OBESITY: A GROWING CONCERN ABOUT FETAL NUTRITION

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Master of Arts

By
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The undersigned, appointed by the Dean of the Graduate School, have examined the Thesis entitled

OBESITY: A GROWING CONCERN ABOUT FETAL NUTRITION

Presented by Benjamin Lloyd Coe
A candidate for the degree of Master of Arts

And hereby certify that in their opinion it is worthy of acceptance.

Dr. Frederick vom Saal

Dr. Joel Maruniak

Dr. Pamela Hinton

DEDICATION

I would like to dedicate this thesis to my parents whose unconditional love, even though I have changed my plans so many times, has always been there to support me. It is because of you, and the way in which you raised me that has allowed me to make it this far in life.

To my extended family, you all have always been there for me, and for that I am eternally grateful. I know that you are all always there for me, whenever I might need you.

I would also like to thank the other graduate students in the lab, whose wit, and humor make everyday in our office one that is not to be missed. Maren and James, you guys have been my comic relief when I needed it the most.

ACKNOWLEDGMENTS

I would like to thank Dr Fred vomSaal for his guidance and assistance throughout my work in his lab. It is through Dr vomSaal's intellect and advice that I have been able to complete this work. If he had not decided to take a chance on me, I honestly do not know if I would have been able to fulfill the requirements to receive this masters' degree.

Additionally, I would like to thank the other members of our lab for their help throughout this project. Whether it was birthing pups, weighing animals, or performing the final collections, I knew that I could count on you all to help me when I needed you. This project would never have been completed without your assistance.

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OBESITY: A GROWING CONCERN ON FETAL NUTRITION

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ABSTRACT

Obesity is a growing epidemic and a major health concern in the United States and elsewhere. The Fetal Basis of Adult Disease (FBAD) theory holds that events that occur during development can permanently alter gene expression throughout the lifetime of the individual. A link has emerged between fetal nutrition, birth weight, and metabolic profile in adulthood. Metabolic diseases represent a host of conditions relating to abnormal "programming" of nutrient management. We have developed a CD-1 mouse model which shows that fetuses within two body weight ranges at birth are at increased risk for developing metabolic diseases such as obesity, hypertension, cardiovascular disease, and Type II diabetes. It is not just individuals that are born with abnormally high birth weights (macrosomia) that are at risk for becoming overweight later in life. Paradoxically, this risk also applies for individuals who are born with extremely low birth weights, which are termed intrauterine growth restricted (IUGR). Mice identified with IUGR at birth show a dramatic increase in body weight during the first week post-weaning, which results in adult obesity and an altered metabolic profile for the remainder of their adult life. Preliminary studies indicate that when examined in adulthood, adipocytes in males identified with IUGR or macrosomia at birth showed evidence of differences in "programming" of expression of genes involved in fat cell differentiation and function. Certain xenoestrogens in the environment are known to interrupt the process of cell differentiation. Utilizing this model, we will be able to test the effects of

these chemicals on individuals that already have a metabolic deficit. By examining the relationship of fetal nutrition and growth with endocrine disruption, greater evidence may emerge for the need of regulation of endocrine disrupting chemicals.

Introduction

With the growing prevalence of obesity and its related metabolic diseases in the United States, the need for an explanation and a working animal model to evaluate treatments is greatly needed. There is an increasing volume of literature on the subject of a fetal basis of obesity and metabolic disease. The studies on this subject point to two fetal conditions that have a higher incidence of obesity and metabolic disease in adulthood, and figure 1 details the visual differences between these two distinct body types. The first of these is Intrauterine Growth Restriction (IUGR). IUGR is characterized by birth in the bottom fifth percentile of full term offspring (Xiu 2003), but this also applies to premature babies. Several factors may lead to IUGR, including maternal malnutrition, and maternal environment, such as endocrine disrupting chemicals. The second is Macrosomia or birth weight in the top fifth percentile for a full term offspring. Just as several factors may increase the incidence of IUGR, several factors also play a role in the development of macrosomia. These characteristics include: gestational diabetes, maternal over nutrition, and the maternal environment including endocrine disrupting chemicals.

Background

Current animal models of metabolic disease typically involve the gross manipulation of maternal diet. The manipulations involve one of two methods: either malnutrition (Jones 1982) or increasing fat intake. The studies using malnutrition showed similar effects to the epidemiological studies performed on the male children of Dutch women who were food deprived during pregnancy due to the German invasion

during WWII (Ravelli 1976). Studies have been performed on sheep, where the mothers were purposefully malnourished to observe the effect on their fetuses. As would be expected, the mothers who were malnourished the longest had the lowest birth weights among their offspring. These offspring also had higher blood glucose levels and increased blood pressure in adulthood (Oliver 2002). The studies where the maternal fat intake was increased produced larger than normal offspring, which retained their increased weight throughout life. Offspring produced through increase in maternal fat intake were defined as being macrosomic (vomSaal, unpublished).

The role that the maternal environment plays in development was noted in higher vertebrates, such as rabbits and horses in the early 1900s. Dr John Hammond of Cambridge University performed experiments in which he reduced the size of the uterus, by either crossing two strains of horses thereby restricting the maximal growth in utero, or by using a larger number of rabbit fetuses to increase litter size (Walton 1938, Hammond 1933). In each Hammond study, fetal growth was limited by the amount of space in the uterus, and all other factors affecting development, i.e. food intake and chemical exposure, were held constant. A further study to explore the restriction of uterine size was performed by transplanting fetuses from a small breed of pig into the uterus of a large breed, and vice versa. The fetuses were collected four days prior to parturition, and substantial changes from the norm were found in both breeds (Nathnielsz 2000). Epidemiological studies have been performed on humans using homozygous twins that shared a single placenta. This condition occurs in roughly 70-80% of all cases of human homozygous twins. The placenta is never shared equally, but is more often an unequal sharing that leads to a whole host of differences including malnutrition and

growth retardation. Typically, when one of the two twins develops diabetes later in life, it is the twin that was born with the lower birth weight and this twin is also more susceptible to cardiopulmonary disease (Machin 1996, Machin et al 1996). Past studies have shown that macrosomia can be induced in mice by selective fetal reduction resulting in singleton litters (Ogata 1988).

