To the Graduate Council:

I am submitting herewith a thesis written by Mary D. Brown entitled "Transcriptional changes in adipose tissue before and after the onset of hyperphagia in the Zucker (*fa/fa*) rat." I have examined the final paper copy of this thesis for form and content and recommend that it be accepted in fulfillment of the requirements for the degree of Master of Science, with a major in Nutrition.

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TRANSCRIPTIONAL CHANGES IN ADIPOSE TISSUE BEFORE AND AFTER THE ONSET OF HYPERPHAGIA IN THE ZUCKER (*fa/fa*) RAT

A Thesis
Presented for the
Master of Science Degree
The University of Tennessee, Knoxville

Mary D. Brown
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DEDICATION

This thesis is dedicated to my parents, Craig and Maureen Brown, invaluable role models and friends, my sister Jenée, and my friends. I would not have been successful in this chapter of my life without your encouragement, inspiration and unending support. All of you made this possible.

ABSTRACT

The Zucker fatty (*fa/fa*) rat inherits a defective leptin receptor with impaired leptin signaling. Fatties grow at the same rate as their littermates for the first 22 days of age, and then suddenly begin to grow at a much higher rate. This corresponds with the emergence of robust hyperphagia in the fatty rat and leads to overt obesity within days. This striking change in energy balance regulation makes it possible to determine which sets of genes are regulated by the fatty mutation before and after the onset of hyperphagia. Genechip microarrays were used to screen inguinal adipose tissue mRNA from wildtype and fatty rats at 20 days of age (before the emergence of hyperphagia) and 24 days of age (after the emergence of hyperphagia). Differentially expressed transcripts from several pathways were validated by real time quantitative reverse transcription polymerase chain reaction (RT-PCR). Transcripts associated with lipogenic pathways, including fatty acid synthase, malic enzyme, transketolase, glycerol 3-phosphate acyltransferase, insulin induced gene 1, and farnesyl diphosphate synthase, were expressed at similar levels in wildtypes and fatty rats before the onset of hyperphagia, but were highly induced in fatty rats after the onset of hyperphagia. A notable exception was stearoyl CoA desaturase 1, which converts saturated fatty acids synthesized by lipogenic pathways into monounsaturated fatty acids. Scd1 was upregulated in fatties 10.6-fold before hyperphagia emerged, and after the onset of hyperphagia, SCD1 expression increased 5.7-fold in fatty rats. This suggests that Scd1 is regulated independent of lipogenesis before the onset of hyperphagia, but is also developmentally upregulated. Leptin, lipoprotein lipase and PPARγ mRNAs were also elevated prior to the onset of hyperphagia and may share a common regulatory pathway with stearoyl CoA desaturase 1.

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NOMENCLATURE

ACTH	Adrenocorticotropin
ADD1	Adipocyte determination and differentiation factor 1
AGRP	Agouti related protein
ATP	Adenosine triphosphate
BAT	Brown adipose tissue
BBS	Bardet-Biedl syndrome
BMI	Body mass index
C	Carbon
C/EBP	CCAAT/enhancer binding proteins
cAMP	Cyclic AMP
CART	Cocaine and amphetamine-related transcript
CCE	Citrate cleavage enzyme
CCK	Cholecystokinin
CoA	Coenzyme A
CRH	Corticotrophin releasing hormone
C_T	Cycle threshold
DEPC	Diethyl pyrocarbonate
DNA	Deoxyribose nucleic acid
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic reticulum
FAS	Fatty Acid Synthase
FATP	Fatty acid transport protein
Fdps	Farnesyl diphosphate synthase
FFA	Free fatty acids
G6PDH	Glucose 6-phosphate dehydrogenase
GHRH	Growth hormone releasing hormone
Gpam	Glycerol 3-phosphate acyltransferase
HMG-CoA	Hydroxy-3-methylglutaryl-CoA
Insig1	Insulin induced gene 1
Insig1	Insulin induced gene 1
Jak	Janus tyrosine kinase
Lep	Leptin
Lpl	Lipoprotein lipase
MAP	Mitogen-activated protein
MCH	Melanin-concentrating hormone
MC-R	Melanocortin receptors
Me1	Malic Enzyme
mM	Milli-molar
MSH	Melanocyte–stimulating hormones
MuLV-RT	Murine leukemia virus reverse transcriptase
NADPH	Nicotinamide adenine dinucleotide phosphate
ng/μL	Nanograms per microliter
NHANES	National Health and Nutrition Examination Survey
NIDDM	Noninsulin dependent diabetes mellitus
Npy	Neuropeptide Y
ob	*obese*
OB-R	Leptin receptors
OB-Rb	Long isoform leptin receptor
PDGF	Platelet-derived growth factor
PEPCK	Phosphoenolpyruvate carboxykinase
PFK	Phosphofructokinase
POMC	Proopiomelanocortin

PPARγ	Peroxisome proliferator activated-receptor γ
PWS	Prader-Willi syndrome
Q	Quencher
R	Reporter
RNA	Ribonucleic Acid
RT-PCR	Reverse transcription polymerase chain reaction
SCAP	Sterol regulatory element-binding protein cleavage-activating protein
Scd1	Stearoyl CoA desaturase 1
SNS	Sympathetic nervous system
SREBP1	Sterol regulatory element binding protein 1
STAT	Signal transducer and activator of transcription
Tkt	Transketolase
TNF-α	Tumor necrosis factor alpha
TZD	Thiazolidinediones
U/μL	Units per microliter
Ucp	Uncoupling protein
UPD	Uniparental disomy
VLDL	Very low density lipoprotein
WAT	White adipose tissue
β-LPH	β-lipotropin hormone
α-MSH	α melanocyte–stimulating hormone
μL	Microliter

CHAPTER 1. LITERATURE REVIEW

Over the past 20 years the obesity crisis has grown to epidemic proportions. Despite heightened interest, little is actually known regarding obesity and its development, particularly the development of childhood obesity. This literature review seeks to shed light on the developmental factors of obesity and their potential roles in the occurrence of obesity. The following literature review begins with an overview of obesity and the resulting epidemic. To provide greater insight into how obesity occurs, the developmental stages of obesity are then discussed. In examining the developmental stages, the importance of the development of obesity becomes apparent. The paper then conveys the field's current understanding of the role of adipose tissue and various hormonal, nutritional and transcriptional regulations that play into the formation of obesity. The literature review concludes with a detailed look into the animal model.

OBESITY: THE NEW EPIDEMIC

Change in the Prevalence of Obesity over Time

It has just been over the past 20 years the obesity epidemic has transpired. Though the origin for the increase in obesity is unclear, a shift has occurred in the primary causes of morbidity and mortality that parallels the spread of obesity. Infectious diseases such as pneumonia and tuberculosis have given way to chronic diseases such as cardiovascular disease and stroke as the primary public health problems (1-3). Researchers claim that the shift of cause of morbidity and mortality is due to modernization and a variety of socioeconomic factors, contributing to the rise in obesity and ultimately resulting in an increase in chronic disease (4,5). Overweight, defined as increased body weight in relation to height compared to a standard of acceptable or desirable weight, and obesity, defined as an accumulation of adipose tissue in relation to lean are known to be directly associated with a large number of chronic illnesses (6). According to the Center for Disease Control, 7 of 10 American deaths are a result of chronic disease a value that parallels the rise of individuals classified as overweight or obese in the United States (7).

The numbers of individuals classified as overweight and obese is increasing to disturbing proportions. Worldwide, over 700 million adults are overweight and more than 300 million are clinically obese. According to 1999-2000 NHANES data, it is estimated that 33% of adult Americans are overweight and that 31% of adults are clinically obese. Thus, over half of all adults in the United States are either overweight or obese, an increase of 8% since NHANES III. Specifically focusing on obesity, the prevalence of adults classified as obese has more than doubled since NHANES II data (8).

Though the statistics for the prevalence of overweight and obesity in adults is alarming, even more frightening are the statistics regarding the prevalence of overweight and obesity of the youth. Worldwide it is estimated that 17.6 million children under the age of 5 are overweight (6). 10% of children aged 2 to 5 years old are overweight and 16% of children (over 9 million) aged 6 to 19 years old are overweight – a 45% increase since NHANES III (1988-1994) (9,10). Since 1980, children classified as overweight aged 6-11 has more than doubled and the rate for 12-19 year olds has more than tripled (7,8). The implications childhood obesity has on adult obesity and its associated correlation with chronic disease has become a major public health concern.

Use of BMI as a Classifier

Determining ones BMI is one of many screening tools used to indicate weight status and predict risk of disease in adults (individuals 20 years of age and older). BMI shows body weight adjusted for height, specifically weight in kilograms is divided by height in meters times itself (kg/m^2). The ranges of BMI are based on the effect body weight has on disease and death (table 1). Overweight, or a BMI greater than 25.0, is correlated with chronic conditions such as hypertension, hypercholesterolemia, cardiovascular disease, diabetes, osteoarthritis, and various cancers. Moreover, a widely held belief is that obesity results in premature death. However, because BMI is calculated from weight and height only, often times it is not an accurate indicator of body fat mass (6).

Prediction of BMI and the classification schema differs from adults in children and teens. With children and teens BMI is based upon age, or 'BMI-for-age', due to the increased rate of

Table 1. BMI classification for adults.

BMI	weight status	obesity class
> 18.5	Underweight	
18.5 – 24.9	Normal	
25.0 – 29.9	Overweight	
30.0 – 34.9	Obesity	I
35.0 – 39.9	Obesity	II
≥40	Extreme Obesity	III

growth that occurs during the developmental years. BMI-for-age is based on gender and age specific charts – BMI-for-age is plotted on gender specific growth charts. Growth charts are then used to identify growth percentiles classifying children as underweight, risk for overweight and overweight (table 2). Though this method simply classifies children, rather than addressing their risk for disease, it allows us to monitor trends in childhood obesity. The monitoring of childhood obesity is pertinent in that it is directly related to risk factors for later development of heart disease, hyperlipidemia, hyperinsulinemia, hypertension, early atherosclerosis, etc. (11).

Health Consequences of Overweight and Obesity

Debilitating disease such as respiratory trouble, musculoskeletal problems, skin problems, infertility, etc. are associated with obesity. Additionally, obesity puts individuals at increased risk for conditions related to cardiovascular disease, insulin resistance and some forms of cancer (3,5,12,13). For example, through a 10 year follow up of the Nurses' Health Study and the Health Professionals Follow-up Study, Field et al found that both men and women with a BMI of ≥35.0 are about 20 times more likely to develop diabetes, compared to those with BMI's of 18.5-24.9. Furthermore, overweight adults (BMI of 25.0-29.9) were more than 3 times as likely to develop diabetes, compared to their leaner peers. The risk of developing diabetes, gallstones, hypertension and heart disease increased in both men and women in response to the severity of overweight (5). The numerous consequences of childhood obesity are now coming to light. Recent studies suggest that obese children are often the target of prejudice and discrimination and thus, more likely to develop physiological problems such as low self-esteem and various behavioral problems. Overweight children have a tendency to be taller with advanced bone age. Overweight girls are at increased risk of early pubertal development while overweight boys are at risk for delayed pubertal development. Furthermore, the influence that childhood obesity has on acquiring adult obesity has been the subject of substantial research (12,14,15). Overweight children are more than two times as likely to become overweight adults compared to non-obese children and concurrently are at increased risk of suffering from the various chronic disease seen in adults discussed above (13,14). Moreover, children are now starting to suffer from what were

Table 2. BMI classification for children.

BMI-for-age	weight status
< 5th Percentile	Underweight
85th – 95th Percentile	At risk for overweight
≥95th Percentile	Overweight

once though to be adulthood disease. For example, an increasing number of children are developing type 2 diabetes, once referred to as "adult onset obesity" (6). Additionally, obese children are at increased risk for asthma, hyperlipidemia, cholecystitis, hypertension, etc. (16).

The biology of obesity is complex and includes overconsumption of energy, the existence of a sedentary lifestyle and various genetic influences. Energy balance is controllable at two levels: intake and expenditure. Thus, if energy intake exceeds energy expenditure excess energy is stored in the form of triglycerides in adipose tissue. Overtime, excess intake coupled with insufficient physical activity results in the accumulation of body fat (1,17,18). Dietz asserts that the occurrence of environmental factors during pre and postnatal development has a permanent influence on normal growth. Accordingly, three specific periods for the development of obesity have been defined: the prenatal period, the period of adiposity rebound and the period of adolescence. Deitz claims that when physiological alterations occur during these critical periods of development individuals are at an increased risk for developing obesity later in life (16,19). It is for this precise reason focus on the development of obesity is crucial.

THE PHYSIOLOGICAL FOUNDATION: GENETICS AND THE DEVELOPMENT OF OBESITY

The obesity epidemic and the health risk associated with obesity warrants the current interest in critical periods for obesity development. Because childhood obesity, more often than not, precedes adulthood obesity it is imperative that we thoroughly understand the mechanisms of obesity during the developmental years. Thorough understanding of the critical periods of development, of their origin, their mechanisms and how obesity ensues, presents a knowledge that may be applied to the treatment and prevention of obesity. Long standing research illustrates that obesity has both a genetic and a behavioral component. In the study of obesity, scholars argue that three critical periods are vital in predicting obesity: prenatal, adiposity rebound and adolescence. The discussion below provides a short definition and summary of each of these stages. Because this research focuses on the early development of obesity and the physiological

mechanisms that influence weight gain, after the brief discussion of the stages attention shifts to a detailed look at the mechanisms influencing anomalies during early gestation.

The Three Critical Periods of Obesity

As mentioned, researchers suggest three specific stages of life that are especially susceptible to risk. Three specific phases of childhood development have been identified as critical periods during which various environmental factors influence growth and development. These periods are: the prenatal period including early infancy, the period of adiposity rebound which occurs between ages five and seven, and the period of adolescence. It is believed that when physiological alterations occur during these periods, individuals are at an increased risk for subsequent obesity (16,19). To prevent the likelihood of obesity it is necessary to understand these critical periods of development and recognize the multiple factors that influence them.

Prenatal and Early Infancy

The first critical period encompasses the prenatal period and early infancy. Increasing evidence is showing a relationship between fetal development and adulthood obesity. The prenatal period encompasses three trimesters: the first, lasting from week one to 13, the second, week 14 to 27 and the third trimester from week 28 to birth. Physiological alterations during any of these periods may result in an underdeveloped fetus and subsequent repercussions resulting in weight gain and fat mass accumulation (19-22).

For example, the development of the brain, specifically the hypothalamus, occurs during the first and second trimester. The hypothalamus is responsible for the control of blood pressure, thermoregulation, energy balance, etc. through its action in is in response to neural inputs and circulating factors. These factors, such as hormones and substrates, inform the hypothalamus on the metabolic status of the body to which the hypothalamus responds. For example, during the fasted state the hypothalamus signals an increase in appetite stimulating factors such as Npy, MCH and glucocorticoids. During the fed state the circulation of appetite suppressing regulators such as CCK, α-MSH and leptin are increased (20). It has been found that an insufficient supply

of nutrient related substrates results in the underdevelopment of the hypothalamus, thus jeopardizing its ability to function properly (19-22).

Another example of a critical period of prenatal development that may be influenced by physiological factors is during the second and third trimester. During this time period there is a 12-fold increase in total body fat and adipocyte replication occurs at an accelerated rate. It has been observed that in early gestation fetal triglyceride stores are of similar composition and influenced by maternal stores. This demonstrates a direct link of the mother's nutritional status during gestation and her unborn child (16).

Adiposity Rebound

The second critical period, the period of adiposity rebound, is defined as the point of life at which a child is at minimum BMI. Adiposity rebound occurs at about six years of age (ranging from three to seven) and is the period before the second rise of adiposity and accelerated development occurs in children. Children experience accelerated growth from month one to month eight resulting in a rapid increase in BMI. At month nine, growth and BMI reach a plateau that continues until age five to six years at which point children experience a gradual increase of BMI through adolescence.

Various studies regarding the age of adiposity rebound have provided valuable information. The Fels Longitudinal Study ultimately found an inverse relationship between the age of adiposity rebound and BMI in those at 18 years – the younger the age of adiposity rebound occurred the higher the BMI at 18. Additionally, adult obesity rates are increased 20% in individuals who experienced early adiposity rebound compared to those who are experience adiposity rebound at an older age (23). Thus, the point at which adiposity rebound occurs influences obesity in adolescence and adulthood – the earlier the adiposity rebound occurs, the higher the BMI in adolescence and adulthood (23,24).

Though several hypotheses have been proposed regarding the influencing factors of adiposity rebound, the most common is associated with environmental factors and learned behavior. Adiposity rebound occurs at a time during which children are learning behaviors associated with energy intake, thus it is believed there is a corresponding relationship between

adiposity rebound and parental control of energy intake. It seems the more parental control regulating energy intake, the less the children are able to regulate energy intake themselves. Children with parents more controlling of their intake have shown less ability to self-regulate energy intake compared to children given the freedom to control the amount they choose to eat (25,26).

Adolescence

The third and last defined period for critical development is adolescence. As discussed earlier, there is a strong correlation between obesity at this age and likelihood of adulthood obesity. Through a retrospective cohort study, Whitaker et al found that the likelihood of obesity as an adult increased more than 50% if the child was older than six years old and obese with no significant differences between boys and girls (15,23). Additionally, Guo et al found that BMI values during adolescence are important risk factors for the presence of adult overweight or obesity – children with BMI values at the 95th percentile had a 62–98% likelihood of becoming overweight by 35 years of age (12). Lastly, the Minneapolis Children's Blood Pressure Study, which monitored children from about 7 years of age to 23 years of age found a direct relation between excess weight gain during childhood and adult cardiovascular disease. This study also showed that height, weight, and BMI measured in early childhood is significantly associated with body size in young adulthood (27). The consequences of childhood obesity encompass psychosocial and medical aspects during the developmental years that continue into adulthood.

There is considerable interest in these three critical periods: prenatal, adiposity rebound and adolescence that has provided useful knowledge regarding the development of obesity, particularly the research regarding prenatal development. This work is necessary because is provides the potential to offer opportunities for obesity prevention, and a way of understanding the mechanisms by which energy balance is regulated. Notwithstanding, the importance of the adiposity rebound and adolescent stages as determinants of obesity, this research focuses on the mechanisms influencing anomalies during early gestation in the development of obesity. Hence, the remainder of this section discusses specific examples regarding conditions occurring during early infancy that contribute to obesity.

Early Infancy Development: A More In-depth Look

With regards to body fat development, adipose tissue hypertrophy's during the first year of extra-uterine life. Size of the child at birth and feeding during the first year is critical in that the adipocytes of obese children continue to undergo hypertrophy, compared to non-obese children who experience little change in adipocyte volume. Because the second and third trimester, as well as the first year of life, is a critical period for the development of adipocyte and fat mass, alterations in normal development as a result of physiological factors can have long lasting affects on fat mass and adipocyte size in children.

A popular example of the effects of physiological alterations during prenatal and early infancy development is demonstrated through the Dutch famine study of 1944-1945. This natural experiment looked at the prevalence of obesity in the offspring's of women exposed to a famine for 5 months in which total caloric consumption ranged from 400 to 1,000 kcal/day. At 19 years of age, the rate of obesity was higher in males who had been exposed to the famine during the first part of gestation compared to males exposed to the famine during the last trimester or immediate postnatal period. From this it has been concluded that the limitation of essential metabolic nutrients during the first and second trimester, specifically during the development of the hypothalamus, puts individuals at an increased risk of obesity later in life. On the other hand, when nutrients are limited during the third trimester and the first few months of life, during adipocyte replication and fat accumulation, individuals have increased chances for leanness later in life (19,28). Alterations of the intrauterine environment may result in a predisposition to obesity. The period of gestation and early infancy is a critical period for the development of obesity proving further investigation into mechanisms developing during this period is warranted.

In sum, three critical periods of development in which environmental factors influence proper development have been identified. This knowledge presents us with a foundation in the basic mechanisms of development. It is from this foundation that knowledge must progress so that there is a more complete understanding of the mechanisms influencing weight gain and subsequently obesity in the developmental years and into adulthood. More complete knowledge provides the opportunity for treatment and prevention of obesity. Additionally, the loss of

significant weight as an adult is rare, thus prevention and treatment of obesity in childhood is essential. In sum, it can be concluded that while some individuals are predisposed to obesity as a result of environmental factors such as the overconsumption of energy and the existence of a sedentary lifestyle, others are predisposed to obesity as a result of genetic influences. This research seeks to extend our understanding of the early developmental influences on obesity with regards to the developmental mechanisms that activate various pathways, contributing to the development of obesity.

The Genetics Behind the Development of Obesity

With regards to genetics, obesity can result from single gene defects or polygenic gene defects in which each gene contributes to the disorder (29,30). Much has been learned with regards to the genetic influences of obesity as a result of the observation of monozygotic twins and the hereditability of fat mass (40-70%) (29). Additionally, various studies have shown there to be a stronger biological influence in the development of obesity compared to environmental in that the weight of adopted children tends to be closer to the biological parents versus the adopted parents (31).

To date, six single-gene mutations and more than 40 Mendelian disorders have been discovered associated with obesity in humans (32). Various genetic disease that exhibit signs of morbid obesity in young children including Prader-Willi syndrome, Proopiomelanocortin, Bardet-Biedl syndrome, Cohen's syndrome and Alstrom syndrome (29). Because environmental factors may be manipulated to prevent weight gain and promote weight loss it is important to identify and assume control of these factors, thus manipulating the outcome. Likewise, because genes are inherited with which we have little control over, it is important to understand the influence of genes to further understand the mechanisms of obesity and thus, develop methods for treatment and prevention of obesity.

PWS

Prader-Willi syndrome, the most common genetic syndrome of obesity, is a disorder that occurs from lack of expression of genes on chromosome 15, specifically region 15q11-q13. With

PWS, the natural expression of maternal alleles in this region is suppressed as a result of genomic imprinting during gametogenesis. Subsequently, it is vital that the genes are inherited paternally for proper expression to occur. However, in PWS the paternal genes are silenced, defective or deleted through maternal uniparental disomy. With maternal UPD two copies of the silenced maternal chromosome 15 are inherited (33).

The major characteristics of those affected by PWS include hyperphagia, hypogonadism and growth hormone deficiency, indicating dysfunction of the hypothalamus, and results in morbid obesity. Development of PWS children is stunted until 6 months of age. From birth to about 6 months, PWS children exhibit feeding difficulties including suckling problems and have little appetite, often times resulting in failure-to-thrive. In contrast, from years one to six, PWS children experience a rapid onset of robust hyperphagia, continuing into the adult years. PWS children eat excessively and do not respond to feelings of satiety as normal children. From these observations it may be assumed that the various pathways that mediate hyperphagia are not developed within the first few months of life. It seems the pathways that mediate hyperphagia are developed within the first few years of life resulting in the onset of hyperphagia after the first year. Without strict diet and weight control, PWS individuals eventually suffer from various obesity-related morbidities including cardiopulmonary disease, type 2 diabetes, premature death, etc.

Little is understood regarding the cause of PWS, however due to the symptoms observed in PWS individuals, there appears to be abnormalities in hypothalamic pathways. PWS individuals experience normal leptin secretion, in proportion to fat mass, CCK secretion, and there are no abnormalities in the distribution of NPY, AGRP and GHRH or the response to obesity. From this it could be assumed that PWS individuals would experience similar responses to satiety as people without the disorder, however hyperphagia continues (33,34). It seems abnormalities in the hypothalamic pathway of those with PWS results in an absence of satiety, despite normal expression of appetite suppressing factors. By more completely understanding the abnormalities associated with PWS we will more completely understand the pathways of the hypothalamus and its role in body weight regulation and possibly other mechanisms influencing appetite.

POMC

Another example of a genetic syndrome resulting in clinical obesity occurs from POMC gene deficiency. The POMC gene is a precursor protein for numerous peptides whose main function is to integrate hormonal signals from adipose tissue. POMC is synthesized in the arcuate nucleus of the hypothalamus when stimulated by Lep, the pituitary, the medulla and different peripheral tissues. POMC deficiency occurs as a result of a mutation of the POMC gene and ultimately results in a deficiency of its products including adrenocorticotropin and β-lipotropin.

ACTH's main function is to maintain adrenal cortex function and production of glucocorticoids. Additionally, when ACTH is produced in the hypothalamus, it is converted to α and β melanocyte–stimulating hormones. ACTH and α-MSH activate the brain melanocortin receptors 1, 2, 3, 4 and 5. The expression and action of the 5 MC-R's vary: MC1-R is expressed in melanocytes; MC2-R is expressed in the adrenal cortex and mediates the activity of ACTH; MC3 and 4-R are involved in energy homeostasis, specifically through the inhibition of energy intake and the activations of energy expenditure. Thus, disruption of the activity of the MC-R's, as through POMC deficiency, results in disruption of the melanocortin pathway. For example disrupting the activation of MC1, 2 and 5-R results in abnormal pigmentation, adrenal function and exocrine gland function. Likewise, disruption of MC3 and 4-R results in a disruption of energy homeostasis. In sum, consequences of POMC gene deficiency are a result of the absence of its derived peptides.

Generalized POMC deficiency ultimately results in inactivation of the hypothalamus and irregulation of pigmentation and weight homeostasis via decreased energy expenditure and increased food intake. Major characteristic of POMC is red hair pigmentation, hyperphagia and early onset obesity that occurs within the first year of life, despite adrenal insufficiency. However, the development of individuals with POMC deficiency is unaffected. From these observations it can be assumed that there is an important role for the central melanocortin system, including POMC and the brain MC-R's, in the maintenance of energy homeostasis. Abnormalities in the melanocortin pathway, such as through POMC deficiency, results in obesity.

