EXPLORING THE EFFECTS OF DIET ON AUTOTAXIN-TRANSGENIC FVB/N MICE AND ABNORMALITIES IN THE NIGHT TIME BOUTS OF FVB/N MICE.

by

MEGAN ANN MACFARLANE

(Under the Direction of Mandi Murph)

ABSTRACT

This study explores some of the effects of a matched high-fat, low-carbohydrate diet versus a low-fat, high-carbohydrate diet on FVB/NJ autotaxin transgenic mice. This study observed abnormal behavior among the leaner mice and explains the rationale behind that observation. The *in vivo* diet study was set up to explore how autotaxin induced obesity impacts the development of cancer. Throughout the course of this study, the male mice on a low-fat, high-carbohydrate diet exhibited signs of stress or aggression towards their littermates, resulting in an abnormal amount of cage separations. From this study, it was concluded that the night time bouts that were observed were caused by the higher available energy to the lean mice, meaning they simply had too much energy and not enough space to avoid clashing with each other, and no opportunity to escape or avoid physical confrontations.

INDEX WORDS: Mouse behavior, FVB/N, Autotaxin, Diet, Aggression, ATF-3, Testosterone, ADP/ATP, UCP2

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B.S., Valdosta State University, 2015

A Thesis Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements of the Degree

MASTER OF SCIENCE

ATHENS, GEORGIA

2018

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December 2018

DEDICATION

To my loved ones. Without your support this work would not have been possible. To all my mentors. Thank you for believing in me.

ACKNOWLEDGEMENTS

I would like to thank: my major Professor, Dr. Mandi Murph and the other members of my committee, Dr. Shelley hooks and Dr. Arthur Roberts, for their guidance. The members of my lab: Sudeepti Kuppa, Charnel Byrnes, Wei Jia, Lindsay Berman, and Poojitha Manne, for their support, knowledge, and insight. I would also like to acknowledge the faculty and staff of the PBS department for all the things in between, thank you!

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CHAPTER 1

INTRODUCTION

Purpose of the Study

This study explores some of the effects of a matched high-fat, low-carbohydrate diet versus a low-fat, high-carbohydrate diet on FVB/NJ autotaxin transgenic mice. In addition, this study observed abnormal behavior among the leaner mice and will attempt to explain the rationale behind that observation. The *in vivo* diet study was set up to explore how autotaxin induced obesity impacts the development of cancer. Obesity, which is associated with several chronic and inflammatory diseases including cancer development (1-3), is related to autotaxin through its role in adipogenesis by catalyzing the production of lysophosphadic acid.

Throughout the course of this study, the male mice on a low-fat, high-carbohydrate diet exhibited signs of stress or aggression towards their littermates, resulting in an abnormal amount of cage separations. Due to links between lysophosphadic acid and the cellular stress marker activating transcription factor 3 (ATF-3) previously observed by our lab, it was an opportunity to explore if autotaxin affected ATF-3 *in vivo.* As this effect was only seen in the male mice of this study hormones were considered, as well as hormone receptor prevalence, as a probable cause for erratic behavior (4). Because of the levels of activity within the mice, the ATP concentration as well as the ratio between ADP and ATP was also explored. To make sure that the results from the ATP concentration and ADP/ATP ratio could be interpreted accurately, expression levels of Uncoupling protein 2 were assessed as this protein is known to reduce ATP

in obese liver tissues. From the results of this study, it was concluded that the effects on stress signals from this type of in vivo study could not be correlated with the cellular stress signaling previously observed in our lab. However, the errant behavior of the mice was found to be induced by increased availability of ATP in liver tissue in the lean male mouse groups.

CHAPTER 2

BACKGROUND

The Problems of Obesity

Access to food high in calories, sugar and fat, and the sedentary lifestyle of modern society has produced an escalation in obesity with medical and socioeconomic implications. The increase in the prevalence of obesity appears to be associated with an increased prevalence of risk factors for cardiovascular disease and type 2 diabetes, including hypertension and reduced glucose tolerance (5). These conditions are associated with chronic inflammatory response characterized by cytokine production, acute-phase reactants, and activation of inflammatory signaling pathways. A feature of the inflammatory response in the presence of obesity is that it is triggered in adipose tissue (6).

Autotaxin and LPA

Autotaxin, an ecto-nucleotide pyrophosphatase/phosphodiesterase (ENPP), is a lysophospholipase D enzyme that can form extracellular lysophospholipids into lysophosphadic acid (LPA) by hydrolyzing the phosphodiester bond of lysophosphatidylcholine (LPC) (figure 1). Autotaxin is typically expressed in the brain, lymph nodes, kidneys, lungs, ovary and testis and is the main source of LPA in the blood. Lysophosphadic Acid, the active

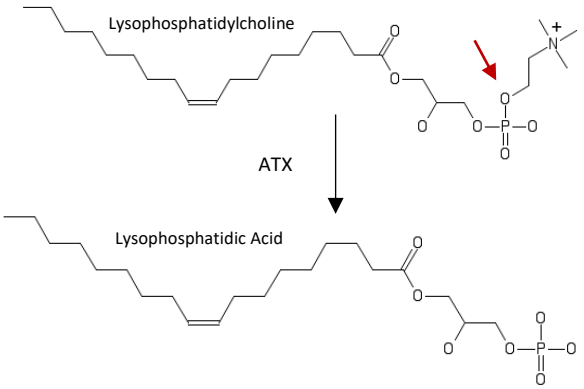


Figure 1: Hydrolysis of Lysophosphatidylcholine by Autotaxin to produce Lysophosphadic Acid

product of autotaxin, is a ligand for specific G-protein coupled receptors, lysophosphadic acid receptors 1-6 (LPAR 1-6), that can activate multiple signaling pathways. The most widely studied effects of lysophosphadic acid include cell migration stimulation, cell proliferation, and cell survival. (7-9)

Autotaxin and Obesity

Adipose tissue is crucial to energy balance due to its ability to store excess energy as triacylglycerol, through lipogenesis, and the ability to release fatty acids and glycerol, known as lipolysis (10). In the obese, there is a breakdown of the balance between lipogenic and lipolytic processes, in adipocytes, that contribute to an excessive accumulation of triacylglycerol (10). Autotaxin is secreted by adipose tissue; it is 2-3 times more expressed in adipocytes than in stromal-vascular cells (11). Autotaxin expression and secretion increase during adipogenesis (11). Levels of autotaxin mRNA increase with adipocyte maturation in culture (12).

Rancoule et. al. found that in mice, fat expression of autotaxin is increased with obesity with a tight association with insulin resistance. In humans, autotaxin expression is increased in visceral adipose tissue from massively obese patients exhibiting diabetic or prediabetic symptoms. In their study, the fat expression of autotaxin in humans is also related to insulin resistance. When comparing obese to non-obese patients, a significant increase (42 %) in autotaxin expression was observed in visceral fat. Visceral obesity is known to be associated with insulin resistance as the result of the inflammation of the adipose tissue and the production of deleterious adipokines. (12)

Adipocyte autotaxin expression and plasma lysophosphadic acid are up-regulated in obese mice fed a high fat diet (13). Lysophosphadic acid receptors are expressed in adipose

tissue with lysophosphadic acid receptor 1 being the most abundant subtype (14). lysophosphadic acid receptor 1 expression is lower in adipocytes than in the stromal-vascular cells containing preadipocytes (15). In human beings, obesity has been shown to have no major influence on lysophosphadic acid receptor 1 expression in adipose tissue (12). The above observations show that, in adipose tissue, obesity alters lysophosphadic acid synthesis. Autotaxin expression and secretion is known to increase when preadipocytes differentiate into adipocytes (adipogenesis) which is associated with a strong accumulation of lysophosphadic acid in the extracellular medium (11).

Behavior of Mice

One of the problems encountered in laboratory animal facilities is aggression in group-housed male mice. In their natural habitat, male mice (Mus musculus) live in social groups existing of one dominant male, females with their progeny, and subordinate males where familiar subordinate males are tolerated inside the territorial boundaries (16). When male mice are group-housed in the laboratory, a certain level of intermale aggression can thus be regarded as normal or natural (17). Laboratory mice that live in a barren confined space may be unable to respond to each other in a suitable societal way where subordinate mice are unable to flee from the dominant's sight or leave of the territory.

Both rats and mice prefer social to individual housing. In rats it has been shown that animals that were reared in group-housed conditions and were later individually housed would choose to feed and sleep near others (18). In general, mice prefer the vicinity of a cage mate during resting periods, especially when mice are familiar to each other. Male mice prefer social housing to individual housing, independent of their level of aggression and social status. (16)

In a review by P. L. Van Loo, both scientific studies and anecdotal evidence from animal caretakers was discussed concerning the modification of housing and husbandry procedures to decrease aggression in group-housed male mice. In summary, it was recommended to transfer used nesting material from the old cage to the new cage reduce male aggression. Social interactions between male mice can be affected both by kinship (19) and by group size. Kinship itself does not affect the level of aggression but more so being reared together from a young age develops familiarity and social status. Group size on the other hand does affect aggression. Groups composed of three mice show the least aggression as compared to groups of Five or eight mice (16). They ended the review with housing tenets to follow to reduce aggression in male mice:

Housing Tenets

Individual housing of male mice should be avoided	Material from the nesting area should be transferred to the clean cage	Best housed in groups of three	Nesting material is the enrichment of choice	Disturbances during an experiment should be limited

According the UGA's guidelines for terrestrial animal care and husbandry, all animals should be housed under conditions that provide enough space as well as supplementary structures and resources required to meet physical, physiologic, and behavioral needs. Social animals should be housed in stable pairs or groups of compatible individuals and bedding for nesting is required. (20) Diet is also known to affect the behavior of mice. A study by Kohsaka et. al., found that a high fat diet can disrupt the circadian rhythms of mice and disrupts several metabolic pathways (21).