Purpose

Due to the need for an animal model of obesity and its related metabolic diseases, an attempt to alter the fetal environment in order to induce either IUGR, or macrosomia was attempted. In this experiment, the birth weight of individual mouse pups are altered by either overcrowding the horn of the uterus through super ovulation, caused by hemi-ovariectomy, creating smaller pups, or by under-crowding the uterus through fetus reduction, giving the fetus the ability to grow to a larger size. There is support to the crowded horn uterus model for inducing IUGR. Figure 2 is a schematic of a mouse with a crowded uterine horn that was induced by the compensatory response to hemiovariectomization. The crowded horn causes a decrease in blood flow to the different uterine segments, and thus decreases fetal weight in the offspring (Yang and vom Saal Unpublished). This is an important observance for the validity of the crowded uterus model for IUGR. The effects of IUGR do not end at the end of fetal life; however, further effects begin developing later in life. Most IUGR individuals exhibit a period of 'catch up' growth. This period of catch up growth has been associated with increased risks of cardiovascular deficit, and with insulin resistance syndrome (IRS). The growth velocity is even more strongly associated with IRS than individual endpoints (Yajnik,

2001). This study evaluated the effect of the crowded uterus as to its validity as an animal model for human adult disease based on its effects on neonatal birth weight, adult weight, and adult glucose tolerance.

Procedure

Animals, Husbandry, and Mating Procedures.

CD-1 mice (*Mus domesticus*) were purchased from Charles River Breeding Laboratories (Wilmington, MA) in December 2004, and were maintained as an out-bred colony since that time. The mice were housed in 18 x 29 x 13-cm polypropylene cages on corncob bedding. Pregnant and lactating mice were fed Purina mouse breeder chow 5008 (soy-based, Ralston-Purina, St. Louis, MO). After weaning, offspring were fed Purina standard lab chow 5001(soy-based). Water was provided *ad libitum* in glass bottles and was purified by ion exchange followed by a series of carbon filters. Rooms were maintained at 25 ± 2°C under a 12:12 light: dark (L: D) cycle, with the lights on at 1030 h.

Two methods of mating were used, depending on group within the study. Group 1 consisted of mice that were to be collected for fetal assays and tissues. Adult female mice that had given birth to one previous litter had the left ovary removed. The females were anesthetized using a cocktail of drugs (Ketamine 25 mg/ml, Xylazine 1 mg/ml, Acepromazine 0.5 mg/ml). The dose is 0.0036 ml/g for an average of 90µl per animal. Once anesthetized, each female had her left ovary removed and the wound was closed with a wound clip. The left ovary was chosen for removal due to the normal anatomy of the female mouse. Normally, the left ovarian artery branches off from the left renal

artery. The right ovarian artery, however, branches from the descending aorta, as demonstrated in figure 3. Five days following surgery, females were placed in groups of 3 with a stud male for 4 hours each day beginning at 0830 h. Mating was verified by the presence of a vaginal plug (day 0 of gestation). After mating, pregnant females were housed three per cage. The pregnant dams were singly housed on gestation day 16. On gestation day 18, the females were killed by asphyxiation followed by decapitation, and blood was collected in 12 x 75 mm borosylicate glass tubes. Fetuses were killed by decapitation, and blood was collected by pipette using 200 µl heparin-coated micro-capillary tubes and then stored in 200 µl Eppendorf tubes. All blood samples were stored overnight at 4°C. The blood was centrifuged, and serum was stored for future analysis. In addition to fetal blood, the following organs and data were collected for each individual pup: liver, brain, pup weight, and intrauterine position of the pup relative to the ovary or cervix as well as in relation to the sex of adjacent pups.

Group 2 consisted of mice that were to be carried to term following hemi-ovariectomy. Adult, hemi-ovariectomized female mice that had given birth to one previous litter (as described above) were singly housed with a stud male for up to 14 days, beginning five days after surgery. Pregnancy was verified by animal weight of at least 40 grams, post pairing. All animals were allowed to deliver naturally on gestation day 19 (postnatal day 1). On the day of weaning on PND 21, all mothers were euthanized with CO_2, and were necropsied to verify location of implantation sites, as well as the location of the blood supply to the uterus. Any abnormalities were noted. All pups were toe clipped, so that each individual could be identified. Each clipping occurred on the back paws of the animal by a set numbering system. Mouse pups were not handled

between the day of birth and the day of weaning. Mouse pups were weaned at 21 days and housed by sex until they were 3-months old. Body weights were determined for all animals at weaning and each week thereafter. At the end of the study, animals were killed by asphyxiation and blood was collected by decapitation in 12 x 75 mm borosilicate glass tubes. Blood samples were stored overnight at 4°C. The blood was centrifuged, and serum was stored for future analysis. In addition to blood, the following organs were collected: liver, kidney, gonadal fat, inguinal fat, adrenal fat, heart, right epididymis, spleen, right gastrocnemius, and both testes. Weights were taken on all of the preceding organs.

Micro MRI analysis of body fat in a lean and fat adult male mouse in relation to fat pad weight and body weight at autopsy

Adult male mice were anesthetized using an isoflourane respirator set at 5% isoflourane. Anesthetization was maintained at 2.5%. Animals were monitored for temperature, breathing rate, and heart rate while being scanned. Adult male mice were placed in the micro MRI, after which body weight and combined weight of 3 fat pads (epididymal, renal and inguinal) were determined at autopsy.

