Effects of Manipulation of the Feeding Window During Gestation on Maternal and Child Health  
  
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# Abstract

Intermittent fasting (IF) is an emerging dietary practice that has shown promise as a method to manage chronic health issues such as insulin resistance and diabetes. Studies in humans and animals find improvements in glycemic health and insulin sensitivity, even without caloric restriction or weight loss through the early time restricted feeding (eTRF) modality of IF. This crucial finding suggests eTRF may be of use to women who are pregnant and have insulin resistance. The use of eTRF in pregnancy is critically understudied, with no human studies to date, and only one animal study that did not investigate implications on maternal glycemia. This project aims to investigate the following: 1) examine the effects of implementation of the early time restricted feeding (eTRF) during gestation in a mouse model on maternal health, 2) determine the effects of gestational eTRF on male and female offspring through adulthood and Western dietary challenge, and 3) to use clinical and observational data from the Biorepository for Understanding Maternal and Pediatric Health (BUMP) cohort to assess the relationships of the duration of the feeding window on child birthweight and odds of developing gestational diabetes. To execute this research, I will employ a mouse model of 6-hour eTRF and will monitor maternal food intake, body composition, insulin sensitivity, and fertility. I will then observe the resulting offspring through adulthood, measuring body weights and composition, food intake, growth and development, and metabolic responses to high fat diet feeding. I will also develop a partnership with BUMP collaborators to initiate collection of feeding window data, and will analyze medical data using both multiple linear and logistic regression techniques. This proposed study will be the first of its kind to observe eTRF in pregnancy while studying maternal glycemic health and body composition, and the first study to examine long term offspring health after gestational exposure to eTRF. Furthermore, the collaboration with the BUMP study will supply the first information on feeding windows in pregnancy outside of the context of Ramadan. This study will contribute to both human and animal literature on the effects and associations that result from periods of fasting, and will supply crucial information on the glycemic effects of IF during pregnancy.

# Specific Aims

There is a well-established body of literature demonstrating the effect of maternal diet and metabolic health during pregnancy on both maternal and child health outcomes, making pregnancy a critical period for both maternal metabolic health and offspring risk of chronic disease. Hyperglycemia is one of the most prevalent pregnancy-associated maternal health complications (Farrar, 2016), with 7.6% of pregnancies in the US being complicated by gestational diabetes. Furthermore, this condition is known to increase maternal risk of chronic disease morbidity (Casagrande, Linder, & Cowie, 2018) and offspring risk of obesity and cardiometabolic disease (Kaseva et al., 2019). Restriction of the eating window is a component of diet that is gaining more traction as an approach for improving metabolic health. Recent studies have detailed the benefits of time-restricted feeding (TRF) in improving chronic disease-related outcomes like insulin resistance (Halberg et al., 2005; Hatori et al., 2012; Kahleova, Lloren, Mashchak, Hill, & Fraser, 2017; Liu et al., 2019; Ravussin, Beyl, Poggiogalle, Hsia, & Peterson, 2019; Sutton et al., 2018; Woodie et al., 2018), and high blood pressure (Gabel et al., 2018; Stote et al., 2007, Sutton et al, 2018) which can occur without weight loss. To date, only one study of TRF during mouse pregnancy has been completed (Upadhyay et al., 2019); however, maternal metabolic health and offspring health in the post-natal period were not evaluated. Therefore, the effects of TRF need to be evaluated during pregnancy to characterize its ability to mitigate insulin resistance in pregnancy and long-term effects on offspring health and development.

The goal of my dissertation is to investigate how fertility and maternal metabolic health are affected by TRF, and whether or not those effects alter the developmental course and health of the resulting offspring. This will be accomplished by employing a mouse model where the early time-restricted feeding (eTRF) technique is used in pregnancy. To translate these findings to humans, I will use observational data from a human pregnancy cohort to understand feeding windows and their associations with maternal and child health outcomes. I will test the hypothesis that *in the setting of intermittent fasting, insulin resistance in pregnancy will be lessened, which will improve insulin sensitivity and confer improved glycemic health to the offspring*. This hypothesis is consistent with reduction of insulinemia and improved glycemic control without adverse effect on body weight and habitus demonstrated in the literature. To test this central hypothesis, I propose the following 3 aims:

**Model Organism Aim 1: Examine the effects of manipulation of the feeding window on female fertility, gestational health, and maternal glycemia during gestation.**

Whether or not eTRF would alter fertility and gestational health or work to alleviate insulin resistance of pregnancy in females has not yet been evaluated in mice or humans. To fill this hole in the literature, age-matched female mice will be randomized to either *ad libitum* (AL) or eTRF regimens before exposure to mating. I will assess fertility, body composition, and maternal insulin sensitivity. Furthermore, hormonal and molecular effects will be investigated to elucidate mechanisms of hypothesized improvements.

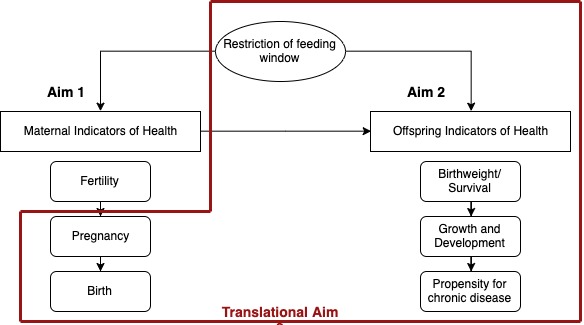
**Model Organism Aim 2: Determine the effects of gestational manipulation of the feeding window on offspring health at birth, during growth and development, and in response to Western dietary challenge.**

The pups of eTRF and AL fed dams will be evaluated for survival, litter size, and weight at birth. Further study of their body mass accretion and metabolic health will be evaluated through adulthood. Once adulthood is reached, response to high fat diet feeding and will be evaluated and molecular targets that drive differences in body mass and metabolic health will be investigated.

**Translational Aim 3: Characterize the prevalence and associations of restricted feeding with maternal and child health in humans.**

I will first characterize the prevalence of pregnancy-associated complications in the biorepository for understanding maternal and pediatric health (BUMP) cohort, then will further investigate the associations of the length of feeding window with perinatal health outcomes including: preterm birth, small for gestational age, intrauterine fetal demise, gestational diabetes, gestational weight gain, and pre-eclampsia.

The proposed study is the first of its kind to monitor eTRF in pregnancy while considering both maternal and child health outcomes. Furthermore, this is the first study of the effect of the length of eating window performed in the BUMP cohort. This work will fill critical gaps in the intermittent fasting literature; elucidating mechanisms the phenomenon of insulin sensitization and further understanding how that may affect pregnancy and the resulting offspring. Based on previous literature in non-pregnant animals and humans, we expect that employment of eTRF during gestation will improve insulin resistance during pregnancy, without effect on bodyweight or body composition, or harm to the offspring during gestation. This study will help us to understand the impacts of TRF as a feeding strategy to inform patient treatment of hyperglycemia during pregnancy; a critical period for lifelong metabolic health.



# Model Organism Aim 1: Examine the effects of manipulation of the feeding window on female fertility, gestational health, and maternal glycemia during gestation

## Background:

### Time-restricted feeding



Figure 1: Depicts popularity of “intermittent fasting” as a google search term from 2009-2019.

Time-restricted feeding, meaning a designated and condensed period in which one consumes their daily calories (usually between 6-10 hours in length), is a dietary strategy that is gaining in popular interest (Figure 1). This method is a modality to implement intermittent fasting, which is distinct from two other modalities, alternate day fasting (ADF) where an individual alternates full days of fasting and *ad libitum (AL)* feeding, and periodic fasting, encompassing a fast of 24 hours or more periodically throughout the month or year, followed by *AL*  feeding for the rest of the time (Stockman, Thomas, Burke, & Apovian, 2018). Studies in humans have demonstrated improvements in insulin sensitivity, hypertension, as well as other molecular markers of health (Halberg et al., 2005; Hatori et al., 2012; Kahleova et al., 2017; Liu et al., 2019; n.d.; Ravussin et al., 2019; Sherman et al., 2012; Sutton et al., 2018; Woodie et al., 2018). An overview of rodent models of TRF demonstrate metabolic improvements in insulin resistance without weight loss (Hatori et al., 2012; Liu et al., 2019; Sherman et al., 2012; Woodie et al., 2018). This suggests that time-restricted feeding may be an appropriate strategy for use in insulin resistant pregnant women. Only one study of time restricted feeding during gestation has been published (Upadhyay et al., 2019). This work demonstrated that HFD-TRF feeding led to similar caloric intake consumed by both HFD-TRF and HFD-AL (*ad libitum*) counterparts, with similar pre-pregnancy body weight gain between these groups. This study did not evaluate body composition, and did not asses maternal insulin sensitivity, fertility or offspring health. For this reason, I propose to study the effect of TRF in mice before and during pregnancy.

