Rescue of Diet-induced Obesity and Estrous Cycle Irregularity with Omega-3 Rich Fish Oil Diet

Simone Marie Godwin
RESCUE OF DIET-INDUCED OBESITY AND ESTROUS CYCLE IRREGULARITY WITH OMEGA-3 RICH FISH OIL DIET

by

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Abstract

Reproduction, an energetically costly process, is subject to nutritional and metabolic control. There is a high occurrence of female infertility in obese women, and Polycystic Ovarian Syndrome (PCOS), a condition characterized by androgen excess and oligo- and amenorrhea, is highly associated with obesity and diabetes mellitus. Obesity is characterized by chronic, low-grade inflammation and increased circulating pro-inflammatory cytokines. These pro-inflammatory molecules can alter insulin signaling, thereby contributing to glucose intolerance and insulin resistance, both hallmarks of type 2 diabetes. This study seeks to elucidate the relationship between obesity, inflammation, and reproductive dysfunction through diet intervention. Diets rich in n-3 poly-unsaturated fatty acids (PUFAs), such as found in fish oil, are considered to have anti-inflammatory effects. We induced obesity and reproductive dysfunction in C57BL/6 female mice by feeding a “Western-type” high fat diet (45% energy from fat) with lard as the source of fat. At 16 weeks of age, the experimental group was switched to a diet of identical composition but with fish oil as a source of fat for an additional 8 weeks. Changes in reproductive function and estrous cycle regularity were assessed by vaginal cytology. Metabolic outcomes were evaluated by measuring body weight, food consumption, glucose tolerance, fasting insulin levels, and adipose and liver histology. Inflammatory status was determined by circulating cytokine and adipokine levels. Reproductive irregularity was successfully established by 16 weeks of age at which time the mice also had increased glucose intolerance and higher circulating leptin levels. At 24 weeks, mice that had remained on the lard based diet weighed more, had a dramatic increase in fat deposition in the liver, showed worse glucose tolerance response, and had higher fasting insulin levels than their fish-oil counterparts. Consumption of the fish oil diet for 8 weeks did not restore estrous cycle regularity for all C57BL/6 female mice, however, cyclicity did improve for fish oil mice that exhibited reduced
weight gain. These findings suggest including fish oil in the diet of obese females will improve metabolic status and improve estrous cycle regularity for those whose weight is responsive to the treatment.
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Introduction

Background

Between 2003 and 2004, 33.2% of adult women in the US were obese. With nearly one third of the female adult population (and a comparable 32.2% of all US adults) having this condition, obesity is justifiably one of the United States’ primary public health concerns (Ogden CL et al.). The etiology of this epidemic cannot be narrowed to one specific cause, but is a consequence of a complex relationship between genetics, environment, lifestyle, and diet. The health complications associated with obesity are abundant: insulin resistance, cardiovascular disease, dyslipidemia, inflammation (Buettner, Schölmerich, and Bollheimer), and there are also other physiological effects downstream of these more direct metabolic consequences. One such system that responds to energetic signals is the reproductive system. Reproduction in female mammals, from ovulation to weaning, is an energetically costly process. It therefore should come as no surprise that reproduction is metabolically controlled, and that metabolic dysfunction as induced during obesity, plays a role in the disruption of normal reproductive function. This project seeks to elucidate the relationship between obesity induced by a high-fat diet, obesity-induced inflammation, and reproductive dysfunction (by way of estrous cycle regularity) using C57Bl/J6 mice as a model.

High-fat Diets and Obesity

High-fat diets (containing more than 30% energy as fat) have successfully been used to generate models of obesity in laboratory animals since as early as the 1940s. Such models have been able to recreate the conditions associated with human obesity: weight gain, insulin resistance and type II diabetes, dyslipidemia, increase in adipocyte number and size, high blood pressure, and high leptin and low adiponectin levels. However, this is only consistently true for
HFD models that use lard or ω-6/ω-9-containing plant oils as the primary fat source, whereas diets that use fish oil (ω-3 poly-unsaturated fatty acids) as a fat source are protective against the weight gain and insulin resistance (Buettner, Schölmerich, and Bollheimer).

**Obesity and Inflammation**

Obesity is characterized by chronic low-grade inflammation, which is the body’s protective response to infection or cellular injury. The inflammatory response begins with the body’s recognition of injury or foreign pathogen, to which it responds with the release of cytokines (small proteins that serve as signaling molecules) that recruit phagocytic cells to the site of injury. These phagocytic cells contain the insult as well as recruit additional immune cells. Such a response is triggered by foreign pathogen or, in the case of obesity, sterile activation. Other examples of sterile inflammatory activators include mechanical trauma, environmental irritants, and ischemia (Lukens, Gross, and Kanneganti). During the development of obesity, there is an increase in the visceral adipose tissue depot and adipocyte hypertrophy that leads to the production of pro-inflammatory mediators such as tumor necrosis factor-α (TNFα), and interleukin-6 (IL-6). Hypertrophic adipocytes also allow for the infiltration of activated macrophages into adipose tissue, which in turn contributes to increased plasma levels of TNFα and IL-6 (Kanda et al.). One mechanism for the development of insulin resistance itself is thought to be caused by the changes in levels of these inflammatory mediators, as TNFα and IL-6 lead to the interruption of insulin signaling by inhibiting insulin receptor (IR) tyrosine kinase activity (Dandona, Aljada, and Bandyopadhyay; Hotamisligil et al.). Additionally, it has been found that high amounts of saturated free fatty acids (FFAs), but not unsaturated fatty acids, can activate Toll-like receptors and directly activate pro-inflammatory cytokine synthesis (Lee et al., 2001).
The adipocyte-derived hormones leptin and adiponectin also influence the chronic pro-inflammatory state in obesity. Leptin, known as the satiety hormone, belongs to the cytokine class I receptor family and acts on the hypothalamic nuclei to reduce food intake and promote energy expenditure (Otero et al.; Meier and Gressner). Although complete leptin deficiency results in an obese phenotype, obese individuals exhibit higher levels of serum leptin than their normal-weight counterparts. In fact, circulating serum leptin levels are positively correlated with amount of body fat, suggesting that obese individuals are insensitive to leptin’s effects (i.e., leptin insensitive), which may be due to defects in leptin’s signal transduction pathway (Considine et al.; Myers, Cowley, and Münzberg). Leptin has been shown to promote phagocytosis and the production of pro-inflammatory cytokines (Loffreda et al.). Adiponectin, a protein also secreted by adipocytes, decreases insulin resistance and blood glucose levels. In obesity and type II diabetes, circulating adiponectin levels are low compared to metabolically normal individuals. Unlike leptin, adiponectin exerts an anti-inflammatory effect by negatively regulating C-reactive protein (CRP), which activates the complement system and TNFα expression in adipose tissues. TNFα and IL-6, however, inhibit the expression of adiponectin (Meier and Gressner; Ouchi and Walsh).

Various immune populations have been implicated in the obese-inflammatory state. Regulatory T cells, also known as Tregs, suppress the immune responses of other cells and are integral in the maintenance of immune homeostasis and self-tolerance. As reviewed in Chen et al., there are a number of associations between Tregs, obesity, and insulin resistance. Tregs accumulate in the visceral adipose tissue of normal, lean mice but not in that of obese mice, and a reduction in Tregs in obese animal models is closely associated with insulin resistance and adipose inflammation. Additionally, adoptive transfer of visceral adipose Tregs into obese mice
was shown to significantly improve insulin sensitivity and glucose tolerance, while deletion of the same population was associated with a substantial decrease of insulin sensitivity and increase in inflammatory mediators (Chen, Wu, and Wang). A more recent study proposed a possible mechanism for this Treg regulation, showing that hyperinsulinemia reduces the ability of Tregs to produce the anti-inflammatory cytokine interleukin-10 (IL-10) as well as their ability to suppress the production of TNFα. (Han et al.).

In addition to Tregs, another population of immune cells has been linked to obesity-induced inflammation and insulin resistance: Gr-1⁺ CD11b⁺ myeloid-derived suppressor cells (MDSCs). These cells also cause immune suppression following inflammatory injury by inhibiting the activation of cytotoxic T cells, reducing natural killer (NK) cell cytotoxicity, promoting the proliferation of Tregs, and skewing macrophage polarization toward M2 macrophages (as opposed to M1 or “angry” macrophages) (Xia et al.; Ostrand-Rosenberg et al.). In high fat feeding animal models, Gr-1⁺ CD11b⁺ MDSCs are highly expressed in adipose tissue and the liver in early stages of obesity. Ablation of this population in obese mice resulted in significantly increased inflammation, insulin resistance, and glucose intolerance, while adoptive transfer of this population into obese animal models improved glucose tolerance (Xia et al.).