Tests Performed on organs collected

Several organs were collected, as previously noted. To date, the only collected tissue to be analyzed is the gonadal fat pad. The left fat pad, which was frozen at the time of necropsy, was used to perform RT PCR to observe the expression of several key genes. Gene specific real-time RT-PCR analysis. mRNA was determined for each gene by reverse transcription (RT) and PCR amplification, which was carried out on an ABI

PRISM 7700 Sequence Detector System. Primers/probe sets were obtained from Applied Biosystems (Foster City, CA). All RNA samples were run in duplicate, Linearity and specificity was confirmed in each run. The genes that were examined were: GLUT4, Lpl, PPARγ, CYP19, 11βHSD, and C/EBPα. The basis for the selection of these genes is presented in table 2.

Fasting Glucose Measurements

Glucose measurements were taken using the methods of Heine et al. A Reli-On Ultima glucometer was used. The glucose challenge concentration was 1.5mg/g body weight glucose in 0.9% saline solution and was given I.P. (Heine 2000).

Statistical Analyses

All analyses were conducted using the Statistical Analyzing System, Mixed Model procedure (SAS Institute, Inc., Cary, NC). Planned comparisons were made using the LSmeans procedure in SAS, using $P < 0.05$ as the criterion for statistical significance.

Litter Information

Through the course of the experiment, 57 litters were produced for the postnatal study. This gave a total of 605 offspring, of which 585 survived to weaning. This provided a neonatal mortality rate of 3.3%. Twenty five percent of all pre-weaning deaths occurred in IUGR animals. Additionally, only 0.05% of the pre-weaning deaths were in macrosomic animals. Two hundred ninety seven (297) females and 308 males made up the total number of animals. Analysis was performed to identify the top and

bottom fifth percentile weights based on calculating the mean and standard deviations for body weight at birth for males and for females (males were significantly heavier than females). In males, the bottom fifth percentile was any animal born weighing less than 1.25g, the average was 1.64g, and the top fifth percentile was any animal that was 2.01g or heavier. Females were identified as being in the lower fifth percentile if they weighed less than 1.24 g, their average weight was 1.58g, and they were in the top fifth percentile if they weighed greater than 1.95g. From the animals born to each group, 16 were randomly selected for collection as representative of all animals with similar characteristics.

Growth Information

Following categorization of birth weight, animals were further segregated based upon their post-weaning growth rate between weaning on PND 21 and PND 28. The birth weight classifications of IUGR, average and macrosomia were further divided into thirds due to the appearance of a tri-modal distribution of rate of growth during this week, placing five to six animals in each subgroup. As can be seen from this data, and in the graphs of figures 5 and 6, it is the IUGR animals that have the highest average post weaning growth rate. These animals undergo a catch-up growth phase where they not only catch up to the animals that are born heavier than them, but surpass all non-macrosomic animals and become as heavy as those identified with macrosomia at birth. This catch-up growth phase in humans has been associated with poor glucose tolerance (Crowther 1998).

It is important to note that the IUGR males that underwent the rapid post-natal growth phase retain their post-weaning growth profile, and in fact that it becomes more pronounced as time goes on. This post-natal growth pattern allows the IUGR animals to become statistically the same in weight as macrosomic animals by as soon as four weeks post-weaning. This can be seen all the way into adulthood. When looking at the adult males in figure 6 the IUGR animals, which grew with at least 100% growth in week 3-4, were equivalent to the macrosomia animals that also had the highest growth rate in the same week. The trend in females was different, with all females, regardless of birth weight, ending up at the same body weight by 7 weeks of age.

Micro-MRI Imaging of Body Fat

Two animals, one characteristic of the overweight animals produced, and one characteristic of the normal weight animals produced were subjected to micro-magnetic resonance imaging (mMRI). The mMRI was used to observe the visual differences in the size of the fat pads in the animals these differences are illustrated in figure 7. The NMR peaks show the difference in fat water ratio of the lean and the fat animal. The mMRI accurately predicted the amount of body fat, this is shown in figure 8 and table 2, which will allow use of this method to determine peak periods of fat accumulation for examination of adipocyte gene activity in future studies.

Epididymal Fat Pad Gene Expression by Real-time RT-PCR

The gene expression within the gonadal fat pad was assayed for six genes. The graphs in figure 9 present the data that were obtained from the PCR analysis. As can be

seen, the results are varied by gene product. The general trend suggests an increase in the expression of all of these genes in the IUGR animal with the exception of CYP19.

Glucose Information

The results presented in figure 10 show that there is no statistical difference between the glucose sensitivity of the low (IUGR) and average birth weight animals, however there is a difference between these two groups and the high birth weight animals (macrosomia group). The high birth weight animals appear to be more sensitive to glucose than the low or average birth weight animals. Additionally, there is no significant statistical difference between animals with a low or average postnatal growth rate, but there is a difference in animals with a rapid rate of post natal growth.

Discussion

The results of these experiments demonstrate the capability of the crowded uterine horn to induce IUGR and macrosomia in siblings. As would be expected, there was a large number of animals that did not meet the requirements for either IUGR or macrosomia, purely by the definition of these conditions representing animals in the bottom fifth percentile and top 95 percentile for body weight at birth. The major finding from this study is that IUGR fetuses go through a period of rapid catch-up growth immediately following weaning. The consequence of this rapid period of growth is that IUGR males end up significantly heavier than males who were not categorized at birth with macrosomia, which were the heaviest animals in adulthood.

Additionally, the results show the induction of obesity and impaired glucose tolerance in the male mouse as a result of experiencing a rapid weight gain during the week following weaning. In contrast, as can be seen in figure 10-A, there was no significant difference between IUGR and normal body weight males in their response to a glucose challenge. In viewing the results, it is important to note the two seemingly necessary factors for induction of obesity and impaired glucose tolerance through this model. The first of these is a low birth weight. The IUGR animals as a whole go through a catch-up growth phase where their weight rapidly increases to that of their litter cohorts. This rapid growth phase makes up the second necessary factor. The results in Figure 10-B show that a postnatal growth rate in the top one third of all of those animals within their particular birth weight class results in impaired glucose tolerance. These data are interesting in that in humans, these same two factors when coupled together appear to create the greatest effect on the metabolic profile of the afflicted individuals (Oken 2003).