### Nutrient Restriction in Pregnancy

Nutrition and nutrient restriction have been well studied in pregnancy. Diet can modulate not only offspring health, but also the health of the mother during, and long after gestation (Walter, 2014; Donnelly, 2019). One such study of maternal food restriction that is largely credited with the burgeoning of the DOHaD field is that of the Dutch Hunger Winter wherein the effects of severe nutrient restriction during pregnancy during extreme rationing in WWII had a profound effect on offspring risk for obesity and cardiovascular disease later in life (Heijmans et al., 2008; Schulz, 2010).

Other, less severe instance of food restriction have also been investigated during pregnancy. Another example is Ramadan fasting. Ramadan fasting takes place over the course of a month in the Islam calendar. During Ramadan, all food and water consumption if confined to after sunset and before morning prayer (1 hour before sunrise). The length of the fast depends on location and time of year when Ramadan takes place in the Islamic calendar. In the United States, this translates to 16 hours of fasting. In most cultures who practice Islam, there are two meals, one larger meal after sundown that breaks the fast, and one before sunrise that is smaller. Conception and gestation during Ramadan fasting has been shown to increase the prevalence of low birth weight babies in some (Opaneye, Villegas, & Abdel Azeim, 1990; Ziaee et al., 2010) but not all (Daley et al., 2017; Hızlı et al., 2012) reports.

Pregnancy is a time of profound physiological change for expectant mothers; including the onset of insulin resistance without hyperglycemia and increases in body weight and food intake. The physiological adaptations to pregnancy are thought to maximize nutrient availability for the fetus. This suggests there is a physiological mechanism to reassign the desired glycemic set point, making the study of pregnancy a relevant and important implication for not only overweight and obese women of childbearing age, but also obese adults in general.

### Fertility and Pregnancy- a critical time for maternal health and physiological adaptation

Fertility represents yet another biological function that demonstrates circadian rhythm (W.-X. Zhang, Chen, & Liu, 2016). As demonstrated by Mereness et al, the coordination of the preovulatory luteinizing hormone surge as well as the coordination of ovary responsiveness to gonadotropins demonstrate entrainment by the CLOCK-BMAL1 system, and even show cell-specific regulation in female mice (Mereness et al., 2016). Swamy and colleagues determined that phase shifting of the period of food availability is sufficient to not only entrain the liver clock in breeding female mice, but also regulate fertility and fecundity (Swamy et al., 2018). In this way, TRF may be differentially affect propensity to become pregnant.

### Gestational weight gain and food intake

Weight gain is expected for a healthy pregnancy. The Institute of Medicine recommended amount of weight to gain is based on pre-pregnancy body mass index (BMI) (Rasmussen et al., 2010). Since these recommendations were published, many studies have evaluated the prevalence of excessive gestational weight gain. This excessive gain of weight during gestation appears to be highly prevalent, approximately 47% of sampled women (Goldstein et al., 2017). Therefore, the prevalent and problematic excessive weight gain in pregnancy is also an urgent public health problems that needs to be addressed to improve health indices of not only child health, but also maternal cardiometabolic health.

### Insulin Resistance

The induction of insulin resistance in the mother during mid and late gestation has evolved to make available extra glucose and free fatty acids in maternal circulation and further prevent maternal storage of these substrates, allowing consistent nutrient flux toward the developing fetus. Although insulin resistance and weight gain are considered normal adaptations to pregnancy, there are many women who experience excessive, pathological insulin resistance and gestational weight gain. Cho and colleagues estimate that globally, gestational diabetes affects 9.8 % of pregnancies in women aged 20-24 years; the prevalence dramatically increases for women of advanced age during pregnancy (45-49 years) to 45.1% (Cho et al., 2018). Furthermore, a meta-analysis of incidence of type 2 diabetes found that women with a history of gestational diabetes are at 7.43 times the risk than women who were normoglycemic during their pregnancies (Bellamy, Casas, Hingorani, & Williams, 2009). This makes insulin resistance during gestation a critical public health problem that deserves research attention. I have shown that pregnant mice have insulin resistance but not hyperglycemia as evaluated by an insulin tolerance test (Figure 2) demonstrating that mice are a tractable system to evaluate pregnancy-associated insulin resistance. This is consistent with previous work on pregnant rodents that find pregnancy to be associated with increased hepatic glucose production and insulin insensitivity as measured by hyperinsulinemic-euglycemic clamps and insulin tolerance tests (ITTs), respectively (S. R. Ladyman, Carter, & Grattan, 2018; Musial et al., 2016).



Figure 2: Pregnancy-induced insulin resistance in age-matched pregnant and non-pregnant female mice.

### Digestive efficiency and chrono-disruption

Energy intake is both ingestion of food, as well as the efficiency by which energy is absorbed. Digestive efficiency many change as a function of genotype, physiological state, or diet. Perturbation of the circadian system can lead to preferential absorption of certain macronutrients; such as preferred carbohydrate to protein metabolism and overall increased fatty acid absorption with disruption of Clock done by pan and colleagues(Pan & Hussain, 2009). It has also been demonstrated that timing of food is sufficient to entrain the circadian system (Chaix, Lin, Le, Chang, & Panda, 2019; Sherman et al., 2012).

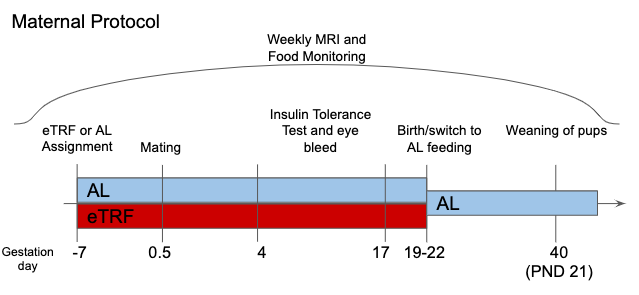


Figure 3: Depicts maternal experimental protocol for this chapter of the proposed dissertation work

Based on the importance of gestational health on the mother and child, and the popularity and effectiveness of time-restricted feeding, it is tempting to speculate that this could be a potential intervention during pregnancy. There are a substantial gaps in our knowledge of the physiology of TRF during pregnancy, and the mechanisms connecting it to maternal glycemia. This chapter will use a rodent model of eTRF to determine effects on maternal fertility, food intake, body composition, and glycemia (Figure 3).

## Specific Aim 1.1 Assess the effects of eTRF on female fertility

While this is not the main focus of my dissertation, our study will evaluate whether eTRF prior to pregnancy affects likelihood of conception. Previous reports have demonstrated that light-cycle feeding can affect fertility (Swamy et al., 2018). Few studies of intermittent fasting have evaluated this in female mice, but those that have only used the ADF modality and find that is disrupts fertility in rodents (Kumar & Kaur, 2013; Nelson, Gosden, & Felicio, 1985). To assess the effects of eTRF on fertility, daily staging of the estrous cycle (Caligioni, 2009) for each dam both before and during the initial exposure to eTRF will be measured. Because timed feeding has previously demonstrated the ability to entrain peripheral tissues, including the female reproductive system, *I predict that feeding during the shortened period of the dark cycle will not result in irregular or prolonged estrous cycles*. Once dams have been mated, daily plug checks will occur until each dam has displayed a copulatory plug. Day of appearance of plug will be coined gestational day 0.5. Days from plug to parturition will be counted. When plug is noted and no litter is born, this will be considered resorption. If resorption occurs, we can furthermore stain collected uteri from dams with 10% ammonium oxide to reveal implantation sites as described by Swamy and colleagues (Swamy et al., 2018). Because one other study has evaluated eTRF in pregnancy, and pups survived to gestational day 18.5, *I expect that fertility will not be compromised by eTRF treatment.* This is supported by my preliminary data, where eTRF dams had similar rates of pup loss compared to AL controls, and both feeding conditions produced litters.

## Specific aim 1.2 Effects on gestational health of the mother

To evaluate gestational health we will determine changes in food intake, body composition, and insulin sensitivity during pregnancy.

### Aim 1.2.1 – Insulin resistance during pregnancy

It has been known that insulin resistance occurs progressively in pregnancy in both animals (Musial et al., 2016) and in humans (Sonagra, Biradar, K., & Murthy D.S., 2014). This insulin resistance is related to having available nutrient to shunt toward the growing feto-placental unit.

To compare the effects of eTRF to that of normal pregnancy, an experiment comparing insulin sensitivity between non-pregnant and pregnant AL and eTRF females. Based on the literature demonstrating more insulin sensitivity in most investigations of TRF in adult subject, I expect that non-pregnant controls will be most insulin sensitive, followed by eTRF dams, and the least insulin sensitive animals will be AL fed dams. Completing this experiment, we will have the first evidence of insulin resistance measures in eTRF pregnancy, but we will also have both a pregnant and non-pregnant controls which will provide context to the level of insulin sensitivity.