As previously mentioned, high-fat diets containing primarily ω-3 poly-unsaturated fatty acids (PUFAs) as a fat source do not induce the same metabolic effects as Western-type diets, which are rich in saturated and monounsaturated fats, and particularly important for this study, ω-6 polyunsaturated fatty acids. This is, in part, due to the systemic inflammation-reducing effects of ω-3 PUFAs, which act to reduce the proportion of arachidonic acid in immune cell membranes thereby reducing the synthesis of pro-inflammatory mediators. Additionally, diets high in ω-3 PUFAs result in increased fatty oxidation and thereby reduced adiposity (and thus
reduced infiltration of adipose tissue by macrophages) and yield higher levels of adiponectin compared with Western-type high-fat diets (Calder).

Reproduction and energetics

The obesity epidemic has prompted researchers to more closely examine the relationship between metabolism and other physiological functions—one of these functions being reproduction. Frisch and Macarthur originally proposed in 1974 that a critical level of stored energy is necessary for the onset and maintenance of reproductive function. This also implies that a female who falls below this critical energy storage, even when reproductive maturity has been reached, will cease to menstruate. Although this theory has been seriously challenged in the years following its proposal, there is some merit to the idea that energy status and reproduction are linked. While the process of ovulation itself may not necessarily be energetically costly, ovulation incurs the risk of pregnancy, lactation, and parenting, which, as any parent will agree, are energetically costly processes. Ovulation status then should reflect an animal’s overall energy status, such that much needed energy for survival is not wasted on attempts at reproduction, which in times of starvation or chronic negative energy balance, would likely be unsuccessful (Bronson and Manning). Indeed, a negative overall energy balance is associated with disruption of normal menstruation, as seen in the amenorrhea of women diagnosed with anorexia nervosa. Upon return to a normal body weight, many women with anorexia nervosa resume menses (American Psychiatric Association; Falk and Halmi). Cessation of normal cycling during times of chronic starvation (normal rat food intake was reduced by 50%) has also been observed in laboratory animal models as shown by Knuth and Friesen.. By as early as the third expected cycle, anestrus was observed in a significant number of rats.

The disruption of normal ovulation and cycling in times of negative energy status is well
established, but less intuitive is this disruption during periods of chronic energy excess (i.e., obesity). It would seem that consistent positive energy balance would mean consistent reproductive success, as excess energy stored in adipose tissue would be more than sufficient for reproductive (and overall) energy demands if mobilized to oxidizable free fatty acids (FFAs). Wade and Schneider proposed that in obese individuals, the body is primed for storage rather than mobilization and oxidation of metabolic fuels, resulting in an effective shortage of energy (Wade and Schneider). This predisposition toward storage would result in reproductive impairment similar to those seen in chronic undernutrition. Support for this appeared in 1952 even before this explanation was proposed when Rogers and Mitchell reported that obesity is associated with amenorrhea in women. In 1974, Hartz et al. showed that with increasing severity of obesity, there is increasing incidence of anovulatory cycles and irregular menstruation. Similar effects have been observed in laboratory animals, including estrous cycle irregularities in overfed rats, fewer overall cycles in high-fat diet induced obese mice, and total sterility of the morbidly obese ob/ob (leptin-deficient) mouse (Glick et al.; Sharma et al.; Ewart-Toland et al.). Similarly, metabolic syndrome and Type 1 Diabetes are also associated with disruption in normal reproductive function, further exemplifying the relationship between metabolism and reproduction (Codner, Merino, and Tena-Sempere; Brothers et al.).

Mechanisms underlying obesity related reproductive dysfunction

If metabolism and reproduction are so highly intertwined, what is the actual link between the two? The hypothalamic-pituitary-gonadal (HPG) axis controls reproduction in humans and other mammals through regulating many endocrine organs. The activation of this axis results in menstruation and cycling and it therefore must be sensitive to metabolic signals.

The regulation of reproduction and the estrous cycle occurs mainly through the
neurosecretory molecule gonadotropin releasing hormone (GnRH), released from the hypothalamus, which acts on the anterior pituitary to stimulate the synthesis and secretion of the gonadotropins leutenizing hormone (LH) and follicle stimulating hormone (FSH). LH and FSH in turn, target the ovaries and uterus in females, stimulating the production of the sex steroids estrogen, progesterone, and testosterone—all of which contribute to the development and maintenance of the genital tract. The sex steroid estradiol functions on this axis in two ways: 1) at low levels, it exerts negative feedback control over FSH and LH and 2) at prolonged, high levels, induces LH and FSH surge. The steroid progesterone enhances the negative feedback effects and blocks the positive feedback effects of estradiol (Johnson; Goldman, Murr, and Cooper). The relationship between these steroids and gonadotropins throughout the phases of the rodent estrous cycle are reviewed in Goldman et al.’s 2007 paper “The Rodent Estrous Cycle: Characterization of Vaginal Cytology and its Utility in Toxilogical Studies” and Johnson’s text *Essential Reproduction* (Goldman, Murr, Cooper; Johnson). The normal rodent estrous cycle lasts approximately 4-5 days and occurs in 4 phases: proestrus, estrus, metestrus, and diestrus. Proestrus and estrus are characterized by epithelial growth in the reproductive tract, with estrus culminating in ovulation. Metestrus is characterized by degenerative epithelial changes, and diestrus is characterized by quiescence and slow cell growth (Quimby).

The hypothalamus, and more specifically the arcuate nucleus, also serves as an important site of regulation for energy intake and expenditure in the body. It is the site of action for the adipocyte-derived hormone leptin, which is thought to be the best candidate for the link between reproduction and metabolism. Leptin, as previously mentioned, is the product of the *ob* gene and suppresses food intake and stimulates energy expenditure. Kennedy and Mitra first showed that both the reproductive axis and leptin signaling occur in the hypothalamus (Kennedy and Mitra).
The leptin-deficient homozygous recessive *ob/ob* mouse, first produced by Jackson Laboratory in 1949, exhibited not only morbid obesity and type II diabetes, but also total sterility and low levels of LH suggesting that leptin plays an important role somewhere along the reproductive axis (Ingalls, Dickie, and Snell; Zhang et al.). Supplementation with exogenous leptin can restore fertility in both male and female *ob/ob* mice (Chehab et al.). Later studies showed that administration of even low concentrations of leptin in vivo were able to stimulate the release of LH via both hypothalamic and pituitary action (Yu et al.).

**Polycystic ovarian syndrome**

In humans, one of the main manifestations of reproductive dysfunction associated with obesity is polycystic ovarian syndrome (PCOS). PCOS is characterized by oligoovulation or anovulation, androgen excess, and sometimes, but not always, polycystic ovaries. In women with PCOS, LH release is increased compared to FSH release, which contributes to increased androgen production. Women diagnosed with PCOS often are obese (sources citing anywhere from 16-80%) and insulin resistant, suggesting that insulin resistance is a major factor in driving the condition. Increased androgen production has been found to drive insulin resistance, but it is thought that genetic predispositions to improper insulin receptor signaling in conjunction with obesity are the main driving factors of the androgen excess and reproductive abnormalities (Dunaif). It has yet to be fully explored how the obese state exacerbates this condition, but the recent findings linking inflammation to insulin resistance implicate obesity-induced inflammation could play a significant role in further driving PCOS.

Although the genetic predispositions to PCOS are not present in laboratory animals, reproductive dysfunction is still seen in obese animals, and thus laboratory animals prone to diet-induced obesity can serve as a model for probing the etiology of the condition. Our hypothesis is
that changes in immune populations and inflammatory mediators associated with insulin resistance will precede insulin resistance. We expect that once insulin resistance is established, reproductive dysfunction by way of irregular cycling will follow.

Study Aims

This first aim of this study is to confirm that inflammation-mediated metabolic and reproductive dysfunction can be established through feeding C57Bl/6 mice a high-fat Western-type diet high in n-6 fatty acids. The second aim of this study is to see if substituting the source of fat with one that is rich in n-3 polyunsaturated fatty acids will result in improved metabolic and reproductive outcomes due to its anti-inflammatory nature.

