**Model Organism Aim 2: Determine the effects of gestational manipulation of the feeding window on offspring health at birth, during growth and development, and in response to Western dietary challenge.**

## Background:

### Childhood origins of metabolic disease

Many studies corroborate the ability of child health markers to modulate risk of chronic disease markers in adulthood. The developmental origins of health and disease hypothesis, introduced by David Barker in the 1980’s, is a nascent field that evaluates the effects of in utero exposure on offspring health, growth, and development from infancy to adulthood (Suzuki, 2018). The canonical example of the ability of nutritional status to modify offspring health comes from studies of the Dutch hunger winter, where in the Netherlands, rations of food were extremely limited (as little as 400-800 kcals per day) and health records of births were extremely thorough. As described by many, children who experienced the famine *in utero* had differential health risk (Schulz, 2010). Babies whose mothers were nutrient restricted in early gestation had normal birth weights, but those whose mothers were experiencing the famine in mid and late gestation had reduced birth weights. Furthermore, effects of the famine on adult health were distinct to the period in which offspring were exposed, early gestation offspring had greater incidence of obesity and dyslipidemia, and those exposed in mid gestation had evidence of reduced kidney function in adulthood. The different effects of famine by periods of gestation may be explained by the main organ system developing during that time. Kidneys experience rapid growth during mid gestation (Rosenblum, Pal, & Reidy, 2017). Further evaluation of this data not only found population incidence-level associations with disease, but molecular changes to the epigenome surrounds the IGF1 gene (Heijmans et al., 2008). This suggests that the gestation and the nutritional environment during gestation can program offspring health later in life.

### Nutrient Restriction in Gestation

Studies of nutrient restriction in gestation in animal models have demonstrated that caloric restriction increases incidence of low birthweight, and may initiate unhealthful catch up growth upon weaning, resulting in excess body weight, body fat, and leptin resistance upon reaching adulthood. Experiments with restrictive feeding in pregnancy have mostly been accomplished using mild to moderate caloric restriction, not time-related restriction. In animals, caloric restriction during pregnancy results in lower birth weights than *ad libitum* feeding (Cunha et al., 2015; Govic, Penman, Tammer, & Paolini, 2016). Having a lower birth weight has been independently associated with greater incidence of metabolic disease. Notably, infants born small for gestational age (SGA) are at increased risk for hypertension, type II diabetes, obesity, heart disease, stroke, renal failure, and even precocious pubertal development (Metrustry et al., 2018; Seckl & Holmes, 2007). This is thought to be related to programming *in utero*  for a nutrient-restricted environment whereas the post-natal environment is not one that is restricted, making those programmed adaptations from gestation inappropriate for the outside food environment.

### Early life exposure to time restricted feeding

Early life is characterized by rapid rates of growth and differentiation and furthermore is a critical period for programming propensity for dysmetabolism. There is substantial evidence that gestation is a critical time for future offspring health. The immediate post-natal life and time preceding adulthood are also crucial in determining risk of ill health in adult life. The largest literature of maternal time-restricted feeding in pregnancy exists in women fasting in observance of Ramadan during their pregnancies. These studies show that gestational age is often similar between those who fasted and those who did not fast during pregnancy (Awwad et al., 2012; Daley et al., 2017; Hizli et al., 2012; Savitri et al., 2014). Furthermore, there may be a greater incidence in low birth weight babies (Awwad et al., 2012; Savitri et al., 2018), especially if the Ramadan fasting took place in the first trimester of pregnancy(Ziaee et al., 2010). However, it is my belief that Ramadan fasting is not a good proxy for TRF during gestation, as it may better model of food entrained chrono-disruption during gestation, which has been shown by Salazar and colleagues to be detrimental to disrupt glucocorticoid stress signaling in rat fetuses, thereby altering their propensity to develop metabolic disease (Salazar et al., 2018).

