights of gizzard, liver, lungs, heart, feet and length of intestine, which had not been studied by other groups.

Orjan found QTL for body weight from 8 through 200 days, growth on day 1- day 200, total and average egg production traits and food consumption on Chromosome 1 at 58-82 cM and 399-431 cM.

Considering jointly the QTL mapping results so far, it is obvious that there are some controversies surrounding the QTL mapping location for production traits in broiler chickens. The main reason for this is that for different study groups, they used different experimental populations that came from multiple chicken lines that were created by different family structures. Even for those who used the same chicken lines, obtained slightly different results. The environment is know to play an important role in chicken growth (Jennen 2004). For some traits, there might be some gene-by-environment interactions. Therefore, different feeding and housing conditions could create the difference in the performance of chickens. In addition, some measurements are not the same for each study. Some of the traits were measured at different states of chicken growth, which might lead to different phenotypic variances, thus providing a different QTL map.

However, past QTL mapping did show some consistencies. In the study of Ikeobi et al, the QTL for skin fatness on Chromosome 3 at 166 cM has a confidence interval that overlaps with the QTL for subcutaneous fat at 170 cM on the same chromosome. This suggests that this QTL might be multifunctional for both skin fatness and subcutaneous fat traits. On Chromosome 1, many QTL were detected by different groups. The QTL at 220 cM detected by Tatsuda & Fujinaka (2001) for body weight at 13 and 16 weeks is located very close to the QTL at 235 cM for feed intake, which was found by Van Kaam et al. (1999).The QTL detected by Jennen (2004) at 241 cM for percentage abdominal fat at 10 weeks is also very close to this region. The overlap of confidence intervals for the first two QTL could be explained by the strong correlation between feed intake and body weight. Abdominal fat and body weight are controlled by different genes (Ankra-Badu et al. 2009). The QTL for feed intake and body weight overlap with abdominal fat, indicating that this QTL region might serve as a pleiotropic QTL containing multiple genes that have different functions.

Fat and lean broiler chicken lines (FL and LL, respectively) were created by the INRA in France (Leclercq 1980). These two lines were genetically selected according to the difference in the proportion of abdominal fat at 9 weeks of age in males. In order to capture as many broiler-type alleles as possible, the F_1 generation contained six strains of commercial broiler chickens (68 females crossed with 23 males). Those broiler chickens were selected for 7 generations according to their abdominal fat (Figure 1). Body weight remained constant between the lines. Chickens with highest deviations from the residual abdominal fat were chosen to make the next generation. After the selection, birds in each line were kept by intercrossing to maintain these two lines. In the FL and LL, the percentage of abdominal fat began to diverge at week 3 of age. The ratio of abdominal fat to body weight in the FL is 2 to 3 times higher than the LL. The difference in abdominal fat percentage between the two lines still remains today (Leclercq 1988). Many differences between these two lines were found in several studies. The FL chickens have heavier livers, higher in vivo fatty acid synthesis and lower blood glucose levels than LL, while LL birds have lower triglycerides levels and showed reduced growth on a low protein diet.
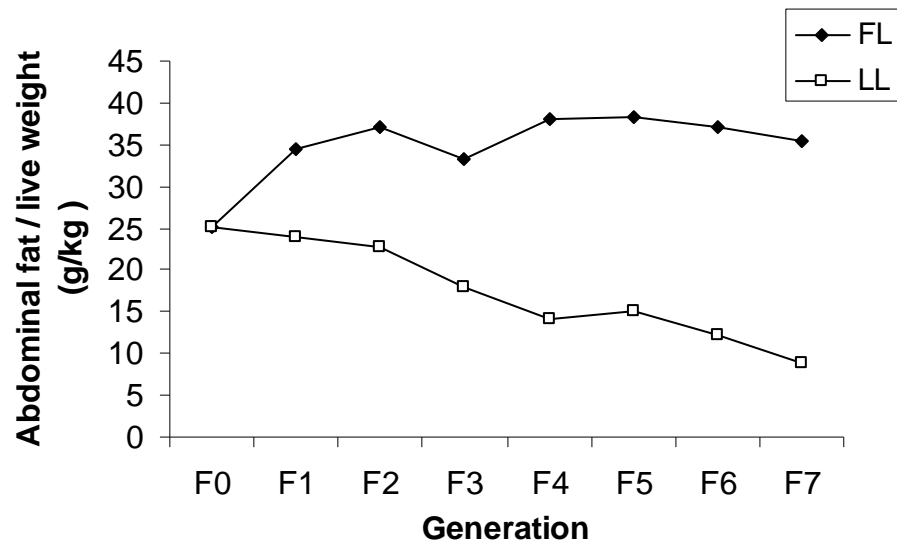
Figure1. Abdominal fat relative to live weight of male chickens in successive generations (Leclercq 1988)

Previous Work

DNA microarray analysis is a high-throughput technique which allows studying the expression of thousands of genes simultaneously (Duggan et al. 1999). It uses base-pairing hybridization. Through covalent attachment, the cDNA or oligonucleotide probes are immobilized on a solid surface. The radioactive isotope- or fluorescence-labeled mRNA or DNA samples hybridize to probes under high-stringency conditions. The mRNA transcript level indicates the expression level of the corresponding gene. Instead of detecting one gene at a time, DNA microarrays make it possible to measure thousands or tens of thousands of genes in a single experiment. DNA microarrays provide the researcher with a highly efficient way to study gene regulation, disease diagnosis and prognosis, bio-marker discovery and drug development.

In previous research, Del-Mar 14k Chicken Integrated Systems microarrays (Cogburn et al. 2003) were used to identify differentially expressed genes in the anterior pituitary glands of fat and lean chickens (Muchow and Porter 2006). Based on these microarray results, five candidate genes located in chromosomes with known QTL for abdominal fat were selected: lysophosphatidic acid (LPA) receptor 1 (LPAR1), Superoxide dismutase 3 (SOD3), Aldo-keto reductase family 1 member 10 (AKR1B10), Glypican 3 (GPC3), and Syndecan 1 (SDC1). Within the promoter region of these four genes, eleven SNPs identified through large scale sequencing of genomic DNA were unevenly distributed between the Fat and Lean chicken lines. One of these SNPs in LPAR1 was investigated further. LPAR1 is involved in regulating adipocyte differentiation (Pages et al. 2001). A T to C transversion which introduces a GATA-1

transcription factor binding site was genotyped in F_2 chickens that had extreme body fat. Significant association between SNP genotype and abdominal fat was found.

The Del-Mar 14K Chicken Integrated Systems Microarray (Cogburn et al. 2004) was printed from cDNAs amplified from expressed sequence tags (EST) sequenced from chicken cDNA libraries created from many tissues, which included neuroendocrine system (pituitary, hypothalamus and pineal), immune system (activated T cells, thymus and activated macrophages), metabolic system (liver and adipose tissue), somatic system (skeletal muscle and growth plate) and reproductive system (oviduct, ovaries and testes). The unique cDNA clone products were purified and then printed onto glass microscope slides. The Del-Mar 14K Chicken Integrated Systems Microarray contains 19200 PCR amplified cDNA spots corresponding to 14053 unique genes and 72 quality control spots.

RNA was extracted from pituitary tissues from eight chickens in the FL and LL at 1, 3, 5 and 7 weeks. By using two-way analysis of variance (ANOVA), 386 genes were determined to have significantly different expression levels in the two lines or have significant line-by-age interaction. Differentially expressed genes are potential genetic markers for body fat. Genomic locations for these genes were determined. Differentially expressed genes that are located within or adjacent the known QTL (Abasht et al. 2006; Ankra-Badu and Aggrey 2005; Ikeobi et al. 2002; Lagarrigue et al. 2006) were selected. Twelve genes were chosen as candidate gene for further studies, of which the gene expression patterns were verified by qRT-PCR. Within 5000 bp upstream of the first exon of these genes, seventeen regions of 12 candidate genes which contain up to 1000 bp with known SNPs were selected. DNA from the FL and LL that had 7 female and 4

22

male birds each were amplified by PCR and sequenced. Eleven SNP in 5 genes that were unevenly distributed in the FL and LL chickens were identified (Table 1).

A T/C SNP at genomic position 32846269 on chromosome Z was in the promoter region of the LPAR-1 gene. The C-SNP introduces a GATA-1 transcription factor binding site (score=87.3). GATA transcription factors function as regulators for adipocyte differentiation. GATA-1 plays an important role in erythropoiesis. LPAR-1 plays an important role in cell differentiation in adipose tissue. There was a significant difference in the SNP frequency between the FL and LL in F_0 chickens. The result was confirmed by allele-specific locked nucleic acid PCR (LNA PCR).

Six hundred and thirty seven F_2 chickens were generated by the intercross of FL and LL. Production traits, such as abdominal fat weight and body weight, were measured in the F_2 generation. The extremes in abdominal fat yield, including 48 fattest females, 48 fattest males, 48 leanest females and 48 leanest males were chosen to be genotyped. Statistical analysis showed that there was a significant association between the SNP genotype and abdominal fat percentage (P<0.05) by using a generalized mixed model.

Table 1. SNP location and distribution among fat and lean chicken lines (Muchow and Porter 2006)

Gene	Sequence	Genomic Location	Lean	Fat
SOD3_SNP1	TCCGTGTGCTT(C/T)GGTGGAGGAC	4:76184498 to 76184519	10C:0T	3C:7T
SOD3_SNP2	AATAATAACCT(A/G)AATGATCTAA	4:76184331 to 76184352	3A:7G	9G:1A
SOD3_SNP1	TTTCTACATTA(C/T)GTATTTAGAT	4:76184289 to 76184310 9	2C:8T	9C:1T
LPA recaptor-1	GGTGGACACAAATCAG(T/C)TCCCAGTTCAAATCTT	Z:65406186 to 65406217	3T:7C	10T:0C
AKR1B10_SNP1	AAAGAATTCAATCCA(A/T)AATACAGAATTATGG	1:64619985 to 64620015	1A:10T	9A:2T
AKR1B10_SNP2	TCAGCACACATA(G/A)CAGCTGTTGAAATG	1:64619705 to 64619731	6G:5A	10G:1A
GPC3_SNP1	CTGAATGTTCCCCT(A/C)TGGAAATACAGCCC	4:3961502 to 3961530	6A:5C	11A:0C
GPC3_SNP2	CAGCAAGCAGTCCTG(T/C)TGTACGACTGCATG	4:3961589 to 3961618	5T:6C	11T:0C
SDC1_SNP1	TTCTCCTTTAACCAGA(G/C)CAGTTCCCTGATCTG	3:104472153 to 104472184	5G:6C	11G:0C
SDC1_SNP2	AATGGCTCCCCAGGG(C/T)GGTGGGCACAGCTCC	3:104471789 to 104471819	4C:7T	9C:2T
SDC1_SNP3	CAGCTCCGAGCTGCC(G/A)GAGCTCAAGGAGCA	3:104471766 to 104471795	5G:6A	11G:0A

The superoxide dismutase (SOD) protein family plays an important role in antioxidant enzyme defense systems against reactive oxygen species (ROS). SODs catalyze the dismutation of two superoxide radicals into hydrogen peroxide and oxygen. They protect brain, lungs and other tissues from oxidative stress. There are three distinct isoforms of SODs in humans (Zelko et al. 2002). SOD1 is present in the cytoplasm. SOD2 is located in mitochondria. SOD3, also known as EC SOD, is secreted into the extracellular space. It was first found in human plasma, lymph, ascites and cerebrospinal fluids. The SOD3 gene is located on chromosome 4 of the chicken and chromosome 4 (region 4p-q21) in humans. It spans 5,900 bp and contains 3 exons. The SOD3 protein is a tetrameric glycoprotein. Like SOD1, SOD3 contains copper and zinc. The expression of SOD3 is cell type- and tissue- specific. High levels of SOD3 were found in human lung, pancreas, thyroid, uterus, heart and placenta. The distinguishing characteristic of SOD3 is its heparin-binding capacity. It binds to the extracellular matrix and cell surfaces through a heparan sulfate proteoglycan anchor. SOD3 eliminates oxygen radicals from the NADP-dependent oxidative system.

The aldo-keto reductase family1, member B10 (AKR1B10) is one of the aldose reductases (Penning 2005). The AKR1B10 gene is located on chromosome 1 of the chicken and chromosome 7 (region 7q33) in humans. The AKR1B10 gene encodes a member of the aldo/keto reductase superfamily. This family contains more than 40 enzymes and proteins. AKR1B10 was discovered as being over expressed in human liver

cancers. It has high expression levels in human small intestine, adrenal gland and colon, and lower levels in liver, thymus, prostate, testis, and skeletal muscle. AKR1B10 can reduce aliphatic and aromatic aldehydes (Crosas et al. 2001; Martin and Maser 2009) and may play an important role in liver carcinogenesis.