### Aim 1.2.2 – Effects of TRF of food intake, body composition, energy expenditure, and digestive efficiency during pregnancy

Aim 1.2.2.1: Food Intake:  
Food intake increases during pregnancy to allow facilitate sufficient nutrient levels to continue maternal healthful living and to provide energy and essential nutrients for the developing fetuses (S. R. Ladyman et al., 2018). This increase in food intake is usually transient, and most pronounced during the last two weeks of gestation in mice (S. R. Ladyman et al., 2018); followed by a sharp uptick during lactation, with up to 254% more food taken in by lactating mice than age-matched, non- lactating controls(Sharon Rachel Ladyman, Khant Aung, & Grattan, 2018).

One such concern about the use of TRF in gestation is that the narrow eating window would provide too little time to consume sufficient calories to support maternal needs and fetal growth. This is especially a concern based on data available from human trials of TRF. In adult humans, when TRF/IF is employed, there is often a reduction in total calorie intake which then leads to weight loss. However, this is often not seen in animal studies even in studies of HFD feeding with TRF, food intake is similar. Furthermore, Upadhyay and colleagues found that TRF of a high fat diet in gestation yielded offspring growth similar to AL chow fed control pups (Upadhyay et al., 2019). For this reason, *I do not hypothesize that dams assigned to eTRF treatment to be unable to consume necessary calories to continue a healthful pregnancy.* Preliminary data suggests that with the eating window of 6 hours, there are no differences in total 24-hour energy intake in the preliminary cohort. In our preliminary data, we observe no differences in overall food intake between AL and eTRF mice, even though we detect a 126% increase in energy intake during the restricted window.

#### Aim 1.2.2.2: Maternal Body Composition

Although only one study has been done in TRF in pregnancy, there have been many studies in non-pregnant adults in humans and in mice that evaluate body weight, body composition, and BMI after treatment with TRF. The literature is divergent in humans and animals. In most studies with humans employing different models of intermittent fasting, there is a moderate reduction of body weight when isocaloric/eucaloric feeding is not employed as part of the study (stote, 2017; Gabel 2018). In rodent models; however, TRF of chow diet usually does not impart weight loss (Liu et al., 2019; Woodie et al., 2018). When High fat diet is given, TRF stimulates body weight loss, in some cases secondary to reduced food intake (Hatori et al., 2012), and in other cases through some other mechanism (Liu et al., 2019; Sherman et al., 2012). We will monitor body composition (Fat mass, lean mass, free water) indirectly by EchoMRI before and during pregnancy. *We hypothesize that we will observe no differences in fat, lean, or free water content compared to gestational-age matched, ad libitum fed control.* This finding would be especially crucial in the state of pregnancy, as progressive and gradual weight gain is expected and necessary for a successful and healthful pregnancy.

#### Aim 1.2.2.3 Maternal Energy Expenditure:

Studies on TRF of humans and animals have demonstrated mixed results with respect to energy expenditure. In some, energy expenditure is increased using this feeding strategy (Halberg, 2005; Gabel, 2018 ), while more either fail to detect any significant increase in daily energy expenditure (Chaix et al., 2019; Ravussin et al., 2019) or leave this unexamined. Based on preliminary results, both the food intake and body composition levels are unchanged; however, these are only proxy measurements of actual energy expenditure. It is possible that while food intake and body weight do not have detectable differences, any changes in one of these indices could be counter-balanced by the other (greater digestive efficiency paired with greater energy expenditure or lower digestive efficiency paired with lower energy expenditure).

Although significant differences in total daily energy expenditure is not often seen, there are often periods where lipid or carbohydrate oxidation is distinct from AL controls. Namely, during the night, the carbohydrate oxidation lowers, resulting in greater fat utilization, and during the day, carbohydrate oxidation predominates – demonstrating greater metabolic capacity for flexibility in those exposed to TRF. I propose to evaluate maternal food intake in the context of body composition and use those data to calculate feeding efficiency. If digestive efficiency is different between eTRF and AL dams (see Aim 1.2.2.4), then I will employ metabolic phenotyping wherein VO2, VCO2, locomotor activity, and food intake would be measured during pregnancy. *I hypothesized that feeding efficiency will not be greatly changed between the two groups.*

#### Aim 1.2.2.4 Maternal digestive efficiency

Although digestion and nutrient utilization are active areas of research in both pregnant animals and humans, the physical and physiological changes of the alimentary canal in pregnancy are not well characterized. The vast majority of work that has been done in both humans and in animals focuses on micronutrient transport and utilization during gestation; especially of iron and calcium (Fisher & Nemeth, 2017; Kovacs, 2000).These studies have demonstrated in animal models that there is hypertrophy of the absorptive surfaces in pregnant animals compared to their non-pregnant counterparts (Sabet Sarvestani, Rahmanifar, & Tamadon, 2015). Still, no measurement of absorptive capacity has been done in the context of 6-hour eTRF on the digestive tract, especially not during pregnancy. This is a critical gap in the literature as circadian rhythms and food restriction have been found to entrain enterocyte nutrient transporters to anticipate caloric intake in animals with intact CLOCK (Pan, 2009). In the case of macronutrient transport, timing of food delivery was found to be a more potent entrainment tool than even light/dark cycle manipulation in mice (Pan & Hussain, 2009). For this reason, I believe that macronutrient and energy absorption will be more efficient in dams fed eTRF. We will determine this by first collecting feces and determining energy and fat content of unabsorbed food, and if this demonstrates a difference we will evaluate macronutrient transporters in the small intestine.

## Specific aim 1.3 Determining how eTRF affects insulin sensitivity and glycemia in pregnant mice

Studies of time restricted feeding have demonstrated improvements in insulin sensitivity in both animals (Chaix et al., 2019; Hatori et al., 2012; Liu et al., 2019; Sherman et al., 2012; Woodie et al., 2018) and humans (Gabel et al., n.d.; Halberg et al., 2005; Jamshed et al., 2019; Sutton et al., 2018). However, these improvements are usually independent to changes in glycemia (Halberg et al., 2005; Hatori et al., 2012; Liu et al., 2019; Sherman et al., 2012; Sutton et al., 2018; Woodie et al., 2018). In fact, reduction in glycemia was only apparent in one human study, and only detectable by the use of continuous glucose monitoring. In this case the authors found that night-time glucose was reduced whereas daytime glycemia was unchanged (Jamshed et al., 2019). For this reason, *I hypothesize that the use of eTRF in pregnancy will result in greater insulin sensitivity compared to pregnant AL fed animals will be improved and that fasting blood glucose will not be affected.* This is supported by our preliminary data showing eTRF dependent insulin sensitization during both the first and third trimester (Figure 4). To test insulin sensitivity, an insulin tolerance test (ITT) will be conducted. Previous work has demonstrated that in pregnancy, insulin tolerance is affected, whereas glycemia is not therefore a glucose tolerance test will not be used (Musial et al., 2016). If the ITT demonstrates improved insulin sensitivity, I propose to conduct a hyperinsulinemic-euglycemic clamp during the mid- pregnancy (between E14.5-E17.5), when insulin resistance is known to be greatest in mice (Musial et al., 2016). This will provide more information to further evaluate the mechanisms that underlie insulin sensitivity; such as contribution from hepatic glucose production, peripheral glucose disposal, and whole organ glucose utilization. We will also determine NEFA levels, as fatty acids are a major contributor to gluconeogenesis. The use of implanted continuous glucose telemetry in mice may also be used to derive more understanding of glycemia over time than fasting blood glucose may provide.

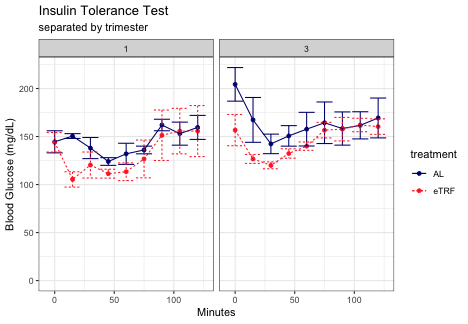


Figure 4: Preliminary results of insulin tolerance test during pregnancy

## Specific aim 1.4 Molecular Mechanisms driving effects of eTRF in pregnancy

After observation of the effects that eTRF have on fertility, food intake, body composition, and maternal insulin resistance, it will be the next goal of this dissertation to detect the molecular drivers of these effects. Although studies have consistently seen insulin sensitivity, that we have replicated, in non-pregnant animals and humans the mechanism driving this phenotype is still unknown. Because of the consistent effect on insulin sensitivity see in published works using TRF, we assume this could be related to a hormonal mechanism that is also present during pregnancy. Candidate hormones will be investigated by ELISA, and if the candidate is altered between feeding regimens in maternal blood samples, evaluation of the role of that hormone in a genetic knockout mouse for that hormone will be considered. I propose three initial candidates for a mechanism of action; glucocorticoids (corticosterone), growth and differentiation factor 15 (GDF15), and a reduction in insulin.

