ABSTRACT Metabolomics is an appealing new approach in systems biology aimed at enabling an improved understanding of the dynamic biochemical composition of living systems. Biological systems are remarkably complex. Importantly, metabolites are the end products of cellular regulatory processes, and their concentrations reflect the ultimate response of a biological system to genetic or environmental changes. In this article, we describe the components of lipid metabolomics and then use them to investigate the metabolic basis for increased abdominal adiposity in 2 strains of divergently selected chickens. Lipid metabolomics were chosen due to the availability of well-developed analytical platforms and the pervasive physiological importance of lipids in metabolism. The analysis suggests that metabolic shifts that result in increased abdominal adiposity are not universal and vary with genetic background. Metabolomics can be used to reverse engineer selection programs through superior metabolic descriptions that can then be associated with specific gene networks and transcriptional profiles.

Key words: genetic selection, adipose, metabolomics, chicken lipid

INTRODUCTION

Poultry have been selected for specific traits for thousands of years. During the past century, there has been intensive selection for production traits such as rapid growth, feed efficiency, and egg laying. Despite tremendous gains in these areas, there is little understanding of the shifts in metabolism that underlie phenotypic improvements. Our current lack of understanding of the metabolic trade-offs arising from specific selection programs has left us with a lack of predictive power with regards to possible collateral outcomes—be they positive or negative—of those selection programs. Feed efficiency, muscle vs. adipose accretion, disease resistance, and immune response are examples of polygenic traits for which improvements could arise by differential modulation of metabolic pathways. Interestingly, many untoward collateral outcomes arising from selection for early rapid growth do not manifest until the bird reaches adulthood. Such deleterious long-term alterations (of reproduction, longevity, fitness, etc.) may, at least in part, result from chronic metabolic changes and reflect a subtle long-term metabolic dysfunction. The recent completion of the avian genome sequence and functional annotation provided by microarray analyses will permit identification of genes responsible for controlling specific traits. However, there are already examples in which studies have not revealed strong candidate or susceptibility genes (Assaf et al., 2004; Bourneuf et al., 2006; Zhang et al., 2006). Furthermore, it is well known that candidate genes for several important human pathologies (i.e., cancer, diabetes, and obesity) are largely influenced by so-called environmental factors, such as diet, which suggests the need for additional approaches other than genomics.

Systems Biology and Metabolomics

This symposium is dedicated to building a bridge between genome and phenotype. Systems biology provides an approach to create just such a bridge, because it seeks to develop a dynamic relational understanding of system components (Kitano, 2002). System-level understanding requires that we understand the parts (i.e., genes, proteins, and metabolites) and their relationships to one another in terms of control and outcomes. The observed
phenotype is an outcome that arises from the dynamic relationships of gene expression, resultant protein activity, and environmental influences. Others in the symposium will describe genomic, transcriptional, and proteomic approaches. This presentation is focused on the use of metabolomics as the final component of a systems biology approach to quantify the interaction of genetics and environment. The metabolomics approach employs an analysis of the entire set of small-molecule metabolites that are involved in primary or intermediary metabolism within any biological system. Simultaneous acquisition of many quantitative metabolite measurements allows for comprehensive statistical testing and expansion of biological networks and pathways. A closely related approach called metabonomics typically employs nuclear magnetic resonance profiling and pattern recognition analysis to identify differences in metabolism. Metabonomics differs from metabolomics in that, identification of individual metabolites is not required for pattern recognition analysis; however, pathway analysis depends on identification and quantitation of the compounds (Sysi-Aho et al., 2007; Wiest and Watkins, 2007). Recently, several analytical platforms have been used to make global assessments of metabolic phenotype (Fiehn, 2002; Morris and Watkins, 2005; Castle et al., 2006).

Metabolomics is now being used for biomarker development, toxicology, pathway analysis, physiological evaluation, and characterization of genetic and environmental modification in the organism (Watkins et al., 2002; Burns et al., 2004; Griffin and Bollard, 2004; Verhoeckx et al., 2004). Metabolomics can be performed on fluids, such as plasma (or serum), ejaculate, yolk, cerebral spinal fluid, or tissue homogenates. Comprehensive and accurate quantification of numerous metabolites allows for a range of biochemical effects induced by a condition or intervention to be determined. Analysis of multiple tissues in addition to serum or plasma is desirable for whole-body pathway analysis. With whole-body pathway knowledge, statistical procedures can be used to create novel biomarkers for specific targets. This information can also be linked to transcriptional profiling to add inference to identified gene networks. In this way, a better appreciation of how gene networks function in different genetic backgrounds at different ages or in different environments can be achieved.

Technologies and applications for vertebrate metabolomics are best developed for human medicine due to the immediate interest for human health, involvement of large pharmaceutical groups, and federal grant programs. In humans, metabolomics is being developed for diagnosis or prediction of disease, to stratify populations by individual specific metabolism, and to determine the safety, efficacy, metabolic consequences, or all three, of therapeutic intervention (Watkins et al., 2002, 2003; Griffin et al., 2004; Moran et al., 2004; Stone et al., 2004; Verhoeckx et al., 2004). Similar applications have been pursued in ruminants (Ametaj et al., 2006; Drackley et al., 2006; Lane, 2006) and could be possible in poultry. Such improved assessment tools (biomarkers) could preclude sib testing or be used to confirm that selected breeders are indeed metabolically equivalent to sibs tested for carcass traits.