The association of these two factors together is shown further by looking at the IUGR animals that do not go through the rapid postnatal growth. The IUGR animals with the lowest level of postnatal growth have a growth rate equivalent to the macrosomia animals with the highest postnatal growth rate. This sub-group of IUGR animals does not put on the amount of body weight that is seen in the other IUGR animals that experience almost a 2-fold increase in body weight during the week after weaning.

The data in this study thus suggest that low birth weight alone does not causes obesity or the impaired glucose tolerance. However, low birth weight can result in the

greatest rate of growth after weaning, and rapid postnatal growth the factor that is associated with impaired glucose tolerance.

The decision of fast the animals overnight prior to collection of fat appears to have resulted in suppression of the activity of a number of the candidate genes we examined in epididymal adipocytes. In future studies we will focus on examining gene activity in adipocytes during the period when animals with IUGR and macrosomia are experiencing markedly different rates of growth. This study was thus valuable in identifying that it is the immediate postnatal period that should be the focus of future studies. The importance of this finding is that this demonstrates that the crowded uterine horn model and the animals with IUGR and macrosomia that result from this model exhibit the same combination of characteristics seen in humans with IUGR and macrosomia. Obesity in humans with IUGR requires a period of rapid postnatal growth that typically occurs in childhood (Oken, 2003), while obesity in babies with macrosomia does not involve this period of rapid postnatal growth.

APPENDIX I

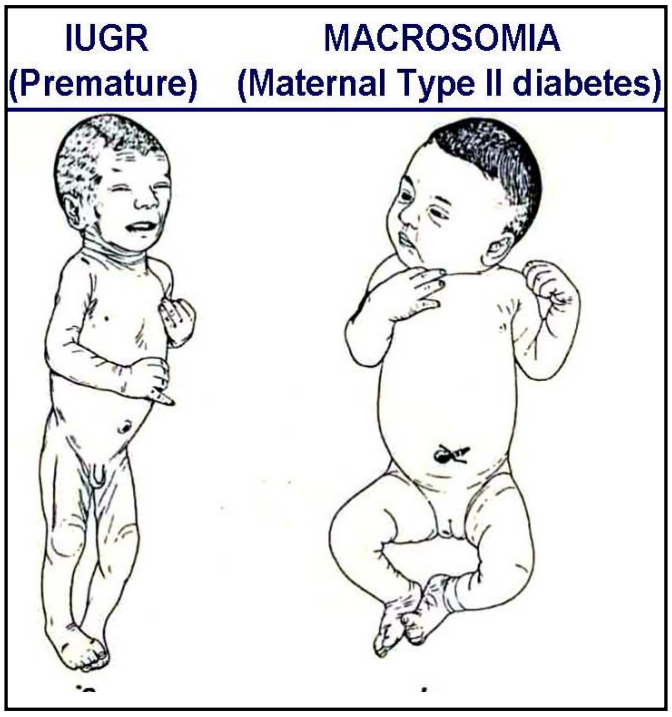


Figure 1
Demonstration of differences between IUGR and macrosomia.

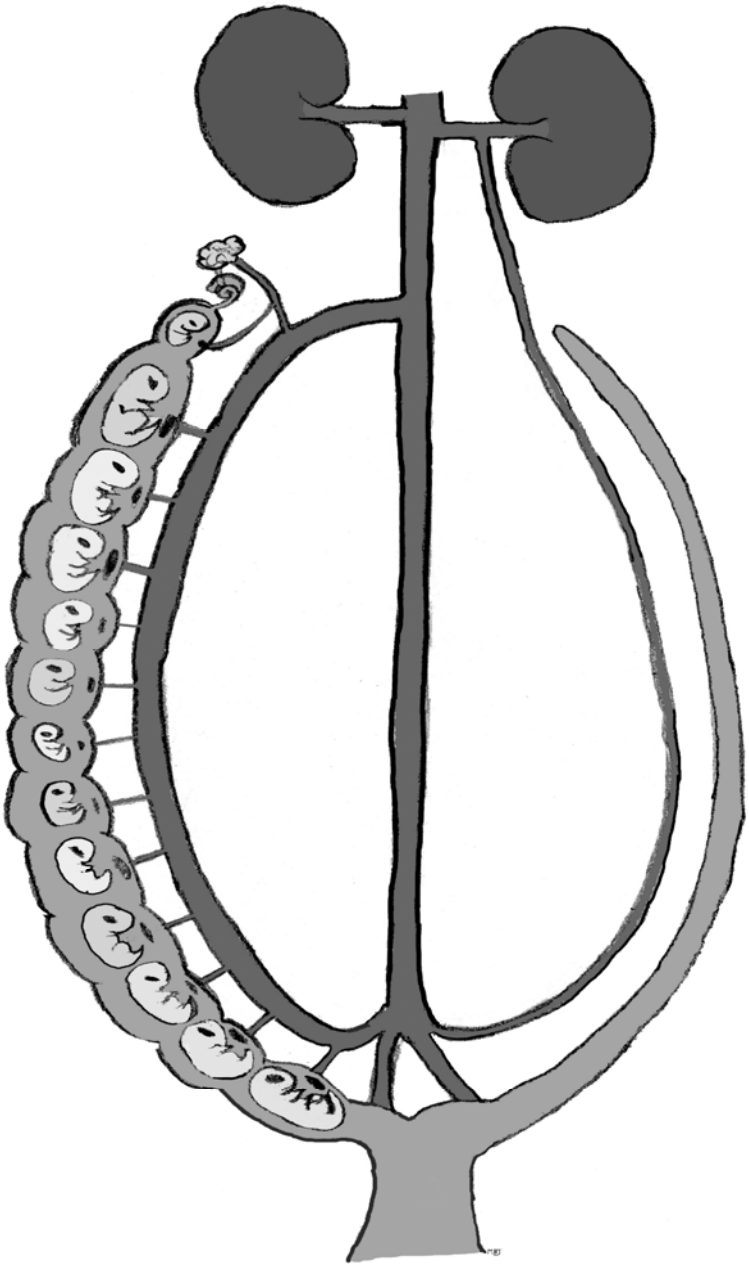


Figure 2
Schematic of the crowded uterine horn model. Note the shunted artery from the ovarian artery to the first fetus.

