Chrononutrition and Endocrine Contributors to Gestational Glycemic Control and Infant Birth Weight

by

Molly C. Mulcahy

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Doctoral Committee:

Associate professor Dave Bridges, Chair
Assistant Professor Erica Jansen
Associate Professor Elizabeth Langen
Professor Darleen Sandoval

Molly C. Mulcahy

mollye@umich.edu

ORCID iD: 0000-0002-8903-7357

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Dedication

This dissertation is dedicated to those who provided me with countless hours of support, encouragement, and motivation. First, I thank my husband, Robert Mulcahy, for the innumerable sacrifices he's made to support my career as a scientist. I thank my parents, Edward and Arlene Carter, for instilling in me an intrinsic curiosity and deep value of continued self-improvement. I thank the other members of my family and dear friends who believed in me when I couldn't. I can say with complete honesty I could not have achieved this milestone without each of your involvement.

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Abstract

Pregnancy is a critical period of development where nutrition choices and endocrine changes can impact the health of the pregnant person and their child long after birth. While poor diet quality and caloric restriction during pregnancy are frequently associated with ill health in both animal and human models, there is very little evidence that speaks to the impact of the timing of food intake and duration of fasting on perinatal health outcomes. There is also evidence that growth differentiation factor 15 (GDF15) levels increase during the course of pregnancy and are associated with nutritional challenges and complications, but the effect of this hormone on critical outcomes in a normal pregnancy are limited to observational studies in humans.

Based on evidence from non-pregnant populations, the manipulation of the timing of eating and duration of fasting can impact both body weight and metabolic health. GDF15 levels have also been evaluated in relation to body weight regulation, food intake, and glycemic health. To understand the impact of these exposures during the normal course of pregnancy in lean individuals we employed a translational approach that incorporated animal and human observational methods. To assess the role of the timing of eating in perinatal health, we restricted food access of laboratory chow to 6 hours in the early dark cycle from one week before mating until birth. We examined outcomes in dams during the course of pregnancy and followed the resultant pups from birth until adulthood, when we challenged them with a high-fat, high-sucrose diet. To assess relationships between the timing of eating in pregnant humans, we surveyed currently pregnant individuals about their habitual timing of eating and fasting habits during the 2nd and 3rd trimester of pregnancy. We then evaluated their responses with respect to mid-

gestation measures of glycemic health and infant birth weights. Finally, to assess the role of GDF15 in normal pregnancy, we used a *Gdf15* knockout mouse model to compare dams and litters where there were normal levels of GDF15 with those who had no circulating levels of GDF15.

These studies showed that gestational eTRF in mice may result in worsened glycemic response to glucose reductions during late gestation in dams and impart lower survival in litters exposed to eTRF *in utero*. Male adult offspring of eTRF dams develop glucose intolerance in after a high-calorie dietary challenge. In pregnant humans, we find that later timing of the 1st meal in the 3rd trimester is associated with modest increases in parent mid-gestation glycemia. Longer fasting durations and later meal timing during the 2nd and 3rd trimester reduces birth weight. We also conclude that normal GDF15 levels are not necessary for healthy weight gain and glycemia during mouse pregnancy. These findings highlight the need for continued research to better understand these exposures during this critical period of development. Taken together, this work suggests that meal timing and fasting duration is an important component of the diet in pregnancy that merits further research.

Chapter 1 Introduction

1.1 Pregnancy is a Critical Period of Development

During the life course, there are periods of critical impact that can change the trajectory of one's health. Pregnancy is one of these critical periods, as complications and behavioral choices that occur during pregnancy not only impact the pregnant person, but also the resultant child for years to come. The study of these critical periods of development, and specifically those that occur during pregnancy have long been coined the Developmental Origins of Health and Disease or (DOHaD) hypothesis. This field was born from Dr. David Barker's epidemiological work that sought to understand why some populations experience higher burden of both infant mortality and adult cardiometabolic morbidity and mortality(Barker et al., 1989, 1993; Barker & Osmond, 1986). Barker and colleagues discovered that individuals who experienced malnutrition or growth restriction *in utero* were experiencing cardiovascular disease and events at greater rates than those who developed with optimal nutrition and growth. Since that those early analyses, the field has exploded to include human clinical, observational, and animal experimental work.
As such, there has been much attention and research focused on optimizing nutrition during this period of life. Especially since maternal diets in utero are also known to impact resultant children's trajectory toward health and disease, even throughout adulthood.

1.2 Pregnancy-Related Changes in Insulin Sensitivity and Endocrine Factors

Pregnancy is an incredibly physiologically demanding process. The body undergoes rapid change in order to facilitate delivery of nutrients to the developing fetus. To facilitate this shift in

nutrient partitioning, there are profound endocrine changes that occur. A state of insulin resistance is considered normal during pregnancy and is well established in both mouse (Ladyman et al., 2018; Musial et al., 2016) and human pregnancies (Sonagra et al., 2014). This state of insulin resistance is related to changes at the level the of pancreas to facilitate hyperinsulinemia (Butte, 2000) and peripheral tissues to interfere with normal insulin signaling (Newbern & Freemark, 2011); these phenomena work in concert to facilitate shunting of vital nutrients towards the developing placenta and fetus. There are many endocrine factors that are known to change in relation to pregnancy. Cortisol (corticosterone in rodents (Barlow et al., 1974)), is one such hormone. Cortisol is one of the major players in coordinating diurnal rhythms and is progressively increased until delivery (Jung et al., 2011). Cortisol also increases insulin resistance of pregnancy and impacts other perinatal complications such as preterm birth(Soma-Pillay et al., 2016).

1.3 GDF15 and Pregnancy

GDF15 is a Transforming Growth Factor (TGF) ß family cytokine that was first discovered in 1997 by Bootcov and colleagues, when it was originally thought to be related to parent immune-tolerance of the feto-placental unit (Bootcov et al., 1997). Since that time, much work has been done to demonstrate that GDF15 has a role in signaling somatic stress in all ages. The hormone increases with age (Welsh et al., 2022), and is elevated by many systemic stressors, such as intense exercise (Klein et al., 2021), metabolic disease (Mullican et al., 2017), liver injury (Hsiao et al., 2000), and pregnancy (Andersson-Hall et al., 2021; Chen et al., 2016; Marjono et al., 2003; Moore et al., 2000). Elevations in GDF15 may occur after periods of nutritional stress, such as overfeeding, caloric restriction, periods of fasting, and unbalanced diets (Patel et al.,

2019). There is evidence avoidance of food intake, reductions in fat preference (Frikke-Schmidt et al., 2019), and conditioned taste aversion (Patel et al., 2019) that result in weight loss when exogenous GDF15 is given in animals models (Mullican et al., 2017; Patel et al., 2019). As such, GDF15 merits study as a contributor toward habitus and nutrition in a wide swath of populations, including pregnant individuals.

The role of GDF15 in pregnancy is incompletely understood. There is great consistency that GDF15 in circulation increases as pregnancy progresses (Andersson-Hall et al., 2021; Moore et al., 2000)and that it is highly expressed in the placenta. More interestingly, elevated GDF15 levels in parent serum during pregnancy is associated with metabolic complications of pregnancy. Recent work in pregnant humans find that GDF15 is reduced in serum from those who would later experience pregnancy loss or miscarriage (Tong et al., 2004). The role of GDF15 on gestational weight gain is less clear, with some showing an inverse relationship (P. Wang et al., 2020)and others finding no differences (Andersson-Hall et al., 2021). Elevations of GDF15 are present in parents diagnosed with gestational diabetes (Yakut et al., 2021), or have pre-existing type1 (Jacobsen et al., 2022) or type 2 diabetes (Sugulle et al., 2009). Recently the effect of GDF15 on nausea and emesis has been of great research interest. Higher levels of emesis and nausea during pregnancy have been related to higher circulating levels of GDF15 in pregnant humans (Petry et al., 2018). Another group demonstrated a relationship between those with excessive nausea and vomiting of pregnancy, or hyperemesis gravidarum, wherein after symptoms subsided there was a normalization in previously elevated levels of GDF15 (Fejzo et al., 2019), and missense genetic variants near *GDF15* were associated with 32% reduced odds of developing the condition. Because of these associations with anorectic behavior and perinatal health complications, more research is needed to understand the role of physiologically normal

levels of GDF15 during routine pregnancy, and moreover to understand the consequences of *Gdf15* ablation during gestation. It may be that GDF15 plays a role in the onset of pregnancy-related complications, or it may serve as a marker of the stress of pregnancy.

1.4 Maternal Nutrition Restriction and Chronodisruption are Public Health Problems

For obvious ethical reasons, much work in DOHaD has been adapted to preclinical models of pregnancy. Poor nutrition in pregnancy is often accomplished in animal models through means of calorie or protein restriction. Frequently, severe nutrient restriction results in more harm for the resultant fetus than for the dam, and studies often find that pups born to restricted dams are growth restricted (Berends et al., 2013; Cunha et al., 2015; Martin-Gronert & Ozanne, 2007). The effects gestational restriction often are more pronounced when offspring reach adulthood, increasing their likelihood for metabolic disease such as glucose intolerance and insulin insensitivity (Radford & Han, 2019; Shahkhalili et al., 2010). Restrictive feeding patterns have also been known to impact endocrine factors that play a role in the circadian rhythm, such a cortisol/corticosterone. Rodent studies have demonstrated that restriction in utero may impart blunted corticosterone responses (Kenny et al., 2014; Levay et al., 2010). Given that it is already established that cortisol/corticosterone is imperative for insulin resistance of pregnancy, dietary strategies that may impact this hormone during pregnancy need to be thoroughly investigated to prevent passing on harmful health effects to gestating infants.

The only human investigations of intentional fasting have occurred within the context of observing Ramadan in Muslim women. These studies have been inconsistent in the ways they quantify Ramadan fasting exposure, making comparison difficult. Results show fasting results in reductions in birth weight (Awwad et al., 2012; Glazier et al., 2018; Savitri et al., 2014; Ziaee et al., 2010), potential for greater rates of small-for-gestational age (Cross et al., 1990; Daley et al.,

2017; Opaneye et al., 1990; Ziaee et al., 2010), and inconsistent effects on parent gestational glycemia (Baynouna Al Ketbi et al., 2014; Ismail et al., 2011).

1.5 Chrononutrition Impact on Metabolic Health

One aspect of eating that has gained interest is not what one eats, but when. Metabolism in mammals occurs in a daily circadian rhythm, where waking hours are predominantly focused on nutrient acquisition and storage and sleeping hours rely on liberation of energy from storage tissues. All mammalian cells have an intrinsic cellular clock mechanism that is coordinated by a series of transcription factors (Panda, 2016). Evaluations of chronodisruption, such as engaging in shift work or sleep disturbances, demonstrate that a disrupted circadian rhythm clock imparts greater risk for poor metabolic health (Vetter et al., 2018). A key hormone for insulin resistance of pregnancy, cortisol/corticosterone, is also critical to the maintenance of a normal circadian rhythm(Potter et al., 2016). This mammalian circadian clock, both central and peripheral, can be entrained be exogenous cues, or zeitgebers. Studies in both humans and in rodents have identified that the timing of food intake is a zeitgeber. Examining or adjusting the temporality of food intake is often called Chrononutrition, and it is a burgeoning area of research. One of the primary interventions evaluated in Chrononutrition is that of time-restricted eating/time-restricted feeding (TRE/TRF). This is a form of intermittent fasting that limits food intake to a consistent period each day, usually less than 12 hours long. In contrast to other calorie restriction methods, TRE does not set stipulations on diet quality, macro- or micronutrients, or even portion size. The ease with which this dietary modality can be employed without repeated counseling from a healthcare practitioner has made it a popular diet. In fact, in a sample meant to reflect typical dietary habits the International Food Council reported 10% of dieters had used intermittent fasting in the last year (International Food Information Council, 2020). A study in

young Canadians also noted that 38.4-52.0% of respondents had used IF in the last 12 months (Ganson et al., 2022). Literature that evaluates TRE/TRF is rarely focused on pregnant populations. Typically, modest weight loss occurs in adults with excess adiposity who engage in TRE, that is comparable to other forms of calorie restriction (Cienfuegos et al., 2021; Gabel, Hoddy, Haggerty, et al., 2018; Gabel et al., 2019; Hutchison et al., 2019; Lowe et al., 2020; Moro et al., 2016). However, some studies find metabolic improvements from the use of TRF in humans(Cienfuegos et al., 2021; Gabel, Hoddy, Haggerty, et al., 2018; Hutchison et al., 2019; Jamshed et al., 2019; Moro et al., 2016; Sutton et al., 2018) and in animal models (Chaix et al., 2019, 2021; Chung et al., 2016; Hatori et al., 2012, 2012; Hua et al., 2020; X.-P. Wang et al., 2020).

Studies that examine TRF interventions during pregnancy are limited to animal models that use TRF to reduce effects of overnutrition on the fetus (Upadhyay et al., 2019, 2020), models that sought to include chronodisruption as well as TRF to model Ramadan fasting (Alkhalefah et al., 2021a, 2022), or focus on the effects of TRF during gestation exclusively on offspring during adulthood (Mulcahy et al., 2022; Prates et al., 2022). Evidence points to interest in this diet in pregnant human populations, as a case study of prolonged fasting to control gestational diabetes (Ali & Kunugi, 2020) and an investigation about the beliefs and attitudes related to intermittent fasting during pregnancy (Flanagan et al., 2022) have recently been published.

Because there are so few studies about this dietary modality in pregnancy, we have an incomplete understanding of its effects. The work of this dissertation seeks to broaden the understanding of how the timing of eating and GDF15 elevations during pregnancy can impact both parent and child health using both animal and human observational methods. Chapter 2

seeks to characterize the effect of TRF of a chow diet during gestation in a mouse model on maternal body weight, food intake, estrus cyclicity and fertility, insulin sensitivity, and early postnatal health indicators. In Chapter 3, I use a *Gdf15* knockout mouse model to evaluate the impact of GDF15 on maternal food intake, gestational weight gain, insulin sensitivity, lactation, and pup postnatal growth. In chapter 4, I use data from an observational cohort study of human pregnancy to assess the relationship between self-reported timing of eating and fasting duration and mid-gestation oral glucose tolerance test values and infant birth weight. Finally, in chapter 5, I evaluate the effect of gestational TRF on the offspring from dams in chapter 2 from weaning until adulthood, and in response to a high-fat, high-sucrose diet in both sexes. These studies together implicate the timing of eating and fasting duration as critical contributors to perinatal health outcomes in both animal model and human participants.

Chapter 2 Gestational Early Time-Restricted Feeding Results in Mild Maternal Glycemic Differences, Reduced Litter Sizes, and Pup Survival

2.1 Abstract

Time-restricted Feeding is an increasingly common diet that may modulate metabolic health. As recent evidence suggests pregnant people have considered or used the diet, and pregnancy is a critical period of development for both parent and child, investigation of the effects of TRF during pregnancy in animal models is warranted. We employed a chow fed daily early time-restricted feeding (eTRF) regimen during the dark during the course of mouse pregnancy and evaluated its effect on maternal body weight and food intake, maternal insulin sensitivity, and offspring early postnatal health. We found that dams who were fed eTRF consumed similar kilocalories during the course of their pregnancies and gained comparable weight to Ad Libitum dams. Dams who were exposed to eTRF had similar insulin sensitivity but had greater rebound from glucose nadir during late gestation. Fertility and pup birth weight were comparable between diets, but there was a significant reduction in litter sizes and rates of survival to postnatal day 3 in eTRF dams. Pups born to eTRF dams had similar growth to postnatal day 21. These data suggest that eTRF during gestation has mild effects in pregnant dams and reductions in offspring survival. Etiology of the reduction in survival is unknown, but may be related to anticipated food restriction in dams after birth. More evaluation of this phenotype is warranted in order to translate to pregnant human populations.

2.2 Introduction

The timing of eating with respect to one's circadian rhythm has become an area of interest as a modifiable component of the diet to alter for health reasons. There are many forms of eating that attempt to manipulate the timing of food; among them is time-restricted eating or feeding (TRF/TRE). With this modality, one confines caloric intake to a predictable and condensed period of time each day, in line with the circadian day, ultimately increasing the number of hours spent fasting.

Rodent models have thoroughly detailed this dietary manipulation. Often, when TRF is employed in rodents provided with a high-fat, high-sucrose diet, that weight gain is reduced compared to ad libitum fed controls (Boucsein et al., 2019; Chaix et al., 2019; Sherman et al., 2012). More importantly, TRF in rodent models has been shown to positively impact glucose homeostasis (Chaix et al., 2019; Chung et al., 2016; Hatori et al., 2012; She et al., 2021; W. Wang et al., 2021; X.-P. Wang et al., 2020; Wilson et al., 2020; Woodie et al., 2018), although this is not present in all studies (Das et al., 2021; García-Gaytán et al., 2020; Sherman et al., 2012). The focus of this body of literature is the ability of TRF to protect from high-fat, high-sucrose diets in adult animals, with few groups working on younger populations or focusing on the effects during critical periods of development.

Human models have evaluated this as a method to treat or prevent accumulation of deleterious amounts of adipose tissue which may result in metabolic illness. Although weight loss is often modest (Gabel, Hoddy, Haggerty, et al., 2018; Hutchison et al., 2019; Lowe et al., 2020; Moro et al., 2016; Stote et al., 2007), there have been comparable health improvements in those with controlled periods of time-restricted eating; such as reductions in blood pressure (Gabel, Hoddy, Haggerty, et al., 2018; Jamshed et al., 2019; Sutton et al., 2018), improved

measures of oxidative stress (Moro et al., 2016; Sutton et al., 2018), reductions in glycemic excursions (Jamshed et al., 2019), or alterations in insulin indices (Hutchison et al., 2019; Jamshed et al., 2019; Moro et al., 2016; Sutton et al., 2018). Some have even found improvement without weight loss (Sutton et al., 2018). Currently, the focus of the majority of TRF/TRE studies have been in preventing or lessening metabolic effects from over-feeding in adults, leaving critical periods of development and lower-calorie diets without evidence. Furthermore, as the popularity of this diet increases, there are critically important populations that develop lasting effects from attempting this diet before its effects are fully characterized; one such population is those who are attempting to become or are pregnant.

Dietary health during pregnancy has long been a topic of intense research interest. This research intensified when David Barker proposed that *in utero* conditions could program the resultant child for health or disease, based on the mismatch they would face once born (Barker & Osmond, 1986). Ultimately, these studies were the first of the developmental origins of health and disease (DOHaD) field. The most prominent DOHaD study examined children who were *in utero* during extreme famine during the "Dutch Hunger Winter" during the second world war. It found that times of food restriction during pregnancy could impart higher risk for cardiometabolic risk in adulthood, even after risk ratios were adjusted for infant birthweights (Heijmans et al., 2008; Rooij et al., 2006; Roseboom et al., 2000). Since that time, many projects have sought to understand the role of adverse nutrition in the womb and its impacts on children, even well after having reached adulthood.