#### Early Post-natal Time-Restricted Feeding

In the field of DOHaD, the early parts of life extend beyond the gestation period and extend into the early post-natal life. Time-restricted feeding has been evaluated in the early postnatal period in one study, in hope it would mitigate the development of obesity later in life. This study began 8-hour, dark cycle TRF immediately after weaning and kept pups on this schedule for 4 weeks. After 4 weeks, they were switched to AL feeding. Instead of the typical protective effects often seen in TRF in adult populations, harmful metabolic effects were noted. Among them are hyperglycemia, reduced size and area of pancreatic islets, reduced insulin production, increased fatty liver, reduced immune competency, and delayed pubertal maturation (Hu et al., 2019). This suggests that there are effects of TRF in the development period. However, the early post-natal life is distinct from the gestational period; as it is the time for behavior, brain, and development, as opposed to the main time of tissue accretion and organogenesis that gestation is. Therefore, post-natal TRF effects are unlikely to be the same as those during gestation.

#### Gestational Time Restricted Feeding

One work has been completed in gestational eTRF. This focused on HFD-TRF feeding in comparison to HFD-AL feeding. This paper focused on in utero and maternal general habitus, and failed to

Upadhyay and colleagues demonstrated the TRF of HFD could be protective compared to AL HFD feeding on fetal development, with a normalization of placetal:fetal ratio, lower liver triglycerides, and improved lung maturity in TRF fed fetuses at E18.5. This suggests that TRF is able to abrogate the effects of high fat diet feeding in utero. It would be worthwhile to see the effects of TRF-NCD. However, the post-natal period, including birth indices, survival, growth, and metabolic health were not evaluated in this study, therefore eTRF effects on the offspring have yet to be characterized in the literature.

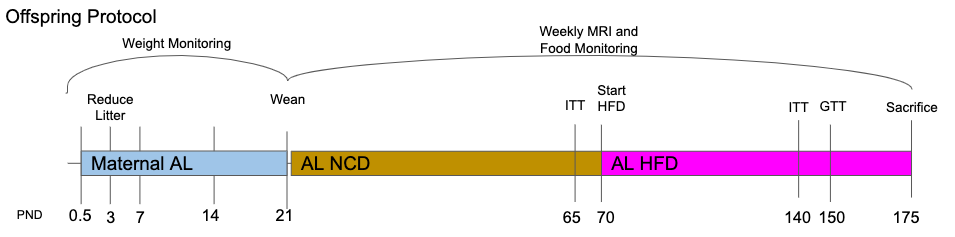


Figure 4. Experimental strategy proposed for aim 2 of this dissertation work

## Specific aim 2.1 Will dam eTRF during gestation affect pup birth indices and survival?

The effects of intermittent fasting on birthweight, gestational age, and offspring survival have not been thoroughly evaluated. This subaim will evaluate the health of the offspring very early in life.

Aim 2.1.1 Body Weight

Birthweight is an important indicator of child health that is associated with infant mortality, and even more recently found to correlate to adult obesity risk (Law, 2002). The effect intermittent fasting during pregnancy has on birth weight has not been examined in either animal or human studies. That being said, the closest proxy to intermittent fasting in pregnant human populations is that fasting that takes place during the month of Ramadan. Some studies find exposure to fasting during Ramadan during pregnancy has no effect on child birthweight (Awwad et al., 2012; Hizli et al., 2012; Savitri et al., 2018), while still others note increased risk for low birthweight (Daley et al., 2017; *Ramadan during pregnancy and birth weight of newborns*, n.d.; Savitri et al., 2014; Ziaee et al., 2010), especially if exposure to fasting was in early gestation.

Other studies of nutrient restriction during gestation have been done and it is often seen that birthweights in nutritionally restricted pregnancies are more likely to be lower than normally fed (Cunha et al., 2015). It is also seen that timing of restriction may play a particularly prominent role in determining risk of low birth weight. Fetuses exposed to the Dutch hunger winter early during gestation had low birth weights, but those who were exposed during late gestation had normal birthweights (Schulz, 2010). One study of moderate caloric restriction (85% of needs) during early gestation in ewes found no differences in birthweight or body weight in either fetuses or in lambs (Hawkins et al., 2000).