Glypicans (GPC) are a family of heparan sulfate proteoglycans that are anchored to the cell membrane by a glycosyl-phosphatidylinositol protein (Filmus 2001). They are expressed mainly during development. Glypicans are involved in cell signaling pathways, cellular proliferation, and tissue growth. There are six family members found in mammals (Filmus 2001). 20-50% of the protein core are identical. Glypican-3 (GPC3) is mutated in the Simpson-Golabi-Behmel syndrome (Cano-Gauci et al. 1999; Lin et al. 1999). GPC3 is located at Xq26.1 in humans (Huber et al. 1998) and on chromosome 4 of the chickens. The gene has 500kb of DNA consisting of 8 exons. GPC3 protein is a 65 kDa protein containing 580 amino acids. GPC3 is expressed ubiquitously in the embryo and over expressed in liver cancers. The expression level is down regulated in most adult tissues. GPC3 plays an important role in morphogenesis and might be a negative regulator of cell proliferation and the progression of malignant tumors. It may modulate the interaction between insulin-like growth factors and their receptors, suggesting a role in growth (Cano-Gauci et al. 1999; Song et al. 2005).

Syndecans are cell surface heparan sulfate proteoglycans (Kolset and Salmivirta 1999). There are four members of the syndecan family in mammals. SDC1 orthologs are also found in many other species including chicken. The SDC1 gene is located on chromosome 3 of the chicken and chromosome 2 (region 2p24.1) in humans. It spans 246,366 bp and contains 9 exons. Several transcript variants exist for this gene. SDC1 is

mainly expressed in epithelial cells and sometimes in plasma cells. SDC1 protein is an integral membrane protein and regulates cell signaling, cell binding, cell proliferation, cell migration and cytoskeletal organization. It can also bind bioactive molecules through its heparan sulfate chains. Core proteins also have functions in a heparan sulfate chain-independent manner.

Chapter 3: Association of Single Nucleotide Polymorphisms with Phenotypic

Production Traits in Broiler Chickens

Introduction

The main meat products in the poultry industry are breasts, wings, thighs and whole bird carcasses. In order to increase the yield of these products, broiler chickens have been selected for rapid growth rate increased breast meat yield and body weight (Havenstein et al. 2003). However, this selection is accompanied by excess fat accumulation, which is a serious problem in the poultry industry due to resulting low feed efficiency and poor carcass quality. Genetic markers for high breast yield, thigh yield, carcass weight and low fat yield are of great commercial value. Moreover, obesity in humans is a major health problem that is controlled by multiple environmental and genetic factors, and 40%-60% of the variation in adiposity among individuals is heritable. The chicken, as a widely used non-mammalian vertebrate model, has not been extensively explored as a model for the genetic basis of human obesity. Genes associated with fatness in chickens could also be involved in obesity in humans. Therefore, identification of genetic markers for body fat in chickens is of direct commercial value and of potential clinical value. The present study was conducted to evaluate potential genetic markers for favorable production traits in broiler chickens with potential as genetic factors for human obesity.

The experimental chickens used in this study were genetically selected according to the deviation from the proportion of abdominal fat weight and body weight at 9 weeks of age. In previous research in our laboratory, Del-Mar 14k Chicken Integrated Systems microarrays (Cogburn et al. 2003) were used to identify differentially expressed genes in

the anterior pituitary glands of Fat and Lean chickens (Muchow and Porter 2006). The pituitary plays an important role in neuroendocrine regulation of adipose tissue accumulation and lean muscle growth. Genes expressed differentially in the pituitary could contribute to the pathways involved in body fat distribution. Based on these microarray results, five candidate genes that were differentially expressed and which were located in chromosomes with known quantitative trait loci (QTL) for abdominal fat were selected. These were lysophosphatidic acid receptor-1 (LPAR1), Superoxide dismutase 3 (SOD3), Aldo-keto reductase family1, member B10 (AKR1B10), Glypican 3 (GPC3), and Syndecan 1 (SDC1). Within the promoter region of these four genes, eleven single nucleotide polymorphisms (SNPs) that were unevenly distributed between 22 individuals of the Fat and Lean chicken lines were identified through large scale sequencing of genomic DNA (Muchow and Porter 2006). The SNP identified in LPAR1 was studied first, and a significant association was found between the LPAR1 SNP and body fat in the experimental Fat and Lean chickens (Muchow and Porter 2006). Based on this finding, we elected to characterize the association of body fat with the SNPs identified in the SOD3, AKR1B10, GPC3, and SDC1 genes.

The superoxide dismutase (SOD) protein family plays an important role in antioxidant enzyme defense systems against reactive oxygen species (ROS). There are three distinct isoforms of SODs. The extracellular SOD3 was first found in human plasma, lymph, ascites and cerebrospinal fluids. The SOD3 gene is located on chromosome 4 of the chicken and chromosome 4 (region 4p-q21) in humans. The AKR1B10 gene encodes a member of the aldo/keto reductase superfamily. It can reduce aliphatic and aromatic aldehydes and may be involved in liver carcinogenesis. The

30

AKR1B10 gene is located on chromosome 1 of the chicken and chromosome 7 (region 7q33) in humans. GPCs are expressed mainly during development. They play a role in cell signaling pathways, cellular proliferation, and tissue growth (Filmus 2001). GPC3 was reported to function in the regulation of insulin-like growth factors (Song et al. 2005). GPC3 is expressed in most tissues and over expressed in liver cancers. The location of GPC3 is on chromosome 4 of the chicken and chromosome X (region Xq26.1) in humans (Lin et al. 1999). Syndecans participate in cell signaling, cell binding and cytoskeletal organization. SDC1 protein is an integral membrane protein and contributes in cell proliferation and cell migration. The SDC1 gene is located on chromosome 3 of the chicken and chromosome 2 (region 2p24.1) in humans.

The current research investigated the association between SNPs in the flanking regions of SOD3, AKR1B10, GPC3, and SDC1 and multiple phenotypic production traits including body fat in an F_2 reciprocal intercross of the experimental FL and LL broiler chicken lines. A factor analysis was performed to take multiple traits into consideration simultaneously. A longitudinal analysis was conducted to estimate the associations between SNPs and body weight from week 1 to week 9. Finally, QTLs on chromosomes 1, 3 and 4 for body fat, initially identified by using 127 microsatellite markers, were refined by incorporating these SNPs into the QTL analysis. Significant associations were found between AKR1B10 SNP1 and fat yield, SDC1 SNP1 and fat yield, SDC1 SNP1 and fat weight as well as SOD3 SNP2 and breast yield. One significant sex-SNP interaction and twelve sire-SNP interactions were detected. These genetic markers may be of great value for marker-assisted selection (MAS) of chickens with less body fat and

improved muscle yield. The same genes are potential candidates for genetic markers of human obesity.

Materials and Methods

Birds

To study genotype-phenotype association in FL and LL broiler chicken lines, a three-generation design was generated from the reciprocal intercross of the FL and LL. In the F_0 generation, 5 males from FL were mated with 14 females from LL to generate the F_1 generation. Three F_1 males and 26 females were chosen to generate a total of 339 F_2 chickens. For the reciprocal cross, 5 LL males were mated with 14 FL females to generate the F_1 birds. Two F_1 males and 17 females were chosen to generate a total of 229 F_2 chickens. Therefore, a total of 568 F_2 chickens were generated for the association analyses.

SNP Genotyping

Genomic DNA was extracted from blood samples using the modified phenol/chloroform method. DNA was quantified according to the saran wrap method (Sambrook J. 1989). Seven SNPs (SOD3 SNP1, AKR1B10 SNP1, AKR1B10 SNP2, GPC3 SNP1, SDC1 SNP1, SDC1 SNP2 and SDC1 SNP3) were genotyped by ABI TaqMan® Genotyping Assays. TaqMan primers and probes were designed by Custom TaqMan SNP Genotyping Assays. The Assays-by-DesignSM service provided with the reagents for SNP genotyping consisted of two unlabeled PCR primers and two TaqMan® MGB probes (FAMSM and VIC® dye-labeled). The primer information is shown in Table 7. The target genomic DNA fragment was amplified in a PCR reaction using

TaqMan Universal PCR master Mix. 5 µL of DNA samples were placed into 384-well

plates and amplified using an ABI Prism Sequence Detection System 7900HT (Applied

Biosystems Inc, Foster City, CA). Thermal cycle parameters were 50 °C for 2min in

Stage 1, 95 °C for 10min in Stage 2, and 45 cycles of 95°C for 15s and 60°C for 1min in

Stage 3. For SDC1 SNP3, Stage 3 of the thermal cycle was changed to 50 cycles of 95°C

for 15s and 58°C for 1min to achieve qualified amplifications. Allelic discrimination

plates were read by an Applied Biosystems Real-Time PCR System. The plotted

fluorescence signals indicating which alleles are in each sample were measured using the

Sequence Detection System (SDS). Allelic discrimination analysis was performed on the

PCR reaction product by using SDS 2.3 software in the ABI Prism Sequence Detection

System 7900HT.

SOD3 SNP2 and SOD3 SNP3 located on chromosome 4 were genotyped by

pyrosequencing. PCR was performed to amplify the two SNPs. Primer sequences used

were SOD_SNP2-3-F1 5' GGGAGTTAACGCAAACACCAA 3' and SOD_SNP2-3-R1

5' GGGACACCGCTGATCGTTTA TTACCAGGATTTCATCTCCACAC 3'. PCR

conditions were 94 °C for 30s, 53 °C for 30s, and 72°C for 20s for 35 cycles using

GoTaq® Green Master Mix (Promega). Biotage PyroMark ID pyrosequencer (Biotage,

Sweden) was used to perform the pyrosequencing assay. Streptavidin-coated beads were

used to capture biotinylated PCR products. The non-biotinylated DNA strand was

removed by washing after alkali denaturation. The beads were then released into

annealing buffer with sequencing primer. After heating at 80-90°C for 2 minutes and then

cooling down to room temperature, DNA sequence was analyzed in the sequencing

reaction.

One of the SNPs, GPC3_SNP2 was not genotyped, due to difficulty in primer design; SNP LPR receptor-1 has already been previously studied.

Statistical Analysis

1. Hardy-Weinberg equilibrium (HWE): HWE was tested as a data quality check. Hardy-Weinberg disequilibrium may be due to selection inbreeding and could also be an implication of disease association. The expected genotype frequencies were calculated by observed allele frequencies according to HWE. Pearson goodness-of-fit test (Chi-square test) was used to test the possible deviations of genotype frequencies.

2. Analysis of Variance (ANOVA): ANOVA was performed to detect the association between SNPs and production traits of the experimental F_2 population. The phenotypic parameter of production traits were estimated with a univariate analysis (

$$y_j = \mu + SNP + sire + hatch + sex + +sire*SNP + sex*SNP + e_j$$), where y_j is the observed fat weight for individual j, μ is the overall population mean, sire is the random sire effect, hatch is the random effect of different hatching group, SNP is the fixed genetic SNP effect, and e_j is the random residual error for individual j. Two meaningful interactions, the interaction between sire and SNP as well as sex and SNP were included in the model. The GLM procedure in Statistical Analysis System software version 9.2 (SAS Institute, Cary, NC) was used to detect the association between SNP, sire and hatch with production traits.

3. SNP effects. Additive effects at each SNP were estimated as half of the difference between the two homozygotes, while dominant effects were calculated as the

difference between heterozygotes and the mean of the two homozygotes. Sex, sire and hatch were also included in the model.

4. Factor Analysis: Factor analysis was used to describe variability among multiple observed variables by using fewer unobserved variables which are known as factors(Martinez et al. 1998). Factors are linear combinations of all observed variables. In order to detect the association between SNP and other multiple correlated carcass production traits, fat yield, breast yield, thigh yield, shank diameter and shank length were analyzed using the FACTOR procedure in SAS 9.2 to determine the factors which contributed to the variation in the production traits. An ANOVA was then performed to determine the associations between these factors and SNP genotypes.

5. Repeated Measurement: The association between SNPs and chicken body weight from 1 week to 9 weeks was examined by longitudinal data analysis. SAS GLM procedure was used to take the correlation within individuals into consideration. The model used here was as follows: weight= SNP| week sire hatch.

Descriptive statistics for female and male chickens in the F_2 population are listed in Table 2 a,b. Combined information for all chickens is listed in Table2 c. Fat yield was calculated as the proportion of fat weight over total carcass weight (body weight at 9 weeks of age). Body weight increased gradually through time for both female and male birds. Male birds had a higher body weight on average (Figure 2). The average fat yield for female birds was higher than for males. The mean thigh yield value for females was slightly lower than for males.