There is evidence to suggest that timing of food intake is an important, yet critically understudied aspect of nutrition during pregnancy. Some of this evidence comes from models of time-restricted feeding in pregnant or reproductively active rodents. These studies find that time-

restricted feeding of high-fat, high-sucrose diets in rodents can reduce oxidative stress in placental tissues that results from overnutrition during pregnancy (Upadhyay et al., 2019), and improve fetal lung development compared to *ad libitum* fed high-fat, high-sucrose dams (Upadhyay et al., 2020). There is also evidence that impaired estrus cyclicity and ovarian follicle development that can occur with overnutrition are rescued with TRF of HFHS feeding compared to *ad libitum* HFHS (Hua et al., 2020). Existing studies in rats have found that TRF during pregnancy has impact for insulin homeostasis in adulthood. In adult offspring of eTRF dams, glucose intolerance developed on a chow diet (Prates et al., 2022), and another from our group finds that glucose intolerance only occurs in male offspring after long term high fat, high sucrose feeding (Mulcahy et al., 2022). Still others have sought to replicate TRF with chronodisruption (as a proxy for Ramadan fasting), and growth restriction was present on a chow diet, where dams ate fewer calories, gained less weight, and pups were smaller in litters randomized to TRF during the light cycle (Alkhalefah et al., 2021b). As the majority of the attention that has been paid to this dietary manipulation focuses on resultant offspring either as adults or in the fetal stage, scientists have failed to comprehensively characterize the effects of TRF during the course of the pregnancy in the dam without chronodisruption as part of the model.

Although animal work is limited, there is evidence that those who are currently pregnant or considering pregnancy would consider manipulation of the timing of food intake as a modality to improve health. Flanagan and colleagues asked participants about their attitudes toward trying time-restricted eating during the course of pregnancy. Of those polled, 24.7% said they would be open to trying a time-restricted regimen during the course of pregnancy to improve their health (Flanagan et al., 2022). There was also a qualitative response from one participant who stated they had practiced intermittent fasting during their pregnancy. Recently, a case study also

identified manipulation of the feeding window and reducing meal numbers to manage gestational diabetes (Ali & Kunugi, 2020). Although epidemiological work on the timing of eating is still limited in pregnant populations, an association between prolonged overnight fasting and fewer meals during the day has been found with a more favorable maternal glycemic response in the second trimester of pregnancy (Loy et al., 2017). Eating overnight, although somewhat common during pregnancy, can also be associated with poorer birth outcomes (Loy, Loo, et al., 2020).

The most robust literature in humans that explores maternal dietary restriction during gestation are studies that evaluate pregnancy outcomes after religious observance of Ramadan in Muslim pregnant populations. Such studies have found that observing Ramadan fasting during pregnancy does not result in reduced gestational age at delivery (Awwad et al., 2012; Safari et al., 2019), does not impact birth weight (Glazier et al., 2018; Safari et al., 2019), and inconsistent results in relation to odds of developing gestational diabetes (Awwad et al., 2012; Daley et al., 2017; Safari et al., 2019). However, Ramadan is an imperfect proxy for TRF, as altered timing of eating is concomitant with sleep disruption and dietary quality changes. Therefore, more direct analyses of altered timing of eating are warranted. Overall, the current literature suggests that there is evidence that human pregnant populations either practice or consider practicing this diet and that we have limited understanding of its implications for safety or efficacy in impacting perinatal health.

In light of the potential use of this diet to improve health during pregnancy and limited characterization of the practice in pregnant populations on the parent, we sought to identify the effect of early time-restricted feeding (eTRF) on maternal insulin sensitivity and early postnatal health in resultant offspring using a mouse model. We hypothesized that maternal glycemic

health would be improved through eTRF of normal chow and that resulting offspring would not be adversely affected.

2.3 Methods

2.3.1 Animal Husbandry

Age-matched (17±0.072 weeks) male and female C57BL/6J mice were obtained from The Jackson Laboratories (RRID:IMSR_JAX:000664). Animals were allowed to acclimatize to our facility for 1 week prior to beginning the experiment. Animals were maintained in a ventilated cages in a temperature and humidity-controlled room. In a 12:12 hour light dark cycle. 4 days before experimental treatment began, dams were single housed with extra enrichment. Every week, mice were weighed, and body composition was assessed using EchoMRI. This experiment was repeated in 3 separate cohorts of animals.

2.3.2 Animal Dietary Intervention

Dams were randomized to either 24-hour access *ad libitum* (AL), or 6-hour early-time restricted feeding (eTRF) access to standard laboratory chow (24% Protein, 5% Fat, 35.7% Carbohydrate). We also measured the food to the nearest 0.1 fram in eTRF and AL dam cages at ZT14. Animals were then allowed to eat freely for 6 hours. At ZT20, food was collected from the hopper and the bottom of the cage and measured again. Cages of all animals were changed at ZT20 to minimize food consumption of the bottom of the cage for eTRF dams and to have similar levels for handling stress in AL dams. Dams randomized to eTRF had empty hoppers placed in their new cages at ZT20, and AL dams had the same hoppers replaced in their new cages. Food intake is determined in both 6-hour (ZT14-ZT20), and 24-hour intervals (ZT14-ZT14).

2.3.3 Estrus Testing

To understand how eTRF affects estrus cycle health, we monitored the estrus stage of females after randomization to dietary treatment each day until copulatory plug appeared in one cohort of the experimental protocol. We assessed this one hour before food was given (ZT13) when a vaginal canal smear was collected for each dam. Using a p20 pipette, 15uL of sterile PBS was lavaged into the vaginal canal and mixed by plunging up and down briefly. Then the same pipette was used to recollect as much of the 15 uL volume as possible which was immediately transferred to a microscope slide. While still wet, slides were visualized at 10X magnification and images were captured. If the sample was dense, dry, or had crystals, more PBS was added and mixed gently with a clean pipette tip. Cell type and proportions were examined and stages were assigned based on methods described previously (Caligioni, 2009; McLean et al., 2012). We calculated the total number of days in each stage for each dam, then averages were taken for each maternal dietary regimen.

2.3.4 Mating, Fertility & Pups

After 6 days of diet, age and diet-matched males were introduced into female cages and were allowed to remain until copulatory plug was discovered (indicating pregnancy and gestational day E0.5). To assess fertility, latency from mating to plug and rates of successful mating events were calculated. When pups were born, they were measured and counted within 24 hours, including those who were dead at birth. Pups were then left to nurse for 3 days. At postnatal day 3, litters were weighed then reduced to 4 pups to each dam (2 males, 2 females when possible) to standardize milk supply between litters. Pups were then reweighed on postnatal days 7, 14, and 21. At postnatal day 21dams and pups were sacrificed by Carbon Dioxide Inhalation and cervical dislocation.

2.3.5 Intraperitoneal Insulin Tolerance Testing

As an index of insulin sensitivity, an intraperitoneal insulin tolerance test (ITT) was performed. On gestational day 16.5, dams were placed in a clean cage free of food with a water bottle at ZT20 (2AM). Dams were fasted for 6 hours. At ZT2, a fasted blood sample was collected via tail clip and handheld glucometer. After assessment of fasting blood glucose, an intraperitoneal injection of insulin (Humulin, 0.75mg/kg body weight) was given. Blood glucose following injection was determined every 15 minutes for 2 hours. Glucose area under the curve (AUC) was calculated by taking the sum of glucose values for each animal. Rates of initial reduction in blood glucose were calculated by limiting the data to 45 minutes after injection. We then modeled the exponential rate of decay in blood glucose for each dam as a slope and took the average by feeding group. We also calculated the rate of rebound after glucose nadir by limiting the data to data collected 75-120 minutes after injection, then modeling the linear rise in glucose as a time:treatment interaction.

2.3.6 Blood Collection and Hormonal Analysis

The day after the insulin tolerance testing, we collected blood samples from dams at ZT1 and ZT13. They were lightly anaesthetized via inhaled isoflurane then whole blood was collected via capillary tube and retroorbital bleed. Whole blood was left to clot on ice for 20 minutes, then was spun down in a cold centrifuge for 20 minutes at 2000G (Eppendorf, 4°C). Serum was pipetted off and stored at -80°C until later use. Insulin was assayed in serum using a commercially available , ultra-sensitive mouse ELISA kit (Crystal Chem, catalog #90080).

2.3.7 Neonatal Life Outcomes

Gestational age was determined by the date of birth subtracted from date of copulatory plug. Litter size was represented as the number of pups delivered per dam, then averaged by feeding

regimen. Percent survival was determined as the number of pups who were present at postnatal day 3 divided by the initial litter size. Birth weight was calculated as the average of all living pups for each dam, then further averaged by feeding regimen.

2.3.8 Statistical Analyses

Values are represented as mean ± standard error. Pairwise values are evaluated by Shapiro test for normality and Levene's Test for equivalence of variance. When values were estimated as normal and of equivalent variance, Student's *t* Test was used; if they were not normal, then we used the appropriate non-parametric test. For fertility measures (estrus staging and success of mating events), chi-square analyses were completed, comparing the proportion of days distributed among estrus stage by maternal dietary treatment, assuming an equal distribution as between stages. For repeated measures, such as food intake, and body composition, linear mixed effect modeling was completed using lme4 (Bates et al., 2015, p. 4). We used random effect of maternal ID and dam ID and fixed effects for feeding regimen, day of gestation or postnatal age, and sex (for pup analyses).

2.4 Results

2.4.1 Early Time Restricted Feeding Does Not Alter Food intake nor Gestational Weight Gain

In order to characterize the effects of early time-restricted feeding (eTRF) during pregnancy, we randomized dams to eTRF between ZT14-ZT20 or *ad libitum* (AL) feeding of laboratory chow (**Figure 1A**) (Mulcahy et al., 2022). After one week acclimating to the diet, males were added to the cage and examined daily until a copulatory plug was identified. Dams were kept on respective timed diets until they gave birth, at which point they were all switched to AL access to chow (**Figure 1B**). During the first week following randomization, there was an evident period

of adaptation, where eTRF dams slowly increased their 6-hour food intake by 1.15±0.32 kcals per day as they habituated to reduced food access time. This resulted in a significant interaction between day of exposure and maternal dietary regimen (**Figure 2A**, $p_{day:diet}$=0.00033). Using linear mixed effect models, we found that in the pre-pregnancy period, eTRF dams consumed 6.63 ± 1.59 more kilocalories during their 6-hour feeding period than AL dams did (**Figure 2B,** p<0.001). There was a significant interaction between gestational age and maternal dietary regimen during pregnancy, where eTRF dams consumed significantly more food at 6 hours during pregnancy, but this difference increased as gestational age advanced (**Figure 2C**, $p_{diet:gest.age}$=0.001). However, when we examined 24-hour intake, we found that during both the pre-pregnancy and pregnancy periods, eTRF dams consumed similar kcals compared to AL dams (**Figure 2D**, p_{diet} = 0.66 and **Figure 2E**, p_{diet} = 0.72). Consistent with their matched food intake, dam body weights remained comparable during pre-pregnancy (**Figure 2F**, p=0.68) and pregnancy (**Figure 2G**, p=0.34). These data suggests that after an adaptation period, dams randomized to eTRF during the perinatal period are able to maintain normal caloric intake and maintain appropriate body weights for pregnancy.

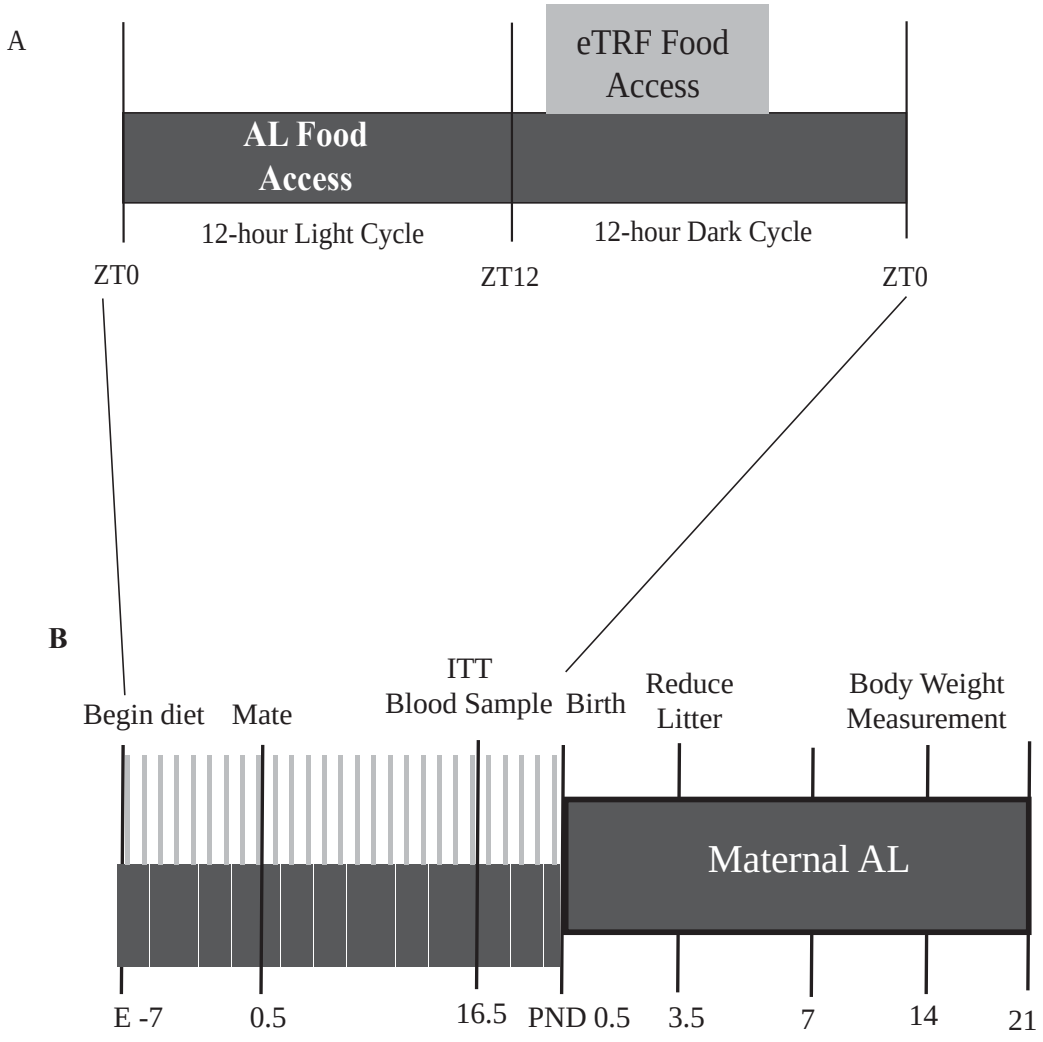

Figure 1:Early Time-Restricted Feeding in a Mouse Pregnancy Model

A) Food availability and light:dark cycle for early time restricted (eTRF) and *Ad Libitum* (AL) dams. Beginning one week before mating and lasting through parturition (eTRF n=16, AL n=14), eTRF dams had food access from chow access between ZT14-ZT20, AL dams had 24-hour chow access. B) Maternal dietary treatment differed in the perinatal period. On E16.5, an intraperitoneal insulin tolerance test was conducted. Blood samples were collected at ZT1 and ZT13 the day after ITT under normal feeding conditions for each dietary regimen. All dams were maintained on chow AL after birth. Pups were reduced to 4 per dam on PND 3. Pups were weighed at birth, PND 3, 7, 14, and 21.

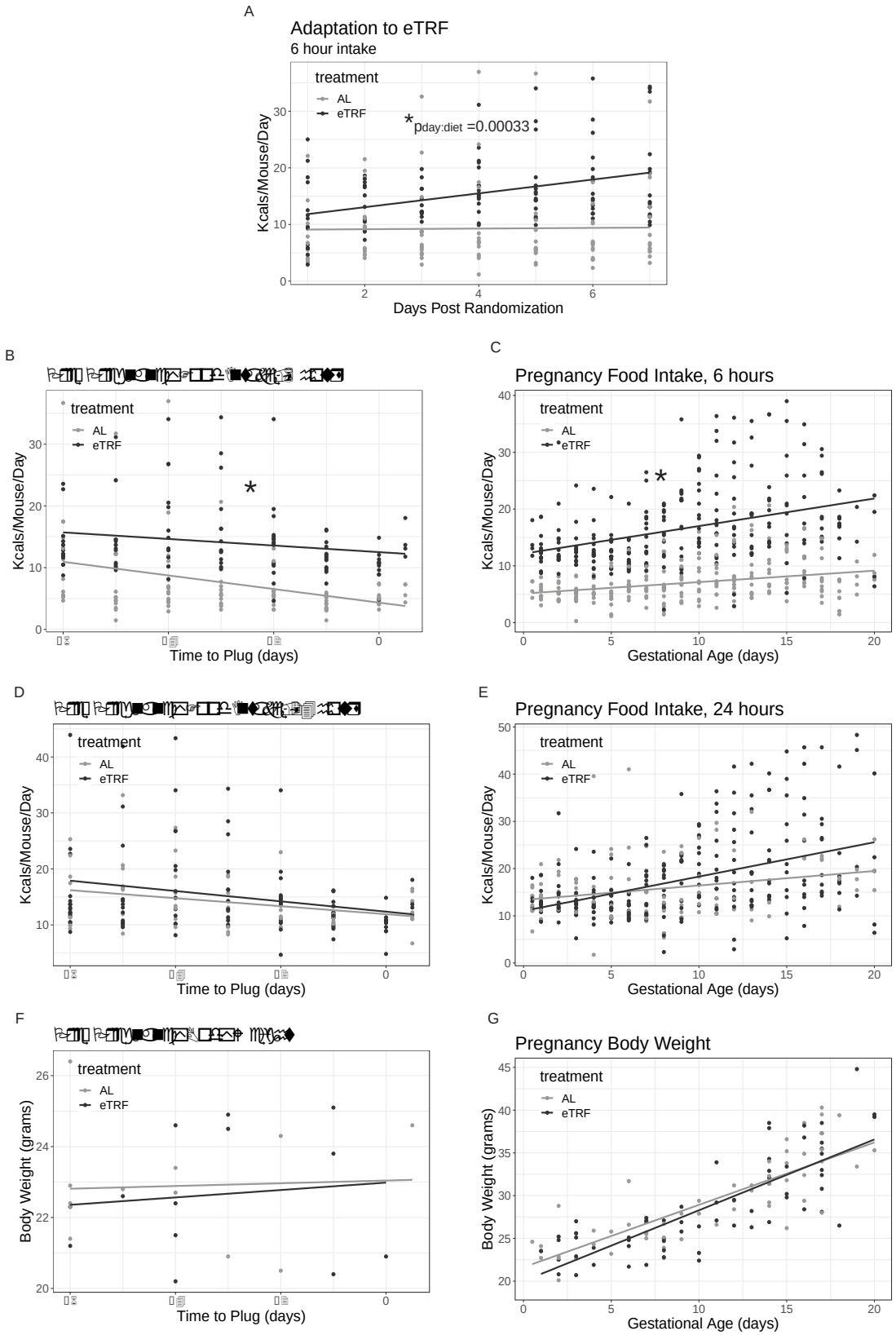
A
Adaptation to eTRF
6 hour intake
treatment
AL
eTRF
* pday:diet =0.00033
Kcals/Mouse/Day
Days Post Randomization
B
treatment
AL
eTRF
*
Kcals/Mouse/Day
Time to Plug (days)
C
Pregnancy Food Intake, 6 hours
treatment
AL
eTRF
*
Kcals/Mouse/Day
Gestational Age (days)
D
treatment
AL
eTRF
Kcals/Mouse/Day
Time to Plug (days)
E
Pregnancy Food Intake, 24 hours
treatment
AL
eTRF
Kcals/Mouse/Day
Gestational Age (days)
F
treatment
AL
eTRF
Body Weight (grams)
Time to Plug (days)
G
Pregnancy Body Weight
treatment
AL
eTRF
Body Weight (grams)
Gestational Age (days)

Figure 2: eTRF in Dams Does Not Affect 24-Hour Food Intake or Body Weight During Pregnancy

A) The adaptation period to eTRF from first day of exposure until day 7 of experiment. Dams randomized to eTRF slower increased food intake in the 6-hour window from AL levels. B) Six-hour food intake (between ZT14-ZT20) in the week before copulatory plug appeared. C) Food intake from ZT14-ZT20 one week before copulatory plug appeared. C) Food intake from ZT14-ZT20 from gestational day 0 until birth. D) Food intake over 24 hours (ZT14-ZT14) one week before copulatory plug appeared. E) Food intake over 24 hours (ZT14-ZT14) during pregnancy (E0-birth). F) Body weights (grams) in dams one week before copulatory plug appeared. G) Body weight in dams (grams) from E0-birth. *Indicates p-value of diet <0.05. If p-value is not for diet alone, it is labeled with interaction. (eTRF n=16, AL n=14)

2.4.2 Insulin Responsiveness is Similar in eTRF Dams, but There is a More Robust Rebound from Glucose Nadir

To test whether dams fed eTRF had improved insulin responsiveness, we conducted intraperitoneal insulin tolerance tests (ITT) on gestational day 16 (**Figure 3A**). We found that fasting blood glucose was similar between eTRF and AL dams at the beginning of the ITT, (**Figure 3B**, p=0.27). Using linear mixed effect models with a random effect for dam ID and fixed effects of time and maternal dietary regimen, we found that eTRF dams averaged 17.6±12.6 mg/dL greater glucose at each time point than AL dams during the course of the full 120 minutes ($p_{diet*time}$ <0.001; **Figure 3A**). As such there was a 19.8% greater area under the curve in eTRF dams (**Figure 3C**, p=0.03) indicating insulin insensitivity. To probe this further, we assessed the initial response to insulin administration. We found eTRF dams and AL dams to be similarly responsive in the initial stages, with comparable rates of glucose drop (**Figure 3D**, p=0.75). eTRF dams seemed to have a more rapid glucose recovery after reaching their lowest glucose value. We evaluated the difference in the rates of glucose recovery after glucose nadir by constructing linear models for each group in just the last 60 minutes of the experiment. We found that eTRF dams recovered glucose at a rate 2.4-fold faster than AL dams, but this did not reach statistical significance (**Figure 3E**, p=0.084). Despite the significant difference in response to

ITT, there were no significant differences in serum insulin concentration between maternal feeding regimens at ZT1 or at ZT13 (**Figure 3F**, p=0.38). These data suggest that insulin sensitivity is similar to normal pregnancies in AL fed dams, but that there is a more robust response from reduced glucose levels in dams who undergo chronic, prolonged overnight fasts during the perinatal period. This change is unlikely to be driven by baseline differences in insulin concentration.