It appears that with POMC deficiency, like PWS, the pathways for hyperphagia are inactive during the first few months of life and emerge later in early development. POMC children are born of normal weight and develop normally until the fifth month of life at which point patients are noted to become hyperphagic. Additionally, strict food restriction fails to prevent weight gain. Thus, it has been hypothesized that POMC signaling may affect metabolic rate (35,36). As in the instance of PWS, a more thorough understanding of the mechanisms of POMC deficiency will provide invaluable knowledge regarding weight gain and obesity, specifically in regards to hyperphagia and energy expenditure.

MC4-R Mutation

The most common monogenic cause of obesity in humans is a result of MC4-R mutations. About 4% of all cases of morbid adult obese are a result of MC4-R mutations. The MC4-R is a 7-transmembrane domain G protein-coupled receptor that is activated by the binding of α-MSH in the hypothalamus. Generation of an MC4-R knockout mouse has provided a great deal of information regarding MC4-R's roles.

As discussed above, MC4-R is highly expressed in the hypothalamus and involved in the hypothalamus's regulation of weight. MC4-R's influence on food intake and energy homeostasis is a result of the peptides it produces, all of which come from POMC. Of all peptides, α-MSH seems to be the most relevant to feeding. Upon activation by α-MSH, MC4-R couples to a G protein and activates adenylyl cyclase, which catalyzes the formation of cyclic AMP from ATP. cAMP is involved in various signal transduction cascades.

Experimenters have found that administration of an α-MSH analogue reduces food while administration of an α-MSH antagonist stimulates feeding, inducing hyperphagia and consequently obesity. An example of an MC4-R antagonist is agouti-related protein of which over expression results in hyperphagia and weight gain. Thus, the balance of AGRP and α-MSH influences either the stimulation or inhibition of eating. To even further confirm the essential functioning of α-MSH, and subsequently MC4-R, leptin has been found to stimulate the melanocortin pathway. This is consistent with the fact that both Lep and MC4-R inhibit feeding

and promote weight loss. Furthermore it has been found that blockage of MC4-R activity impairs leptins function.

Individuals with an MC4-R mutation suffer from hyperphagia as early as 4 months of age, constantly seeking food and displaying distress when food is not provided. Additionally, those with a mutation in MC4-R also experience a marked increase in linear growth are hyperinsulinemic and hyperglycemic. However, unlike other obesity related diseases, pubertal development and reproductive function are unimpaired and individuals do not develop diabetes (37-40).

THE METABOLISM OF LIPIDS: LIPOGENESIS AND ADIPOGENESIS

As defined by the National Library of Medicine, adipose tissue is dense connective tissue, consisting primarily of adipocytes, in which fat is stored (41). The fat stored in the human body, specifically in the adipocytes of adipose tissue, is stored in the form of triglycerides. Adipose tissue accumulates in various sites around the body including the subcutaneous layer between the muscle and dermis, around the heart, kidney and other organs and is vital to daily body functions. There are two forms of adipose tissue, each with its own unique functions: white adipose tissue and brown adipose tissue.

WAT is the predominant form of fat in developed humans, however during the neonatal period humans have significant depots of BAT. Triglycerides are stored in the adipocytes of WAT and during periods of dietary deficiency adipose tissue acts as a source of energy by releasing free fatty acids. WAT also has the ability to act as an endocrine organ, secreting hormones and cytokines that regulate various functions. Additionally, WAT protects joints and organs, regulates body temperature, stores vitamins, etc. (42)

Contrary to the function of WAT as storage of energy, BAT dissipates energy in the form of heat through mitochondrial biogenesis. Additionally, while the lipid incorporated in the adipocytes of WAT is stored as a few large droplets, the lipid stored in BAT is stored as numerous minute droplets. BAT is characterized by its high content of mitochondria in which

uncoupling protein 1 is stored. When induced by the sympathetic nervous system, UCP1 uncouples substrate oxidation and electron transport through the respiratory chain from ATP synthesis, thus generating heat. BATs primary function is thermogenesis, providing a defense against cold and protection from obesity (43).

WAT is essential to the normal functioning of the body. Thus, storage of triglycerides in adipocytes and the presence of WAT is critical to good health. Hence, the production of adipocytes and WAT has been the subject of substantial interest in hopes of isolating potential treatment options for syndromes resulting from too little or too much fat. Research to date has illustrated that adipose tissue is produced in two ways: lipogenesis and adipogenesis. Because of the importance of the development of adipose tissue and its implications in obesity, a more in depth look into these two processes follows.

Lipogenesis – The Production of Fatty Acids

The body has a small capacity for the storage of carbohydrates and no ability to store protein. Thus, the storage of triglycerides in WAT is essential during periods of energy restriction in that it provides a constant source of energy: triglycerides are used as an energy source. During a fed state, triglycerides are stored in adipose tissue for later use (44). The triglycerides stored in WAT come from two main sources: diet and de novo synthesis or lipogenesis.

Roughly 95 percent of fat consumed through diet is in the form of triglycerides. A triglyceride is a molecule consisting of three fatty acids (long chains of carbon atoms bound to each other with hydrogen's with a carboxyl group at one end) attached to a glycerol backbone. Triglycerides are digested and eventually incorporated into chylomicrons at which point they are carried from the intestine into the lymphatic system where they are released into the bloodstream. Additionally triglycerides are packaged and transported in the form of VLDLs in the liver. Lipoprotein lipase hydrolyzes the triglycerides in the chylomicron and VLDL releasing individual free fatty acids, which are then transported into the adipocytes of adipose tissue. The fatty acids combine with coenzyme A, resulting in a thioester, which can then be re-esterified to a glycerol molecule forming a triglyceride. During periods of starvation, the stored triglycerides undergo

lipolysis and are metabolized to glycerol and fatty acids for further oxidation. During the fed state, adipocytes reform the fatty acids into triglycerides and store the triglycerides for later use.

De novo lipogenesis is the production of fat that occurs when total caloric consumption exceeds need. The main sites for de novo synthesis are the cytosol of adipose tissue and the liver. During the fed state (such as when consuming a high carbohydrate diet or when total caloric consumption exceeds the body's immediate requirements) glucose is converted to its storage form glycogen through glycogenesis. De novo lipogenesis occurs when glycogen stores are filled to capacity ultimately resulting in the production of palmitate.

Phosphofructokinase is regulated allosterically and plays a major role in the regulation of glycolysis. Once glycogen stores reach capacity, the excess ATP and citrate inhibits phosphofructokinase production and stimulates the pentose phosphate pathway. Inhibition of PFK results in the channeling of glucose 6-phosphate to the pentose phosphate pathway where it is ultimately converted to glyceraldehydes 3-phosphate after undergoing a series of transformations. Glyceraldehyde 3-phosphate is then able to re-enter glycolysis, bypassing the regulatory effects of phosphofructokinase, and is converted to pyruvate.

After the conversion of glyceraldehyde 3-phosphate to pyruvate, pyruvate is transported via a carrier into the mitochondria. In the mitochondria, pyruvate is oxidized by pyruvate dehydrogenase to produce acetyl CoA that is then converted to citrate by citrate synthase. Citrate is then incorporated into the Krebs cycle. During periods of high energy consumption, the activity of the Krebs cycle is inhibited through the suppression of isocitrate dehydrogenase. Consequently, the reduction in the flow of metabolites through the Krebs cycle results in an accumulation of citrate in the mitochondria. The accumulating citrate then diffuses through the mitochondria into the cytosol via a citrate shuttle: the tricarboxylate carrier located in the inner mitochondrial membrane.

Now cystolic, citrate is cleaved by citrate lyase forming acetyl CoA and oxaloacetate. Malonyl-CoA is synthesized from the carboxylation of acetyl-CoA via acetyl-CoA carboxylase. The fatty acid synthase complex then catalyzes the final steps of lipogenesis. The fatty acids synthase complex is a multienzyme complex that catalyzes 7 successive cycles ultimately

producing palmitate. The first cycle produces a 4-carbon acyl-ACP. The subsequent cycles act to build the fatty acid by the serial addition of 2 carbons to the growing fatty acid chain. After each addition of carbons, they are condensed, reduced, dehydrated and reduced again until, for example, the 16-C fatty acid palmitate has been formed. Once the acyl chain reaches 16 carbons, the thioesterase domain of the fatty acid synthase complex cleaves the free fatty acid from the enzyme (45,46).

As highlighted, lipogenesis is the process by which fat is produced. The lipogenesis process relies on the presence of several critical transcripts. To date, little is known about how transcript levels might alter lipogenesis. Nonetheless, the presence or absence of these transcripts may be important determinants of hyperphagia and hyperinsulinemia and may ultimately contribute to obesity. The following section examines 10 specific transcripts that are thought to play critical roles in lipogenesis: fatty acid synthase, lipoprotein lipase, malic enzyme, transketolase, glycerol 3-phosphate acyltransferase, stearoyl CoA desaturase 1, farnesyl diphosphate synthase and leptin.

Transcripts of Special Interest

Because increased lipogenesis activity results in adiposity, the study of the transcripts that under gird lipogenesis may prove fruitful in providing new insight into the problem of obesity. The following discussion examines the role that 10 key transcripts play in lipogenesis. One of the transcripts appear critical to energy homeostasis and seven are involved in lipogenesis and cholesterol synthesis. The following discussion examines the lipogenic enzymes first and then shifts attention to the transcripts involved in energy regulation. In short, the following transcripts are discussed below: fatty acid synthase, lipoprotein lipase, malic enzyme, transketolase, glycerol 3-phosphate acyltransferase, stearoyl CoA desaturase 1, farnesyl diphosphate synthase and leptin.

Fatty Acid Synthase

FAS is a multifunctional enzyme expressed mostly in the lipogenic tissues liver and adipose tissue. Fatty acid synthase primarily synthesizes the long-chain saturated fatty acid

palmitate (16 carbon fatty acid), but also stearate (18 carbon fatty acid) and myristate (14 carbon fatty acid), from acetyl-CoA, malonyl-CoA and NADPH. These long-chain fatty acids can then be used for the synthesis of triglycerides and phospholipids.

High insulin levels (often resulting from a high carbohydrate diet) stimulate FAS synthesis. Additionally, high FAS levels are correlated with high carbohydrate, low-fat diets. Conversely, FAS expression is inhibited by glucagon (a hormone secreted in response to the fasted state) and consumption of polyunsaturated fatty acids, amino acids, and sterols (47).

Lipoprotein Lipase

Also discussed earlier, Lpl is responsible for the release of free fatty acids from triglycerides. Increased levels of insulin, such as those occurring during the fed state, stimulates the transcription and synthesis of lipoprotein lipase in adipose tissue. Lpl is then translocated to the walls of blood capillaries at which point it attaches to the endothelium where it can hydrolyze triglycerides in chylomicrons and VLDLs into free fatty acids. Fatty acids can then be incorporated into white adipose tissue or oxidized as fuel, and glycerol. Thus, lipoprotein lipase assists in the clearance of chylomicrons and VLDL from the blood, protecting the body from hyperlipidemia, as well as the accumulation of triglycerides in WAT (48).

Transketolase

The flux of glucose 6-phosphate into the pentose phosphate pathway is stimulated once excess glucose has surpassed levels needed for glycogen storage. Transketolase is a thiamin dependent enzyme linking glycolysis with the pentose phosphate pathway by catalyzing reactions in the non-oxidative branch of the pathway. Specifically, Tkt catalyzes the conversion of xylulose 5-phosphate and ribose 5-phosphate to glyceraldehyde 3-phosphate and sedoheptulose 7-phosphate by transferring a two-carbon unit from a ketose to an aldose. With the help of transaldolase, transketolase can ultimately form fructose 6-phosphate and erythrose 4-phosphate from glyceraldehyde 3-phosphate and sedoheptuloase 7-phosphate (49). The pentose phosphate pathway produces NADPH, a necessary constituent of lipogenesis and cholesterol synthesis. Tkt activity is upregulated under the influx of glucose 6-phosphate and the need for NADPH for fatty acid synthesis.

Malic Enzyme

Cystolic malic enzyme catalyzes the reversible oxidative decarboxylation of malate forming pyruvate, CO_2 and NADPH. Specifically, citrate formed in the Krebs cycle is translocated from the mitochondrion to the cytosol via the tricarboxylate carrier. When under the presence of CoA and ATP, citrate is cleaved into acetyl CoA and oxaloacetate. Oxaloacetate can then be reduced to malate. Finally, malate undergoes oxidative decarboxylation by $NADP^+$ and ME1, forming acetyl CoA and NADPH (50). It has been found that fatty acid synthesis requires 0.62 μmole of NADPH per minute per gram of adipose tissue. The pentose phosphate pathway provides 0.39 μmole of NADPH per minute per gram of adipose tissue leaving a need for 0.23 μmole of NADPH (51). Pyruvate carboxylase activity runs at about 0.22 μmole/g of adipose tissue per minute, providing a sufficient amount of oxaloacetate that ultimately can produce the required 0.23 μmole of NADPH per gram of adipose tissue per minute needed for fatty acid synthesis through malic enzymes action (52). Thus, it can be assumed that during lipogenesis malic enzyme activity would be induced dramatically, generating NADPH to support lipogenesis.

Glycerol 3-phosphate acyltransferase

Mitochondrial Gpam catalyzes the initial step in triglycerides and phospholipid synthesis. Similar to FAS, Gpam is expressed primarily in liver and adipose tissue. The synthesis of glycerophospholipids begins with the acylation of Gpam with fatty acyl-CoA, generating monoacylglycerol 3-phosphate. Monoacylglycerol 3-phosphate is then acylated further, forming triacylglycerols to be used for storage in adipose tissue.

Like other lipogenic enzymes, Gpam mRNA is stimulated by insulin during fasting/refeeding and high carbohydrate diets. Additionally, Gpam is predicted to play a role in the regulation of cellular triacylglycerol and phospholipid levels. Mice deficient in glycerol 3-phosphate acyltransferase experience decreased fat pad mass, body weight, hepatic and plasma triacylglycerol content and VLDL triacylglycerol secretion (53,54).

Stearoyl CoA desaturase 1

Scd1 is a lipogenic enzyme that catalyzes the delta-9-*cis* desaturation of fatty acids and forms the monounsaturated fatty acids palmitoleate and oleate. In detail, stearoyl CoA desaturase 1 removes a hydrogen atom at the delta 9 position and then an additional hydrogen atom at the delta 10 position of the fatty acid. Thus, a double bond is formed between carbons number 9 and 10 in the *cis* position.

Insulin has been found to upregulate the expression of Scd1, while leptin and glucagon inhibit expression. Additionally, while glucose and fructose upregulate activity, polyunsaturated fatty acids have been found to down regulate activity. Recent studies have demonstrated that inhibition of Scd1 results in decreased levels of cholesterol, triglycerides, and monounsaturated fatty acids. Additionally, SCD1 deficient mice are resistant to diet induced weight gain and experience increases in β-oxidation, metabolic rates, and down-regulation of lipogenic enzymes. Consequently, deficiency in Scd1 facilitates loss of fat mass and body weight (55,56).

Farnesyl diphosphate synthase

Farnesyl diphosphate synthase is a peroxisomal enzyme involved in the synthesis of cholesterol. Specifically, Fdps catalyzes the formation of farnesyl diphosphate from isopentenyl diphosphate and dimethylallyl diphosphate. Farnesyl diphosphate is a precursor for many molecules including bile acids, steroid hormones and cholesterol. Transcription of Fdps is regulated by sterols and coordinately regulated with HMG-CoA reductase and HMG-CoA synthase (57).

Leptin

Leptin is a hormone secreted from adipose tissue involved in energy homeostasis. Leptin is secreted in direct proportion to white adipose tissue in the fed state. However, during periods of fasting leptin gene expression diminishes markedly. In the fed state leptin is expressed via receptors that results in the inhibition of feeding and increase of thermogenesis (58).

In the hypothalamus, Lep binds to two distinct sets of neurons. One set of neurons influenced by leptin is the orexigenic neuropeptides NPY and AGRP. Lep has been found to

suppress expression of these neuropeptides. The other set of neurons of which leptin induces expression of is the anorexigenic α-MSH and CART. Thus, the influence of leptin on the induction of feeding results from the balance of orexigenic to anorexigenic neuropeptides. In rodent models, administration of leptin has been found to reduce food intake (59). A more explanatory focus of leptin occurs in the following discussion of the rodent model.

Adipogenesis – The Production of Adipose Tissue

Adipogenesis, or adipose cell differentiation, is the conversion of preadipocytes into adipocytes containing lipid. Adipogenesis occurs as a result of normal cell turnover and a requirement for additional fat mass due to excess total caloric consumption, requires a balance between the activation and repression of adipocyte-specific genes. In order for adipogenesis to occur a proliferating network of capillaries in an area of loose connective tissue must be present.

Adipocytes develop from mesenchymal stem cells in two major steps (figure 1). In the first step differentiation occurs – the stem cells are dedicated to the production of adipocytes, rather than in the production of muscle or bone, resulting in cells with fibroblast-like morphology or "pre-adipocytes." The adipocyte differentiation process directly influences the total amount of adipocytes produced. In the second step the pre-adipocytes fully differentiate through mitogenic and adipogenic signal, resulting in the progressive acquisition of the morphological and biological characteristics of the mature adipocyte. Some suggest that insulin/insulin like growth factor 1 (IGF-1), glucocorticoid and cyclic AMP signaling pathways play roles in the differentiation process (60).

After cycles of DNA replication and expansion of cells the adipocyte doubles in size and transforms into a spherical shape. Additionally, changes occur in the composition of the adipocyte that are required for the development of the mature adipocyte. Degradation of the extracellular matrix occurs and the presence of cytoskeletal components changes thus resulting in alterations in shape, lipid accumulation and adipocyte-specific gene expression. The progressive acquisition of the adipocyte phenotype corresponds with changes in expression of more than 2000 genes. Additionally, it seems the transition in cell size and composition may be

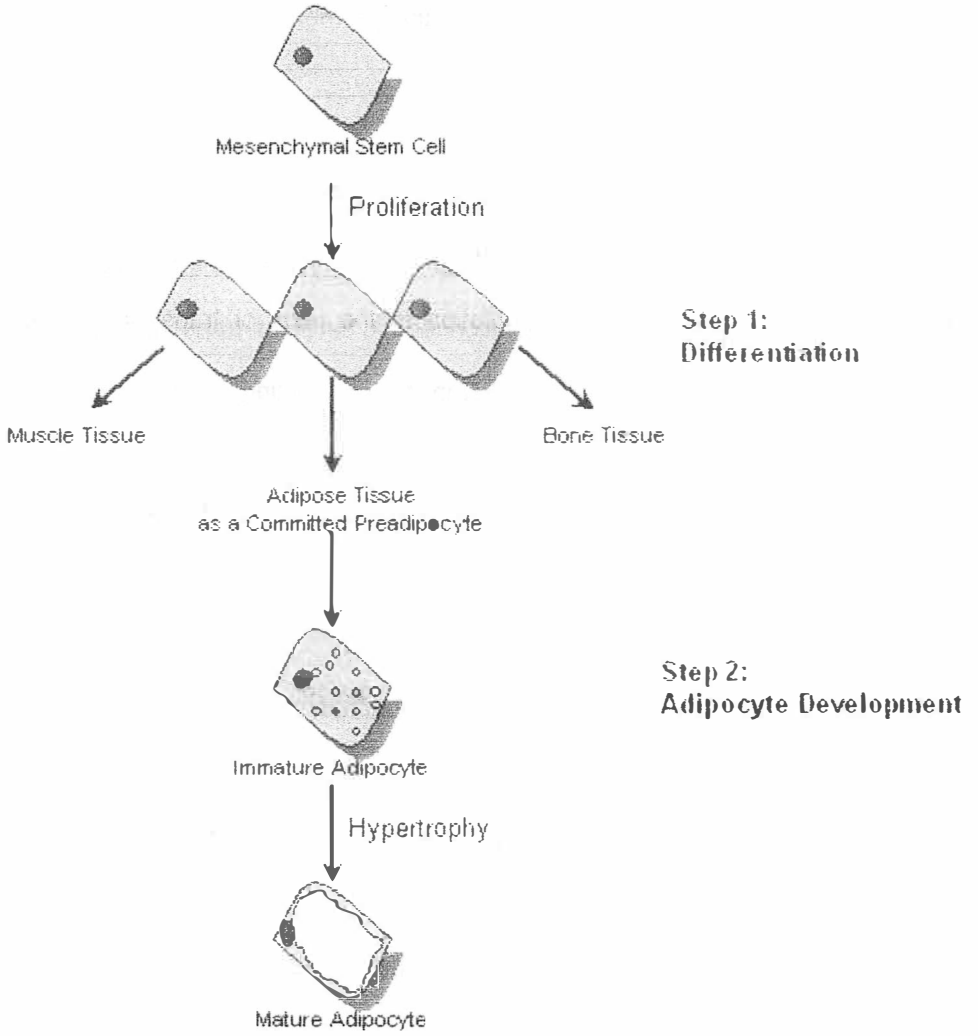


Figure 1. Adipogenesis. Adipocytes develop from mesenchymal stem cells in two steps: differentiation and adipocyte development. In differentiation occurs the stem cells are dedicated to the production of adipocytes and become "pre-adipocytes." In the second step the pre-adipocytes fully differentiate ultimately resulting in the morphological and biological characteristics of the mature adipocyte.

a point of adipogenesis regulation possibly through the promotion of adipogenic transcription factors (42,60,61).

Transcriptional Regulation of the Differentiation Process

Adipose cell differentiation is influenced by various methods of regulation including nutrient stimuli, hormonal regulation and regulation at the level of transcription. Peroxisome proliferator activated receptor gamma, CCAAT/enhancer binding proteins and adipocyte determination and differentiation factor 1/sterol regulatory element binding protein 1 are families of transcription factors that play key roles in the regulation of adipogenesis as well as the development of the mature adipocyte.

PPARγ

PPARγ, found primarily in WAT, is a member of the nuclear hormone receptor superfamily is responsible for the regulation of gene transcription. PPARγ is hypothesized to be a key player in the adipocyte differentiation process because PPARγ expression is induced prior to the activation of most adipogenic genes. Expression of PPARγ seems to induce growth arrest and initiate adipogenesis in fibroblasts. On the other hand, studies regarding the loss of PPARγ function have demonstrated that PPARγ is necessary to promote fat cell differentiation and antagonists that phosphorylate PPARγ, such as MAP kinase, lead to its inactivation and subsequent suppression of differentiation. Additionally, natural ligands to PPARγ such as TZDs and prostaglandins have been found to induce adipogenesis (62). A more detailed discussion of PPARγ occurs below.

C/EBP

C/EBP's belong to the basic-leucine zipper class of transcription factors and were the first transcripts that was found to play a pivotal role in adipocyte differentiation. C/EBPα, β and δ are expressed at specific pointes during adipogenesis. C/EBPβ and d are present in preadipocytes and their expression increases during early differentiation (60). C/EBPβ and d influence adipocyte differentiation through their induction of PPARγ and are induced by

glucocorticoids, cAMP and insulin. C/EBPα is expressed late in differentiation, prior to the transcription of adipocyte-specific genes such as Scd1, leptin and the insulin receptor. C/EBPα has been found to sustain the elevated levels of PPARγ (influenced by C/EBPβ and δ). Loss of function studies have also found that C/EBPβ and δ induce determination and differentiation whereas overexpression of C/EBPα induces preadipocyte differentiation into mature adipocytes (62).

ADD1/SREBP1

ADD1/SREBP1 is also believed to be a key determinant of adipocyte differentiation and lipogenesis, however to a lesser extent than PPARγ and the C/EBPs. ADD1/SREBP1 mRNA levels are increased during preadipocyte differentiation, suggesting its role in the differentiation process. Additionally, coexpression of ADD1/SREBP1 increases PPARγ expression, independent of ligand activation, possibly through its inductions of endogenous ligands for PPARγ. Though little is understood, SREBP1s induction of PPARγ is believed to be the mechanism underlying SREBP1s actions (60,63).

Hormonal Regulation of the Differentiation Process

Various hormones have been found to induce differentiation as well. Insulin has been found to increase the percentage of cells that differentiate. It also increases the amount of lipid accumulation in adipocytes through its cross activation of the IGF-1 receptor, though the exact mechanisms are not clear. Both insulin and IGF-1 stimulate ras, which in turn induces adipogenesis. On the other hand, reductions in adipocyte differentiation appears positively related to reductions in ras expression. Glucocorticoids have also been found to induce differentiation through its induction of C/EBPδ. Lastly, growth hormone has been found to induce adipocyte differentiation, however very little is understood regarding the mechanisms.

Conversely, cytokines such as TNF-a and growth factors such as PDGF are responsible for the suppression of differentiation as well as the dedifferentiation of mature fat cells. It is

hypothesized differentiation is mediated by MAP kinase. MAP kinase phosphorylates PPARγ, thus inhibiting its adipogenic activity and the resulting differentiation it promotes (42,60).

Transcriptional Regulators of Special Interest

In addition to the previously discussed transcripts, PPARγ and insulin induced gene 1 are of special interest to this research. Of particular interest is the role they play in differentiation and the regulation of gene expression involved in glucose utilization and lipogenesis.