Total nutrient restriction and daytime fasting are not good proxies for IF, as they either reduce total number of calories and crucial macronutrients or introduce a disruption to the natural circadian cycle of eating and sleeping. The only study to date of gestational TRF was conducted by Upadhyay and colleagues and demonstrated that HFD-TRF feeding during pregnancy generated produced pups with comparable birth weights to AL fed controls (Upadhyay et al., 2019). This dietary strategy also corrected large birthweight traditionally seen in HFD feeding (Upadhyay et al., 2019). Therefore*, I anticipate that birth weight of pups will be similar in both eTRF and AL fed dams*. To determine the effect of maternal dietary feeding strategy on pup birth weight, each pup’s weight will be taken immediately after delivery (PND 0.5). Pup birth weight will be averaged by litter and then by dam to present an average pup weight. Preliminary cohort data demonstrates that average birthweight per pup does not differ between maternal feeding groups (p = 0.7).

Aim 2.1.2 Gestational age

Another crucial measure of early life health is gestational age. Gestational age is often expressed as either reaching term or failing to reach term before birth (pre-term birth), and has been linked to worsened early child health (Boyle et al., 2012). The effect of Ramadan fasting in pregnancy on gestational age is more consistent in the literature, with studies finding no effect of maternal fasting on gestational age (Awwad et al., 2012; Daley et al., 2017; Hizli et al., 2012; Savitri et al., 2014). No study in animals has been done to date on TRF and pregnancy and the effects of gestational age. To assess gestational age, we will count the number of days between appearance of copulatory plug and birth. *I hypothesize that gestational age will not differ between maternal treatment groups.* The impact of this study will be that we will have the first evidence for iso-caloric time restricted feeding in animals and its influence on risk for pre-term birth.

Aim 2.1.3 Will Gestational exposure to eTRF affect offspring early post-natal survival?

Offspring survival is one aspect of offspring health that is often overlooked in maternal nutrition studies, and hasn’t been reported using TRF in pregnancy. The literature often doesn’t report reduced survival in nutrition restriction studies. This may be related to the lack of human translation or the fact that some pup loss is often expected in the maintenance of a rodent colony.

Work done in dairy cows has demonstrated that restrictive feeding practices initiated before mating resulted in smaller calves, and fewer female calves surviving compared to AL fed controls (Vinsky, Novak, Dixon, Dyck, & Foxcroft, 2006). However, the majority of animal models find that TRF rarely induces caloric deficit when compared to AL fed controls (Anson et al., 2003; Chaix, Lin, Le, Chang, & Panda, 2019). Based on preliminary data and a lack of mention of poor survivorship in the available literature, *I suspect that survival of pups to be similar in both eTRF and AL fed groups.* In order to assess survival of the pups, offspring will be counted on PND 0.5 and sexed as soon as possible. This number will be tracked daily until selective reduction at PND 3.5.

## Specific aim 2.2 Will gestational exposure to eTRF alter growth and development of the offspring?

Growth encompasses many factors including the trajectory of body composition, the propensity for food intake and energy expenditure, and of sexual maturation. This subaim will follow the metabolic health of the offspring throughout life, including a diet-induced obesity challenge.