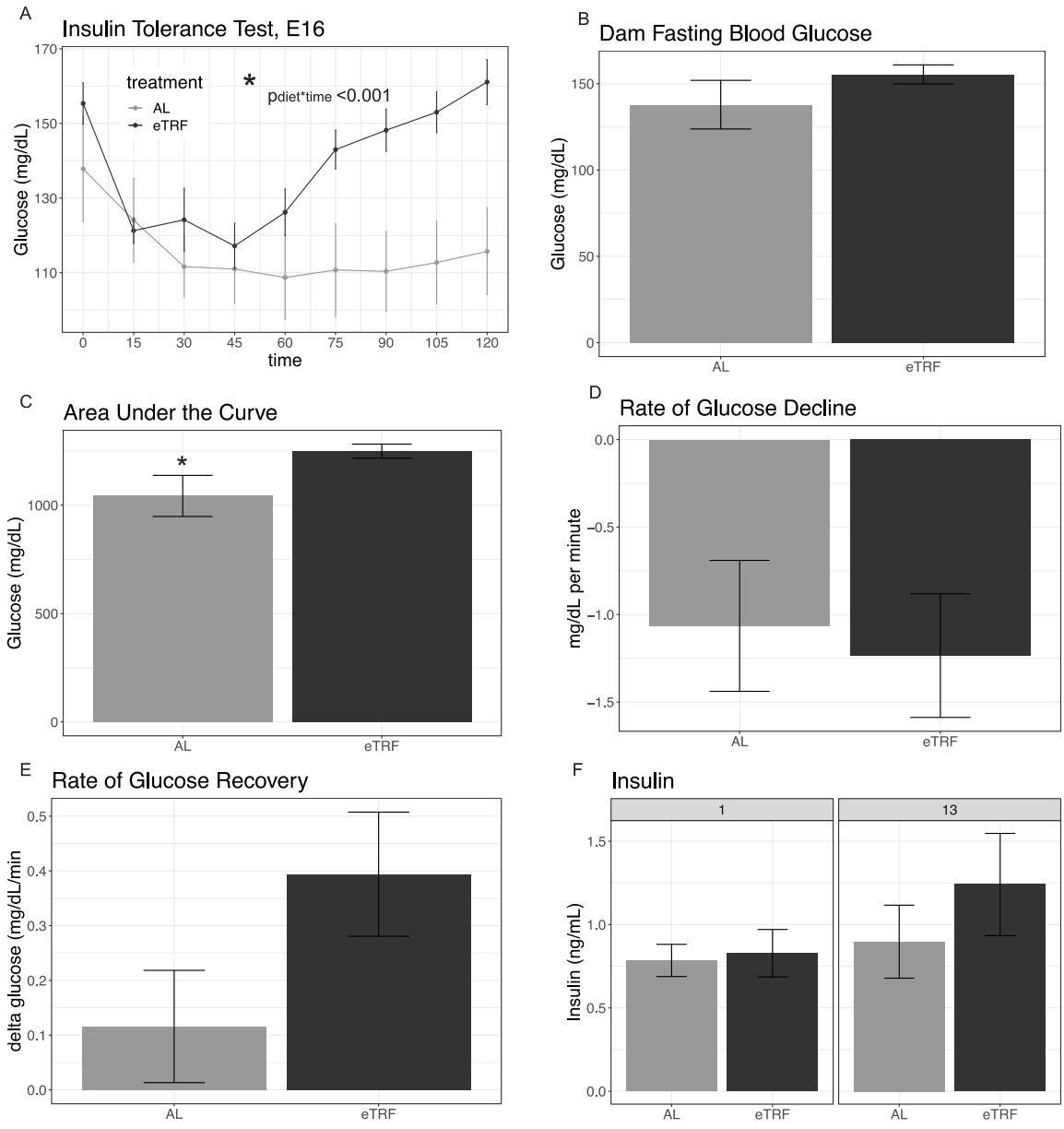

Figure 3: Rebound After Glucose Nadir is Increased in eTRF Dams During Late Gestation

A) Intraperitoneal Insulin Tolerance Test (ITT) in dams on E16.5 after fasting 6 hours (ZT2-ZT8). B) Fasting blood glucose (mg/dL) before insulin administration. C) Area Under the Curve of ITT. D)Rate of glucose decline from modeling exponential rate of drop in glucose from time 0 to 45 minutes. E) Rate of linear increase in glucose from 60-120 minutes during ITT. F) Serum insulin concentration at ZT1 and ZT13 in dams the day after ITT. *Indicates p-value of diet <0.05. If p-value is not for diet alone, it is labeled with interaction. (eTRF n=11, AL n=11)

2.4.3 Fecundity, Birthweights and Growth are Similar between AL and eTRF Pregnancies

We assessed fertility by evaluating the time spent in each stage of the estrus cycle, the latency to copulatory plug appearance after pairing, and rate of successful pairings. We found that the average number of days spent in each estrus stage was similar despite the dam undergoing eTRF (**Figure 4A**, p=0.70). The latency to copulatory plug was less than one day longer (2.29 vs 2.94, AL vs eTRF respectively) in eTRF dams (**Figure 4B**, p=0.39). When comparing mating pairs who were successful and had litters to those that did not, there was no difference in the rates of pregnancy between feeding regimens (not pictured, p=0.99). This suggests that despite fairly restrictive dietary regimen was adopted, fertility and estrus cycling was not disrupted by eTRF. To evaluate the effect of gestational eTRF on reproductive outcomes that are similarly observed and often impacted by gestational food restriction, we calculated litter size, average rates of survival during postnatal days, and weights of pups in the first 24 hours of life. We calculated gestational age for each dam as the average number of days between copulatory plug discovery and parturition. We found that eTRF and AL dams had similar gestational ages within anticipated normal range for mouse pregnancy (**Figure 4C**, p=0.20). There was a 28% reduction in the number of pups surviving to PND3 in eTRF litters (**Figure 4D**, p=0.039). Litter sizes were 15.3% smaller in eTRF dams: though this did not reach statistical significance (**Figure 4E**, p=0.072). Despite smaller litter sizes in eTRF dams, the average weight of each pup was similar between maternal dietary treatments (**Figure 4F**, p=0.13). This suggests that there may be adverse effects for dams fed eTRF, who may cannibalize their pups at greater rates, resulting in worse survival. We suspect that reduced survival may be due to maternal cannibalization, which is common in mice undergoing nutrient restriction. We suspect this because litters were monitored daily, and the majority of the pup loss occurred within 48 hours of discontinuation of

the eTRF regimen. As stated previously, it is evident that transitioning onto eTRF takes a number of days for animals to anticipate this feeding pattern and compensate with appropriate calorie intake. We therefore think it is likely that dams upon giving birth were anticipating continued restriction, and cannibalized pups more frequently than dams that were fed AL and did not experience restriction during pregnancy.

2.4.4 Pup growth to PND 21 is unchanged in offspring of eTRF dams

To assess if there was an early postnatal effect of gestational eTRF on pup body weights, we weighed pups at birth, and on postnatal days 3, 7, 14, and 21. Then, using linear mixed effect modeling with random effects of pup and maternal id and fixed effects of postnatal age, pup sex, and maternal dietary regiment, we found no differences in body weight in the first 21 days of life (**Figure 4G**, p=0.073). This suggests that despite the restrictive nature of this dietary exposure, there was no evidence of growth restriction during early life in either male or female pups.

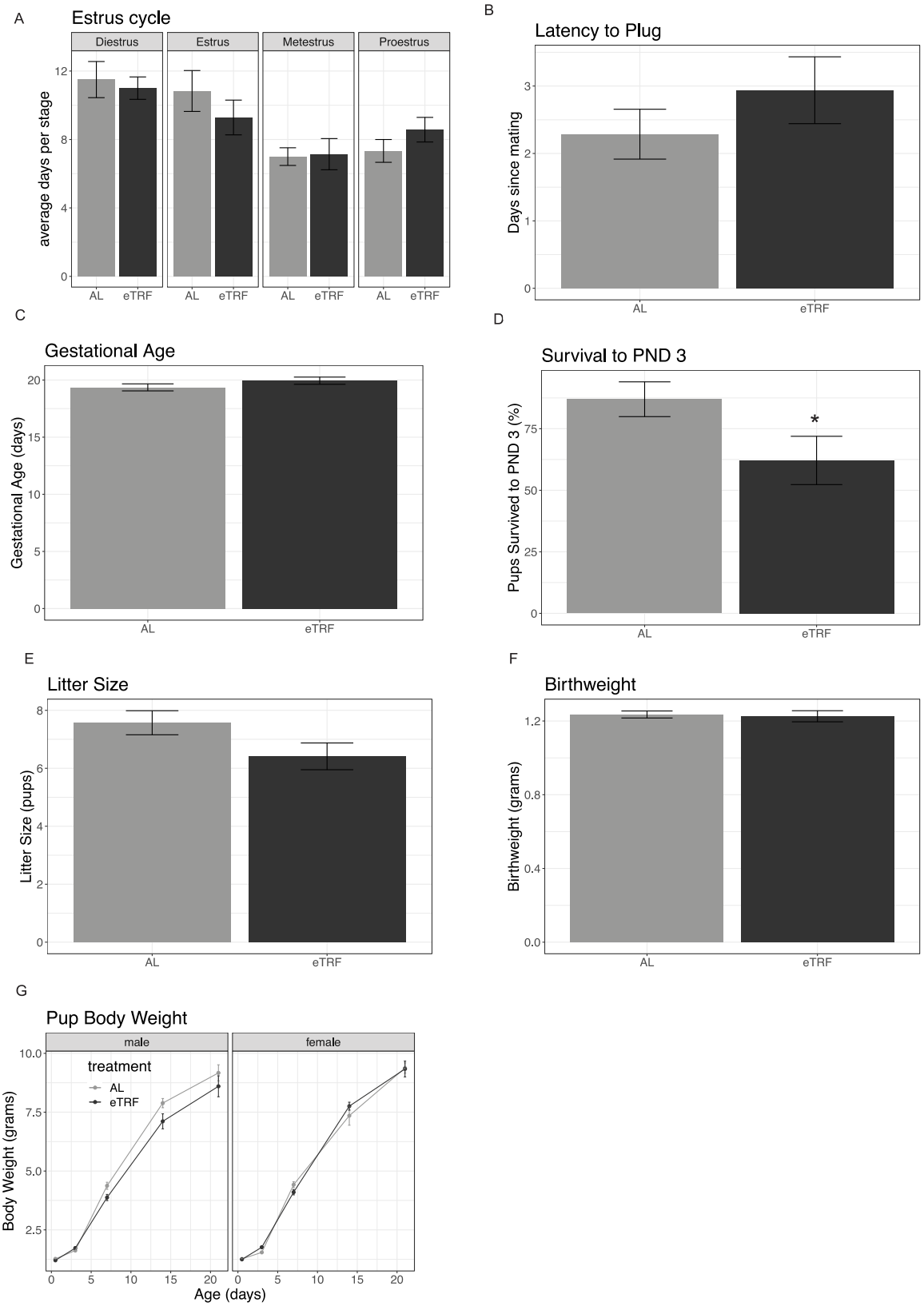
A
Estrus cycle
Diestrus
Estrus
Metestrus
Proestrus
average days per stage
AL
eTRF
B
Latency to Plug
Days since mating
C
Gestational Age
Gestational Age (days)
D
Survival to PND 3
Pups Survived to PND 3 (%)
*
E
Litter Size
Litter Size (pups)
F
Birthweight
Birthweight (grams)
G
Pup Body Weight
male
female
treatment
Body Weight (grams)
Age (days)

Figure 4: eTRF Does Not Affect Fertility but Reduces Pup Postnatal Survival and Litter Size

A) Total days spent in each stage of the estrus cycle before initiation of mating in dams (eTRF n=7, AL = 6). B) Time in days from pairing of male and female to copulatory plug appearance. C) Total days from copulatory plug appearance to birth of litter D) Percent of pups per litter who survived from birth until postnatal day 3. E) Number of pups per dam. F) Average weight of pup per litter on PND 0 (grams). G) Body weight of pups by maternal dietary treatment and pup sex (Male eTRF n=23, Male AL n= 33, Female eTRF n= 34, Female AL n= 27, eTRF pups culled before sexing at PND 3 n=53, AL pups culled before sexing at PND 3 n=40). Sample numbers for latency to plug, gestational age, 3-day survival, litter size, and birthweight are the same (eTRF n=16, AL n=14).

2.5 Discussion

To our knowledge, this is the first report of the effects of 6-hour eTRF on maternal food intake and insulin sensitivity. We find that despite the very narrow window of food availability, there are negligible effects on the dam. There is no evidence of reduced weight gain, calorie restriction, or fasting glucose values in dams who are exposed to eTRF. The effect on offspring also appears to be mild, but difficult to translate to humans; these included smaller litters and reduced pup survival rates. The more comparable indices of gestational age and birthweight remained unchanged in eTRF dams. This study contributes to our understanding of the implications of eTRF during pregnancy on gestating parents as previous studies, namely in rats, that evaluated time restricted feeding either exclude findings in the dam (Upadhyay et al., 2019, 2020) or find significant reduction in food intake and more modest gains in body weight during pregnancy (Alkhalefah et al., 2021a). However, the latter work was meant to model Ramadan fasting, and as such, food intake was outside of the nocturnal eating window in rodents. So results must be interpreted carefully, as they are also in the presence of chronodisruption during pregnancy, which is thought to cause adverse fetal outcomes (Salazar et al., 2018) including increased risk of miscarriage (Begtrup et al., 2019). Despite normal levels of insulin resistance of pregnancy being present, we found that eTRF during the perinatal period in dams resulted in no improvements in insulin sensitivity. We did find that there was more robust recovery in blood glucose after insulin-mediated glucose nadir in eTRF dams, which may suggest that there could be more gluconeogenesis and glycogenolysis in these dams. However, we were not able to evaluate this in the current study. We did not find evidence of reduced fasting blood glucose from gestational eTRF, which is in line with studies in humans that find no differences in glycemia (Hutchison et al., 2019; Jamshed et al., 2019; Sutton et al., 2018).

The similar pup weights in eTRF dams and continued normal body weights in both sexes after birth is in opposition to other studies where either male offspring of TRF dams weights at birth are reduced (Prates et al., 2022), or male and female fetal weights are smaller than AL counterparts (Alkhalefah et al., 2021a). Studies that find reduction in birth weights also find that dams are calorie restricted during pregnancy, resulting in reduced maternal weight gain, which could explain the lack of this phenotype in our current work.

Although the sample size for our estrus data is limited to one cohort, we found no impact on estrus cyclicity. One study that more rigorously evaluated fertility in response to TRF found it resulted in greater follicle counts, and increased number of estrus cycles compared to AL, in both chow and HFD females(Hua et al., 2020), although this was this in the context of pre-conceptional dietary changes (Hua et al., 2020). Where we found reductions in litter sizes, Hua and colleagues found that litter sizes were increased in high-fat diet fed dams who had previously been pregnant (Hua et al., 2020). Results from the current study must be interpreted with caution, as latency to plug and estrus staging are less robust assessments of fertility than is ovarian sectioning, continued monitoring of breeding success rates, and counting of ovarian follicles or corpus lutea. Of note, we are the first to report reduced survival rates in eTRF offspring. We suspect this may be related to cannibalization, which is common in the strain we used in the study (Brajon et al., 2021). Dams who were fed eTRF were likely anticipating continued food restriction after birth, as is evident about the adaptation period stated previously. Moreover, the reduction of survival in pups born in large litters is difficult to translate to human pregnancy.

Animal models are an imperfect proxy for human pregnancy for many reasons, but the evidence from this study may have translational value in pregnant human populations. Given the lack of

growth restriction in offspring in early life and absent maternal weight loss, nutrient restriction, and insulin dysmetabolism, this may warrant further evaluation in pregnant humans in controlled spaces. Other outcomes such as latency to plug, 3-day pup survival, and litter sizes are not as easily translated to human populations. However, as the majority of the deleterious effects that arise from this dietary treatment in the current study are difficult to translate, it would be presumptuous to say that negative effects are unlikely in human pregnancy.

As with any experimental model, this work has some limitations. One such limitation is the lack of molecular mechanisms investigated at the level of both dam and pup. We sought to evaluate the phenotype at a basic level in this study and as such were not able to investigate changes at the tissue level in either dams or pups. It is possible that despite the lack of overt metabolic differences in dams and body weights in pups, that there were more nuanced differences in metabolically active tissues or even within the central circadian clock. Future work should devote attention to these analyses. Another limitation is that fertility outcomes were only cursory in assessment, and in a reduced number of dams. As the effect of intermittent energy restriction on fertility and reproductive health is a concern in non-pregnant females, more stringent study and in greater numbers should be devoted to this question.

The current study has many strengths. First, the design was carefully considered to ensure handling stress was minimized and the timing of eating was in line with natural mouse rhythms. As such, results can be separated from effects from chronodisruption. This was repeated in 3 separate cohorts resulting in large samples sizes for both dams and pups. This suggests the observed phenotype is likely to be reproducible with other groups using a similar paradigm.