Nine SNPs (SOD3 SNP1, AKR1B10 SNP1, AKR1B10 SNP2, GPC3 SNP1, SDC1 SNP1, SDC1 SNP2, SDC1 SNP3, SOD3 SNP2 and SOD3 SNP3) were genotyped by ABI TaqMan® Genotyping Assays and pyrosequencing. Seven of the SNPs had two alleles and three SNP genotypes: SOD3 SNP1 (AA, AG and GG), SOD3 SNP2 (AA, AG and GG) and SOD3 SNP3 (CC, CT and TT), AKR1B10 SNP1 (AA, AT and TT), GPC3 SNP1 (AA, AC and CC), SDC1 SNP2 (AA, AG and GG) and SDC1 SNP3 (AA, AG and GG). Two of the SNPs had two alleles and only two SNP genotypes: AKR1B10 SNP2 (AG and GG), SDC1 SNP1 (GG and GC). The genotyping results showed that SOD3 SNP2 and SOD3 SNP3 were completely linked. For this reason, SOD3 SNP3 was removed from the pool of SNPs.

Table 3 shows the results of the Hardy-Weinberg equilibrium test. Two DF was adopted in the analysis. The observed SOD3 SNP2 genotype frequencies were: AA (65.37%), AG (29.87%) and GG (4.76%). The corresponding observed allele frequencies

were A (0.8030) and G (0.1970). According to Hardy-Weinberg equilibrium, the expected genotype frequencies were AA (64.49%), AG (31.63%) and GG (3.88%). To test whether the population was in Hardy-Weinberg equilibrium, a Chi-square test was performed to calculate the possible deviations of the expected genotype frequencies from the observed values. The χ^2 value was 1.3242, which indicates that the population was in Hardy-Weinberg equilibrium. The results of Chi-square tests for the other SNPs were SOD3 SNP1 (P<0.0001), AKR1B10 SNP 1 (P=0.2230), AKR1B10 SNP2 (P=0.4003), GPC3 SNP1 (P=0.2445), SDC1 SNP1 (P=0.3046), SDC1 SNP2 (P=0.7674) and SDC1 SNP3 (P=0.9963). Except for SOD3 SNP1, all of the other SNPs were in Hardy-Weinberg equilibrium.

The ANOVA results indicated that some of the SNPs had significant associations with production traits. Many of them had significant interaction with sire or sex. (Figure 3 and Figure 4). AKR1B10 SNP1 and SDC1 SNP1 had a significant association with fat yield (P< 0.0001 and P=0.0406, respectively). SOD3 SNP2 had a significant interaction with sire (P=0.0295), while GPC3 SNP1 had a significant interaction with sex on fat yield (P=0.0239). Similarly, SDC1 SNP1 had a significant association with fat weight (P=0.0154). The interactions between SOD3 SNP2 and AKR1B10 SNP1 with sire on fat weight were significant (P=0.018 and 0.0495, respectively). There was a significant association between SOD3 SNP2 and breast yield (P=0.0022). The Sire-GPC3 SNP1 interaction was significant at P=0.0005 for breast yield. For thigh yield, SDC1 SNP1 had a significant interaction with sire (P=0.0116).

A Tukey test was conducted to compare the mean values for the genotypes for those SNPs with a significant major effect (Figure 5). SDC1 SNP1 had significant associations with fat yield and fat weight. It only had two genotypes. Chickens with genotype GG had significant lower body fat (3.27±0.05%, 71.48±1.19g) than genotype GC (3.50±0.09%, 77.65±2.24g). The association between AKR1B10 and fat yield was also significant. Birds with genotype TT (3.06±0.09%) had the lowest fat yield compared to genotype AA (3.50±0.07%) and AT (3.26±0.06%). SOD3 SNP2 was significantly associated with breast yield. Genotype GG had a significantly higher mean (12.21±0.20%) compared to genotype AA (12.12±0.06%).

The association between GPC3 SNP1 and fat yield was gender-specific (Figure 6). For males, chickens with genotype CC had significantly higher fat yield (3.62±0.21%) than genotype AA (2.74±0.07%) and AC (2.99±0.10%). However, for females, there were no significant differences between chickens with the three genotypes.

Figure 7 shows family-specific associations between SNPs and carcass production traits. There are five sire families in total. Significant Sire-SNP interactions were detected in body weight at week 1, 3, 5 and 9. But since broiler chickens are slaughtered at 6-7 weeks of age, only body weight at week 7 was graphed and analyzed. The associations between SNPs and traits were different among each sire family for SDC1 SNP1, AKR1B10 SNP1, SOD3 SNP2 and GPC3 SNP1. Standard errors and number of chickens were shown in Table 8 a, b and c. A Tukey test was condected within each sire family. The association of AKR1B10 SNP1 with fat weight was sire-specific. For sire 7826, chickens with genotype TT for AKR1B10 SNP1 had significantly lower fat weight (41.89±6.39g) than genotype AT (65.01±3.57g) and AA (65. 68±3.43g). The other

39

comparisons within the other sire families were not significant. The association of SOD3 SNP2 with fat weight was also sire-specific. Genotype AG for SOD3 SNP2 was significantly lower (59.93± 5.52g) of fat than AA (75.39±2.18g) within sire 7712.The difference between the mean values was 15.46g. No other Tukey tests showed significant results. GPC3 SNP1 had a significantly association with breast yield within sire 7712. Chickens with genotype AC had significant higher breast yield (12.52±0.23%) than genotype AA (11.95±0.12%), with a difference of 0.57% of the means. All of the other comparisons did not detect any significant difference. When the sire was family 7734, SDC1 SNP1 was significantly associated with thigh yield. Birds with GG (24.03±0.16%) had significantly lower values than GC (24.94±0.29%) thigh yield. The difference between their mean thigh yields was 0.91%. All of the other Tukey tests within each sire family were not significant.

Backward selection (Table 4) was used to select the best prediction model for fat weight and fat yield. The factor with the highest P-value, which means the lowest association with the production traits, was taken out the model in successive analyses until all factors remaining were significant. In the final model, hatch, sire, sex, AKR1B10 SNP1, GPC3 SNP1 and SDC1 SNP1 remained. The result is consistent with the ANOVA presented in Figure 3. The alpha level for staying in the model was 0.05.

Figure 8 shows differences in fat yield for genotype combinations of AKR1B10 SNP1 and SDC1 SNP1, which had significant associations with fat yield. Chickens with genotype GG for SDC1 SNP1 and TT for AKR1B10 SNP1 had significantly lower fat yield (2.94±0.11%) than the ones with genotype GC for SDC1 SNP1 and AA for

AKR1B10 SNP1 (3.61±0.15%), as well as GG for SDC1 SNP1 and AA for AKR1B10 SNP1 (3.41±0.07%).

The SNP effect test for additive and dominant effects on fat yield was performed on six SNPs (Table 5) with all three SNP genotypes. The additive effect of SOD1 SNP1 was 0.33. The t-test showed that this effect was not significant. The dominance effect was -0.19, which was also not significant. The additive and dominance effects of SOD1 SNP2 were also not significant. AKR1B10 SNP1 had a significant additive effect, -0.41 (P=0.001). The additive effect of GPC3 SNP1 was significant, with an estimation 0.43, P=0.0432. The additive and dominance effects for SDC1 SNP2 and SNP3 were not significant.

Factor analysis (FA) included 463 female and male chickens for four production traits (thigh weight, breast weight, shank diameter and shank length, which are correlated with each other). The first factor calculated represents 83.83% of the total variance and was retained for further analysis. Factor 1 was highly correlated with thigh weight (TW), breast weight (BW), shank diameter (SD) and length (SL). The correlation coefficients between them were 0.9096, 0.9619, 0.8750 and 0.9136, respectively. Therefore, this factor was labeled as muscle-related factor. Muscle-related factor (MF) was calculated as a linear combination of standardized values of the four production traits as follows:

$$MF = 0.2713 * \frac{(TW - \overline{TW})}{Std_{TW}} + 0.2869 * \frac{(BW - \overline{BW})}{Std_{BW}} + 0.2610 * \frac{(SD - \overline{SD})}{Std_{SD}} + 0.2725 * \frac{(SL - \overline{SL})}{Std_{SL}} .$$

$\overline{TW}$ is the mean value of TW for the entire population, and Std_{TW} is the standard deviation of TW. Other traits in the calculation are similarly represented. Since female

41

chickens have relatively low TW, BW, SD and shank SL, the standardized values of these traits for females were negative. Thus, the values of factor 1 were negative for females and were positive for males. To examine the associations between SNPs and muscle-related factor, ANOVA was performed by including SNP, sire, hatch, Sire*SNP interaction and Sex*SNP interaction in the model (Figure 9). SDC1 SNP3 had a significant interaction with sex on muscle related factor (P=0.033). For males, chickens with genotype AG had significantly higher muscle-related factor (0.9612±0.07) than genotype AA (0.6569±0.11), which indicated that birds with genotype AG had a relatively higher combination of TW, BW, SD and SL values (Figure 10). For female birds, no significant differences were detected among the three genotypes.

Repeated Measurement was used on the set of longitudinal data for chicken body weight for week 1 to 9 weeks (Table 6). The results show that GPC3 SNP1 and time had a significant interaction. The association between SNP genotypes and body weight were different at each time point. SDC1 SNP1 did not interact with time. It had a significant association with body weight (P=0.0444). Chickens with SDC1 SNP1 genotype GC had higher average body weight from week 1 through week 9. AKR1B10 did not have significant association with body weight through time.

The QTL mapping for abdominal fat was initially identified by using 127 microsatellite markers through F_2 Analysis in QTL express (Seaton G. et al, 2002). This was initially performed by our colleagues at INRA. SNPs in this study which are located on chromosomes 1, 3 and 4 were incorporated into the new QTL analysis. First, the centimorgan (cM) locations of the SNPs were estimated by comparing existing markers with the closest physical location. Some the SNPs are too close to each other on the

42

chromosome level and thus share the same location. AKR1B10 SNP1 is located at 197cM on chromosome 1. The location for SDC1 SNP1 is at 277 cM on chromosome 3. GPC3 SNP1 and SOD3 SNP1 are located on chromosome 4 at 12 cM and 210 cM, respectively. The SNP genotype, abdominal fat measurements for each individual, and SNP location information were added to the new QTL analysis. On Chromosome 1, one QTL location was detected at 144 cM in both QTL analyses. The F value for this QTL was 10.15, and the LOD was 4.299 after adding in the information on AKR1B10 SNP1. By comparison, in the QTL analysis lacking the AKR1B10 results, the F value was 8.58, and the LOD was 3.652. In figure 11, the new mapping (Figure b) has a higher Var Ratio for the power calculation of QTL detection than the original mapping (Figure a). The peak that has a variance ratio above 5 disappeared in the new mapping at the location where we added in a new SNP. On Chromosome 1, adding information of AKR1B10 SNP1 made the QTL mapping more powerful by increasing the variance ratio and eliminating one marginally significant region. SDC1 SNP1 was added on chromosome 3 at 277 cM (Figure c and d). The new mapping detected a QTL region at 308 cM, which is the same as the original. The F value for the new mapping increased slightly from 1.32 to 1.38, and the LOD score increased from 0.572 to 0.599. On chromosome 4, adding GPC3 SNP1 (12 cM) and SOD3 SNP1 (210 cM) moved the detected QTL from 169 cM to 171 cM (Figure e and f). The F value decreased from 4.45 to 4.17 and the LOD decreased from 1.913 to 1.792. A new peak which has a variance ratio greater than 3 was detected. QTLs on chromosomes 1, 3 and 4 for body fat were refined by incorporating these SNPs into the QTL analysis.