Aim 2.2.1 Body weight, body composition, and food intake

It is well documented that maternal diet during gestation can alter offspring body composition. The ability of an animal to gain weight and length is correlated to its propensity for disease (CITE). There is potential for catch-up growth. This is most easily seen by observation of body weight, with low initial bodyweight, followed by rapid accumulation of body fat, and even surpassing body weight of normally fed control pups (Berends, Fernandez-Twinn, Martin-Gronert, Cripps, & Ozanne, 2013). Furthermore, catch up growth in rodents has been demonstrated to program insulin-insensitivity in the adipose tissue of young mice and reduce lifespan (Berends, Fernandez-Twinn, Martin-Gronert, Cripps, & Ozanne, 2013). However because of we do not anticipate calorie intake reduction in eTRF dams, *I predict that eTRF offspring will have a similar pattern of weight gain and fat mass accumulation as their AL counterparts*. To capture sufficient information to be able to identify normal or catch up growth, I propose frequent measurement of body weights; on PND days 0.5, 3, 7, 14, 21, and weekly thereafter until sacrifice in adulthood. Body composition will be assessed weekly after PND 21 by EchoMRI until sacrifice to detect differences in compartmentalization of body mass.

Aim 2.2.2 Sexual development and maturation

The only study to date of eTRF in early post-natal life resulted in delayed sexual maturation (Hu et al., 2019). Less significant induction of the integrated stress response suggests that he in utero environment is not one that is inhospitable to fetuses, but may be one that is slightly stressed, as the integreated stress response (ISR) was moderately upregulated compared to NCD-AL feeding (Upadhyay et al., 2019). The effect of gestational TRF on the rate of sexual maturation hasn’t been evaluated to date. *I expect there will be* *no* *impairment in the progression of eTRF offspring toward sexual development.* To test this, I will monitor vaginal opening and testicular descent in offspring daily beginning at PND 25 (Mello et al., 2014).

## Specific aim 2.3 Will gestational exposure to eTRF improve insulin sensitivity and glycemia of offspring?

The many studies in humans and in animals of TRF demonstrate a consistent propensity for improvement in insulin and glucose homeostasis. Notably, human studies find a reduction in glycemia (Halberg et al., 2005; Hutchison et al., 2019; Jamshed et al., 2019; Moro et al., 2016) and in insulinemia (Jamshed et al., 2019; Moro et al., 2016; Sutton et al., 2018)with TRF. Animal models exhibit similar reductions in HOMA-IR (Sherman et al., 2012; Woodie et al., 2018), fasting insulin (Chaix, Lin, Le, Chang, & Panda, 2019; Sherman et al., 2012; Woodie et al., 2018), and blood glucose (Chaix, Lin, Le, Chang, & Panda, 2019). Based on the evidence for improved insulin function and glycemic health with TRF employment and because normal chow diets fail to produce metabolic disturbance, *I expect offspring of eTRF dams to be more insulin sensitive that pups of AL dams.* In order to assess metabolic health, this will assess insulin sensitivity by insulin tolerance test after reaching adulthood (PND 65).

One model of maternal nutrient restriction that is often used in DOHaD is a low-protein diet, as it is known to cause IUGR and altered offspring health (Hawkins et al., 2000). One study of nutrient restriction in animals (low protein diet *in utero*) found that both blood glucose and insulin secretion are elevated in adult rats whose mothers were protein restricted compared to protein-replete fed dams (Hales, Desai, Ozanne, & Crowther, 1996). Because insulin does not precipitously affect the fetus and is prevented from entering fetal circulation (Widness, Goldman, Susa, Oh, & Schwartz, 1983), it is unlikely that insulin signaling affects the developing fetus *in utero.* Therefore, the transfer of glycemic health from mother to offspring may be more related to glycemia that can cross the placenta and enter fetal circulation. Furthermore, this may be mediated by Incretins, one such study found that in offspring whose mothers were diabetic during gestation demonstrated lower start GLP 1 and reduced GLP 1 secretion as well as a more profound increase in glucagon response to an OGTT (Kelstrup et al., 2015). This could mean that offspring of dams who are more insulin sensitive may see the opposite effect, a glucose sensitization. In fact, GLP-1 is known to modulate adaptations of pancreatic beta cells to pregnancy (PAPER I’M READING RIGHT NOW). To assess insulin sensitivity that was seen in the dams that produced these offspring, an insulin tolerance test will be conducted after 10 weeks of age fed NCD. Based on preliminary data, *I do not hypothesize that offspring of eTRF dams will be more insulin sensitive than those of AL dams.* Because the only study to date of TRF in gestation culled the pups before delivery, this study will be the first indicator of offspring glycemic health. Furthermore, the result of this study will provide evidence whether or not gestational TRF disrupts glucose homeostasis and metabolic function, like early life TRF was seen to do (Hu et al., 2019) .