Glucocorticoids are stress-induced hormones derived from steroids and is released in a diurnal pattern. Elevations in glucocorticoids are known to worsen insulin sensitivity, whether from endogenous production (Pivonello, De Leo, Cozzolino, & Colao, 2015) or exogenous administration (Dube, Slama, Basu, Rizza, & Basu, 2015). Corticosterone concentration in the blood increases steadily over rodent pregnancies until late term (Barlow, Morrison, & Sullivan, 1974; Jafari, Mehla, Afrashteh, Kolb, & Mohajerani, 2017). This rise in corticosterone is also known to overlap with the steady rise in insulin resistance of pregnancy in mice (Musial et al., 2016). Therefore, it may be that levels of corticosterone in the circulation could be affected by feeding strategy and may further affect insulin resistance of pregnancy. Cortisol has been responsive to eTRF in humans (Jamshed et al., 2019). Jamshed and colleagues found that morning cortisol was higher in eTRF, and evening cortisol was lower. This could mean that the circadian pattern of cortisol secretion is enhanced by eTRF. This enhanced rhythm of cortisol secretion was seen alongside eTRF individuals having fewer glycemic excursions over 24 hours (Jamshed et al., 2019). To test for circadian entrainment of corticosterone secretion in mice (the predominant glucocorticoid in rodent circulation), we will collect serum from pregnant dams, and non-pregnant controls at both ZT0 and ZT12. This serum will then be tested for corticosterone concentration to understand the relationship between corticosterone and insulin resistance in pregnant mice who have been exposed to eTRF.

Another hormonal candidate mechanism is growth and differentiation factor, GDF15. The effects of GDF15 are known to be exclusively mediated through the GFRAL receptor (Hsu et al., 2017) in the brainstem, and it plays a role in weight and appetite regulation (Macia et al., 2012, p. 15; Patel et al., 2019). Furthermore, GDF15 appears to promote ketogenesis and fatty acid catabolism (Hsu et al., 2017), both of which are elevated in IF (Anson et al., 2003; Jamshed et al., 2019; M. Zhang, Sun, Qian, & Tang, 2018). It is known to increase during gestation, and is associated with reduced food intake, leanness, and improvements in glucose tolerance (Macia et al., 2012). Sugulle and colleagues demonstrated that GDF15 is elevated in human pregnancies that are complicated by pre-eclampsia and diabetes (Sugulle et al., 2009). It has also been demonstrated that overexpression of GDF15 in adult mice fed both chow or high fat diet reduced glycemic response to IPGTT challenge and had greater insulin sensitivity compared to wildtype controls (Macia et al., 2012). Evaluation of serum levels of GDF15 in eTRF and AL fed dams will be conducted. If meaningful differences in GDF15 are detected in concert with improvements in insulin sensitivity, future experiments may investigate the effects of eTRF in GDF15 knockout mice.

One of the few fairly consistent findings of time-restricted feeding trials in both humans and animals is a reduction in insulin. Insulin concentration increases are known to be a part of the natural history of type II diabetes disease progression. To test the role of insulin in the effects of eTRF, serum insulin will be measured in both eTRF and AL dams and compared. The tissues specific-mechanisms of insulin signaling can also be investigated.

The proposed work in this dissertation project will help to elucidate the mechanism for the efficacy of TRF and will represent the early stages of consideration of this dietary practice for use in a new population, expectant mothers.

## 

## Potential Pitfalls and Alternative Approaches

### Fertility-specific pitfalls

Due to the restrictive nature of this feeding regimen, we may observe reduced litter size or litter numbers in eTRF fed dams. If this becomes apparent, further examining the reason for reduced pups and litters will be of great importance. It could be that the reduction in litters is behavioral, in that there is reduced mating drive in mating pairs exposed to eTRF. Swamy and colleagues noted that implantations were not reduced in chrono-disrupted mating pairs, but fewer plugs and litters resulted in their union. To test if the problem is implantation related – staining of dams’ uteri post mortem for implantations will be critical. If implantations largely correllates with number of pups produced per dam, it will be necessary to observe and characterize mating behavior and frequency.

### Maternal nurturing behavioral pitfalls

Maternal behavior is a critical modulator in both maternal and offspring health. In mice, it is known that there is a risk of cannibalism of offspring(Weber, Algers, Würbel, Hultgren, & Olsson, 2013). Unfortunately, this effect is largely unavoidable, in that C57BL/6J mice are known to partake in this behavior. In order to minimize this effect, handling of pups for counting and weighing will be minimal and animals who are not nulliparous will be used as breeders, in the hopes of maternal instinct being more developed after having had one litter before mating under eTRF or AL conditions.

### Null Findings

Although there is an anticipated insulin-sensitive and normal body composition phenotype, it may be that eTRF fails to impart any effect on fertility, feeding, body composition, and maternal insulin sensitivity. If this is found, it will still be of great public health importance, as there would be no preliminary evidence to suggest harm for mother or child when the dyad observes this feeding paradigm. This would require further experimentation and phenotyping to confirm.

### Circadian rhythm of hormones and metabolism

As much of metabolism and humoral release is coordinated by the circadian clock system, the timing of samples must be considered. Because continuous sampling for blood is impractical and create hypovolemic stress for both the dam and the gestating offspring, beginning the sampling for hormones, glucose, and other candidates must first be undertaken as static measures. If there is a circadian pattern to the effect of eTRF, like there is known to be for cortisol, a morning and evening sample should be compared. If the diurnal relationship for these indices is unclear with two samples, then continuous sampling may need to be employed. The use of glucose or blood pressure telemetry can help to give a more accurate picture of the circadian rhythm of hormone and metabolism. However, as these methodologies are costly and may exert additional stress on recently impregnated dam, this method will be employed only if necessary to clarify the effects of eTRF.

## Methods:

### Animals:

C57BL6/J mice were previously used in the insulin resistance of pregnancy experiment were used in this experiment. At 134 days of age, age matched females were randomized to either *ad libitum* (AL) or early time-restricted eating (eTRF). Dams randomized to AL feeding had 24-hour access to chow (5% fat, 24% protein, 3.7% sucrose, 32% starch). Dams randomized to eTRF feeding were allowed *ad libitum* access to chow during 6 hours of the dark cycle (8pm-2am). At 2 am, all dams were moved to clean cages to standardize stress and handling between feeding regimens (Hatori, 2012). Animals were held in a 12:12 light dark cycle, in a temperature and humidity-controlled facility. Food intake was monitored daily, with 6 hour and 24-hour intake calculated as total grams of food consumed per day multiplied by utilizable energy in the provided diet. Dams were switched to AL feeding upon parturition.

### Mating:

Dams were singly housed for the course of the experiment. After a one-week acclimation period, males were added to the cages in monogamous pairs. Males were allowed to remain in cages until copulatory plug appeared, which was noted as day 0.5 of pregnancy. At gestation day 19, males were removed to prevent second pregnancies after delivering. During the course of the birth and post-natal period until PND 21, dams were singly housed with their litters.

### Body Composition:

Once a week, Dams weight was measured weekly using an electronic scale (Mettler Toledo). Body composition including fat mass, lean mass, and free water was assessed indirectly via magnetic resonance imaging (EchoMRI). This technique has been described previously by members of our lab (Harvey et al., 2018).

### Insulin Sensitivity:

#### Insulin tolerance test:

Insulin sensitivity was assessed by insulin tolerance test 16 days after mating began. Gestational age during ITT was determined using plug data, body weight gain, and date of delivery. As a result, most dams were in the 1st or 3rd week of gestation during this time. After 6-hour fast, blood glucose was taken using a glucometer and tail clip. Females were given 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 cold sterile filtered PBS was administered and subsequent blood glucose measurements were omitted from the ITT.

#### Hyperinsulinemic-euglycemic clamp:

After mating and confirmation of pregnancy by weight gain of 1.75g signaling 7 days of pregnancy (Heyne et al., 2015), and pending the results of the insulin tolerance tests, animals will be placed singly housed into a special cage unit. Dams will be cannulated and exogenous insulin will be administered, inducing a state of hyperinsulinemia. Glucose will be infused and rate of infusion required to maintain steady blood glucose will be recorded for each dam. Greater glucose infusion rates represent more insulin sensitive animals. This method also allows for understanding of tissue-specific glucose disposal through the use of radiolabeled glucose. This technique has been employed previously in our lab (Harvey et al., 2018).

### Glycemia:

#### Fasting blood glucose

Fasting blood glucose will be assessed with a tail vein blood collection and a glucometer immediately preceding the insulin tolerance test.

#### Continuous Glucose Monitoring

However, as Jamshed and Hutchison have previously demonstrated, the use of continuous glucose monitoring may demonstrate more significant trends in glycemia that static blood glucose and terminal blood glucose measurement are able to capture (Hutchison et al., 2019; Jamshed et al., 2019). For this reason, if no clinically significant differences in glycemia between dams arises, I propose to use continuous glucose telemetry during pregnancy to collect 24-hours of continuous glucose measurements without the need for serial sampling and reduction in maternal blood volume. With the collaboration and expertise provided from the animal phenotyping core, implantable glucose telemetry units may be implanted into dams during early pregnancy. The telemetry units collect glucose data can collect data anywhere from 28 to 45 days; therefore, glycemia during the entire pregnancy can be captured with this implantable device.