2.6 Conclusion

In summary, we find that eTRF feeding of dams during the perinatal period results in very few changes in the physiology of the dam, only a greater rebound in glucose after insulin challenge. There are similar rates of pregnancy and fecundity in dams fed eTRF. We find that pups born to eTRF dams are of similar size and grow in comparable ways to AL offspring. The deleterious effects noted are a reduction in litter size and in pup survival to postnatal day 3, although the reason for these reductions in not clear but could be due to maternal behavior. Further work must be done to scrutinize the safety of this practice and efficacy for ameliorating metabolic illness during pregnancy in higher risk populations.

Chapter 3 Gdf15 Knockout Does Not Impact Perinatal Body Weight or Neonatal Outcomes in Mice

3.1 Abstract

Growth differentiation factor-15 (GDF15) is known to increase in circulation during pregnancy and has been implicated in food intake, and weight loss, complications of pregnancy, and dysmetabolism. We used a *Gdf15* knockout mouse model to assess the role of *Gdf15* in body weight regulation and food intake during pregnancy. We found that *Gdf15*$^{-/-}$ dams consumed a similar amount of food and gained similar amounts of weight during the course of pregnancy as their *Gdf15*$^{+/+}$ counterparts. Insulin sensitivity on gestational day 16.5 was also comparable between dams. In the postnatal period, pups were of similar birthweight, litter size, and had similar survival rates in both genotypes. There were also no detectable differences in milk volume production, milk fat percentage, or in offspring postnatal body weights until day 14.5 of life. These data suggest that elimination of GDF15 is inessential for differences in food intake, weight gain, and dysmetabolism during pregnancy in a mouse model. Further research is warranted to evaluate the role of GDF15 in pregnancy, outside of its role in body weight and food intake regulation.

3.2 Introduction

Growth-like differentiation factor-15 (GDF15), a Transforming Growth Factor-ß superfamily member, placental derived growth factor, and cytokine, was discovered in 1997 and dubbed macrophage-inhibiting cytokine-1 (MIC-1) (Bootcov et al., 1997). Circulating levels of

Gdf15 in adults vary based on sex, age, disease status, and physiological state. In a large sample from Scotland, they found that levels of circulating GDF15 increase with age in both men and women and tended to be higher in those who had cardiovascular disease, cancer, or diabetes (Welsh et al., 2022). GDF15 increases in response to many stressors including cardiac injury (Kempf et al., 2006), cachexia of cancer (Suriben et al., 2020), mitochondrial stress (Ost et al., 2020), intense exercise (Klein et al., 2021), and most relevant to this work, during pregnancy (Andersson-Hall et al., 2021; Chen et al., 2016; Marjono et al., 2003; Sugulle et al., 2009; Welsh et al., 2022).

Animal work with knockout or knockdown models have highlighted the role of GDF15 in body weight regulation (Hsu et al., 2017, p. 15), appetite (Tsai et al., 2019), and emesis (Borner et al., 2020). These models show that GDF15 acts through the GFRAL receptor found in the area postrema of the brain (Mullican et al., 2017; Yang et al., 2017). The effect of GDF15 antagonism through antibodies or knockout on food intake depends on the diet the rodents are fed. When consuming a high fat, high sucrose diet food intake and body weight increases (Tran et al., 2018; Tsai et al., 2019); however, when consuming a chow diet, they remain similar to wildtype animals (Tran et al., 2018). Although the studies of food intake and body weight regulation are inconsistent in the effect of the knockout of GDF15 signaling, the relationship is clear when examining the effect of supraphysiological levels of GDF15.

Pharmacologic administration or overexpression of *Gdf15* induces weight loss through reductions in food intake (Hsu et al., 2017; Mullican et al., 2017; Yang et al., 2017). It also results in nausea-like behavior in mice and emesis in shrews (Borner et al., 2020), reduced fat preference (Frikke-Schmidt et al., 2019), and decreased meal size (Emmerson et al., 2017). Inconsistencies in the magnitude of the reduction in food intake while on a high fat diet may be

related to reduced fat preferences; as models employing a 60% high fat diet found reductions in food intake (Mullican et al., 2017) while another using 45% high fat diet did not see reduced food intake (Yang et al., 2017). However as such, evaluating GDF15 for its capacity to ameliorate metabolic illness is currently being explored.

During pregnancy GDF15 increases across gestation and reaches its highest levels during the third trimester of pregnancy (Andersson-Hall et al., 2021; Chen et al., 2016; Moore et al., 2000; Sugulle et al., 2009). It is heavily expressed in the placental trophoblasts, and secreted into parental circulation, and is present in the amniotic fluid (Moore et al., 2000). In spite of these pregnancy-related increases, details on the functional role of GDF15 in pregnancy are just emerging. GDF15 has been linked to several complications and conditions that can arise in pregnancy. Lower levels of GDF15 during early pregnancy were present in patients who later suffered a miscarriage (Tong et al., 2004). GDF15 levels have also been linked to gestational weight gain, as elevations were negatively associated with cumulative gestational weight gain (P. Wang et al., 2020). Petry and colleagues found pre-pregnancy BMI was inversely related to GDF15 levels during pregnancy (Petry et al., 2018). Different levels of GDF15 are secreted in concert with complications of pregnancy. In several cases, the epidemiological data is in conflict. For example, pre-eclampsia, a life-threatening complication involving critically high blood pressure and protein loss in urine, has been found to be associated with reductions (Chen et al., 2016), increases (Sugulle et al., 2009; L. Wang & Yang, 2022), and no changes (Marjono et al., 2003) in GDF15 in serum compared to non-preeclamptic, normotensive parents. Similarly, some studies find that GDF15 is higher in pregnancies complicated by gestational diabetes (GDM) (Yakut et al., 2021), or type 2 diabetes (T2DM) (Sugulle et al., 2009) while others find it is only significantly increased in pregnancies that are complicated by T1DM but not T2DM or GDM

(Jacobsen et al., 2022). GWAS have indicated that *GDF15* variants in humans are associated with hyperemesis gravidarum, an extreme form of nausea and vomiting of pregnancy (Fejzo et al., 2018, 2019). Given the sometimes-conflicting human data, we sought to understand more about the effects of *Gdf15* loss of function during the course of murine pregnancy, including effects on weight gain, food intake, insulin sensitivity, and neonatal outcomes.

3.3 Materials and Methods

3.3.1 Animal Husbandry

Animals from both studies described below were housed in a temperature and humidity-controlled facility with a 12-hour light: dark cycle, with lights on being zeitgeber time (ZT) 0 and lights off being ZT 12. All protocols were approved by the Institutional Animal Care and Use Committee of the University of Michigan.

Insulin Resistance of Pregnancy Study

Virgin female C57BL/6J (RRID: IMSR_JAX:000664) mice were ordered from The Jackson Laboratories. Mice were allowed to acclimatize for two weeks to the temperature and humidity-controlled facility with free access to water and laboratory chow (CD, Picolab Laboratory Rodent diet 5L0D; 5% of Calories from fat, 24% from protein, 71% from carbohydrates). After acclimatizing, females were randomized into three groups, non-pregnant females (n=7), pregnant females (n=7), and pregnant females exposed to dexamethasone (1mg/kg/day Sigma-Aldrich catalog #D2915-100MG) in drinking water (n=7). One week after experimental treatment began, males were introduced to the dam's cage and allowed to remain until gestational day 19. Body weight and food intake measurements occurred weekly from randomization until birth.

GDF15 Study

Male and female *Gdf15* null animals are described elsewhere (Frikke-Schmidt et al., 2019). Null animals were generated using CRISPR Cas-9 deletion of Exon 2 of *Gdf15*. Exon 2 (translational start site), which we ablated, is present in every known *Gdf15* transcript. We chose to study $Gdf15^{+/+}$ mated pairs compared to $Gdf15^{-/-}$ pairs because comparing littermates of $Gdf15^{+/-}$ pairs would result in potential placental contributions to GDF15 in dam serum as the fetus provides a substantial amount of the placenta. To limit genetic drift, all homozygous parents were direct offspring of heterozygous crosses. We combined homozygous pairs, resulting in homozygous genotype progeny and placentae. Adult virgin female mice ($Gdf15^{-/-}$n=8 , $Gdf15^{+/+}$n=6), between 45 and 119 days old (mean 82 days), were singly housed with *ad libitum* access to water and a CD. Once single-housed, weekly food intake and body weight measurements began and continued throughout the experiment. After one week of food and body weight monitoring, males of like-genotype for *Gdf15* were introduced into the dam's cage. Males were allowed to remain in the breeding cage until a copulatory plug was identified, indicating pregnancy (E0.5). Body weight and food intake measurements continued weekly through gestation and postnatal day 14.5. Their resultant offspring and their placentae were homozygous $Gdf15^{+/+}$ $Gdf15^{-/-}$ and were studied until postnatal day 14 (PND14).

3.3.2 Genotyping

At 14 days of age, a small section of the tail was collected and digested in 100uL of lysis buffer (10 mM Tris pH 8.0, 150 mM NaCl, 10 mM EDTA, 0.1% SDS and 1 mg/ml proteinase K) at 55°C for 4 hours. Digested DNA samples were amplified with DreamTaq Green to generate PCR product (ThermoFisher Scientific, Catalog #K1081). Genotyping by PCR was conducted with 2 forward and one reverse primer sets (forward 1: 5' GAT TCC CGC CCG AAT TAG C 3',

forward 2: 5' CCG AAT TAG CCT GGT CAC CC 3', Reverse: 5’ ATC CGT CCT ACT CTG GCT AAG 3'). Initiation of PCR was at 95 °C for 3 minutes, followed by 38 cycles of denaturation (95°C for 30 seconds), annealing (60°C for 40 seconds), and elongation (72°C for 1 minute), and a final amplification step at 72°C for 5 minutes. PCR product resulted in 2 visible bands, one at 200bp *Gdf15*$^{-/-}$ and another at 600bp *Gdf15*$^{+/+}$. Mice with both bands were considered *Gdf15*$^{+/-}$. Dam genotype was secondarily confirmed via maternal serum ELISA.

3.3.3 Intraperitoneal Insulin Tolerance Tests

On E16.5, dams underwent intraperitoneal insulin tolerance testing (Bridges et al., 2022). Dams were placed in clean cage without access to food but with ad libitum access to water at ZT 2. Dams were fasted for 6 hours (ZT2-ZT8). Baseline blood glucose was assessed using a tail clip and a handheld glucometer (OneTouch Ultra). After initial blood glucose measurement, an intraperitoneal injection of insulin was administered (Humulin, u-100; 0.75U/kg lean mass). Blood glucose was measured in 15-minute intervals for 2 hours. Area under the curve was calculated by taking the sum of all glucose values for each animal and averaging by genotype. We then calculated the rate of initial drop in blood glucose after insulin administration. We limited data to the first 45 minutes after injection and modeling the exponential rate of decay in glucose for each animal as a slope. This rate was then averaged by genotype.

24 hours after ITT, we collected two fed blood samples: at ZT1 and ZT13. Dams were lightly anesthetized via inhaled isoflurane and whole blood was collected by retroorbital bleed in a heparinized capillary tube. Blood was allowed to clot on ice for 20 minutes then was spun down in a cold centrifuge (4°C, Eppendorf microcentrifuge, model 5415R) for 20 minutes at 2000 g. Serum was pipetted off after centrifugation and stored at -80°C until used for analysis.

3.3.4 Serum GDF15 Quantification

Serum GDF15 determinations were completed using maternal serum collected 24 hours after insulin tolerance tests on E16.5 in the Gdf15 and maternal comparator C57BL/6J studies. Gdf15 levels were determined via ELISA according to manufacturer guidelines (R&D system, catalog # MGD150).

3.3.5 Offspring Assessments

Pups were counted and body weights were recorded within 24 hours of birth, postnatal day (PND 0.5). Latency to copulatory plug was defined as the number of days between the introduction of the male and appearance of a copulatory plug. Gestational age was determined as difference between birth dates and dates of appearance of copulatory plug. At PND 3.5, litter sizes were culled to 2 male and 2 female pups, to standardize amount of nutrition provided to each pup. Survival of pups to PND 3.5 was assessed by comparing the number of pups present at PND 3.5 to the number present on PND 0.5 and is expressed as a percentage. Body weight was assessed for each pup on PND 0.5, 3.5, 7.5, 10.5, and 14.5. Pups were euthanized by decapitation on the 2 hours before milk collection began (PND 14.5-17.5).

3.3.6 Weigh-Suckle-Weigh, Milk Volume Production

On postnatal day 10.5, we assessed milk volume production by the weigh-suckle-weigh method (Boston et al., 2001; El Habbal et al., 2021). Dams were weighed using an analytical scale to the nearest 10 mg and placed in a clean cage with free access to food and water. Pups were then weighed in aggregate and placed in a clean cage on top of a heating pad without access to food or water. Dam and pups remained separated for 2 hours. After 2 hours, weight measurements were repeated, and pups were reintroduced to the dam's cage where they remained for 1 hour. After one hour, the final weights were taken for both dams and pups in aggregate. The volume of

milk produced is expressed as the average weight lost by each dam after 1 hour of nursing divided by the number of pups in the litter.

3.3.7 Milk Collection

Milk collection took place on PND 14.5-17.5. Pups were separated from dams and sacrificed 2 hours before milk collection began. Dams were allowed to *ad libitum* access to food and water in a clean cage during that time. Dams were anesthetized with intramuscular injection of Ketamine/Xylazine (0.13g/kg body weight) into forelimb muscle. Once the dam was fully anesthetized, an oxytocin injection (2U per dam) was given in the forelimb muscle to begin let-down. Milk was collected with a pipette after manually expressing milk from nipples and stored in a 1.5 mL Eppendorf tube. Following milk collection, dams were immediately euthanized via isoflurane inhalation and cervical dislocation.

3.3.8 Statistical Analyses

Data were analyzed in R Studio version 4.2.0 (R Core Team, 2021) and are presented as mean ± standard error. Longitudinal analyses, such as food intake, body composition, and insulin tolerance testing were assessed using linear mixed effects modeling with R package lme4 (Bates et al., 2015) with random slopes and intercepts for the dam and pup with respect to time and fixed effects of genotype, age, and sex. Models for offspring body weight were assessed for interaction of sex with time and genotype but neither were significant, so sex remained a fixed effect. Pairwise values were assessed for normality by the Shapiro-Wilk test and equivalence of variance by Levene's test. Variables that were not normally distributed or of equivalent variance underwent non-parametric testing via Mann-Whitney U test. Those that were normally

distributed and of equivalent variance were assessed via Student's *t*-test as noted in the figure legends. For this study, p-values <0.05 were considered statistically significant.

3.4 Results

3.4.1 GDF15 is Elevated During Pregnancy in Mice

Previous work has shown that pregnancy in mice results in maternal insulin resistance (Ladyman et al., 2018; Musial et al., 2016), so we sought to understand if GDF15 levels related to either pregnancy or a model of excess insulin resistance in pregnancy. We tested compared age-matched pregnant and non-pregnant females using an intraperitoneal insulin tolerance test on day 16 of pregnancy (**Figure 6A**). Consistent with prior work, we found that pregnant dams responded less to insulin than non-pregnant females, though this did not reach statistical significance (**Figure 6A**, p=0.23 via mixed linear models). Inconsistent to Musial and colleagues, there were no significant differences in their fasting blood glucose (**Figure 6B**, p=0.020). We found that GDF15 is 49% (54 ±18.8 pg/dL) elevated in pregnant animals compared to non-pregnant mice (**Figure 6C,** p=0.007). As expected, body weights in pregnant females were 1.57± 0.55 grams heavier than non-pregnant females (**Supplemental Figure 2A**, p=0.0039).

To enhance insulin resistance in pregnancy, we leveraged prior work from our lab demonstrating that administration of the glucocorticoid dexamethasone (dex) in their drinking water impairs insulin sensitivity in non-pregnant mice (Gunder et al., 2020; Harvey et al., 2018). We treated dams with 1 mg/kg dexamethasone one week before mating and it continued for the length of the pregnancy. We compared dexamethasone-treated dams to age-matched pregnant dams who were provided normal drinking water. We found that dexamethasone dams did not respond to insulin compared to pregnant dams with plain drinking water (**Figure 6D**,

$p_{dex*time}$=0.02 via linear mixed effect models). Dexamethasone-treated dams had 33% lower fasting blood glucose (**Figure 6E**, p_{dex}=0.007) consistent with our findings in non-pregnant mice. GDF15 levels were not further increased by dexamethasone administration in pregnant dams (**Figure 6F**, p=0.11). Body weights in pregnant dams were 2.77±0.58 grams lighter in those treated with dex compared to water dams (**Supplementary Figure 2B**, p<0.0001).We were interested to see how pregnancy and dexamethasone administration in pregnancy related to GDF15 levels in these mice. Based on these data we conclude that while GDF15 is increased during pregnancy, it is not elevated in insulin resistant dexamethasone treated dams.

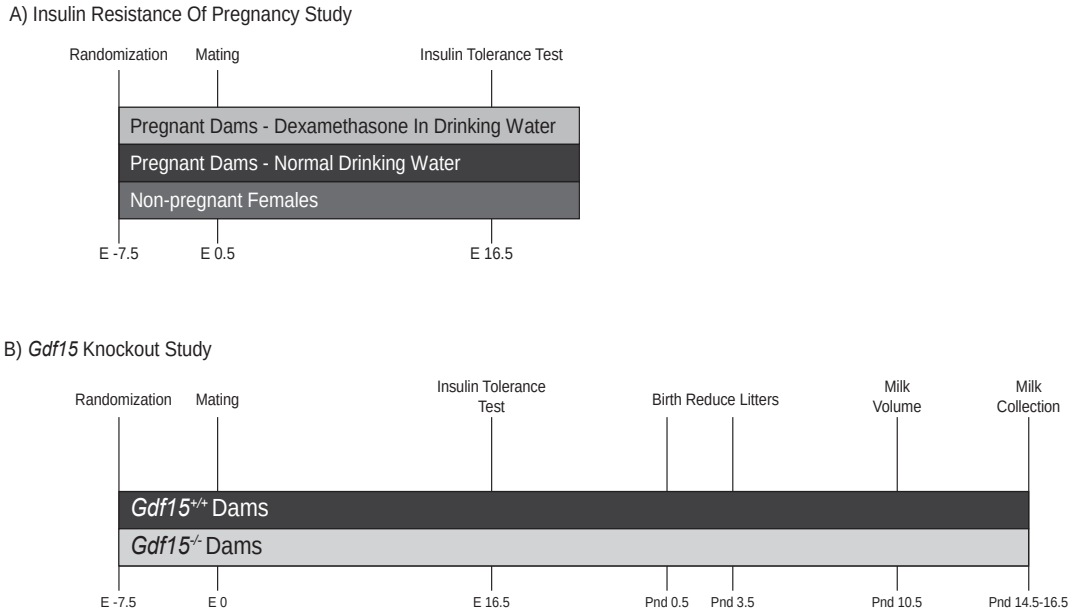

Figure 5: Gdf15 Knockout in Mouse Pregnancy

A) Insulin resistance of pregnancy study, comparing age-matched females in 3 groups; non-pregnant females (n=7), pregnant females given plain drinking water (n=7), pregnant females given 1.0 mg/kg dexamethasone in drinking water (n=7). B) *Gdf15* Knockout study in pregnancy. $Gdf15^{+/+}$ females (n=6) were mated with $Gdf15^{+/+}$ males. $Gdf15^{-/-}$ females (n=7) were mated with $Gdf15^{-/-}$ males. Food intake and body weight was measured weekly from one week before mating until 14-16 days after pups were born.