## Specific aim 2.4 Will gestational exposure to eTRF confer metabolic benefit when challenged with a high fat diet?

Initiation of high fat diet feeding is consistent in the literature in creating the appropriate milieu to generate the metabolic syndrome in mice. Among the characteristics of the metabolic syndrome, are many individual organ shifts away from healthy tissue with good function. Such as increases in liver fat, leading to non-alcoholic fatty liver disease (NAFLD), Increases of adipose tissue, increases in blood lipids, insulin insensitivity, glucose intolerance, and higher insulin concentrations that healthy controls. We do this to mismatch the adult environment from the environment *in utero*.

TRF exposed mice may be more resistant to diet induced obesity, manifesting as a lower body fat percentage. To test this, at adulthood (70 days of age) all offspring will be begin *ad libitum* 45% HFD feeding. This diet treatment will remain for 10-12 weeks. Weekly measurements of body weight, fat mass, lean mass, and food intake will be assessed.   
Whether or not TRF can program offspring to be protected from diet-induced obesity and metabolic syndrome has yet to be evaluated

NAFLD

Non-alcoholic fatty liver disease is a common outcome from high fat diet feeding (CITE THESE). Some animal studies of TRF have demonstrated effects on indices of NAFLD. In general, high fat diet feeding ad libitum generates significant liver triglyceride and fat accumulation in the liver tissue (CITE). TRF studies have evaluated this paradigm in both high fat (Chaix, Lin, Le, Chang, & Panda, 2019; Sherman et al., 2012; Upadhyay et al., 2019; Woodie et al., 2018)and normal chow feeding (Sherman et al., 2012). Reductions in total liver size (Woodie et al., 2018) and in liver triglyceride accumulation (Chaix, Lin, Le, Chang, & Panda, 2019; Upadhyay et al., 2019; Woodie et al., 2018) has been seen with TRF of HFD. This suggests that induction of fasting-refeeding cycles lowers the propensity for fat storage in the liver. This is corroborated by increased levels of inhibited lipogenic enzymes in TRF animals’ livers, such as p-ACC (Sherman et al., 2012). Liver fat accumulation was also observed in gestational TRF exposure, and they found that TRF feeding of HFD was able to reduce fetal liver TG almost to the same level as control diet AL fed fetuses (Upadhyay et al., 2019). Furthermore, TRF has been shown to reduce NAFLD score while on a high fat diet, even with a short treatment period of 4 weeks. Because TRF consistently reduces liver triglycerides and has even been found to do so with gestational TRF, *I expect that liver triglyceride content will be reduced in offspring whose mothers were eTRF during gestation even after challenge with high fat diet.*

Serum Triglyceridemia

Animal studies of TRF have been very consistent in their findings in blood lipids, in that there is a consistent lowering of fasted triglyceride and total cholesterol levels after exposure to TRF (Chaix, Lin, Le, Chang, & Panda, 2019; Sherman et al., 2012). Because the literature is very consistent in the effect of TRF on triglyceride content, *I expect that offspring of eTRF dams will have lower circulating triglyceride levels than pups of AL dams*. For this reason, I will collect serum from offspring after high fat diet treatment and assess the triglyceride content.