### 

### Energy Expenditure:

As body composition and food intake are similar in both eTRF and AL maternal groups, it is unlikely that we will need to do metabolic phenotyping of these animals, as differences in their energy expenditure would likely manifest as differences in food intake and body composition. If warranted indirect calorimetry will be performed at the MMPC animal phenotyping core.

### 

### Digestive Physiology:

#### Energy Absorption

To determine if there exist any differences in the amounts of energy consumed from food consumed between eTRF dams and ad libitum fed dams, fecal calorimetry will be performed. Full 24-hour fecal samples will be collected from dams individually and then dried. Dried fecal matter will be assessed by a bomb calorimeter to determine total energy content in the stool as described by Murphy and colleagues (Murphy et al., 2010). Results will be expressed as total energy intake for that day – energy found in stool.

#### Macronutrient absorption into portal circulation

Macronutrient absorption will be assessed in vivo, pending the results of the energy absorotion experiments. This can be accomplished through the use of *in situ* looping of the intestinal tract. Anaesthetized dams at day 17.5 will have two small incisions made on the abdomen, and peritoneal cavity will be flushed with PBS. A proximal jejeunal loop will be made and a mixture of PBS/radiolabeled macronutrient solution (PBS/ [3H] Triolein/[14C] Cholesterol and cholesterol for lipid absorption, and [14C] alpha Methylglucoside (αMG) for carbohydrates, and [3H]glycylsarcosine (gly-sar) for protein) will be introduced to the lumen of the loop via microsyringe. After 1-hour elapses, loops will be collected as well as blood samples from the portal vein. Blood will then be centrifuged at 4 degrees C at 5000 RCF for 20 minutes to collect serum. Serum will be analyzed by scintillation counter to quantify nutrient absorption into portal circulation. Given that maternal food intake is similar between treatment groups, it is unlikely we will need to run this set of experiments

### Maternal Blood ELISAs

Maternal blood will be used determine insulin, corticosterone, and GDF15 concentrations. An enzyme-linked immunosorbent assay (ELISA) will be run specific to each hormone and manufacturer protocol will be followed.

# 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 did not evaluate offspring health. 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.

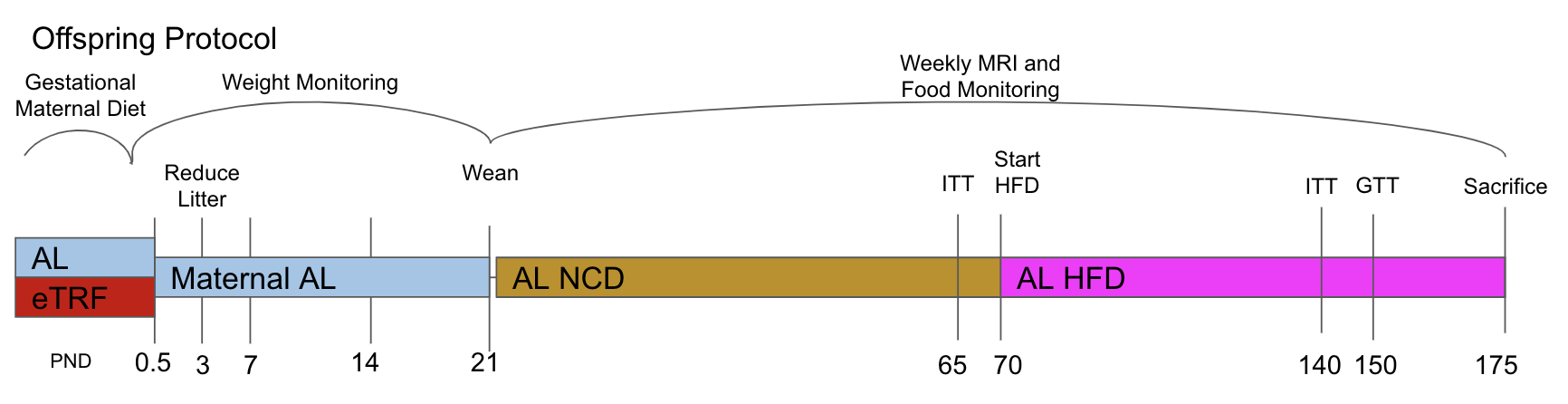


Figure 1. Experimental strategy proposed for aim 2 of this dissertation work

## 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 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 et al., 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 (Harada et al., 1999). 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 et al., 2013). However, because 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 integrated 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 et al., 2019; Sherman et al., 2012; Woodie et al., 2018), and blood glucose (Chaix et al., 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.

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 (Moffett, Vasu, Thorens, Drucker, & Flatt, 2014). 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 (Velázquez et al., 2019). 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 (Velázquez et al., 2019). TRF studies have evaluated this paradigm in both high fat (Chaix et al., 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 et al., 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 Triglycerides

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 et al., 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 (Chaix et al., 2019; Sherman et al., 2012; 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 et al., 2019; Sherman et al., 2012; Woodie et al., 2018) and a slight improvement in glucose tolerance(Chaix et al., 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.

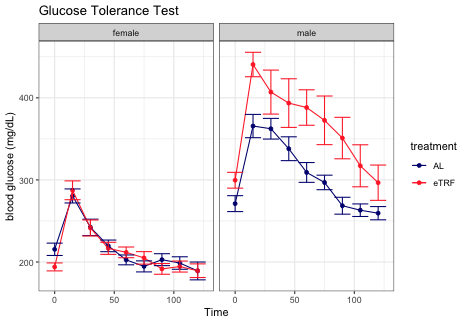
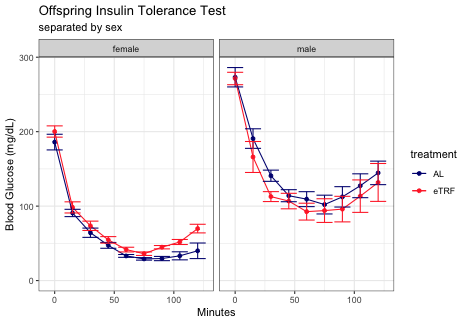


Figure 2: The figure depicts HFD ITT and GTT values in male and female offspring

## Potential pitfalls and alternative approaches

### Intrauterine growth restriction

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 2). 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 *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.

# Translational Aim 3: Characterize the prevalence and associations of restricted feeding with maternal and child health in humans

## Background:

### Intermittent fasting and time-restricted feeding

Time-restricted feeding (TRF), meaning a designated and condensed period in which one consumes their daily calories (usually between 6-10 hours in length).This method is a modality to implement intermittent fasting, which is distinct from two other modalities, alternate day fasting (ADF) where an individual alternates full days of fasting and *ad libitum (AL)* feeding, and periodic fasting, encompassing a fast of 24 hours or more periodically throughout the month or year, followed by *AL*  feeding for the rest of the time (Stockman et al., 2018). Studies in adult humans show improvements in insulin sensitivity, hypertension, as well as other molecular markers of health (Halberg et al., 2005; Hatori et al., 2012; Kahleova et al., 2017; Liu et al., 2019; n.d.; Ravussin et al., 2019; Sherman et al., 2012; Sutton et al., 2018; Woodie et al., 2018). There is evidence that these metabolic improvements can be achieved without weight loss or caloric restriction in both humans (Sutton et al., 2018), and animals (Hatori et al., 2012). This suggests that time-restricted feeding may be an appropriate strategy for use in insulin resistant pregnant women. Although it should be said that IF is a controversial dietary practice, which could contribute to the lack of research of this practice in pregnant women. The goal of this portion of the proposed dissertation work is to observe eating practices without assigning an intervention, be begin the lengthy process of understanding whether or not this could be applied to expectant female humans.

### Fasting and in pregnancy

Intermittent fasting during pregnancy has not been examined in humans, even at the observational level. The closest analogue to the TRF paradigm of IF would be fasting in Ramadan. Ramadan fasting takes place over the course of a month in the Islam calendar. During Ramadan, all food and water consumption if confined to after sunset and before morning prayer (1 hour before sunrise). The length of the fast depends on location and time of year when Ramadan takes place in the Islamic calendar. In the United States, this translates to 16 hours of fasting. In most cultures who practice Islam, there are two meals, one larger meal after sundown that breaks the fast, and one before sunrise that is smaller. 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). Ramadan fasting is an imperfect proxy for the TRF paradigm of IF, as it often is accompanied by changes in sleep patterns and food choices, both of which could independently affect disease risk and health.