Materials and Methods

Animals and diets

C57BL/6 female mice were bred and housed at the University of Memphis campus. (Breeding pairs purchased from Harlan Laboratories, Inc., Indianapolis, IN). All animals received food and water ad libitum under a 12-hour dark/12-hour light cycle. To establish our model, female mice were weaned at 3 weeks of age onto a standard rodent chow diet (Chow, n=13) or a Western-type diet (HFL, n=13) composed of 45% energy from fat (45% kcal from lard-based fat, 41% carbohydrate, 20% sucrose, 9% cornstarch, 12% Maltodextrin 10) and were exposed to their respective diets until 20 weeks of age. For our intervention, female mice (n=18) were weaned onto the HFL diet through 16 weeks age, at which point half of the mice (n=9) were converted to a high-fat diet of 45% kcal which substituted Menhaden (fish) oil (HFO) as the fat source while the other half (n=8) remained on HFL until sacrifice at 24 weeks of age (Table 1A). Mice were group housed with no more than 5 mice per cage. Food intake and total body weight were measured twice per week. The high-fat experimental diets were identical in overall
composition but differed in their fatty acid profiles (Table 1B). The HFL diet contained a higher ω-6 to ω-3 fatty acid ratio than the HFO diet (13.1 to 0.3 respectively). The HFL diet contained high amounts of stearic, oleic, and linoleic acid while the HFO diet contained greater amounts of stearidonic, eicosapentaenoic (EPA), docosapentaenoic, and docosahexaenoic acid (Table 1B). All experiments were conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Experimental animals and were approved by the University of Memphis Institutional Animal Care and Use Committee.
Table 1. Composition of Experimental Diets. HFL n-6 with 45% kcals/g from lard; HFO n-3 with 45% kcals/g from menhaden oil. (A) ingredients in HFL-n-6 and HFO n-3 diets; (B) fatty acid composition of HFL n-6 and HFO n-3 diets.

A

<table>
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<th>HFL n-6</th>
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<tr>
<td></td>
<td>gm (%)</td>
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*BHQ: tert-Butylhydroquinone
Table 1. Composition of Experimental Diets. HFL n-6 with 45% kcals/g from lard; HFO n-3 with 45% kcals/g from menhaden oil. (A) ingredients in HFL-n-6 and HFO n-3 diets; (B) fatty acid composition of HFL n-6 and HFO n-3 diets.

<table>
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<th>Ingredients (g)</th>
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<tr>
<td>Lard</td>
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<td>Menhaden Oil, ARBP-F</td>
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Tissue collection and histology

Tissues were harvested and weighed at sacrifice for each animal. A portion of liver, adipose tissue, and ovaries were fixed in formalin solution (Fisher Scientific Co. LLC), embedded in paraffin, and 4μm sections were stained with hemotoxylin and eosin. Adipocyte size (average surface area of 10-15 randomly selected adipocytes from each mouse were determined using Axiovision r4.8.2 software. Analysis and documentation were performed using an imager M2 microscope (Axiocam MRC, Zeiss, Oberkochen, Germany). A second portion of liver, adipose tissue, and ovaries were snap-frozen in liquid nitrogen and stored at -80°C for cytokine and adipokine analysis. The spleen, mesenteric lymph nodes (MLNs), and the remainder of liver and adipose tissue were collected and immediately processed for immune cell population analysis. Spleens and MLNs were placed into RPMI 1640 solution containing 2% FBS. Liver was placed into MACS buffer containing 2% FBS. Adipose tissue was placed in to DMEM (HyClone, GE Healthcare Life Sciences, Logan, Utah).

Glucose tolerance test and fasting insulin measurement

At 8, 12, 16, 20, and 24 weeks of age, half of the mice were subjected to a glucose tolerance test (GTT). Mice were fasted for 6 hours and a baseline fasting blood glucose level was measured from a tail vein sample using a glucometer (Onetouch Ultra 2 Meter, Bayer Healthcare, Tarrytown, New York). Mice then received an intraperitoneal injection of glucose (2 g/kg body weight), and blood glucose readings were measured again at 30, 60, and 90 minutes after the injection. For fasting insulin measurements, the mice that did not undergo GTT were fasted for 6 hours, and serum samples were collected from the cheek vein and stored at -80°C and subsequently analyzed by ELISA (Thermo Fisher Scientific, Waltham, MA).
Cytokine and adipokine analysis

Plasma samples were collected from the cheek vein of mice and stored at -80°C. Lysates were made from previously snap-frozen tissue samples by lysing and homogenizing in the presence of a protease inhibitor (Thermo Fisher Scientific) and centrifuged at 15000 RPM for 10 minutes. Plasma and tissue cytokine and adipokine levels were measured via magnetic bead assay (R&D Systems, Minneapolis, MN) and read on the Luminex MAGPIX platform.

Measurement of Estrous Cycle Regularity

Vaginal lavage was performed on mice to determine estrous cyclicity. To establish our model, the mice that remained on a single diet for 20 weeks were evaluated for 14 days starting at 16 weeks of age. To test intervention effects, mice weaned onto high-fat lard were evaluated for 14 days at 14 weeks of age until their intervention (16 weeks of age). These mice were then evaluated again for 14 days beginning at week 22 until sacrifice. Lavage fluid was placed onto slides and allowed to dry. Smears were stained with methylene blue and classified as proestrus, estrus, metestrus, or diestrus based on the proportion of nucleated epithelial cells, cornified epithelial cells, and leukocytes present.

Cell suspensions

Livers, spleens and MLNs were isolated by mashing with the end of a syringe plunger and filtered through a 40 µm nylon mesh filter. Spleens and whole blood was treated with red blood cell lysis buffer. Spleens, MLNs, and blood were spun at 1700 RPM for 7 minutes and resuspended in PBS containing 2% FBS. Liver cells were diluted into 8ml of MACS buffer containing 2% FBS, overlayed onto 3 ml of Lympholyte cell separation media (Cedarlane, Burlington, NC) and centrifuged without brakes for 25 minutes at 2250 RPM. The buffy coat above the lympholyte was removed and diluted into 15 ml PBS with 2% FBS and centrifuged at
1700 RPM for 7 minutes and resuspended in PBS with 2% FBS. Adipose tissue was minced and treated with 2 mg/ml type II collagenase (Worthington, Lakewood, New Jersey) in DMEM incubated and shook for 40 minutes at 37°C and filtered through a 40 µm nylon mesh filter. Cells were then centrifuged at 500 G for 10 minutes and resuspended in PBS with 2% FBS.

Flow cytometry

Cells were counted using a Neubauer improved hemocytometer and plated onto a 96-well-plate such that each well contained between 1 and 5 million cells. Cells were centrifuged at 1500 RPM for 5 minutes, the supernatant was removed. Cells were then incubated with fragment crystallizable block (FcX, Biolegend, San Diego, California) diluted in PBS with 2% FBS (1:50) on a plate shaker in the dark at room temperature for 10 minutes to prevent nonspecific antibody binding. Cells were then incubated with population specific antibodies at optimum concentrations (Table 2) for 30 minutes on a plate shaker in the dark at room temperature. Cells were spun at 1500 RPM for 5 minutes, the supernatant was removed, and the cells were suspended in fixative solution (eBioscience, San Diego, California) overnight at 4°C. In the morning, fixative was removed by centrifuging at 1500 RPM for 5 minutes, and supernatant was discarded. Cells receiving intracellular stains were incubated with Fc block diluted in permeabilization buffer (eBioscience, San Diego, California) for 15 minutes at room temperature on a plate shaker in the dark. Cells were then incubated with the intracellular antibody diluted in permeabilization buffer for 30 minutes at room temperature on a plate shaker in the dark. Cells were centrifuged once more at 1500 RPM for 5 minutes, supernatant was discarded, and cells were resuspended in PBS with 2% FBS for analysis using the LSR II Flow Cytometer (BD Biosciences, San Jose, California).
Table 2. Antibodies used for flow cytometric analysis

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Statistics

Data are presented as means ± SEM. Statistical significance between different experimental groups was determined using the non-parametric Mann-Whitney U test due to small n values. *p*-values less than 0.05 were considered statistically significant.