Glycemic health

Many studies have evaluated insulin under HFD time restricted feeding. Most studies find that TRF reduces fasting insulin levels in animal models (Woodie et al., 2018). This reduction in fasting insulin brings about more insulin sensitivity. These studies are less consistent in their evaluations of glycemia, where some studies show HFD TRF has no effect on fasting blood glucose (Chaix, Lin, Le, Chang, & Panda, 2019; Sherman et al., 2012; Woodie et al., 2018) and a slight improvement in glucose tolerance(Chaix, Lin, Le, Chang, & Panda, 2019; Woodie et al., 2018). Based on the literature, *I hypothesize that offspring of eTRF dams will be more insulin sensitive than AL counterparts after high fat diet treatment.* To test this hypothesis, we will conduct both insulin and glucose tolerance tests after 10 weeks of high fat diet feeding.

**Specific aim 2.5 Mechanisms driving differences in offspring metabolic health**

The mechanisms driving phenotypic differences between gestational eTRF and AL fed pups could be numerous. As stated in the previous aim, this could be mediated by the hormonal milieu of the mother during gestation. This could affect the development of metabolically active tissues *in utero*; such as the liver, pancreas, or muscle tissues.

One mechanism that has not been studied in TRF of pregnancy is the epigenetic changes associated with this feeding paradigm. Studies that use models of IUGR by way of protein restriction have found differences in methylation in promotor regions of nutrient metabolism-related genes like PPAR and glucocorticoid receptor(Lillycrop, Phillips, Jackson, Hanson, & Burdge, 2005). Salazar and colleagues also found that circadian disruption of the mother during gestation was sufficient to initiate changes in the adrenals and corticosterone secretion of offspring (Salazar et al., 2018). A similar exposure, gestational eTRF could be enough to entrain molecular pathways that are stress-responsive, like nutrient signaling and amino acid biosynthesis (Salazar et al., 2018).

Our preliminary data shows that there is sex-specific development of increased fat mass, insulin sensitivity and glucose intolerance in males (Figure XX). Based on a lack of insulin resistance, I predict that glucose intolerance will be traced back to the pancreas. To test this, we will conduct a glucose-stimulated insulin secretion (GSIS) test *in vivo.* If GSIS is consistent and demonstrates glucose intolerance, then collection and culture of the pancreas will be done and GSIS will be repeated *in vitro* on isolated pancreatic islets from the offspringin collaboration with Dr. Brigid Gregg’s laboratory. If cultured islets still demonstrate glucose intolerance, beta cell physiology can further be studied to identify what drives this glucose intolerance. If GSIS in vitro is inconsistent with in vivo results, then it is reasonable that there is another physiological phenomenon responsible that is not related to beta cell function, potentially a hormonal mediator.

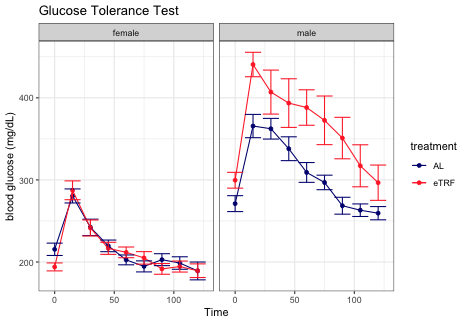
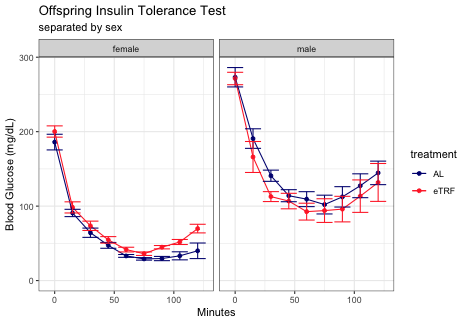


Figure XX:

The figure depicts HFD ITT and GTT values in male and female offspring

*Potential pitfalls and alternative approaches*

One of the most obvious concerns with a restrictive dietary intake for the gestational period is the risk of development of intrauterine growth restriction (IUGR). This would be demonstrated initially by low offspring birthweights, which we do not expect based on our preliminary data. However if this changes with repeat cohorts, one measure to determine if IUGR secondary to poor placentation has occurred is to measure the late term fetus to placenta ratio (FPR. Based on dams following this strategy in a previous study, pups at day E 18.5 who resulted from dams who were TRF HFD, there was a resolution in the placental insufficiency seen with AL HFD feeding; however, it is important to note that FPR was not quite the same as AL-NCD fed controls (Upadhyay et al., 2019), it may be that IUGR is not likely with TRF as long as caloric needs are met in the restricted feeding period. Furthermore, it was seen that lung development in the TRF-HFD group was more advanced than in AL-HFD group pups, meaning that development was more complete, despite a moderate phenotype of partially altered FPR. If that measure isn’t appropriate, we could also make it more translatable by comparing birth weights of pups to other growth curves generated in the C57/B6J mouse (Dilworth et al., 2011).

Poor lactation/maternal attentiveness

One unintentional consequence of altering maternal feeding strategy could be that stress would affect maternal attentiveness or lactation. These effects are difficult to gauge, as most studies that evaluate stressors from diet or the psychosocial atmosphere also continue that stressor during lactation. This study does not plan to do so, in order to be able to tell if an offspring phenotype that is generated is directly related to the gestational exposure alone. One such way to determine in lactation is affected, is to determine maternal milk production in relation to fetal suckling (Boston, Bleck, Conroy, Wheeler, & Miller, 2001). This has been done before by our group and was able to detect lower weight gained from nursing in TSC-KO pups (Unpublished data, Noura El Habbal, 2019). Again, we do not predict this to be an issue based on our preliminary data showing similar early growth trajectories and our restrictive feeding ending prior to the lactational phase.

Sex differences in phenotype

Our design is testing for potential sex-modifying effects for all outcomes, some of which are already apparent (see Figure XX). Sexual dimorphism, as early as *in utero* is known to exist in mouse species. This has been contributed to differences such as placental differences between male and female fetuses. Furthermore effects of maternal undernutrition has also demonstrated sex-specific phenotypes (Gabory, Roseboom, Moore, Moore, & Junien, 2013). If this is the case, we can account for this by genotyping the nascent offspring for Sex-determining Region Y (SRY), indicating male sex (Larney, Bailey, & Koopman, 2014). This would allow for us to group offspring not only by litter and maternal dietary regimen, but also sex, making sex-specific differences in all early life indices detectable.

Deleterious Developmental Adaptation to Feeding

It is possible that eTRF *in utero* is insufficient exposure to be protective from HFD feeding. This may occur due to a mismatch of the *in* *utero* environment of fasting/feeding cycles, making TRF exposed mice more likely to be hyperglycemic or obese. The proposed study design is appropriate to reflect either adaptive or maladaptive responses of HFD feeding. If this is the phenotype that we see, it could be related to many of the sub-attributes of metabolic syndrome, all of which this project proposes to monitor.

## Methods:

### Animal care and use:

Upon birth, litters were counted and individual pups were weighed to the nearest 0.1 gram within 24 hours. At postnatal day 3, litters were reduced to four (two males and two females, when feasible) to standardize milk supply. At 21 days, pups were weaned by sex and maternal treatment group. Upon weaning, animals are allowed 24-hour access to chow (5% fat, 24% protein, 3.7% sucrose, 32% starch, 2.91 kcal per gram) and water.

### Body composition:

Body weight was assessed using a scale to the nearest 0.1 gram. This was assessed at birth, 7, 14, and 21 days of life. At PND 21, weekly indirect body composition assessment using EchoMRI was conducted, generating fat mass, lean mass, and free water measurements in addition to body weights.

### Survival:

Survival of pups will be assessed by counting the number of pups in each litter each day until PND 3.

### Determination of sex:

In order to determine sex, at PND3, anogenital distance of each pup will be evaluated. Those pups with greater anogenital distances will be designated male, and those with lesser distances, female. This will be confirmed by genotyping the fetal tissue for expression of SRY, which is carried on the Y chromosome and is causal in phenotypic sexual determination (Larney et al., 2014).