### 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 et al., 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.

### Neonatal Health Outcomes

Preterm birth is a significant health risk for neonates. It has been demonstrated that infants born before term (37 weeks’ gestation), are at greater lifetime risks for higher total cholesterol, triglycerides, glucose and insulin as well as high blood pressure (Suzuki, 2018)(Lewandowski Adam J. et al., 2015). Because pregnancy is a complex period of rapid adaptation for the mother, the etiological drivers of pre-term birth have been difficult to isolate and study. Mothers with short stature, lower educational attainment, who smoke, or have diabetes are more likely to deliver before term (Kramer, McLean, Eason, & Usher, 1992). Some of these risk factors can directly be tied to nutritional status, such as diabetes. However, many cannot be directly corrected by nutrition, but would likely have consequences for maternal nutritional status, such as increased need for water soluble vitamins in those who smoke, lower fruit and vegetable intakes in those with lower incomes, and lower food security for women who have lower educational attainment. This can be difficult to disentangle from other conditions that are associated with pre-term birth, such as infant birth weight.

### Maternal Health Outcomes

The appropriate amount of weight that is to be gained for a healthful pregnancy is drawing attention from both clinicians and researchers in recent years, and recommendations have been tailored to pre-pregnancy BMI to optimize offspring health outcomes (Rasmussen et al., 2010). Gestational weight gain has been associated with offspring body mass index and risk of obesity from infancy all the way through adulthood (Schack-Nielsen, Michaelsen, Gamborg, Mortensen, & Sørensen, 2010). Although insulin resistance and weight gain are considered normal adaptations to pregnancy, there are many women who experience excessive, pathological insulin resistance and gestational weight gain; which manifests as gestational diabetes. Cho and colleagues estimate that globally, gestational diabetes affects 9.8% of pregnancies in women aged 20-24 years; the prevalence dramatically increases for women of advanced age during pregnancy (45-49 years) to 45.1% (Cho et al., 2018).

## Main exposure and outcome variables:

Because the insulin-resistance is a well-document association for both maternal fasting in Ramadan and intermittent fasting, the primary outcome of interest for this study for mothers will be the development of gestational diabetes. Other analyses for preeclampsia, hyperemesis gravidarum will also be conducted.

In terms of the offspring, because birth weight is seen in women who observe Ramadan fasting and because intermittent fasting is may affect body weight, the main outcome variable for this study will be child birth weight. The exposure that will be evaluated in relation to these outcomes is the duration of the feeding window in expectant mothers.

## Translational Aim 3.1: Examine the baseline characteristics of the BUMP cohort

Because no previous study has utilized BUMP cohort data, there must be some descriptive statistics done in order to understand what confounding variables and collinearities exist in the cohort.

### Study Population:

In brief, recruitment is done in the Vonn Voigtlander Women’s clinic, with special focus on the maternal and fetal medicine clinic days, who serve high risk obstetric patients. As of August 2019, this sample consists of roughly 800 women enrolled at different stages in their pregnancies. Eligible women are those who are 18 years or older, who van read and understand the consent form in English, and receive their prenatal care at the VVWH and plan to deliver at VVWH. Research assistants are told by physicians during prenatal care visits if patients are interested in enrolling in the BUMP study. The study is explained, a pamphlet is given, and if a patient is interested, the research assistant obtains written informed consent and gives the participant the questionnaire (appendix 1). Inclusion criteria will be women with live, singleton births. Pregnancies complicated by fetal anomaly, congenital birth defects, or poor placentation/placental defects, or multiple gestation will be excluded. This will result in a population of women that could be quite heterogeneous; who may or may not have obesity, may or may not have experienced gestational hypertension, gestational diabetes, preterm birth, cesarean delivery, or taken glucocorticoid drugs during the course of their pregnancy.

### Power analysis:

A power analysis was conducted for linear regression models using G\*power (Faul, Erdfelder, Lang, & Buchner, 2007). Analyses were conducted by calculating Cohen’s D using mean data from maternal Ramadan fasting studies of the main outcomes (gestational diabetes and low birth weight), a power of 0.80, and a significance level of 0.5. As both effect sizes (Cohen’s D) were quite large, >0.50, additional power analyses were conducted using a medium effect size (arbitrary, 0.3), and small effect size (arbitrary 0.15). As the true effect size of maternal feeding window has not been calculated before and hasn’t been characterized yet in this study population, it is uncertain whether or not the calculated effect sizes will be replicable in this study. The multiple power analyses provide a range of sample sizes, with as few as 31 participants and as many as 203 participants. As the BUMP recruitment has been active for one year and >800 participants have been recruited, I propose to recruit the full 203 participants required to identify a small effect size.

Table 1: Gestational Diabetes

|  |  |  |  |
| --- | --- | --- | --- |
| **Effect size** | **Sample size for each group** | **Seeking to recruit** | **Source of Cohen’s D** |
| 0.621 (large) | 27 | 54+10% | (Baynouna Al Ketbi, Niglekerke, Zein Al Deen, & Mirghani, 2014) |
| 0.3 (medium) | 49 | 98+10% | arbitrary |
| 0.15 (small) | 92 | 184 +10% | arbitrary |

Table 2: Low Birth Weight

|  |  |  |  |
| --- | --- | --- | --- |
| **Effect size** | **Sample size for each group** | **Seeking to recruit** | **Source of Cohen’s D** |
| 0.5864 | 14 | 28+10% | (Savitri et al., 2014) |
| 0.3  (medium) | 49 | 98+10% | arbitrary |
| 0.15 (small) | 92 | 184 +10% | arbitrary |

### Ethical Approval, Data Acquisition, and Data management:

The recruitment of participants and collection of medical information and biospecimens has been approved by the University of Michigan Institutional Review Board (HUM00118179). I will prepare, submit, and be approved for a secondary use permit before beginning any analysis described below. I will submit requests for the full medical information I seek from charts and will be supplied with de-identified participant data. This data will be held on a secure server, with access only to those who are part of the study team and named in the secondary use IRB application. Participant data will not be downloaded on personal computers, and will be backed up on MBOX. As biological assays are conducted on biorepository tissues, they will be merged with the original dataset to maintain a single, de-identified dataset available for statistical analysis.

Collection of Biological Samples  
By participating in the study, women consent to collection of urine, blood, placenta, and cord blood. During each trimester, women who consent to be part of the study are given urine containers and asked to provide up to 100 mL of urine. Urine is then frozen and kept at the Michigan Medicine Central Biorepository under a unique study ID. Blood samples that are drawn for research purposes are coordinated to occur at the same time as prenatal lab draws to minimize participant burden. Present in the research kit are vacutainers for blood draw, which usually takes place at a Michigan Medicine laboratory. Trained phlebotomists collect 40 mL of whole blood each trimester. Blood samples are then picked up by research assistants, aliquoted, and stored Because blood samples are in coordination with prenatal labs, there may be inconsistency in fasting state of these samples. The mid-gestation blood draw is usually done in combination with the oral glucose tolerance test screen for gestational diabetes, which is recommended to occur between 24 and 28 weeks gestation (Randel, 2014). Therefore, indices like insulin will need to be interpreted with caution. After delivery and cutting of the umbilical cord, cord blood will be collected by labor and delivery nurses for clinical and research purposes, up to 40 mL of which can be used for research purposes. Upon delivery of both the infant and the placenta, a labor and delivery nurse will collect two (each sized 1x1x3 cm) placental samples. One sample will be stored in RNAlater, and another will be fixed and embedded in paraffin for histological analysis. All biological samples are stored in the Michigan Medicine Central Biorepository under a unique study ID/barcode. Study samples will not be stored with identifying information.

### Medical Chart Data

Medical chart data is accessible to the research team and can be compiled at the request of the secondary use IRB protocol. This process allows this to access diagnosis codes for the pregnancy and child health in the subsequent infant’s chart. The proposed medical data to be retrieved from the medical chart will include: last measured pre-pregnancy height and weight, maternal age at conception, last measured gestational weight and height, maternal medication use (glucocorticoid, insulin, metformin, progesterone, aspirin, statins, ADHD medications), maternal smoking and drug use history, pre-existing diabetes or hypertension, gestational diabetes, hyperemesis gravidarum, hypertensive disorders of pregnancy, intrauterine fetal demise, birth weight of offspring in grams, gestational age at birth, and APGAR score. These medical data will then be compiled for each participant and added to the demographic and feeding window data compiled from the intake questionnaire.

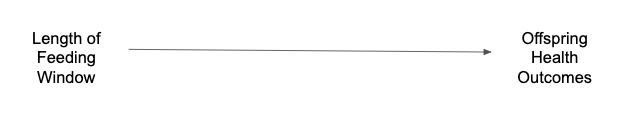
### Assessment of Feeding Window

In order to ascertain the window within each participant consumes their meals each day, the following questions were added to the intake questionnaire, “On a typical day during your pregnancy, when was the first time in the day you had something to eat?” to determine the beginning of the feeding period, and “On a typical day during your pregnancy, when was the last time you had something to eat before going to bed?” to assess the closing of the feeding period. Ideally, the responses will be grouped into a categorical variable consisting of 2-hour differences in length of the feeding window (below). These feeding windows are all reflected in the TRF literature (Rothschild, Hoddy, Jambazian, & Varady, 2014).

* >12 hours
* 10-12 hours
* 8-10 hours
* 6-8 hours,
* <6 hours.