Results

*A diet rich in ω-6 fatty acids induces obesity, glucose intolerance, and liver damage*

Mice exposed to the HFL diet containing 45% kcal from lard gained more weight than their chow fed counterparts (Figure 1). Significant differences in total weight were observed by 9 weeks of age, and at sacrifice HFL mice weighed on average 50.8% more than chow mice.
Figure 1. Time course for body weight gain. Mice were fed a chow (n=12) or HFL (n=13) diet and total body weight measured weekly from weaning until 20 weeks of age. HFL mice gained more weight over time than chow mice. Results represent mean ± SEM (Chow SEMs included not visible). * p<0.05

Chow mice consumed more food by weight over the study period (Figure 2), but gained less weight per gram of food consumed (Figure 3), consistent with the HFL diet being more energy dense. The HFL diet contained 45% fat and has an energy value of 4.72 kcal/g, while the chow diet contains only 6% fat and has an energy value of 3.2 kcal/g.
Figure 2. Total amount food consumed per mouse. Food consumption was measured twice weekly for each cage of mice. Total food consumption per mouse (Chow, n=8; HFL, n=13) was obtained by summing food intake over the study period and dividing by the number of mice in the cage. Chow mice consumed more food by weight than HFL mice. Results represent mean ± SEM. *p<.0001

Figure 3. Weight gained per kcal of food consumed. Change in each mouse’s weight from weaning until sacrifice was divided by total amount of food consumed per mouse for that mouse’s cage (Chow, n=8; HFL, n=13). HFL mice gained more weight per gram of food consumed than chow mice. Results represent mean ± SEM. *p<.0001
At sacrifice visceral adipose tissue was harvested and weighed. HFL mice exhibited increased fat in the mesentery (data not shown) and a significant increase visceral adipose as seen in Figure 4.

![Figure 4](image)

**Figure 4. Total adipose tissue weight.** Visceral adipose tissue was weighted at sacrifice. Adipose tissue was greater in HFL mice compared to chow (Chow, n=12; HFL n=13). Results represent mean ± SEM *p<.0001

In addition to increase in total fat content, adipocytes were also enlarged compared to chow mice (Figures 5a and b). This is consistent with previous data showing that excess energy causes adipocyte hypertrophy (Bays et al.).
Figure 5a. Adipocyte Area. Representative images of visceral adipose tissue H&E stained section demonstrate the hypertrophy that occurs with HFL feeding. (10X magnification)

Figure 5b. Adipocyte area. Random images of adipose histology were selected for adipocyte size analysis. The area of 15-20 adipocyte per slide per mouse were determined. Adipocyte size was larger for HFL mice than chow (Chow, n=7; HFL, n=9). Results represent mean ± SEM *p<.0001
Dysfunctional carbohydrate metabolism as demonstrated by insulin resistance and glucose intolerance is a hallmark of diet-induced obesity. Fasting glucose levels were determined and a glucose tolerance test performed. There was no difference in fasting blood glucose (data not shown), but mice consuming a HFL diet showed a dramatic decrease in glucose clearance at both 8 and 20 weeks, but the difference between groups was only significant when measured at 20 weeks (Figure 6).

**Figure 6. Glucose tolerance test over time.** Blood glucose concentration was measured over a 90 minute period after IP injection with 2 g glucose/kg body weight at 8 (Chow, n=4; HFL, n=5) and 20 weeks of age (Chow, n=4; HFL, n=7). HFL mice (red lines) showed worse glucose tolerance response than chow mice (black lines). Results represent mean ± SEM *significantly different at each time point, p<.05

Non alcoholic fatty liver disease is associated with a high fat diet. Even though there was no difference in liver weight between the two diets (Figure 7a), there was a dramatic increase in fat deposition liver exposed to HFL (Figure 7b), indicative of liver steatosis.
Figure 7a. Liver health. Livers were harvested and weighed at sacrifice. HFL mice showed a greater range of liver weights than chow mice (Chow, n=12; HFL, n=13). Results represent mean ± SEM.

Figure 7b. Liver Health. Representative image of H&E stained liver section. HFL mice exhibit a dramatic increase in fatty deposits in the liver seen as round white openings (indicated by black arrows).
A diet rich in ω-6 fatty acids alters circulating leptin but not cytokine levels

The adipokine leptin is secreted by the adipose tissue and has been shown to be systemically increased during obesity. Circulating plasma leptin levels were higher in HFL mice and increased over time (Figure 8). It is also known that hypertrophied adipose tissue releases inflammatory cytokines, but we did not detect increased levels of TNFα, IL-6 or IL-1β in tissue lysates or in the blood (data not shown).

![Figure 8. Circulating plasma leptin over time.](image)

Plasma samples were obtained at 8 (Chow, n=2; HFL, n=5), 16 (Chow, n=3; HFL, n=5), and on the day of sacrifice at 20 weeks of age (Chow, n=3; HFL, n=5). HFL mice showed increased leptin over time, whereas chow mice showed little detectable circulating plasma leptin. Results represent mean ± SEM *week 20 comparison between HFL and Chow, p=0.036.

Normal Estrous Cyclicity is altered in mice fed a high-fat diet rich in ω-6 fatty acids

Cycling was followed by vaginal lavage. Mice fed a chow diet demonstrated a normal 4-5 day cycle as seen in Fig 9a. Mice fed a high-fat diet exhibited significantly longer estrous cycles than their chow-fed counterparts (Figures 9a and 9b). HFL mice also showed a shortened proestrus phase and a slightly lengthened estrus phase compared to chow mice (Figure 9c).
Figure 9a. Estrous Cyclicity. Five consecutive days of observation reveal normal 4-5 day cycle length in chow mice and elongated cycle and altered vaginal cytology in HFL mice.
**Figure 9b. Estrous Cyclicity.** HFL mice exhibited longer cycles than chow mice over the course of cycle monitoring (Chow, n=4; HFL, n=8). Results represent mean ± SEM *p=.0182

**Figure 9c. Estrous Cyclicity.** Phase length was normalized to cycle length and number of days observed. HFL mice exhibited shortened proestrus phase but this difference was not significant. There were no differences in any other phases (Chow, n=4; HFL, n=5). Results represent mean ± SEM *trending toward significance, p=.069
Intervention with an isocaloric diet containing fish oil diet rich in ω-3 polyunsaturated fatty acids rescues metabolic outcomes and liver damage

Mice that were switched from the lard-based to the isocaloric fish oil based diet after 16 weeks of age showed reduced weight gain compared to mice that remained on HFL (Figure 10).

**Figure 10. Intervention total body weight gain.** Total body weight was measured weekly from weaning until 24 weeks of age for mice fed either HFL or switched to HFO after 16 weeks of age (HFL, n=5; HFO Switch, n=7). HFL mice gained more weight over time than HFO mice. Results represent mean ± SEM *significantly different at each timepoint; p<.05

During the intervention (week 16 -24), the mice consuming the HFO gained less weight/kcal of food as compared to the mice consuming the HFL diet. The HFO mice did consume slightly but not significantly less food (data not shown). This is consistent with data showing that a fish oil
based diet induces an increase in the expression of transcription driving the fatty acid oxidation program as compared to fatty acid synthesis when consuming the HFL diet (Figures 10 and 11).

**Figure 11. Weight gained per kcal of food consumed after intervention.** Weight gained during the intervention was normalized to the amount of energy consumed during this time period. (HFL, n=5; HFO Switch, n=7). HFL mice gained more weight per kcal of food consumed than HFO mice. Results represent mean ± SEM *p=.048

The weight of visceral adipose tissue was significantly lower in mice switched to HFO (Figure 12a). Despite the differences in adipose weight, adipocyte size was comparable between the two groups (Figures 12b and c). To determine changes in carbohydrate metabolism, glucose tolerance tests were performed at the beginning of intervention and at the end. At week 24 (end of intervention) there was improvement in glucose tolerance (Figure 13a) and fasting insulin levels (Figure 13b) for mice on the HFO diet. There was no difference observed in liver weights between the two groups (Figure 14a), but a dramatic improvement could be seen in fat deposition of mice switched to HFO (Figure 14b).
Figure 12a. Intervention adiposity. Intervention adipose tissue weight shown as fraction of total body weight. Visceral adipose tissue was weighted at sacrifice. Adipose tissue comprised a greater fraction of total body weight in HFL compared to HFO (HFL, n=5; HFL n=7). Results represent mean ± SEM

Figure 12b. Intervention adiposity. Representative histology of HFL and HFO Switch mice. Adipocyte size did not differ between HFL and HFO mice.
Figure 12c. Intervention adiposity. Random images of adipose histology were selected for adipocyte size analysis. Adipocyte size comparable between the two groups (HFL, n=3; HFO Switch, n=3). Results represent mean ± SEM.