Metabolite measurements have historically been used to assess health and production outcomes; therefore, metabolomics is not a completely revolutionary approach. However, the use of metabolic measurements to assess production outcomes or health status has traditionally used only single (or a few) biomarkers known to be associated with a specific trait or a final concentration of the target compound being increased (e.g., n-3 fatty acids) or decreased (e.g., saturated fatty acids). Metabolomics offers a fresh perspective on this approach because of the broad scope of measurements and their interpretation by sophisticated statistical modeling. Instead of measuring a single metabolite, a highly comprehensive set of metabolite measurements is obtained by multiple, parallel analyses. In such analyses, metabolism itself is describable in breadth, depth, and time. These metabolic platforms and databases make it possible to assess productive potential, fitness, disease resistance, or development on a global scale.

The first priority in metabolic profiling is to develop analytical platforms capable of generating quantitative data on a significant fraction of metabolites. The massive amounts of data generated by these analyses must then be statistically analyzed and interpreted. Many companies make metabolomic measurements (Table 1). Terminology and data-mining strategies are not identical among companies. These emerging technologies are based upon well-established analytical platforms and often use novel visualization tools to facilitate interpretation.

**Rationale for Focusing on Lipids**

Lipid metabolomics can use several different analytical platforms (German et al., 2007), and focused efforts to document and organize gene and protein networks responsible for lipid metabolism are making rapid progress (Cotter et al., 2006). Physiologically, lipids are a unique class of biological metabolites in that they provide critical structural elements as components of cellular membranes as well as mediating extensive signaling functions within and between cells (German et al., 2007). Lipids signal primarily between similar and nearby cells as autacoids (i.e., hormones that act locally and acutely to regulate responses to cellular stresses). Lipids also constitute the major energy source and fuel repository of higher animals. Unlike dietary amino acids and carbohydrates, dietary fatty acids strongly influence the fatty acid composition of nonruminant body lipids (constitutive and depot components), because n-3 and n-6 double bonds of essential fatty acids retain their molecular identity during metabolism (Walzem, 1996). The proportion of individual fatty acids ultimately entering either constitutive or depot compartments is regulated by both structural requirements of specific lipid molecules and biochemical pathway controls (Gurr et al., 2002). This regulation in turn reflects the lipid and energy intake, energy expenditure,
fatty acid oxidation, and endogenous lipogenesis from carbon chains derived from carbohydrates and amino acids in a bird. An ultimate goal in animal production is to select and rear animals in whom regulation favors muscle development at the expense of adipose tissue. In humans, disregulation at any step of this chain leads to several pathologies (obesity, diabetes, cardiovascular diseases, reproductive and bone dysfunctions, and Alzheimer’s disease; Saltzman et al., 2006). In animal species, particularly in chickens, excessive fattening is observed following intensive selection for rapid growth (Griffin and Goddard, 1994; Buyse et al., 1999; Julian, 2005; Chen et al., 2006) in conjunction with impaired performance, livability, and feed efficiency. Because of the potential for disregulation and involvement in multiple functions, lipids are distinctive in virtually every physiological state. Accurate measurement of specific lipid species provides a means to resolve the question of how genetic selection, environmental factors (including diet), and disease affect large segments of metabolism. Table 2 describes some of the analyses that can be used to characterize the lipid metabolome.

### Table 2. Types of analyses used in lipid profiling

<table>
<thead>
<tr>
<th>Analysis</th>
<th>Description and use</th>
</tr>
</thead>
<tbody>
<tr>
<td>True mass</td>
<td>Utilizes liquid and gas chromatography (LC-GC-FID) to quantify approximately 400 individual metabolites involved in structural and energetic lipid metabolism in 200 mL of serum or plasma or 50 mg of tissue. Analysis includes triacylglycerols, cholesteryl esters, and phospholipids. Useful in profiling structural and energetic lipids that are central to metabolism and virtually every biological process in the body.</td>
</tr>
<tr>
<td>Eicosanoid</td>
<td>Utilizes a proprietary reverse-phase LC-negative electrospray ionization triple-quadrupole mass spectrometry (RP-LC-nESI-QQQ-MS) method and a 250-mL sample to generate quantitative data on approximately 40 products of the cyclooxygenase, lipoxygenase, and P450 pathways of n-6 and n-3 fatty acid metabolism. Useful in profiling eicosanoids that are central to inflammatory processes, immune responses, vasodilation and constriction, macrophage recruitment, blood clotting, and pain and swelling. Eicosanoids are the metabolites targeted for inhibition by COX-2 inhibitors (i.e., aspirin, naproxen, and other steroid and nonsteroidal antiinflammatory agents).</td>
</tr>
<tr>
<td>Acylcarnitines</td>
<td>Utilizes reverse-phase LC-positive electrospray ionization triple-quadrupole mass spectrometry (RP-LC-pESI-QQQ-MS) and 25 mL of serum or plasma to produce quantitative data on 16 short- and long-chain acylcarnitines and free carnitine. Acylcarnitines are intermediate products of mitochondrial μ-oxidation of fats and are useful indices of energy balance and fat utilization.</td>
</tr>
<tr>
<td>Bile acids and sterols</td>
<td>This dual platform generates quantitative data on 16 intermediates of sterol biosynthesis and bile acid metabolism. Bile acids are assayed by RP-LC-nESI-QQQ-MS, whereas sterols are assayed by GC chemical ionization mass spectrometry (GC-CI-MS). These measurements are useful in profiling processes involved in synthesis, absorption, and excretion of cholesterol.</td>
</tr>
</tbody>
</table>

1. Readers are encouraged to visit Web sites due to nonstandardized language used to describe analytical and interpretive capabilities.