PPARγ

The binding of ligands to PPARγ results in the transcription of various target genes through the release of corepressors and recruitment of coactivators. With regards to adipogenesis, PPARγ manipulates the influx and efflux of fatty acids in adipose tissue through the activation and suppression of various genes. PPARγ stimulates LPL in adipocytes resulting in the generation of FFA from circulating lipoproteins. The uptake of FFAs is increased by PPARγ stimulation of fatty acid transporters fatty acid transport protein a molecule that facilitates entry, acylation and esterification of FFAs in adipocytes. FFA efflux is reduced by PPARγ induction of glycerol-3-phosphate, phosphoenolpyruvate carboxykinase and glycerol kinase, all of which promote the production and storage of triglycerides. These actions of PPARγ ultimately result in increased uptake and reductions in total serum FFA levels. Additionally, ectopic expression of PPARγ has been found to induce the differentiation of fibroblastic cells into preadipocytes. Thus, PPARγ activity influences the composition and number of adipocytes as well as circulating fatty acids.

Unsaturated fatty acids such as oleate and linoleate, arachidonic acids, prostaglandins, and members of the thiazolidinediones family have all been found to activate PPARγ. PPARγ activation has been found to play a role in adipogenesis as well in mediating the insulin-sensitizing action of TZDs. TZDs are synthetic compounds developed as insulin sensitizers for the treatment of insulin resistance in patients with NIDDM through regulation of hyperglycemia, hyperinsulinemia, and hyperlipidemia. It was later discovered the target of TZDs was PPARγ – TZD is ligand of adipocyte-specific PPARγ. TZDs antidiabetic affects are primarily elicited by

their improvement of muscle insulin sensitivity. One hypothesis regarding the mechanisms of TZDs, and subsequently PPARγ, is related to its impact on fatty acids. Activation of PPARγ may result in channeling fatty acids into adipose tissue, thus decreasing plasma concentration levels. The reduction of circulating fatty acids leads to increased insulin sensitivity in muscle tissue (64,63).

Insulin induced gene 1

Insig1, a protein located in the endoplasmic reticulum, is one of the most highly induced genes in liver cells (65). Insig1, whose expression increases in response to a insulin levels, binds to the sterol-sensing domain of sterol regulatory element-binding protein cleavage-activating protein when in the presence of sterols (66). On the other hand, when cells are low in sterol's SCAP binds to SREBP and escorts SREBP from the ER to the golgi complex enabling SREBPs stimulation of cholesterol synthesis. Insig1s binding with SCAP inhibits the movement of the SCAP/SREBP complex from the ER to the golgi complex thus, inhibiting the activation of SREBP and consequently cholesterol synthesis. The greater the expression of Insig1, the greater the influence on cholesterol homeostasis (67). Additionally, the inhibition of SREBP1-c specifically was found to result in reduced expression of SREBP1-c target lipogenic enzymes, such as Scd1 and FAS, arguing insig1s antilipogenic action (68).

Sever et al. found that Insig1 is also capable of regulating cholesterol through its actions on HMG CoA reductase. In a similar manner as SCAP, Insig1 binds to the sterol-sensing domains of HMG CoA reductase stimulating accelerated degradation of HMG CoA reductase and inhibiting cholesterol synthesis. Additionally, SCAP and HMG CoA reductase have been found to compete for the same binding site of Insig1, a mechanism that may play a crucial role in cholesterol homeostasis (69).

Lastly, it has also been observed that over expression of Insig1 inhibits the differentiation of preadipocytes and limits triglyceride synthesis and accumulation in adipocytes. In mice, Insig1 mRNA rose in response to the feeding of a high-fat diet. On the other hand, Insig1 mRNA decreased in mice in response to a low-fat diet. The upregulation observed in Insig1 mRNA is believed to be a result of the increased fat mass that accompanies feeding of a high fat diet. On

the other hand, Insig1 expression was downregulated in response to decreased fat mass ocurring as a result of the low fat diet (66).

THE RODENT MODEL

The rodent model has proven to be invaluable in its role in human obesity research. The genetics of obesity is a complicated syndrome that is a result of various mechanisms, some of which occur simultaneously. Insight into various obesity related candidate genes have been identified through use of animal models. Specifically, use of rodents has provided invaluable information regarding the role of leptin, NPY, CRH, MC3, etc. all of which are directly related to the development of obesity. The following paragraphs will go into detail regarding the animal model, the Zucker fatty (*fa/fa*) rat, employed in this research effort.

The Zucker fatty (fa/fa) Rat

The fatty rat, a rat homolog of the *db/db* mouse displaying the same phenotype, is used as a model of early onset obesity. The fatty rat was discovered by Theodore and Lois Zucker in 1961 through research on the susceptibility for the inheritance of obesity. A gene was found that was responsible for the development of obesity and was subsequently labeled the "fatty gene" (*fa*). The *fa* mutation was mapped to rat chromosome 5, further arguing that the *fa* and *db* mutation are homologous genes (70).

The fatty rat inherits the *fa* gene as an autosomal recessive mutation. Because the *fa* gene is inherited, the mutation is present at the time of conception and its influence occurs during the early stages of life. The mutation associated with the *fa*-gene encodes for a shortened leptin receptor protein that cannot interact with leptin, thus the leptin receptors do not function properly. The mutant leptin receptor appears to influence the hypothalamus' ability to regulate feelings of satiety (71).

The *fa* gene acts on leptin receptors impacting the hypothalamus. Leptin is a hormone whose primary physiological function is its anorectic effects. The fatty rat inherits an amino acid

substitution at position 269 of the leptin receptor protein sequence. Specifically, the leptin receptors of those with the *fa* mutation contain a mutation at nucleotide position 880 resulting in a conversion of adenine to cytosine. This causes amino acid 269, glutamine, to change to proline in the extracellular domain (72). Thus, though the fatty rat experiences high levels of circulating leptin, the mutant leptin receptor is not capable of binding the leptin for transport across the blood-brain barrier to the hypothalamus. Deficiencies in the hypothalamus ultimately result in reduced sensations of satiety and thus, hyperphagia (73). Fatty rats grossly overeat to the point of obesity.

Fatty rats are hyperphagic, obese, infertile and suffer from hyperinsulinemia, however blood glucose levels are normal. Fatty rats also suffer from hypertriglyceridemia due to increased production of VLDL by the liver. Fatty rats experience increases in adipocyte size and number with subcutaneous fat pads showing the largest increase in fat cells compared to nonmutants. Additionally, leptin secretion occurs in proportion to fat mass but is ineffective in regards to its anorectic effects due to the defect in the leptin receptor gene. The phenotypic characteristics of fatty rats are comparable to obese rats with hypothalamic dysfunction.

Interestingly, though the weight gain in the fatty rat is significant, rat pups with the *fa* mutation are indistinguishable from littermates until 23 days of age. Truett et al found that when rat pups were left with their dam until 28 days of age (rather than the customary 21 days of age) the fatty rat experiences a developmental change that is manifested by 23 days of age. Fatty rats weigh significantly more than lean littermates on day 23, stomach contents and liver weight are increased and plasma insulin levels are elevated, thus displaying the phenotypic characteristics of the *fa* gene mutation. Additionally, this genotype effect is apparent through visual inspection of their bellies at 24 days of age which protrude do to the onset of hyperphagia. The dramatic increase in growth rate at 23 days of age is attributed to the decreased responsiveness to leptin after which the fatty rat develops hyperphagia and hyperinsulinemia. The authors concluded that a change occurs in the genes associated with growth and obesity during the 4^{th} week of life during which the activation of a "developmental switch" takes place resulting in hyperphagia (74).

The observation that the onset of hyperphagia in the fatty rat occurs after the third week of life leads one to believe the pathways that mediate the onset of hyperphagia are underdeveloped or inactive, later emerging during the fourth week of life. Because the development of hyperphagia occurs during the first few weeks of life, the fatty rat is a useful animal model with regards to the early development of obesity. The following paragraphs will discuss the influence of the *fa* gene in those possessing the mutation with regards to leptin and the leptin receptor. The focus will then shift to the characteristics of the Zucker fatty rat including hyperphagia, the implications of weaning and thermogenesis.

Leptin and Leptin Receptors

The actions of leptin were first discovered through studies of *ob/ob* and *db/db* mice, which suffered hyperphagia, early onset obesity and decreased energy expenditure. It was found that parabiosis of *ob/ob* mice and wildtype mice suppressed weight gain in mutants. Alternatively, parabiosis of *db/db* mice and wildtype mice resulted in hypophagia in wildtype mice. The observation led researchers to believe that *ob/ob* mice secrete a factor *db/db* mice respond to – this factor was later named leptin.

Leptin, a product of the *ob* gene, is a hormone synthesized and excreted predominantly in white adipose tissue. Expression of leptin is proportional to energy stores and is responsible for anorectic effects. Additionally, leptin levels fluctuate in response to fasting and feeding: leptin levels rise during the fed state and fall during the fasted state. Leptin exerts its effects on energy balance through its actions on the hypothalamic region of the brain. Expression of leptin results in a reduction in food intake, increases in energy expenditure and increase in physical activity. As might be assumed, disruption of leptin's actions, as those that occur through receptor mutations, result in hyperphagia, increased adiposity, decreased energy expenditure, etc. Recent research has also found leptin to have an effect on the regulation of lipid metabolism and protection from lipotoxicity (75).

The regulation of leptin appears to be mediated by insulin – leptin expression increases after insulin levels peak following a meal. On the other hand, leptin expression decreases during periods of low insulin levels, such as while fasting. Glucocorticoids also mediate leptin's actions

through stimulation of synthesis in adipocytes. Additionally, acute infections and cytokines increase leptin expression in adipose tissue while testosterone and exposure to cold decrease leptin expression.

Leptin-sensitive neurons are found in the hypothalamus in nuclei that express neuropeptides (such as NPY, AGRP, POMC and CART) that are involved in the regulation of energy balance (76). Leptin interacts with its receptor OB-R, a member of the cytokine receptor superfamily. There are five receptor isoforms: OB-Ra, OB-Rb, OB-Rc, OB-Rd, OB-Re, OB-Rf. However the long isoform leptin receptor is believed to be the functioning receptor. OB-Rb is highly expressed in the hypothalamus. The principal signaling transduction pathway of leptin is the Jak/STAT pathway. Because OB-R do not have enzymatic domains they must bind with cytoplasmic tyrosine kinases of the janus kinase family. In brief, circulating leptin binds to the leptin receptor activating Jak kinases. Jak kinases then phosphorylate other janus kinases as well as specific tyrosine residues of the receptor (77). Phosphorylation of the tyrosine residues provides docking stations downstream for STAT proteins. STAT proteins eventually translocate to the nucleus and act as transcription factors by binding specific response elements in the promoter of their target genes.

Although OB-Rb is the functioning receptor, other receptor isoforms may still be necessary for mediating leptin's biological effects. For example OB-Ra is highly expressed in the brain and blood-brain barrier. Additionally, the fatty rat lacks OB-Ra and experiences reduced levels of leptin in the cerebrospinal fluid. As such, it has led researcher to suggest that OB-Ra may be involved in the transport of leptin across the blood brain barrier to reach the hypothalamus, thereby validating the role of the short form leptin receptor in leptin transport to the hypothalamus (58,59,78).

Hyperphagia

As discussed earlier, fatty rats develop hyperphagia resulting in severe weight gain. Several studies have focused on diet consumption, timing of the onset of hyperphagia and weight gain. Despite the efforts, the findings are largely ambiguous and contradictory in nature. For the most part, the research lacks a definitive understanding of the relationship between pathway

activities and hyperphagic onset. Moreover, researchers have yet to determine the nature of how diet composition influences hyperphagic behavior. Much of the lack of definitive findings is further complicated by the lack of generalization deriving from the use of multiple research methodologies.

As a case in point, Turkenkopf, Maggio and Greenwood found that while all fatty rats became hyperphagic, those weaned at day 16 to a standard chow diet (high in carbohydrate) experienced lower overall body weight and fat pad mass compared to those weaned to a high fat diet (79). Yet, Ramirez and Friedman found that fatty rats weight gain was associated with a diet rich in carbohydrates (80). Others suggest that a high fat diet influences the onset, duration and magnitude of hyperphagia in developing obesity (81).

With regards to onset of hyperphagia, Kowalski et al attempted to determine the period during which hyperphagia emerges. To do this, researchers examined the ingestion of preweanling rats independent of the dam. They found that hyperphagia to emerged between day 9 and 12 of life, with significant consumption of food occurring at day 12 (71). However, Truett et al found a significant increase in daily gain, stomach contents, liver weight and plasma insulin levels occurring at 23 days of age. Additionally Bazin and Lavau reported no difference in body weight between fatty rats and wildtypes at 22 days of age, but a significant difference by 30 (82).

Despite these conflicting reports, one finding appears to consistently surface -- fatty rats develop hyperphagia after the first week and before the fourth week of life. Thus, research indicates that the pathways influencing hyperphagia are not fully developed in the rat pup in the first week of life. The pathways mediating hyperphagic behavior appear to exhibit a delayed onset with manifestation occurring later in life.

Little is known about the physiological changes that occur early in development that trigger the manifestation of hyperphagia. Researchers have found that lipogenic enzyme activity of adipose tissue is significantly increased in fatty pups, prior to the onset of hyperphagia. Bazin et al found a 50% increase in the activity of adipose lipogenic enzymes, including FAS, Me1, CCE and G6PDH, in fatty rats at 9 days of age followed by a doubling of adipose mass by 17 days of age. An increase in lipogenic enzyme expression in the liver did not occur. The authors asserted

these findings are in accordance with the "pull" theory of obesity first recognized by Cleary, Vasselli and Greenwood (82,83). This theory asserts that the increase in adipocyte lipoprotein lipase activity in the early development of fatty rats results in an increase in the funneling of nutrients into the adipose tissue, leading to hyperphagia. It was concluded that the primary alteration in the fatty rat would be an increase in lipogenic enzyme activities in adipose tissue, channeling ketone bodies, glucose and amino acids into adipose tissue. Apparently, low levels of circulating hunger-suppressor substrates increases the drive to eat in fatty pups. Hyperphagia coupled with hyperinsulinemia appears to stimulate hyperlipogenesis in the liver and increased lipogenic enzyme activities in adipose tissue. However, it was also found that lipogenic enzyme activity in adipose tissue continued to increase after the onset of hyperphagia. Thus, it seems that though increased expression of FAS, CCE, Me1 and G6PDH are perhaps correlated with hyperphagia they do not cause the onset of hyperphagia.

Lastly, in a study conducted by Cheverud et al (1996), the set of genes that regulate growth during the first three weeks of life differs from the set of genes that regulate growth in later life. Thus, the timing of developmental changes are in fact regulated by different sets of genes (84). Overall, the findings tend to suggest that the various pathways that mediate hyperphagia are not completely developed within the first weeks of life. A more in depth understanding of this animal model will provide valuable information and a more thorough understanding of the specific role the pathways play in the development of hyperphagia and obesity.

Weaning

Impact of weaning on rat pups must be considered when assessing onset of hyperphagia and subsequent weight gain. During the first two weeks of life, rat pups consume dam's milk exclusively. Dam's milk is high in fat (68%) and low in carbohydrate. Additionally, suckling does not appear to be influenced by circadian rhythms, which are oftentimes important in determining the sleeping and feeding pattern in animals. After about two weeks of life, rat pups begin to consume small quantities of rat chow in addition to dam's milk. Standard chow diets are high in carbohydrate (60%) and low in fat. By the end of the fourth week of life pups are weaned and their diet is exclusive to rodent chow. Thus, the diet of rat pups changes during weaning from

high fat, low carbohydrate to low fat, high carbohydrate. It has been noted that hyperphagia occurs during the weaning transition when pups experience a marked shift in their macronutrient intake. It is believed that this shift from the high fat dam's milk to a standard high carbohydrate chow diet could contribute to the onset of hyperphagia at this age (85,86).

As discussed above, rats are dependent on dam's milk until roughly the 4th week of life. When left with the dam to wean naturally and gradually, rat pups can nurse up to 5 weeks of age. Common practice in research laboratories includes the abrupt weaning of rat pups from their dam at 3 weeks of age. The abrupt weaning transition is required to prevent consumption of dam's milk from contaminating consumption findings. The premature weaning results in both immediate and delayed effects.

With respect to the gastrointestinal tract, the increase in production of enzymes that digest carbohydrates (such as pancreatic amylase and intestinal sucrase) occurs gradually in pups left to wean on their own. However when weaned early, a large increase in these enzymes occurs instantaneously, via adrenal stimulation. Another consequence of premature weaning is the induction of stress responses. Rat pups loose a significant amount of weight when weaned prematurely, which can take up to 4 days to recover. Moreover, premature weaning during this crucial time period results is known to lead to stunted growth. Finally, there appears to be a relationship between weight loss after premature weaning and the immaturity of food intake mechanisms and digestive processes later in life (86,87). In all, the switch from a high fat diet to a high carbohydrate diet is accompanied by various developmental changes including an induction of enzymes for carbohydrate metabolism. These factors may influence the development of hyperphagia, hyperinsulinemia and lipogenesis in the fatty rat. Thus, weaning pups prematurely at three weeks of age may render current findings regarding the onset of hyperphagia suspect, if not moot.

Thermogenesis

Rat pups are dependent on their dam for maintenance of body temperature through the second week of life. The removal of the dam prematurely has been found to delay the maturation of the thermoregulatory functions of the pups (86). Additionally, it has been found that fatty rats

experience a reduction in energy expenditure before the onset of hyperphagia. Apparently, brown adipose tissue plays a role in both heat dissipation and thermogenesis. The hypothalamus, in concert with the sympathetic nervous system, controls thermogenesis of BAT in response to factors such as the environment and diet. Effective thermogenesis of BAT is associated with leanness and decreased metabolic efficiency while defective thermogenesis is associated with increased metabolic efficiency resulting in weight gain. The Zucker fatty rat experiences defective BAT in response to diet. However, environment induced thermogenesis functions normally. BAT possesses the ability to uncouple protons, thus activating the β-oxidation pathway. This results in non-shivering thermogenesis.

Preobese fatty rats often maintain a lower body temperature that lean littermates resulting in higher amounts of adipose mass accumulation. However, researches have found that when fatty rats are artificially reared in a neutral thermal environment their body-fat is indistinguishable from littermates before the onset of hyperphagia (88,89). Fatty rats, when compared to their wildtype littermates, tend to experience a reduced rate of thermogenesis, which apparently influences weight gain before the onset of hyperphagia. Hence, the diminished rate of energy expenditure may prove to be a significant contributor to the early development of obesity in the fatty rat.

CHAPTER 2. TRANSCRIPTIONAL CHANGES IN ADIPOSE TISSUE

INTRODUCTION

The Zucker fatty (*fa/fa*) rat is an animal model of obesity that has provided a means to study genetics and its role in the development of obesity (30). The fatty rat inherits a defective leptin receptor which results in impaired leptin signaling (71). Fatty rats grow at the same rate as their lean littermates for their first 22 days of age -- then suddenly begin to grow at a much higher rate. This sudden increase in growth corresponds with the emergence of robust hyperphagia rat and leads to overt obesity within days. By 24 days of age fatty rats are visibly distinguishable from their lean littermates by the distension of their bellies (74).

The fact that the onset of hyperphagia occurs at a specific age, when animals are without any environmental changes, suggests that a developmentally regulated mechanism may determine the timing of the onset of hyperphagia. For example, previous work found that fatty rats are insulin resistant prior to the onset of hyperphagia and insulin sensitive afterwards (90). The increase in insulin sensitivity may be attributable to an increase in lipogenesis, which enables glucose to be cleared from circulation. The increase in lipogenesis may in turn be related to a developmental increase in expression of PPARγ (90), a nuclear receptor that regulates the expression of lipogenic enzymes.

The rapid increase in growth of adipose tissue after the onset of hyperphagia is likely to alter the sets of gene regulating adipose tissue growth and metabolism. The objective of the project was to identify the sets of genes differentially expressed between wildtypes and fatties before and after the onset of hyperphagia. Transcripts that are differentially expressed after the onset of hyperphagia will include those that are primarily affected by reduced leptin signaling and those secondarily affected by hyperphagia and the physiological responses that accompany it, such as hyperinsulinemia. Transcripts that are differentially expressed prior to the onset of hyperphagia will include the secondary responders. Instead, this set of transcripts will include those primarily affected by reduced leptin signaling, increased energy efficiency, increased

adipose mass and adipocyte development. This analysis will identify which pathways are active under these two physiological conditions and mighty identify pathways that are critical for the development of hyperphagia.

MATERIALS AND METHODS

Experimental Design

We have previously demonstrated that hyperphagia emerges at 23 days of age in the fatty rat. An experiment was designed to generate inguinal adipose tissue samples for RNA extraction at two points in development, one shortly before the onset of hyperphagia (20 days of age) and one shortly after the onset of hyperphagia (24 days of age). Other data were collected to verify that animals were not hyperphagic at the first age, but were hyperphagic at the second age.

Animals

Animals were produced in the principal investigator's colony of inbred Zucker-*fa* rats. They were housed in plastic breeder boxes with wood shavings as bedding and *ad libitum* access to Teklad 8640 Rodent Diet and water. The light cycle was 12 hours of dark followed by 12 hours of light, room temperature was 68 to 74°F and humidity was 55 to 60%. All animal studies were approved by the University of Tennessee Institutional Animal Care and Use Committee.

Animals were produced with brother by sister matings. Sires were removed from the breeder box when the dams were visibly pregnant. The day of birth was considered 0 days of age. Neonates were marked by toe-clipping during the first few days of life, and a single toe from each pup was saved to prepare DNA by the HotSHOT method (91). Briefly, toes were collected into 200 μl thermal cycler tubes. Seventy-five microliters of 25 mM NaOH/ 0.2 mM EDTA/ pH 12 were added to the tubes. Samples were heated to 95 degrees C for 30 minutes, and then cooled to room temperature. Seventy-five microliters of 40 mM Tris HCl/ pH 5 were added to each tube to adjust the pH to 8, generating PCR quality DNA.

The *fa* mutation creates a restriction site for *Msp* I that can be used to detect *fa* genotype (92). However, *Msp* I occasionally fails to completely cut DNA, which can cause genotype misclassification. To overcome this problem, we engineered an alternative restriction site by a strategy that has been described previously (93). PCR primers were designed to amplify 101 base pair (bp) of DNA flanking the *fa* mutation. The reverse primer, RLepr, was selected to anneal adjacent to the site of the mutation, and a single base substitution (C→G) was made in the second base from the 3' end of the primer to create a Pvu II restriction site in the wildtype allele and no restriction site in the mutant allele. The primer sequences were fLepr, 5'-CGTATGGAAGTCACAGA-3', and RLepr, 5'GAATTCTCTAAATATTTCAGC-3'; the base substitution in RLepr is underlined. Primers were mixed at 500 nM each in 50mM KCl, 10mM Tris-HCl (pH 9 at 25°C), 0.1% Triton X-100, 1.5 mM $MgCl_2$, 0.2 mM each dATP, dCTP, dGTP and dTTP, 0.25 units Taq DNA polymerase and 2 µl of HotSHOT DNA in a 22 µl volume. PCR amplification was carried out in a Robocycler thermal controller (Stratagene, La Jolla, CA) at 95°C for two min, followed by 40 cycles of 95°C for 30 sec, 45°C for 45 sec and 72°C for 30 sec. The PCR product was digested with 1 unit *Pvu* II (New England Biolabs, Beverly MA) in a 30 µl volume for 1 h at 37°C. Thirty µl of *Pvu* II treated PCR product were loaded onto a 3% 3:1 high-resolution blend agarose (Amresco) gel and electrophoresed 30 minutes at 5 volts/cm. Gels were stained with ethidium bromide and exposed to UV light. The wildtype genotype produces an 80 bp band, and the mutant genotype produces a 101 bp band; both bands are visible in heterozygotes.

After genotyping, twelve litters were reduced to 8 pups per litter (96 pups) by decapitating excess heterozygotes. Pups were weighed daily from 14 days of age; daily weighing acclimates the pups to handling and reduces stress when they are killed. Body weight was recorded to the nearest 0.01g in a file linked to a Mettler PM2000 balance via BalanceLink software (VWR Scientific, West Chester, PA). In this experiment, pups remained with their dam until they were killed. It should be noted that many investigators routinely separate the pups from the dam at 21 days of age. This practice interferes with growth and creates an investigator-induced increase in the variation in data collected at this age. Sex-matched littermate wildtypes

and fatty rats were killed at 20 and 24 days of age for collection of tissue samples and heterozygotes were removed from the experiment. After removing heterozygotes, 45 subjects remained. The distribution of these subjects by age, genotype and sex is given in table 3. Stomachs, liver and inguinal adipose pads were dissected and weighed after the pups were decapitated. Stomach contents were then removed and empty stomachs were reweighed. The difference between full stomach weight and empty stomach weight was calculated to estimate the weight of the stomach contents. Inguinal adipose pads were frozen (in foil packets) in liquid nitrogen and stored at -80°C to be used for RNA extraction.

RNA Extraction

RNA was extracted from inguinal adipose tissue using modifications to the Totally RNA Total RNA Isolation kit (Ambion Inc., Austin, TX). Samples were homogenized in a guanidinium thiocyanate denaturation buffer, according to kit instructions. Then, in a departure from the kit instructions, the aqueous homogenate was extracted twice with 1 volume of chloroform to remove lipids. The remaining extraction and precipitation steps followed the kit instructions. After the RNA was precipitated with isopropanol, it was dissolved in DEPC water and precipitated with LiCl. One-half volume of 5 M LiCl was added to each sample. Samples were incubated 20 minutes at -20° C and centrifuged at 10,000 x g for 15 minutes. The supernatant was discarded and the RNA pellet was washed with 300 μl 70% ethanol. The RNA was pelleted again and then dissolved in 300 μl of nuclease free water/ 0.1 mM EDTA. Samples were stored at -80° C.