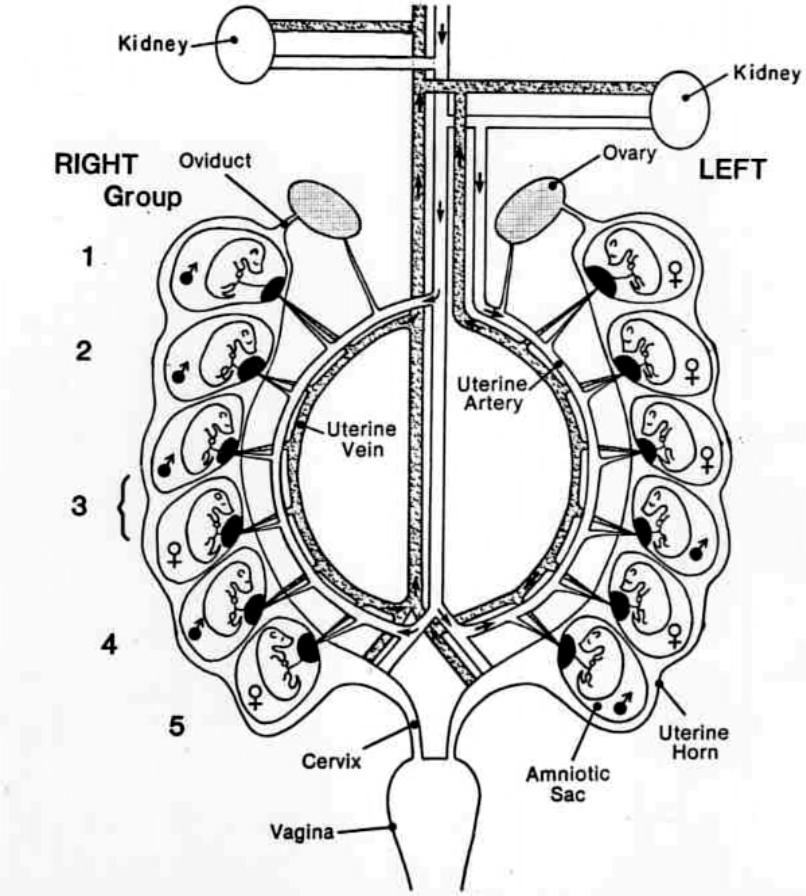
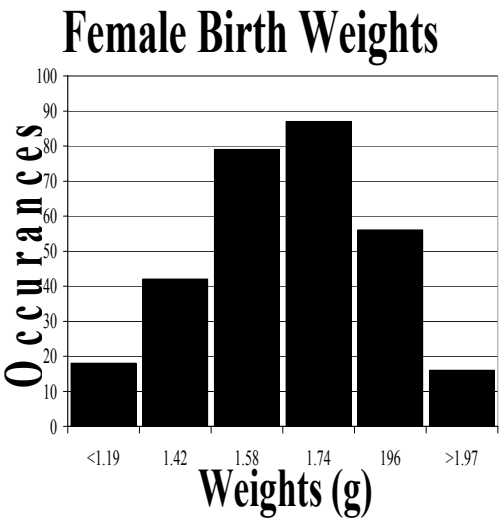


Figure 3.
The normal reproductive tract of an adult female mouse (Even et al, 1994).