Figure 13a. Intervention glucose tolerance test over time. Blood glucose concentration was measured over a 90 minute period after IP injection with 2 g glucose/kg body weight prior to intervention at 16 weeks (HFL, n=11), and prior to sacrifice at 24 weeks (HFL, n=5; HFO Switch, n=7). HFO mice showed improved glucose tolerance compared to HFL mice. Chow mice shown for reference (n=4).
Figure 13b. Intervention fasting serum insulin before and after intervention. Fasting serum sampled were collected prior to intervention at 16 weeks of age (HFL, n=3) and prior to sacrifice at 24 weeks of age (HFL, n=2; HFO Switch, n=2). HFO mice showed lower fasting serum insulin levels than HFL mice. Results represent mean ± SEM.

Figure 14a. Intervention liver health. Livers were harvested and weighed at sacrifice. There was no difference in mean liver weights, but HFL mice showed a greater range of liver weights than HFO mice (HFL, n=5; HFL, n=7). Results represent mean ± SEM.
Figure 14b. Intervention liver health. Representative images of liver sections stained with H&E after intervention. HFL mice exhibit fatty deposits (white holes) in the liver (indicated by black arrows) while very few deposits could be detected in the mice consuming the HFO diet.

*Intervention with fish oil diet alters leptin but not cytokine levels*

Circulating plasma and adipose leptin levels were reduced in HFO mice compared to HFL (Figures 15a and b). There were no detectable differences in adipose, liver, or circulating plasma cytokine levels (data not shown).
Figure 15a. Circulating plasma leptin levels after intervention. Plasma samples were obtained on the day of sacrifice at 24 weeks of age. Leptin concentration were determined using a multiplex bead assay, HFO mice showed lower plasma leptin levels than HFL mice (HFL, n=4; HFO Switch, n=6). Results represent mean ± SEM

Figure 15b. Adipose tissue leptin levels after intervention. Adipose tissue leptin determined from lysates of adipose tissue samples collected at sacrifice. Leptin concentration was determined using a multiplex bead assay. HFO mice showed lower adipose leptin levels than HFL mice (HFL, n=3; HFO Switch, n=4). Results represent mean ± SEM
**Intervention with fish oil diet somewhat alters immune populations**

There were slight alterations in immune populations of interest in adipose tissue with fish oil treatment (Figure 16). CD11b⁺ cells were reduced in fish oil intervention mice compared to HFL mice, as were F4/80⁺ cells. Changes in T regulatory cell populations could not be determined due to the lack of CD3⁺ cells in the sample (data not shown).

![Figure 16. Intervention changes in adipose immune populations.](image)

**Figure 16. Intervention changes in adipose immune populations.** Intervention mice exhibited reduced percentage of A) CD11b⁺ cells and B) F4/80⁺ cells as compared to HFL mice.

**Intervention with fish oil diet alters estrous cyclicity**

At the end of intervention the mice could be classified as being high weight (greater than 30 g total body weight) or reduced weight (less than 30 grams total body weight) groups. The high weight group did not have any alteration in the cycle length, while the reduced weight group exhibited shorter cycles compared to mice that remained on HFL (Figure 17a). More similar to what is seen when mice consume a show diet. HFL mice spent less time in proestrus and longer in estrus compared to reduced weight mice switched to HFO (Figure 17b).
Figure 17a. Intervention estrous cyclicity. HFL mice exhibited longer cycles than reduced weight (total body weight less than 30 grams) HFO mice over the course of cycle monitoring (HFL, n=7; HFO Switch, n=6). Results represent mean ± SEM *trending toward significance, p=.069.

Figure 17b. Intervention cyclicity. Phase length was normalized to cycle length and number of days observed. HFL mice exhibited shortened proestrus phase and lengthened estrus phase compared to HFO mice (HFL, n=7; HFO Switch, n=9). Results represent mean ± SEM.
Discussion, Conclusions, and Recommendations

Discussion

The relationship between metabolic dysfunction and infertility, particularly in the case of PCOS, has long been known. Metabolic dysfunction has many manifestations, including obesity with a high deposition of visceral fat and the development of insulin resistance. Interestingly, many females diagnosed with PCOS have also been diagnosed with type II diabetes.

It is now well established that hypertrophic adipose tissue leads to low grade chronic inflammation and that it is the release of these pro-inflammatory molecules that are driving many of the pathologies associated with obesity, including type-II diabetes and cardiovascular disease. The question therefore remains as to what affect a diet high in anti-inflammatory molecules, such as contained in fish oil (omega 3 fatty acids) will have on the reproductive dysfunction associated with obesity.

Few studies have been conducted to date on the effects of dietary supplementation with omega-3 PUFAs and reproductive outcomes. Most studies focused on long term consumption and not on the use of omega-3 PUFAs as an intervention at therapeutic levels. Nehra et al. saw that a life-long consumption of a diet high in omega-3 PUFAs compared to omega-6 PUFAs (ω-6:ω-3 ratio of 1:20 and 10% kilocalories from fat) elongated the reproductive lifespan of female C57Bl/6 mice, and short term treatment near the age-related decline in murine reproductive life with omega-3 PUFAs improved oocyte quality compared to omega-6 treatment, which in fact reduced oocyte quality. Wakefield et al. fed female C57Bl6 mice a diet high in omega-3 PUFAs (ω-6:ω-3 ratio of 1:5.14 and 7% kilocalories from fat) compared to a chow diet and found adverse effects in not only the maturing oocyte in the form of reactive oxygen species but also in the morphological appearance and developmental ability of the embryo at the blastocyst stage.
An earlier study in female rats showed increased ovulation with a low fat diet rich in ω-3 PUFAs, (10% fat and ω-6:ω-3 ratios of 1:0.2 and 1:1 for their low ω-3 and high ω-3, respectively), (Trujillo and Broughton).

Based on this information, we hypothesized that a high fat diet (containing mostly ω-6 PUFAs) would generate a pro-inflammatory milieu, such that it would drive the development of metabolic and reproductive dysfunction and that these dysfunctions could be rescued by a diet rich in ω-3 PUFAs from fish oil possibly through its anti-inflammatory action.

We induced obesity, metabolic dysfunction, and cycle irregularity by feeding C57Bl/6 female mice a Western-type high-fat diet containing 45% fat from lard. Mice on this high-fat diet gained 50.8% more total body weight than mice on a standard chow diet and demonstrated fatty liver development and glucose intolerance from week 16 onwards. These female mice exhibited a trend toward a shortened proestrus phase in agreement with previous diet-induced obesity studies in C57Bl/6 mice (Sharma et al.).

To determine if the increase in weight has generated an increase in pro-inflammatory cytokine secretion, we used a bead-based multiplex assay to determine protein levels. Low levels of pro-inflammatory cytokines were detected in tissue lysates and plasma, but there were no observable differences between the lard-based or fish oil intervention diet. However, there were dramatic increases in leptin levels systemically and in the adipose tissue lysates for mice consuming the high fat lard diet, with a decrease when consuming the fish oil diet. Systemic leptin levels correlated with the amount of visceral adipose tissue. In addition to sending satiety signals, leptin also promote the production of pro-inflammatory cytokines and phagocytosis (Loffreda et al.), suggesting that the physiological environment created by the omega-6 fatty acids from the lard diet, is in a pro-inflammatory state. In addition to increased leptin levels,
glucose tolerance also deteriorated over time when consuming the lard diet. Glucose intolerance results from attenuation of signaling through the insulin receptor. It is well-established that pro-inflammatory molecules such as TNF-α and IL-6 can alter the insulin signaling culminating in insulin resistance. As various immune populations play a role in the increase of pro-inflammatory molecules, immune cells in the adipose tissue were phenotyped. There were no differences in the T cells compartments, including Foxp3+ regulatory T cells in the visceral adipose tissue. Although not significant, there was an observable reduction in infiltrating CD11b+F4/80+ macrophages (these cells are associated with generating a pro-inflammatory environment) when consuming the fish oil diet. Even though the differences in cell numbers were small, there might be alterations in the functionality of these cells in regards to their activating or suppressor capacity. As the fish oil diet was able to stop the exacerbation of glucose intolerance, it suggests that the anti-inflammatory activity of this diet, especially induced by EPA and DHA, reduces the inflammatory milieu, returning metabolic functionality and improve lipid metabolism as suggested by Kopecky et al.