FVB/N strain Comparison

There is some contradiction in the literature on the level of displayed aggression in FVB/N mice. According to Pugh, FVB/N mice are considered aggressive (22). This may be due to the breeds tendency to startle easily (23). However, according to Jackson Laboratories, FVB/NJ mice are even tempered with a slightly elevated appetite compared to their other breeds. Their temperament, or wildness score, which was calculated by the sum of the capture and hold means, was compared to 21 other mouse breeds (24). The higher the score, the wilder the breed is considered. Wildness scores of mice were ascertained in a third-party study by Wahlsten et. al. (24). Out of the 21 other breeds, FVB/NJ breed is just below the average of 0.7 with a score of 0.6 for females and 0.5 for males. From the data from Jackson, FVB/N mice are below the average wildness, for holding, of laboratory mice for both the males and the female groups and well below the average of other laboratory breeds on biting. A second parameter of wildness is determined from the number of holding tests that resulted in bites. Of the 21, FVB/NJ breed is well below the average of the mice breeds for both males and females. In a 2nd third party study by Backmanov et. al. (25), the food intake for the same 21 breeds were observed. On average, the FVB/NJ male mice consume 4.75 g/day which is slightly above the average of 4.45 g/day. FVB/NJ males rate on the high end of the average for grams of food intake on a daily average (25).

ATF-3 and Stress

ATF-3 is part of a large group of basic region leucine zipper transcription factors which are part of the ATF/cAMP CREB cycle. This basic region is responsible for DNA binding because the leucine zipper acts as a platform for dimerization with itself or other basic-zip proteins (26).

The ability to act as both a homodimer and a heterodimer allows ATF-3 to perform multiple functions depending on the stressors on the cellular system (i.e. cytokines, genotoxic agents, or physiological) (26). ATF-3 is an adaptive response gene that is an indicator of a cellularly stressed system (27). It is induced when cells are exposed to physiological and pathological stimulus, including seizure, toxic chemicals, anti-cancer drugs, proteasome inhibitor, genotoxic agents, homocysteine, and in rapidly regenerating liver (28,29). In our lab, we previously found that autotaxin expression levels regulate the production of lysophosphadic acid and that lysophosphadic acid mediates ATF-3 expression (30). The mRNA level of ATF-3 is relatively low in most cell types, however it has the most expression in liver tissues due to its role supporting regeneration.

Testosterone

Testosterone is a steroid (figure 2) from the androstane class containing a keto and hydroxyl groups at the three and seventeen positions respectively (31). It exerts its action through binding to and activation of androgen receptors. High expression of testosterone, the male sex hormone, is typically associated with higher levels of aggression in mice, rats, primates, and other animals. Testosterone promotes dominance behaviors in small animals and has been observed to increase responsiveness to adverse stimuli and potential aggression (32). The androgen receptor, a type of nuclear receptor, is activated by binding any of the androgen hormones, including testosterone and dihydrotestosterone, in

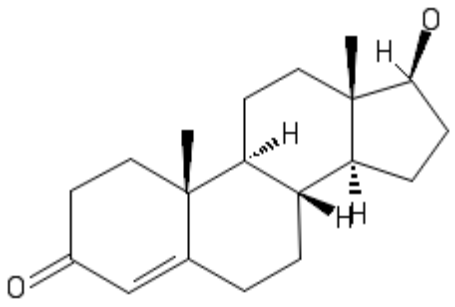


Figure 2: Structure of testosterone

the cytoplasm which are translocated into the nucleus (33).

ADP/ATP and Energy

Adenosine triphosphate (ATP) consists of three components: a nitrogenous base (adenine), the sugar ribose, and the triphosphate. Since ATP occurs in all forms of life, it is referred to as the "molecular unit of currency" of intracellular energy transfer (34). ATP can be produced by three main pathways: glycolysis, the citric acid cycle/oxidative phosphorylation, and beta-oxidation (35). When ATP is expended in metabolic processes it converts to adenosine diphosphate (ADP), through hydrolysis, which is then used to recreate ATP (36). Measurement of the adenosine diphosphate (ADP)/adenosine triphosphate (ATP) ratio has proved to be a reliable indicator of cell viability, necrosis, and apoptosis (37). The ADP/ATP assay is used as a quantitative assay that shows the energy status of the cells by an objective measurement of proliferation and necrosis. From the ADP/ATP ratio the concentration of ATP can be determined. The hepatocytes of an organism, which store glucose for glycolysis, are constantly metabolizing various biomolecules and therefore constantly require this molecular energy. Determining the ADP/ATP ratio and concentration from tissue is used in this study to quantify the total energy available to the organism.

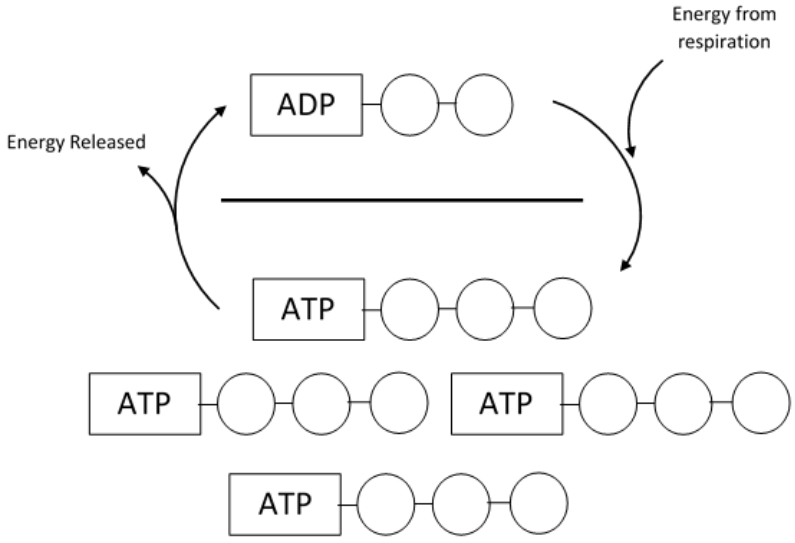


Figure 3: ATP cycle, in relation to the ADP/ATP ratio, where a phosphate is affixed to ADP through cellular respiration to form ATP. This process requires energy and indicates that the cell is continuing to thrive and proliferate due to the continuous metabolic process needed to fuel the cycle.

Figure 3 represents the key principle behind the ADP/ATP ratio's meaning. Shown above is a basic representation of the ATP cycle where a phosphate is affixed to ADP through cellular respiration to form ATP. This process requires energy and indicates that the cell is continuing to thrive and proliferate due to the continuous metabolic process needed to fuel the cycle. If this cycle slows down or halts the available ATP is used up in cellular processes and is not replenished leaving a surplus of ADP. The cessation of the production of ATP indicates cell death, necrosis and apoptosis. So, in relation to the ADP/ATP ratio, the higher the concentration of ATP, or lower amounts of ADP, in a cell, the smaller the ratio. A small ratio difference indicates more ATP than ADP. In the case where there is more ADP than ATP the ratio number will increase.

Mitochondrial uncoupling protein 2 (UCP2) separates the transmembrane proton fluxes of oxidative phosphorylation from ATP synthesis. The uncoupling of this process results in less ATP in a cellular system as it halts ATP synthesis. In fatty liver tissues UPC2 has been shown to be upregulated which results in a lower amount of ATP synthesized compared to normal liver tissues (38).

CHAPTER 3

METHODS

Protein assay and quantification

A BCA Protein Assay is a formulation based on bicinchoninic acid (BCA) for the colorimetric detection and quantitation of total protein. This method combines the well-known reduction of Cu^{+2} to Cu^{+1} by protein in an alkaline medium, the biuret reaction, with the highly sensitive and selective colorimetric detection of the cuprous cation (Cu^{+1}) using a reagent containing bicinchoninic acid. The purple-colored reaction product of this assay is formed by the chelation of two molecules of BCA with one cuprous ion. This water-soluble complex exhibits absorbance at 562nm that is nearly linear with increasing protein concentrations over a broad working range (20-2000µg/mL) (39).