Table 2. General characteristics of female and male chickens in this study.

a)

Female	N	Mean ± Std Dev
bw_1week (g)	231	90.42 ± 12.55
bw_3weeks (g)	233	404.29 ± 47.51
bw_5weeks (g)	234	892.24 ± 96.69
bw_7weeks (g)	234	1363.97 ± 140.64
bw_9weeks (g)	234	1946.88 ± 195.07
breast_yield (%)	234	12.29 ± 0.99
fat_yield (%)	234	3.65 ± 0.93
fat_weight (g)	234	71.81 ± 21.73
thigh_yield (%)	234	23.26 ± 1.21
shank_diameter (mm)	234	10.96 ± 0.63
shank_length (mm)	234	108.02 ± 4.71

b)

Male	N	Mean ± Std Dev
bw_1week (g)	228	94.26 ± 13.12
bw_3weeks (g)	227	452.64 ± 52.71
bw_5weeks (g)	229	1045.16 ± 106.09
bw_7weeks (g)	226	1657.04 ± 168.72
bw_9weeks (g)	229	2445.45 ± 261.33
breast_yield (%)	228	11.94 ± 0.86
fat_yield (%)	229	2.81 ± 0.78
fat_weight (g)	229	69.05 ± 21.12
thigh_yield (%)	229	24.02 ± 1.25
shank_diameter (mm)	229	12.62 ± 0.91
shank_length (mm)	229	122.24 ± 5.56

c)

Combined	N	Mean ± Std Dev
bw_1week (g)	459	92.28 ± 12.95
bw_3weeks (g)	460	428.51 ± 55.66
bw_5weeks (g)	463	968.35 ± 127.31
bw_7weeks (g)	463	1506.86 ± 215.82
bw_9weeks (g)	463	2190.70 ± 343.18
breast_yield (%)	462	12.11 ± 0.94
fat_yield (%)	463	3.24 ± 0.96
fat_weight (g)	463	70.44 ± 21.45
thigh_yield (%)	463	23.63 ± 1.29
shank_diameter (mm)	463	11.78 ± 1.15
shank_length (mm)	463	115.00 ± 8.88

a.
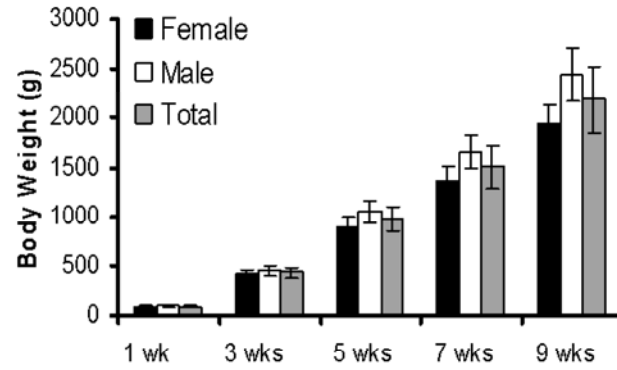
b.

Figure 2. Body weight and Carcass Yield. a) Body weight from week 1 to week 9 for female and male chickens. b) Breast yield, fat yield and thigh yield for female and male chickens.

Table 3. Observed and expected numbers and percentages of SNP genotypes and allele frequencies in the population.

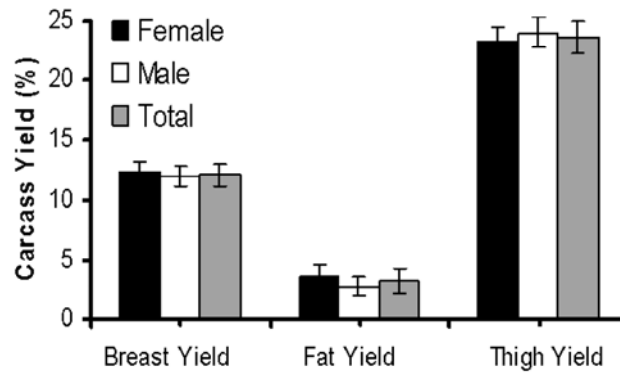|	Genotype			Allele Frequency	
SOD3 SNP1	AA	AG	GG	A	G
Observed	5 (1.16%)	5(1.16%)	422 (97.68%)	0.0174	0.9826
Expected	13 (0.03%)	15 (3.41%)	417 (96.56%)		
χ^2 = 189.52	P<0.0001				
SOD3 SNP2	AA	AG	GG	A	G
Observed	302 (65.37%)	138 (29.87%)	22 (4.76%)	0.8030	0.1970
Expected	298 (64.49%)	146 (31.63%)	18 (3.88%)		
χ^2 = 1.3242	P=0.5158				
AKR1B10 SNP1	AA	AT	TT	A	T
Observed	173 (38.11%)	200 (44.05%)	81 (17.84%)	0.6013	0.3987
Expected	164 (36.16%)	217 (47.95%)	72 (15.89%)		
χ^2 = 3.0013	P=0.2230				
AKR1B10 SNP2	AA	AG	GG	A	G
Observed	0 (0.00%)	80 (17.58%)	375 (82.42%)	0.0879	0.9121
Expected	4 (0.77%)	73 (16.04%)	379 (83.19%)		
χ^2 = 0.7075	P=0.4003				
GPC3 SNP1	AA	AC	CC	A	C
Observed	301 (66.01%)	132 (28.95%)	23 (5.04%)	0.8048	0.1952
Expected	295 (64.77%)	143 (31.42%)	17 (3.81%)		
χ^2 = 2.8174	P=0.2445				
SDC1 SNP1	GG	GC	CC	G	C
Observed	367 (80.13%)	91 (19.87%)	0 (0.00%)	0.9007	0.0993
Expected	371 (81.18%)	82 (17.90%)	5 (0.99%)		
χ^2 = 1.0541	P=0.3046				
SDC1 SNP2	AA	AG	GG	A	G
Observed	52 (11.40%)	192 (42.11%)	212 (46.49%)	0.3246	0.6754
Expected	48 (10.53%)	200 (43.84%)	208 (45.62%)		
χ^2 = 0.5294	P=0.7674				
SDC1 SNP3	AA	AG	GG	A	G
Observed	83 (18.12%)	225 (49.13%)	150 (32.75%)	0.4269	0.5731
Expected	83 (18.22%)	224 (48.93%)	150 (32.85%)		
χ^2 = 0.0074	P=0.9963				
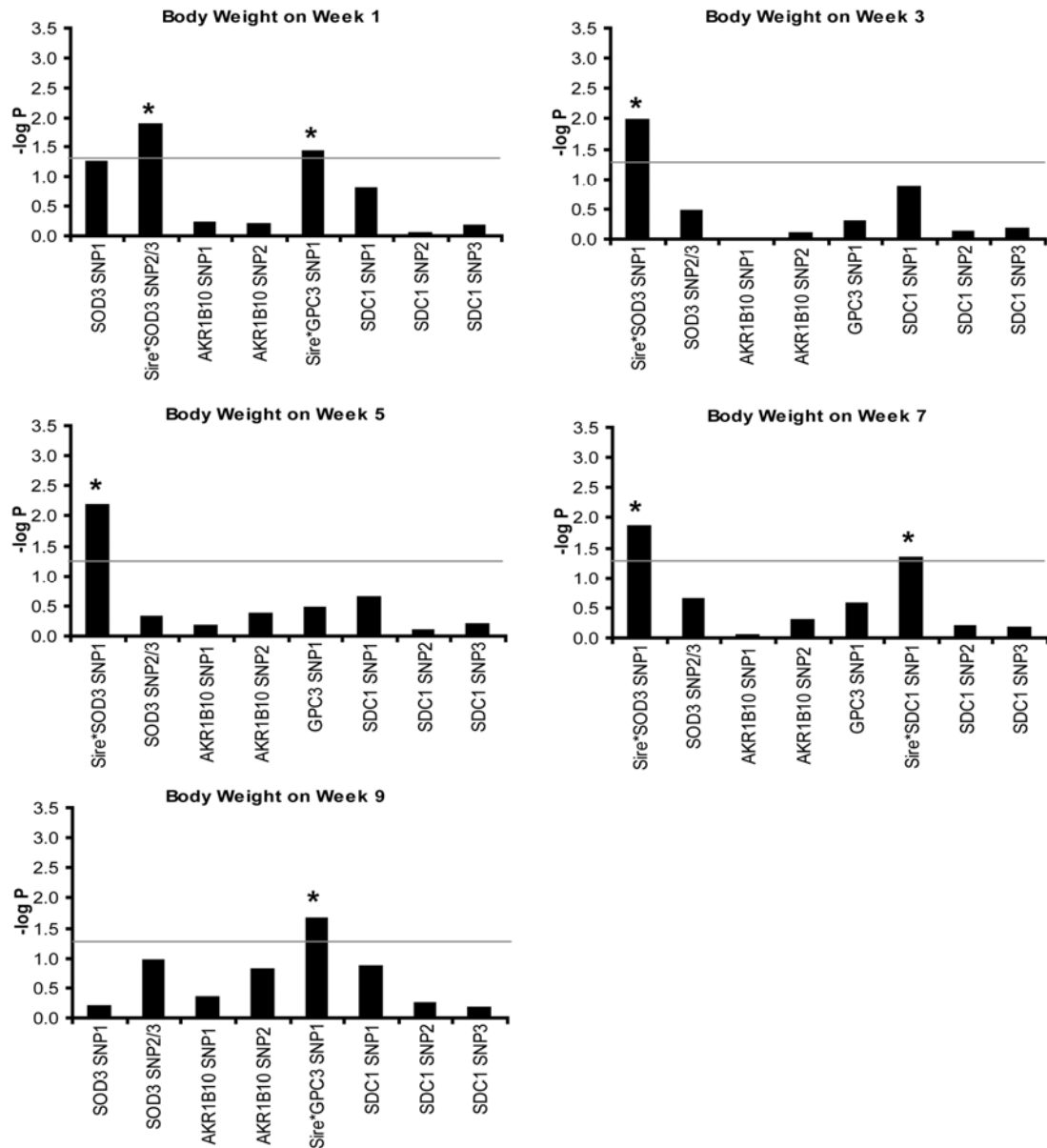
Figure 3. Association between SNPs and body weight from week 1 to week 9. Significant associations were detected at all time points. X-axis indicates Sire-SNP interactions, Sex-SNP interactions and SNP effects, when there was no significant interaction. P-values were calculated from ANOVA. Negative log P values were used for y-axis. The grey line indicates –log P=1.3, which is also P=0.05. Value that is higher than the grey line have a P-value smaller than 0.05.
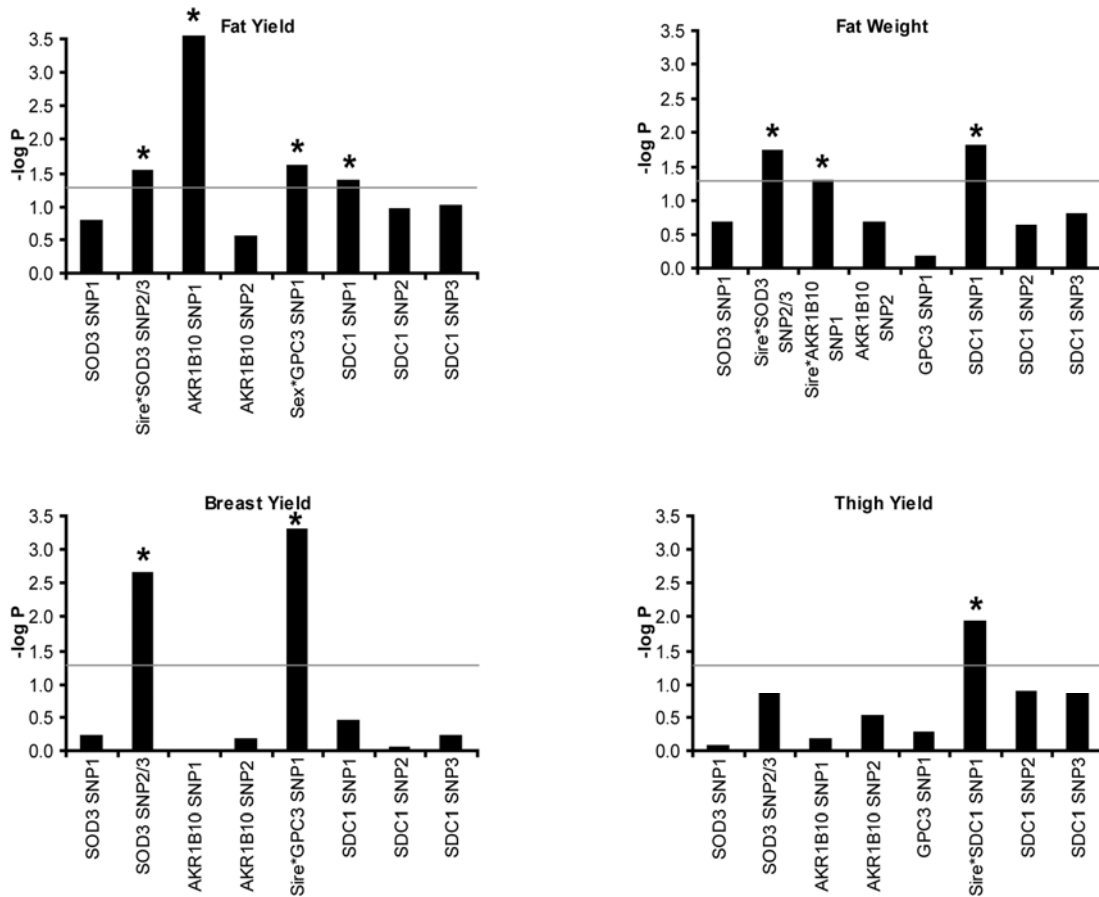
Figure 4. Association between SNPs and carcass traits. Significant associations were detected on four production traits: fat yield and fat weight, breast yield and thigh yield.
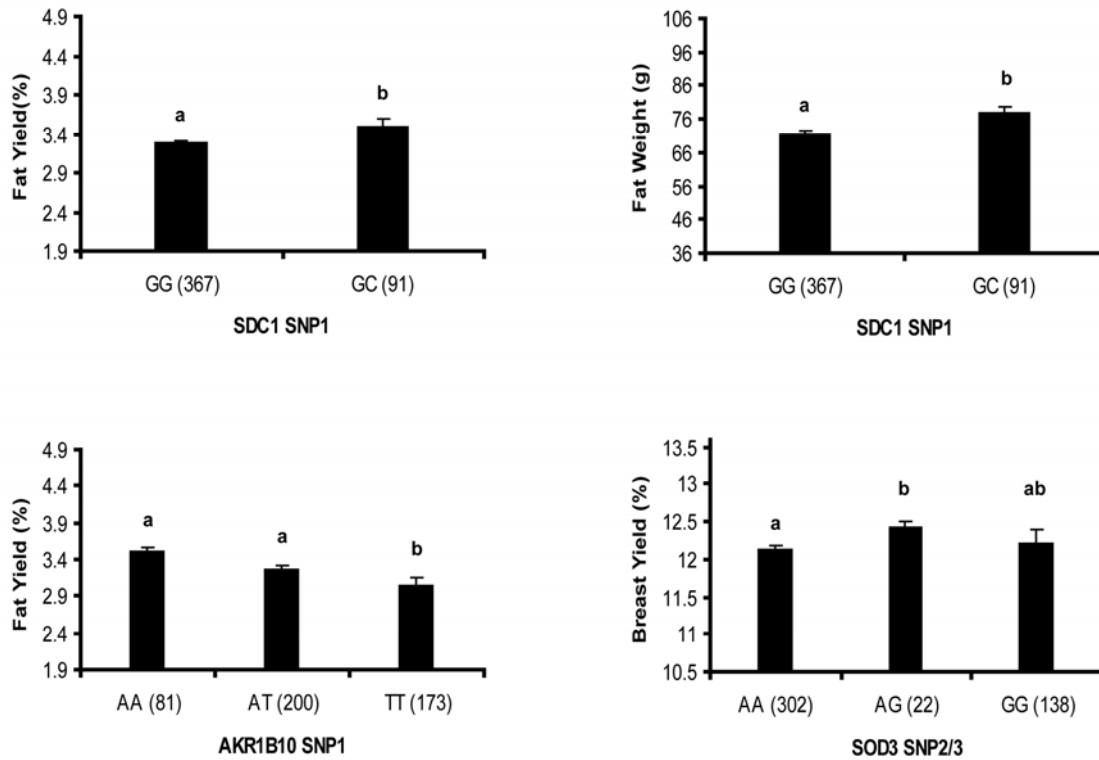
Figure 5. Association Analysis between SNP genotypes and production traits. X-axis indicates the possible SNP genotypes. Y-axis is the values of the corresponding production traits. The ranges of the Y-axis were calculated as 5% to 95% of all of the measured values. For example, for fat yield, only 5% of chickens had fat yield that were lower than 1.9%, while 95% of the chickens had fat yield lower than 4.9%. The numbers of chickens with each SNP genotype are shown in the legend below each figure. Tukey test results are shown above each bar. There were no chickens with genotype of CC for SDC1 SNP1.
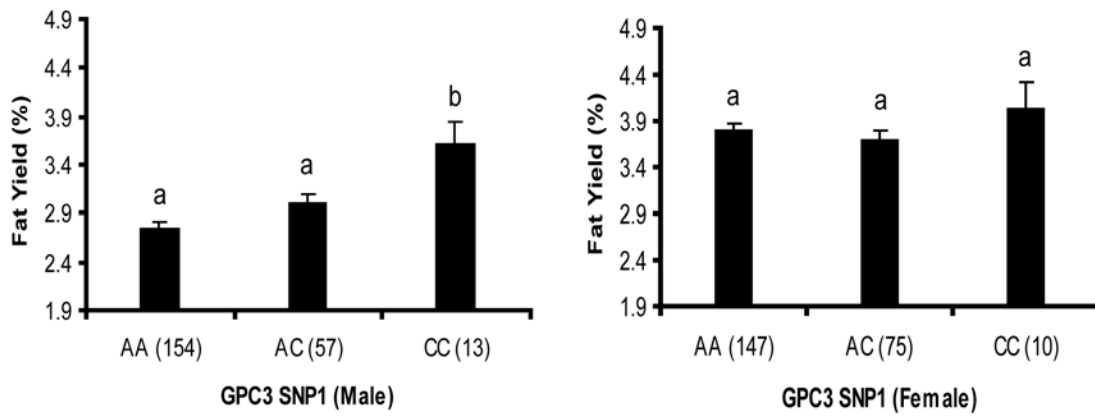
Figure 6. Gender-specific association between GPC3 SNP1 and fat yield. Tukey results are shown over each bar.
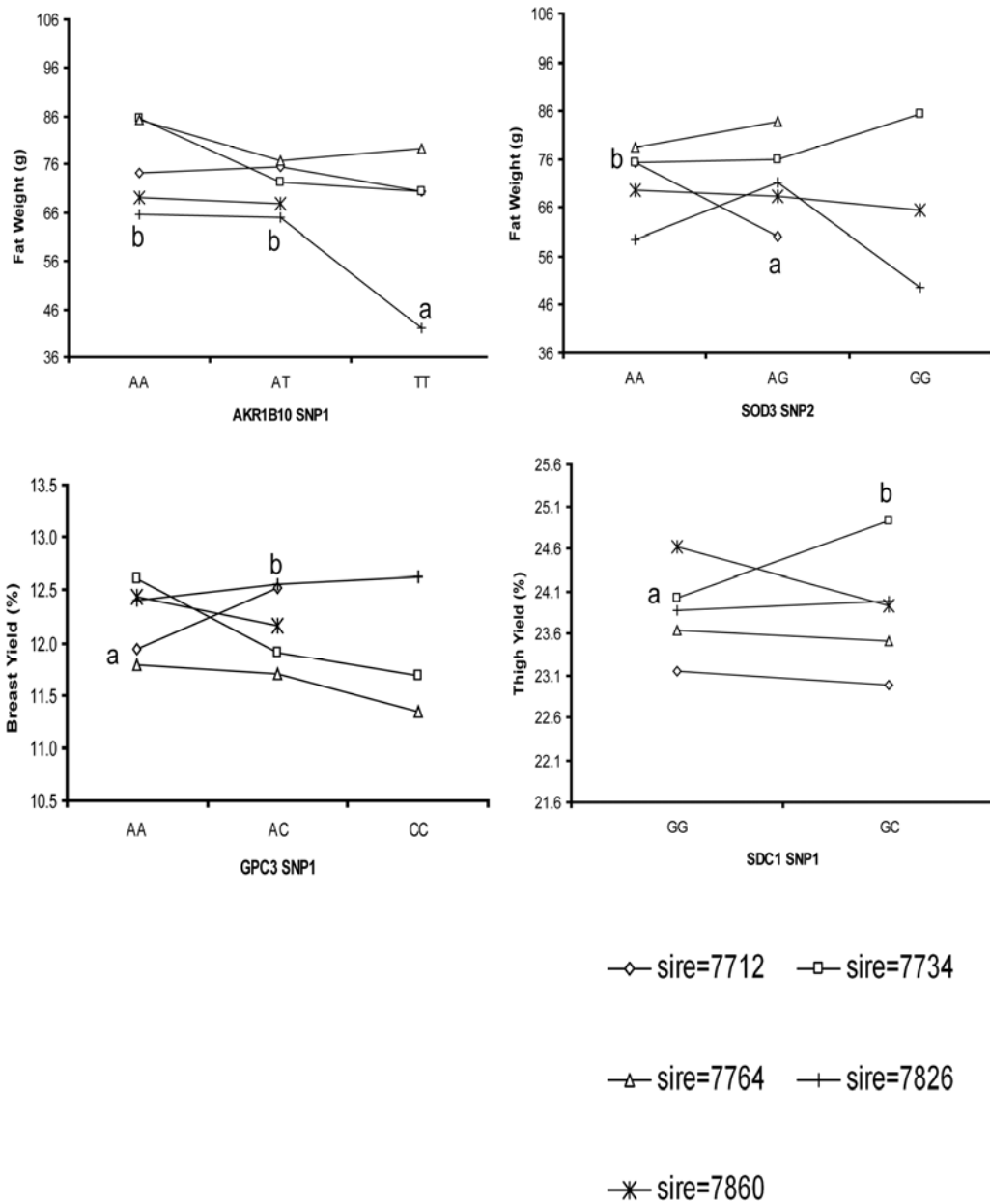
Figure 7. Family-specific associations between SNPs and production traits. X-axis indicates the possible SNP genotypes. Each series indicates one sire family. For some sire families, there were no offspring with certain SNP genotypes. Standard Errors are not shown in this figure, for graphical clarity.