In addition to cytokines, sex hormones can also alter the inflammatory state, sex hormone binding globulin (SHBG) is responsible for regulating the biological action of sex hormones and binds with higher affinity to testosterone than to estradiol (Anderson). Insulin sensitivity has been shown to be positively correlated with the production of SHGB, as insulin inhibits the synthesis of SHBG in the liver (Lee et al.). Previous studies have shown that circulating SHBG is reduced in individuals with greater amounts total body, visceral, and liver fat (Peter et al.). Reduced circulating SHBG can result in increased circulating free testosterone, which is implicated in the pathogenesis of both PCOS and type II diabetes mellitus (Le et al.; Dunais). We saw reduced visceral adiposity and liver fat deposition in conjunction with improved insulin
sensitivity in fish oil intervention mice. Although not measured, it is possible that improved liver SHBG synthesis in fish oil intervention mice influenced the improvement seen in estrous cyclicity. Because we also saw improvement in hepatic steatosis in our histological analyses for all intervention mice, it is unlikely that improvement in SHBG levels is the only factor in improving reproductive cyclicity.

Metformin has served as a therapy for both women with PCOS presenting with infertility as well as those with type II diabetes mellitus. Current dogma for PCOS asserts that insulin resistance plays a major role in the pathogenesis and maintenance of PCOS, and it has long been thought that metformin can improve fertility through its insulin sensitizing effects (Misso and Teede). Metformin inhibits gluconeogenesis in the liver and improves insulin inhibition of endogenous glucose production. Furthermore, metformin has been shown to reduce hepatic steatosis and lipogenesis while increasing fatty acid oxidation (Foretz et al.). We saw a reduction in hepatic steatosis, fasting insulin, and lipogenesis in fish oil intervention mice, suggesting a diet containing large amounts of ω-3 fatty acids can provide many of the same benefits as metformin, including improving the insulin sensitivity that may influence reproductive dysfunction. It is worth noting that more recently, however, the efficacy of metformin in the treatment of infertility related to PCOS has been disputed, as both lifestyle changes and other pharmacological interventions have been shown to be more effective than metformin alone (Abu Hashim).

Conclusions

We conclude that diet-induced obesity and obesity-related reproductive dysfunction can be established through feeding a Western-type diet containing 45% kcal fat from with a high amount of ω-6 PUFAs. Although we saw overall improvement in metabolic and reproductive
outcomes, considerable variability of response to fish oil intervention treatment in terms of individual metabolic outcomes and estrous cyclicity was observed (Table 3), and there was no discernible relationship between one specific metabolic outcome or all metabolic outcomes taken together and estrous cycle regularity. From this we can conclude that intervention with a fish oil diet rich in ω-3 versus ω-6 PUFAs can rescue diet-induced obesity and estrous cycle irregularities in females, but not all individuals may be responsive to this treatment to the same degree.

**Table 3. Variability in individual response to fish oil treatment.** Individual metabolic and reproductive outcomes varied amongst the fish oil mice. Means for chow and lard mice at 24 weeks of age are shown for reference.

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<tr>
<td>Lard (mean)</td>
<td>40.176</td>
<td>492.8</td>
<td>7.07</td>
<td>1.34</td>
</tr>
</tbody>
</table>

**Recommendations**

A larger intervention group and subsequent multivariate statistical analysis may help to elucidate relationships between specific metabolic outcomes and estrous cyclicity such that a more focused approach toward specific biomolecular pathways can be taken. Additionally, future studies should be conducted altering the ω-3:ω-6 fatty acid ratio to find the most clinically relevant and effective dietary intervention with ω-3 PUFAs.
References


IACUC Approval

IACUC PROTOCOL

FOR USE OF LIVE VERTEBRATES FOR RESEARCH, TEACHING OR DEMONSTRATION

UNIVERSITY OF MEMPHIS

IACUC Protocol # 0761 Date Submitted to IACUC 1/27/15

Dates Protocol will be in effect: from 3/3/15 to 3/2/17
(not to exceed three years including two yearly renewals)

Is this protocol related to an external grant or contract application? Yes □ No X

If yes, complete the following:

Agency: ___________________________ Date Submitted ___________________________

Grant #

University account for Animal Care Facility per diem charge:

If the protocol is not related to an external grant or contract application, complete the following:

University account for Animal Care Facility per diem charge: 211700

Project Title: (If project relates to a grant or contract application, give that title; if the project is related to a class, give the course name and number):

High-fat diet induced inflammation and reproductive dysfunction in mice

I. Personnel

Investigator/Instructor: Marie van der Merwe

Department: Health and Sport Sciences

Academic Rank: Assistant Professor

Campus phone: 901 678 3476 Emergency phone: 901 406 7458

Attending Veterinarian: Karyl Buddington

Phone: 901 678 2359 Emergency phone: 901 258 1232

List all individuals that will handle animals using this protocol and their level of expertise (e.g. relevant qualifications). If the protocol applies to a class then so specify.
If additional personnel become involved in handling animals used in this protocol, it is the responsibility of the principal investigator to notify the Animal Care Facility in writing before they start.

If applicable, has the investigator/instructor and all personnel listed above received the appropriate vaccinations (tetanus, rabies)?

Yes X No □

Is it necessary for personnel listed on this protocol to be tested for TB?

Yes □ No X

If you have questions about the kind of vaccination or about TB, call the Animal Care Facility at 678 2034.

All U of M personnel involved in this protocol must complete the animal care and use training program (satisfactory completion of, or concurrent registration in Biol 7006/8006), or have completed a comparable training program at another institution before animals can be procured or before the experiments/teaching or demonstration. In submitting this protocol, I, as Principal Investigator/Instructor accept the responsibility for compliance with this requirement.

In addition, the Principal Investigator/Instructor must be willing to provide appropriate supervision for all persons working on this protocol. In the case of a class, the Instructor must be responsible for training any students in classes involved prior to using animals.

II. Project Description

A. Summary (Enter a brief description below of your project, using lay terminology):
Energy status and reproductive status are closely related, as energy stores must be adequate to fuel such energetically costly processes such as pregnancy, birth, and lactation. Obesity, which can be considered chronic energy excess, and its associated metabolic conditions (chronic low-grade inflammation, type II diabetes, glucose intolerance, dyslipidemia, increased adiposity, high blood pressure, and high leptin and low adiponectin levels) has been shown to influence female reproductive function in both humans and research animals, mainly by disrupting or ceasing cycling altogether. The aims of this experiment are 1) to establish whether reproductive dysfunction (measured via estrous cycle regularity) can be induced by a high-fat Western-type diet in C57Bl/6 mice, 2) determine if the source of fat has any ability to protect or reverse this dysfunction and 3) determine the relationship between cycle regularity and inflammatory status over time.

We will use a mouse model (C57BL/6) that will receive either a 45% fat (lard) diet, typical of a “Western” diet, or a 45% fish oil diet which is high in omega-3 fatty acids. Control mice will be on a “normal” 10% fat diet. Omega-3 fatty acids have previously been shown to reduce inflammation and decrease risk of development of glucose intolerance.

After weaning, mice will be exposed to one of the aforementioned diets, and vaginal smears will be conducted daily to assess estrous cycle phase. Eating habits (amount of food consumed) and weights of mice will be monitored twice a week. Mice will be bled biweekly to look at adipokine and cytokine changes that are associated with the different diets. Because inflammation and type 2 diabetes are highly associated, mice will also undergo a glucose tolerance test on the same schedule. After mice on the high fat lard diet begin to exhibit irregular cycles (approximately 12 weeks but not to exceed 16 weeks), they will be euthanized, at which point the brain, ovaries, adrenal glands, liver, spleen, mesenteric lymph nodes and adipose tissue will be harvested for analysis.

B. Describe IN DETAIL the procedures you will follow. You may do this in either of two ways (Check one of the options below and follow the associated instructions):

1. Accompanying documentation (include documentation in box below). X

OR

2. By reference to previously published work (provide a complete bibliographic citation in the box below, and describe any variations from the published technique).
Mice: C57BL/6 female mice will be used for this study and obtained from breeder pairs purchased from Harlan Labs and that are bred and housed at the animal facility on the University of Memphis campus.