For this technique, lysates of flash frozen tissues are created by homogenizing samples in 500 µL of lysis buffer (RIPA buffer with a protease inhibitor in a 1:100 ratio). Buffered tissue was sonicated, centrifuged and the supernatant extract was obtained. The supernatant is then quantified through BCA assay. To perform this, assay a Pierce BSA protein assay kit (Pierce BSA protein assay kit, Thermo Fisher Scientific, Rockford, IL, USA) was used. Per the manual a solution of 50:1 of solution A to Solution B was prepared. The volume of solution used is dependent of the number of samples and standards to test. A 96-well plate is used where the blank wells contained 200 µL of water, the unknown wells contained 200 µL of the 50:1 solution and 10 µL of protein containing supernatant, and the standard wells contained 200 µL of the

BSA Standard Ratios

Well	1	2	3	4	5	6	7	8	9	10	11
BSA µL	0	1	2	3	4	5	6	7	8	9	10
H_2O µL	10	9	8	7	6	5	4	3	2	1	0
BSA conc.	0	200	400	600	800	1000	1200	1400	1600	1800	2000

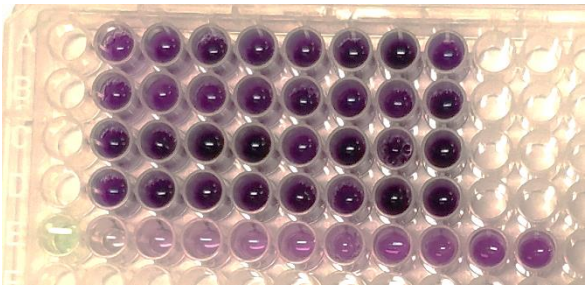


Table 1: BSA ratios used in preparation of the standards to determine quantification of protein samples. The BSA standard used contains 200 µg of BSA per µL resulting in the concentrations shown above

Image 1: Sample of a prepared and incubated plate were the samples run from A-D with blanks in the first column and standards are along the bottom. You can see the gradient of concentration in the change from green 50:1 solution with no protein turning more and more purple as the concentration increases.

50:1 solution as well as a gradient mix of BSA standard and water as described in table 1. Once the 96-well plate was prepared with samples, it was incubated at 37.0 °C for 30 min. The incubated plate, which has turned from green to purple shades, is imaged by a plate reader and quantified using a BCA quantification program. The program creates a standard curve from the absorbance of the standard wells. Using the standard curve, the concentrations of the samples are determined from the absorbance of the samples. Figure 4 is the standard curve from the

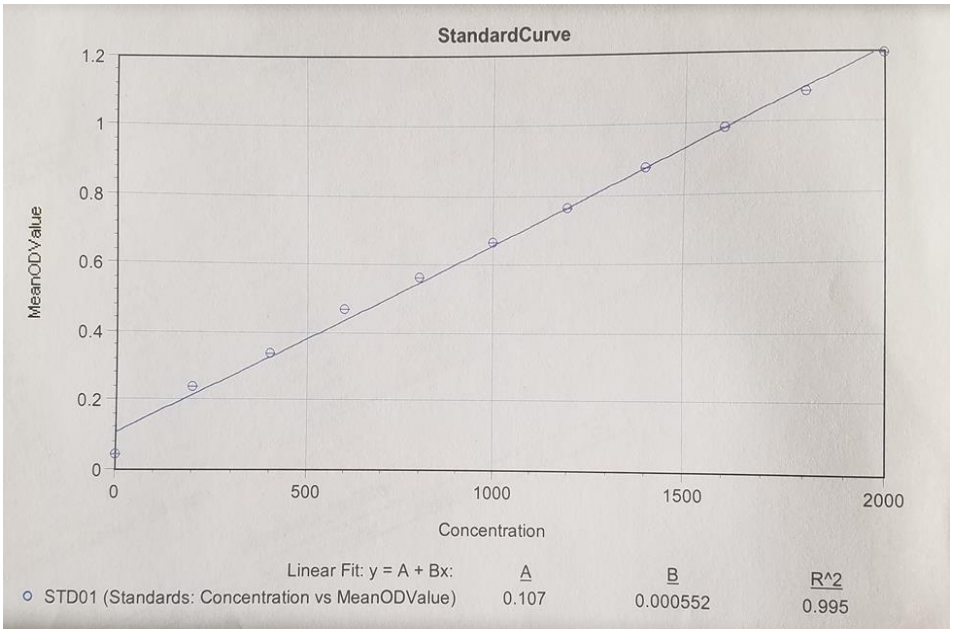
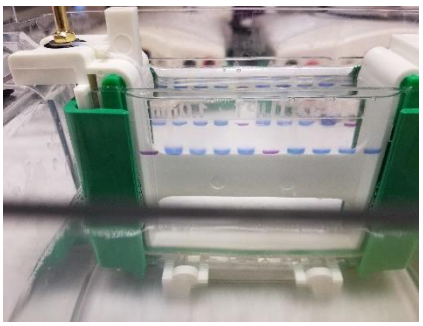


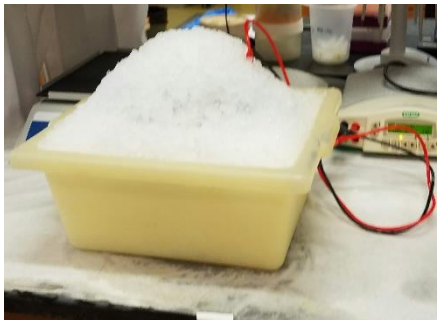
Figure 4: This figure shows the Linear trend of the BSA concentrations for the BCA assay. On the Y-axis is the mean optical density, or absorbance of the standards. Below on the x-axis is the concentrations provided. Under the graph is the calculated linear equation of the line that is used to determine the concentrations of the samples. The R^2 value is a statistical value that provides insight into correlation of the data to the line. The closer to1 the more related the data is.

plate above. In image 1, the samples are darker than the standards shown. When this happens the protein quantification is repeated with either a diluted aliquot of sample tissue lysate or a wider range of standard concentrations can be used.

Western blotting

The Western blot or immunoblotting is an analytical technique used to detect specific proteins in a sample of tissue extract. The tissue extract undergoes protein denaturation through heating which is followed by gel electrophoresis to separate the proteins by molecular weight. Antibodies are created that identify and bind to a target protein, known as the primary antibody. After electrophoresis, the proteins contained within the gel are transferred to a membrane of either PVDF or nitrocellulose. Both varieties of membrane are chosen for their non-specific protein binding. After the transfer, the membrane is washed in a solution containing the primary antibody. A secondary antibody is added which recognizes and binds to the primary antibody. The secondary antibody is visualized through various methods chemiluminescence allowing indirect detection of the specific target protein. For the immunoblots performed, liver extracts were used. Liver tissue from mice was homogenized in





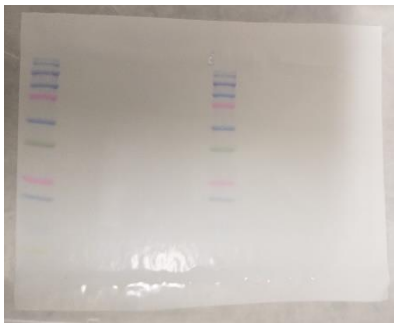


Image 2: Electrophoresis chamber with loaded SDS-Page gels. The ladder is the darker purple in the 1st and 4th positions on the gel. The volumes loaded into the lanes contain 30 µg of protein per sample.
Image 3: Transfer set up where the bath chamber is on ice to mitigate the heat produced
Image 4: A transferred membrane in washing buffer. The bands of the ladder are clearly visible indicating a successful transfer.

500 µL of lysis buffer (RIPA buffer with a protease inhibitor in a 1:100 ratio). Buffered tissue was sonicated, centrifuged and the supernatant extract was obtained. Protein concentrations were determined by a BCA protein assay (Pierce BSA protein assay kit, Thermo Fisher Scientific, Rockford, IL, USA). Thirty µg per sample were analyzed by SDS-PAGE electrophoresis (Image 2) for 120 min at 110V. Proteins were transferred to nitrocellulose membranes on ice for 90 min at 100V (Image 3 and Image 4). Membranes were blocked in milk buffer at room temperature for 1 h. Antibody incubation with ATF-3 antibody or Androgen receptor AR441 (MA5-13426, Thermo Fisher Scientific, Rockford, IL, USA) were incubated on a shaker plate overnight at 4°C. Membranes were washed with TBST three times, 10 min each, then incubated with secondary for 120 min at room temperature and washed again, as stated previously. Chemiluminescent substrate DURA (SuperSignal West Dura extended duration substrate, Thermo Fisher Scientific, Rockford, IL, USA) was applied for 5 min then the membranes were imaged for 6 min. GAPDH image was obtained the same way with a regular chemiluminescent substrate (SuperSignal West Pico, Thermo Fisher Scientific, Rockford, IL,USA) and applied for 3 min and imaged for 2 min. Imaging was obtained with a Li-cor OdysseyFC, Li-cor Biosciences, Lincoln, NE, USA).

For Testosterone ELISA

Testosterone content was determined using a colorimetric competitive enzyme linked immunosorbent assay (ELISA) kit (Testosterone ELISA kit, Enzo Life Sciences, Farmingdale, NY). The kit uses a monoclonal antibody against testosterone to bind testosterone in the standard or in the sample of the provided plate. After simultaneous incubations at room temperature, the excess reagents are washed off and the substrate is added, which after a short incubation period a yellow color is generated (405nm). The experimental plate was prepared according to

the manufacturer's instructions and was read at an absorbance of 405nm. A calibration plot was made with testosterone standard included in the kit with a range from 2000 pg/mL to 7.81 pg/mL. The samples' testosterone concentration was determined from the standard curve and the absorbance values from the test.