## Translational Aim 3.2. Investigate the associations of feeding window length on maternal and child health outcomes

No studies to date have evaluated the effects of time-restricted feeding on the incidence of maternal and child outcomes in human populations. There have been studies on the effects of Ramadan fasting during pregnancy on these outcomes. As stated previously, Ramadan fasting is inconsistent in its findings. Some studies associated fasting with lower birth weight, whereas others see no effect. Studies on gestational age are somewhat more consistent in that there is no apparent relationship between Ramadan fasting and preterm birth. Although imperfect, one such cross-sectional study evaluated a consistent exposure to Ramadan fasting and categorized participants based on the level of fasting completed (1-10d, 11-20d, 21-29 days in the 2017 month of Ramadan). They found that Ramadan fasting only affected two of their nine outcomes. The odds of gestational diabetes were lower in expectant mothers who participated in the Ramadan fast (2.6% vs 8.3%)(Safari, Piro, & Ahmad, 2019). The strength of the association remained after adjusting for maternal education, maternal age, maternal occupation, parity, and pre-pregnancy BMI (OR = 1.51 (0.06+/-1.74)). There were no observed differences in rates of preterm labor, preeclampsia, low birth weight, fetal height, fetal head circumference, or APGAR score at 5 minutes. Based on the work done during Ramadan fasting in pregnancy and other TRF literature, *I anticipate that women who have shorter eating windows are less likely to develop pregnancy related maternal health issues (diabetes, preeclampsia, hyperemesis gravidarum) and that there will be no effect on child birthweight.* (Figures 1 and 2)

Figure 1: Directed acyclic graph for aim 3.2

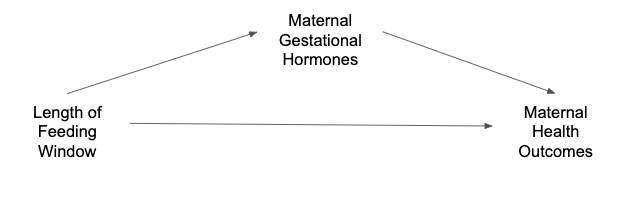
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Figure 2: Directed acyclic graph for aim 3.3

To assess the associations of feeding window length with maternal and child health outcomes, multiple linear regression analysis will be used for the categorical and continuous outcomes; such as offspring birth weight, gestational age, and APGAR score. For dichotomous outcomes, such as gestational diabetes, hypertensive disorders of pregnancy, and hyperemesis gravidarum logistic regression will be employed.

## Translational Aim 3.3 Examine the molecular basis for feeding window association with maternal and child health in biological samples

The metabolic and biochemical effects of fasting during pregnancy are critically understudied. It is known that a large proportion of the Islamic world chooses to fast when pregnancy, for many reasons (Safari et al., 2019). However, as pregnant mothers are a particularly difficult group to enroll and study, there is very little data on what fasting does to maternal and child hormonal and nutritional signals. Of those few studies, the majority exist as case studies or observational studies of women with existing diabetes during pregnancy (Azlin et al., 2011; Ismail et al., 2011). Observational studies of pregnant, normo-glycemic women who observed Ramadan fast noticed that women had higher post-prandial glucose responses after breaking the fast than pregnant controls (measured one hour after first meal in the morning) (Baynouna Al Ketbi et al., 2014). This sample is imperfect in a few ways. The two samples were taken at different times of day, which is known to affect glycemia and insulinemia in humans. Furthermore, the measurement of fasting and post-prandial glucose alone is insufficient to begin to understand the mechanisms that may be activated by maternal fasting. A baseline understanding of the associations of habitual dietary fasting and markers of metabolic health in the blood of pregnant women is a critical need. The BUMP cohort biological samples (blood, and urine) can help to fill that gap. The hormones to be assayed in the blood samples is largely to be determined by the results of the model organism aims of this proposed dissertation work. The initial list will be comprised of blood insulin, glucose, GDF15, and cortisol provide the strongest candidates. Each will require 50uL of plasma, totaling 200uL minimum of each sample. To provide translatability to the animal study, human maternal blood samples may be tested for other contributing hormones that demonstrate effect in the animal cohort.

Univariate and Bivariate Analyses

In order to assess the distributions of the maternal feeding window data and gestational and infant outcomes, I will conduct a univariate analysis of each variable independently. This will determine whether or not these variables need to be normalized for use in multiple regression and will determine cutoffs for the categorical variables. Bivariate analyses will then be conducted by examining the distributions of the outcome variables across socio-demographic and maternal health indices. This will identify variables that should be considered confounders in the relationship between the feeding window during pregnancy and maternal and child health outcomes. As seen in table 3, these are the current proposed variables to be considered in the bivariate analyses.

Table 3: Potential confounders:

|  |  |
| --- | --- |
| **Proposed confounder** | **Source of variable** |
| Maternal gestational age at enrollment | Medical chart |
| Maternal race/ethnicity | Enrollment questionnaire |
| Household income | Enrollment questionnaire |
| Maternal pre-pregnancy BMI | Medical chart |
| Gestational weight gain | Medical chart |
| Maternal smoking status | Enrollment questionnaire |
| Maternal sleep quality | Enrollment questionnaire |
| Offspring sex | Medical chart |

Multivariate Analyses

To assess the associations of feeding window length with maternal and child health outcomes, multiple linear regression analysis will be used for the categorical and continuous outcomes; such as offspring birth weight, gestational age, and APGAR score. For dichotomous outcomes, such as gestational diabetes, hypertensive disorders of pregnancy, and hyperemesis gravidarum logistic regression will be employed.

Proposed models:

Logistic Regression Models:

**Exposure:** Maternal length of the feeding window **Outcome:** Gestational Diabetes

Model 1: Logit(Pr(Y=1)) = maternal feeding window + maternal education

Model 2: Logit(Pr(Y=1)) = Model 1 + maternal pre-pregnancy BMI  
Model 3: Logit(Pr(Y=1)) = Model 2 + Sleep quality

Model 1, the most simplistic will consider only the exposure variable and a well-recognized measure of socioeconomic status, maternal education. Models 2 and 3 will consider Model 1’s covariates as well as inclusion of maternal pre-pregnancy BMI, which can be an indicator of baseline risk of developing diabetes (Torloni et al., 2009), independent of pregnancy and sleep quality, which is known to be associated with risk of developing gestational diabetes (Facco et al., 2017). Models may be subject to change after univariate and bivariate analysis of cohort data.

Multiple Linear Regression Models:

**Exposure:** Maternal length of the feeding window **Outcome:** child birth weight

Model 1: maternal feeding window + maternal education

Model 2: Model 1 + gestational weight gain  
Model 3: Model 2 + Offspring sex

Model 1 comprises the most simplistic model and will account only for the exposure variable and socioeconomic status. Model 2 will account for the same covariates as model 1, but will also take include gestational weight gain, which has been proven to be associated with larger birth weights and child fat mass (Schack-Nielsen et al., 2010). Model 3 will include child sex, as differences of sex-specific intrauterine growth and adaptation to numerous environmental stimuli (Alwasel et al., 2011; Clifton, 2010) have been seen in human pregnancy. Models proposed are subject to change based on initial analyses of cohort data.

**Potential Pitfalls and alternative approaches:**

### Low recruitment/underpowered in the feeding windows

Because recruitment is part of an observational instead of interventional study, we will not know the length of the feeding window until after the recruitment process. Because of this, there may not be an even distribution of women in each of the designated feeding window groups. If this is the case, there are two alternative approaches. The first is to continue recruitment, which may introduce selection bias and for that reason is not recommended. The second is to analyze the distributions of the feeding window variable collected and to use more iterative cut offs that are apparent in the data.

### Lower or unrepresentative incidence of disease states

As this study proposes to use participant data that is recruited in an untargeted manner, it is possible that recruitment could result in lower than expected incidence of the maternal outcomes investigated. It is also possible that because of the prestige in neonatal care associated with this hospital could attract a greater than expected incidence in maternal health outcomes. Either situation could affect the generalizability of the results of this study.

### Confounding of feeding variable by dietary quality

While the purpose of this proposed dissertation work is to further the understanding of the relationship between length of time fasting and maternal and child health, using only the length of the fast as the only dietary measure is a limitation. There are many components to consider when attempting to understand dietary adequacy, and timing of meals is only one. Others such as dietary quality, macro- and micronutrient adequacy, macronutrient distribution, energy intake, and food safety are all concerns that may affect the relationship. Because dietary quality data is not collected for this sample, we cannot say that associations determined are exclusively because of the feeding window. However, the literature on TRF has shown some robust effects in models that utilize Western, high-fat dietary exposures, meaning there could still be some relationship of the feeding window to outcomes independent of diet quality and calorie intake.

### Poor reliability of fasting state in blood samples

The BUMP cohort design makes it reasonable and easy for participants to enroll in the study, and one fantastic example is in the drawing of neonatal labs. It may be unreliable to assume a similar level of feeding or fasting in these samples. For that reason, insulin and glycemic health data sensitive to fasting/refeeding cycles will be interpreted with caution. It may be of benefit to specifically choose the mid-gestation collection point, as this is done in concert with the oral glucose tolerance test, and therefore is likely to be more uniform in the feeding level (75g of glucose within 1 hour of blood draw).