Samples of purified RNA were diluted 100-fold in 1mM Na_2HPO_4 buffer (pH 8.5) and UV absorbance was quantified spectrophotometrically at 260 and 280 nm (94). The ratio of absorbance at 260 nm to absorbance at 280 nm was calculated as an estimate of purity. The mean absorbance ratio was 1.99 ± 0.01 for these samples, which is close to the value of 2.00 expected for pure RNA. The concentration of nucleic acid was then calculated by the following formula: [RNA] μg/mL = $Absorbance_{260}$ x 40.0 μg RNA per absorbance unit x 100 (dilution). The quality of the RNA was evaluated by agarose gel electrophoresis. RNA samples were prepared

Table 3. Distribution of subjects by age, genotype and sex.

days of age	genotype	female	male
20	+/+	5	5
	fa/fa	5	5
24	+/+	6	7
	fa/fa	6	6

and enough DEPC water to make a final volume of 20 μl. The RNA was loaded on a 1% agarose gel prepared with 0.2 M MOPS (pH 7.0), 50 mM sodium acetate, 10 mM EDTA, 0.22 M formaldehyde (added after the agarose is melted) and 0.5 μg/μl ethidium bromide. The same buffer was used as the electrophoresis buffer and the gel was electrophoresed at 75 volts for 3 hours. Clear 28S and 18S ribosomal RNA bands indicated that the RNA was not degraded or contaminated with excess salts (figure 2). RNA was stored at -80°C for use later in microarray analysis and quantitative real-time RT-PCR.

After isolation and assurance of intactness, four pools of RNA were prepared by mixing 5 μg of RNA from 5 males in each genotype by age group: wildtypes at 20 days of age, fatty rats at 20 days of age, wildtypes at 24 days of age and fatty rats at 24 days of age. RNA samples were shipped on dry ice to Genome Explorations (Memphis, TN) for Affymetrix GeneChip hybridization and quantification. U34A Rat Expression Arrays were used for hybridization. Because the expression data was based on pooled RNA samples with no replication, no statistical analysis of the data is possible. However, the magnitude of the gene expression changes are large enough that 2-fold changes in gene expression are likely to represent real changes. Thus, transcripts that were differentially expressed a minimum of 2-fold by genotype within age or 2-fold by age within genotype were identified for further investigation. Of the transcripts identified, specific transcripts of interest were selected for their involvement as key regulatory steps in pathways of lipogenesis and cholesterol synthesis. Primers and probe sets were ordered for selected transcripts through Applied Biosystems Assays by Demand.

Real Time Quantitative RT-PCR

The results of the GeneChip analysis were validated by real time RT-PCR of a selection of transcripts including: farnesyl diphosphate synthase, stearoyl CoA desaturase 1, malic enzyme, transketolase, lipoprotein lipase, leptin, insulin induced gene 1, glycerol 3-phosphate acyltransferase and fatty acid synthase. Peroxisome proliferator activated-receptor γ was not present on the Genechip, but was chosen for RT-PCR because it is an important transcription

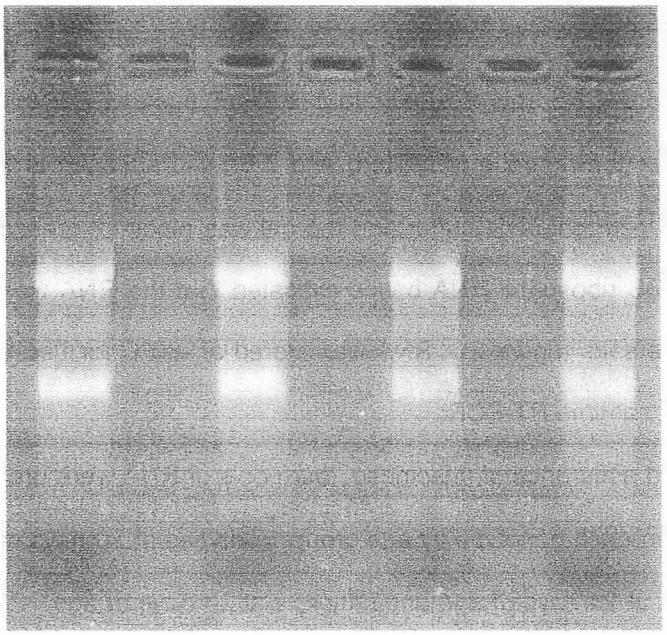


Figure 2. Example of 28S and 18S ribosomal RNA. RNA was run on 1% denaturing agarose gel. The clear bands demonstrate that RNA is not degraded or contaminated with excess salts. All RNA samples were evaluated by this method to ensure quality.

factor in lipogenesis. Reverse transcription polymerase chain reaction allows RNA to be converted into DNA and to be amplified by PCR. This is a sensitive method for detection of specific transcripts, particularly of RNA in low abundance or in small sample amount. The first stage of reverse transcription PCR is mediated by a reverse transcriptase that converts RNA into its complementary single-stranded DNA, called first strand synthesis. Using the first strand as a template, the first strand of DNA is converted into a double strand by synthesis of the second strand.

In the final stage, cDNA undergoes exponential amplification in the PCR reaction. Protocols and instruments to monitor the accumulation of PCR product continuously (real time RT-PCR) have made the techniques highly quantitative. A dual-labeled fluorescent probe, in this case a Taqman probe, forms the basis of the detection system (figure 3). At the 5' end of the Taqman probe is the reporter dye and at the 3' end is the quencher dye. The intact probe hybridizes to the target amplicon downstream from the primer. At this point the reporter and quencher dyes are in close enough proximity that the reporter emission is quenched. However, during the extension phase of the PCR cycle, the DNA polymerase with 5 ' to 3 ' nuclease activity degrades the probe, releasing the reporter dye and the quencher from the DNA strand. Because they are no longer close enough for the reporter to be quenched, the amount of fluorescence in solution increases as the PCR product accumulates. It is the fluorescence emission of the reporter that is monitored continuously during RT-PCR amplification. The first cycle in which a significant increase in fluorescence above the background, or fluorescent signal derived from unbound or non-specific cleavage of probe, occurs is termed the cycle threshold (figure 4). C_T is proportional to the log of the concentration of the RNA, so simple linear regression between C_T and the log of a series of dilutions of an RNA pool generate a straight line that can be used to estimate the relative concentration of RNA in unknown samples (figure 5).

Core reagents for RT-PCR, including 25 mM $MgCl_2$, 10 mM dATP, 10 mM dCTP, 10 mM dGTP, 10 mM dTTP, MuLV-RT and Rnase inhibitor were obtained from GeneAmp RNA PCR kits

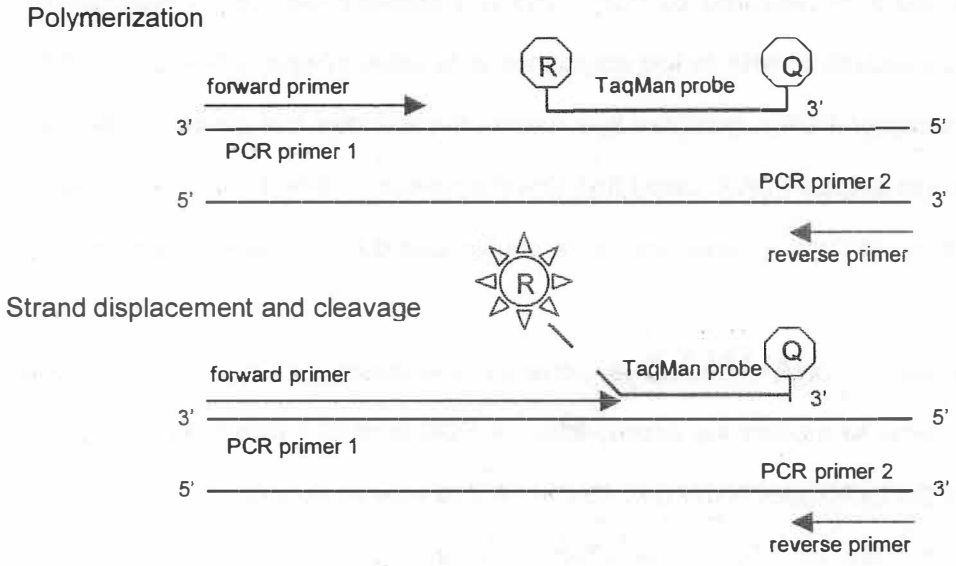


Figure 3. Depiction of reverse transcription PCR. PCR primers and a Taqman probe, containing a reporter dye (R) at the 5' end and a quencher dye (Q) at the 3' end, bind to the DNA template. The Taq DNA polymerase allows the primer to extend eventually cleaving the Taqman probe. In cleaving the probe, the reporter dye is cleaved from the quencher dye by the DNA polymerase resulting in the emission of fluorescence by the reporter. The accumulation of PCR products is monitored by the increasing fluorescence of the reporter dye.

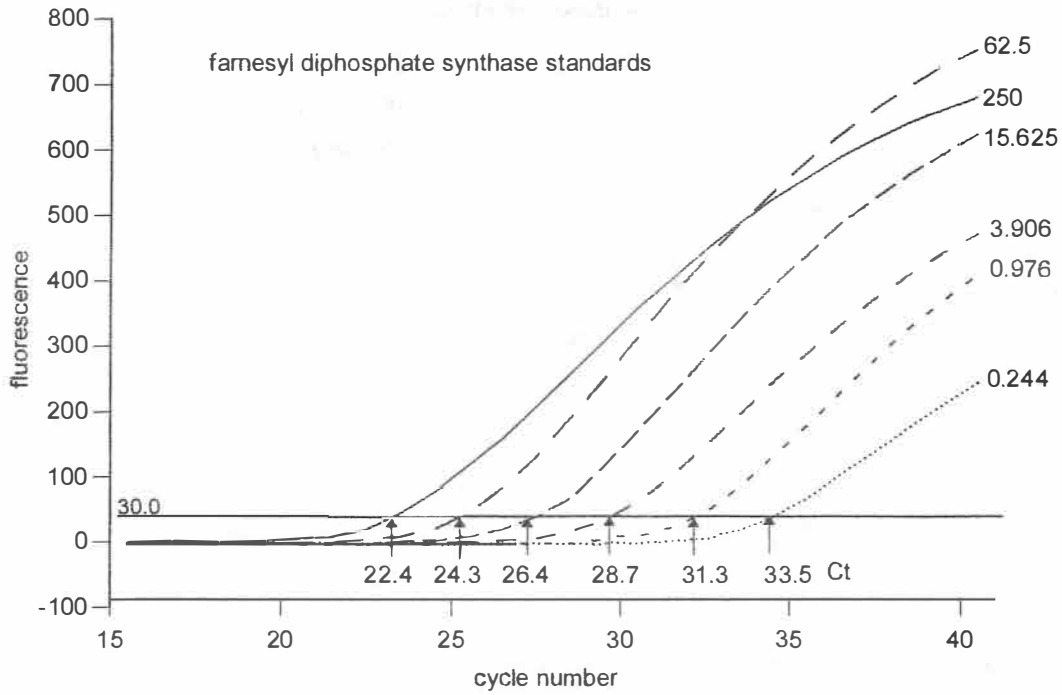


Figure 4. Threshold cycles for standard curve of farnesyl diphosphate synthase. A serial dilution was performed generating 6 RNA concentrations from which the dilution curve was developed. The concentrations of each of the six points of the standard curve were: 250, 62.5, 15.625, 3.906, 0.976, 0.244 ng/μl.

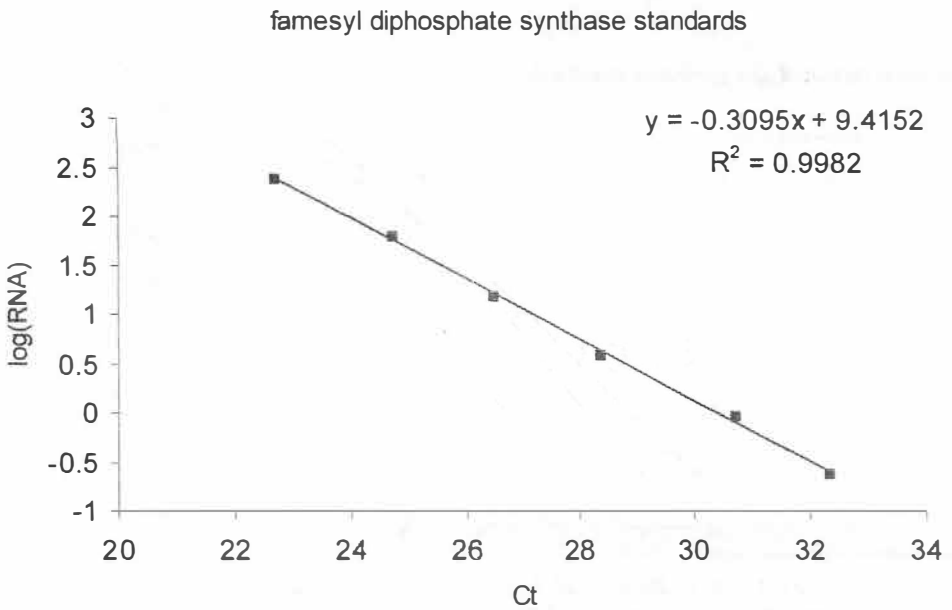


Figure 5. Standard curve for farnesyl diphosphate synthase. A serial dilution was performed generating 6 RNA concentrations from which the dilution curve was developed. The concentrations of each of the six points of the standard curve were: 250, 62.5, 15.625, 3.906, 0.976, 0.244 ng/μl.

(Applied Biosystems, Foster City, CA). AmpliTaq Gold and 10X Taqman buffer A were obtained from a Taqman 1000 reaction Gold/ Buffer A Pack purchased from Applied Biosystems. Assays on Demand primers and probes for most assays were obtained from Applied Biosystems (table 4). Primers and probes for PPARγ, FAS and 18S RNA were synthesized commercially (table 5).

For each set of RT-PCR reactions, a master mix was prepared by mixing 7.35 μL DEPC water, 2.5 μL 10X Taqman buffer A, 5.5 μL of 25 mM $MgCl_2$, 0.75 μL of each 10 mM dNTP, 0.125 μL of 50U/μL MuLVRT, 0.125 μL of 20U/μL RNase inhibitor, 0.15 μL of 50U/μL AmpliTaq Gold and 1.25 μL of primer and probes mix to make a 20 μL volume. A standard dilution curve was established by performing a serial dilution of pooled RNA resulting in 6 concentrations of RNA at 50, 12.5, 3.125, 0.781, 0.195 and 0.049 ng/μL. Five μL of standard and unknown RNAs diluted to 2.5 ng/μL were added to separate tubes for a final volume of 25 μL. Samples were centrifuged and loaded into a Smart Cycler Real Time PCR System (Cepheid, Sunnyvale, CA). Amplification was performed in a Smart Cycler Real Time PCR System (Cepheid, Sunnyvale, CA). The reactions was performed using a 3-stage cycling protocol: stage 1 at 48.0° C for 30 minutes, stage 2 at 95.0°C for 10 minutes, and stage 3 which consisted of 50 two-temperature cycles, 95°C for 15 seconds followed by 60°C for 60 seconds. Reverse transcription occurs in stage 1, inactivation of reverse transcriptase and denaturation occurs in stage 2 and denaturation, annealing and extension occurs during stage 3.

After completion of the RT-PCR reaction, linear regression was performed with C_T values for the standards and the log of the RNA amount in the given tube was determined. This regression line was then used to estimate the relative expression of the unknowns based on their C_T values. The units are arbitrary. To adjust for variation in starting RNA amount, the estimates were divided by 18S RNA expression levels for the same sample.

Data Analysis

A control group pre-test/post-test design was used to examine the research questions. Twenty- two wildtype rats served as the control group and the test group was comprised of 23

Table 4. Applied Biosystems Assays by Demand primers and probes.

transcript name and symbol	affymetrix assay ID
malic enzyme (Me1)	Rn00561502_m1
transketolase (Tkt)	Rn00574981_m1
glycerol 3-phosphate acyltransferase, mitochondrial (Gpam)	Rn00568620_m1
farnesyl diphosphate synthase (Fdps)	Rn00821389 _g1
insulin induced gene 1 (Insig1)	Rn00574380_m1
leptin (Lep)	Rn00565158_m1
lipoprotein lipase (Lpl)	Rn00561482_m1
stearoyl CoA desaturase 1 (Scd1)	Rn00594894_g1

Table 5. RT-PCR primer and probe concentrations and compositions.

oligo	concentration	sequence
FAS for	200 nM	CCCAGAGGCTTGTGCTGACT
FAS rev	400 nM	CGAATGTGCTTGGCTTGGT
FAS probe	100 nM	TET d (CCGATCTGGAATCCGCACCGG) TAMRA
PPAR for	200 nM	TGCGAGTGGTCTTCCATCAC
PPAR rev	400 nM	GCCTTGAGCACTTCACAAGAAATT
PPAR probe	100 nM	CAL RED d (TTTGCCAAGCTGCTCCAGAAAATGA) BHQ-2
18S for	200 nM	AGTCCCTGCCCTTTGTACACA
18S rev	400 nM	GATCCGAGGGCCTCACTAAAC
18S probe	100 nM	6-FAM d (CGCCCGTCGCTACTACCGATTGG) BHQ-1

fatty rats. The findings are based on data derived from both groups at 20 and 24 days of age. If hyperphagia is developmentally related, differences between the two genotypes should be exhibited within the four-day period. Independent-samples t-tests were employed to analyze the difference of means between the two genotypes on inguinal fat pad mass, body weight, stomach mass, and stomach content and 20 and 24 days of age.

Additionally, SAS's "proc mixed" routine was used to control for the effects of litter and to determine the presence of interactive effects. "Proc mixed" was designed to model both random and mixed effect data. The procedure is built on the analysis of variance (ANOVA) statistical routine, and returns F values and probability values for each model. It also provides means and standard errors for each fixed effect factor. For the models discussed below, transcript expression level, inguinal fat pad mass, body weight, stomach mass, and stomach content served as the dependent variables of interest. Genotype, age and sex were treated as fixed effects factors and litter was defined as a random effects factor (figure 6).

RESULTS

Development of Hyperphagia

It has previously been demonstrated that the fatty rat grows at the same rate as their littermates for the first 22 days of age, and then begins to grow at a higher rate because of the sudden onset of hyperphagia (74). This pattern was repeated in this experimental population. Body weights were affected by genotype, age, genotype by age interaction and by sex (table 6A). The effect of sex is quantitative and not qualitative, the pattern of change is similar in males and females. Because we are not particularly interested in the sex effects unless they are qualitatively different between the sexes, the graphs present the sex-averaged means for wildtypes at 20 and 24 days of age and fatty rats at 20 and 24 days of age (figure 7).

The body weights of wildtypes and fatty rats were similar at 20 days of age, but by 24 days of age fatty rats were heavier than wildtypes (figure 7). Daily gain was affected by age and

```
options linesize = 72; options pagesize = 20000;
  options nodate; options nonumber; run;
  data one;
infile 'i:/Mary/switchdata.txt' FIRSTOBS=2;
input id lit age geno sex $ body gain liver ing stom MT contents r18s fas pparg Scd1
Me1 Lep Gpam Tkt Fdps Insig1 Lpl;
run;
%macro mixed (yvar);
proc mixed;
  class id lit geno age sex;
  model &yvar=geno|age|sex;
  random lit;
  lsmeans geno*age /slice=age slice=geno;
run;
%mend mixed;
%mixed (body);%mixed (gain);%mixed (liver);%mixed (ing);%mixed (stom);
%mixed (MT); %mixed (contents); %mixed (r18s);
%mixed (fas);%mixed (pparg);%mixed (Scd1);%mixed (Me1); %mixed (Lep);
%mixed (Gpam); %mixed (Tkt); %mixed (Fdps); %mixed (Insig1); %mixed (Lpl);
```

Figure 6. SAS code used for statistical analysis of all data.

Table 6A. Type 3 tests of fixed effects for body weight.

effect	num DF	den DF	F Value	Pr > F
geno	1	27	7.41	0.0112
age	1	27	99.79	<.0001
geno*age	1	27	14.06	0.0009
sex	1	27	11.94	0.0018
geno*sex	1	27	0.17	0.6818
age*sex	1	27	0.15	0.7026
geno*age*sex	1	27	0.91	0.3489

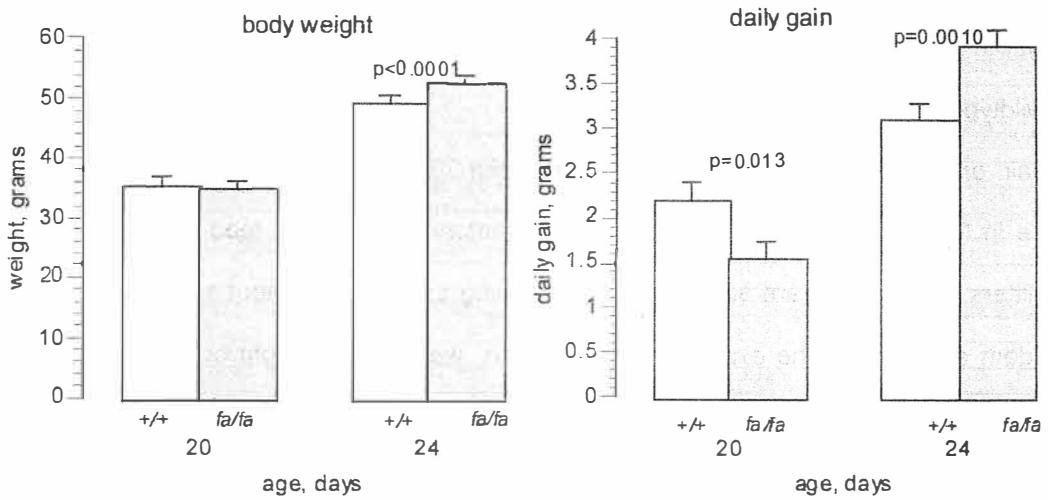


Figure 7. Body weights and daily gain of wildtype and fatty rats at 20 and 24 days of age. Body weights of fatty rats and wildtypes were similar at 20 days of age. By 24 days, fatty rats were significantly heavier than wildtypes ($p<0.0001$). Daily gain of wildtypes at 20 days of age was significantly higher than fatty rats ($p=0.013$), but fatty rats gained significantly more than wildtypes at 24 days of age ($p=0.0010$).

genotype by age interaction (table 6B). The interaction between age and genotype is such that daily gain is lower in fatty rats compared to wildtypes at 20 days of age, but daily gain is greater in fatty rats than wildtypes by 24 days of age (figure 7).

Daily gain of the fatty rat more than doubles between 20 and 24 days of age suggesting that an increase in food intake could be occurring in this interval. Because food intake is not measurable in litters of pups that are suckling and consuming solid food, without separating the pups from the dam and altering the experimental conditions, we use the weight of the stomach contents as a proxy estimator of food intake at these ages. The genotype by age effect is the major influence on stomach weight (table 7A) and weight of the stomach contents (table 7B). In contrast, the weight of the stomach itself is affected only by age (table 7C). These differences reflect the fact that full stomach weight and stomach contents are similar between wildtypes and fatty rats at 20 days of age, but are increased in fatty rats compared to wildtypes at 24 days of age. Furthermore, the weight of the empty stomach is similar between wildtypes and fatty rats at both 20 and 24 days of age. Thus, the increase in stomach weight is accounted for by an increase in stomach contents and not by an increase in the stomach tissue proper (figure 8). These data suggest that fatty rats are not hyperphagic at 20 days of age, but are hyperphagia by 24 days of age.

The genotype by age effect is also the major influence on liver weight (table 8A) because the liver weights are similar between wildtypes and fatty rats at 20 days of age but greater in fatty rats at 24 days of age (figure 9). The increase in liver weight is another indicator that fatty rats are hyperphagic at this age. Finally, genotype is the major influence on inguinal adipose pads weight (table 8B). This is consistent with the observation that fatty rats accumulate excess fat mass before the emergence of hyperphagia through reduced energy expenditure. Age is also an important influence on inguinal adipose mass and the genotype by age effect is relatively small (table 8B). These effects indicate that the fatty rat has much greater fat mass even before the onset of hyperphagia, fat mass continues to expand in both genotypes at this age, and there is an increase in the rate of gain of adipose mass in fatty rats compared to wildtypes in this interval (figure 9).