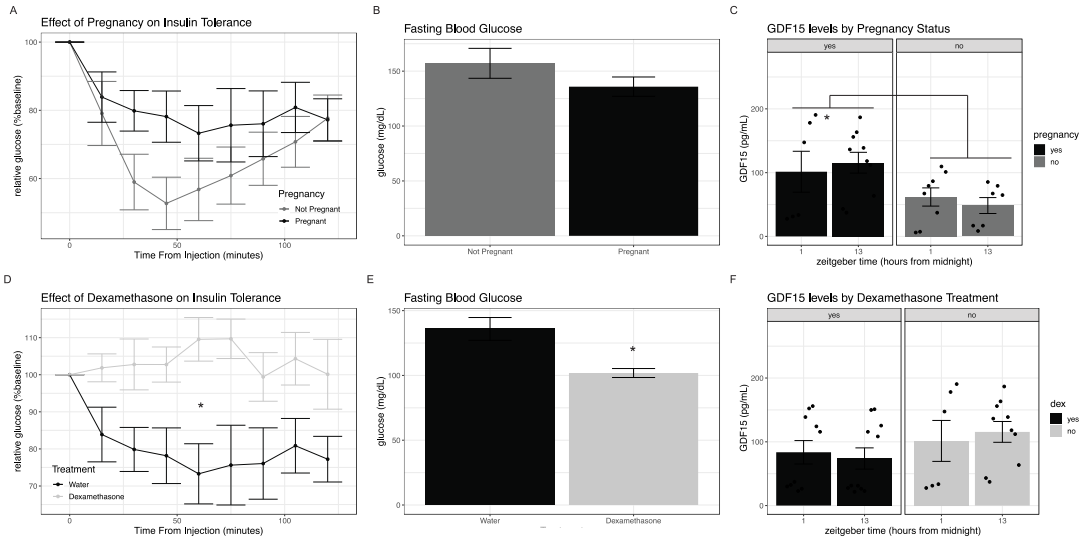

Figure 6: Insulin Resistance of Pregnancy Co-occurs with Elevations in GDF15

A) Intraperitoneal insulin tolerance testing on E16.5 in pregnant mice given plain water and age-matched non-pregnant females. Values are relative to fasting blood glucose and were assessed using a linear mixed effect model. B) Fasting blood glucose values in pregnant dams given water and non-pregnant females, assessed using student's T test. C) GDF15 levels at ZT1 in pregnant and non-pregnant females, assessed as paired t tests. D) Intraperitoneal insulin tolerance testing on E16.5 in pregnant dams given water or 1mg/kg dexamethasone in drinking water, assessed via linear mixed effect modeling. Values are relative to fasting blood glucose levels. E) Fasting blood glucose values in pregnant dams given plain drinking water or dexamethasone in drinking water, assessed via student's t test. F) GDF15 ELISA evaluating serum levels at ZT1 and ZT13 in pregnant dams given plain drinking water, pregnant dams given dexamethasone in drinking water, assessed as paired t tests.

3.4.2 Gdf15$^{-/-}$ Dams Have Normal Weight Gain and Modestly Reduced Food Intake During Pregnancy and Lactation

To evaluate the role of *Gdf15* ablation in maternal food intake and body weight accretion during mouse pregnancy, we mated *Gdf15*$^{+/+}$ dams with *Gdf15*$^{+/+}$ males and compared them to *Gdf15*$^{-/-}$ mated pairs (**Figure 5B**). Dam body weight and food intake were measured weekly, beginning one week before mating and continued until pups reached 14 days of age (PND14.5).

Gdf15$^{-/-}$ dams consumed similar cumulative kilocalories during the prenatal period (**Figure 7A**, p=0.52). They also had a similar weight change when compared to *Gdf15*$^{+/+}$ dams during the course of pregnancy (**Figure 7B**, p=0.99). Both strains consumed similar calories weekly (**Figure 7E**, $p_{genotype}$=0.23). Both genotypes had a rapid increase in food intake in the final trimester of pregnancy, with smaller increases in the *Gdf15*$^{-/-}$ dams. In the postnatal period, cumulative food intake was similar between genotypes (**Figure 7C**, p=0.94). *Gdf15*$^{-/-}$ dams had 54% lower postnatal weight loss than *Gdf15*$^{+/+}$ dams, but this failed to reach statistical significance (**Figure 7D**, p=0.20; **Figure 7F**). This suggests that *Gdf15* is not a major determinant of either body weight or food intake during first pregnancy in the mouse.

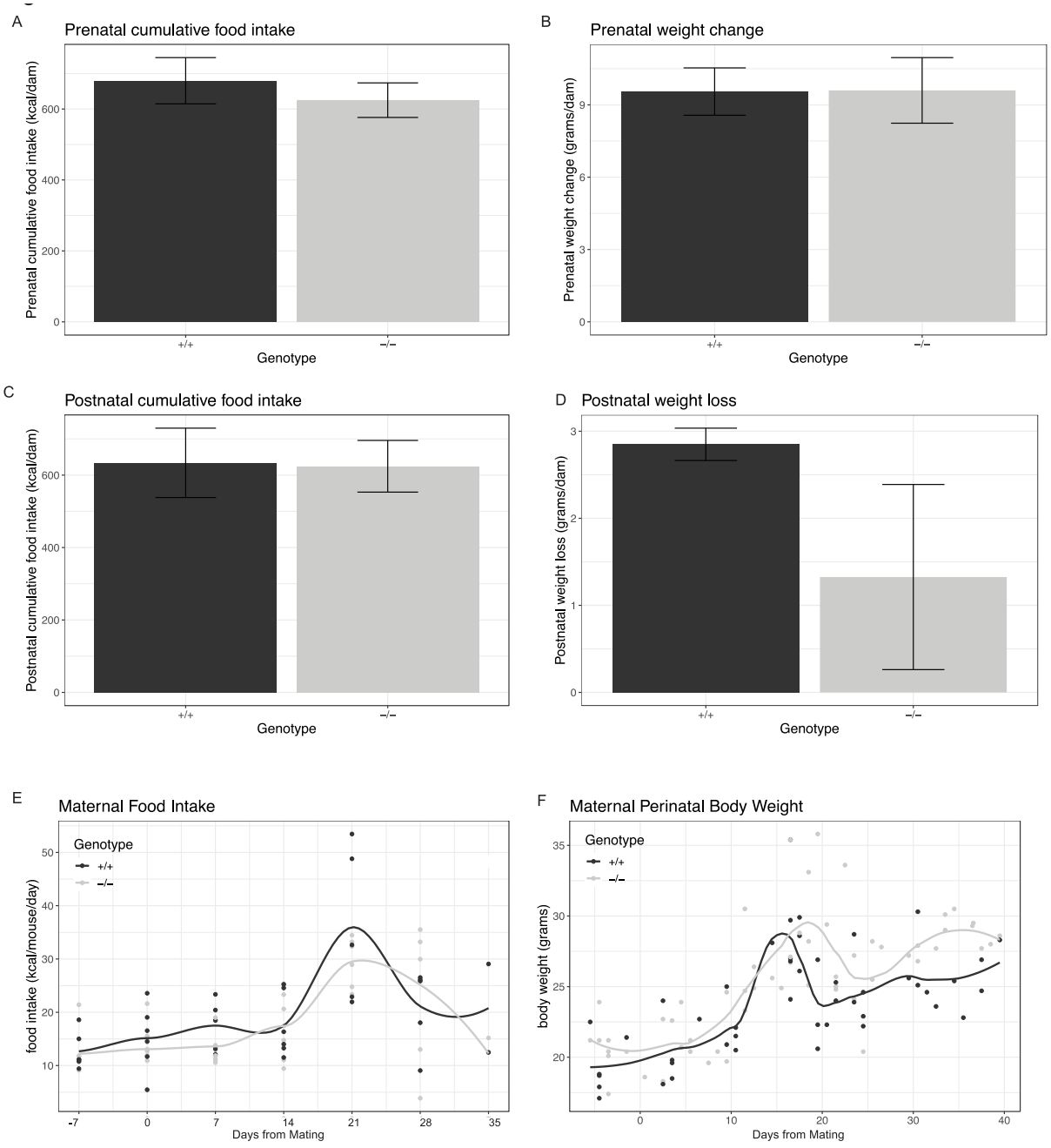

Figure 7: Gdf15 Knockout Does Not Impact Food Intake or Body Weight During Mouse Pregnancy

A) Cumulative food intake during the prenatal period (pre-mating through final measurement before birth), assessed via student's *t* test. B) Weight gained during prenatal period, assessed via student's t test. C)Postnatal cumulative food intake (after birth of pups-end of experiment), assessed via student's t test. D) Weight lost in the postnatal period, assessed via students' t test. E) Plot of the weekly food intake in both genotypes from 1 week before mating until end of the experiment. F) Plot of maternal body weight throughout the experimental period.

3.4.3 Gdf15$^{-/-}$ Dams Have Normal Insulin Tolerance During Pregnancy

On Gestational day 16.5, we conducted an intraperitoneal insulin tolerance test to assess the effect of *Gdf15* ablation on maternal insulin sensitivity during pregnancy (**Figure 8A**). Fasting blood glucose was slightly but insignificantly lower in *Gdf15*$^{-/-}$ dams compared to *Gdf15*$^{+/+}$ dams (**Figure 8B**, p = 0.20). Overall, linear mixed effect modeling revealed no effect of the genotype ($p_{genotype}$ = 0.71). This was confirmed by determining the area under the ITT curve, again showing similar responses (**Figure 8C**, p=0.74). Often an informative measure of the insulin response is the initial rate of drop of blood glucose. The initial rate of glucose decline was 9.3% less in *Gdf15*$^{-/-}$ dams compared to *Gdf15*$^{+/+}$ dams but again, did not reach statistical significance (**Figure 8D**, p=0.082). These data suggest that ablation of *Gdf15* is not sufficient to substantially affect insulin sensitivity in the pregnant mouse.

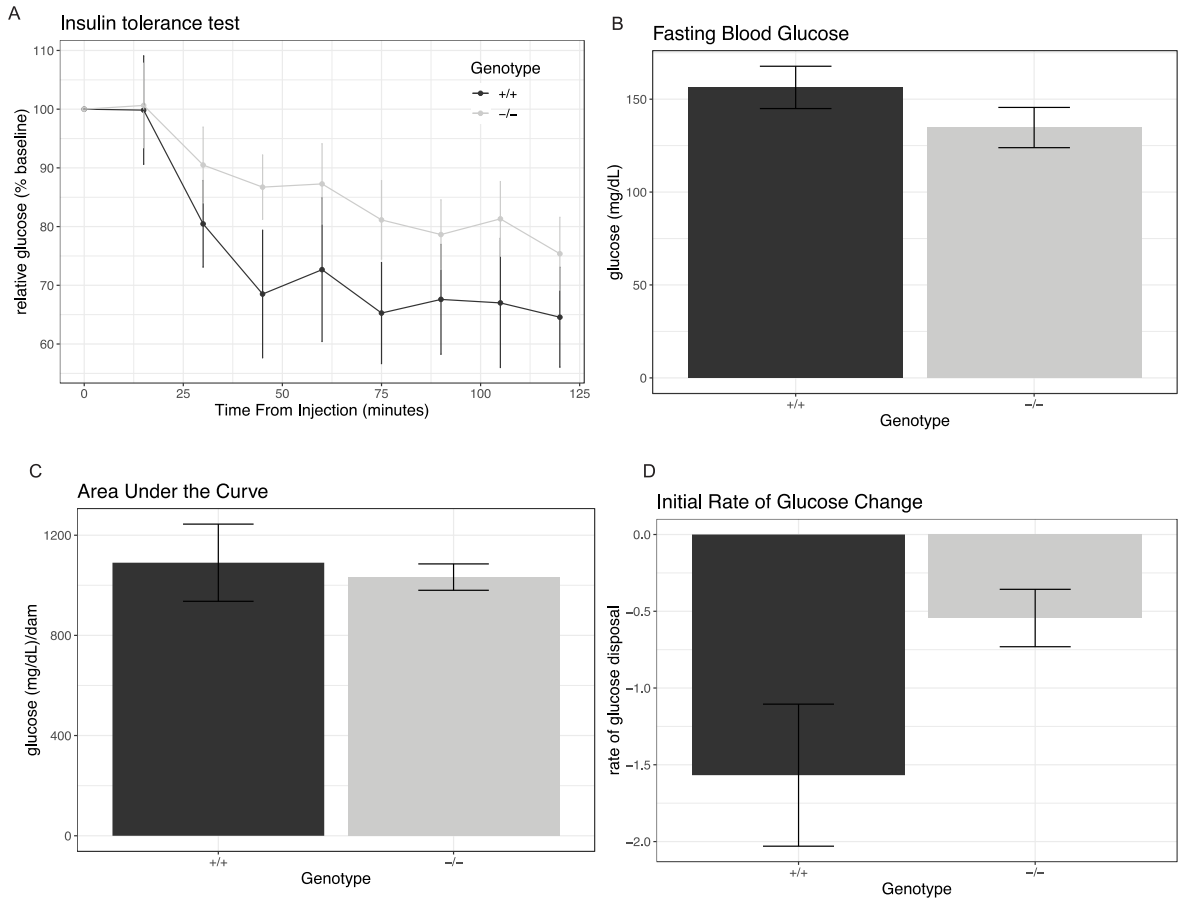

Figure 8: Gdf15 Knockout Has No Effect on Gestational Insulin Tolerance

A) Intraperitoneal insulin tolerance test in *Gdf15*$^{+/+}$ and *Gdf15*$^{-/-}$ dams at E16.5. Values are relative to fasting blood glucose levels. Assessed via linear mixed effects modeling. B) Fasting Blood glucose levels in dams, assessed by students t test. C) Area under the curve defined as sum of all glucose values for each animal, assessed by student's t test. D) Rate of drop in blood glucose in the first hour of the insulin tolerance test, assessed by student's *t* test.

3.4.4 Gdf15$^{-/-}$ Dams Have Normal Fertility, Gestational Age, Postnatal Survival, and Pup Birth Weights

To understand the role of *Gdf15* knockout on pregnancy and early post-partum outcomes in the pups, we calculated latency to plug, gestational age and measured litter size, birth weight, and 3-day survival in all mated dams. Pups from *Gdf15*$^{-/-}$ dams were 3.4% smaller than those from *Gdf1*$^{+/+}$ dams (**Figure 9C**, p=0.05). The latency to copulatory plug was similar between genotypes, averaging 3 days (**Figure 9A**, p=0.74). Gestational age was similar between genotypes, averaging 20 days (**Figure 9B**, p=0.76). The total number of pups born in a litter was 27% greater in *Gdf15*$^{-/-}$ dams (1.6 pups greater on average) compared to *Gdf1*$^{+/+}$ dams (**Figure 9D**, p=0.15). When comparing litter size, counting only pups who were born alive, that difference was only 7.8% larger (**Figure 9E**, p=0.70, or 0.46 pups/litter greater on average). The total pups who were born alive that lived to postnatal day 3 was variable within genotypes, resulting in 91.7% survival for *Gdf15*$^{+/+}$ dams and 90% for *Gdf15*$^{-/-}$ dams which did not reach statistical significance (**Figure 9F**, p=0.99). Together these data show that aside from modest decreases in birthweights, *Gdf15*$^{-/-}$ mice are similarly fertile, and carry pregnancies to a similar effectiveness as their wild-type counterparts.

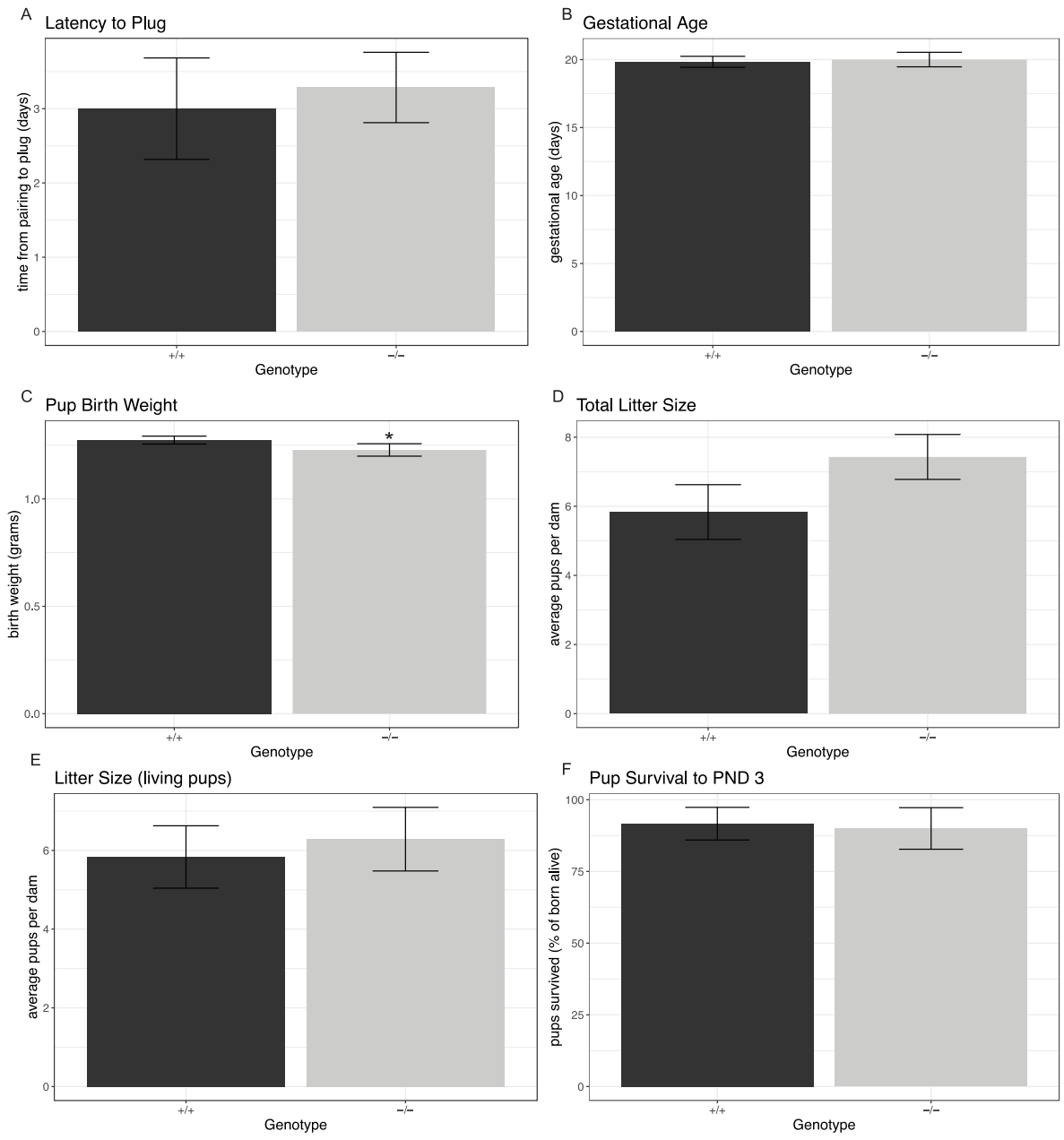

Figure 9: Birth Weight is Reduced in *Gdf15* Knockout Pregnancies

A) Latency to copulatory plug (time from introduction of male into cage until copulatory plug is discovered), assessed via student's t test. B)Gestational age in days, calculated as the number of

days from appearance of copulatory plug until birth of the litter. Assessed via Mann-Whitney test. C)Average birth weight of pups, calculated as the average birth weight for each dam, then averaged by genotype. Assessed by student's t test. D)Total litter size (including those who were dead), assessed via student's t test. E)Number of pups born per litter that were alive, assessed via student's t test. F) Percentage of pups in each litter who were dead by postnatal day 3.5, assessed by Mann Whitney test.