### Reduction of litters:

Because maternal milk supply may differ based on number of pups, milk supply will be standardized after the initiation of the lactational period. At PND 3, litters will be reduced to 4 when possible (2 male, 2 female). This will help to ensure each dam can supply sufficient and equal amounts of milk to each pup.

### Food intake:

Food intake monitoring began at weaning. Weekly food intake was measured in grams for each cage, and food intake in calories was computed by taking the total food intake per week and dividing by number of animals in each cage. At 65 days of age, animals were switched to *ad libitum* feeding with high fat diet (HFD) (45% fat, 20% protein, 17% sucrose, and 7% starch, 4.73 kcal per gram). Animals will remain on HFD for 10 weeks.

### Insulin Sensitivity:

*Insulin tolerance test:*

After 6-hour fast, blood glucose was taken using a glucometer and tail clip. Animals were given intraperitoneal insulin injections (0.75 units/kg body weight; Humulin U100 in cold, sterile-filtered phosphate buffered saline (PBS)) and blood glucose was tested using a glucometer at 15-minute intervals for 2 hours. If animals began to exhibit moribund behaviors, 300 units of 10% glucose in PBS was administered, the animal was then removed from the experiment, and subsequent blood glucose measurements were omitted from data analysis.

*Glucose tolerance test:*

After 6- hour fast, animals will have a small cut placed at the distal end of their tails. Fasting blood glucose will be assessed by glucometer. After measuring fasting blood glucose, animals will be given an injection of 10% glucose in cold, sterile-filtered phosphate buffered saline (PBS)) (1.0 uL/g lean weight). Blood glucose will be taken every 15 minutes for 2 hours.

*Glucose-Stimulated Insulin Secretion:*

Animals will be fasted for 6 hours and fasting blood glucose and insulin will be determined by retroorbital bleed. This will be followed by an injection of 10% glucose in cold, sterile-filtered phosphate buffered saline (in PBS; 1.0 uL/g lean weight), administered intraperitoneally. Blood will be collected by retro-orbital bleed 15 minutes after injection. Blood collected will be spun down and analyzed for insulin content using an ultrasensitive mouse insulin ELISA.

### Sacrifice and tissue collection:

Offspring will be sacrificed after the high fat diet glucose tolerance test (See study figure). Animals will be fasted for 16 hours with *ad libitum* access to water. Animals will be lightly anesthetized by isofluorane inhalation. Blood glucose will be determined by glucometer and a fasted blood sample will be collected by retro-orbital bleed and immediately put on ice to clot. Once clot is formed, whole blood was spun down in a cold centrifuge (4 degrees C) for 20 minutes at 5000 RCF. Serum was pipetted off and placed in the -80 degrees C freezer until analysis. After blood collection, animals were euthanized by isofluorane overdose and cervical dislocation. Animal body weight was taken immediately after euthanasia on an electronic scale to the nearest 0.1 gram. Liver, inguinal white adipose tissue (iWAT), gonadal white adipose tissue (gWAT), and quadriceps femoris muscle were dissected from the right side of each mouse and snap frozen in liquid nitrogen.

### Liver Triglyceride Content:

30-50mg of snap frozen liver tissue will by lysed and total triglyceride content will be determined using the Sigma Triglyceride assay kit (catalog TR0100).

### Liver Histology:

A portion of the liver was designated for histology by placement in a cassette and fixed in 10% formalin for 24 hours. After fixation, samples were switched to 70% ethanol. Samples will be fixed in paraffin and stained with H & E and evaluated under the microscope.

### Statistical Analyses:

All statistical analyses were completed in R. Repeated measures, such as body weight, body composition, food intake, and insulin tolerance testing utilized mixed linear modeling (LME4 package) with each maternal feeding group assessed as a random effect. All models were tested for sex-interaction. Models were built bottom up and were tested in pairs using ANOVA. Models where ANOVA p value was <0.05 were considered statistically significant.

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