Diets: All of the mice will be weaned at 3 weeks of age (which is the standard weaning time for C57BL/6 mice) onto one of the following diets: 1) control (standard chow) diet, 2) western diet with 45% lard and 41% carbohydrate (20% sucrose, 9% corn starch and 12% Maltodextrin 10), or 3) 45% fish-oil and 41% carbohydrate (20% sucrose, 9% corn starch and 12% Maltodextrin 10). For aims 1 and 2 the mice will remain on their respective diets throughout the test period. To determine if the fish oil diet can reverse the irregular cycling, the lard-based diet group will be switched to the fish-oil diet at the onset of irregular cycles. The diets will be purchased from Research Diets, which has experience in producing the western diet for rodent studies.

Experimental design:
For aim 1, 18 female mice will be divided into 3 groups. At weaning, group 1 will receive the lard diet, group 2 the fish oil diet, and group 3 the control diet (8 mice/group). Once mice reach puberty (determined by vaginal opening), vaginal smears will be conducted daily to assess estrous cycle phase. Blood samples will be collected from all mice biweekly via the facial vein to determine alterations in adipokines, cytokines and regulatory immune populations such as Regulatory T cells and Myeloid Derived Suppressor cells. Mice will be anesthetized with isoflurane (2-4%) via inhalation for less than 10 minutes during facial vein blood collections (Collecting 100-200ul of blood). The glucose tolerance test will also be performed biweekly (on a different day as blood collection), by fasting the mice for 6 hours and then administering glucose at 1g glucose/kg body weight intraperitoneally. 10-20 ul of blood will be collected via the tail vein at 30 minute intervals for 90 minutes to measure glucose levels. No anesthetic will be used for tail vein blood sampling as it alters the systemic glucose levels, but mice will be held in hand and the tail gently “milked”. Before this procedure the mice will be held often to get them used to being handled. Once cyclicity becomes consistently irregular in the lard diet group (or at 16 weeks, whichever comes first), all mice will be euthanized (CO2 inhalation) to harvest the brain, ovaries, adrenal glands, liver, spleen, mesenteric lymph nodes, intestines and adipose tissue for histology, biochemistry and immune cells harvest.

For aim 2, female mice will be divided into 2 groups: one group (n=8) will receive the control diet and the second group (n=12) will receive the high fat lard diet. Once cycle irregularity has been established (~12 weeks), the high fat lard diet group will be transferred to the fish oil diet and followed for an additional 6 weeks. Vaginal smears will be conducted to establish whether the fish oil intervention can rescue diet induced reproductive dysfunction. For this group, blood samples collected via the facial vein will be done on the second day of diestrus phase of each cycle to control for hormonal variation. The glucose tolerance test will be performed biweekly on the second day of diestrus. At the end of intervention and second day of diestrus (~18 weeks), all mice will be euthanized to harvest brain, ovaries, adrenal glands, liver, spleen, mesenteric lymph nodes, intestines and adipose tissue for histology, biochemistry and immune cells harvest.

For aim 3, we would like to establish a timeline for the onset of reproductive disturbances, metabolic syndrome, and inflammatory status in various organs. Blood parameters would have been already determined in aim 1, therefore this aim will focus on changes on tissues. For this experiment, 8 female mice will be placed on the chow diet, 6 on high fat lard diet, and 6 on the fish oil diet at weaning for 12 weeks. Half of each group will be euthanized early, 6-8 weeks of age (depending on when irregular cycles start as determined in Aim 1) to examine any early changes in the metabolic and reproductive phenotype. The other half will continue until 12-14 weeks (timepoint when irregularity is often seen), at which point they will be euthanized to harvest brain, ovaries, adrenal glands, liver, spleen, mesenteric lymph nodes, intestines and adipose tissue for histology, biochemistry and immune cells harvest.
C. Rationale for Involving Animals and the Appropriateness of Species and Number Used.

Indicate (here) briefly the short and/or long-term benefits (to humans and/or other animals) of this use of animals for research, teaching or demonstration. In addition, state briefly why living animals are required for this study, rather than some alternative model.

The goal of this experiment is to mimic the metabolic syndrome and associated reproductive complications characteristic of obese humans. The cause of this obesity-induced reproductive dysfunction has yet to be fully elucidated, but degree of obesity have been highly correlated with degree of cycle irregularity. Given that chronic low-grade inflammation is characteristic of diet-induced obesity, we hope to establish a link between inflammatory status and reproductive dysfunction. We are interested in the kinetics of the onset of metabolic syndrome and reproductive dysfunction and also what role the immune system plays in setting up the environment for the disorder and estrus irregularities.

The C57BL/6 diet induced obesity (DIO) mouse model has been used previously to look at the effect of excess weight on various organ systems. As we are interested in the interaction between the immune system what role it plays in the onset of metabolic phenotype and estrous cycle irregularities in mammals, we thus cannot use isolated cell lines or model organisms such as yeast. Many previous studies have used obese rodent models to examine the downstream effects of metabolic syndrome on reproductive function. Additionally, many reagents have been developed for the use of mouse tissues, especially antibodies that will be used to identify immune populations.

There will be 6-10 mice per group for each experiment (2-3 groups/experiment) and 3 experiments. This number should be sufficient to determine statistical significance and account for any unanticipated issues that may arise.

D. Do the procedures described in B above, have the potential to inflict more than momentary pain or distress (this does not include pain caused by injections or other minor procedures)?

Yes [ ] No [X]

If yes, please address the following:
I have considered alternatives to procedures that might cause more than momentary or slight pain/distress, and I have not found such alternatives. As such, I have used one or more of the following methods and sources to search for such alternatives: (check below each method used)

- Agricola Data Base
- Medline Data Base
- CAB Abstracts
- TOXLINE
- BIOSIS
- Animal Welfare Info Center
- Lab. Animals Journal
- Lab Animal
- ATLA (Alternatives to Laboratory Animal Journal)
- Quick Biblio. Series
- Lab Animal Welfare Bibliography (QL55L27311988)
- "Benchmarks"
- "Alternatives to Animal Use in Research, Testing and Education"
- Current Contents
- CARL
- Direct contact with colleagues (if selected, you MUST document this below)
List search words for the literature search:

| Reproduction and metabolism, diet induced obesity, inflammation, estrous cycle, C57Bl/6, reproductive dysfunction, inflammation |

What is the length of time that the literature search covers? 1983 - present

III. Animal Use

A. List all animal species to be used (example below).

<table>
<thead>
<tr>
<th>Species</th>
<th>Number</th>
<th>Age</th>
<th>Sex</th>
<th>Weight</th>
<th>Where Housed (Bldg./Rm#)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6 mice</td>
<td>90</td>
<td>21 days</td>
<td>Female</td>
<td>20-25 gm</td>
<td>Psychology Bld./422I</td>
</tr>
</tbody>
</table>

1Individuals using ectotherms need to only approximate numbers.
2Individuals using fish or other ectotherms need not answer this question.

Is any species threatened or endangered? Yes ☐ No X

B. Source of animals

☒ Commercial vendor (Source: Harlan Labs) Female mice and male mice will be purchased for breeding
☒ Bred at The University of Memphis
☐ Captured from wild
☐ Transferred from another study (IACUC Protocol Number)
☐ Donated (Source)
☐ Tennessee Wildlife Resources Agency
☐ Purchased and supplied by TMGC

Is the supplier a USDA approved source? Yes X No ☐

If not, explain why:

☐ Animals are already in residence at U of M

C. Will surgery be conducted on animals? Yes ☐ No X

If yes, complete this section:

☐ Non Recovery Surgery ☐ Recovery Surgery
☐ Multiple Survival Surgery (if the latter is checked, complete section F)

Surgeon(s) (Name/Job/Title/Academic Rank) Location of Surgery (Bldg. & Room #)

<table>
<thead>
<tr>
<th>Species &amp; Sex</th>
<th>Agent</th>
<th>Dose</th>
<th>Route</th>
<th>Performed by (Name/Title/Academic Rank)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6 mice</td>
<td>Isoflurane</td>
<td>2-4%</td>
<td>Inhalation</td>
<td>Marie van der Merwe/ Assistant Professor</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Simone Godwin/Masters Student</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>LeeAnnaBeech/Masters Student</td>
</tr>
</tbody>
</table>

E. Will euthanasia be carried out? Yes X No ☐

If yes, complete this section (example below).