3.4.5 Gdf15$^{-/-}$ Dams Have No Differences in Milk Production or Milkfat Percentage

To determine the effect of *Gdf15* knockout during in pregnancy on lactation in the postnatal period, we conducted a milk volume assessment at postnatal day 10. We found no differences between *Gdf15*$^{+/+}$*and Gdf15*$^{-/-}$ dams in the volume of milk produced at peak lactation. The amount of weight lost by dams after nursing (**Figure 10A**, p=0.7) or and weight gained by pups during nursing (**Figure 10B**, p=0.7) was similar between genotypes, though highly variable between dams. Next, we evaluated whether the major macronutrient in milk, fat, was changed by *Gdf15* knockout. To do this, we collected whole milk between PND 14-17 and evaluated milk fat percentage. We found that milk fat percentage was similar between strains (**Figure 10C**, p=0.93). Despite reductions in maternal levels of *Gdf15* in the *Gdf15*$^{-/-}$ dams during pregnancy, mammary gland development, and lactation there is no apparent impact lactational volume milk fat content.

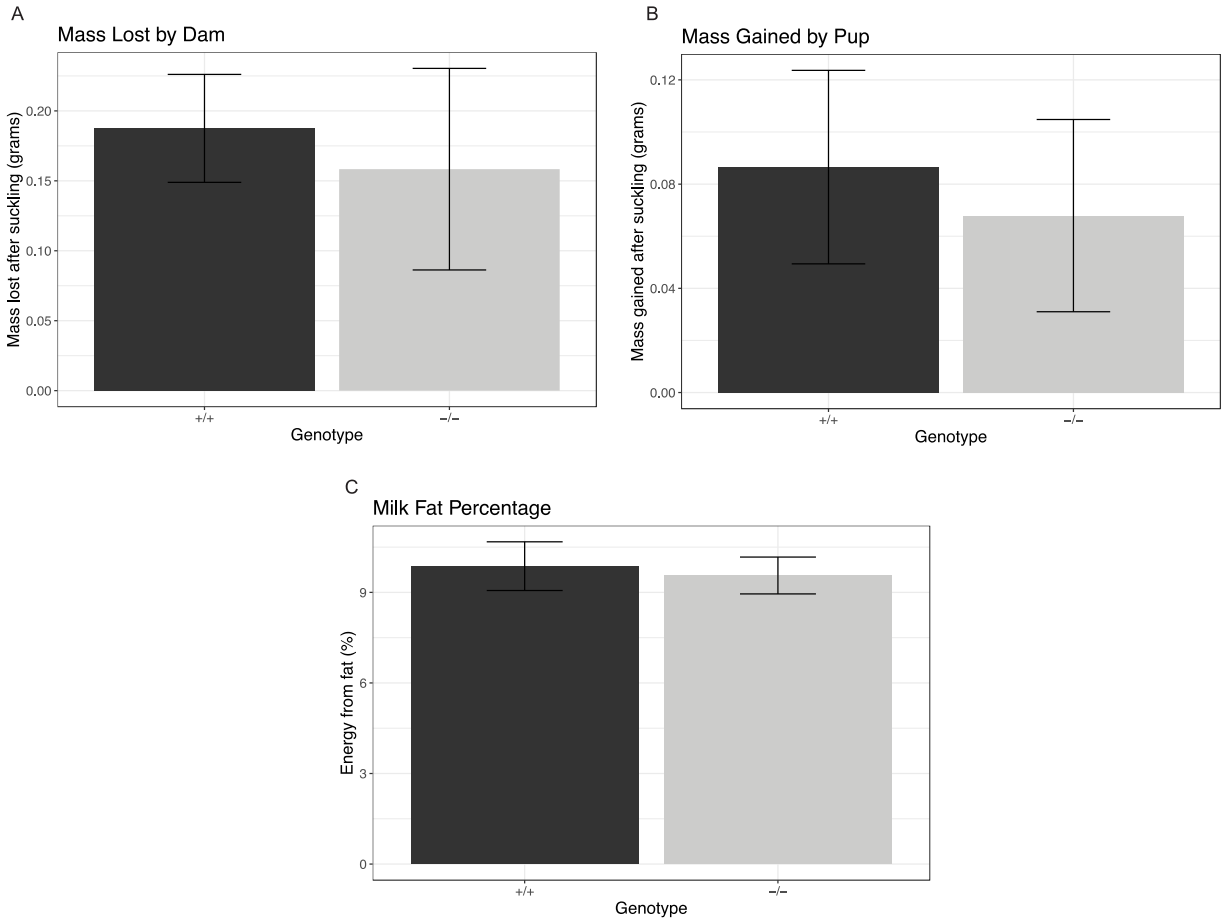

Figure 10: Milk Volume and Milkfat Percentage are Not Changed in *Gdf15* Knockout Dams

A) Total mass (in grams) lost by dam during the suckling period of the weigh-suckle-weigh test on PND10.5, assessed by student's t test. B)Total mass (in grams) gained cumulatively between all pups in the litter during suckling period during weigh-suckle-weigh test, assessed by Mann Whitney test. C)Percentage of fat found in mouse milk collected PND 14-16.5, assessed by student's t test.

3.4.6 Gdf15$^{-/-}$ Pups Accrete Body Mass at Similar Rates Compared to Gdf15$^{+/+}$ Pups

To assess the effect of *Gdf15* knockout during pregnancy and lactation on early pup postnatal growth, we weighed male and female offspring of *Gdf15*$^{+/+}$ and *Gdf15*$^{-/-}$ dams on PND 0.5, 3.5, 7.5, and 14.5. We used linear mixed effect modeling which detected no differences in body weight between birth and 14 days of age in *Gdf15*$^{+/+}$ and *Gdf15*$^{-/-}$ pups (**Figure 11A**, $p_{genotype}$=0.81 after adjusting for sex differences). There was also no statistically significant modifying effect of sex on body weight from birth to PND 14.5 (p_{sex}=0.16). Therefore, consistent with similar milk production and composition, we did not detect any effects of GDF15 ablation on perinatal growth.

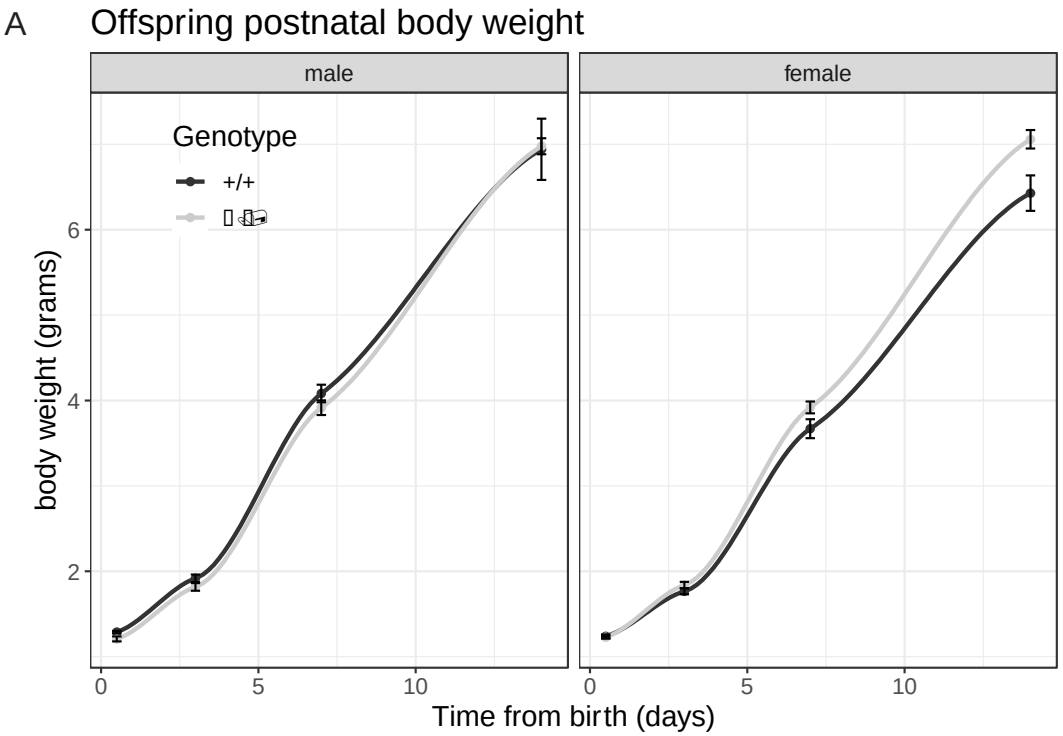

Figure 11: Offspring Postnatal Growth is Normal in *Gdf15* Knockout Litters

A) Postnatal bodyweight measurements from birth through PND14.5 in male and female pups, assessed via linear mixed effect models.

3.5 Discussion

GDF15 has recently been tied to several complications of pregnancy in addition to its better understood role in signaling somatic stress throughout the body. In fact, pregnancy itself is an oft-underappreciated stressor on the body, an effect that is consistent with elevations in GDF15. The goal of this study was to understand the role of GDF15 in gestational health. To date there are very few studies that evaluate GDF15 in human pregnancy. One study found no differences in circulating GDF15 between mothers with obesity and mothers of normal weight status (Andersson-Hall et al., 2021). Another that found that GDF15 was negatively associated with total gestational weight gain (P. Wang et al., 2020). The lack of prominent changes in gestational outcomes, although contrary to our prediction, is novel in the literature. Previous reports of *Gdf15* or *Gfral* null mouse models have generally not reported pregnancy or gestational outcomes in null mice during breeding or maintenance, but only describe differences as adults when used in experimental models. One study evaluated transgenic expression of human *GDF15* in mice and found that there was early involution and reduced milk production, reduced survival in pups, and lower weight gain in the postnatal period born to transgenic dams (Binder et al., 2016). Previous work shows that external administration of GDF15, similar to levels that are seen in the rise that accompanies pregnancy, in mice results in reductions in food intake (Mullican et al., 2017; Patel et al., 2019). The current study found that ablation of *Gdf15* and the resulting loss of GDF15 in maternal circulation (**Supplementary Figure 1A**) does not result in any differences in body weight accretion during the prenatal period and resulted in non-statistically significant higher body weights during the postnatal period in mice, with only small reductions in pup birth weight. This suggests that GDF15 in pregnant mice is altered but it is not

necessary for changes in weight accretion during a normal pregnancy. It is possible that under conditions of elevated somatic stress, GDF15 plays a larger role.

Taken together, the lack of evidence of differences in food intake, body weight, insulin sensitivity, and lactation in our *Gdf15* null model suggests that there may be a threshold effect for GDF15 during pregnancy. Only those studies that overexpress, deliver exogenous, or induce long-term highly disruptive stressors to their model show differences in GDF15 in relation to food intake and body weight. Therefore, it might be that pregnancy-related inductions of GDF15 are insufficient to meet the threshold to elicit an effect. *Gdf15* may act as a less acute stressor during pregnancy and more as a long-term indicator or feto-placental implantation. It could also imply that in the observational human studies, GDF15 is a biomarker of pregnancy related complications but not part of a causal pathway.

There are several limitations to our study. Murine pregnancy is not entirely comparable to human pregnancy. The majority of human pregnancies are singleton and mice are multi-parous, the placental structure is also different when compared with human pregnancy in the level of invasion of the tissue into the maternal uterus and the structure of the zones of the placenta itself (Schmidt et al., 2015). The approach we took eliminated feto-placental contribution of GDF15 to maternal serum during pregnancy by the use of homozygous breeding pairs. As a result, all knockout pups had knockout dams and sires, and all wild-type pups had wild-type dams and sires. Even though we did not detect any differences in offspring growth, the genotypes of these mice are not the same. A larger sample size could have provided more statistical power to detect differences in the outcomes evaluated. For example, via a reverse power analysis, we cannot rule out an effect size smaller than 15.3% difference in body weight gain during pregnancy between strains, but such a small effect would likely be physiologically insignificant. We also

followed the pups for a relatively short period of time after birth. So, any effect that would have manifested after the second week of life was not evaluated. Finally, we did not evaluate two other GDF15-associated complications, hypertension or nausea-related behavior in these mice. In contrast to the human findings, this study had several strengths including strong environmental, genetic and experimental consistency. Dams and sires were homozygous, they were derived from heterozygous crosses to limit genetic drift. In contrast to human observational studies demonstrating connections to pregnancy complications, we do not observe any detectable differences in litter sizes, glucose homeostasis, or gestational weight gain in the knockout mice. This is the first report of the loss of GDF15 in pregnancy and provides strong evidence for a lack of effect on body weight, food intake, or offspring health.

3.6 Conclusion

Despite the well-known, multi-fold rise in GDF15 during mouse and human pregnancy, we found no evidence that *Gdf15* ablation during mouse pregnancy and lactation causes metabolic, body weight, appetite, or lactational differences compared to $Gdf15^{+/+}$ counterpart dams. In the neonatal period, we did not observe any differences in survival, gestational age, litter size or birth weight between genotypes. Despite monitoring growth for 14 days after birth, there were no differences in body weight accretion in $Gdf15^{-/-}$ pups of either sex; indistinguishable from age-matched $Gdf15^{+/+}$ pups. More studies with larger sample sizes are needed to confirm these findings.

Chapter 4 Later Timing of Eating and Longer Fasting Duration During Pregnancy are Associated with Lower Infant Birth Weight and Greater Parent Glycemia

4.1 Abstract

The timing of eating and duration of overnight fast are emerging components of the diet that are gaining popularity as factors to manipulate for health reasons. Little research has been conducted on the timing of eating in pregnant populations, despite pregnancy being recognized as a critical period in development. We assessed the relationship between the timing of eating and duration of fasting during the second and third trimester of pregnancy and parent mid-gestation oral glucose tolerance test results and infant birthweight. In 102 parent-child dyads of the Pregnancy Related Eating Sleeping and Stress (PRESS) study, we found later timing of first meal in the second and third trimesters were associated with slightly higher OGTT test values.
Later timing of first meal and later fasting midpoint in the 2nd trimester was associated lower birth weight. During the third trimester, longer fasting duration and later fasting midpoint were associated with significantly lower infant birth weight, although these associations were attenuated when individuals who delivered preterm were excluded from the analysis. These data suggest that the timing of eating and duration of fasting during pregnancy could be important and under-recognized components of eating behavior that impact perinatal health.

4.2 Introduction

There are few times in life when nutritional intake matters as much as during the course of pregnancy. Modern science has demonstrated that pregnancy is a critical period of vulnerability that can impact trajectory toward health or disease, in both the birthing parent and the child. As such, much attention has been paid in scientific discourse on identifying modifiable behaviors that can improve the likelihood that results in a healthy pregnancy. The majority of the research in nutrition on this topic are focused on dietary quality, and nutrient adequacy. It is unsurprising that caloric restriction and poor diet quality are both strongly associated with worse outcomes in both parents and children (Marshall et al., 2022). However, recent evidence has pointed toward a previously under-recognized modifiable component of the diet, the timing and duration of eating. Initial evidence about the impact of the timing of eating on human health came from the field of sleep research. Routinely, researchers found that workers whose shifts are in opposition to the normal circadian rhythm have greater risks of ill health, including higher rates of miscarriage (Begtrup et al., 2019), pre-term birth (Cai et al., 2019; Davari et al., 2018), and odds of developing preeclampsia (Cai et al., 2019).

New attention has been called to all health behaviors that impact or are impacted by one's circadian rhythm. As such, modifying or compressing the timing of ones eating schedule is gaining popularity as a way to modulate health. One such modality is time-restricted eating (TRE). Evidence from human studies finds that condensing the eating window is effective for weight loss (Gabel, Hoddy, & Varady, 2018; Hutchison et al., 2019; Lowe et al., 2020). There have also been studies that find that metabolic health markers, such as blood pressure and cholesterol can be improved from TRE without the reduction in body weight (Sutton et al.,

2018). However, experimental data from human pregnant populations does not currently exist and observational evaluations are extremely limited.

These few studies that evaluate the timing and duration of eating in relation to perinatal health in humans find that there are small, but significant elevations in parent fasting glucose when fasting duration is greater during pregnancy (Loy et al., 2017). There is also evidence that eating earlier in the day during pregnancy is associated with more favorable dietary quality (Gontijo et al., 2020). Interest in this topic is mounting as future cohort studies are planned to further assess the role of chronobiology and timing of meals during pregnancy on perinatal health outcomes (Kaur et al., 2020). One study has also investigated the attitudes surrounding engaging in time-restricted eating during pregnancy. In their population of pregnant or recent post-partum parents, nearly 25% reported they would be open to trying this modality during pregnancy to improve health (Flanagan et al., 2022). Although, not all participants endorsed the diet as appropriate for pregnancy. There is also a clinical case study that employed intermittent fasting to improve postprandial blood glucose concentrations in a pregnant woman with gestational diabetes(Ali & Kunugi, 2020).

The most complete literature about the timing of food intake and duration of fasting during pregnancy is studies that evaluate Ramadan observance in pregnant Muslim populations. Although the characterization of participating in fasting differs by study, there is conflicting evidence about the impact on infant birth weight and maternal glycemia. Some studies identify greater risk of small-for-gestational age in infants whose parent fasted during gestation (Cross et al., 1990; Daley et al., 2017; Opaneye et al., 1990; Ziaee et al., 2010), or smaller birth weights (Savitri et al., 2014, 2018). The effect of fasting during pregnancy on glycemia is less frequently studied, but demonstrates there may be small elevations in glycemia (Baynouna Al Ketbi et al.,

2014). These data should be interpreted with caution, as the month of Ramadan not only results in altered timing of eating, but also changes in sleeping patterns and dietary quality. Therefore, more research is warranted to disentangle the relationship between chrononutritional factors during pregnancy and perinatal health outcomes.

Because pregnancy is a critical period of development with opportunity to impact health of the pregnant person and their child and because evidence surrounding the timing of eating in these populations is minimal, we sought to examine the association between the timing of eating and duration of fasting and mid-gestation glycemia and birth weight in a pregnancy cohort. Based on the available literature, we hypothesized that those who have earlier meal timing and longer duration of overnight fast would greater mid-gestation glucose tolerance test results and modest reductions in infant birth weights.

4.3 Methods

4.3.1 Study Population

The Pregnancy Related Eating Sleeping, and Stress (PRESS) cohort was developed as a longitudinal, survey-based, clinical research study. This study was designed to understand nutritional and behavioral contributors to perinatal health. Participants were recruited into PRESS after being invited to enroll through email based on their status as a pregnant patient receiving care at Michigan Medicine between June 2022 and October 2022 . Individuals who were interested in the study were directed toward a public REDCap link that results in a screening questionnaire. Those who were 18 years old, currently pregnant, in weeks 1-30 of pregnancy, and were currently receiving care and planning to deliver at Michigan Medicine we deemed as eligible and were invited to join the study.

Because individuals were eligible to join the study between 1-30 weeks of gestation, participants joined the study at various timepoints. Gestational age at enrollment was self-identified by answering the question, *"what week of your pregnancy are you currently in?"* which was verified in the medical chart upon enrollment. Survey information for the present analysis included those who participated at least once during the second trimester (14-28 weeks gestation, sent between 20-24 weeks) and third trimester (29-42+ weeks gestation, sent between 30-34 weeks). As participants could enroll in any trimester, they only received the surveys that were in line with target gestational weeks after enrollment. At the time of medical chart data abstraction in December 2022, the majority of participants who had delivered had entered the study during the 2nd trimester, and thus only completed survey information for trimesters 2 and 3. For this reason, the present analysis excludes data from trimester 1.

4.3.2 Participant Exposures, Outcomes, and Covariates

The bulk of the survey instruments sent to participants were repeated for each data collection event, with the exceptions being an additional sociodemographic and lifestyle factor questionnaire and anticipated gestational weight gain instrument in trimester 1 and question about most recent body weight in pounds and ounces in trimester 3. The detailed list of questionnaires sent for each trimester follow up is listed in **Figure 12**.