Table 4: Backward selection of multiple variables in the model (Alpha= 0.05).

| Fat yield Source	P value					
hatch	0.0006	0.0007	0.0004	0.0004	0.0003	0.0002
sire	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
sex	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
SOD3 SNP2/3	0.5537	0.467	0.4629			
GPC3 SNP1	0.0267	0.029	0.0322	0.0302	0.0224	0.0125
SDC1 SNP1	0.0234	0.0334	0.0386	0.0314	0.0399	0.021
AKR1B10 SNP1	0.0012	0.0012	0.0016	0.0011	0.0007	0.0005
AKR1B10 SNP2	0.6294					
SDC1 SNP2	0.3881	0.3757	0.3825	0.4066		
SDC1 SNP3	0.5087	0.5706				
SOD3 SNP1	0.244	0.2274	0.2394	0.2447	0.2376	

Fat weight Source	P value					
hatch	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
sire	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
sex	0.0925	0.0912	0.1036	0.1107	0.0879	0.0914
SOD3 SNP2/3	0.887					
GPC3 SNP1	0.0509	0.0495	0.0384	0.0338	0.0343	0.0212
SDC1 SNP1	0.053	0.0482	0.0393	0.0217	0.0255	0.0142
AKR1B10 SNP1	0.0112	0.0094	0.0088	0.0081	0.0069	0.0046
AKR1B10 SNP2	0.4848	0.4431	0.4425	0.5203		
SDC1 SNP2	0.7815	0.7914				
SDC1 SNP3	0.8063	0.7834	0.67			
SOD3 SNP1	0.2701	0.2607	0.2648	0.2512	0.2418	
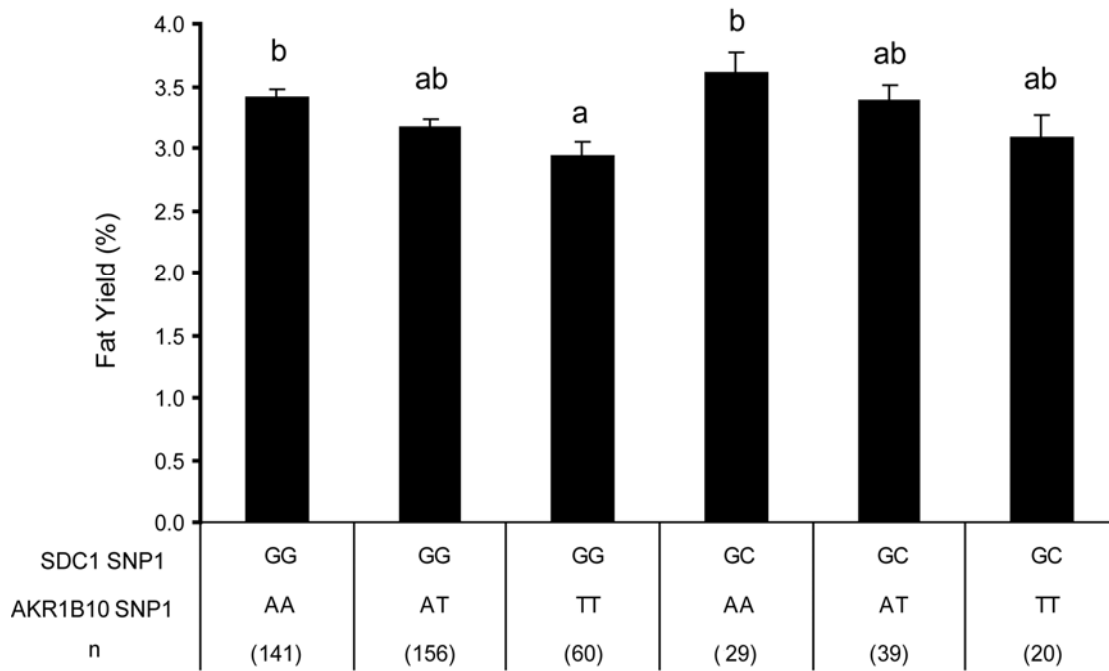
Figure 8. Best combination of AKR1B10 SNP1 and SDC1 SNP1 genotypes in the entire F_2 population. There are 6 combinations of different SNP genotypes. The SNP genotypes and the number of chickens are all shown under the X-axis.