Table 6B. Type 3 tests of fixed effects for daily gain.

effect	num DF	den DF	F Value	Pr > F
geno	1	27	0.23	0.6345
age	1	27	68.31	<.0001
geno*age	1	27	19.78	0.0001
sex	1	27	2.26	0.1441
geno*sex	1	27	0.05	0.8201
age*sex	1	27	0.02	0.8924
geno*age*sex	1	27	0.05	0.8196

Table 7A. Type 3 tests of fixed effects for stomach weight.

effect	num DF	den DF	F Value	Pr > F
geno	1	27	10.77	0.0029
age	1	27	11.06	0.0026
geno*age	1	27	29.53	<.0001
sex	1	27	0.68	0.4173
geno*sex	1	27	0.25	0.6224
age*sex	1	27	0.01	0.9241
geno*age*sex	1	27	0.01	0.9409

Table 7B. Type 3 tests of fixed effects for stomach contents.

effect	num DF	den DF	F Value	Pr > F
geno	1	27	9.29	0.0051
age	1	27	7.22	0.0122
geno*age	1	27	24.77	<.0001
sex	1	27	0.74	0.3986
geno*sex	1	27	0.05	0.8255
age*sex	1	27	0.01	0.9116
geno*age*sex	1	27	9.29	0.0051

Table 7C. Type 3 tests of fixed effects for empty stomach.

effect	num DF	den DF	F Value	Pr > F
geno	1	27	0.05	0.8220
age	1	27	27.66	<.0001
geno*age	1	27	0.71	0.4057
sex	1	27	0	0.9561
geno*sex	1	27	1.73	0.1993
age*sex	1	27	0	0.9854
geno*age*sex	1	27	1.2	0.2824

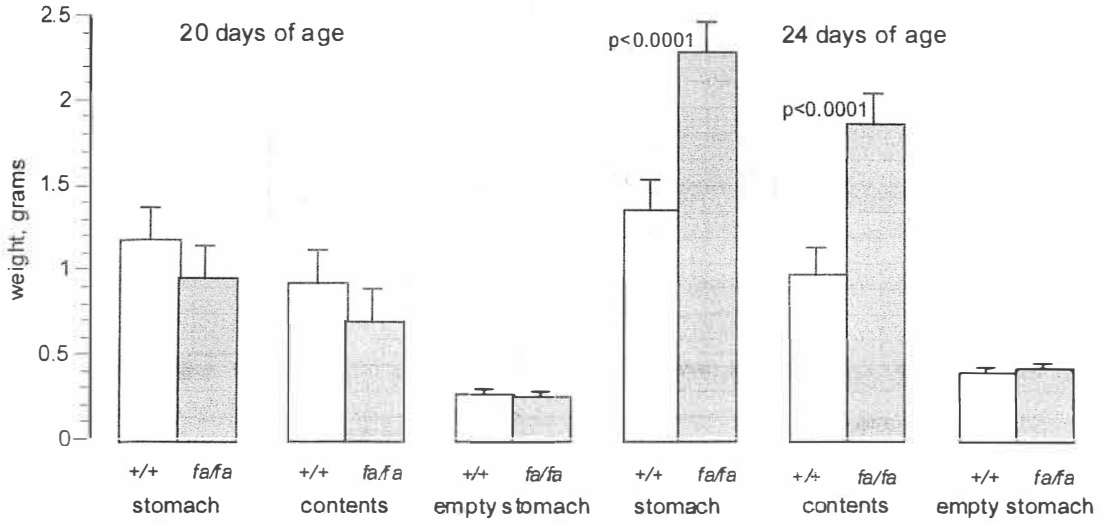


Figure 8. Stomach, stomach contents and empty stomach weights of wildtype and fatty rats. Stomach, stomach contents and empty stomach weights are similar between wildtypes and fatty rats at 20 days of age. By 24 days, fatty rats stomach contents were significantly heavier than wildtypes ($p<0.0001$) while empty stomach weights are the same. The increased stomach weight fatty rats experience at 24 days of age is a result of increased stomach contents compared to wildtypes ($p<0.0001$). These data suggest that the onset of hyperphagia occurs in the fatty rat after 20 days of age but before 24 days of age.

Table 8A. Type 3 tests of fixed effects for liver weight.

effect	num DF	den DF	F Value	Pr > F
geno	1	27	17.37	0.0003
age	1	27	76.98	<.0001
geno*age	1	27	15.51	0.0005
sex	1	27	13.93	0.0009
geno*sex	1	27	2.03	0.1653
age*sex	1	27	0	0.9746
geno*age*sex	1	27	0.24	0.6259

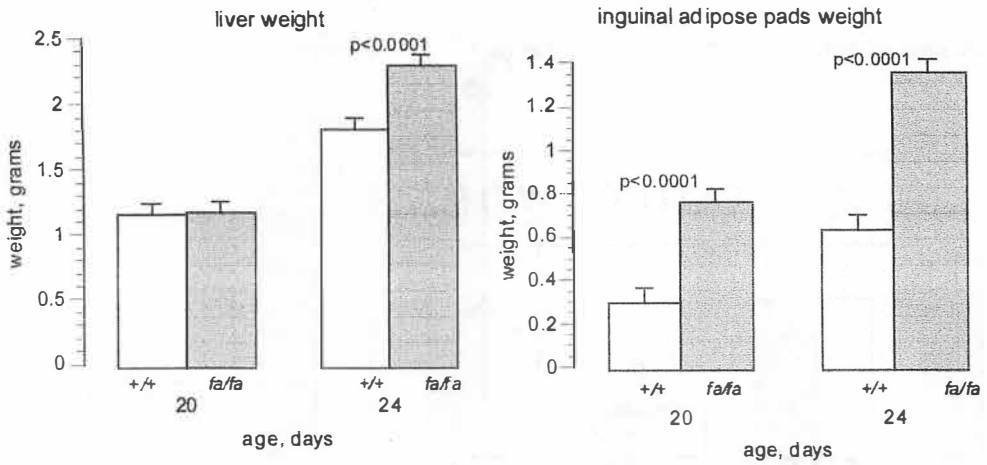


Figure 9. Liver and inguinal adipose pads weight of wildtype and fatty rats at 20 and 24 days of age. Liver weight are similar in rats at 20 days of age, however by 24 days of age fatty rats livers weight significantly more than wildtypes ($p<0.0001$). Fatty inguinal adipose pads weighed significantly more at both 20 days of age and 24 days of age ($p<0.0001$).

Table 8B. Type 3 tests of fixed effects for inguinal adipose pads weight.

effect	num DF	Den DF	F Value	Pr > F
geno	1	27	283.53	<.0001
age	1	27	34.19	<.0001
geno*age	1	27	12.6	0.0014
sex	1	27	0.53	0.4725
geno*sex	1	27	0.58	0.4530
age*sex	1	27	0.08	0.7740
geno*age*sex	1	27	0	0.9830

Transcriptional Profiling

The onset of hyperphagia might be expected to be associated with changes in pathways in adipose tissue of fatty rats. Pathways that are differentially expressed in fatty rats before the onset of hyperphagia are likely to be more closely related to leptin signaling than those that are differentially expressed after the emergence of hyperphagia and hyperinsulinemia. To identify which pathways are differentially expressed prior to the onset of hyperphagia and after the onset of hyperphagia, transcriptional profiling was performed using Affymetrix Genechips on inguinal adipose tissue in male wildtype and fatty rats at 20 and 24 days of age.

At 20 days of age, before the onset of hyperphagia, 52 transcripts were downregulated by -2-fold or more in fatty rats and 25 transcripts were upregulated by 2-fold or more in fatty rats (table 9). Fifteen transcripts that were differentially expressed were involved in the immune system or inflammation, consistent with the known role of leptin signaling in regulation of the immune system. Eleven of these were downregulated in fatty rats and 4 were upregulated. Another 13 transcripts related with the cytoskeleton were all downregulated in fatty rats. These and many other downregulated transcripts (β-enolase 3, calcium ATPase, creatine kinase, etc.) are likely to be involved with muscle cell phenotype during differentiation. Leptin, as expected, was upregulated 2.8 fold in fatty rats already at 20 days of age. The transcripts that had the greatest levels of upregulation were stearoyl CoA desaturase 1 (6.5-fold), stearoyl CoA desaturase 2 (6.5-fold) and natriuretic factor receptor C (9.2-fold). Natriuretic factor receptor 3 is involved in the regulation of blood pressure, and might be directly regulated by leptin signaling. Stearoyl CoA desaturases are principally considered to be responsible for the conversion of saturated fatty acids to monounsaturated fatty acids, and can be considered to be components of lipogenic pathways. However, no other lipogenic enzymes are upregulated at 20 days of age in fatty rats, suggesting alternative roles for Scd1 at this age. By 24 days of age, 27 transcripts are downregulated in fatty rats and 49 transcripts are upregulated (table 10). Prominent among the upregulated transcripts are enzymes involved in growth, glucose metabolism, lipogenesis, fatty acid oxidation and insulin signaling. When the lists of differentially expressed transcripts at 20 and 24 days of age are compared, 10 transcripts are differentially expressed at both ages. These

Table 9. Genotype effects on inguinal adipose tissue transcripts at 20 days of age.

Probeset	*	Transcript	D	I	Pathway
X53054_at		MHC RT1 class II E-β chain	-4.6		immune system
U39609_s_at	1	immunoglobulin κ light chain variable	-4.3		immune system
M64795_f_at		MHC class I antigen gene (RT1-u haplotype)	-3.7		immune system
X14254cds_g_at		MHC class II-associated invariant chain	-2.5		immune system
U75411cds_g_at		anti-idiotype immunoglobulin M light chain gene	-2.1		immune system
M18528cds_f_at	2	Ig germline κ-chain C-region gene	-2.1		immune system
M15562_g_at		MHC class II RT1.u-D-α chain	-2.1		immune system
X13044_g_at		MHC-associated invariant chain gamma	-2.1		immune system
S79711_g_at	3	CD3 γ-chain	-2.0		immune system
M57276_at		leukocyte antigen MRC-OX44	-2.0		immune system
X14319cds_g_at	4	T-cell receptor β chain	-2.0		immune system
rc_AI176307_at		T-cell receptor γ chain		2.5	immune system
M23889_g_at		T-cell receptor β-chain mRNA V-region		3.0	immune system
Z22812_at		interleukin-1 receptor type 2		3.0	immune system
AJ132230_g_at		B1 bradykinin receptor		2.3	inflammation
rc_H33461_at		oxidation resistance 1	-5.7		oxidative stress response
rc_AA894029_at		cytochrome b-245 (Cybb)	-2.0		oxidative stress response; angiogenesis
X60328_g_at		cytosolic epoxide hydrolase	-5.7		xenobiotic metabolism
M74439mRNA_i_at		UDP glucuronosyltransferase		3.0	xenobiotic metabolism
M15474cds_s_at		α-tropomyosin	-2.1		cytoskeleton
M34134_s_at		brain α-tropomyosin	-2.0		cytoskeleton
X02412_at		striated muscle α-tropomyosin	-3.5		cytoskeleton
rc_AA925473_g_at		cell division cycle 42 homolog	-2.0		cytoskeleton
L00088expanded_cds#2_at		fast myosin alkali light chain	-4.3		cytoskeleton
X00975_g_at		muscle myosin light chain 2	-4.0		cytoskeleton
L24897_s_at		myosin heavy chain	-4.3		cytoskeleton

continued next page

Table 9 Continued.

Probeset	*	Transcript	D	I	Pathway
rc_AI639096_at		similar to myosin binding protein C	-4.3		cytoskeleton
rc_AI639532_at		similar to troponin C	-4.6		cytoskeleton
J00692_at		skeletal muscle α-actin gene	-2.8		cytoskeleton
rc_AI231821_at		stathmin 1	-2.0		cytoskeleton; differentiation
rc_AA851223_at		β-enolase 3, muscle	-16.0		glycolysis and gluconeogenesis
rc_AI175539_at		parvalbumin	-2.5		calcium binding
M99223_at		calcium transporting ATPase	-4.0		calcium transport
X99326_at		striatin		2.5	calmodulin binding
M64986_g_at		high-mobility group box 1	-2.6		DNA binding protein; muscle development
M10140_at		skeletal muscle creatine kinase	-2.5		ATP buffer
L08100_at		glycam 1	-2.3		cell adhesion
M84488_at		vascular cell adhesion molecule-1	-3.7		cell adhesion
X76985_at		latexin	-3.2		protease inhibitor; inflammation
D00753_at		contrapsin-like protease inhibitor related protein		3.0	protease inhibitor; regulated by GH
A03913cds_s_at		glia-derived neurite-promoting factor		2.5	protease inhibitor
L13445_at		sialyltransferase gene family related		3.7	glycosylation of proteins
U00964_at		common salivary protein 1		2.3	secreted protein
X80395cds_s_at		vesicular acetylcholine transporter		2.3	acetylcholine transport
AB017655_s_at		muscarinic receptor m2		3.5	acetylcholine receptor
rc_AA874794_at		nerve growth factor receptor associated protein 1	-3.0		cholinergic neuron growth factor
AF091573_f_at		isolate HGL-SL2 olfactory receptor		3.2	olfactory receptor
rc_AA893690_g_at		EST197493	-2.1		unknown
rc_AI639422_at		moderately similarity to protein NICE-1 protein	-2.1		unknown
rc_AI638986_s_at		Rat mixed-tissue library cDNA clone rx00108	-11.3		unknown
rc_AA891651_g_at		similar to NADH-ubiquinone oxidoreductase	-2.3		unknown
L35558_s_at		neuronal glutamate/aspartate transporter		2.6	amino acid transporter

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Table 9 Continued.

Probeset	*	Transcript	D	I	Pathway
S69160_s_at		thyrotropin releasing hormone receptor		2.6	TRH receptor
S79624_at	5	natriuretic peptide receptor subt *		9.2	natriuretic receptor
rc_AI012275_at		developmentally regulated protein rpb1	-3.0		polyamine transporter
D84418_s_at		chromosomal protein HMG2	-2.1		transcription
AF055286_g_at		organic cation transporter 3; Oct3	-5.3		transcription
X60769mRNA_at		sfb mRNA for silencer factor B	-2.0		transcription; inhibits GST expression
M83679_at		RAB15, member RAS onocogene family		2.8	transcription; protein transport
J03190_g_at		5-aminolevulinate synthase	-3.2		heme synthesis
L32601_s_at		20 α-hydroxysteroid dehydrogenase		2.8	progesterone metabolism
D28560_g_at		phosphodiesterase I		2.8	phosphodiesterase; adenosine synthesis
D14015_g_at		cyclin E		2.3	kinase, cell cycle
X95986mRNA#1_f_at		carbonyl reductase 1	-2.8		NADPH-dependent oxidoreductases
D14046_at		DNA topoisomerase IIB		2.1	DNA topological change
X04139_s_at		protein kinase C	-5.7		cell signaling; growth
rc_AA800315_at		similar to PxF protein (LOC289233)	-3.7		peroxisome biogenesis
J05592_g_at		protein phosphatase inhibitor-1 protein	-2.0		cell signaling; glycogen metabolism
S63521_i_at	6	glucose-regulated protein GRP78	-7.0		protein trafficking; regulated by glucose
X13411cds_s_at		elk protein		3.0	tyrosine kinase; insulin signaling
M64301_at		mitogen activated protein kinase 6 (MAPK6)	-2.3		cell signaling; cell cycle
M81183Exon_UTR_g_at	7	insulin-like growth factor I	-2.5		growth regulation
D45862_g_at	8	leptin		2.8	lipid metabolism; immune system
rc_AI175764_s_at	9	stearoyl-Co A desaturase 1		6.5	lipogenesis; fatty acid desaturation
AF036761_g_at	10	stearoyl-CoA desaturase 2		6.5	lipogenesis; fatty acid desaturation
Number affected			52	25	

* Transcripts that are differentially expressed by genotype at 20 and 24 days of age are marked with these numbers in the two tables.
D, fold decrease in fatty rats compared to wildtypes. I, fold increase in fatty rats compared to wildtypes.

Table 10. Genotype effects on inguinal adipose tissue transcripts at 24 days of age.

Probeset	*	Transcript	D	I	Pathway
X07189cds_at		Ig ε chain constant region	-3.2		immune system
U39609_s_at	1	immunoglobulin κ light chain variable	-3.0		immune system
M18528cds_f_at	2	Ig germline κ-chain C-region gene	-2.6		immune system
X07551cds_s_at		B-alpha gene for class II antigen	-2.1		immune system
S79711_g_at	3	CD3 γ-chain	-2.1		immune system
X14319cds_g_at	4	T-cell receptor β chain	-2.1		immune system
U75411cds_g_at		anti-idiotype immunoglobulin M light chain	-2.0		immune system
U86379_at		anaphylatoxin C3a receptor		4.0	immune system
rc_AI171243_at		similar to replication protein A3	-2.3		DNA replication
U75689_s_at		DNase γ	-7.0		apoptosis
S61865_s_at		syndecan	-2.0		cell adhesion; apoptosis
rc_AI011706_at		similar to splicing factor SRP20	-2.1		mRNA synthesis
Y17295cds_s_at		peroxiredoxin 6		2.0	antioxidant
M84719_at		flavin-containing monooxygenase 1	-2.1		xenobiotic metabolism
S72505_f_at		glutathione S-transferase Yc1 subunit		2.1	xenobiotic metabolism
U36895_at		vomeronasal receptor 3		4.9	vomeronasal receptor
AF034899_i_at		olfactory receptor gene Olr1699		4.0	olfactory receptor
S79624_at	5	natriuretic peptide receptor subtype C *		2.8	natriuretic receptor
rc_AI072634_at		keratin complex 1, acidic, gene 18	-2.5		cytoskeleton
rc_AA892248_g_at		EST196051	-2.5		cytoskeleton
rc_AA893032_at		similarity to β-galactosidase (lactase)		2.0	lactase
J00711_at		β-casein		2.1	calcium binding
rc_AI010725_g_at		calnexin	-2.3		calcium binding, protein trafficking
S63521_i_at	6	glucose-regulated protein GRP78	-2.0		calcium binding, protein trafficking
AJ001044cds_at		EGP-314 protein homologue	-2.1		calcium signal transducer
L13445_at		sialyltransferase gene family related mRNA		2.0	glycosylation of proteins

continued next page

Table 10 Continued.

Probeset	*	Transcript	D	I	Pathway
U50717_s_at		synaptic density protein PSD-93		4.3	component of synapses
U07971_at		L-arginine-glycine amidinotransferase		3.2	ATP buffer; creatine synthesis
M59460mRNA#2_at		liver glycogen phosphorylase		2.0	glycogen metabolism
S56464mRNA_g_at		hexokinase II		2.1	glycolysis
rc_AA924326_s_at		aldolase A		2.3	glycolysis and gluconeogenesis
D00092_s_at		pyruvate dehydrogenase		2.3	glycolysis and gluconeogenesis
U32314_at		pyruvate carboxylase		2.0	glycolysis and gluconeogenesis
L22294_at		pyruvate dehydrogenase kinase		2.1	glycolysis and gluconeogenesis
M81183Exon_UTR_g_at	7	insulin-like growth factor I	-2.3		growth regulation
J04486_at		insulin growth factor-binding protein		-4.3	growth regulation
A09811cds_s_at		IGF binding protein 2	-5.7		growth regulation
S49003_s_at		short isoform growth hormone receptor		2.0	growth regulation
Z83757mRNA_at		growth hormone receptor		2.0	growth regulation
S56481_s_at		β_3 adrenergic receptor	-2.0		lipolytic regulator
rc_AI639525_at		adiponutrin		4.6	adipocyte specific lipase
rc_AA893242_g_at		fatty acid CoA ligase, long chain 2		2.3	fatty acid oxidation
D43623_g_at		carnitine palmitoyltransferase I like protein		2.0	fatty acid oxidation
D45862_g_at	8	leptin		2.5	lipid metabolism; immune system
L27075_g_at		citrate lyase		3.0	lipogenesis
J03808_at		acetyl CoA carboxylase		4.6	lipogenesis,
X13527cds_s_at		acyl carrier protein domain of fatty acid synthase		3.2	lipogenesis;
M76767_s_at		fatty acid synthase		2.5	lipogenesis; fatty acid synthesis
M26594_at		malic enzyme		8.6	lipogenesis; NADPH production
rc_AI175764_s_at	9	stearoyl-Co A desaturase 1		3.2	lipogenesis; fatty acid desaturation
AF036761_g_at	10	stearoyl-CoA desaturase 2		4.0	lipogenesis; fatty acid desaturation
AB002558_at		glycerol 3-phosphate dehydrogenase		2.1	lipogenesis; glycerol 3-phosphate synthesis
U36773_g_at		glycerol-3-phosphate acyltransferase		2.6	lipogenesis; triglyceride synthesis

continued next page

Table 10 Continued.

Probeset	*	Transcript	D	I	Pathway
rc_AI180442_at		farnesyl diphosphate synthase		2.3	lipogenesis; cholesterol synthesis
rc_AI236145_at		hydroxysteroid dehydrogenase 17 beta, type 7		5.3	lipogenesis; cholesterol synthesis
rc_AI059508_s_at		transketolase		2.6	pentose phosphate pathway
rc_AA875025_at		cellular retinoic acid binding protein I	-3.2		retinol binding; adipocyte differentiation
K03045cds_r_at		retinol-binding protein gene		2.6	retinol binding; adipocyte differentiation
AJ223355_at		mitochondrial dicarboxylate carrier		2.0	adipocyte development
K01934mRNA#2_at		hepatic product spot 14		2.0	transcription of lipogenic genes
L13619_g_at		insulin-induced growth-response, insig-1		2.1	transcription of lipogenic genes
U09540_at		cytochrome P450	-2.5		mixed function monooxygenase
X99477cds_at		phosphotriesterase-related protein7		3.7	hydrolysis of phosphotriesters
D84667_g_at		phosphatidylinositol 4-kinase	-7.0		PI 4,5-bisphosphate synthesis
U02553cds_s_at		dual specificity phosphatase 1		2.1	insulin signaling; PI signaling
X94185cds_s_at		dual specificity phosphatase 6	-2.3		cell signaling; differentiation
X16476cds_at		drk1 gene mRNA for potassium channel protein		4.0	insulin regulated potassium channel
J00801_at		whey acidic protein		3.7	protease inhibitor
rc_AI639241_at		similar to ubiquitin carboxyl-terminal hydrolase 12		2.5	protein turnover
rc_AI102868_g_at		phosphoserine aminotransferase 1		2.1	serine synthesis
S59158_at		glutamate transporter	-2.0		glutamate transport
AF104399_g_at		melanocyte-specific gene 1 protein	-2.0		transcription
AF036335_g_at		NonO/p54nrb homolog	-2.8		transcription
rc_AA892863_at		similar to mitochondrial carrier homolog 2		2.3	unknown
rc_AI639074_at		Rat mixed-tissue library cDNA clone rx01925		24.3	unknown
rc_AI639112_at		Rat mixed-tissue library cDNA clone rx04824		7.5	unknown
Number affected			27	49	

* Transcripts that are differentially expressed by genotype at 20 and 24 days of age are marked with these numbers in the two tables. D, fold decrease in fatty rats compared to wildtypes. I, fold increase in fatty rats compared to wildtypes.

include four transcripts with immune system functions: immunoglobulin κ light chain variable region, immunoglobulin κ-chain constant region, CD3 γ-chain, and T-cell receptor β-chain, all downregulated at both ages. Natriuretic peptide receptor type C is upregulated 9.8 fold in fatty rats at 20 days of age and 2.8 fold at 24 days of age. Glucose regulated protein 78, an endoplasmic reticulum protein that is normally upregulated in cells under starvation, is downregulated -7-fold at 20 days of age and -2-fold at 24 days of age. Insulin-like growth factor I, a growth factor involved in adipocyte development, is downregulated -2.5-fold at 20 days of age and -2.3-fold at 24 days of age. Leptin is upregulated at both ages, as expected. Stearoyl CoA desaturase 1 and stearoyl CoA desaturase 2 are upregulated 6.5-fold at 20 days of age, and 3.2-fold and 4.0-fold, respectively, at 24 days of age.

Validation of differential expression results

Real-time RT-PCR was performed to confirm the results of the microarray analysis. Lipogenic enzymes fatty acid synthase, malic enzyme, transketolase, glycerol 3-phosphate acyltransferase and farnesyl diphosphate synthase were first tested. Afterwards, transcriptional regulators peroxisome proliferator activated-receptor γ and insulin induced gene 1 were confirmed with RT-PCR. Lastly, transcripts differentially expressed at both 20 days of age and 24 days of age, including stearoyl CoA desaturase 1, leptin and lipoprotein lipase were selected and the microarray results were confirmed.

18S ribosomal RNA is involved in protein synthesis and accounts for a large proportion of the RNA in any total RNA sample. 18S RNA was measured in all samples to serve as a control. To confirm that 18S RNA was not affected by any variables under investigation, we analyzed the effect of age, genotype and sex on 18S RNA expression (table 11). None of these variables ortheir interactions had any significant effect on 18S RNA expression, verifying that it is an appropriate control. The remaining transcripts were all expressed as ratios relative to 18S expression to improve the power of the statistical analysis by adjusting for amount of RNA in each sample.

Table 11. Type 3 tests of fixed effects for 18S RNA.

effect	num DF	den DF	F Value	Pr > F
geno	1	27	0.02	0.8969
age	1	27	0.9	0.3508
geno*age	1	27	1.19	0.2840
sex	1	27	0.47	0.4969
geno*sex	1	27	0.02	0.8847
age*sex	1	27	0.09	0.7618
geno*age*sex	1	27	0.22	0.6462

Fatty acid synthase (FAS) is a transcript that represents the regulation of synthesis of saturated fatty acids (47). FAS expression was effected by genotype, age, sex and all interactions (table 12A). FAS expression was low in wildtypes and fatty rats at 20 days of age, and there were no differences between the genotypes at this age (figure 10). By 24 days of age, FAS expression was 13.5-fold greater in fatty rats than in wildtypes (figure 10). In comparison, the arrays detected a 2.5-fold difference in expression at 24 days of age, suggesting that the arrays underestimate expression of this transcript (table 10).