For ADP/ATP ratio test

ADP/ATP ratio was determined in single cell suspensions with a luciferin–luciferase bioluminescent assay (ADP/ATP ratio assay kit, Abcam, Cambridge, MA) according to manufacturer's instructions, and calculated based off a calibration plot made with a standard ATP solution with a range from 1 nM to 10 nM (ATP Disodium Trihydrate, Amresco, Solon, Ohio). Single cell suspensions were created using 200 mg of flash frozen tissues and dissociated with collagenase type I (Stemcell Technologies, Vancouver, Canada) and suspended in PBS. Imaging was obtained with a Li-cor OdysseyFC, (Li-cor Biosciences, Lincoln, NE, USA).

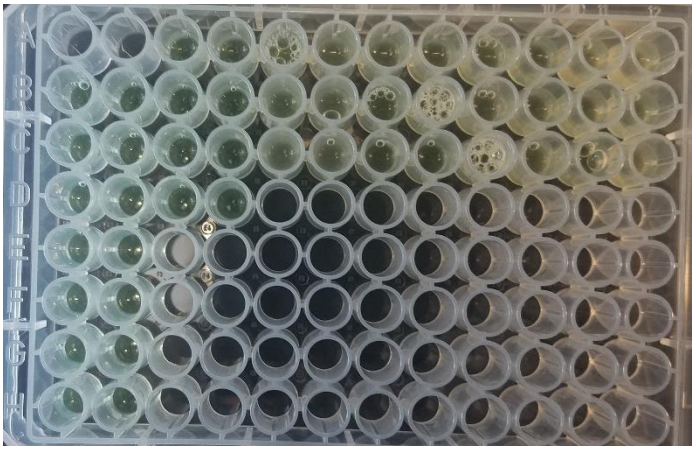


Image 5: Sample of a prepared and run ADP/ATP test. The standards and samples were run in duplicate. The standards are in the first four columns along with a blank of 200 µL of water. From column 5-12 contains the samples in duplicate.

CHAPTER 4

RESULTS AND DISCUSSION

Since obesity and chronic disorders often show a correlation, our lab devised an *in vivo* study of the effects of obesity on autotaxin using ATX-transgenic mice. FVB/NJ inbred mice were either ATX-Transgenic (ATX-T) or wild type (WT) and were further divided by diet. Some were fed a lean feed diet and others were fed a high fat feed diet, resulting in four mice groupings. The Diets were matched feeds with the Fat diet (Research Diet D12451) which had a nutrient breakdown of 45% kcal % fat, 35% kcal % carbohydrates, and 20% kcal % protein compared to the Lean diet (Research Diet D12450H) 10 % kcal % fat, 70 % kcal % carbs, 20 % kcal protein (table 1). Aside from the carbs and fat, the diets proteins and nutrients were matched to provide full nutrition to the mice. These types of diet differ from a regular chow diet which consists of 13.38 % kcal % fat, 57.94 % kcal % carbohydrates, and 28.67% kcal % protein (40). This regular chow, from these base values seems to be the median of the two diets chosen for this study, meaning the both the Fat diet and the Lean diet are considered out of the normal

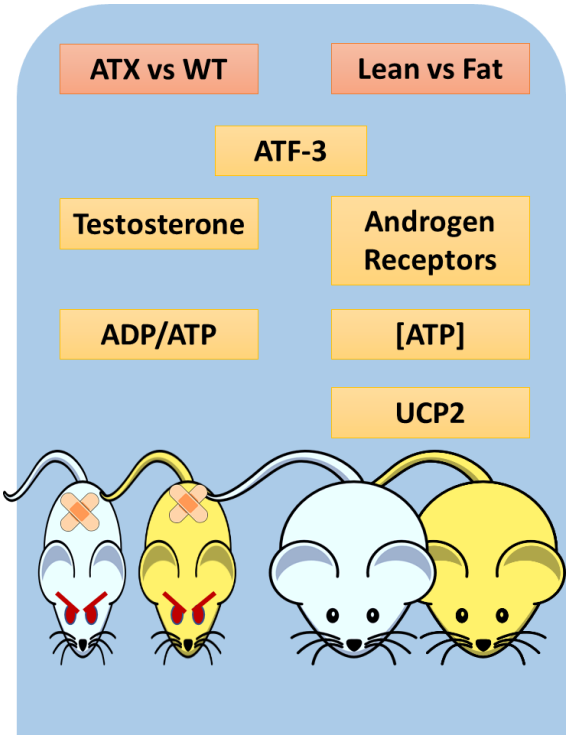


Figure 5: Representative layout for the study. White mice above represent wild type mice and the yellow mice represent the ATX-transgenic mice. Displayed above are the various pathways examined.

Physiological Fuel Values	Fat Diet D12451	Control diet D12450H
Protein	20 % kcal	20 % kcal
Fat	45 % kcal	10 % kcal
Carbohydrate	35 % kcal	70 % kcal
Energy Density	**4.7 kcal/g**	**3.82 kcal/g**

Table 2: represented above are the Physiological fuel values for the two diets used in this mouse study. The Diets are matched where the only variance in the diet come from the Fat and Carbohydrates within the feed.

Image 6: Results of a night bout in male lean mice

physiological fuel distributions for adult mice. Cages were grouped by gendered littermates and nesting material transferred between cage changes to reduce aggression. During this study it became apparent that the male mice under the lean diet were becoming more aggressive where the cage separations between the littermates were significantly higher compared to the other diet groups. Over the course of the study, the lean male mice group went from 5-6 cages to 25-26 individual cages due to severe, near fatal, interactions (Image 6).

ATF-3 in tissues

To determine what happened to these lean mice in our lab's *in vivo* study, various tests were performed to determine whether it was stress or aggression that caused them to have cage fights. Since our lab was still interested in the effects of obesity and stress on autotaxin, stress signals, like ATF-3 and cortisol, were thought to be present in the tissues and were considered first because the lean diet was thought to act as a stressor in the tissues to promote ATF-3 production. The expectation was that there should be an increase in ATF-3 expression level in the lean mice over the mice on the fat diet because of the implied stress. Also because

of our previous study with ATF-3 and LPA (30), expression levels of ATF-3 in the ATX-T mice in the lean group were expected to be even higher than the WT lean mice. Western blots for ATF-3 were performed on liver, kidney, heart, and lung tissues where liver tissues showed quantifiable expression of ATF-3 (Figure 6) by the procedure listed in materials and methods. In figure 6, the expression levels of ATF-3 in liver tissue is observed to be roughly equal across the groups when normalized to GAPDH content in the samples; WT mice represented by - and ATX-T by +. Unfortunately, cortisol, which is a prominent stress hormone, could not be tested due to limited blood serum samples that were used for analyzing other research questions, prior to the initiation of this study.

ATF-3 Expression levels within liver tissues

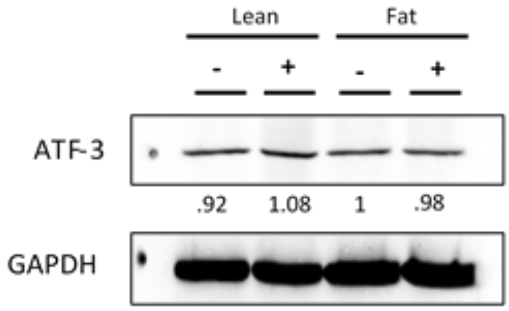


Figure 6: ATF-3 Western blot of liver tissue lysate from the 4 mice groups. By column Lean WT, Lean ATX, Fat WT, and Fat ATX mice. The upper blot is of ATF-3 antibody results and the lower image represents GAPDH present in the samples.

Testosterone as an Aggression Promoter

Hormone-driven aggression was the next possible cause considered to explain the increased night bouts, since the observed aggression was only in the male population. Because of the links to aggressive behavior, serum from the male mice was analyzed for its testosterone in comparison to their counterparts which were on different diets. To ascertain expression levels of testosterone, blood serum was analyzed by a testosterone ELISA kit from Enzo. The Testosterone ELISA is a competitive immuno-assay for the quantitative determination of the hormone in biological fluids, which uses a monoclonal antibody against testosterone to bind testosterone in the standard or in the sample to the provided plate.

Figure 7 shows the results from the testosterone ELISA in blood serum. From this data it appears that the male mice across both groups have approximately equal testosterone levels. The lean and fat groups on average have the same levels, whereas the WT vs ATX-T mice have a statistically significant difference. In the figure, a few outliers exist in the ATX-T populations of both the lean and the fat diet types as well as only two subjects for the lean WT serum levels (due to limitations in serum samples remaining) can be observed. Given the results of this test, testosterone itself was not the sole contributor to the behavior that was observed in the lean population.

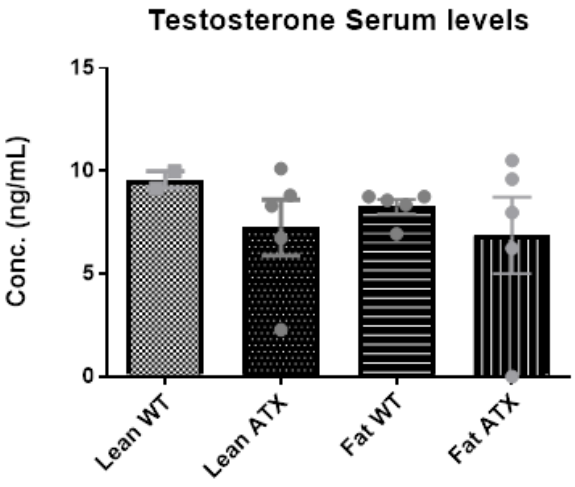


Figure 7: Testosterone blood serum levels for male mice from all 4 groups. The concentration in ng/mL within the serum is displayed on the y-axis. The lowered concentration in the ATX groups is either the result of two outliers or could represent a trend. The serum available to this test was limited so no further tests could be made to verify a correlation.