### A Case Study of Lipid Profiling in Divergently Selected Broiler Chickens

The plasma samples used for lipid profiling in this case study originated from a functional genomics consortium project (awarded to J. Simon, T. E. Porter, and L. A. Cogburn) focused on transcriptional profiling of multiple tissues during juvenile development (1 to 11 wk of age) of 4 divergently selected broiler lines (Cogburn et al., 2003, 2004; Carre et al., 2006). One set of broiler chickens was selected for either high growth rate (HG) or low growth rate (LG) based on BW at 9 and 36 wk of age (Ricard, 1975). The other set of broiler chickens was divergently selected for either high [fat line (FL)] or low [lean line (LL)] body fat content (Leclercq et al., 1980) at equivalent 9-wk BW, using abdominal fat weight of collaterals. These divergent genetic lines were chosen as models for transcriptional profiling and gene network modeling because of extreme differences in growth rate and body composition (Cogburn et al., 2003). This case study shows that as in other systems (Trethewey, 2001), metabolomic analysis can improve our understanding of avian lipid metabolism.
and serve as a discovery tool that complements gene expression profiling.

**MATERIALS AND METHODS**

**Birds and Methods**

At hatching, chicks were wing-banded, sexed, and vaccinated against Marek’s disease virus. The FL and LL cockerels were raised together in floor pens in a 4.4 × 3.9 m room located at the Station de Recherches Avicoles, Institut National de la Recherche Agronomique, Nouzilly, France. The HG and LG cockerels were similarly housed in an adjacent room, except that the HG birds were initially separated from LG birds by a wire partition due to the small size of the LG birds. Birds were fed ad libitum with a conventional starter ration from hatching to 3 wk of age (22% CP and 3,050 kcal of ME/kg), with a grower pelleted ration from 3 to 11 wk of age (20% CP and 3,100 kcal of ME/kg). Pellets were crushed for the LG chickens for the first 3 wk. At the time of these plasma lipid measurements (5 wk), birds had been consuming the grower ration for 2 wk; this ration contained 7.5% total fat, consisting of 12.9% saturates, 29.5% monounsaturates, and 51.3% polyunsaturates, with n-6 and n-3 fatty acids = 5.6 (on a calculated basis). After continuous light for the first 2 d, the birds were maintained on a 14L:10D cycle. Supplemental heat was provided by infrared gas, and ambient temperature was progressively decreased from 32°C at hatching until 22°C was reached at 22 d. Birds had free access to water from nipple fountains. At 1, 3, 5, 7, 9, and 11 wk of age, 8 fed birds from each genetic strain were selected at random and weighed before blood tissue sampling. Briefly, blood was drawn from the heart using 21-gauge (0.8 × 30 cm) needles for LG chickens into 5-mL syringes rinsed with heparin:saline (1:5, 5,000 IU/mL, Choay, Sanofi-Aventis, Le Plessis-Robinson, France) by aspiration and ejection before venipuncture. Blood was held on ice ≤ 1 hr before centrifugation at 3,000 × g for 15 min. Plasma was harvested by pipet and aliquoted before storage at −80°C. Plasma glucose and insulin levels were determined at the Institut National de la Recherche Agronomique (J. Simon) as previously described (Simon et al., 2000). Aliquots of plasma were shipped frozen on dry ice to the University of Delaware for additional metabolite and hormone analyses and to Lipomic Technologies Inc. for lipid metabolite analysis. The majority of strain-related differences in gene expression occurred in 7-wk-old birds (L. A. Cogburn, J. Simon, T. E. Porter, unpublished data). True mass analysis (Table 2) was performed on plasma on 5-wk-old fed chickens to determine whether metabolic changes preceded changes in gene transcription. (n = 4 chickens/line, the same birds used in microarray analysis).

**Lipid Profiling**

Plasma lipid profiles were performed as described previously (Watkins et al., 2002). Briefly, the lipids from plasma (200 μL) were extracted with CHCl3:CH3OH (2:1, vol/vol; Folch et al., 1957) in the presence of a panel of internal standards (Ohta et al., 1990; Walzem et al., 1995). Individual lipid classes within the extract were separated by preparative HPLC (Watkins et al., 2001b). Isolated lipid classes were transesterified in 3 N methanolic HCl in a sealed vial under a N atmosphere at 100°C for 45 min. Resultant fatty acid methyl esters were extracted with hexane containing 0.05% butylated hydroxytoluene and sealed under N before separation. A gas chromatograph (model 6890, Hewlett-Packard, Wilmington, DE) equipped with a 30-m DB-225MS capillary column (J&W Scientific, Folsom, CA) and a flame-ionization detector was used to separate and quantitate fatty acid methyl esters. Sterols were similarly separated and quantified using a 30-m J&W DB-35MS capillary column. Quantitative measurements of fatty acids in various lipid classes were determined as nanomoles of fatty acid per gram of plasma. Lipid classes included in this study were unesterified cholesterol (FC), cholesteryl esters, diacylglycerols (DG), free fatty acids (FFA), lysophosphatidylcholine, phosphatidylcholine (PC), phosphatidylethanolamine (PE), and triacylglycerols (TG). Both lipid class and fatty acid moiety characterized lipid metabolite data. Specifically, fatty acids were identified first by the number of carbons in the molecule (e.g., 20), the number of double bonds in the molecule (e.g., 4), and lastly, the position of the double bonds (e.g., n-6). Thus, PC20:4n6 would be a 20-carbon fatty acid with 4 double bonds commencing at the sixth carbon counting from the methyl end of the molecule located on a PC molecule. Relative amounts of individual fatty acids within a lipid class were expressed as a percentage of the total moles of fatty acids in each lipid class. Expression of fatty acid data as an absolute molar quantity (nmol/g of plasma) or an absolute molar fraction (mole percentage) of sample mass, rather than a weight percentage of recovered lipid, is required for metabolic modeling (Watkins, 2000; Watkins et al., 2001a). Rigorously quantitated metabolite measurements are requisite to create valid databases for comparison of studies conducted at different times or by different investigators (Dixon et al., 2006; Mehrotra and Mendes, 2006).