Farnesyl diphosphate synthase (Fdps) is an enzyme required in the synthesis of cholesterol and its derivatives (57). Fdps was affected by genotype, age and all genotype interactions (table 12B). Fdps expression was similar at 20 days of age in wildtypes and fatty rats (figure 10). At 24 days of age, fatty rat expression was upregulated 2.2-fold (figure 10). Additionally, the array results estimated a 2.3-fold increase in fatty rats, compared to wildtypes at 24 days of age (table 10).

Malic enzyme (Me1) catalyzes the reversible oxidative decarboxylation of malate forming pyruvate and NADPH, which provides reducing equivalents for lipogenesis (50). Malic enzyme expression was affected by genotype, age and all genotype interactions (table 13A). Me1 expression at 20 days of age illustrated no statistical difference between wildtype and fatty rats (figure 11). However, by 24 days of age, fatty rats experienced a nearly 7.5-fold increase in expression compared to wildtypes (figure 11). The array results detected a 8.6-fold increase at 24 days of age (table 10).

Transketolase (Tkt) is a transcript linking glycolysis with the pentose phosphate pathway, which also produces NADPH to serve as reducing equivalents for fatty acid synthesis (49). Tkt was expressed at similar levels in wildtype and fatty rats at 20 days of age (figure 11). While the array analysis depicted a 2.6-fold increase in Tkt expression (table 10), RT-PCR data depicted an upregulation of 3.2-fold in fatty rats at 24 days of age, compared to wildtypes (figure 11). Transketolase was affected by genotype, age and the genotype by age interaction (table 13B).

Glycerol 3-phosphate acyltransferase (Gpam) catalyzes the initial step in triglyceride and phospholipid synthesis converting glycerol 3-phosphate to monoacylglycerol 3-phosphate (54).

Table 12A. Type 3 tests of fixed effects for fatty acid synthase.

effect	num DF	den DF	F Value	Pr > F
geno	1	27	81.19	<.0001
age	1	27	90.26	<.0001
geno*age	1	27	76.61	<.0001
sex	1	27	14.35	0.0008
geno*sex	1	27	18.06	0.0002
age*sex	1	27	13.31	0.0011
geno*age*sex	1	27	16.16	0.0004

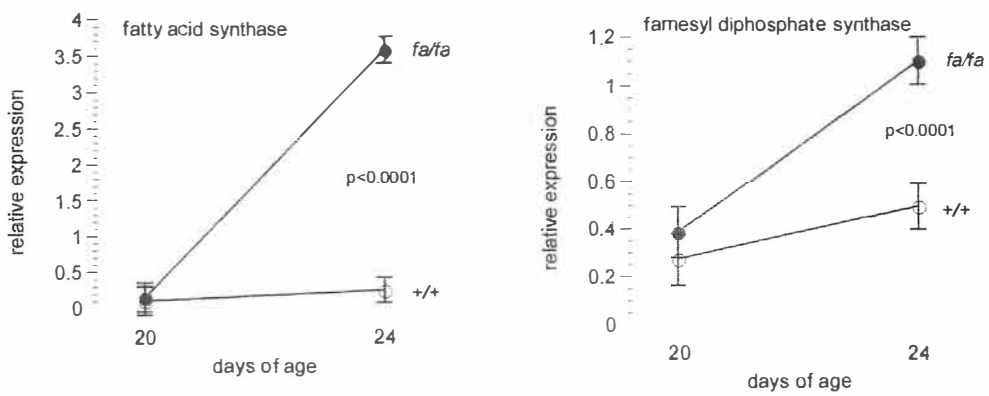


Figure 10. Relative expression of fatty acid synthase and farnesyl diphosphate synthase. At 20 days of age expression of fatty acid synthase and farnesyl diphosphate synthase is similar between fatty and wildtype rats. At 24 days of age the expression of fatty rats is upregulated, compared to wildtypes, in both fatty acid synthase and farnesyl diphosphate synthase ($p<0.0001$).

Table 12B. Type 3 tests of fixed effects for farnesyl diphosphate synthase.

effect	num DF	den DF	F Value	Pr > F
geno	1	27	28.81	<.0001
age	1	27	13.4	0.0011
geno*age	1	27	13.58	0.0010
sex	1	27	0.94	0.3420
geno*sex	1	27	13.49	0.0010
age*sex	1	27	3.11	0.0891
geno*age*sex	1	27	9.69	0.0043

Table 13A. Type 3 tests of fixed effects for malic enzyme.

effect	num DF	den DF	F Value	Pr > F
geno	1	27	34.81	<.0001
age	1	27	42.76	<.0001
geno*age	1	27	28.34	<.0001
sex	1	27	2.38	0.1342
geno*sex	1	27	4.68	0.0395
age*sex	1	27	2.24	0.1462
geno*age*sex	1	27	5.39	0.0280

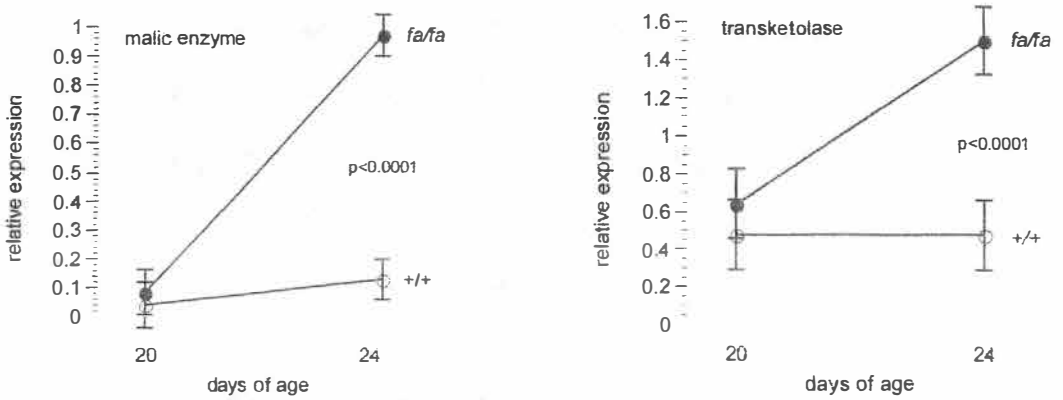


Figure 11. Relative expression of malic enzyme and transketolase. At 20 days of age expression of fatty malic enzyme and transketolase is similar between fatty and wildtype rats. At 24 days of age the expression of fatty rats is upregulated, compared to wildtypes, in both transcripts ($p<0.0001$).

Table 13B. Type 3 tests of fixed effects for transketolase.

Effect	Num DF	Den DF	F Value	Pr > F
geno	1	26	18.2	0.0002
age	1	26	4.38	0.0464
geno*age	1	26	8.89	0.0061
sex	1	26	0.06	0.8057
geno*sex	1	26	2.33	0.1389
age*sex	1	26	2.53	0.1239
geno*age*sex	1	26	2.56	0.1219

Gpam is influenced by genotype, age and all genotype interactions (table 14A). At 20 days of age Gpam expression in wildtype and fatty rats were not significantly different (figure 12). As with other transcripts involved in lipogenic pathways, fatty Gpam expression was upregulated compared to wildtypes at 24 days of age (figure 12). At 24 days of age, fatty Gpam expression was upregulated 4.9-fold (figure 12). This was similar to the array results with which fatty rats experienced a 2.6-fold increase in Gpam expression at 24 days of age compared to wildtypes (table 10).

Insulin induced gene 1 (Insig1) is a transcriptional regulator involved in cholesterol homeostasis and is proposed to "break" lipogenesis (66). While average Insig1 expression was similar between wildtype and fatty rats at 20 days of age, expression in fatty rats was upregulated 2.3-fold at 24 days of age (figure 12). The array results depicted an upregulation of 2.1-fold in fatty rats, compared to wildtypes, at 24 days of age (table 10). Insig1 was affected by genotype, age, genotype by age and genotype by age by sex interactions (table 14B).

Peroxisome proliferator activated-receptor γ (PPARγ) is a nuclear receptor that regulates the expression of genes for glucose utilization and lipogenesis in adipose tissue and is involved in adipocyte differentiation (64). Although PPARγ was not among the transcripts detected by the Genechip, we analyzed PPARγ expression because of its role in the regulation of lipogenic enzymes. PPARγ expression was influenced by genotype, age and sex and by all interactions expect age by sex (table 15A). Unlike the transcripts discussed earlier, PPARγ expression was differentially expressed at both 20 and 24 days of age (figure 13). At 20 days of age, fatty rats experienced a 1.6-fold increase in PPARγ expression and at 24 days of age, fatty rats experienced a 1.8-fold increase in PPARγ expression (figure 13). Because PPARγ was not present on the Genechip, there is no estimated expression available through the microarray results.

Stearoyl CoA desaturase 1 (Scd1) is a lipogenic enzyme that catalyzes the delta-9-*cis* desaturation of fatty acids, thus forming monounsaturated fatty acids palmitoleate (primarily) and oleate(55). Tests of fixed effects showed that stearoyl CoA desaturase 1 was influenced by

Table 14A. Type 3 tests of fixed effects for mitochondrial glycerol 3-phosphate acyltransferase.

effect	num DF	den DF	F Value	Pr > F
geno	1	27	57.09	<.0001
age	1	27	7.55	0.0106
geno*age	1	27	38.30	<.0001
sex	1	27	0.39	0.5350
geno*sex	1	27	8.65	0.0066
age*sex	1	27	0.21	0.6493
geno*age*sex	1	27	7.72	0.0098

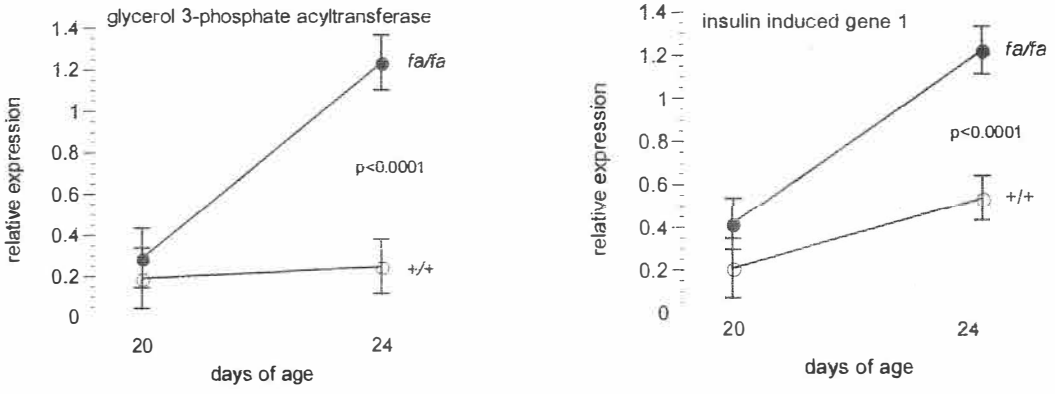


Figure 12. Relative expression of glycerol 3-phosphate acyltransferase and insulin induced gene 1. Expression of glycerol 3-phosphate acyltransferase and insulin induced gene 1 is similar between fatty and wildtype rats at 20 days of age. Both transcripts are upregulated in fatty rats at 24 days of age, compared to wildtypes ($p<0.0001$).

Table 14B. Type 3 tests of fixed effects for insulin induced gene 1.

effect	num DF	den DF	F Value	Pr > F
geno	1	25	14.19	0.0009
age	1	25	22.76	<.0001
geno*age	1	25	4.11	0.0533
sex	1	25	0.99	0.3293
geno*sex	1	25	8.65	0.0070
age*sex	1	25	4.01	0.0562
geno*age*sex	1	25	9.44	0.0051

Table 15A. Type 3 tests of fixed effects for PPARγ.

effect	num DF	den DF	F Value	Pr > F
geno	1	27	41.29	<.0001
age	1	27	6	0.0210
geno*age	1	27	7.6	0.0103
sex	1	27	4.69	0.0394
geno*sex	1	27	10.27	0.0035
age*sex	1	27	3.54	0.0709
geno*age*sex	1	27	11.07	0.0025

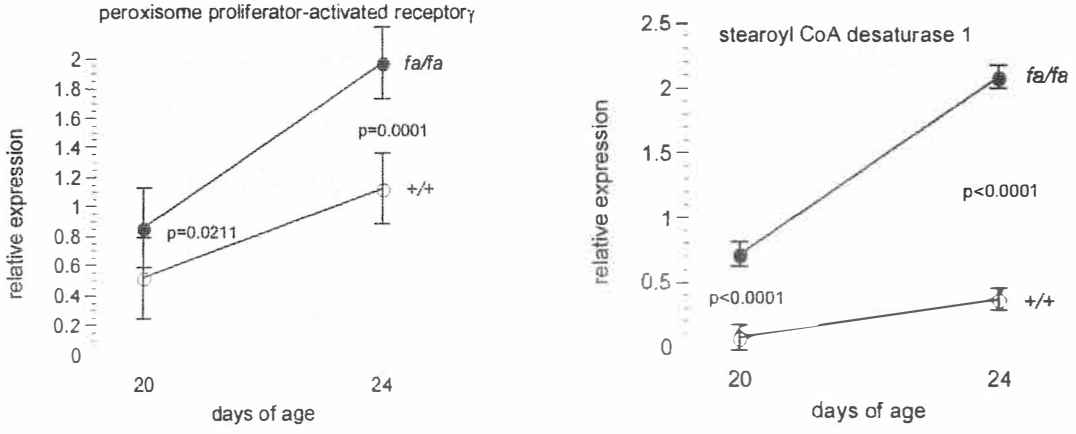


Figure 13. Relative expression of peroxisome proliferator-activated receptor γ and stearoyl CoA desaturase 1. Expression of PPARγ and Scd1 in fatty rats at 20 days of age is significant, compared to wildtypes ($p=0.0211$ and $p<0.0001$, respectively). Additionally, both transcripts are upregulated in fatty rats at 24 days of age, compared to wildtypes ($p=0.0001$ and $p<0.0001$, respectively).

genotype, age and by the genotype by age interaction (table 15B). In contrast to other lipogenic enzymes discussed above, Scd1 expression was highly induced in fatty rats at both 20 and 24 days of age (figure 13). At 20 days of age, Scd1 expression was upregulated 10.6-fold in fatty rats, compared to wildtypes (figure 13). Scd1 was upregulated to an even greater extent in fatty rats at 24 days of age: 5.7-fold compared to wildtypes (figure 13). Because expression increases 5.4-fold in wildtypes and 2.9-fold in fatty rats with age, Scd1 expression in fatty rats is only 5.7-fold greater than in wildtypes at 24 days of age. Array results drastically underestimated Scd1 expression at 20 and 24 days of age, depicting a 6.5-fold (table 9) and 3.2-fold increase (table 10), respectively.

Leptin, a hormone secreted from adipose tissue involved in energy homeostasis (59). Leptin expression was influenced by genotype, sex and the genotype by age interaction (table 16A). At 20 days of age fatty rats leptin expression was upregulated 2.3-fold compared to wildtypes (figure 14). Fatty rats experienced an even further upregulation of leptin expression at 24 days of age, 3.5-fold, compared to wildtypes (figure 14). Array results showed an upregulation in fatty rats, compared to wildtypes, of 2.8-fold at 20 days of age (table 9), overestimating the expression, and 2.5-fold at 24 days of age (table 10), underestimating the expression.

Lipoprotein lipase (Lpl) is a transcript responsible for the release of free fatty acids from triglycerides in circulation and is a regulator of triglyceride deposition (48). Lpl expression was influenced by genotype and the genotype by age interaction (table 16B). At 20 days of age Lpl expression was upregulated 3.3-fold in fatty rats, compared to wildtype rats (figure 14). Fatty Lpl expression was upregulated 6.4-fold compared to wildtypes at 24 days of age (figure 14). Moreover, while wildtype rats experienced a reduction in Lpl expression at 24 days of age, compared to 20 days of age, fatty rats experienced an increase from 20 to 24 days of age (figure 14).

These data demonstrate that the onset of hyperphagia in fatty rats occurs between 20 and 24 days of age. Fatty rats accumulate more adipose mass than wildtypes at 20 and 24 days of age, a reflection of the *fa* mutation. At 20 days of age fatty rats are similar to wildtype rats in

Table 15B. Type 3 tests of fixed effects for stearoyl CoA desaturase 1.

effect	num DF	den DF	F Value	Pr > F
geno	1	24	179.59	<.0001
age	1	24	78.33	<.0001
geno*age	1	24	37.02	<.0001
sex	1	24	0.85	0.3654
geno*sex	1	24	4.04	0.0544
age*sex	1	24	0.13	0.7214
geno*age*sex	1	24	1.19	0.2849

Table 16A. Type 3 tests of fixed effects for leptin.

effect	num DF	den DF	F Value	Pr > F
geno	1	26	44.27	<.0001
age	1	26	3.2	0.0854
geno*age	1	26	9.87	0.0042
sex	1	26	4.98	0.0345
geno*sex	1	26	0.34	0.5673
age*sex	1	26	1.4	0.2476
geno*age*sex	1	26	0.02	0.8931

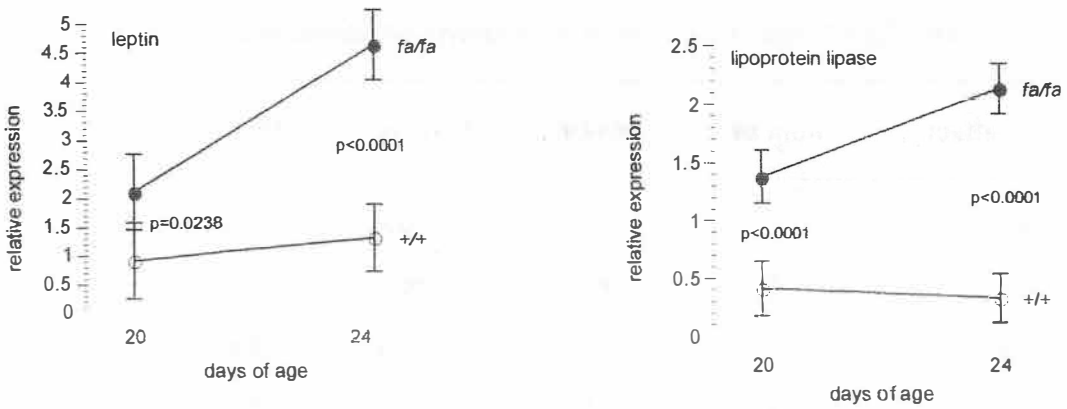


Figure 14. Relative expression of leptin and lipoprotein lipase. Leptin and lipoprotein lipase expression are upregulated in fatty rats, compared to wildtypes, at both 20 (p=0.0238 and p<0.0001, respectively) and 24 days of age (p<0.0001).

Table 16B. Type 3 tests of fixed effects for lipoprotein lipase.

effect	num DF	den DF	F Value	Pr > F
geno	1	24	125.88	<.0001
age	1	24	1.31	0.2638
geno*age	1	24	11.32	0.0026
sex	1	24	2.09	0.1613
geno*sex	1	24	0.06	0.8033
age*sex	1	24	0.21	0.6519
geno*age*sex	1	24	2.53	0.1248

weight and food intake, as demonstrated through their stomach contents. However, by 24 days of age fatty rats weigh significantly more than wildtype rats. Additionally, daily gain is significant in fatty rats at 24 days of age, compared to wildtypes. Liver weight is the same in animals at 20 days of age, but is significantly greater in fatty rats by 24 days of age. This weight gain, increased stomach contents and increased liver mass can all be attributed to the onset of hyperphagia.

When considering the transcripts fatty acid synthase, malic enzyme, transketolase, glycerol 3-phosphate acyltransferase, farnesyl diphosphate synthase and insulin induced gene 1, expression was similar in wildtypes and fatty rats prior to the onset of hyperphagia, but upregulated in fatty rats after the onset of hyperphagia. However, expression of leptin, stearoyl CoA desaturase 1, lipoprotein lipase, and to a lesser extent peroxisome proliferator-activated receptor γ, was upregulated before and after the onset of hyperphagia in fatty rats (compared to wildtype rats).

DISCUSSION

The most striking feature of this analysis is the changes in expression of transcripts associated with lipogenesis before and after the onset of hyperphagia. The lipogenic enzymes fatty acid synthase, malic enzyme, and glycerol 3-phosphate acyltransferase are expressed at similar levels in wildtype and fatty rats at 20 days of age. The enzyme farnesyl diphosphate synthase, which is involved in cholesterol synthesis (57), is also expressed at similar levels in wildtype and fatty rats at 20 days of age. Transketolase, which is involved in the pentose phosphate pathway (49), a source of NADPH for lipogenesis, is also expressed at similar levels between wildtype and fatty rats at 20 days of age. These data suggest that lipogenic pathways do not contribute significantly to the fatty phenotype before the onset of hyperphagia. However, each of these transcripts are upregulated in fatty rats after the onset of hyperphagia; suggesting that the development of hyperphagia and increased lipogenesis are tightly linked.

The onset of hyperphagia is associated with increased insulin levels (74). Insulin stimulates lipogenesis by increasing transcription of lipogenic enzymes through the transcription factor SREBP-1c. SREBP-1c is tethered to the endoplasmic reticulum by insig1; insulin causes SREBP-1c to be cleaved off and transported to the nucleus to bind to the promoter region of genes for lipogenesis and cholesterol synthesis (95). Although Insig1 is stimulated by insulin, its functional role actually appears to counter the effects of insulin on lipogenesis. Selective overexpression of Insig1 in livers of fatty rats actually inhibits lipogenesis in fatty rats (66). Thus, the induction of Insig1 after the onset of hyperphagia might be a mechanism to maintain control of accelerated lipogenesis.

PPARγ is also upregulated after the onset of hyperphagia. PPARγ is a nuclear receptor that upregulates genes for lipogenesis, including Insig1 (96). The PPARγ activator rosiglitazone stimulates lipogenesis in type II diabetes (97). As a result, rosiglitazone increases insulin sensitivity (63). Work in this lab has shown that fatties are insulin resistant before the onset of hyperphagia and become insulin sensitive after the onset of hyperphagia (90). This suggests that the onset of hyperphagia is closely linked with changes in insulin sensitivity. In the current study, PPARγ was also slightly upregulated in fatty rats prior to the onset of hyperphagia. However, Durham (2004) has shown that PPARγ protein expression is low prior to the onset of hyperphagia, and increases substantially with the development of hyperphagia. In fact, PPARγ protein level increase in lean littermates of fatty rats over the same interval (90). These observations suggest that developmental changes in PPARγ signaling may be critical for the development of hyperphagia in fatty rats.

Leptin was upregulated before the onset of hyperphagia and was further upregulated after the onset of hyperphagia. These results are consistent with the fact that leptin and the leptin receptor normally contribute a feedback loop that maintains adipose mass (58). Because the fatty mutation disrupts the signaling abilities of the leptin receptor, leptin expression remains high (71). Truett et al. (2000) have shown the circulating leptin levels are high before the onset of hyperphagia and increase further afterward (74).

Lipoprotein lipase was also upregulated before and after the onset of hyperphagia. In addition to its roles in delivering fatty acids from the circulation to adipose tissue, lipoprotein lipase is an early marker of adipocyte differentiation, increasing in expression before significant lipid stores are detectable in preparation for lipid filling (98). The early increase in lipoprotein lipase might represent a direct effect of the lack of leptin signaling. However, Ramsay (2003) has shown that leptin suppresses lipogenesis without affecting lipoprotein lipase expression (99). Instead, leptin may have an indirect effect on lipoprotein lipase expression by inhibiting adipocyte differentiation. In this case, the elevation of Lpl in fatty rats would represent the lack of inhibition of adipocyte differentiation.

Stearoyl CoA desaturase 1 was the transcript that was most strongly influenced by fatty genotype. Scd1 was upregulated in fatties prior to the onset of hyperphagia and was further upregulated after the onset of hyperphagia. Although Scd1 can be considered to be a lipogenic enzyme because of its role in forming monounsaturated fatty acids (56), it may be performing a different role prior to the onset of hyperphagia. Cohen et al. (2002) pair-fed *ob/ob* mice to match the intake of leptinized *ob/ob* mice, then identified differentially expressed genes in liver. Scd1 was the gene with the largest differences between these two. They suggested that Scd1 may play an important role in mediating leptin's effects on energy expenditure, independent of food intake. Furthermore, they also crossed Scd1 deficient mice with *ob/+* mice to produce double mutants that were leaner than *ob/ob* mice (100). These data suggest that Scd1 is a major mediator of leptin signaling. Asilmaz et al. (2004) showed that Scd1 is an important mediator of leptin's effects on glucose and lipid metabolism in a model of lipodystrophy that lacks expression of SREBP1c in adipose tissue. These mice develop fatty livers and are diabetic. Leptin injected into the central nervous system inhibited Scd1 and improved glucose homeostasis in these mice (101).

While the mechanisms of action of Scd1 in mediating the effects of leptin on metabolism are unknown, it is possible that Scd1 generates an intracellular lipid that acts as a signaling molecule (102). This signaling molecule could be a ligand for PPARγ or other lipid binding

nuclear receptors. The onset of hyperphagia in fatty rats could be dependent of the interaction of this signal with developmentally regulated PPARγ.

This project has identified a set of major regulators that are proposed to work together to regulate the onset of hyperphagia in fatty rats and drive the induction of lipogenesis. One hypothesis is that these changes in metabolism lead to an induction in lipogenesis and secondary hyperphagia. An alternative explanation might be that a central mechanism activates hyperphagia, and lipogenesis is upregulated in response. Because rosiglitazone can stimulate the development of hyperphagia by inducing lipogenesis, we favor the first alternative. This hypothesis may be testable by treating young fatty rats with rosiglitazone to promote and earlier onset of hyperphagia.