### Residual Confounding

As is the case with any observational study, the inclusion of any confounding variables is a best attempt at reducing the relationship between the outcome and the exposure through the causal pathway, but there is also potential for residual confounding. For example, perhaps the intake questionnaire assessment of sleep, by asking about snoring, captures poor sleep quality in the form of sleep apnea, but fails to capture women who wake up multiple times a night or get very little sleep which would also be a measure of quality. Furthermore, as the intake questionnaire is both quite simplistic and could simply not measure a confounding variable that could occlude the relationships we are looking for.

## Methods:

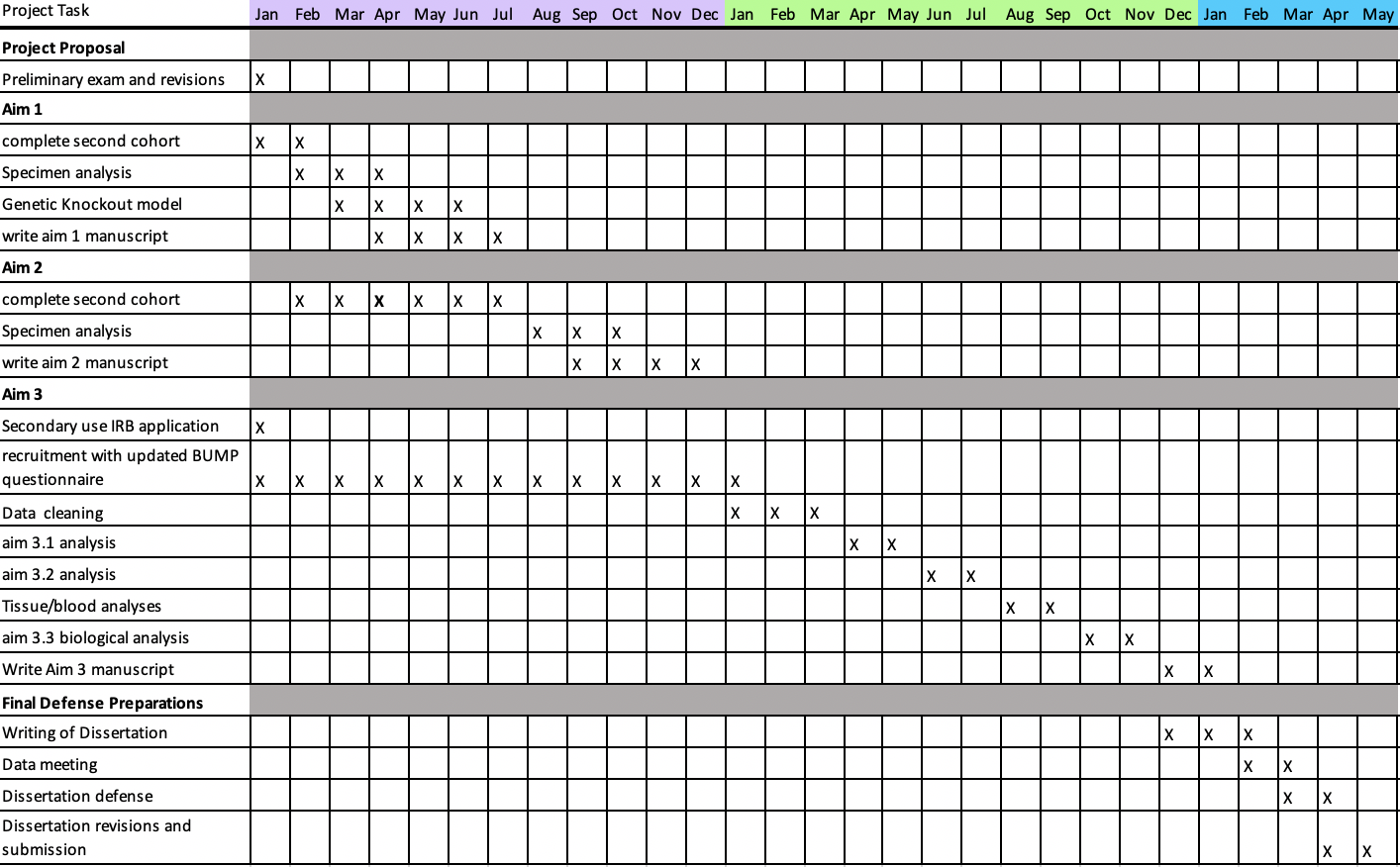
### Human blood hormone determination: ELISA

Human blood insulin concentration will be determined by ELISA (Crystal Chem catalog #90095). This highly reactive and non-cross-reactive assay for C-peptide will be done in duplicate for each sample. Insulin concentration will be calculated using a standard curve fit to known concentrations of a set solution. Individual observations will be reported as the mean concentration (pg/mL) of the two replicates.

### Statistical Analysis

Statistical analysis will be conducted in R. Logistic regression analyses will result in odds ratios (OR) and 95% confidence intervals (CI). Multiple linear regression analyses will be expressed as beta coefficients (β) and 95% confidence intervals.

# Project Gant Chart



Appendix 1: BUMP Study Intake Questionnaire  
  
Page 1: University of Michigan Pregnancy Biorepository Study ID: \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_

HUM00118179 Date: \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_

1. **What is your Ethnicity:**

⃞ Hispanic or Latino or Spanish Origin

⃞ Not Hispanic or Latino or Spanish Origin

⃞ Unknown

⃞ Prefer not to say

1. **What is your Race (check all that apply):**

⃞ American Indian or Alaska Native

⃞ Asian

⃞ Black or African American

⃞ Native Hawaiian or Other Pacific Islander

⃞ White

⃞ Unknown

⃞ Prefer not to say

⃞ Other 🡪 Please describe: \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_

1. **What is the highest level of education you have completed:**

⃞ Some high school, no diploma

⃞ High school graduate, diploma or equivalent (for example: GED)

⃞ Some college credit, no degree

⃞ Trade/technical/vocational training

⃞ Associate degree

⃞ Bachelor’s degree

⃞ Master’s degree

⃞ Doctorate or Professional degree

⃞ Prefer not to say

1. **What is your annual household income:**

⃞ $11,999 or less

⃞ $12,000 to $24,999

⃞ $25,000 to $49,999

⃞ $50,000 to $99,999

⃞ $100,000 to $149,999

⃞ $150,000 or more

⃞ Prefer not to say

1. **How would you best describe your marital or partnership status:**

⃞ Single, never married

⃞ Married or domestic partnership

⃞ Widowed

⃞ Divorced

⃞ Separated

⃞ Prefer not to say

⃞ Other 🡪 Please describe: \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_

Page 2: University of Michigan Pregnancy Biorepository Study ID: \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_

HUM00118179 Date: \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_

1. **How many people are in your household (including yourself):** \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_
2. **Do you currently snore 3 or more nights a week?**  ⃞ Yes ⃞ No ⃞ I don’t know
3. **Before your pregnancy did you snore more than 3 nights a week?**  ⃞ Yes ⃞ No ⃞ I don’t know
4. **Are you currently a smoker?**  ⃞ Yes ⃞ No

9a. If yes, how much do you smoke per day? \_\_\_\_\_\_\_\_\_

1. **Are you a former smoker?**  ⃞ Yes ⃞ No

10a. If yes, when did you quit? \_\_\_\_\_\_\_\_\_\_

1. **Are you regularly exposed (several times/week) to someone else’s smoke during the past 3 months?**

⃞ Yes ⃞ No

1. **Do you live near a landfill (less than 2 miles)?**  ⃞ Yes ⃞ No
2. **Please let us know if you use any of the following personal care products on a regular basis:**

Perfumes and cosmetics  ⃞ Yes ⃞ No

Hair care products ⃞ Yes ⃞ No

1. **Have you had dental fillings in the past 3 months?**  ⃞ Yes ⃞ No
2. **Do you eat canned foods (at least once a week)?**  ⃞ Yes ⃞ No

**15a. If yes, how often do you eat canned food?**

⃞ 1 serving or less/day ⃞ 2-3 servings a day ⃞ 4 servings or more/day

1. **Do you eat at fast food restaurants (at least once a week)?**  ⃞ Yes ⃞ No

16a. **If yes, how often?**

⃞ once a week ⃞ 2-3 times/week ⃞ 4 times or more/week

1. **Do you eat fresh vegetables (at least once a week)?**  ⃞ Yes ⃞ No

17a. **If yes, how often?**

⃞ 1-3 servings/day ⃞ 4-5 servings/day ⃞ 6 or more servings/day

1. **Do you feel stressed?**  ⃞ Yes ⃞ No

**18a. If yes, how often do you feel stressed?**

⃞ Never ⃞ Almost Never ⃞ Some Days ⃞ Most Days ⃞ Every Day

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