**Statistical Analyses**

Significant differences in phenotypic measurements between individual strains of each strain were assessed by paired t-tests. Evaluation of line and line × strain effects was assessed by 2-way ANOVA. All statistics were done using R with the following functions: t.test, anova. (Team, 2005). To address the issue of multiple comparisons and the generation of false positive results, change detection analysis was used to determine if observed signals were greater than that which could be expected by chance (noise) before beginning statistical analyses. The chance distribution of probability values was determined by permuting the outcome groupings as described (Golub et al., 1999). Briefly, probability values for the appropriate
comparison were calculated for each metabolite using a Student’s t-test. The metabolites were ranked by probability value from smallest to largest. The log of the rank vs. the 
log of the probability value for each comparison of interest was plotted as a black line (Figure 1). The plotting convention adopted plotted values for birds that were either fatter (FL, group 4) or larger and fatter (HG, group 3) as the black line. The distribution of probability values expected by chance at each rank was indicated by the shaded area (Figure 1). The chance distribution was determined by a Monte Carlo permutation method applied to a data set in which the posttreatment and controls had been scaled to the same mean value and combined. The Z-scores were calculated from the area under the curve of the treatment group compared with the distribution of the area under the curve for the random permutations. The Z-values were used to evaluate if there was a treatment effect (Golub et al., 1999). Values were significantly different at $P \leq 0.05$, unless specifically noted.

**Visualization of Lipid Profiles**

Surveyor software (Lipomic Technologies Inc., West Sacramento, CA) was used to create heatmaps by testing the significance of each comparison between genetic strains within each line on the metabolite concentration using a paired Student’s t-test (Figure 2). If the treatment effect was significant at $\alpha = 0.05$, a mean percentage difference in the concentration of the metabolite induced by strain was calculated. Heatmaps can be read as follows: the column headers display the fatty acid family or individual fatty acid, as specified by the user, whereas the row headers indicate the lipid class. Each cell in the heatmap represents the comparison for a particular metabolite or metabolite family if a summary value is appropriate. Metabolites that were significantly greater in 1 strain compared with the other were displayed in red, whereas significantly lower concentrations or fractional amounts were displayed in green. The brightness of each color corresponds to the magnitude of the difference in quartiles. The larger the difference in the compared values, the brighter the color of the square. Surveyor outcomes for data presented here can be accessed at http://go.lipomics.com/PoulSci2006 for additional comparisons not shown in Figure 2.

Signature equations (Lipomic Technologies Inc.) use quantitative metabolite data to estimate the steady-state
Figure 2. Surveyor heat maps showing differences in individual lipid metabolites in the plasma. Lipid metabolites found to increase are shown in red, whereas those found to decrease are shown in green. The intensity of the color is proportional to the significance of the change, where the minimum statistical difference was \( P < 0.05 \). A: Quantitative data (nmol/g) of the low growth rate (LG) strain compared with the high growth rate (HG) strain. B: Mole percentage data of the LG strain as compared with the HG strain. C: Quantitative data of the low lean line (LL) strain as compared with the high-fat line (FL) strain. D: Mole percentage data of the LL strain as compared with the FL strain. The column headers indicate fatty acid metabolites as they appear in each distinct lipid class (rows).

flux of lipid metabolites through key lipid metabolism pathways. Signature equations generate semiquantitative values that have no units. Equations are empirically tested using the extensive database of human and animal experimental subjects of the company. Animal subjects include mice, rats, monkeys, apes, cats, hamsters, and chickens. At present, equations are not optimized for avian metabolism due to a lack of species-specific data, and generation of such data is needed for further optimization. Signature estimates can be presented as means (e.g., Table 3) or in a heatmap format similar to that generated by Surveyor or used as part of an Insight pathway map (e.g., Figures 3 and 4).

Insight pathway maps (Lipomic Technologies Inc.) assemble the results of quantitative lipid metabolite measurements and Signature outcomes in a graphical representation of lipid metabolism along a specified pathway. Insight depicts the estimated bulk flow of metabolites
is different between strains of each line. No differences of the LG birds, whereas the abdominal fat content of age, the BW of HG birds was 2.8-fold greater than that difference in 9- and 36-wk BW (Ricard, 1975). At 5-wk of HG and LG lines were divergently selected for a large was 2.8-fold higher than that of the LL (Table 4). The FL and LL genotype at 5 wk of age show that BW were significantly different among all 4 genotypes (Table 4). The FL and LL for a large differences in visceral fat through specific metabolic pathways and treatment-induced increase or decrease in either the metabolite concentration or estimated flux relative to the comparison group. Such visualization tools distill data into visual metabolic relationships.