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APPENDICES

APPENDIX A1: DETAILED METHODS

RNA Extraction Protocol by Totally RNA kit (Ambion Inc., Austin, TX)

Day 1:

1. Homogenize frozen adipose tissue in 30 ml Oak Ridge tube with 5 ml Denaturation Solution.
2. Add 5 ml Chloroform and mix thoroughly to extract excess lipids.
3. Centrifuge at 10,000 to 12,000 x g at 7°C for 20 minutes.
4. Transfer upper aqueous phase to a new vessel.
5. Add 5 ml Chloroform and mix thoroughly.
6. Centrifuge at 10,000 to 12,000 x g at 7°C for 20 minutes.
7. Transfer upper aqueous phase to a new vessel.
8. Transfer 5 ml of Phenol:Chloroform:IAA solution to homogenate and mix.
9. Store preparation in ice bath for 15 minutes.
10. Centrifuge at 10,000 to 12,000 x g at 7°C for 30 minutes.
11. Transfer upper aqueous phase and white interface to a new vessel.
12. Centrifuge at 10,000 to 12,000 x g at 7°C for 5 minutes.
13. Transfer upper aqueous phase to a new vessel.
14. Add 1/10 of volume of step 13 of Sodium Acetate Solution and mix.
15. Transfer 5 ml of Acid-Phenol:Chloroform solution to homogenate and mix.
16. Store preparation in ice bath for 15 minutes.
17. Centrifuge at 10,000 to 12,000 x g at 7°C for 30 minutes.
18. Transfer upper aqueous phase and white interface to a new vessel.
19. Centrifuge at 10,000 to 12,000 x g at 7°C for 5 minutes.
20. Transfer upper aqueous phase to an RNase-free vessel avoiding transfer of interface.
21. Add an equal volume of step 20 of isopropanol (stored at 4°C) and mix.
22. Store preparation overnight at -20°C to allow RNA to precipitate.

Day 2:

1. Centrifuge at 10,000 to 12,000 x g at 7°C for 30 minutes.
2. Remove and discard supernatant solution.
3. Re-suspend pellet in 300 µL DEPC H_2O and dissolve pellet in hot water bath with intermittent vortexing.
4. Add 150 µL of 0.5M LiCl Precipitation Solution to the preparation and mix. This step removes excess carbohydrates and small RNA molecules.

5. Store preparation at -20°C for 30 minutes.
6. Centrifuge at 10,000 to 12,000 x g at 7°C for 30 minutes.
7. Remove supernatant and add 300 µL of room temperature 70% ethanol prepared with nuclease free water to wash pellet.
8. Dislodge pellet by flicking tube and gently agitate for 1 minute.
9. Centrifuge at 3,000 x g at 4°C for 20 minutes.
10. Remove ethanol supernatant thoroughly and allow pellet to dry completely under fume hood.
11. Re-suspend pellet in 300 µL DEPC H_2O/ 0.1 mM EDTA. Store RNA at -80°C for long term or at -20° C for short-term.

Quantification of RNA

1. Prepare 1 mM Na_2HPO_4, pH 8.5 by mixing 0.14196 grams Na_2HPO_4 with 1 L deionized H_2O.
2. Load 500 µL 1 mM Na_2HPO_4, pH 8.5 in 1.5 ml eppendorf tube for each sample to be quantified plus one additional tube to be used as the blank.
3. Load 5 µL of unknown RNA samples in the eppendorf tubes, skipping the tube to be used as the blank.
4. Quantify with use of Spectrophotometer reading each RNA sample at both 260 nm and 280 nm.

Denaturing gel electrophoresis

Preparation of 10X MOPS buffer

1. Weigh 41.90 g of MOPS, pH 7.0 (final concentration = 0.2 M).
2. Weigh 4.70 g of EDTA, pH 8.0 (final concentration = 10mM).
3. Weigh 4.10 g of sodium acetate (final concentration = 50mM).
4. Mix MOPS, EDTA and sodium acetate in 1 liter DEPC treated water.
5. Insure a pH of 7.0.

Preparation of 1% agarose gel

1. Prepare a 1.2% to 1.5% LE agarose gel in a volume of 100 ml.
2. Weigh 1.2-1.5 g agarose.
3. Mix agarose with 80 ml DEPC water in a baked flask.
4. Microwave until all agarose is cooked (about 3 minutes)
5. Cool gel solution to 50° C in water bath.
6. Add 10 ml 10X MOPS buffer (final concentration = 1X) to gel solution.

7. Add 1.79 ml formaldehyde and mix (final concentration = 0.22M).
8. Add 5 μl EtBr and mix thoroughly.
9. Keep the gel solution at 50° C until ready to pour.
10. Carefully pour the gel into the gel tray trying to minimize bubbles and immediately add gel combs.
11. Allow gel to cool about 20-30 minutes before loading samples.

Preparation of 1X MOPS / 0.22M formaldehyde running buffer

1. Mix 70 ml 10X MOPS, 12.5 ml formaldehyde and 617.5 ml DEPC treated water.
2. Add running buffer to completely solidified and cooled gel until running buffer covers the gel by about 3-4 mm.

Preparation of samples to load on gel

1. Samples will be diluted to a final volume of 20 μl.
2. Add 10 μl of formaldehyde loading buffer.
3. Calculate the volume of sample necessary for 2-3 □g of RNA per well and add this to a sterile 1.5 ml tube.
4. Add DEPC water to make the final volume 20 μl.
5. Heat samples 10 minutes at 65 degrees.
6. Load 20 μl of prepared samples.
7. Run gel at 75 volts for 3-4 hours.

Quantitative Real-Time Polymerase Chain Reaction

Preparing the Smart Cycler

1. Turn on computer.
2. Turn on PCR machine(s).
3. Double click on "Smart Cycler" icon.
4. Select "Create Run."
5. Label run name: (Initials)(Date)(Target).
6. Select "Add Remove Sites" – select run protocol "Truett1" and select sites A1 -... for corresponding numbers of samples to run and select "OK."
7. Select sample type for each site labeling the standards as "STD" and the unknowns as "UNKN."
8. Assign concentrations for the standards in the "FAM Std CONC" column.
9. Assign sample id's in the "Sample ID" column
10. Prepare samples for quantification.

Preparation of RNA dilution curve

1. Dilute concentration of RNA sample with high levels of targets expression to 50 ng/μl.
2. Perform serial dilutions to generate 6 RNA concentrations from which a dilution curve will be developed. The concentration will range from 0.244140625 to 50 ng/μl (table 20).
3. Add 5 μl of dilution ID's #1-6 to the first 6 Cepheid Smart Cycler reaction tubes.
4. Add 5 μl of unknown RNA samples to reaction tubes #7-(32).

Preparation of master mix

1. Prepare the PCR mix (master mix). Be sure to add primers and probe and subsequent reagents in dim light (table 21).
2. Add 20 μl of master mix to each of the reaction tubes.

Running the Cepheid Smart Cycler

1. Centrifuge reaction tubes thoroughly and check that no bubbles are visible.
2. Carefully insert reaction tubes into corresponding wells of PCR machine, avoid touching the optical excitation/detection window.
3. Close lids of PCR machine and select "Start Run" – the PCR Machine will run for about 2 hours.

Table 17. RNA serial dilutions to prepare dilution curve.

dilution ID	concentration (ng/μl)	RNA(μl)	DEPC H_2O (μl)	ng RNA per reaction (when used in 5 μl volume)
#1	50	0	0	250
#2	12.5	25 μl of #1	75	62.5
#3	3.125	25 μl of #2	75	15.625
#4	0.78125	25 μl of #3	75	3.90625
#5	0.1953125	25 μl of #4	75	0.9765625
#6	0.048828125	25 μl of #5	75	0.244140625

Table 18. Preparation of master mix.

reagent	µl per reaction	µl per 32 reactions (with 20% excess)
DEPC Water	7.350	282.240
10X Taqman Buffer A from Taqman Gold Kit	2.5	96.000
MgCl2, 25 mM GeneAmp kit	5.5	211.200
dATP, 10 mM GeneAmp kit	0.75	28.800
dCTP, 10 mM GeneAmp kit	0.75	28.800
dGTP, 10 mM GeneAmp kit	0.75	28.800
dTTP, 10 mM GeneAmp kit	0.75	28.800
20X Applied Biosystems Assays on Demand primers and probes	1.25	48.000
MuLV Reverse transcriptase, 50 U/ml GeneAmp kit	0.125	4.800
RNase inhibitor, 20 U/ml GeneAmp kit	0.125	4.800
AmpliTaq Gold from Taqman Gold kit	0.15	5.760
Total	7.350	768.000

APPENDIX A2. SAS DATA

Table 19. Physical traits of wildtype and fatty rats at 20 and 24 days of age.

					weight in grams						
id	lit	age	genotype	sex	body	gain	liver	inguinal	stomach	empty stomach	stomach contents
12380	1142	24	0	F	45.71	2.87	1.630	0.600	0.639	0.372	0.267
12381	1142	24	2	F	48.97	4.98	2.034	1.261	2.100	0.344	1.756
12382	1142	24	2	F	50.25	3.31	2.086	1.397	1.852	0.569	1.283
12383	1142	24	0	F	47.51	3.10	1.617	0.623	1.406	0.317	1.089
12384	1142	24	2	F	44.81	2.96	1.956	0.965	2.183	0.337	1.846
12385	1142	24	0	F	45.43	3.25	1.55	0.603	1.194	0.313	0.881
12418	1146	24	0	F	48.44	3.32	1.411	0.315	1.846	0.396	1.450
12419	1146	24	2	F	57.03	3.13	2.741	1.467	3.185	0.502	2.683
12420	1146	24	2	F	54.92	4.11	2.268	1.276	2.316	0.520	1.796
12421	1146	24	0	F	51.22	2.90	1.946	0.735	1.453	0.406	1.047
12423	1146	24	2	F	53.40	4.18	2.371	1.385	2.428	0.387	2.041
12425	1146	24	0	F	50.26	2.77	1.76	0.765	1.628	0.42	1.208
12427	1146	24	2	M	56.92	5.05	2.604	1.354	3.353	0.423	2.930
12428	1146	24	0	M	53.81	3.31	2.181	0.666	1.258	0.388	0.870
12478	1150	24	0	M	49.13	3.45	1.903	0.54	0.961	0.65	0.311
12485	1150	24	2	M	49.44	4.88	2.376	0.988	2.767	0.389	2.378
12487	1151	20	0	F	36.42	1.85	1.212	0.316	1.284	0.266	1.018
12492	1151	20	2	F	32.86	1.98	1.026	0.77	0.975	0.130	0.845
12494	1151	20	0	M	36.30	1.85	1.300	0.302	1.222	0.234	0.988

continued next page

Table 19 Continued.

					weight in grams						
id	lit	age	genotype	sex	body	gain	liver	inguinal	stomach	empty stomach	stomach contents
12495	1151	20	2	M	37.70	1.88	1.220	0.838	0.750	0.251	0.499
12498	1152	20	2	F	35.30	1.70	1.244	0.829	1.055	0.452	0.603
12499	1152	20	0	F	37.12	2.38	1.270	0.322	1.013	0.275	0.738
12504	1152	20	2	M	38.90	1.83	1.330	0.856	1.122	0.275	0.847
12506	1152	20	0	M	38.43	3.01	1.359	0.339	1.258	0.379	0.879
12511	1153	20	2	F	30.13	0.68	0.939	0.558	0.728	0.210	0.518
12614	1162	20	0	M	33.68	2.15	1.178	0.244	1.384	0.241	1.143
12616	1162	20	2	M	35.28	1.38	1.279	0.758	1.079	0.291	0.788
12617	1162	20	0	M	35.04	1.51	1.257	0.293	1.395	0.259	1.136
12618	1162	20	2	M	34.62	1.76	1.102	0.706	1.048	0.209	0.839
12639	1164	24	2	M	48.95	3.54	1.824	1.406	1.869	0.326	1.543
12641	1164	24	0	M	48.18	4.54	1.977	0.566	1.831	0.38	1.451
12646	1165	24	0	M	55.86	3.10	2.073	0.903	2.059	0.375	1.684
12651	1165	24	2	M	58.33	4.07	2.584	1.816	2.331	0.443	1.888
12652	1165	24	0	M	53.97	2.50	2.131	0.849	1.669	0.384	1.285
12662	1166	24	0	M	48.95	2.71	2.009	0.679	1.876	0.365	1.511
12665	1166	24	2	M	56.04	3.86	2.558	1.459	2.853	0.406	2.447
12675	1167	24	0	M	52.06	2.79	1.960	0.630	0.853	0.426	0.427
12676	1167	24	2	M	55.34	3.23	2.387	1.358	0.851	0.396	0.455

Table 20. Expression data for wildtype and fatty rats at 20 and 24 days of age.

id	r18s	FAS	PPARγ	Scd1	Me1	Lep	Gpam	Tkt	Fdps	Insig1	Lpl
12511	22.982	0.091	0.475	0.193	0.047	0.326	0.105	0.194	0.396	0.249	0.468
12513	20.791	0.132	0.507	0.067	0.042	0.284	0.134	0.125	0.244	0.202	0.132
12514	20.708	0.148	0.660	1.050	0.113	0.474	0.170	0.528	0.706	0.298	0.615
12554	18.556	0.214	0.669	1.138	0.128	0.790	0.212	0.426	0.309	0.445	1.126
12557	24.657	0.129	0.470	0.050	0.103	0.311	0.116	0.285	0.481	.	0.137
12611	27.594	0.071	0.472	0.035	0.012	1.758	0.132	0.367	0.134	0.106	0.333
12614	24.929	0.094	0.617	0.064	0.010	0.755	0.266	0.082	0.144	0.141	0.341
12616	27.026	0.107	1.294	0.499	0.054	0.950	0.402	0.672	0.312	0.334	1.760
12617	33.23	0.081	0.451	0.032	0.018	2.470	0.201	0.516	0.202	0.162	0.456
12618	21.069	0.140	1.053	0.624	0.065	1.505	0.384	0.462	0.351	0.402	2.036
12639	21.417	0.848	1.121	1.989	0.110	2.024	0.579	0.732	0.622	0.243	2.486
12641	24.177	0.436	0.788	0.276	0.271	1.017	0.352	0.299	0.641	1.061	0.467
12646	12.745	0.084	0.438	0.433	0.279	0.501	0.162	0.017	0.300	1.139	0.242
12651	31.432	2.825	1.491	1.018	0.011	.	0.166	.	0.025	0.175	.
12652	21.214	0.193	1.170	0.364	0.103	0.641	0.151	0.346	0.213	0.022	0.394
12662	24.695	0.248	0.682	0.477	0.128	0.974	0.241	0.580	0.488	0.369	0.347
12665	24.804	2.134	1.122	1.918	0.770	7.594	0.797	1.262	1.485	0.401	1.415
12675	17.058	0.378	0.505	0.735	0.201	3.980	0.333	0.072	0.646	1.519	.
12676	22.566	1.761	0.523	2.240	1.002	8.537	0.797	0.449	0.985	0.459	0.861

APPENDIX A3. STATISTICAL ANALYSIS

SAS Program Code

```
Program

options linesize = 72; options pagesize = 20000;
   options nodate; options nonumber; run;
   data one;
infile 'i:/Mary/switchdata.txt' FIRSTOBS=2;
input id lit age geno sex $ body gain liver ing stom MT contents r18s
fas pparg Scd1 Me1 Lep Gpam Tkt Fdps Insig1 Lpl;
run;
%macro mixed (yvar);
proc mixed;
  class id lit geno age sex;
  model &yvar=geno|age|sex;
  random lit;
  lsmeans geno*age /slice=age slice=geno;
run;
%mend mixed;
%mixed (body);%mixed (gain);%mixed (liver);%mixed (ing);%mixed
(stom);%mixed (MT); %mixed (contents); %mixed (r18s);
%mixed (fas);%mixed (pparg);%mixed (Scd1);%mixed (Me1); %mixed (Lep);
%mixed (Gpam); %mixed (Tkt);
%mixed (Fdps); %mixed (Insig1); %mixed (Lpl);
```

SAS LOG

```
Log

35      options linesize = 72; options pagesize = 20000;
36      options nodate; options nonumber; run;
37      data one;
38    infile 'i:/Mary/switchdata.txt' FIRSTOBS=2;
39    input id lit age geno sex $ body gain liver ing stom MT contents
39 !  r18s fas pparg Scd1 Me1 Lep Gpam Tkt Fdps Insig1 Lpl;
40    run;
NOTE: The infile 'i:/Mary/switchdata.txt' is:
      File Name=i:\Mary\switchdata.txt,
      RECFM=V,LRECL=256
NOTE: 45 records were read from the infile 'i:/Mary/switchdata.txt'.
      The minimum record length was 166.
      The maximum record length was 197.
NOTE: The data set WORK.ONE has 45 observations and 24 variables.
NOTE: DATA statement used (Total process time):
      real time           0.09 seconds
      cpu time            0.01 seconds
41    %macro mixed (yvar);
42    proc mixed;
43      class id lit geno age sex;
44      model &yvar=geno|age|sex;
45      random lit;
46      lsmeans geno*age /slice=age slice=geno;
47    run;
48    %mend mixed;
49    %mixed (body);%mixed (gain);%mixed (liver);%mixed (ing);%mixed (stom);
49 !  %mixed (MT); %mixed (contents); %mixed (r18s);
NOTE: Convergence criteria met.
NOTE: PROCEDURE MIXED used (Total process time):
      real time           0.94 seconds
      cpu time            0.07 seconds
NOTE: Convergence criteria met.
NOTE: PROCEDURE MIXED used (Total process time):
      real time           0.07 seconds
      cpu time            0.07 seconds
NOTE: Convergence criteria met.
NOTE: PROCEDURE MIXED used (Total process time):
      real time           0.08 seconds
      cpu time            0.06 seconds
NOTE: Convergence criteria met.
NOTE: PROCEDURE MIXED used (Total process time):
      real time           0.07 seconds
      cpu time            0.06 seconds
NOTE: Convergence criteria met.
NOTE: PROCEDURE MIXED used (Total process time):
      real time           0.09 seconds
      cpu time            0.08 seconds
NOTE: Convergence criteria met.
NOTE: PROCEDURE MIXED used (Total process time):
      real time           0.08 seconds
      cpu time            0.08 seconds
NOTE: Convergence criteria met.
NOTE: PROCEDURE MIXED used (Total process time):
      real time           0.08 seconds
      cpu time            0.08 seconds
NOTE: Convergence criteria met.
NOTE: PROCEDURE MIXED used (Total process time):
      real time           0.09 seconds
      cpu time            0.08 seconds
50    %mixed (fas);%mixed (pparg);%mixed (Scd1);%mixed (Me1);
50 !  %mixed (Lep); %mixed (Gpam); %mixed (Tkt);
NOTE: Convergence criteria met.
NOTE: PROCEDURE MIXED used (Total process time):
      real time           0.14 seconds
      cpu time            0.06 seconds
NOTE: Convergence criteria met.
NOTE: Estimated G matrix is not positive definite.
NOTE: PROCEDURE MIXED used (Total process time):
      real time           0.08 seconds
      cpu time            0.06 seconds
NOTE: Convergence criteria met.
NOTE: PROCEDURE MIXED used (Total process time):
      real time           0.07 seconds
      cpu time            0.06 seconds
```

```
NOTE: Convergence criteria met.
NOTE: PROCEDURE MIXED used (Total process time):
      real time           0.08 seconds
      cpu time            0.06 seconds
NOTE: Convergence criteria met.
NOTE: Estimated G matrix is not positive definite.
NOTE: PROCEDURE MIXED used (Total process time):
      real time           0.07 seconds
      cpu time            0.06 seconds
NOTE: 1 observation is not included because of missing values.
NOTE: Convergence criteria met.
NOTE: PROCEDURE MIXED used (Total process time):
      real time           0.07 seconds
      cpu time            0.06 seconds
NOTE: Convergence criteria met.
NOTE: PROCEDURE MIXED used (Total process time):
      real time           0.08 seconds
      cpu time            0.07 seconds
NOTE: 1 observation is not included because of missing values.
NOTE: Convergence criteria met.
NOTE: PROCEDURE MIXED used (Total process time):
      real time           0.07 seconds
      cpu time            0.07 seconds
51   %mixed (Fdps); %mixed (Insig1); %mixed (Lpl);
NOTE: Convergence criteria met.
NOTE: PROCEDURE MIXED used (Total process time):
      real time           0.18 seconds
      cpu time            0.07 seconds
NOTE: 2 observations are not included because of missing values.
NOTE: Convergence criteria met.
NOTE: Estimated G matrix is not positive definite.
NOTE: PROCEDURE MIXED used (Total process time):
      real time           0.08 seconds
      cpu time            0.06 seconds
NOTE: 3 observations are not included because of missing values.
NOTE: Convergence criteria met.
NOTE: PROCEDURE MIXED used (Total process time):
      real time           0.07 seconds
      cpu time            0.06 seconds
```

SAS Output

Output

The SAS System

The Mixed Procedure

Model Information

Data Set	WORK.ONE
Dependent Variable	body (body weight, grams)
Covariance Structure	Variance Components
Estimation Method	REML
Residual Variance Method	Profile
Fixed Effects SE Method	Model-Based
Degrees of Freedom Method	Containment

Class Level Information

Class	Levels	Values
id	45	12380 12381 12382 12383 12384 12385 12418 12419 12420 12421 12423 12425 12427 12428 12478 12485 12487 12492 12494 12495 12498 12499 12504 12506 12507 12509 12511 12513 12514 12554 12557 12611 12614 12616 12617 12618 12639 12641 12646 12651 12652 12662 12665 12675 12676
lit	12	1142 1146 1150 1151 1152 1153 1157 1162 1164 1165 1166 1167
geno	2	0 2
age	2	20 24
sex	2	F M

Dimensions

Covariance Parameters	2
Columns in X	27
Columns in Z	12
Subjects	1
Max Obs Per Subject	45

Number of Observations

Number of Observations Read	45
Number of Observations Used	45
Number of Observations Not Used	0

Iteration History

Iteration	Evaluations	-2 Res Log Like	Criterion
0	1	199.28107931	
1	3	175.33458031	0.02032755
2	2	174.96201255	0.00270359
3	1	174.79484295	0.00022675
4	1	174.78202153	0.00000206
5	1	174.78191096	0.00000000

Convergence criteria met.

Covariance Parameter Estimates

Cov Parm	Estimate
lit	6.2425
Residual	2.6546

Fit Statistics

-2 Res Log Likelihood	174.8
AIC (smaller is better)	178.8
AICC (smaller is better)	179.1
BIC (smaller is better)	179.8

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
geno	1	27	7.41	0.0112
age	1	27	99.79	<.0001
geno*age	1	27	14.06	0.0009
sex	1	27	11.94	0.0018
geno*sex	1	27	0.17	0.6818
age*sex	1	27	0.15	0.7026
geno*age*sex	1	27	0.91	0.3489

Least Squares Means

Effect	geno	age	Estimate	Standard Error	DF	t Value	Pr > |t|
geno*age	0	20	35.3427	1.2478	27	28.32	<.0001
geno*age	0	24	49.3127	1.0958	27	45.00	<.0001
geno*age	2	20	34.8341	1.2377	27	28.14	<.0001
geno*age	2	24	52.5145	1.1042	27	47.56	<.0001

Tests of Effect Slices

Effect	geno	age	Num DF	Den DF	F Value	Pr > F
geno*age		20	1	27	0.47	0.4982
geno*age		24	1	27	23.83	<.0001
geno*age	0		1	27	70.77	<.0001
geno*age	2		1	27	113.63	<.0001

Dependent Variable gain (daily gain on the last day of life, grams)

Iteration History

Iteration	Evaluations	-2 Res Log Like	Criterion
0	1	77.62838352	
1	2	77.33088728	0.00000147
2	1	77.33088041	0.00000000

ConVergence criteria met.

Covariance Parameter Estimates

Cov Parm	Estimate
lit	0.02880
Residual	0.3053

Fit Statistics

-2 Res Log Likelihood	77.3
AIC (smaller is better)	81.3
AICC (smaller is better)	81.7
BIC (smaller is better)	82.3

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
geno	1	27	0.23	0.6345
age	1	27	68.31	<.0001
geno*age	1	27	19.78	0.0001
sex	1	27	2.26	0.1441
geno*sex	1	27	0.05	0.8201
age*sex	1	27	0.02	0.8924
geno*age*sex	1	27	0.05	0.8196

Least Squares Means

Effect	geno	age	Estimate	Standard Error	DF	t Value	Pr > |t|
geno*age	0	20	2.1973	0.1930	27	11.38	<.0001
geno*age	0	24	3.1019	0.1713	27	18.11	<.0001
geno*age	2	20	1.5374	0.1917	27	8.02	<.0001
geno*age	2	24	3.9218	0.1766	27	22.21	<.0001

Tests of Effect Slices

Effect	geno	age	Num DF	Den DF	F Value	Pr > F
geno*age		20	1	27	7.08	0.0130
geno*age		24	1	27	13.67	0.0010
geno*age	0		1	27	12.29	0.0016
geno*age	2		1	27	83.68	<.0001

Dependent Variable liver (liver weight, grams)

Iteration History

Iteration	Evaluations	-2 Res Log Like	Criterion
0	1	11.18038481	
1	3	6.01659125	0.00041316
2	1	6.00295648	0.00000459
3	1	6.00281297	0.00000000

Convergence criteria met.