Table 5. Additive and dominant effects of SNPs with significant association with fat yield.

SNP Effect	Estimate	S.E.	t Value	Pr.
SOD3 SNP1 additive	0.329034	0.373456	0.88	0.3788
SOD3 SNP1 dominance	-0.1893	0.449381	-0.42	0.6738
SOD3 SNP2 additive	-0.22608	0.219531	-1.03	0.3037
SOD3 SNP2 dominance	0.159361	0.120941	1.32	0.1884
AKR1B10 SNP1 additive	-0.40671	0.122173	-3.33	0.001
AKR1B10 SNP1 dominance	0.004338	0.084589	0.05	0.9591
GPC3 SNP1 additive	0.43347	0.213717	2.03	0.0432
GPC3 SNP1 dominance	-0.20765	0.128744	-1.61	0.1076
SDC1 SNP2 additive	-0.5145	0.400967	-1.28	0.2002
SDC1 SNP2 dominance	-0.08238	0.125635	-0.66	0.5124
SDC1 SNP3 additive	0.310607	0.385662	0.81	0.4211
SDC1 SNP3 dominance	-0.09775	0.112943	-0.87	0.3873
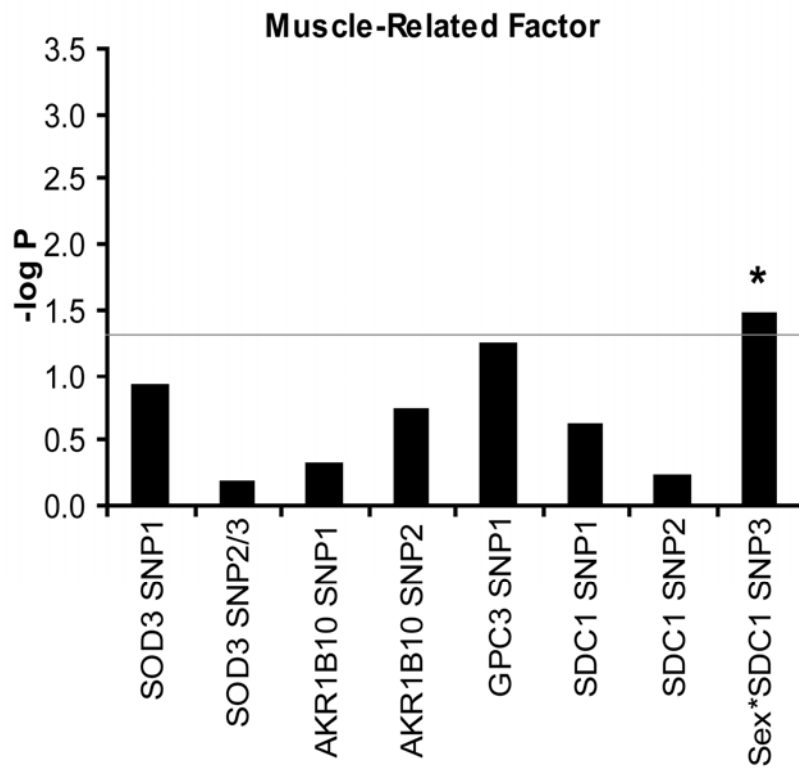
Figure 9. The association between SNPs and Muscle related factor for male and female chickens.
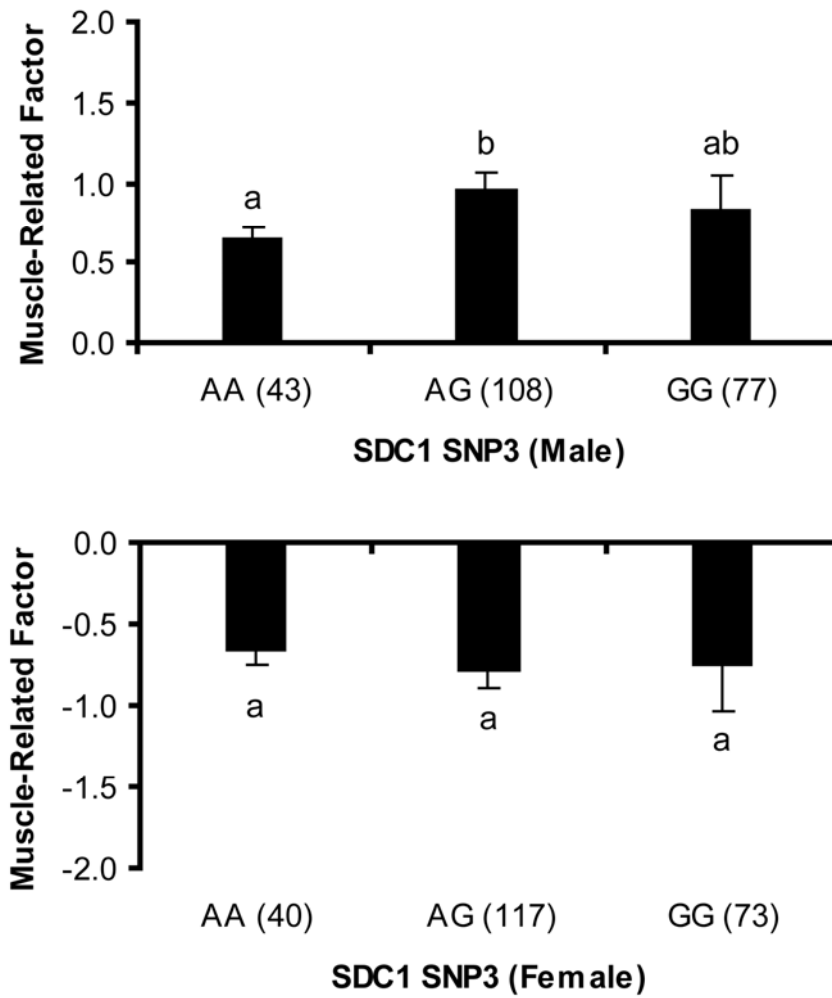
Figure 10. Gender-specific association between SDC1 SNP3 and muscle related factor.

Tukey grouping (a<b, P<0.05) are shown over each bar.

Table 6. Body weight growth for different SNP genotypes from week 1 to week 9.

GPC3 SNP1	week1	week3	week5	week7	week9
AA (301)	91.39	429.70	975.53	1508.10	2214.94
AC (132)	92.24	414.50	951.87	1481.66	2144.12
CC (23)	93.52	437.91	991.00	1479.09	2214.22
Difference	2.13	23.41	39.13	29.01	70.82

SDC1 SNP1	week1	week3	week5	week7	week9
GG (367)	90.77	421.69	941.00	1497.53	2181.16
GC (91)	94.30	441.72	984.49	1502.50	2241.31
Difference	3.53	20.03	43.49	4.97	60.15

* Difference for GPC3 SNP1 is the absolute value of the difference between the genotype with the biggest body weight and the genotype with the smallest body weight at each time point. Difference for SDC1 SNP1 is the difference between the body weight of chickens with GC and chickens with GG for SDC 1SNP1.
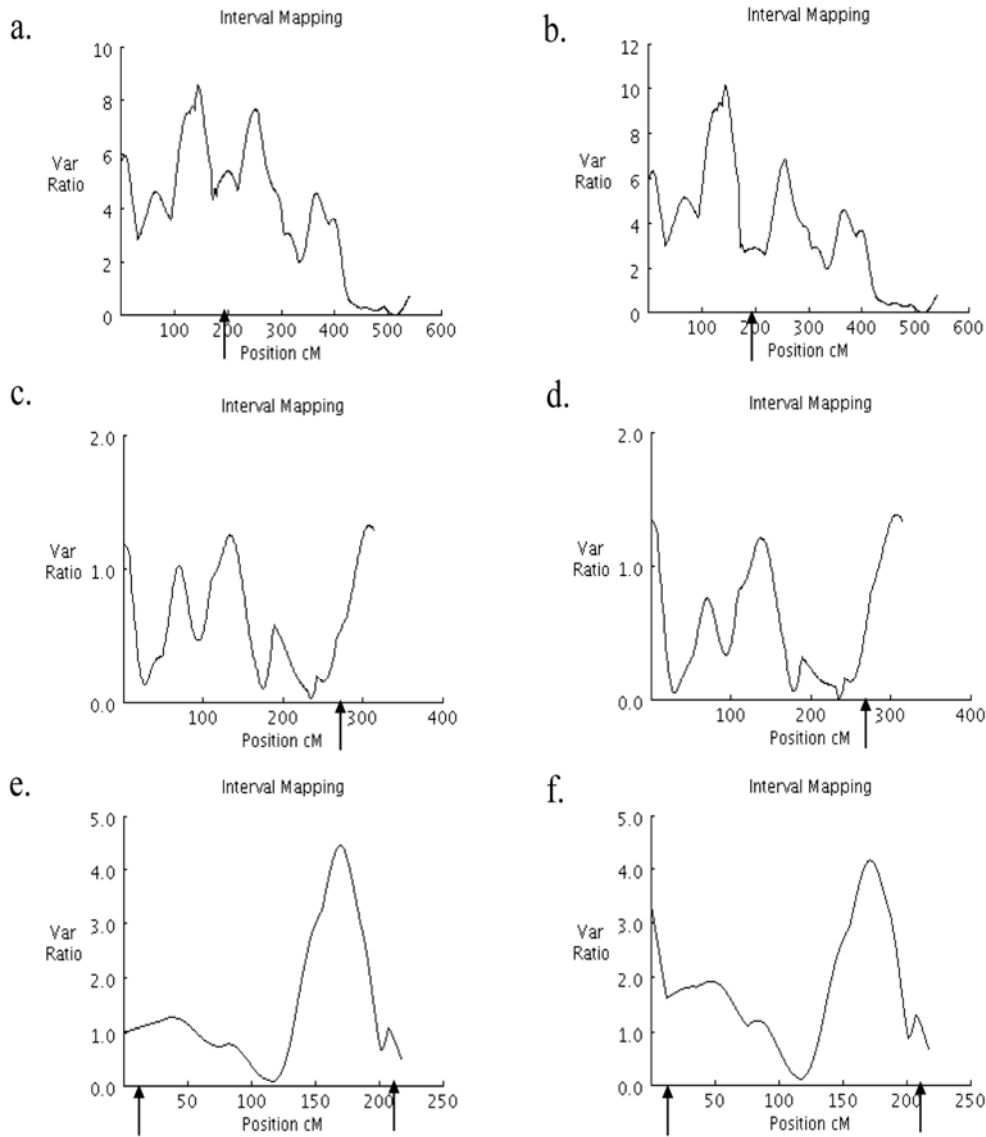
Figure 11. QTL mapping for body fat. Figures a and b show the QTL mapping on chromosome 1. c and d are for chromosome 3, while e and f are for chromosome 4. Figures on the left side are the original QTL mapping, and the ones on the right side are the new mapping. AKR1B10 is located at 197 cM on Chromosome 1, SDC1 SNP1 at 277 cM on Chromosome 3, GPC3 SNP1 at 12 cM and SOD3 SNP1 at 210 cM on Chromosome 4, as indicated by the arrows.

Table 7. Primer sequence for Taqman genotyping.