### RESULTS AND DISCUSSION

The phenotypic measurements taken from 4 birds/ genotype at 5 wk of age show that BW were significantly different among all 4 genotypes (Table 4). The FL and LL birds were selected on large differences in visceral fat content at the same 9-wk BW (Leclercq et al., 1984). At 5 wk of age, FL birds were 13% heavier than LL birds, whereas the abdominal fat content (g) of the FL birds was 2.8-fold higher than that of the LL (P < 0.05). The HG and LG lines were divergently selected for a large difference in 9- and 36-wk BW (Ricard, 1975). At 5-wk of age, the BW of HG birds was 2.8-fold greater than that of the LG birds, whereas the abdominal fat content of HG birds was 37.5 times greater than that of the LG birds. Similarly, abdominal fat expressed as a percentage of BW is different between strains of each line. No differences were found among genotypes for plasma glucose and insulin levels at 5 wk of age. Lack of strain differences in these measurements is likely due to the small sample size, because significant differences were found in these parameters when all 8 birds/genotype and age were included in a time series (1 to 11 wk of age) ANOVA (J. Simon and L. A. Cogburn, unpublished data).

Multiple comparisons of data within large data sets that are generated from a limited number of samples, such as the 4 birds per strain used in the present study, can result in the generation of false positives. For this reason, a change detection analysis (Golub et al., 1999) was used to determine if the measured responses were greater than that observed by chance (Figure 1). Both the quantitative and mole percentage data were evaluated. For the comparison of FL vs. LL groups, the number of significant differences was very close or the same as the number of false positives for both the quantitative (Z-score = 4.96) and the mole percentage data (Z-score = 3.09). Therefore, there were few true positives in this data set, and the data were evaluated conservatively. For the HG vs. LG groups, there were far more significant comparisons than there are false positives for both the quanti-

<table>
<thead>
<tr>
<th>Item</th>
<th>LG</th>
<th>FL</th>
<th>LG</th>
<th>HG</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW, g</td>
<td>332.8 ± 15.2d</td>
<td>937.8 ± 20.9e</td>
<td>1,197 ± 25.4b</td>
<td>1,356.5 ± 42.5s</td>
</tr>
<tr>
<td>Abdominal fat tissue, g</td>
<td>0.4 ± 0.2d</td>
<td>15.0 ± 1.3e</td>
<td>13.8 ± 2.8b</td>
<td>39.6 ± 2.5b</td>
</tr>
<tr>
<td>Plasma glucose, mg/dL</td>
<td>264.3 ± 10.2</td>
<td>249.3 ± 5.4</td>
<td>243.8 ± 6</td>
<td>238 ± 3.6</td>
</tr>
<tr>
<td>Plasma insulin, ng/mL</td>
<td>1.1 ± 0.2</td>
<td>1.3 ± 0.2</td>
<td>1.8 ± 0.4</td>
<td>2.0 ± 0.5</td>
</tr>
</tbody>
</table>

*<sup>a</sup>*Means not sharing a common superscript are significantly different (P ≤ 0.05).

<sup>1</sup>Values are means ± SEM of chickens used for lipid profiling, n = 4. LG = low growth rate; HG = high growth rate; LL = low lean line; FL = high-fat line.
Figure 3. Insight pathway map comparing the estimated metabolism of the low lean line (LL) strain chickens with the high-fat line (FL) strain chickens. The percentage of increase or decrease in lipids was calculated as described in the methods. The activities of each of the enzymes were estimated utilizing the Lipomics Signature analyses as indicated in the methods. Coding for statistical significance is shown in the lower left corner of the figure. Figure boxes and arrows shown with gray fill are unchanged. Nonsignificant increases and decreases (P = 0.1 to 0.05) are indicated by vertical and horizontal fill, respectively. Significant decreases are indicated by progressively fine diagonal line fill, with the greatest decrease indicated by the finest diagonal fill. Significant increases are indicated by progressively darker stippling as fill, with the greatest increase indicated by the darkest stippling. FAS = fatty acid synthetase; Δ9 = Δ9 desaturase; Δ6 = Δ6 desaturase; Δ5 = Δ5 desaturase; DGAT = diacylglycerol acyl transferase; PEMT = phosphatidylethanolamine methyl transferase; CDP-CT = cytosine diphosphatecholine cytidylyltransferase; ACAT = acyl-coenzyme A:cholesterol acyltransferase; TG = triacylglycerol; PC = phosphatidyl choline; CE = cholesteryl ester; PE = phosphatidyl ethanolamine; 1-LY = 1-lysophosphatidyl choline; Lipase = lipoprotein lipase; PLA = phospholipase A; DG = diacylglyceride; LCAT = lecithin cholesterol acyl transferase; FA = unesterified fatty acids; 2-LY = 2-lysophosphatidyl choline; SP = sphingomyelin; FC = unesterified cholesterol.

tative (Z-score = 9.37) and mole percentage data (Z-score = 8.42). Therefore, the significant changes that are found are likely to be true positives and more numerous in the HG-LG pair than in the FL-LL pair.

Utility of Data Expression on a Molar Basis

External standards are adequate to define retention times of individual fatty acids. However, internal standards are necessary for quantitation, because this provides a correction for differences in extraction efficiency and losses during separation and derivatization. Historically, fatty acid data has been expressed as weight (e.g., μg) and weight percentages of total fatty acid weight contributed by an individual fatty acid (weight %). Fatty acids differ in molecular weight depending on chain length and number of double bonds; for example, myristic acid (C14:0) has a molecular weight of 228.38, whereas that of docosahexaenoic acid (C22:6, n-3) is 328.49. The key physiological implication is that for a given weight of material within a sample, the compound with the lowest molecular weight will contribute the greatest number of molecules. Molar concentrations more accurately convey differences in numbers of fatty acid molecules present in a tissue or metabolic compartment. Enzymes and transfer proteins operate in accord with molecular concentrations (e.g., nmol/L). For this reason, it is desirable to express data on a molar basis for modeling or cross-experiment comparison purposes. The parallel physiologically relevant expression of the relative amounts of fatty acid within a lipid class is mole percentage.