<table>
<thead>
<tr>
<th>Species &amp; Sex</th>
<th>Agent</th>
<th>Dose</th>
<th>Route</th>
<th>Performed by (Name/Title/Academic Rank)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6 mice</td>
<td>CO2</td>
<td>3L/min</td>
<td>Inhalation</td>
<td>Marie van der Merwe/ Assistant Professor</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Simone Godwin/Masters Student</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>LeeAnnaBeech/Masters Student</td>
</tr>
</tbody>
</table>

If no, describe disposition of animal(s) at conclusion of this study in box below.

F. Will special housing, conditioning, diets or other conditions be required? Yes X No ☐

If yes, please explain in box below.
G. Will animals be removed from the U of M campus at any time?  Yes ☐  No X

If yes, please indicate to where and for how long in box below.

H. If they are to be housed for more than 24 hours outside approved facilities at U of M, provide a scientific justification in box below.

IV. Toxic and Hazardous Substances

A. Check off any of the following below that will be used in these experiments?

☐ Infectious agents (Fill out a, b)
☐ Radioisotopes (Fill out a, b, e)
☐ Toxic chemicals or carcinogens (Fill out a, b)
☐ Recombinant DNA (Fill out a)
☐ Experimental drugs (Fill out a)
☐ Malignant cells or hybridomas (Fill out a, c)
☐ Adjuvants (Fill out a)
☐ Controlled substances (Fill out a, d, e)

For each checked off category, answer the questions indicated below:

a. Identify the substance(s) and completely describe their use, including how will be injected or given to the animal(s):

b. Describe all procedures necessary for personnel and animal safety including biohazardous waste, carcass disposal and cage decontamination:

c. If transplantable tumors or hybridoma cells are to be injected into the

Mice will be on special high fat (either lard/fish oil) diets acquired from Research diets. The diets must be refrigerated or frozen.
animals, have the tissues/cells been tested for inadvertent contamination by viruses or mycoplasma?  
Yes □   No □

If yes, what was the result (indicate in box below).


d. In the box below, provide a complete list of these substances, and if their use is not explicitly explained in the materials already provided, explain their use and role in the research.  


Provide DEA license # covering the use of these substances:

To whom (or what entity) is the license issued?  


e. Provide Radioisotope License Number:

To whom is the license issued?  


V. Categories of Animal Experimentation Based Upon Level of Manipulation and Pain: (check off each category that is applicable to this application)

X  A. Animals will be involved in teaching, research, experiments or tests involving no pain, distress, or use of pain-relieving drugs.

X  B. Animals will be subject to mild stress only (e.g., food or water deprivation of less than 24 hours for use in behavioral studies such as operant conditioning; physical restraint for less than 30 minutes), and will not be subject to surgery, painful stimuli, or any of the other conditions described below. Procedures described in this protocol have the potential to inflict no more than momentary or slight pain or distress on the animal(s)---that is, no pain in excess of that caused by injections or other minor procedures such as blood sampling.

X  C. Animals will have minor procedures performed, blood sampling, etc. while anesthetized.

X  D. Live animals will be humanely killed without any treatments, manipulations, etc. but will be used to obtain tissue, cells, sera, etc.

☐  E. Live animals will have significant manipulations, surgery, etc. performed while anesthetized. The animals will be humanely killed at experiment termination without regaining consciousness.

☐  F. Live animals will receive a painful stimulus of short duration without anesthesia (behavior experiments with flight or avoidance reactions--e.g., shock/reward) resulting in a short-term traumatic response. Other examples in this category are, blood sampling, injections of adjuvants, or drugs, etc.
G. Live animals will have significant manipulations performed, such as surgery, while anesthetized and allowed to recover. Such procedures cause post-anesthetic pain/discomfort resulting from the experiment protocol (e.g., chronic catheters, surgical wounds, implants) which cause a minimum of pain and/or distress. Also included are mild toxic drugs or chemicals, tumor implants (including hybridomas), tethered animals, short-termed physically restrained animals (up to 1 hour), mother/infant separations.

H. Live animals will have significant manipulations or severe discomfort, etc. without benefit of anesthesia, analgesics or tranquilizers. Examples to be included in this category are: toxicity testing, radiation sickness, irritants, burns, trauma, biologic toxins, virulence challenge, prolonged: restrictions of food or water intake, cold exposure, physical restraint or drug addiction. All use of paralytic agents (curare-like drugs) must be included in this category. Describe any abnormal environmental conditions that may be imposed. Describe and justify the use of any physical restrain devices employed longer then 1 hour.

VI. Justifications for Category G Studies and Deviations from Standard Techniques

Describe in the box below any steps to be taken to monitor potential or overt pain and/or distress during the course of this study and how such pain or distress will be alleviated. Be as detailed as necessary to justify your procedure.

VII. Certifications

(By submitting this protocol, I am acknowledging that I comply with the certifications included in Section VII.) (check one)

X Animal Use for Research. I certify that the above statements are true and the protocol stands as the original or is essentially the same as found in the grant application or program/project. The IACUC will be notified of any changes in the proposed project, or personnel, relative to this application, prior to proceeding with any animal experimentation. I will not purchase animals nor proceed with animal experimentation until approval by the IACUC is granted.

Animal Use for Teaching/Demonstration. I certify that the information in this application is essentially the same as contained in the course outline and a copy of the laboratory exercises using animals is on file in the IACUC office. The IACUC will be notified of any changes in the proposed project, or personnel, relative to this application, prior to proceeding with any animal experimentation. I will not proceed with animal experimentation until approval by the IACUC is granted.

Estimate the cost of maintaining animals used in this protocol based on current per diem charge at University of Memphis.

Please specify cost per unit of time: $0.25/day/cage

$ 1680
Specify anticipated total costs for project duration:

**As supervisor of this project it is required that you inform your department chair concerning any animal per diem costs related to this project that are to be paid by the department.**

By submitting this protocol, the Principal Investigator/Course Director indicates that the following have been considered:

1. Alternatives to use of animals.
2. Reduction of pain and stress in animals to the lowest level possible.
3. The proper needs of the animals with respect to housing and care.
4. The lowest number of animals used that will give the appropriate experimental results.
5. Use of the most primitive species that will give the appropriate experimental results.
6. Proper training of all personnel in the care and handling of the species used and in the procedures called for in this protocol before beginning the experiment/teaching or demonstration.
7. That this protocol is not an unnecessary repeat of results already in the literature or in the case of teaching/demonstrations, results that can be demonstrated using models or video material.

Principal Investigator/Course Director (Type Name)          Marie van der Merwe

E-mail address          mvndrmrw@memphis.edu

Date              7/21/14

Federal Law requires that members of the IACUC be given adequate time to read and review protocols including any changes or revisions in them.

The University of Memphis IACUC evaluates protocols on a continuous basis. Any protocols or modifications or renewals to any protocols to be considered at this time must be received by the Animal Care Facility no later than the end of the second week of the previous month.

Incomplete protocols will be returned to the principal investigator. We will not accept a FAXed protocol, renewal form or changes to a protocol.

E-mail the completed protocol to Dr. Guy Mittleman (Dept. Psychology):

mailto:<g.mittleman@mail.psyc.memphis.edu>?subject=RE: IACUC Application

January, 2008
IACUC PROTOCOL ACTION FORM

To: Marie van der Merwe
From: Institutional Animal Care and Use Committee
Subject: Animal Research Protocol
Date: March 3, 2015

The institutional Animal Care and Use Committee (IACUC) has taken the following action concerning your Animal Research Protocol No. 0761:

High-fat diet induced inflammation and reproductive dysfunction in mice

☐ Your protocol is approved for the following period:
   From: March 3, 2015 To: March 2, 2017

☐ Your protocol is not approved for the following reasons (see attached memo).

☐ Your protocol is renewed without changes for the following period:
   From: To:

☐ Your protocol is renewed with the changes described in your IACUC Animal Research Protocol Update/Amendment Memorandum dated for the following period:
   From: To:

☐ Your protocol is not renewed and the animals have been properly disposed of as described in your IACUC Animal Research Protocol Update/Amendment Memorandum dated

Amy L. de Jongh Curry, PhD, Chair of the IACUC

Karyl Buddington, University Veterinarian
And Director of the Animal Care Facilities