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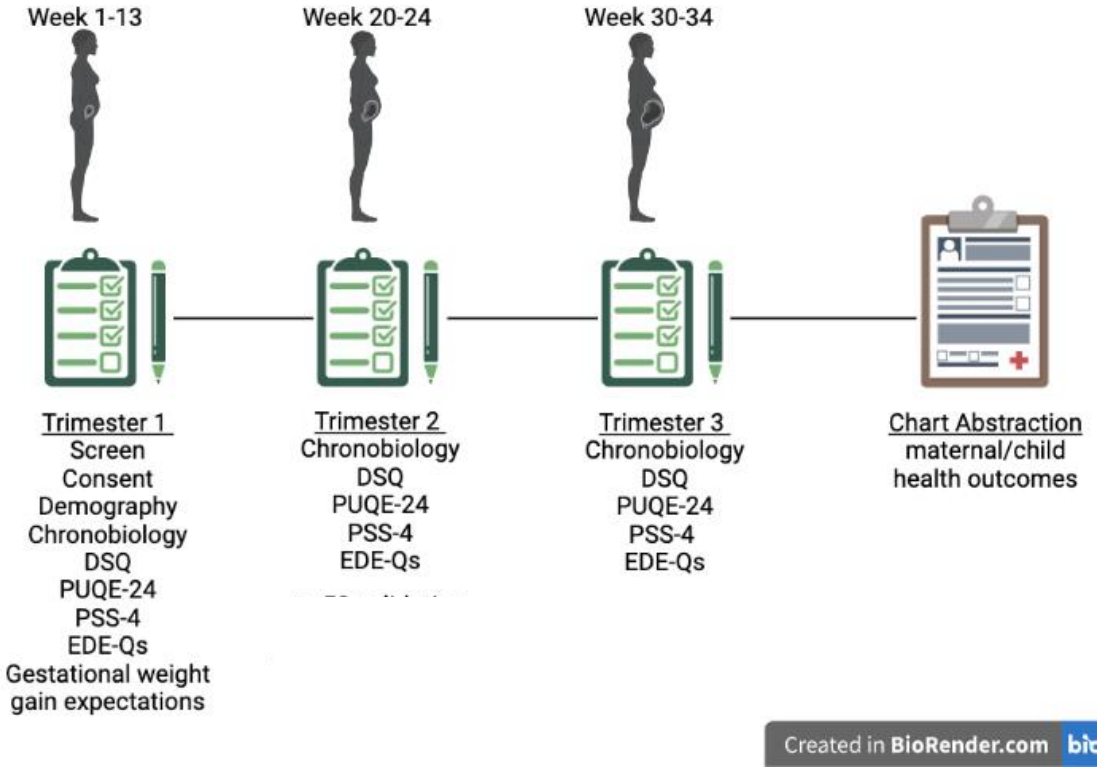


Figure 12: Survey Instrument and Data Abstraction Design of the PRESS Study

Survey instruments used and target gestation weeks for each contact event for participants of the PRESS study.

Timing of First and Last Meal

The timing of eating during pregnancy was assessed during each trimester using a questionnaire that asked participants *"On a typical day during this trimester, when was the first time in the day you had something to eat? (This includes beverages that have calories; like coffee or tea with cream or sugar)"* to indicate the beginning of an eating window, and *"On a typical day during this trimester, when was the last you had something to eat before going to bed? (This includes beverages that have calories; like coffee with cream or sugar)"* to indicate the end of the eating window. We also collected timing of sleep onset and wake time. Participants provided answers to these questions with respect to both weekdays and weekends, for all eligible trimesters.

Participants who did not report timing of eating or sleeping in military time were manually converted when necessary. Timing of eating data were inspected for overlap with reported sleeping intervals. Participant responses were evaluated for evidence of shift work, which was not apparent in the current sample.

Fasting Duration and Fasting Midpoint

To determine fasting duration, we subtracted eating duration (the difference between the last eating occasion and first eating occasion, expressed hours and minutes) from 24 hours. The fasting midpoint was calculated as time of last meal plus half the fasting duration.

Abstractions From Medical Chart Data

Trained research staff accessed the participant's medical charts to collect objective medical information about their current pregnancy. This included laboratory values for oral glucose tolerance tests and blood pressure readings, diagnoses of complications of pregnancy, parity, infant birth weight, sex of infant assigned at birth, gestational age at delivery, and delivery method. Participants who did not have pre-existing diabetes underwent 1-hour oral glucose tolerance test (OGTT) during mid gestation (24-28 weeks' gestation) according to Michigan Medicine guidelines. These labs were collected at a Michigan Medicine laboratory, where participants were instructed to consume a 50-gram liquid glucose drink in under 5 minutes. One hour later, blood was collected via venipuncture and glucose was determined by Michigan Medicine laboratory personnel. Primary outcomes of interest in this analysis were parent OGTT in mg/dL during mid-gestation and infant birth weight in grams.

Other Covariates

Sociodemographic and lifestyle information was collected upon enrolling in the study. This included data about self-reported pre-pregnancy BMI, physical activity, relationship status, smoking exposure, race/ethnicity, annual household income, and pregnant person's level of education. Covariates were considered based on sociodemographic information available as well as *a priori* biological associations.

4.3.3 Statistical Analyses

Univariate analysis was completed on all sociodemographic, eating exposure, and health outcome data were assessed for normality through histograms and residual plots. Measures that are normally distributed are expressed as mean ± standard deviation (SD) and those that were not normally distributed are expressed as median ± inter-quartile range (IQR). After initial investigation of variables, we assessed the associations between covariates of interest and primary exposures and outcomes identified in this study, grouped by weekday or weekend and trimester. Due to low subject numbers in some categories potential confounders, we collapsed them into fewer categories; such as dichotomizing self-reported race and ethnicity, parent educational attainment, annual household income. When data were distributed normally, association was determined through ANOVA. When data was not normally distributed, associations were assessed through Kruskal-Wallis test. Associations with a P-value <0.10 were considered as statistically relevant confounders or precision covariates in multivariable-adjusted models. Final models for OGTT were adjusted for pre-pregnancy BMI, physical activity, sleep duration, annual household income, and dichotomized race/ethnicity. Infant birth weight models were adjusted for gestational age at birth, infant sex assigned at birth, sleep duration, annual

household income, dichotomized race/ethnicity, and physical activity. All statistical analyses were conducted in R Studio, version 4.2.2.

4.3.4 Sensitivity Analysis

In models of infant birth weight, gestational age at delivery could theoretically be a mediator or a precision covariate. Thus, we conducted sensitivity analyses where we restricted models to not include those with preterm delivery status (born before 37 weeks gestation), to evaluate this relationship. Specifically, we evaluated trimester 3 weekday exposures in relation to infant birth weight in the full sample and then again in a sample without individuals who delivered preterm. We selected restricted analysis as we were underpowered to complete a full mediation analysis.

4.4 Results

4.4.1 Timing of Eating During Pregnancy Differs Based on Sociodemographic Factors

While recruiting for this study, we excluded individuals with pre-existing diabetes, inaccurate or missing timing data, who were lost to follow up, those who delivered multiples, and those without outcome data at the time of the analysis. This resulted in 102 unique individuals, 54 of whom had survey responses for both trimesters 2 and 3 (**Figure 13**). PRESS participants were 32.1 ± 0.54 years old, highly educated (78.4% had at least one college degree), wealthy (63.7% >$100,000 a year), and had similar proportions of male and female infants (56.9% male).
In this population the timing of the first eating occasion on weekdays during the second trimester was associated with parent-reported race and ethnicity, maternal education, sleep duration, and marital status. Participants from historically excluded racial and ethnic groups tended to eat their first and last meal later in the day, as well as have a later fasting midpoint (**Table 1**).

*Table 1:*Second trimester timing of eating on weekdays in relation to sociodemographic and lifestyle factors

Maternal Characteristics	n=88	First Eat Median (IQR)	Last Eat Median (IQR)	Fasting Midpoint Median (IQR)	Fasting Duration Median (IQR)
Maternal race/ethnicity					
White or Caucasian	70	7:33 (0:30)	20:00 (1:45)	2:00 (0:02)	11.5 (2.00)
Asian American or Asian	8	8:22 (1:30)	20:40 (1:15)	2:22 (0:01)	12.4 (1.91)
Hispanic, Latinx, or Spanish Origin	5	8:30 (2:15)	21:45 (1:00)	2:52 (0:01)	11.5 (1.75)
Middle Eastern or North African	2	9:28 (0:58)	21:00 (1:00)	3:14 (0:02)	12.5 (0.03)
Black or African American	2	8:22 (1:30)	20:45 (00:15)	2:37 (0:02)	11.8 (1.25)
P value		**0.04**	0.14	**0.043**	0.62
Race (Dichotomized)					
White or Caucasian	70	7:33 (0:30)	20:00 (1:45)	2:00 (0:02)	11.5 (2.00)
Not White or Caucasian	18	8:30 (2:00)	21:00 (1:13)	2:45 (0:03)	12.1 (2.04)
P value		**0.003**	**0.040**	**0.002**	0.32
Maternal Age					
< 35yo	64	8:00 (1:00)	20:30 (1:42)	2:07 (0:03)	11.5 (2.00)
more than 35 years	24	7:48 (1:30)	20:30 (1:32)	2:30 (0:02)	11.8 (2.31)
P value		0.64	0.98	0.39	0.7
Parity					
0	39	7:45 (1:05)	20:30 (1:29)	2:15 (0:02)	11.5 (1.72)
1+	47	8:00 (1:15)	20:00 (2:28)	2:150:03)	12.0 (1.94)
P value		0.45	0.040	0.84	0.08
Maternal education					
< Bachelor's Degree	17	9:00 (2:00)	20:36 (1:26)	2:55 (0:03)	12.8 (1.83)
Bachelor's Degree	22	7:52 (00:30)	20:30 (2:12)	2:00 (0:02)	11.5 (2.06)
Master's Degree	33	8:00 (00:45)	20:30 (1:30)	2:07 (0:02)	11.5 (2.00)
Doctorate or Professional Degree	16	7:30 (1:00)	20:00 (1:07)	2:00 (0:02)	11.5 (1.75)
P value		**0.019**	0.84	0.14	0.074
Household Annual Income					

<$100,000	26	8:00 (1:53)	21:00 (1:34)	2:30 (0:02)	12.0 (2.30)
$100,000 - 149,999	27	8:00 (00:57)	20:30 (1:30)	2:15 (0:03)	11.5 (1.50)
$150,000 or more	32	7:30 (00:30)	20:00 (1:14)	1:45 (0:02)	11.5 (2.06)
P value		8:00 (1:53)	21:00 (1:34)	2:30 (0:02)	12.0 (2.30)
Number of persons in household					
2 or fewer	41	8:00 (1:10)	20:30 (1:20)	2:07 (0:03)	11.0 (1.50)
3+	46	8:00 (1:22)	20:00 (2:30)	2:21 (0:03)	12.0(1.97)
P value		0.24	0.35	0.95	**0.039**
Sleep Duration					
<8 hours	13	7:30 (1:30)	21:30 (0:55)	2:30 (0:02)	10.2 (0.50)
8 + hours	75	8:00 (1:30)	20:00 (1:30)	2:15 (0:03)	12.0 (1.94)
P value		**0.042**	**0.012**	0.75	**0.0002**
Physical Activity					
0-2 times per week	43	8:00 (1:31)	20:30 (1:41)	2:15 (0:03)	12.0 (2.00)
3-4 times per week	33	8:00 (1:00)	20:30 (1:30)	2:15 (0:02)	11.5 (2.00)
5-7 times per week	12	7:30 (0:33)	20:15 (2:00)	2:03 (0:02)	10.8 (2.00)
P value		0.49	0.89	0.64	0.058
PSS score					
<12	21	7:45 (00:30)	20:00 (2;26)	2:00 (0.02)	12.0 (1.75)
12 +	67	8:00 (1:30)	20:30 (1:33)	2:30 (0:02)	11.5 (2.00)
P value		0.37	0.39	0.23	0.56
Maternal BMI					
Underweight	2	8:30 (1:00)	21:15 (0:15)	2:52 (0:00)	11.5 (1.25)
Normal weight	37	7:45 (1:00)	20:30 (1:30)	2:15 (0:02)	11.5 (1.75)
Overweight	22	7:45 (1:07)	20:00 (1:52)	1:55 (0:02)	11.5 (2.05)
Obesity Class 1	13	8:00 (0:30)	20:00 (2:26)	2:28 (0:03)	12.0 (2.00)
Obesity Class 2	7	9:00 (1:30)	20:30 (1:45)	2:30 (0:03)	13.0 (1.25)
Obesity Class 3	5	8:00 (0:55)	21:00 (1:15)	2:42 (0:02)	11.4 (1.00)
P value		0.62	*0.83*	0.68	0.45
Marital Status					
Married or Long-term Partnership	82	8:00 (1:00)	20:07 (1:59)	2:07 (0:03)	11.5 (2.00)
Not Married or Partnered	6	10:00 (0:56)	21:00 (1:03)	3:26 (0:00)	13.0 (1.80)
P value		**0.002**	0.086	**0.001**	0.073

Child Characteristics

Delivery type					
Vaginal	55	8:00 (1:00)	20:30 (2:26)	2:07 (0:03)	12.0 (2.44)
Cesarean Section	32	8:00 (1:07)	20:30 (1:06)	2:21 (0:03)	11.5 (1.10)
P value		0.62	0.39	0.40	0.41
Gestational Age at Delivery					
<37 weeks	9	8:00 (1:53)	20:00 (1:06)	2:00 (0:05)	12.0 (1.90)
37+ weeks	79	8:00 (1:00)	20:30 (2:00)	2:15 (0:03)	11.5 (2.12)
P value		0.46	0.87	0.98	0.9
Infant Sex at Birth					
Male	54	8:00 (1:22)	20:30 (1:45)	2.1 (0:02)	12.0 (1.75)
Female	33	7:30 (1:00)	21:00 (1:30)	2.5 (0:02)	11.5 (2.50)
P value		0.54	0.25	0.54	0.25

P values were calculated from Kruskal-Wallis test

Factors with fewer than 102 individuals reflect missing data for participants

This was also true with respect to measures of socioeconomic status , including household income and maternal education, where those who were wealthier and more highly educated tended to begin and finish eating earlier in the day than those with fewer resources. Participants who were married or in a partnership tended to consume meals earlier, begin fasts earlier and have earlier fasting midpoints. Sleep duration greater than 8 hours was significantly associated with later timing of the first and last meal, as well as longer fasting durations, but not fasting midpoint. Similar associations were seen for weekend values during the 2nd trimester. Delivering before 37 weeks gestation was associated with later timing of first meal on weekend, and the timing of eating for both first and last meals tended to be later than on weekdays, although there were few individuals who delivered preterm (**Table 2**).

Table 2: Second trimester timing of eating on weekends in relation to sociodemographic and lifestyle factors

Maternal Characteristics	n=88	First Eat Median (IQR)	Last Eat Median (IQR)	Fasting Midpoint Median (IQR)	Fasting Duration Median (IQR)
Maternal race/ethnicity					
White or Caucasian	70	8:30 (1:30)	21:00 (1:56)	2:30 (0:03)	12.0 (2.00)
Asian American or Asian	8	9:15 (1:07)	20:55 (1:15)	2:52 (0:01)	12.8 (2.13)
Hispanic, Latinx, or Spanish Origin	5	9:00 (1:30)	22:00 (1:30)	3:22 (0:00)	11.5 (0.50)
Middle Eastern or North African	2	10:13 (0:00)	21:00 (0:00)	3:36 (0:00)	13.2 (0.22)
Black or African American	2	9:00 (1:00)	20:45 (0:15)	2:52 (0:01)	12.2 (0.75)
P value		**0.085**	0.30	0.15	0.33
Race (Dichotomized)					
White or Caucasian	70	9:45 (1:22)	21:00 (1:56)	2:30 (0:03)	12.0 (2.00)
Not White or Caucasian	18	8:30 (1:30)	21:00 (1:00)	3:18 (0:01)	12.6 (1.87)
P value		**0.009**	0.402	**0.034**	0.22
Maternal Age					
<35yo	64	9:00 (2:00)	21:00 (1:39)	2:45 (0:03)	12.0 (2.00)
more than 35 years	24	8:45 (1:31)	20:55 (2:07)	2:37 (0:03)	12.0 (2.12)
P value		0.63	0.71	0.67	0.98
Parity					
0	39	9:00 (2:00)	21:00 (1:30)	2:45 (0:03)	12.0 (2.30)
1+	47	8:30 (1:35)	21:00 (2:00)	2:30 (0:03)	12.0 (2.00)
P value		0.46	0.330	0.35	0.46
Maternal education					
< Bachelor's Degree	17	10:00 1:15)	20:54 (2:00)	3:30 (0:03)	13.4 (2.50)
Bachelor's Degree	22	8:30 (1:00)	21:00 (1:33)	2:45 (0:02)	11.5 (1.00)
Master's Degree	33	9:00 (1:30)	21:00 (1:37)	2:30 (0:03)	12.0 (2.00)
Doctorate or Professional Degree	16	8:15 (1:08)	21:00 (1:00)	2:22 (0:02)	12.0 (1.06)
P value		**0.004**	0.92	0.15	**0.036**
Household Annual Income					

<$100,000	26	9:30 (2:00)	21:00 (2:00)	3:15 (0:02)	12.0 (2.40)
$100,000 - 149,999	27	9:00 (1:48)	21:00 (2:00)	2:45 (0:03)	12.0 (1.75)
$150,000 or more	32	8:00 (1:07)	20:30 (1:30)	2:30 (0:02)	12.0 (2.12)
Not Reported	3	10:00 (1:07)	20:30 (0:33)	3:22 (0:01)	12.4 (1.12)
P value		0.057	0.58	0.15	0.65
Number of persons in household					
2 or fewer	41	9:00 (2:00)	21:00 (1:30)	3:00 (0:02)	12.0 (2.00)
3+	46	8:22 (1:52)	20:45 (2:32)	2:30 (0:03)	12.0 (2.00)
P value		0.12	0.26	0.054	0.87
Sleep Duration					
<8 hours	13	9:00 (1:37)	21:30 (1:00)	3:15 (0:01)	11.5 (1.50)
8 + hours	75	8:30 (2:00)	21:00 (1:33)	2:30 (0:03)	12.0 (1.95)
P value		0.75	0.100	0.17	0.19
Physical Activity					
0-2 times per week	43	9:00 (2:00)	21:00 (2:26)	2:45 (0:03)	12.0 (2.37)
3-4 times per week	33	9:00 (1:30)	21:00 (1:30)	2:45 (0:02)	12.0 (1.50)
5-7 times per week	12	8:00 (1:07)	20:45 (2:00)	2:45 (0:03)	11.5 (1.62)
P value		0.304	0.99	0.81	0.44
PSS score					
<12	21	8:30 (2:00)	20:15 (1:56)	2:45 (0:03)	12.2 (1.50)
12 +	67	9:00 (2:00)	21:00 (2:00)	2:45 (0:03)	12.0 (2.00)
P value		0.87	0.20	0.43	0.28
Maternal BMI					
Underweight	2	9:15 (0:45)	21:00 (1:00)	3:07 (0:00)	12.2 (1.75)
Normal weight	37	9:00 (1:40)	21:00 (2:00)	2:33 (0:03)	12.0 (2.08)
Overweight	22	8:30 (1:52)	21:00 (1:30)	2:45 (0:03)	12.0 (1.31)
Obesity Class 1	13	8:00 (2:00)	21:00 (2:18)	2:45 (0:05)	12.0 (1.02)
Obesity Class 2	7	9:00 (1:00)	20:30 (1:45)	2:30 (2:02)	13.0 (1.62)
Obesity Class 3	5	8:00 (1:00)	21:45 (1:30)	2:52 (0:03)	11.5 (0.50)
P value		0.99	0.93	0.98	0.89
Marital Status					
Married or Long-term Partnership	82	8:30 (1:35)	21:00 (1:43)	2:31 (0:03)	12.0 (2.00)
Not Married or Partnered	6	10:07 (0:26)	20:48 (1:13)	3:26 (0:00)	13.6 (1.21)

P value		**0.002**	0.55	**0.03**	0.026
Child Characteristics					
Delivery type					
Vaginal	55	8:30 (2:00)	21:00 (1:41)	2:45 (0:03)	12.0 (2.08)
Cesarean Section	32	9:00 (1:42)	21:00 (2:00)	2:37 (0:03)	12.0 (1.93)
P value		0.35	0.97	0.68	0.51
Gestational Age at Delivery					
<37 weeks	9	10:00 (0:23)	21:00 (1:00)	3:30 (0:02)	13.0 (2.00)
37+ weeks	79	8:30 (1:30)	21:00 (1:52)	2:33 (0:03)	12.0 (2.00)
P value		**0.006**	0.96	0.06	0.072
Infant Sex at Birth					
Male	54	9:00 (2:00)	20:55 (1:30)	2:31 (0:03)	12.0 (1.50)
Female	33	9:00 (1:30)	21:00 (2:00)	3:00 (0:02)	11.5 (2.50)
P value		0.82	0.11	0.20	0.071

P values were calculated from Kruskal-Wallis test

Factors with fewer than 102 individuals reflect missing data for participants

The timing of eating in the 3rd trimester on weekdays had fewer significant associations than did the 2nd trimester (**Table 3**). Later sleeping midpoint was associated with higher self-reported stress levels, which was driven by first meal. There were also more associations with gestational factors, such as earlier timing of first meal and higher likelihood of vaginal delivery, and longer fasting duration occurring more in pregnancies that resulted in children assigned male sex at birth.