Mole percentages are appropriate for comparisons both within and between pairs of lines. An example of how mole percentage values can be used is provided by the compositions of PC and PE (Figure 2 and Table 5). Significant differences appear in the mole percentage comparisons for both pairs of strains. However, those in contrasts between FL and LL (Figure 2) occurred in minor fatty acids, whereas contrasts between HG and LG show significant differences in major fatty acids (Figure 2 and Table 5). In this latter line comparison, PC and PE have the same pattern of fatty acid changes. A portion of PC is derived from PE (Vance, 1991; Mayes, 2000). The equivalence of changes in the mole percentage distribution of PE and PC strongly suggests that the changes in PC reflect the precursor-product relationship between PE and PC. This raises the question of whether the LG birds have a different source of fatty acids for PE production than HG birds to be able to alter the fractional composition of PE, and subsequently, PC, so extensively. Interestingly, the change in PE mole percentage composition of LG birds could be a function of the low rate of growth of these chickens. All 3 of the faster-growing strains (HG, LL, and FL) had similar compositions for the PC and PE, whereas
Figure 4. Insight pathway map comparing the estimated metabolism of the low growth rate (LG) strain chickens with the high growth rate (HG) strain chickens. The percentage of increase or decrease in lipids was calculated as described in the methods. The activities of each of the enzymes were estimated utilizing the Lipomics Signature analyses as indicated in the methods. Coding for statistical significance is shown in the lower left corner of the figure. Figure boxes and arrows shown with gray fill are unchanged. Nonsignificant increases and decreases (P = 0.1 to 0.05) are indicated by vertical and horizontal fill, respectively. Significant decreases are indicated by progressively fine diagonal line fill, with the greatest decrease indicated by the finest diagonal fill. Significant increases are indicated by progressively darker stippling as fill, with the greatest increase indicated by the darkest stippling. FAS = fatty acid synthetase; Δ9 = Δ9 desaturase; Δ6 = Δ6 desaturase; Δ5 = Δ5 desaturase; DGAT = diacylglycerol acyl transferase; PEMT = phosphatidylethanolamine methyl transferase; CDP-CT = cytosine diphosphateicholine cytidylyltransferase; ACAT = acyl-coenzyme A:cholesterol acyltransferase; TG = triacylglycerol; PC = phosphatidyl choline; CE = cholesteryl ester; PE = phosphatidyl ethanolamine; 1-LY = 1-lysophosphatidyl choline; Lipase = lipoprotein lipase; PLA = phospholipase A; DG = diacylglyceride; LCAT = lecithin cholesterol acyl transferase; FA = unesterified fatty acids; 2-LY = 2-lysophosphatidyl choline; SP = sphingomyelin; FC = unesterified cholesterol.

the strain with the slowest growth and lowest abdominal fat content (LG) had a different composition.

**Did Differences in Adiposity Between Genetic Lines Arise from Different Metabolic Shifts?**

Circulating lipid class concentrations are shown in Table 6, and calculated Signature values in Table 3. Surveyor visualized differences in concentrations, and fractional compositions of fatty acids between line pairs in each lipid class are shown in Figure 2. The first most notable differences are seen in comparisons between the 2 lines of birds (Tables 3 and 6) produced using very different selection criteria. Birds of the HG and LG lines resulted from divergent selection for high and low BW at 9 and 36 wk of age from an initial population of Bresse-Pile meat-type birds (issued from the crossing of Bresse-Blanche, New Hampshire, and White-American birds). Birds of the LL and FL lines resulted from divergent selection for high and low abdominal fat weight from an initial population created from birds from 6 different meat-type lines to form as complex a gene pool as possible (Leclercq, 1988). Segregation of founder strains into lines with differing degrees of adiposity resulted in additional changes in lipid metabolism within each strain. Fundamental differences in both phospholipid metabolism and pathways associated with hepatic lipid synthesis and secretion exist in the 2 different founder strains. For example, plasma concentrations of PC and PE were 20 to 25% higher (P ≤ 0.01) in the FL-LL birds compared with HG-LG birds (Table 6). Signature estimates predict a general upregulation of lipid synthesis and secretion in the FL-LL strain compared with HG-LG, because flux estimates for fatty acid synthetase (FAS), stearoyl coenzyme A desaturase (SCD16), DG acyltransferase (DGAT1), and acylcoenzyme A:cholesterol acyltransferase were all significantly increased (Table 3; Brown et al., 1999; Liang et al., 2004). The FL-LL birds are also heavier overall than the LG-HG birds. At present, it is unknown if a generalized increase in lipid synthesis and export capability as suggested by the metabolomic analysis is essential for the generally increased overall growth rate of the FL-LL strain compared with that of the HG-LG strain. Interestingly, plasma glucose concentrations were significantly lower (P < 0.038) in the LL-FL strain than the LG-HG strain, and although not significant (P < 0.057), plasma insulin concentrations were higher in LL-FL birds. Insulin is permissive in the long term for TG-rich lipoprotein assembly and secretion (Leclercq et al., 1988), perhaps through indirect effects (Xu et al., 2006; Tsai et al., 2007). However, differences in the lipid metabolism of these 2 lines are also apparent in Figure 2, in which an increase in adiposity (and enhanced growth rate for LG-HG) is
Associated with very different patterns of fatty acid utilization. The lack of similarity in either relative or quantitative fatty acid use in overall line comparisons as well as strain comparisons within a line in which abdominal adipose increases both absolutely and as a fraction of BW strongly supports 2 conclusions. The first is that founder lines have fundamental differences in lipid metabolism. The second is that differences in adiposity develop by different rather than similar metabolic shifts in the 2 lines. Due to the fundamental differences in growth rate and different rather than similar metabolic shifts in the 2 lines.