SNP	Sequence	
GPC3 SNP1	Forward Primer Seq. AACTGGAGATGCCATTGGGAAT	Reverse Primer Seq. CTAAGGAAGCATGGCCTGACA
	Reporter 1 Dye (VIC) ATGTTCCCCTATGGAAAT	Reporter 2 Dye (FAM) TTCCCCTCTGGAAAT
SOD3 SNP1	Forward Primer Seq. AGTGCGAGTGTGCACAGT	Reverse Primer Seq. TGTTGCCCACATGCAGACT
	Reporter 1 Dye (VIC) CTCCACCGAAGCAC	Reporter 2 Dye (FAM) TCCACCAAAGCAC
SDC1 SNP2	Forward Primer Seq. GGTTGGGCCCTGGAATGG	Reverse Primer Seq. CAGTGTCCAAATGCTCCTTGAG
	Reporter 1 Dye (VIC) CCCACCGCCCTGGG	Reporter 2 Dye (FAM) CCCACCACCCTGGG
SDC1 SNP3	Forward Primer Seq. GGTTGGGCCCTGGAATGG	Reverse Primer Seq. TCTGAGAGCAGTGTCCAAATGC
	Reporter 1 Dye (VIC) CTGCCGGAGCTCA	Reporter 2 Dye (FAM) CTGCCAGAGCTCA
SDC1 SNP1	Forward Primer Seq. GCATGACAGTGTTGGTTTTCTTTGT	Reverse Primer Seq. CCCCGTATTCACACAGTCTTGT
	Reporter 1 Dye (VIC) TTAACCAGAGCAGTTCC	Reporter 2 Dye (FAM) TTAACCAGACCAGTTCC
AKR1B10 SNP1	Forward Primer Seq. GTGGGAAAGTGAAAGGAACATGTG	Reverse Primer Seq. GTGTCTTCTAAATGCTTCCCACCAT
	Reporter 1 Dye (VIC) AATTCAATCCAAAATAC	Reporter 2 Dye (FAM) AGAATTCAATCCATAATAC
AKR1B10 SNP2	Forward Primer Seq. ACATCACTCTTGGAAGTGGTCAG	Reverse Primer Seq. CTGTGCTTGTCACTATGGTATCTGA
	Reporter 1 Dye (VIC) ACACATAGCAGCTGTT	Reporter 2 Dye (FAM) CACACATAACAGCTGTT

Table 8 a. Family-specific SNP effects (Tukey groups were formed with alpha=0.05).

	AKR1	fat weight		sodsnp2	fat weight	
	AA	AT	TT	AA	AG	GG
sire=7712	74.21	75.54	70.41	75.3943	59.9315	
SE	4.236	3.0188	3.784	2.1786	5.5219	
Tukey	a	a	a	b	a	
n	23	45	27	84	12	0
sire=7734	85.51	72.29	70.38	75.3969	76.0017	85.2856
SE	4.8713	4.4517	5.0368	4.101	4.6163	13.5757
Tukey	a	a	a	a	a	a
n	24	37	26	45	40	3
sire=7764	85.37	76.59	79.12	78.5869	83.9828	
SE	4.0261	2.836	4.7911	2.4366	5.5741	
Tukey	a	a	a	a	a	
n	26	51	18	84	14	0
sire=7826	65.68	65.01	41.89	59.5342	71.1649	49.4733
SE	3.4341	3.5684	6.3903	3.1197	4.2502	20.4154
Tukey	b	a	a	a	a	a
n	35	34	10	57	23	1
sire=7860	69.21	67.87		69.5951	68.2866	65.3546
SE	2.5567	3.3349		3.5051	2.8663	4.7087
Tukey	a	a		a	a	a
n	65	33	0	32	50	18

Table 8 b. Family-specific SNP effects (Tukey groups were formed with alpha=0.05).

	sodsnp2	fat yield		gly1	breast yield	
	AA	AG	GG	AA	AC	CC
sire=7712	3.4565	2.9868		11.945	12.515	
SE	0.09731	0.2466		0.1209	0.2275	
Tukey	a	a		a	b	
n	84	12	0	76	19	0
sire=7734	3.5401	3.5648	3.6753	12.6126	11.904	11.686
SE	0.1752	0.1972	0.5799	0.1844	0.195	0.3789
Tukey	a	a	a	a	a	a
n	45	40	3	44	33	7
sire=7764	3.464	3.5707		11.7822	11.7082	11.3317
SE	0.1131	0.2588		0.1032	0.4088	0.8039
Tukey	a	a		a	a	a
n	84	14	0	93	4	1
sire=7826	2.6955	3.3156	2.6203	12.3966	12.5494	12.62
SE	0.1376	0.1875	0.9005	0.1752	0.1308	0.2122
Tukey	a	a	a	a	a	a
n	57	23	1	23	43	15
sire=7860	3.083	3.1582	3.0129	12.444	12.1586	
SE	0.1713	0.1401	0.2302	0.1042	0.1365	
Tukey	a	a	a	a	a	
n	32	50	18	65	33	0

Table 8 c. Family-specific SNP effects (Tukey groups were formed with alpha=0.05).

	syn1	thigh yield	Syn1	body weight at week 7
	GG	GC	GG	GC
sire=7712	23.154	22.9871	1500.74	1443.43
SE	0.1137	0.347	25.0018	76.3198
Tukey	a	a	a	a
n	88	8	88	8
sire=7734	24.0251	24.9362	1464.39	1553.69
SE	0.1623	0.2891	30.902	55.042
Tukey	a	b	a	a
n	72	15	72	15
sire=7764	23.642	23.5113	1572.96	1613.34
SE	0.1675	0.2266	28.9738	39.9307
Tukey	a	a	a	a
n	69	27	69	27
sire=7826	23.8676	23.9898	1535.04	1517.76
SE	0.1974	0.2649	28.9847	38.8967
Tukey	a	a	a	a
n	46	33	46	33
sire=7860	24.6095	23.9294	1556.47	1642.18
SE	0.1235	0.3969	23.0768	74.1663
Tukey	a	a	a	a
n	92	8	92	8

Discussion

In this study, we examined the association between eight SNPs and production traits, including body fat, in broiler chickens. We found that four SNPs out of the eight were associated with body fat; two SNPs were associated with breast yield, while one was associated with thigh yield. These SNPs are potentially useful for the identification of genetic markers for human obesity. They are also of potentially great use in marker-assisted selection of chickens with less abdominal fat in the broiler breeder industry.

Table 7 shows the best genotypes for favorable production traits, including low fat weight and fat yield, high thigh yield and high breast yield. Four SNPs, SDC1 SNP1, GPC3 SNP1, AKR1B10 SNP1 and SOD3 SNP2 were significantly associated with at least one of the production traits. For SDC1 SNP1, chickens with genotype GG had the lowest fat weight and fat yield. This suggests that SDC1 SNP1 could be used as a genetic marker for the selection of body fat. Chickens with genotype GC in sire family 7734 had the highest thigh yield. Male chickens that have genotype of AA and AC for GPC3 SNP1 had the lowest fat yield, while genotype AC also had the highest breast yield in sire family 7712. The fat yield of chickens with genotype TT of AKR1B10 SNP1 was the lowest. The fat weight was also significantly lower than the other two genotypes in sire family 7826. For SOD3 SNP2, chickens with genotype AG had the highest breast yield and the lowest fat weight in sire family 7712. Therefore, the selection of favorable production traits such as less body fat, more thigh yield and greater breast yield can be

potentially based on four SNPs in this study. The sire and sex effects should be taken into consideration.

Table 9. Best genotypes for favorable production traits (Tukey groups were formed with alpha=0.05).

SDC1 SNP1	GG	GC	CC
	Fat weight Fat yield	Thigh yield (sire=7734)	
GPC3 SNP1	AA	AC	CC
	Fat yield (sex=male)	Fat yield (sex=male) Breast yield (sire=7712)	
AKR1B10 SNP1	AA	AT	TT
			Fat yield Fat weight (sire=7826)
SOD3 SNP2	AA	AG	GG
		Breast yield Fat weight (sire=7712)	

Some of the SNP associations were family-specific. There were significant interactions between sire and SNP on fat weight, fat yield, breast yield, thigh yield and body weight at week 7. The associations between the SNP and production traits varied within each sire family. Specific genetic background plays an important role in the selection of those traits based on the corresponding SNP markers. In the application of marker-assistant selection, the interaction between sire and SNP should be taken into consideration. Within some certain sire families the efficiency of the selection might be much higher than in other sire families. Interaction between gender and SNP was found in GPC3 SNP1 on fat yield. The difference in fat yield between genotype AA, AC and genotype CC was only significant in males. Genetic differences in abdominal fat between female and male chickens have been found in many studies (Broadbent 1982; Leenstra 1986). This interaction might be of great importance in poultry breeding. Broiler chickens are selected within gender, with separate male and female lines maintained for breeding of production birds. Thus, SNPs that were significant only for one gender could easily be adopted.

The favorable phenotypic values for carcass traits, including thigh weight, breast weight, shank diameter and shank length, are correlated with each other. Changes in one trait could be accompanied by changes in the other traits. As a result, there is a risk that when selection is based on one single trait, the selected chicken as a whole might not be favorable in the chicken industry. In order to detect an association between SNP and these multiple correlated production traits together, factor analysis was used in this study. The muscle related factor indicated a combination of those four traits as a whole. SDC1 SNP3 showed a significant interaction with sex on this factor. Instead of selecting

towards only one production trait, the selection based on muscle related factor would be predicted to result in chickens with higher thigh weight, breast weight and larger shank diameter and shank length at the same time, at least in males.

The chicken lines used in this study are Fat and Lean chickens, which had significantly different body fat percentage. In previous work in our laboratory, four candidate genes were selected that were expressed differentially in the anterior pituitary glands of these chickens. Eleven SNPs were identified in the promoter region of these genes. In the present study, four of the SNPs turned out to have some association with fat weight or fat yield. Four of them were associated with body weight at different ages. Two of the SNPs were associated with breast yield and one of them was associated with thigh yield. Choosing FL and LL and identifying differentially expressed SNPs in FL and LL lines was an efficient way to search for potential markers associated with fat yield. In this study, the efficiency of identification of genes or markers associated with body fat was about 36%. In other words, 36% of the SNPs analyzed were significantly associated with production traits. The SNP selection method used in this study combined genomic and functional approaches together. The genomic approach of DNA microarray can provide investigators with a broad view of all differentially expressed genes. Checking QTL regions for body fat helped to efficiently select genes that might be involved in the process of fat deposition according to the QTL analysis.

In previous studies in our laboratory, one SNP in the promoter region of LPAR1 was investigated. A T-to-C transversion was found which introduces a GATA-1 transcription factor binding site. The SNP was genotyped in 192 F_2 fattest and leanest female and male chickens. The SNP genotype was found to be significantly associated

with abdominal fat. This association study proved the possibility of identifying genetic markers for body fat by using the method described in the previous study. It was a rational foundation for our current research on the association between the rest of the eleven SNPs and production traits in boiler chickens.

In summary, eight SNPs that are in the promoter regions of four genes were genotyped in the F_2 generation created by intercrossing FL and LL chickens. Four SNPs, SOD3 SNP2, AKR1B10 SNP1, GPC3 SNP1 and SDC1SNP1 were found to have significant associations with body fat. Two SNPs, SOD3 SNP2 and GPC3 SNP1 were found to be associated with breast yield, while SDC1 SNP1 was associated with thigh yield. SDC1 SNP3 was significantly associated with muscle-related factor. The additive and dominant effects for these SNPs were determined. These SNPs can serve as important genetic markers for abdominal fat in the poultry industry and selection of superior animals for breeding using marker-assisted selection.

Chapter 4: Identification of Putative Transcription Factor Binding Sites

Gene expression level can be regulated when a transcription factor (TF) binds to the promoter region of a gene. TESS, Transcription Element Search System (Schug 2008), was used to search for possible transcription factor binding sites in SNP-specific consensus sequences by browsing the data from TRANSFAC database (http://www. cbil.upenn.edu /cgi-bin/tess/tess). The default threshold was La score >10, Sc score>1 and Sm score>1.

The C-SNP consensus sequence of SOD3 SNP3 introduced putative TF binding sites of insulin promoter factor 1 (IPF1), Opaque-2, TGA1a and common plant regulatory factor 2 (CPRF-2). The IPF1 may be involved in tissue-specific control of insulin expression (Boam and Docherty 1989), pancreas development (Stoffers et al. 1997), maturity-onset diabetes of the young and type II diabetes (Hani et al. 1999; Macfarlane et al. 1999). Opaque-1 is associated with decreased translation efficiency of the O2 mRNA (Lohmer et al. 1993). TGA1a enhances transcription through as-1, which regulates auxin- and salicylic acid-inducible transcription (Klinedinst et al. 2000; Niggeweg et al. 2000). CPRF-2 is involved in the activation of light-responsive chalcone synthase (Weisshaar et al. 1991). The T-SNP returned binding sites to Murine hepatocyte nuclear factor-3B (HNF-3B), HNF-6alpha, HNF-6beta and OC-2. HNF-3B participates in the regulation of gene expression in liver and intestine (Pani et al. 1992; Rausa et al. 1997). HNF-6 (HNF-6alpha and HNF-6beta) play a role in developmental programs. They regulate sex-specific effects of growth hormone in rat liver (Lahuna et al. 1997). OC-2 may be involved in liver differentiation and metabolism (Jacquemin et al. 1999). Both C and T allele introduced the same E4BP4 and POU3F2 binding sites. In our study, chickens with genotype CC of SOD3 SNP2 were shown to have more fat weight in sire family 7712 and

less breast yield than chickens with genotype CT. The reason of the better performance

of the heterozygous chickens might due to the possibility of introducing TF binding sites

by both C-SNP and T-SNP.