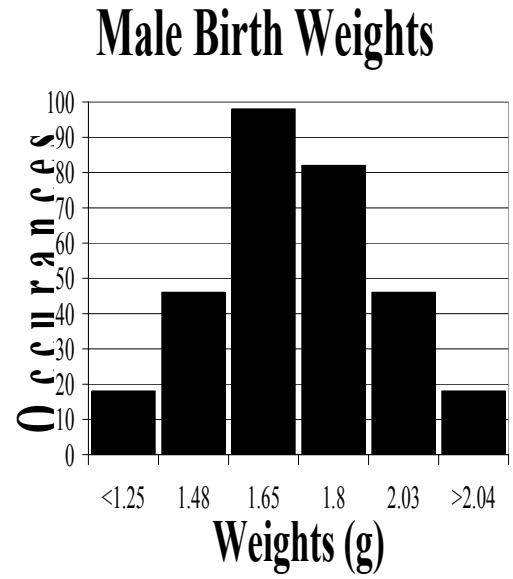


Figure 4.
Distribution of Birth Weights

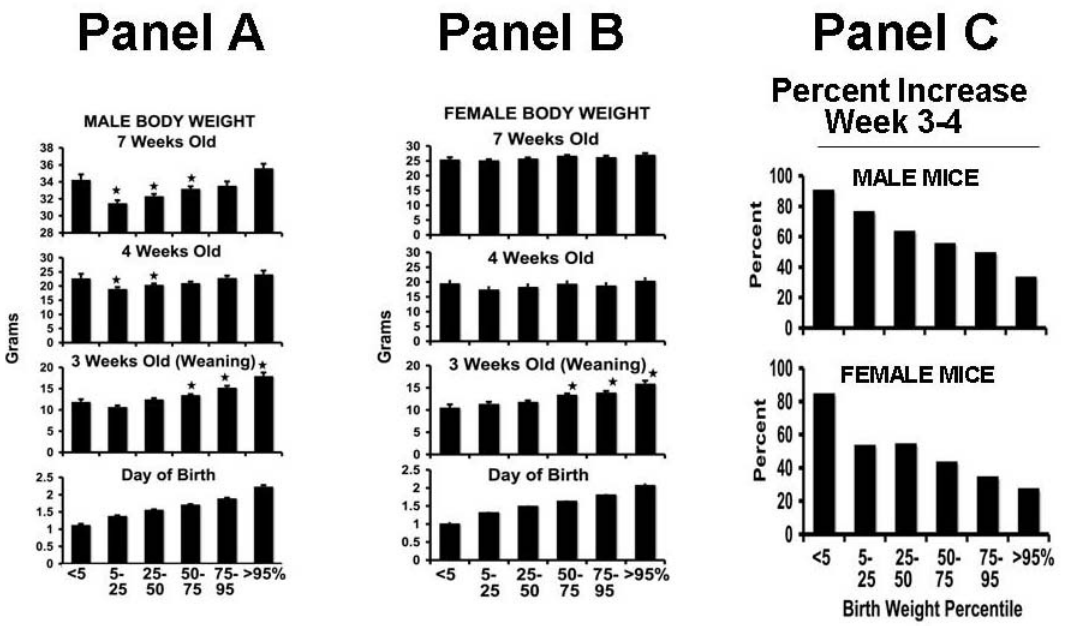


Figure 5
Panel A is the male weight profile during the experiment.
Panel B is the female weight profile during the experiment
Panel C is the percent weight gain of the different weight classifications during the first week post-weaning.

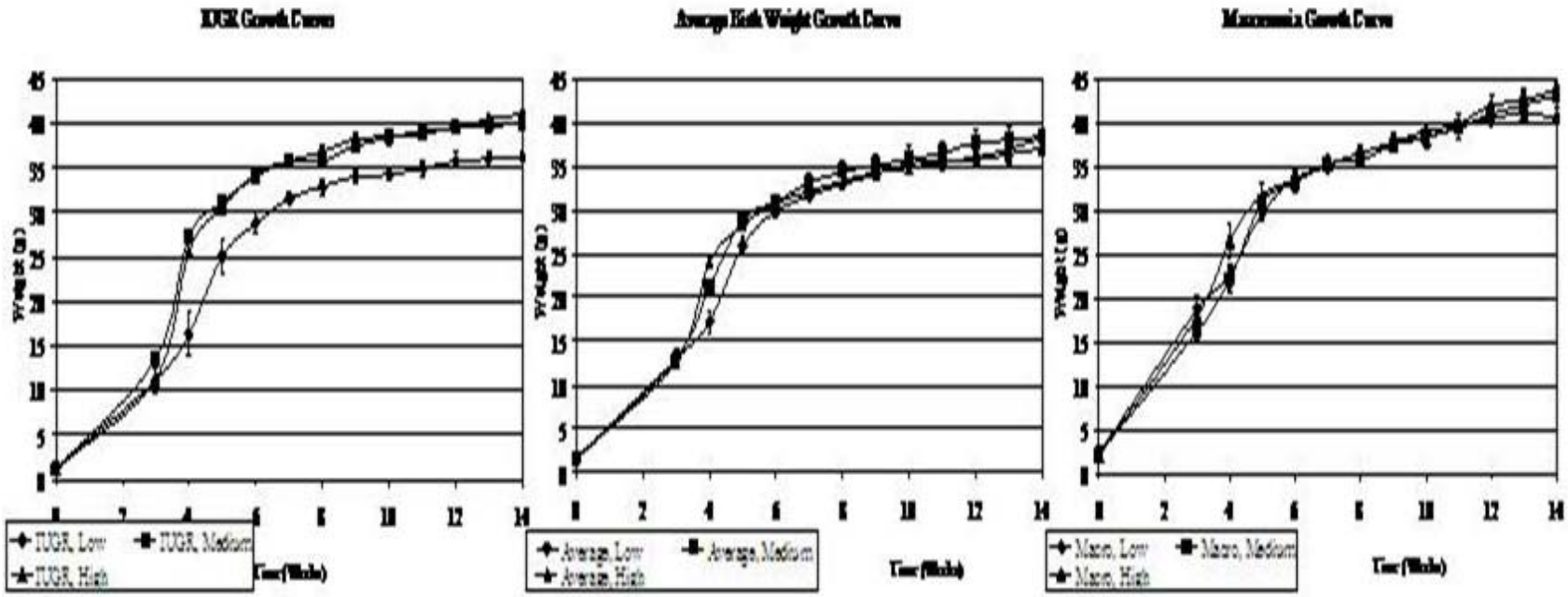
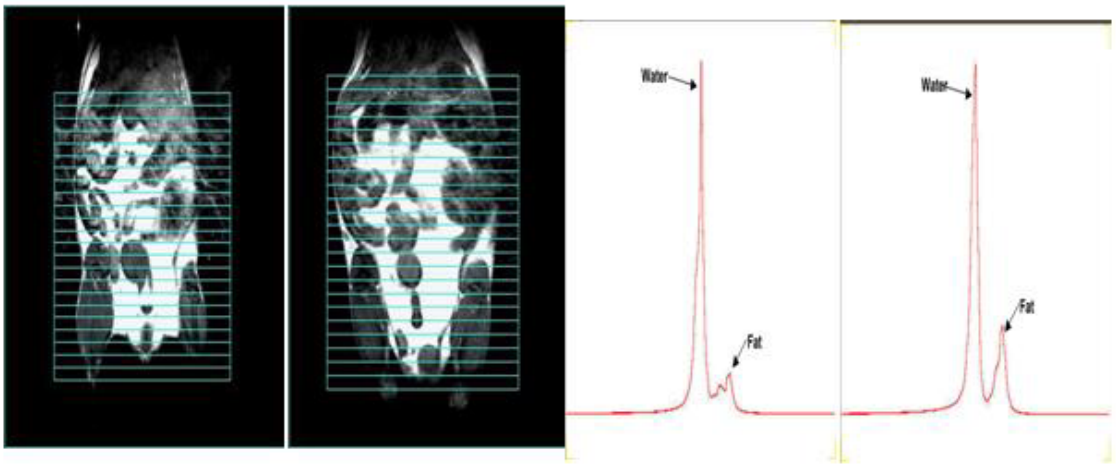


Figure 6
Growth Curves demonstrating the Subgroup Divisions. The Left Panel is the IUGR Animals. The middle panel is the Average Birth Weight Animals. The right panel is the Macrosomia animals.