The second is that differences in adiposity develop by a particular genetic background.

To resolve questions related to catalytic or transfer protein activity, information on other lipid classes can be helpful, particularly if known metabolic relationships can be used to create estimates of relevant enzyme activities. Plasma DG and FFA are the products of lipase action on TG. Comparison of the concentrations of the products to those of precursor TG was used to evaluate the relative amount of lipase activity in these birds. As shown in Table 3, Signature estimates for lipoprotein lipase (LPL) were marginally higher in HG than LG. Because these are not actual LPL rate measurements, the increase suggests that either the rate of LPL action is greater in HG birds than in LG birds or the efficiency of uptake of both of the products is lower. It is unlikely that the rate of uptake of both FFA and DG is reduced; therefore, it could be expected that there is a slight increase in LPL activity in the HG birds. As with other Signature predictions, metabolomic or transcriptional profiling of peripheral tissues or actual enzyme activity measurements would be necessary to confirm this prediction. Moreover, these slight changes in apparent LPL activity do not eliminate the possibility of increased production of VLDL. A small increase in VLDL together with very small changes in LPL activity could result in the increased circulating concentrations of TG and FC due to a reduced VLDL lipoprotein clearance.

A decreased clearance rate of lipoproteins might also result in increased oxidation of the lipids. An increased oxidation of the lipids. An increased oxidation of the lipids.

Comparison of Lipid Profiles in LG and HG Strains.

In this study, plasma samples were collected from fed birds and interpreted accordingly. The LG birds had higher TG (65% increase, P < 0.02) but similar FC (18% increase, P < 0.09) levels compared with the HG birds (Table 6). Because the bulk of the TG is transported in very low density lipoprotein (VLDL) and FC in low-density lipoprotein (LDL; Hermier et al., 1985), this implies that there was an increase in VLDL and perhaps a small increase in LDL concentration. Any increase in LDL could be a secondary outcome to increased VLDL concentrations, because LDL is an end product of VLDL metabolism (Walzem, 1996). This implies that either VLDL production was higher or its clearance was decreased.

Table 6. Concentrations of plasma lipids per class1

<table>
<thead>
<tr>
<th>Item</th>
<th>Plasma</th>
<th>LG (n = 4)</th>
<th>HG (n = 4)</th>
<th>LL (n = 4)</th>
<th>FL (n = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutral lipids, nmol/g</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cholesteryl ester</td>
<td>1,897 ± 99</td>
<td>1,792 ± 164</td>
<td>2,049 ± 165</td>
<td>1,984 ± 110</td>
<td></td>
</tr>
<tr>
<td>Triaclylglycerol</td>
<td>514.2 ± 68ab</td>
<td>309.8 ± 12</td>
<td>601.7 ± 76c</td>
<td>330.4 ± 54bc</td>
<td></td>
</tr>
<tr>
<td>Diacylglycerol</td>
<td>52.8 ± 2.0a</td>
<td>40.1 ± 4.0b</td>
<td>53.9 ± 4.0a</td>
<td>50.2 ± 7.0ab</td>
<td></td>
</tr>
<tr>
<td>Free fatty acids</td>
<td>288 ± 37</td>
<td>238 ± 24</td>
<td>251 ± 13</td>
<td>248 ± 16</td>
<td></td>
</tr>
<tr>
<td>Polar lipids, nmol/g</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphatidylcholine</td>
<td>2,091.3 ± 154c</td>
<td>2,124.6 ± 169bc</td>
<td>2,605.5 ± 137c</td>
<td>2,673.7 ± 193abc</td>
<td></td>
</tr>
<tr>
<td>Phosphatidylethanolamine</td>
<td>461.6 ± 27bc</td>
<td>419.1 ± 29b</td>
<td>549.0 ± 27abc</td>
<td>599.7 ± 34bc</td>
<td></td>
</tr>
<tr>
<td>Sphingomyelin</td>
<td>286 ± 20</td>
<td>260 ± 25</td>
<td>303 ± 8</td>
<td>301 ± 44</td>
<td></td>
</tr>
<tr>
<td>Free cholesterol</td>
<td>1,289 ± 66</td>
<td>1,087 ± 76</td>
<td>1,289 ± 38</td>
<td>1,212 ± 52</td>
<td></td>
</tr>
<tr>
<td>Lysophosphatidylcholine</td>
<td>165 ± 23</td>
<td>167 ± 17</td>
<td>154 ± 7</td>
<td>155 ± 13</td>
<td></td>
</tr>
</tbody>
</table>

1Means not sharing a common superscript are significantly different (P ≤ 0.05).
2Values are means ± SEM, n = 4 per strain. LG = low growth rate; HG = high growth rate; LL = low lean line; FL = high-fat line.
VLDDL production coupled with decreased lipoprotein clearance in the LG birds could result in reduced lipoprotein lipid uptake into the adipose tissue. In such a scenario, circulating VLDL and LDL would return to the liver, be taken up, and the content lipids would be resecreted as VLDL. Although speculative, this is a readily testable hypothesis supported by the decreased absolute and relative amounts of abdominal fat in LG birds. If feed intake per metabolic body size was similar in LG and HG birds, a reduction in fat storage as adipose would require that the TG either be used as fuel or be stored in other tissues such as muscle. Again, metabolomic analysis of peripheral tissues could provide key information on these alternatives.