Covariance Parameter Estimates

Cov Parm	Estimate
lit	0.01754
Residual	0.03791

Fit Statistics

-2 Res Log Likelihood	6.0
AIC (smaller is better)	10.0
AICC (smaller is better)	10.4
BIC (smaller is better)	11.0

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
geno	1	27	17.37	0.0003
age	1	27	76.98	<.0001
geno*age	1	27	15.51	0.0005
sex	1	27	13.93	0.0009
geno*sex	1	27	2.03	0.1653
age*sex	1	27	0.00	0.9746
geno*age*sex	1	27	0.24	0.6259

Least Squares Means

Effect	geno	age	Estimate	Standard Error	DF	t Value	Pr > |t|
geno*age	0	20	1.1572	0.08750	27	13.23	<.0001
geno*age	0	24	1.8174	0.07829	27	23.21	<.0001
geno*age	2	20	1.1708	0.08634	27	13.56	<.0001
geno*age	2	24	2.2947	0.07981	27	28.75	<.0001

Tests of Effect Slices

Effect	geno	age	Num DF	Den DF	F Value	Pr > F
geno*age		20	1	27	0.02	0.8789
geno*age		24	1	27	37.19	<.0001
geno*age	0		1	27	31.62	<.0001
geno*age	2		1	27	91.38	<.0001

Dependent Variable ing (inguinal adipose pads weight, grams)

Iteration History

Iteration	Evaluations	-2 Res Log Like	Criterion
0	1	-21.34400323	
1	2	-27.59817381	0.00575461
2	1	-27.92553852	0.00078918
3	1	-27.96699643	0.00002063
4	1	-27.96800458	0.00000002
5	1	-27.96800537	0.00000000

Convergence criteria met.

Covariance Parameter Estimates

Cov Parm	Estimate
lit	0.01346
Residual	0.01323

Fit Statistics

-2 Res Log Likelihood	-28.0
AIC (smaller is better)	-24.0
AICC (smaller is better)	-23.6
BIC (smaller is better)	-23.0

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
geno	1	27	283.53	<.0001
age	1	27	34.19	<.0001
geno*age	1	27	12.60	0.0014
sex	1	27	0.53	0.4725
geno*sex	1	27	0.58	0.4530
age*sex	1	27	0.08	0.7740
geno*age*sex	1	27	0.00	0.9830

Least Squares Means

Effect	geno	age	Estimate	Standard Error	DF	t Value	Pr > |t|
geno*age	0	20	0.2997	0.06472	27	4.63	<.0001
geno*age	0	24	0.6397	0.05764	27	11.10	<.0001
geno*age	2	20	0.7630	0.06394	27	11.93	<.0001
geno*age	2	24	1.3505	0.05839	27	23.13	<.0001

Tests of Effect Slices

Effect	geno	age	Num DF	Den DF	F Value	Pr > F
geno*age		20	1	27	78.90	<.0001
geno*age		24	1	27	235.93	<.0001
geno*age	0		1	27	15.39	0.0005
geno*age	2		1	27	46.03	<.0001

Dependent Variable stom (full stomach weight, grams)

Iteration History

Iteration	Evaluations	-2 Res Log Like	Criterion
0	1	59.96214305	
1	2	53.14850482	0.00033651
2	1	53.14593199	0.00000071
3	1	53.14592672	0.00000000

Convergence criteria met.

Covariance Parameter Estimates

Cov Parm	Estimate
lit	0.1063
Residual	0.1220

Fit Statistics

-2 Res Log Likelihood	53.1
AIC (smaller is better)	57.1
AICC (smaller is better)	57.5
BIC (smaller is better)	58.1

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
geno	1	27	10.77	0.0029
age	1	27	11.06	0.0026
geno*age	1	27	29.53	<.0001
sex	1	27	0.68	0.4173
geno*sex	1	27	0.25	0.6224
age*sex	1	27	0.01	0.9241
geno*age*sex	1	27	0.01	0.9409

Least Squares Means

Effect	geno	age	Estimate	Standard Error	DF	t Value	Pr > |t|
geno*age	0	20	1.1834	0.1870	27	6.33	<.0001
geno*age	0	24	1.3636	0.1668	27	8.17	<.0001
geno*age	2	20	0.9556	0.1847	27	5.17	<.0001
geno*age	2	24	2.2859	0.1692	27	13.51	<.0001

Tests of Effect Slices

Effect	geno	age	Num DF	Den DF	F Value	Pr > F
geno*age		20	1	27	2.07	0.1616
geno*age		24	1	27	43.09	<.0001
geno*age	0		1	27	0.52	0.4783
geno*age	2		1	27	28.21	<.0001

Dependent Variable MT (empty stomach weight, grams)

Iteration History

Iteration	Evaluations	-2 Res Log Like	Criterion
0	1	-73.48329865	
1	3	-74.69446273	0.00001124
2	1	-74.69528030	0.00000001
3	1	-74.69528104	0.00000000

Convergence criteria met.

Covariance Parameter Estimates

Cov Parm	Estimate
lit	0.000909
Residual	0.004760

Fit Statistics

-2 Res Log Likelihood	-74.7
AIC (smaller is better)	-70.7
AICC (smaller is better)	-70.3
BIC (smaller is better)	-69.7

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
geno	1	27	0.05	0.8220
age	1	27	27.66	<.0001
geno*age	1	27	0.71	0.4057
sex	1	27	0.00	0.9561
geno*sex	1	27	1.73	0.1993
age*sex	1	27	0.00	0.9854
geno*age*sex	1	27	1.20	0.2824

Least Squares Means

Effect	geno	age	Estimate	Standard Error	DF	t Value	Pr > |t|
geno*age	0	20	0.2664	0.02615	27	10.19	<.0001
geno*age	0	24	0.3978	0.02331	27	17.06	<.0001
geno*age	2	20	0.2536	0.02587	27	9.80	<.0001
geno*age	2	24	0.4201	0.02393	27	17.56	<.0001

Tests of Effect Slices

Effect	geno	age	Num DF	Den DF	F Value	Pr > F
geno*age		20	1	27	0.17	0.6823
geno*age		24	1	27	0.65	0.4279
geno*age	0		1	27	14.06	0.0009
geno*age	2		1	27	22.32	<.0001

Dependent Variable contents (weight of stomach contents, grams)

Iteration History

Iteration	Evaluations	-2 Res Log Like	Criterion
0	1	61.24710155	
1	2	56.18741132	0.00168990
2	1	56.17686587	0.00001392
3	1	56.17678310	0.00000000

Convergence criteria met.

Covariance Parameter Estimates

Cov Parm	Estimate
lit	0.09857
Residual	0.1371

Fit Statistics

-2 Res Log Likelihood	56.2
AIC (smaller is better)	60.2
AICC (smaller is better)	60.5
BIC (smaller is better)	61.1

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
geno	1	27	9.29	0.0051
age	1	27	7.22	0.0122
geno*age	1	27	24.77	<.0001
sex	1	27	0.74	0.3986
geno*sex	1	27	0.05	0.8255
age*sex	1	27	0.01	0.9116
geno*age*sex	1	27	0.02	0.9016

Least Squares Means

Effect	geno	age	Estimate	Standard Error	DF	t Value	Pr > |t|
geno*age	0	20	0.9220	0.1871	27	4.93	<.0001
geno*age	0	24	0.9670	0.1672	27	5.78	<.0001
geno*age	2	20	0.7057	0.1847	27	3.82	0.0007
geno*age	2	24	1.8665	0.1698	27	10.99	<.0001

Tests of Effect Slices

Effect	geno	age	Num DF	Den DF	F Value	Pr > F
geno*age		20	1	27	1.66	0.2080
geno*age		24	1	27	36.50	<.0001
geno*age	0		1	27	0.03	0.8590
geno*age	2		1	27	21.41	<.0001

Dependent Variable r18s (18s ribosomal RNA)

Iteration History

Iteration	Evaluations	-2 Res Log Like	Criterion
0	1	247.20634815	
1	2	242.96477398	0.00000007
2	1	242.96476801	0.00000000

Convergence criteria met.

Covariance Parameter Estimates

Cov Parm	Estimate
lit	11.3594
Residual	22.6420

Fit Statistics

-2 Res Log Likelihood	243.0
AIC (smaller is better)	247.0
AICC (smaller is better)	247.3
BIC (smaller is better)	247.9

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
geno	1	27	0.02	0.8969
age	1	27	0.90	0.3508
geno*age	1	27	1.19	0.2840
sex	1	27	0.47	0.4969
geno*sex	1	27	0.02	0.8847
age*sex	1	27	0.09	0.7618
geno*age*sex	1	27	0.22	0.6462

Least Squares Means

Effect	geno	age	Estimate	Standard Error	DF	t Value	Pr > |t|
geno*age	0	20	22.5364	2.1813	27	10.33	<.0001
geno*age	0	24	18.5418	1.9516	27	9.50	<.0001
geno*age	2	20	20.7749	2.1523	27	9.65	<.0001
geno*age	2	24	19.9268	1.9882	27	10.02	<.0001

Tests of Effect Slices

Effect	geno	age	Num DF	Den DF	F Value	Pr > F
geno*age		20	1	27	0.67	0.4200
geno*age		24	1	27	0.52	0.4753
geno*age	0		1	27	1.86	0.1836
geno*age	2		1	27	0.08	0.7745

Dependent Variable fas (fatty acid synthase)

Iteration History

Iteration	Evaluations	-2 Res Log Like	Criterion
0	1	83.86814094	
1	2	83.86772763	0.00000000

Convergence criteria met.

Covariance Parameter Estimates

Cov Parm	Estimate
lit	0.001111
Residual	0.3885

Fit Statistics

-2 Res Log Likelihood	83.9
AIC (smaller is better)	87.9
AICC (smaller is better)	88.2
BIC (smaller is better)	88.8

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
geno	1	27	81.19	<.0001
age	1	27	90.26	<.0001
geno*age	1	27	76.61	<.0001
sex	1	27	14.35	0.0008
geno*sex	1	27	18.06	0.0002
age*sex	1	27	13.31	0.0011
geno*age*sex	1	27	16.16	0.0004

Least Squares Means

Effect	geno	age	Estimate	Standard Error	DF	t Value	Pr > |t|
geno*age	0	20	0.1136	0.1978	27	0.57	0.5706
geno*age	0	24	0.2656	0.1741	27	1.53	0.1387
geno*age	2	20	0.1619	0.1977	27	0.82	0.4202
geno*age	2	24	3.5908	0.1806	27	19.88	<.0001

Tests of Effect Slices

Effect	geno	age	Num DF	Den DF	F Value	Pr > F
geno*age		20	1	27	0.03	0.8638
geno*age		24	1	27	177.06	<.0001
geno*age	0		1	27	0.33	0.5687
geno*age	2		1	27	163.97	<.0001

Dependent Variable pparg (peroxisome proliferator activated receptor gamma)

Iteration History

Iteration	Evaluations	-2 Res Log Like	Criterion
0	1	70.16382794	
1	2	58.18365917	0.44501442
2	1	55.36791433	0.15055178
3	1	54.17569461	0.04124819
4	1	53.83609670	0.00561167
5	1	53.79250837	0.00015134
6	1	53.79141272	0.00000013
7	1	53.79141180	0.00000000

Convergence criteria met.

Covariance Parameter Estimates

Cov Parm	Estimate
lit	0.3189
Residual	0.09211

Fit Statistics

-2 Res Log Likelihood	53.8
AIC (smaller is better)	57.8
AICC (smaller is better)	58.1
BIC (smaller is better)	58.8

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
geno	1	27	41.29	<.0001
age	1	27	6.00	0.0210
geno*age	1	27	7.60	0.0103
sex	1	27	4.69	0.0394
geno*sex	1	27	10.27	0.0035
age*sex	1	27	3.54	0.0709
geno*age*sex	1	27	11.07	0.0025

Least Squares Means

Effect	geno	age	Estimate	Standard Error	DF	t Value	Pr > |t|
geno*age	0	20	0.5219	0.2731	27	1.91	0.0667
geno*age	0	24	1.1255	0.2380	27	4.73	<.0001
geno*age	2	20	0.8601	0.2714	27	3.17	0.0038
geno*age	2	24	1.9722	0.2394	27	8.24	<.0001

Tests of Effect Slices

Effect	geno	age	Num DF	Den DF	F Value	Pr > F
geno*age		20	1	27	6.00	0.0211
geno*age		24	1	27	48.01	<.0001
geno*age	0		1	27	2.78	0.1072
geno*age	2		1	27	9.44	0.0048

Dependent Variable Scd1 (stearoyl CoA desaturase 1)

Iteration History

Iteration	Evaluations	-2 Res Log Like	Criterion
0	1	29.04786022	
1	3	29.00604105	0.00000001
2	1	29.00604076	0.00000000

Convergence criteria met.

Covariance Parameter Estimates

Cov Parm	Estimate
lit	0.002500
Residual	0.08642

Fit Statistics

-2 Res Log Likelihood	29.0
AIC (smaller is better)	33.0
AICC (smaller is better)	33.4
BIC (smaller is better)	34.0

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
geno	1	27	179.59	<.0001
age	1	27	78.33	<.0001
geno*age	1	27	37.02	<.0001
sex	1	27	0.85	0.3654
geno*sex	1	27	4.04	0.0544
age*sex	1	27	0.13	0.7214
geno*age*sex	1	27	1.19	0.2849

Least Squares Means

Effect	geno	age	Estimate	Standard Error	DF	t Value	Pr > |t|
geno*age	0	20	0.06768	0.09611	27	0.70	0.4873
geno*age	0	24	0.3631	0.08483	27	4.28	0.0002
geno*age	2	20	0.7142	0.09585	27	7.45	<.0001
geno*age	2	24	2.0849	0.08783	27	23.74	<.0001

Tests of Effect Slices

Effect	geno	age	Num DF	Den DF	F Value	Pr > F
geno*age		20	1	27	24.12	<.0001
geno*age		24	1	27	213.28	<.0001
geno*age	0		1	27	5.31	0.0291
geno*age	2		1	27	111.15	<.0001

Dependent Variable Me1 (malic enzyme)

Iteration History

Iteration	Evaluations	-2 Res Log Like	Criterion
0	1	16.19376478	
1	1	16.19376478	0.00000000

Convergence criteria met.

Covariance Parameter Estimates

Cov Parm	Estimate
lit	0
Residual	0.06253

Fit Statistics

-2 Res Log Likelihood	16.2
AIC (smaller is better)	18.2
AICC (smaller is better)	18.3
BIC (smaller is better)	18.7

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
geno	1	27	34.81	<.0001
age	1	27	42.76	<.0001
geno*age	1	27	28.34	<.0001
sex	1	27	2.38	0.1342
geno*sex	1	27	4.68	0.0395
age*sex	1	27	2.24	0.1462
geno*age*sex	1	27	5.39	0.0280

Least Squares Means

Effect	geno	age	Estimate	Standard Error	DF	t Value	Pr > |t|
geno*age	0	20	0.03981	0.07908	27	0.50	0.6187
geno*age	0	24	0.1311	0.06956	27	1.88	0.0703
geno*age	2	20	0.08312	0.07908	27	1.05	0.3025
geno*age	2	24	0.9739	0.07219	27	13.49	<.0001

Tests of Effect Slices

Effect	geno	age	Num DF	Den DF	F Value	Pr > F
geno*age		20	1	27	0.15	0.7016
geno*age		24	1	27	70.68	<.0001
geno*age	0		1	27	0.75	0.3938
geno*age	2		1	27	69.21	<.0001

Dependent Variable Lep (leptin)

Iteration History

Iteration	Evaluations	-2 Res Log Like	Criterion
0	1	146.38819674	
1	2	136.65225391	0.00009341
2	1	136.64885208	0.00000025
3	1	136.64884313	0.00000000

Convergence criteria met.

Covariance Parameter Estimates

Cov Parm	Estimate
lit	1.4867
Residual	1.1968

Fit Statistics

-2 Res Log Likelihood	136.6
AIC (smaller is better)	140.6
AICC (smaller is better)	141.0
BIC (smaller is better)	141.6

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
geno	1	26	44.27	<.0001
age	1	26	3.20	0.0854
geno*age	1	26	9.87	0.0042
sex	1	26	4.98	0.0345
geno*sex	1	26	0.34	0.5673
age*sex	1	26	1.40	0.2476
geno*age*sex	1	26	0.02	0.8931

Least Squares Means

Effect	geno	age	Estimate	Standard Error	DF	t Value	Pr > |t|
geno*age	0	20	0.9315	0.6586	26	1.41	0.1691
geno*age	0	24	1.3290	0.5850	26	2.27	0.0316
geno*age	2	20	2.1231	0.6512	26	3.26	0.0031
geno*age	2	24	4.6518	0.6075	26	7.66	<.0001

Tests of Effect Slices

Effect	geno	age	Num DF	Den DF	F Value	Pr > F
geno*age		20	1	26	5.76	0.0238
geno*age		24	1	26	51.62	<.0001
geno*age	0		1	26	0.20	0.6556
geno*age	2		1	26	8.06	0.0087

Dependent Variable Gpam (glycerol 3-phosphate acyltransferase)

Iteration History

Iteration	Evaluations	-2 Res Log Like	Criterion
0	1	33.05888350	
1	2	28.65300436	0.03532677
2	1	27.79040136	0.00900110
3	1	27.57877271	0.00091757
4	1	27.55889803	0.00001273
5	1	27.55863814	0.00000000

Convergence criteria met.

Covariance Parameter Estimates

Cov Parm	Estimate
lit	0.07571
Residual	0.05606

Fit Statistics

-2 Res Log Likelihood	27.6
AIC (smaller is better)	31.6
AICC (smaller is better)	31.9
BIC (smaller is better)	32.5

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
geno	1	27	57.09	<.0001
age	1	27	7.55	0.0106
geno*age	1	27	38.30	<.0001
sex	1	27	0.39	0.5350
geno*sex	1	27	8.65	0.0066
age*sex	1	27	0.21	0.6493
geno*age*sex	1	27	7.72	0.0098

Least Squares Means

Effect	geno	age	Estimate	Standard Error	DF	t Value	Pr > |t|
geno*age	0	20	0.1904	0.1468	27	1.30	0.2055
geno*age	0	24	0.2473	0.1302	27	1.90	0.0683
geno*age	2	20	0.2886	0.1452	27	1.99	0.0571
geno*age	2	24	1.2344	0.1317	27	9.37	<.0001

Tests of Effect Slices

Effect	geno	age	Num DF	Den DF	F Value	Pr > F
geno*age		20	1	27	0.83	0.3692
geno*age		24	1	27	107.32	<.0001
geno*age	0		1	27	0.08	0.7742
geno*age	2		1	27	23.28	<.0001

Dependent Variable Tkt (transketolase)

Iteration History

Iteration	Evaluations	-2 Res Log Like	Criterion
0	1	66.76063509	
1	2	65.71710139	0.06421459
2	1	65.70152914	0.00128234
3	1	65.70122878	0.00000057
4	1	65.70122865	0.00000000

Convergence criteria met.

Covariance Parameter Estimates

Cov Parm	Estimate
lit	0.06094
Residual	0.2119

Fit Statistics

-2 Res Log Likelihood	65.7
AIC (smaller is better)	69.7
AICC (smaller is better)	70.1
BIC (smaller is better)	70.7

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
geno	1	26	18.20	0.0002
age	1	26	4.38	0.0464
geno*age	1	26	8.89	0.0061
sex	1	26	0.06	0.8057
geno*sex	1	26	2.33	0.1389
age*sex	1	26	2.53	0.1239
geno*age*sex	1	26	2.56	0.1219

Least Squares Means

Effect	geno	age	Estimate	Standard Error	DF	t Value	Pr > |t|
geno*age	0	20	0.4578	0.1868	26	2.45	0.0213
geno*age	0	24	0.4764	0.1669	26	2.85	0.0084
geno*age	2	20	0.6391	0.1845	26	3.46	0.0019
geno*age	2	24	1.5007	0.1779	26	8.44	<.0001

Tests of Effect Slices

Effect	geno	age	Num DF	Den DF	F Value	Pr > F
geno*age		20	1	26	0.76	0.3902
geno*age		24	1	26	28.50	<.0001
geno*age	0		1	26	0.01	0.9414
geno*age	2		1	26	11.30	0.0024

Dependent Variable Fdps (farnesyl diphosphate synthase)

Iteration History

Iteration	Evaluations	-2 Res Log Like	Criterion
0	1	20.83749854	
1	2	17.54310505	0.00446460
2	1	17.41398343	0.00031731
3	1	17.40555616	0.00000202
4	1	17.40550477	0.00000000

Convergence criteria met.

Covariance Parameter Estimates

Cov Parm	Estimate
lit	0.03052
Residual	0.04930

Fit Statistics

-2 Res Log Likelihood	17.4
AIC (smaller is better)	21.4
AICC (smaller is better)	21.8
BIC (smaller is better)	22.4

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
geno	1	27	28.81	<.0001
age	1	27	13.40	0.0011
geno*age	1	27	13.58	0.0010
sex	1	27	0.94	0.3420
geno*sex	1	27	13.49	0.0010
age*sex	1	27	3.11	0.0891
geno*age*sex	1	27	9.69	0.0043

Least Squares Means

Effect	geno	age	Estimate	Standard Error	DF	t Value	Pr > |t|
geno*age	0	20	0.2730	0.1076	27	2.54	0.0172
geno*age	0	24	0.4928	0.09618	27	5.12	<.0001
geno*age	2	20	0.3860	0.1061	27	3.64	0.0011
geno*age	2	24	1.1011	0.09781	27	11.26	<.0001

Tests of Effect Slices

Effect	geno	age	Num DF	Den DF	F Value	Pr > F
geno*age		20	1	27	1.27	0.2704
geno*age		24	1	27	46.43	<.0001
geno*age	0		1	27	2.32	0.1393
geno*age	2		1	27	24.55	<.0001

Dependent Variable Insig1 (insulin induced gene 1)

Iteration History

Iteration	Evaluations	-2 Res Log Like	Criterion
0	1	44.38680636	
1	1	44.38680636	0.00000000

Convergence criteria met.

Covariance Parameter Estimates

Cov Parm	Estimate
lit	0
Residual	0.1425

Fit Statistics

-2 Res Log Likelihood	44.4
AIC (smaller is better)	46.4
AICC (smaller is better)	46.5
BIC (smaller is better)	46.9

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
geno	1	25	14.19	0.0009
age	1	25	22.76	<.0001
geno*age	1	25	4.11	0.0533
sex	1	25	0.99	0.3293
geno*sex	1	25	8.65	0.0070
age*sex	1	25	4.01	0.0562
geno*age*sex	1	25	9.44	0.0051

Least Squares Means

Effect	geno	age	Estimate	Standard Error	DF	t Value	Pr > |t|
geno*age	0	20	0.2086	0.1379	25	1.51	0.1428
geno*age	0	24	0.5336	0.1050	25	5.08	<.0001
geno*age	2	20	0.4146	0.1194	25	3.47	0.0019
geno*age	2	24	1.2203	0.1090	25	11.20	<.0001

Tests of Effect Slices

Effect	geno	age	Num DF	Den DF	F Value	Pr > F
geno*age		20	1	25	1.28	0.2695
geno*age		24	1	25	20.58	0.0001
geno*age	0		1	25	3.52	0.0725
geno*age	2		1	25	24.84	<.0001

Dependent Variable Lpl (lipoprotein lipase)

Iteration History

Iteration	Evaluations	-2 Res Log Like	Criterion
0	1	62.25696858	
1	2	58.74797299	0.17645505
2	1	58.34160656	0.03528263
3	1	58.25859092	0.00200552
4	1	58.25418206	0.00000779
5	1	58.25416553	0.00000000

Convergence criteria met.

Covariance Parameter Estimates

Cov Parm	Estimate
lit	0.1857
Residual	0.1465

Fit Statistics

-2 Res Log Likelihood	58.3
AIC (smaller is better)	62.3
AICC (smaller is better)	62.6
BIC (smaller is better)	63.2

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
geno	1	24	125.88	<.0001
age	1	24	1.31	0.2638
geno*age	1	24	11.32	0.0026
sex	1	24	2.09	0.1613
geno*sex	1	24	0.06	0.8033
age*sex	1	24	0.21	0.6519
geno*age*sex	1	24	2.53	0.1248

Least Squares Means

Effect	geno	age	Estimate	Standard Error	DF	t Value	Pr > |t|
geno*age	0	20	0.4155	0.2362	24	1.76	0.0913
geno*age	0	24	0.3328	0.2119	24	1.57	0.1293
geno*age	2	20	1.3842	0.2295	24	6.03	<.0001
geno*age	2	24	2.1315	0.2139	24	9.96	<.0001

Tests of Effect Slices

Effect	geno	age	Num DF	Den DF	F Value	Pr > F
geno*age		20	1	24	29.00	<.0001
geno*age		24	1	24	113.59	<.0001
geno*age	0		1	24	0.07	0.7966
geno*age	2		1	24	5.67	0.0255

VITA

Mary D. Brown was born in Beaufort, SC on November 13, 1980. She graduated from Winthrop University in December 2003 with a Bachelors of Science degree in Human Nutrition with a concentration in Dietetics. She then went on to accomplish her Masters of Science degree in Nutrition at the University of Tennessee at Knoxville in May of 2005. Mary will be attending the dietetic internship at OSF St. Francis Medical Center in Peoria, Illinois in the fall of 2005.

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