Other than testosterone circulating in the mice systemically, effects on the mice could still be skewed from testosterone in either an upregulation or downregulation of androgen receptors. An androgen receptor antibody, AR441, was used to target the level of androgen receptor expression in the mice liver tissues. Liver tissue was chosen due to the marked expression rate of androgen receptors for that organ, according to the NCBI, from the other available organs.

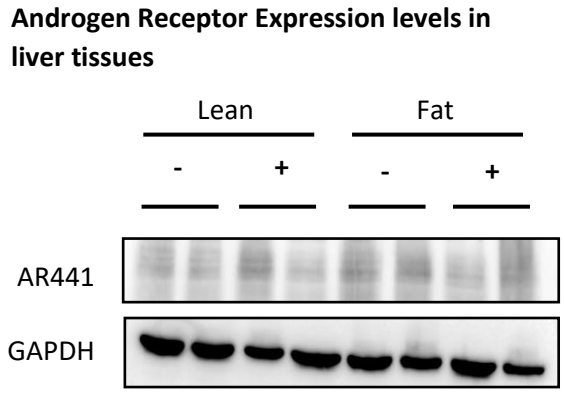


Figure 8: Western blot of liver tissue lysate from the 4 mice groups. By column Lean WT, Lean ATX, Fat WT, and Fat ATX mice. The upper blot is of AN441 androgen receptor antibody results and the lower image represents GAPDH present in the samples.

In figure 8, expression levels of AR441 in liver tissue is observed to be again roughly equal across the groups when normalized to GAPDH content in the samples; WT mice represented by - and ATX-T by +. Because there is no marked increase of expression between the groups of mice, it was extrapolated that testosterone and its receptors are not responsible for the increased night time activity of the mice.

Energy and ATP levels in liver tissues

Changes in the levels of ATP have been correlated to variances in total energy levels of an organic system (40). Changes in the ADP/ATP ratio have been used to differentiate between different modes of cell death and viability. Increased levels of ATP and decreased levels of ADP have been recognized in proliferating cells. In contrast decreased levels of ATP and increased levels of ADP are recognized in apoptotic cells (40). To measure the ATP concentration and ratio for the mice tissues, an ADP/ATP ratio assay kit, from abcam, was used on liver cells in single cell suspensions. This assay is based on the bioluminescent detection of the ADP and ATP levels in the sample through a luciferase substrate. Luciferase catalyzes the conversion of ATP and luciferin to light, which in turn can be measured using a luminometer. The ADP level is measured by its conversion of ATP that is detected through the same reaction.

This assay works best with fresh cells and tissue samples but can also work with liquid nitrogen flash frozen tissue. The liver tissue was dissociated using collagenase type I to digest the connective tissue and native fibrils of the tissues to obtain single cell suspensions. The assay was carried out in the manner proscribed for the kit and analyzed on a Glow capable luminometer as described in the methods section. Figure 9-A shows the determined ratio of ADP to ATP within the suspended liver cells of the four mouse groups. The ADP/ATP ratio

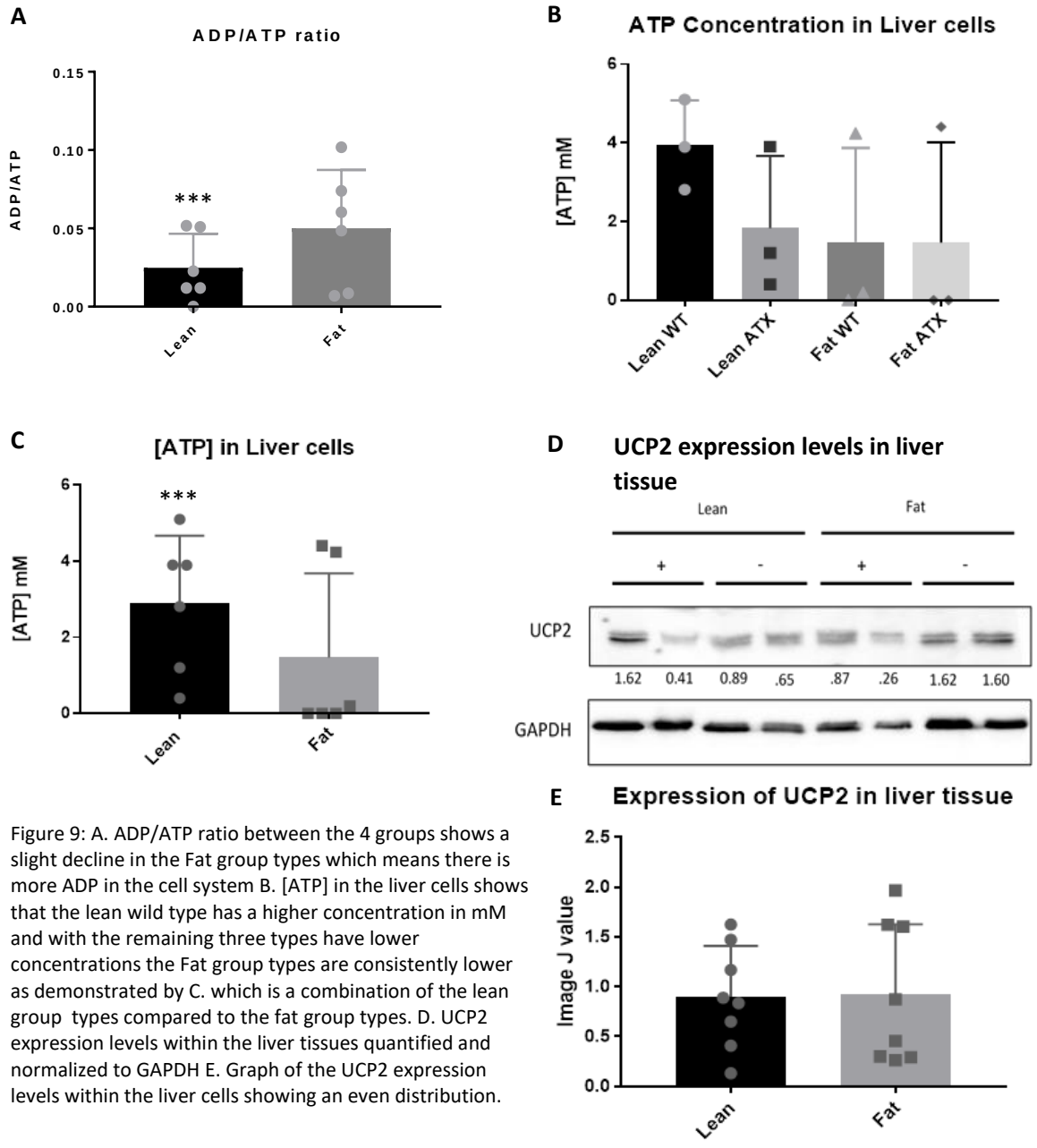


Figure 9: A. ADP/ATP ratio between the 4 groups shows a slight decline in the Fat group types which means there is more ADP in the cell system B. [ATP] in the liver cells shows that the lean wild type has a higher concentration in mM and with the remaining three types have lower concentrations the Fat group types are consistently lower as demonstrated by C. which is a combination of the lean group types compared to the fat group types. D. UCP2 expression levels within the liver tissues quantified and normalized to GAPDH E. Graph of the UCP2 expression levels within the liver cells showing an even distribution.

between the 2 groups shows a decline towards zero in the fat groups which means there is more ADP in the cell system than ATP. To extrapolate more from the ratio, the concentrations were determined from the ATP standard used in the assay. In figure 9-B the concentration of total ATP for the cells in the lean WT group shows a marked increase above the fat types and while the lean ATX group has a lower average than the WT, it seems to be trending higher than

the fat types. To illustrate this better, the lean groups and the fat groups are shown together in figure 9-C. This figure shows a clear difference between the energy available to the lean group over the mice on the fat diet. Now, since UCP2 effects ATP concentrations in fatty liver tissues, as discussed earlier, the expression of UCP2 was checked by western blot analysis and quantification (figure 9-D). Figure 9-E further illustrates the even expression of UCP2 in the liver tissue from the quantified western blots. From this analysis, it was determined that the expression of UCP2 in these specimen were equal and therefore did not contribute to the marked decrease in ATP shown in 9-C.

CHAPTER 5

CONCLUSIONS

This study explored some of the effects of a matched high-fat, low-carbohydrate diet to a low-fat, high-carbohydrate diet on FVB/NJ background transgenic and wild-type mice. Autotaxin transgenic mice as well as well as wild-type displayed the observed abnormal behavior among lean diet-fed mice, but not mice fed a high-fat diet. To note on the differences observed between the transgenic mice and the wild type mice, ATF-3 expression was even across the groups which indicated that this type of cellular stress was not impacted by the physical stress perceived. The previous data from our lab was performed in female ovarian cancer cells. So, this bring the questions: Is a change in diet enough of a physiological stressor to provoke production of ATF-3 in this situation? Also, does the upregulation of ATF-3 depend on stress signals only relevant to the tumor microenvironment when considering autotaxin and lysophosphadic acid upregulation? There could also be a difference in the testosterone blood serum level of the transgenic mice that was observed compared to their wild-type counterparts. These inquiries were not investigated further in this study but could be of some interest. It was also interesting to see a relative decrease in the [ATP] of the transgenic mice on both diets compared to their wild type counterparts. As the samples were limited in this test more data would be needed to make further conclusions.