For AKR1B10 SNP1, the A-SNP introduced multiprotein bridging factor-1

(MBF-1) binding site. MBF-1 participates in lipid metabolism (Brendel et al. 2002). The

T-SNP sequence introduced HNF-6alpha, HNF-6beta, OC-2, aristaless homeobox like 4

(Alx-4), cartilage homeoprotein 1 (Cart-1) and double homeobox protein 1 (DUX1)

binding sites. Alx-4 determines anterior-posterior patterning of the limb bud (Qu et al.

1997). Cart-1 plays an essential role in forebrain mesenchyme survival (Zhao et al. 1996).

DUX1 might participate in facioscapulohumeral muscular dystrophy (Ding et al. 1998;

Ostlund et al. 2005). Broiler chickens that have genotype TT tend to have lower fat yield

than AA and AT. Since transcription factor MBF-1, introduced by the A-SNP, is

involved in lipid metabolism, it might be associated with the increased fat percentage in

chickens with AA and AT genotype by regulating AKR1B10 expression.

The G-SNP sequence in AKR1B10 SNP2 returned a FACB binding site, while

the A-SNP returned GT-IIBalpha and GT-IIBbeta binding sites. FACB plays a role in

acetamide and acetate utilization (Todd et al. 1997). Galactosyltransferase isozyme II

(GT-II) transfers galactose to N-acetylglucosamine from UDP-galactose (Nozawa et al.

1989). In our association study, AKR1B10 SNP2 did not show any significant association

with important production traits in broiler chickens.

For GPC3 SNP1, the C-SNP sequence introduced three possible binding sites, interleukin (IL-6), H-APF-1 and C/EBPbeta. IL-6 regulates the expression of albumin and several genes in liver cells and interacts with glucocorticoid receptor (Hocke et al. 1992; Majello et al. 1990). H-APF-1 is expressed in hepatic cells and interacts with C-reactive protein. C/EBPbeta plays an important role in adipocyte differentiation (Tanaka et al. 1997). However, the A-SNP consensus sequence was not shown to return any TF binding site. Broiler chickens that have genotype CC were shown to have higher fat yield than AA and AC. Two putative transcription factors, IL-6 and C/EBPbeta introduced by only the C-SNP, which are associated with GR and adipocyte differentiation, might cause the differences in body fat in the birds.

Only one potential TF binding site, FACB (Todd et al. 1997) was introduced by G-SNP sequence in SDC1 SNP1. FACB is involved in acetamide and acetate utilization (Todd et al. 1997). In our study, chickens with genotype GG had significantly lower fat yield and fat weight than genotype GC. It is possible that the FACB transcription factor might regulate the gene expression level of SDC1, thereby having an effect on body fat accumulation in broiler chickens.

For SDC1 SNP2, binding sites for activator protein 2 (AP-2), AP-2alpha, AP-2alpha A, AP-2 alphaB, TFAP2A, EGFR-specific transcription factor (ETF) and CP1 were introduced by the C-SNP sequence. AP-2 is a regulator of vertebrate development (Mitchell et al. 1991). AP-2 alpha is able to transactivate estrogen receptor (McPherson and Weigel 1999). TFAP2A regulates differentiation in an early phase (Barrallo-Gimeno et al. 2004). ETF participates in the overexpression of epidermal growth factor receptor (EGFR) (Kageyama et al. 1988). AP-2, also known as nuclear factor Y (NF-y), regulates

cell-type-specific gene expression (Kim and Sheffery 1990). The T-SNP returned a CACCC-binding factor binding site. The CACCC-binding factor plays an important role in T-cell receptor gene expression (Wang et al. 1993). There was no significant association between SDC1 SNP2 and production traits detected in our study.

In all, SNPs that exist in the promoter region of a gene can introduce transcription factor binding sites and thereby regulate gene expression. The SNP-specific sequences of seven SNPs in our study were found to return some important TF binding sites. Five of the SNPs have significant associations with production traits such as fat weight, fat yield and breast yield. The changes in gene expression level caused by TF might be the reason for the differences in the production traits.

Chapter 5: Discussion

The fat accumulation in broiler chickens is a serious problem in the poultry industry. This study investigated the association between SNPs and multiple phenotypic production traits, including abdominal fat, in experimental Fat and Lean chicken broiler lines. cDNA microarrays were used in the previous study to identify differentially expressed genes in the anterior pituitary glands of Fat and Lean chickens. Based on these microarray results, four candidate genes located in chromosomes with known QTL for abdominal fat were selected (SOD3, AKR1B10, GPC3), and SDC1. Within the promoter region of these four genes, eight SNPs identified through large scale sequencing of genomic DNA were unevenly distributed between the Fat and Lean chicken lines. A three-generation experimental population produced through a reciprocal intercross of the FL and LL was genotyped in this study. The general character for the experimental population was examined by Hardy-Weinberg Equilibrium test. A univariate model was used to estimate several production traits, with sire and hatch as random effects and SNP as a fixed effect. Significant associations were detected between AKR1B10 SNP1 and SDC1 SNP1 and fat yield. SDC1 SNP1 was significantly associated with fat weight. SOD3 SNP2 was significantly associated with breast yield. Five sire-SNP interactions and one sex-SNP interactions were found to be significant. A factor analysis was performed to take multiple traits into consideration at the same time. There was a significant interaction between sex and SDC1 SNP3 on muscle-related factor. A longitudinal analysis on body weight growth revealed that GPC3 SNP1 interacted with time period on body weight from week 1 to week 9. QTLs analysis was performed. QTL on chromosomes 1, 3 and 4 for body fat, initially identified by using 127 microsatellite markers, were refined by incorporating these SNPs into the QTL analysis. Canonical

75

correlation analysis was conducted to predict the carcass traits from body weight growth. SNP associations were also tested in other chickens lines, specifically high growth and low growth chicken lines. No significant associations were found, and subsequent power analysis showed that the sample size we had was not enough for further study to detect significant associations.

To examine the properties of the experimental population, Hardy-Weinberg Equilibrium test was performed. Except for SOD3 SNP1, all of the other SNPs were in Hardy-Weinberg Equilibrium. There are five conditions for the Hardy-Weinberg Equilibrium: No mutations; no migration; random mating; large population; no selection. For SOD3 SNP1, one or more of the conditions had not been met. But since different sources can cause the same discrepancy, we can not identify which condition had not been fulfilled. For example, the less-than-expected observed frequency of AA and AG genotypes might due to the selective elimination of allele A, or due to different gene frequency in male and female birds of the parental generation. For all of the SNPs in our study except for SOD3 SNP1, the population fulfilled the five conditions for HWE.

Carcass yield was significantly different among SNP genotypes. The leanest chickens with genotypes TT for AKR1B10 SNP1 and GG for SDC1 SNP1 had the lowest mean value of fat yield (2.94%), while the mean fat yield for the fattest chickens with AA genotype for AKR1B10 SNP1 and GC for SDC1 SNP1 was 3.61%. Chickens with genotype AG for SOD3 SNP2 had significantly higher breast yield than chickens with genotype AA. The importance of these differences would be magnified in the broiler industry, due to the large scale of the industry. According to data from the National Agricultural Statistics Service, 140,455,000 broiler chickens were slaughtered in

76

Maryland in 2008. The average body weight of the birds was 4.9 pounds. If the leanest chickens with genotypes TT for AKR1B10 SNP1 and GG for SDC1 SNP1 were selected by marker-assistant selection, the potential fat reduction compared with the fattest chickens will be 4.9 million pounds. If chickens with genotype AG for SOD3 SNP2 were selected for higher breast yield, a maximum of 2.1 million pounds of breast weight could be potentially gained. If chicken breast was sold on the market at a price of three dollars per pound, the potential commercial value for this selection is 6.3 million dollars in Maryland in 2008 alone. However, this estimate is based on a complete conversion of the broiler population to the AG genotype for SOD3 SNP2. This genetic selection could take several generations, and the possibility exists that other undesirable phenotypes might result from the selection.

Many genome-wide studies for body fat and other production traits in broiler chickens have been conducted. Several experimental populations were produced, and many QTL mapping method were applied. Tatsuda & Fujinaka. (2001) found a QTL for the ratio of abdominal fat deposition to live body weight (abdominal fat %) at 16 weeks of age on Chromosome 7 at 38 cM. Ikeobi et al. (2002) detected QTLs for abdominal fat weight on Chromosome 3 at 40 cM, Chromosome 5 at 51 cM, Chromosome 7 at 41 cM, Chromosome 15 at 0 cM and Chromosome 28 at 17 cM. They also found QTL for abdominal fat weight adjusted for carcass weight on Chromosome 1 (126 cM), 5 (50 cM), 7 (39 cM) and 28 (17 cM), QTLs for subcutaneous fat on Chromosome 3 (170 cM), 7 (78 cM), and 13 (35 cM), QTL for skin fatness on Chromosome 3 (166 cM) and Chromosome 28 (0 cM). QLTs for fat distribution (skin fat weight adjusted for abdominal fat weight) were located on Chromosome 5 (51 cM), 7 (36 cM) and 15 (0 cM).

Jennen et al. 2004 found two genome-wise significant QTL for fatness. One is for percentage abdominal fat at 10 weeks of age. It was located on Chromosome 1 at 241 cM. The other was for body weight at 10 week on Chromosome 13 at 9 cM. They also found multiple suggestive QTL on Chromosome 1, 2, 4, 13, and 18. Lagarrigue et al. (2005) found 6 QTL for abdominal fat weight adjusted for live body weight on Chromosome 1 (449 cM), 3 (84 cM and 121 cM), 5 (68 cM and 150 cM), and 7 (32 cM). In our study, we found QTL for body fat on Chromosome 1 (144 cM), Chromosome 3 (308 cM) and Chromosome 4 (169 cM or 171 cM), which had not been found in those earlier studies.

There were two sets of variables measured on each chicken through 9 weeks of age. The first set was body weight from week1 to week 9. The second set was the correlated carcass production traits. To further explore the relationship between these two set of variables, canonical correlation analysis was performed. The first canonical variable of the growth measurement accounted for 97.72% of the total variance in the data. This variable, body-growth variable was a very good predictor of thigh weight (Pearson correlation coefficient= 0.9067), a fairly good predictor of breast weight (0.7286) and a poorer predictor of fat weight (0.6286), shank diameter (0.5157) and shank length (0.6245). Canonical correlation analysis showed that growth rate measurement could be a good predictor for carcass production traits. It is possible to gain some information about production traits by measuring body weights from week 1 to week 9 without killing the chickens, which could be very important if the birds need to be kept for breeding.

In order to verify the value of these SNPs to the poultry industry, SNP associations should be tested in other chicken lines or populations. High growth (HG) and Low growth (LG) chicken lines have been selected based on the growth rate at juvenile and adult ages by Poultry Research Center (Nouzilly, France). SNPs that showed significant association with fat weight or fat yield previously were genotyped in an F_2 intercross of the HG and LG lines. Because of the family structure, one of the SNPs, GPC3 SNP1 had only one genotype, and thus could not be used in the association study. Two SNPs, AKR1B10 SNP1 and SDC1 SNP1 were genotyped in 96 F_2 chickens that were in the extremes of the population for body fat (24 fattest females, 24 fattest males, 24 leanest females and 24 leanest males). Significant associations between SDC1 SNP1 and body weight at hatch was detected (P=0.01423). However, body weight at hatch is not considered as an important production trait in our study. There also was a trend for an association between SDC1 SNP1 and fat yield (P=0.3670). It is possible that this association might turn out to be significant if more chickens were to be genotyped. A power analysis was conducted to calculate the number of F_2 chickens needed in order to detect a significant association. In FL and LL F_2 chickens, a significant association between SDC1 SNP1 and fat yield was detected with a difference of d=0.2229 between genotype GG and GC. The number of chickens were calculated by using n=s2t2/d2 (s2= SE2*n, t is the table value). Since the variation in the fat yield value in HGLG F_2 chickens is relatively large (SE=0.9307), the total number of chickens needed turned out to be 4564, which is much bigger than the population size we currently have.

In all, this study focused on detecting genetic markers that may be of great value in the poultry industry world-wide for marker-assisted selection of chickens with less

abdominal fat and improved meat yield. We found four SNPs that could be used as

genetic markers for body fat, two for breast yield, one for thigh yield and one for muscle-

related factor in broiler chickens.

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