Comparison of Lipid Profiles in LL and FL Strains. In contrast to previous studies (Hermier et al., 1984; Hermier and Chapman, 1985), the LL strain exhibited an 82% higher TG concentration ($P < 0.03$) in the fed state than the FL strain. The reason for this difference is not immediately apparent; however, some earlier TG measurements used a colorimetric assay (Biggs and Edwards, 1975) that did not specifically measure TG. Moreover, the difference in TG concentration between these 2 strains has not always been apparent (Leclercq et al., 1984). Importantly, Signature estimates predicted significantly higher rates of fatty acid synthesis (2.56-fold increase) and steroloyl coenzyme A desaturase activity toward palmitic acid (1.57-fold increase) in FL birds, consistent with previous reports (Saadoun and Leclercq, 1986; Legrand and Hermier, 1992) and microarray outcomes (L. A. Cogburn, T. E. Porter, and J. Simon, unpublished data). Estimated LPL activity was also significantly higher in FL compared with LL (Table 3). Direct measurements of LPL activity per adipocyte are similar in these 2 strains (Leclercq, 1988). However, because abdominal adipose is 2.8-fold more abundant in FL than LL, total LPL activity is potentially 3-fold greater in FL than LL birds. Indeed, FL chickens were shown to have a 3-fold greater clearance rate of VLDL-TG into adipose compared with LL birds (Leclercq et al., 1990). Taken with the results of the present analysis, it is possible that in the postprandial state, the LL chickens have higher TG, because these birds are better at exporting the lipid from the liver than they are at TG deposition into adipose. If the LL birds were also more efficient at releasing FFA from adipose and $\beta$-oxidation of those fatty acids for energy, substantially less TG-rich VLDL would be secreted from the livers of LL birds than FL birds. As a result, plasma TG or VLDL levels could become much lower during fasting in LL than FL birds. However, adipocytes isolated from 14-d-old chickens from the LL have a similar lipolytic response to glucagon as those of FL birds (Leclercq, 1988). Therefore, enhanced FFA oxidation in liver is the more reasonable hypothesis to test. This will require specific and direct measurements; at present, indirect measurements in the form of carnitine-palmitoyl transferase-1 messenger levels and ketone body use did not show marked differences in FL and LL chickens (Skiba-Cassy et al., 2007).

Insight pathway maps summarizing differences in lipid metabolism in LL vs. FL (Figure 3) and LG vs. HG (Figure 4) were constructed using available data. Although the current figures are based on data from plasma samples alone, these cartoons never the less provide an integrated perspective on changes in bulk lipid movements brought about by genetic selection programs and places them into a physiological context. These visualizations have utility in bringing key metabolic shifts into focus while also identifying research needs and generating hypotheses or predictions for future studies (Trethewey, 2001). Overall, the impression made is that in FL birds, increased adiposity is due to increased hepatic conversion of feed to VLDL-TG and peripheral capacity for uptake and storage of that TG. In contrast, the reduced adiposity of LG compared with HG birds appears to arise from an inability to utilize and store VLDL-TG. Lipid metabolomics were used to demonstrate the utility of this approach because of their diverse and pervasive roles in metabolism and physiological importance.

Intermediary metabolism is proximal to phenotype, and as a result, directly profiling metabolites (metabolomics) has distinct advantages over other “omic” approaches (Thomas and Ganji, 2006). Metabolomics can be used to discover or confirm metabolic relationships and to functionally annotate transcriptional, proteomic, or both, data (Schilling et al., 1999; Cotter et al., 2006; Wiest and Watkins, 2007). This is particularly true for assignment of functional outcomes to observed changes in transcription or protein profiles. The caveat to this conclusion is that no single platform accurately measures all metabolites. Thus, all-inclusive platforms such as expression arrays used in transcriptomics are unlikely for metabolite measurements. Never the less, in-depth targeted analysis, such as the structural and energetic lipids interrogated in this study, to produce quantitative data on known metabolites is a logical and productive approach that builds on over a century of biochemical knowledge. Importantly, as a more global understanding of avian metabolism emerges from comparisons of multiple strains within and between lines, and this understanding is linked to transcriptional and proteomic detail, our ability to predict the metabolic and phenotypic outcomes of changes in gene networks through selection should markedly improve (Csete and Doyle, 2002). Such an improvement in biological understanding will enable continued improvement in consumer, producer, and bird health outcomes.

ACKNOWLEDGMENTS

The longitudinal study and sampling of the 4 divergent lines required the contribution of many scientists and technicians from the laboratories and breeding facilities at the Station de Recherches Avicoles, Institut National de la Recherche Agronomique, Nouzilly, France. Although each person cannot be named, their essential contribution is gratefully acknowledged. Special thanks is given to E. Duval and M. Duclos (Station de Recherches Avicoles,
Institut National de la Recherche Agronomique) for their invaluable participation in all aspects of the functional genomics consortium project.

REFERENCES