As for the increased night time activity of the lean diet fed mouse groups, from the ATF-3 expression we could not ascertain if the activity was caused by stress factors, as the

expression of this signal was considered even for the groups. Cortisol as a stress signal was considered but could not be tested due to low sample amounts of blood serum. Testosterone was again considered because this behavior was limited to the male mice of this study. Between the lean and the fat groups they were considered on average the same. Androgen receptor expression was then checked to see if the level testosterone could still cause the difference by receptor activation. This too was found to be evenly expressed across the lean and fat group types. Because the lean diet's energy component is predominantly carbohydrates, ATP concentration and ADP/ATP ratio were explored to see cellular energy could be related to the observed night time bouts. From this test between the lean diet-fed and the high-fat diet-fed groups, a clear difference was observed in the concentration of ATP present in the liver cells. To make sure that the difference wasn't caused by UCP2 the expression levels were analyzed and found to be even between the two groups. From this study, it was concluded that the night time bouts that were observed were caused by the higher available energy to the lean mice, meaning they simply had too much energy and not enough space to avoid clashing with each other, and no opportunity to escape or avoid physical confrontations.

If this study could be performed again as a behavioral study, selecting a third regular chow diet as a control to see if the behavioral traits observed by Jackson laboratories could be repeated would be necessary. Once preformed, along with verifying previous results, testing cortisol in the blood serum to indicate psychological stressors as well as testing glucose levels, since the lean diet mice were on a diet consisting predominantly of carbohydrates, should be analyzed. Finally, the differences between the autotaxin transgenic mice, in both Lean and Fat,

in testosterone expression compared to their wild-type counterparts should be explored further to determine what processes an upregulation of autotaxin affects that can cause lower testosterone in male mice.

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APPENDIX A

FIGURES

Figure 1:

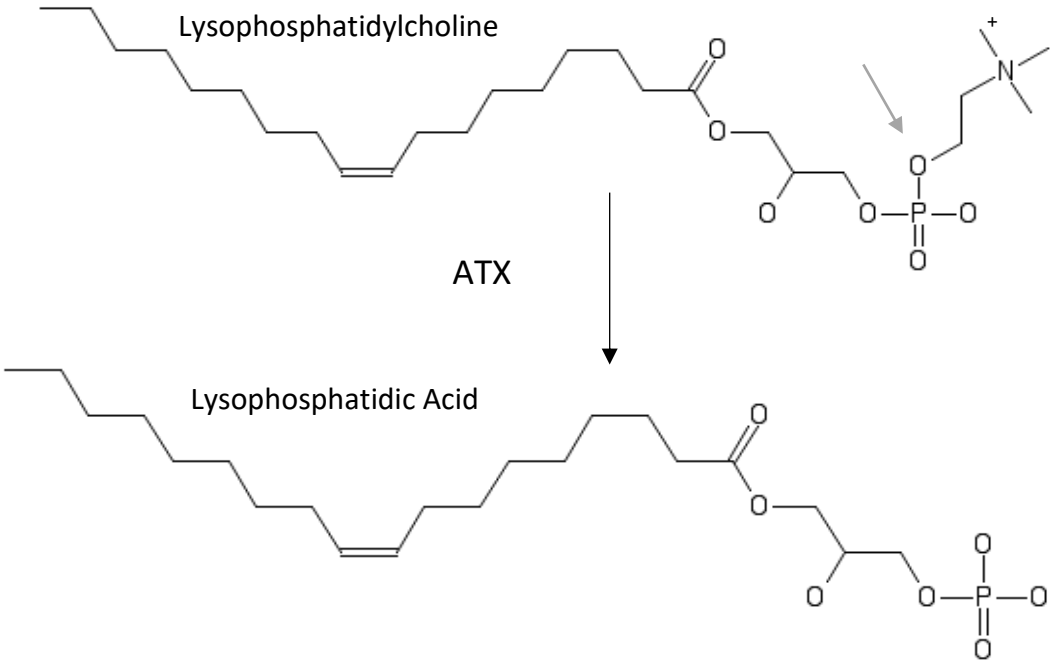


Figure 2:

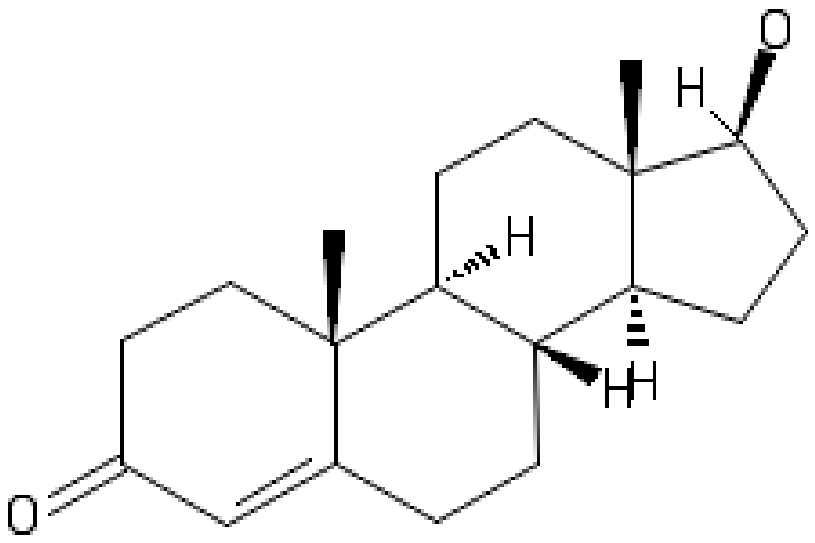


Figure 3:

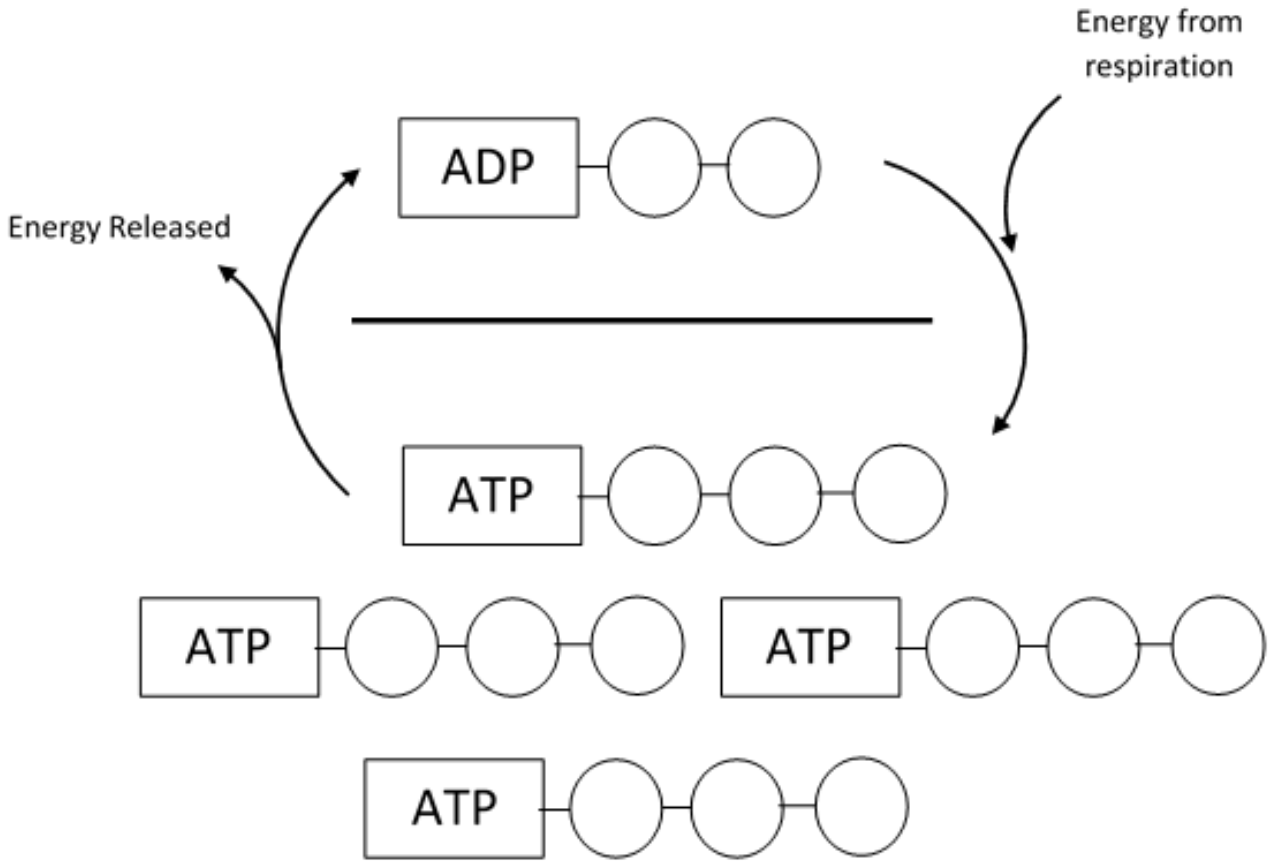

Energy from respiration
ADP
Energy Released
ATP
ATP
ATP
ATP

Figure 4:

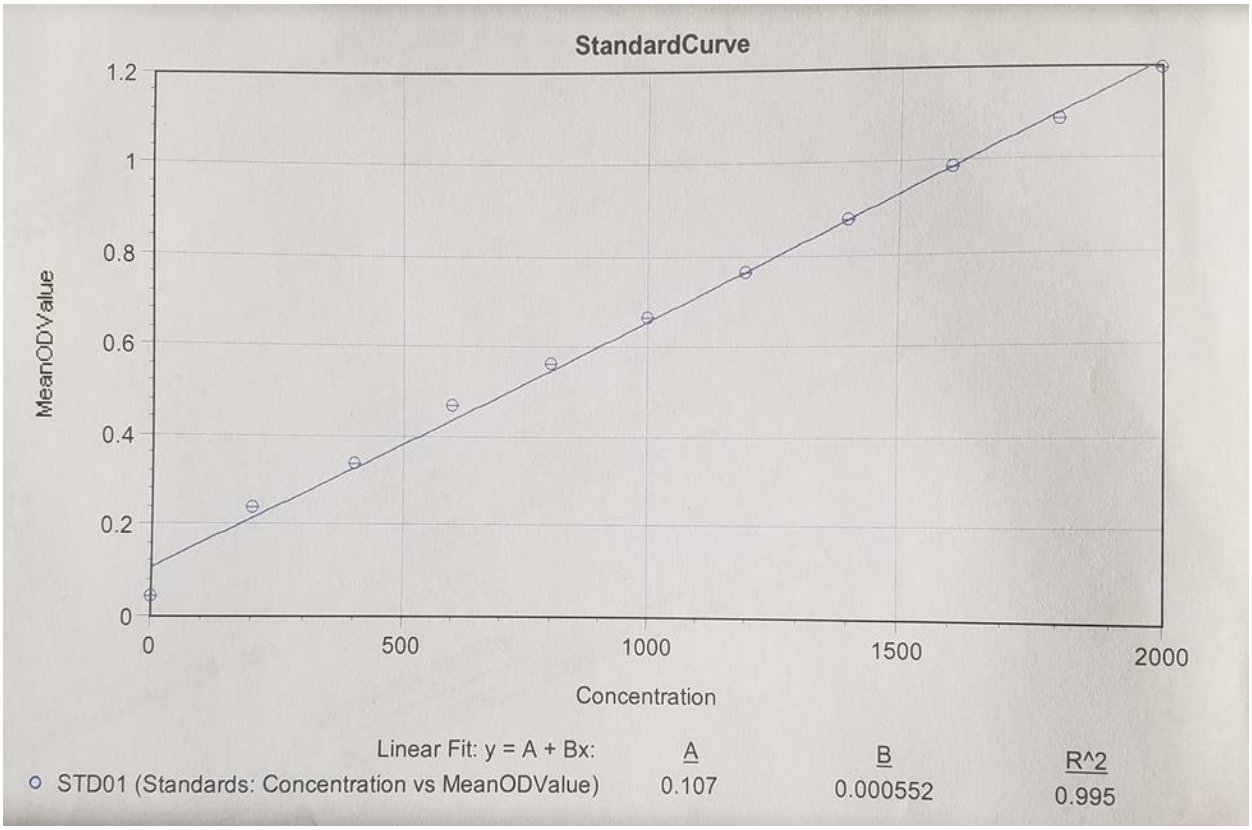

StandardCurve
MeanODValue
1.2
1
0.8
0.6
0.4
0.2
0
0
500
1000
1500
2000
Concentration
Linear Fit: y = A + Bx:
A
B
R^2
STD01 (Standards: Concentration vs MeanODValue)
0.107
0.000552
0.995

Figure 5:

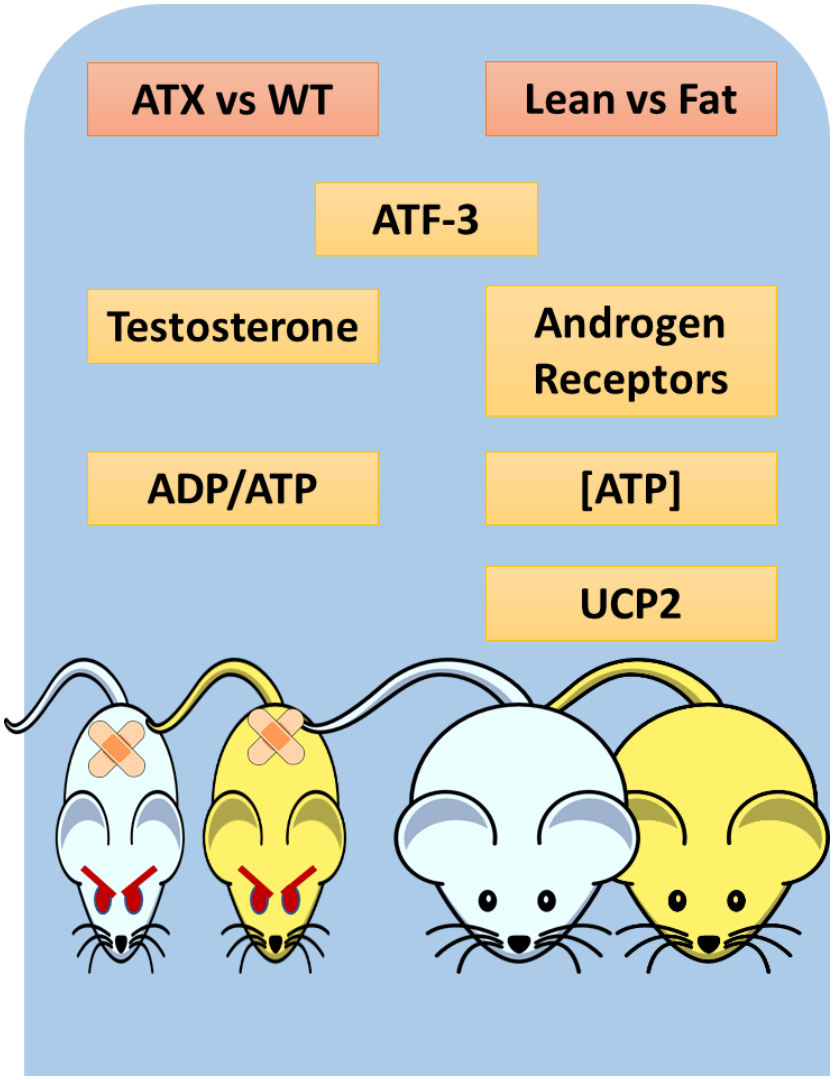
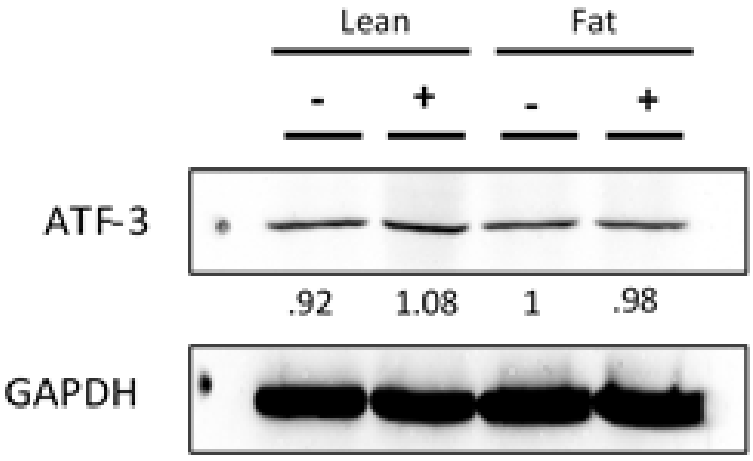
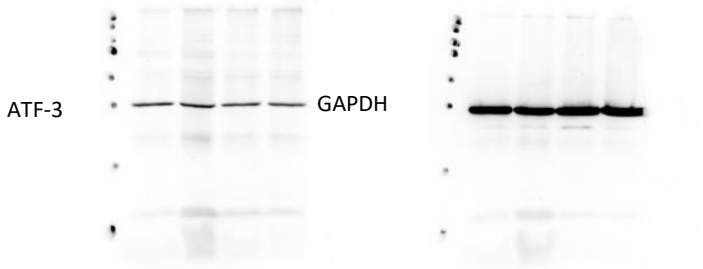

ATX vs WT
Lean vs Fat
ATF-3
Testosterone
Androgen Receptors
ADP/ATP
[ATP]
UCP2

Figure 6: w/ suplimentary whole membranes from western



Liver Whole Membrane



Kidney Whole Membrane

Figure 7:

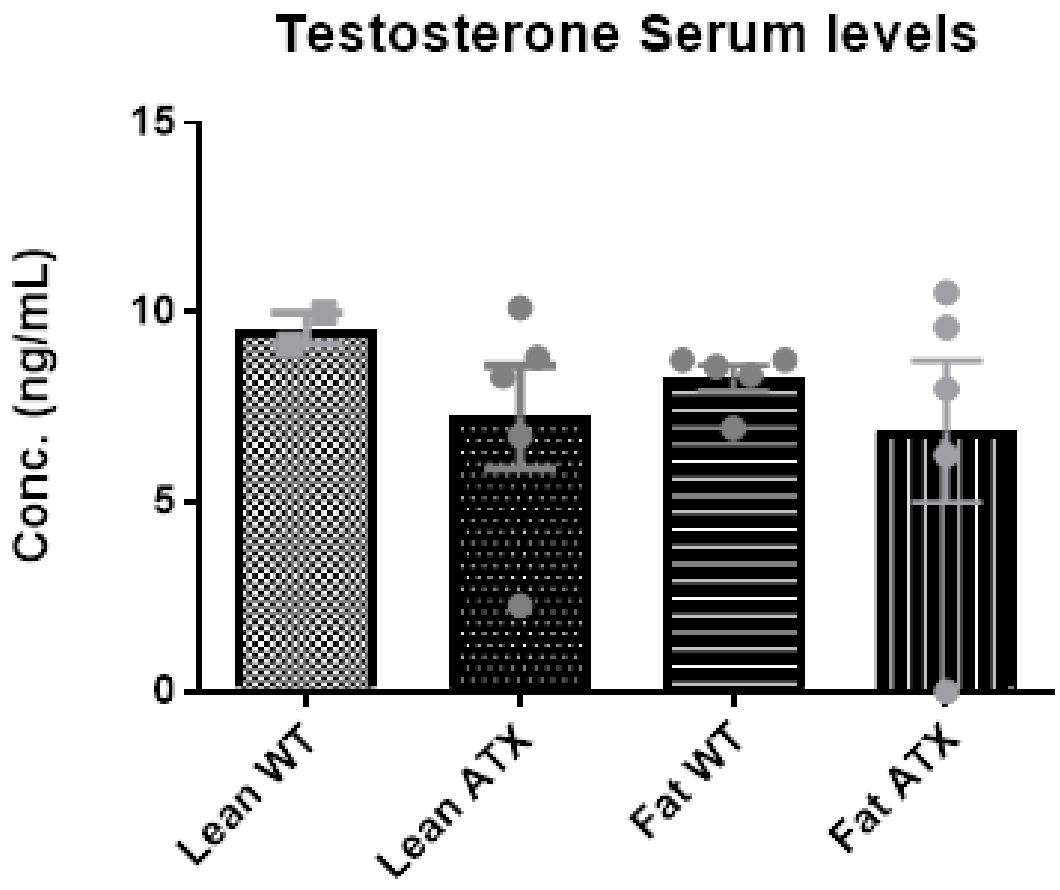

Testosterone Serum levels
Conc. (ng/mL)
15
10
5
0
Lean WT
Lean ATX
Fat WT
Fat ATX

Figure 8:

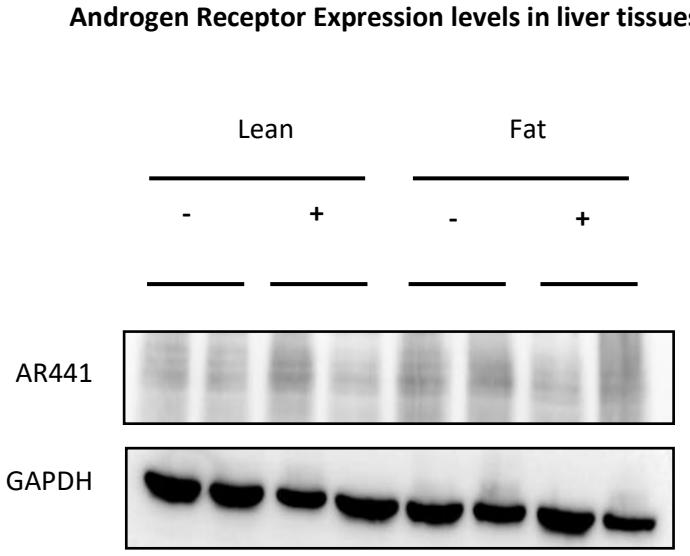
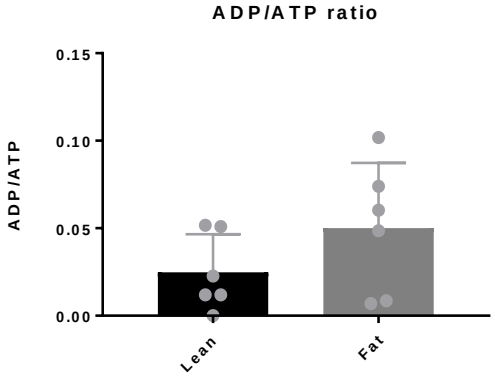
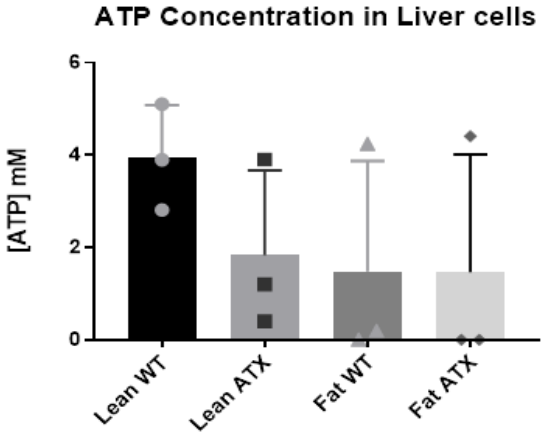
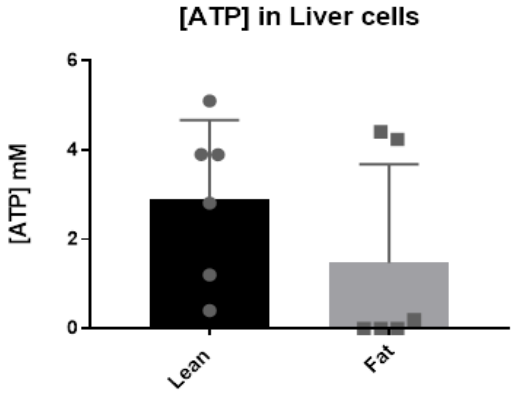
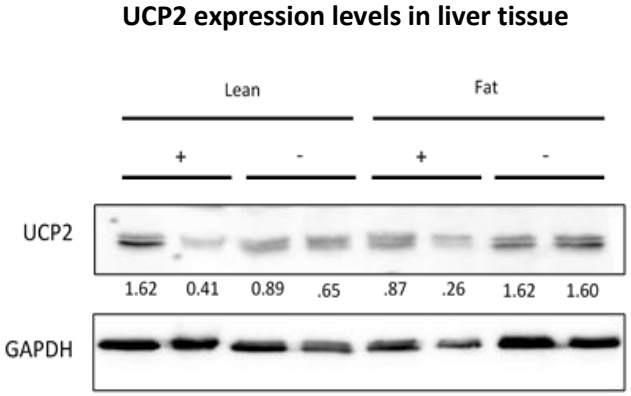

Androgen Receptor Expression levels in liver tissues
Lean
Fat
-
+
-
+
AR441
GAPDH

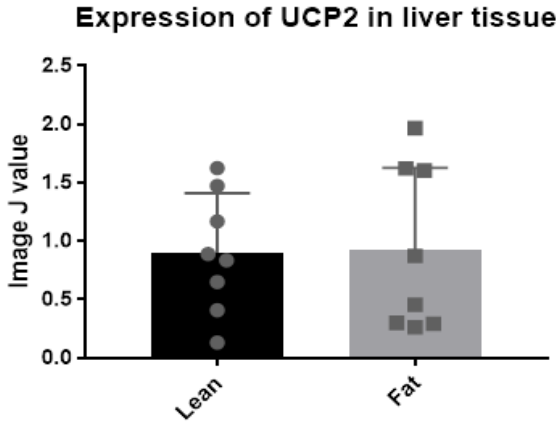
Figure 9:


ADP/ATP ratio
ADP/ATP
0.15
0.10
0.05
0.00
Lean
Fat


ATP Concentration in Liver cells
[ATP] mM
6
4
2
0
Lean WT
Lean ATX
Fat WT
Fat ATX


[ATP] in Liver cells
[ATP] mM
6
4
2
0
Lean
Fat


UCP2 expression levels in liver tissue
Lean
Fat
+
-
+
-
UCP2
1.62
0.41
0.89
.65
.87
.26
1.62
1.60
GAPDH


Expression of UCP2 in liver tissue
Image J value
2.5
2.0
1.5
1.0
0.5
0.0
Lean
Fat

APPENDIX B

TABLES

Table 1:

BSA Standard Ratios

Well	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
BSA µL	0	1	2	3	4	5	6	7	8	9	10
H_2O µL	10	9	8	7	6	5	4	3	2	1	0
BSA conc.	0	200	400	600	800	1000	1200	1400	1600	1800	2000

Table 2:

Physiological Fuel Values	**Fat Diet D12451**	**Control diet D12450H**
Protein	20 % kcal	20 % kcal
Fat	45 % kcal	10 % kcal
Carbohydrate	35 % kcal	70 % kcal
Energy Density	**4.7 kcal/g**	**3.82 kcal/g**