Figure 7: **Panel A** **Panel B**

LEAN **FAT** **LEAN** **FAT**



Panel A. Abdomen Coronal Spin-density-weighted MRI showing fat (in white) in a lean and fat male mouse. **Panel B.** NMR peaks of body water and fat proportion calculated from data collected during the scans shown in Panel A.

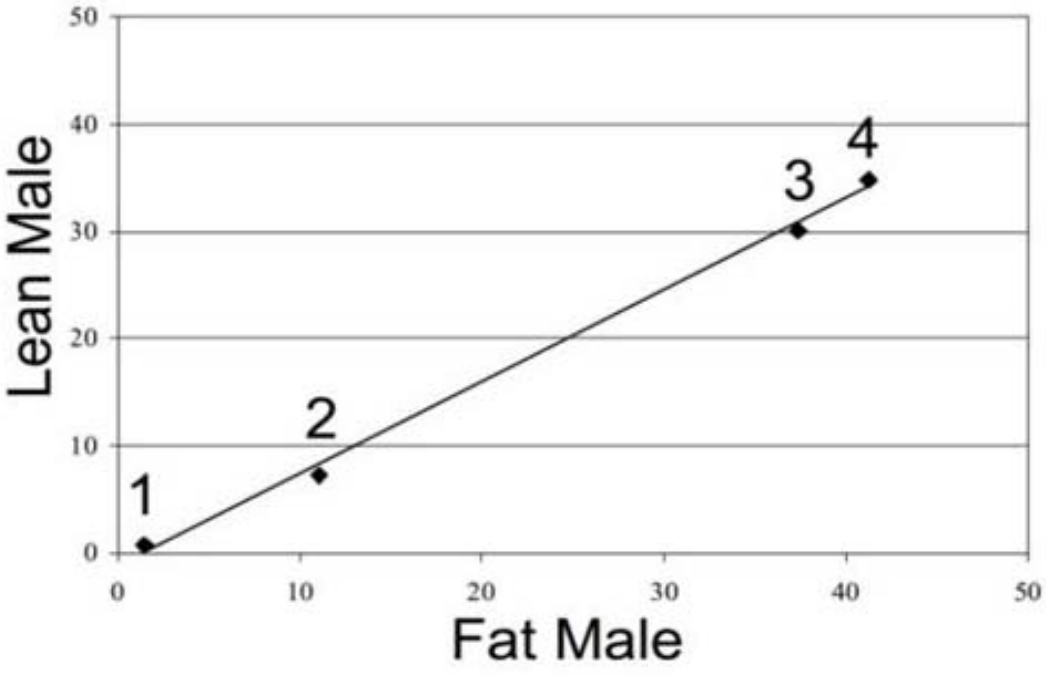


Figure 8. Comparison of data for body weight and body fat from lean and fat adult male mice measured by micro MRI and immediately after at autopsy. Specific measures based on data in

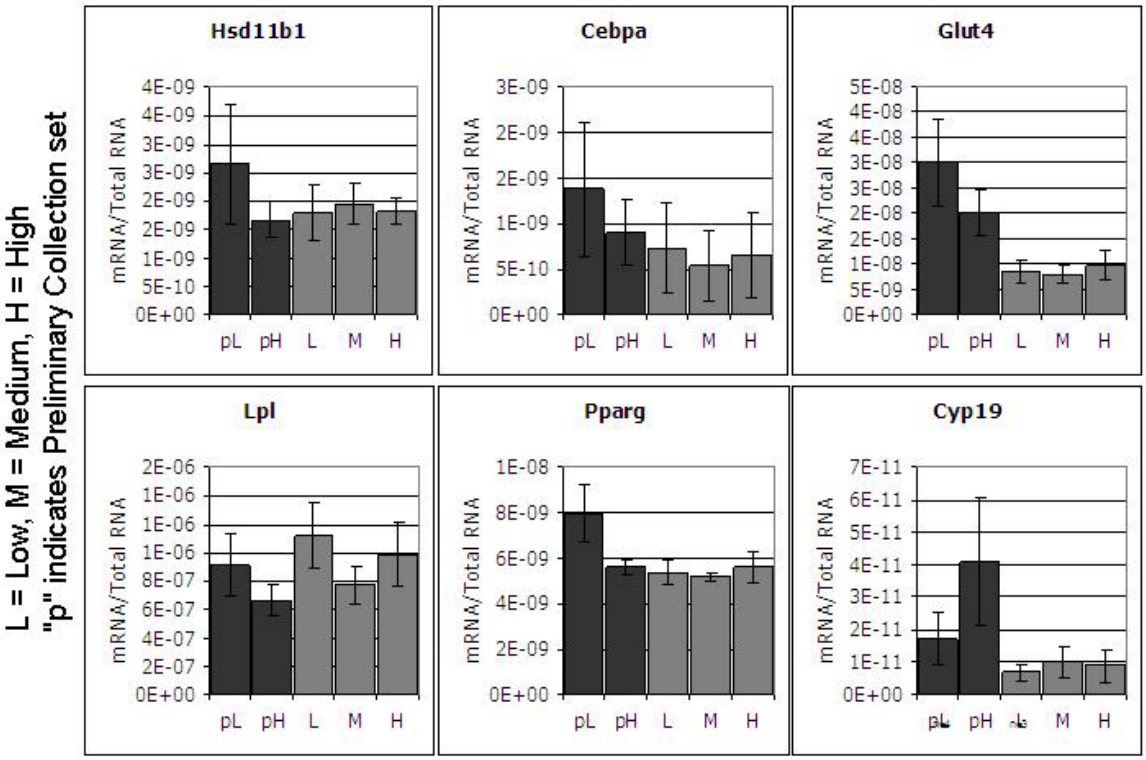


Figure 9
Gene expression results from RT PCR analysis
The darker shaded bars were collected at an earlier time, as a preliminary collection. No other data is available for these animals. The lighter bars are the animals collected at the closure of the experiment. All animals that were collected at the closure of the experiment were fasted overnight. These animals show a profile that is characteristic to that fact.

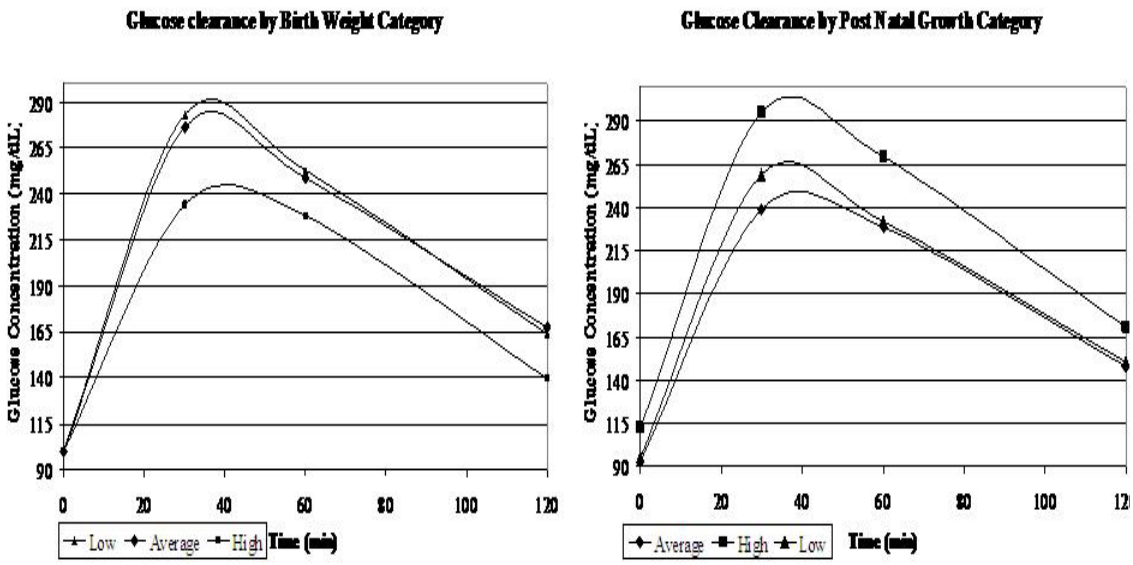


Figure 10
The left figure, figure a, is a graph of glucose clearance distributed by birth weight category.
The right figure, figure b, is a graph of glucose clearance distributed by post natal growth rate.

Gene	Reason
GLUT4	GLUT4 encodes Glucose transporter 4, a glucose transporter vital to normal glucose metabolism. GLUT4 causes increased basal and insulin modulated uptake of glucose by the adipocytes (Deems, 1994)
Lpl	Lpl encodes lipoprotein lipase, a protein that catalyzes the reaction to remove fatty acids and glycerol from VLDL as it passes across the capillary wall. (Gregoire, 1998)
PPARγ	The PPARγ gene encodes PPAR-γ and is a regulator of adipocyte differentiation. Additionally, PPAR-γ has been implicated in the etiology of numerous diseases including obesity, diabetes, and atherosclerosis.
CYP19	CYP19 encodes the protein aromatase, which converts testerone to estradiol. Lpl is regulated by serum estrogen, so an increase in CYP19 suggests an increase in estrogen. (Misso, 2003)
11βHSD	11βHSD encodes the protein 11ketoreductase, which converts cortisone to its active form of cortisol.
C/EBPα	C/EBPα encodes a protein has been shown to modulate the expression of the gene encoding leptin, a protein that plays an important role in body weight homeostasis. C/EBPα also aids in adipocyte differentiation.

Table 1

Genes analyzed by RT PCR and the reasoning for their testing

	MICRO MRI AND AUTOPSY MEASURES	LEAN	FAT
1	Total fat at autopsy (grams)	0.6959	1.401
2	Micro MRI estimated fat proportion (relative units)	7.3	11.05
3	Body weight at autopsy (grams)	34.8	41.3
4	Micro MRI estimated water proportion (relative units)	30.05	37.4

Table 2:

1. Autopsy weight of epididymal, renal and inguinal fat pads,
2. Micro MRI accurately estimated total body fat,
3. Autopsy body weight,
4. Micro MRI determination of body water proportion accurately predicted body weight.

APPENDIX II

Gene sequences used for PCR analysis or where they were purchased.

11β-HSD

HSD11β1-04: GCA GCA TTG CCG TCA TCTC
HSD11β1-05: GAA CCC ATC CAG AGC AAA CTTG
HSD11β1-P1: 6FAM-TGG CTG GGA AAA TGA CCC AGC CTA TG-TAMRA

CYP19

CYP19-1: CCG AGC CTT TGG AGA ACA ATT
CYP19-2: TCC ACA CAA ACT TCC ACC ATT C
CYP19-1-P: 6FAM-TTT CTT TAT GAA AGC TCT GAC GGG CCC T-TAMRA

The following gene primers were all purchased as pre-optimized and validated primer-probe gene expression assays from Applied Biosytems.
cEBP-α
PPAR-γ
GLUT-4
LPL

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VITA

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