Table 3: Third trimester timing of eating on weekdays in relation to sociodemographic and lifestyle factors

	First Eat	Last Eat	Fasting Midpoint	Fasting Duration

Maternal Characteristics	n=63	Median (IQR)	Median (IQR)	Median (IQR)	Median (IQR)
Maternal race/ethnicity					
White or Caucasian	45	8:00 (0:55)	20:30 (1:00)	2:15 (0:50)	11.2 (2.00)
Asian American or Asian	7	8:00 (0:45)	21:00 (1:14)	2:30 (0:30)	11.6 (1.17)
Hispanic, Latinx, or Spanish Origin	5	8:30 (1:15)	21:00 (3:30)	2:45 (1:07)	11.5 (1.25)
Other	6	8:30 (2:18)	20:30 (1:22)	2:08 (1:47)	12.2 (1.25)
P value		0.31	0.80	0.65	0.65
Race (Dichotomized)					
White or Caucasian	45	8:00 (0:55)	21:00 (1:51)	2:15 (0:50)	11.2 (2.00)
Not White or Caucasian	18	8:00 (1:26)	20:30 (1:00)	2:30 (1:12)	11.7 (1.31)
P value		0.11	0.33	0.23	0.50
Maternal Age					
<35yo	43	8:00 (1:00)	21:00 (1:22)	2:17 (0:56)	11.2 (1.50)
more than 35 years	18	7:34 (0:57)	20:00 (1:45)	2:15 (0:48)	11.8 (2.01)
P value		0.92	0.48	0.45	0.55
Parity					
0	26	8:00 (0:53)	20:47 (0:52)	2:18 (0:47)	11.3 (1.01)
1+	36	7:45 (1:02)	20:15 (1:30)	2:15 (1:15)	11.5 (2.00)
P value		0.46	0.28	0.51	0.41
Maternal education					
< Bachelor's Degree	4	8:30 (1:26)	20:30 (1:45)	2:15 (1:50)	11.4 (1.56)
Bachelor's Degree	18	8:00 (0:58)	20:45 (1:00)	2:16 (1:01)	11.5 (1.00)
Master's Degree	25	8:00 (1:00)	20:30 (2:00)	2:15 (0:43)	11.6 (2.25)
Doctorate or Professional Degree	11	7:34 (0:45)	21:00 (1:30)	2:30 (0:39)	11.0 (1.88)
P value		0.72	0.82	0.76	0.55
Household Annual Income					
<$100,000	17	8:00 (1:00)	21:00 (1:49)	2:15 (1:00)	11.5 (1.50)
$100,000 - 149,999	17	7:45 (0:55)	21:00 (1:00)	2:30 (1:00)	11.0 (1.08)
$150,000 or more	23	8:00 (1:00)	20:30 (1:00)	2:15 (0:55)	11.5 (2.12)
P value		0.72	0.48	0.80	0.35
Number of persons in household					

2 or fewer	24	8:00 (1:00)	20:30 (1:00)	2:30 (0:54)	11.3 (1.19)
3+	34	7:47 (0:58)	21:00 (1:33)	2:15 (1:14)	11.5 (1.88)
P value		0.46	0.97	0.20	0.79
Sleep Duration					
<8 hours	10	7:22 (0:52)	20:45 (1:15)	2:15 (0:52)	10.6 (2.00)
8 + hours	53	8:00 (1:00)	20:30 (1:00)	2:15 (1:00)	11.5 (1.52)
P value		**0.018**	0.530	0.97	0.10
Physical Activity					
0-2 times per week	25	8:00 (1:00)	20:30 (1:49)	2:15 (1:15)	11.8 (1.50)
3-4 times per week	26	8:00 (1:22)	20:47 (1:00)	2:23 (0:30)	11.2 (1.50)
5-7 times per week	11	7:30 (0:45)	21:00 (2:30)	2:30 (1:15)	11.2 (2.75)
P value		0.48	0.37	0.46	0.33
PSS score					
<12	18	7:30 (0:41)	20:30 (1:52)	1:49 (0:45)	11.1 (1.44)
12 +	44	8:00 (1:07)	20:47 (1:18)	2:30 (0:43)	11.5 (2.00)
P value		0.063	0.37	**0.035**	0.42
Maternal BMI					
Normal weight	27	7:30 (0:37)	21:00 (1:00)	2:15 (0:52)	10.8 (2.12)
Overweight	14	8:30 (1:03)	20:00 (1:15)	2:15 (0:52)	12.0 (1.44)
Obesity Class 1	8	80 (0:27)	20:17 (2:00)	2:02 (1:03)	11.5 (1.13)
Obesity Class 2	4	8:30 (1:17)	20:07 (2:48)	2:08 (0:57)	12.0 (3.23)
Obesity Class 3	2	9:12 (0:47)	21:45 (0:45)	3:28 (0:46)	11.5 (0.04)
P value		0.058	0.13	0.31	0.09
Marital Status					
Married or Long-term Partnership	57	8:00 (1:00)	20:30 (1:00)	2:15 (1:00)	11.5 (2.0)
Not Married or Partnered	1	9:30 (-)	23:00 (-)	4:15 (-)	10.5(-)
P value		0.33	0.23	0.28	0.52
Child Characteristics					
Delivery type					
Vaginal	39	7:45 (0:40)	21:00 (1:00)	2:15 (1:05)	11.3 (1.50)
Cesarean Section	23	8:00 (1:20)	20:30 (1:15)	2:04 (1:04)	12.0 (1.75)
P value		**0.025**	0.33	0.93	0.06
Gestational Age at Delivery					
<37 weeks	2	8:30 (1:30)	15:45 (6:45)	12:07 (7:52)	16.8 (5.25)

37+ weeks	61	8:00 (1:00)	20:30 (1:00)	2:15 (0:57)	11.5 (2.00)
P value		0.81	0.94	**0.019**	0.21
Infant Sex at Birth					
Male	34	8:00 (1:22)	20:30 (1:00)	2:18 (0:50)	12.0 (1.50)
Female	28	7:39 (0:40)	21:00 (1:33)	2:15 (1:02)	11.0 (1.66)
P value		0.12	0.20	0.65	**0.021**

P values were calculated from Kruskal-Wallis test

Factors with fewer than 102 individuals reflect missing data for participants

Timing of eating variables in the 3rd trimester on weekends were also later than weekday, and with fewer associations (**Table 4**). Timing of first and last meal was again associated with parent race/ethnicity. Having greater amounts of physical activity during pregnancy was associated with lower oral glucose tolerance test in mid-gestation (**Table 5**). Infant birth weight positively associated with gestational age at delivery.

Table 4: Third trimester timing of eating on weekends in relation to sociodemographic and lifestyle factors

Maternal Characteristics	n=63	First Eat Median (IQR)	Last Eat Median (IQR)	Fasting Midpoint Median (IQR)	Fasting Duration Median (IQR)
Maternal race/ethnicity					
White or Caucasian	45	8:30 (1:00)	21:00 (1:30)	2:45 (1:22)	11.5 (2.50)
Asian American or Asian	7	9:00 (0:30)	21:30 (1:15)	3:15 (0:38)	11.0 (1.97)
Hispanic, Latinx, or Spanish Origin	5	10:00 (0:30)	21:00 (2:00)	3:30 (1:15)	13.0 (1.00)
Other	6	9:00 (1:52)	21:00 (1:30)	2:45 (1:54)	13.0 (0.75)
P value		**0.039**	0.60	0.25	0.086
Race (Dichotomized)					
White or Caucasian	45	8:30 (1:00)	21:00 (1:30)	2:45 (1:19)	11.5 (2.50)
Not White or Caucasian	18	9:00 (0:53)	21:00 (2:00)	3:07 (1:22)	12.5 (1.62)
P value		**0.017**	**0.480**	0.093	0.11
Maternal Age					
<35yo	43	8:45 (1:30)	21:00 (1:37)	3:00 (1:22)	12.0 (2.12)
more than 35 years	18	9:00 (0:45)	21:00 (1:45)	2:45 (1:00)	11.5 (2.50)
P value		0.99	0.67	0.78	0.32
Parity					
0	26	9:00 (1:38)	21:00 (1:22)	3:07 (0:52)	12.0 (2.00)
1+	36	8:45 (1:00)	20:45 (1:30)	2:30 (1:15)	12.0 (2.12)
P value		0.17	0.19	0.16	0.27
Maternal education					
< Bachelor's Degree	4	9:00 (0:22)	21:15 (1:22)	3:07 (0:52)	11.8 (1.00)
Bachelor's Degree	18	8:30 (1:30)	21:00 (1:22)	2:52 (1:30)	12.0 (1.38)
Master's Degree	25	9:00 (1:30)	21:00 (1:30)	2:44 (1:00)	13.0 (2.50)
Doctorate or Professional Degree	11	8:30 (0:52)	21:30 (1:30)	3:15 (1:26)	11.0 (1.25)
P value		0.78	0.62	0.95	0.12
Household Annual Income					
<$100,000	17	9:00 (1:31)	21:00 (1:30)	2:44 (1:15)	12.0 (2.45)
$100,000 - 149,999	17	9:00 (1:30)	21:30 (1:30)	3:15 (0:30)	12.0 (1.00)
$150,000 or more	23	8:30 (1:00)	21:00 (1:30)	2:30 (1:07)	12.0 (2.50)
P value		0.56	0.32	0.26	0.63

Number of persons in household					
2 or fewer	24	9:00 (1:32)	21:00 (1:15)	3:07 (0:56)	12.0 (2.00)
3+	34	8:30 (1:00)	21:00 (1:41)	2:37 (1:00)	11.8 (2.50)
P value		0.14	0.68	0.19	0.53
Sleep Duration					
<8 hours	10	8:45 (0:52)	21:15 (1:30)	3:00 (0:41)	11.2 (1.89)
8 + hours	53	9:00 (1:30)	21:00 (1:30)	2:45 (1:15)	12.0 (2.00)
P value		0.82	0.34	0.64	0.24
Physical Activity					
0-2 times per week	25	9:00 (1:00)	21:00 (1:30)	2:45 (1:15)	12.0 (2.00)
3-4 times per week	26	9:00 (1:53)	21:00 (1:18)	3:00 (1:00)	12.0 (2.50)
5-7 times per week	11	8:30 (1:15)	21:00 (2:00)	2:35 (1:22)	11.2 (1.50)
P value		0.60	0.37	0.40	0.66
PSS score					
<12	18	9:00 (1:00)	20:30 (1:22)	2:30 (1:00)	12.0 (1.62)
12 +	44	9:00 (1:30)	21:00 (2:00)	3:00 (1:00)	11.5 (2.46)
P value		0.63	0.10	0.11	0.28
Maternal BMI					
Normal weight	27	8:30 (1:00)	21:00 (1:15)	3:00 (1:07)	11.0 (1.50)
Overweight	14	9:00 (0:30)	21:00 (1:15)	2:52 (0:41)	12.5 (1.38)
Obesity Class 1	8	8:15 (1:15)	20:00 (1:45)	2:07 (1:15)	12.2 (1.25)
Obesity Class 2	4	8:30 (1:17)	20:07 (2:48)	2:08 (0:57)	12.0 (3.23)
Obesity Class 3	2	9:30 (0:30)	22:00 (0:00)	3:45 (0:15)	11.5 (0.50)
P value		0.46	0.08	0.18	0.13
Marital Status					
Married or Long-term Partnership	57	9:00 (1:30)	21:00 (1:30)	2:45 (1:15)	12.0 (2.25)
Not Married or Partnered	1	9:30 (-)	23:00 (-)	4:15 (-)	10.5 (-)
P value		0.66	0.20	0.27	0.19
Child Characteristics					
Delivery type					
Vaginal	39	8:30 (1:00)	21:00 (1:52)	2:45 (1:07)	11.5 (2.22)
Cesarean Section	23	9:00 (1:45)	21:00 (1:30)	3:00 (1:37)	12.0 (1.75)
P value		0.21	0.46	0.73	0.14

Gestational Age at Delivery					
<37 weeks	2	9:00 (1:00)	21:00 (1:00)	3:00 (1:00)	12.0 (0.00)
37+ weeks	61	9:00 (1:30)	21:00 (1:30)	2:45 (1:15)	12.0 (2.25)
P value		0.75	0.91	0.86	0.87
Infant Sex at Birth					
Male	34	9:00 (1:00)	21:00 (1:30)	2:45 (1:11)	12.0 (1.96)
Female	28	8:37 (1:39)	21:07 (2:00)	3:00 (1:13)	11.1 (2.52)
P value		0.97	0.11	0.33	0.12

P values were calculated from Kruskal-Wallis test

Table 5: Parent mid-gestation 1-hour oral glucose tolerance test values and infant birth weight in relation to sociodemographic and lifestyle factors

Maternal Characteristics		1-hour Oral Glucose Tolerance Test Value (mg/dL)	Infant Birth Weight (grams)
	n=102	n=99	n=102
Maternal race/ethnicity			
White or Caucasian	76	115 ± 26.3	3401 ± 534.9
Asian American or Asian	10	130 ± 30.8	3290 ± 573.2
Hispanic, Latinx, or Spanish Origin	6	140 ± 24.4	3248 ± 6498.1
Middle Eastern or North African	2	148 ± 48.1	3058 ± 830.9
Black or African American	2	116 ± 19.1	2855 ± 42.4
P value		0.066	0.68
Maternal Age			
18-35	71	119 ± 28	3356 ± 572
more than 35 years	26	119 ± 27	3386 ± 458
P value		0.51	0.96
Parity			
0	45	121 ± 26.4	3315 ± 529
1+	55	116 ± 28.7	3385 ± 560
P value		0.67	0.44
Maternal education			
< Bachelor's Degree	17	118 ± 29.7	3234 ± 414
Bachelor's Degree	24	125 ± 26.3	3444 ± 548
Master's Degree	37	120 ± 28.5	3371 ± 526
Doctorate or Professional Degree	19	111 ± 25.5	3366 ± 673
P value		0.41	0.83
Household Annual Income			
<$100,000	29	120 ± 26.3	3315 ± 404
$100,000 - 149,999	29	116 ± 28.4	3390 ± 602
$150,000 or more	36	118 ± 28.7	3351 ± 598
P value		0.95	0.88
Number of persons in household			
2 or fewer	43	121 ± 25.0	3274 ± 593
3+	53	118 ± 29.8	3446 ± 490

P value		0.59	0.12
Sleep Duration			
<8 hours	15	126 ± 34.3	3170 ± 686
8 + hours	87	117 ± 26.1	3395 ± 512
P value		0.24	0.14
Physical Activity			
0-2 times per week	47	126 ± 25.1	3396 ± 467
3-4 times per week	36	114 ± 29.2	3467 ± 523
5-7 times per week	18	107 ± 25.2	3074 ± 696
P value		**0.018**	0.079
PSS score			
<12	27	124 ± 30.9	3426 ± 588
12 +	74	116 ± 26.1	3341 ± 532
P value		0.22	0.73
Gestational Age at Enrollment			
trimester 1 (0-13 weeks)	1	159	4380
trimester 2 (14-26 weeks)	88	118 ± 27.5	3369±540
trimester 3 (27+ weeks)	13	115±26.6	3237 ± 520
P value		0.43	0.17
Maternal BMI			
Underweight	2	99 ± 18.4	2912 ± 499
Normal weight	43	115 ± 25.4	3296 ± 593
Overweight	24	122 ± 29.9	3371 ± 515
Obesity Class 1	13	122 ± 32.4	3620 ± 347
Obesity Class 2	8	119 ± 23.0	3481 ± 469
Obesity Class 3	5	149 ± 21.8	3171 ± 753
P value		0.18	0.44
Marital Status			
Married or Long-term Partnership	91	118 ± 27.6	3375 ± 554
Not Married or Partnered	6	133 ± 23.9	3193 ± 277
P value		0.21	0.72
Child Characteristics			
Delivery type			
Vaginal	65	117 ± 28.9	3376 ± 521

Cesarean Section	36	121 ± 24.1	3331 ± 595
P value		0.25	0.88
Gestational Age at Delivery			
<37 weeks	9	129 ± 31.2	2489 ± 444
37+ weeks	93	117 ± 27.0	3446 ± 475
P value		0.22	**<0.001**
Infant Sex at Birth			
Male	58	120 ± 28.48	3451 ± 542
Female	42	115 ± 26.5	3253 ± 522
P value		0.48	0.069

P value calculated by ANOVA

Not all participants had values for the glucose tolerance test, resulting in fewer individuals than for birth weight

4.4.2 Later Timing of Eating is Marginally Related to Parent Glycemia in the Second and Third Trimester

During the second trimester of pregnancy, the association between timing of eating variables and parent mid-gestation OGTT were comparable between weekdays and weekends. Each hour later timing of the first meal (**Figure 14A; Tables 6 & 7**) was related to a 5.14 mg/dL 95% CI (-0.09, 9.25) higher OGTT (p=0.058) which was not statistically significant. A similar association was seen for longer fasting duration being related to 3.71 mg/dL 95% CI (-0.019, 7.44) OGTT (p=0.055). However, after adjustment with covariates, the significance of timing of the first meal was reduced but estimates retained similar direction and magnitude 2.89 CI (-3.48, 9.29) p=0.38 and fasting duration 4.49 CI (-1.74, 10.7) p=0.16.

During the third trimester, timing of eating was more significantly related to OGTT values on weekdays than on weekends. For each hour the first meal of the weekday was delayed, there was a 6.67 mg/dL 95%CI (0.30, 13.07) greater 1-hour glucose value (**Tables 6 & 7; Figure**

14B, p=0.045). After adjustment for covariates, this association lost statistical significance, but the estimate remained similar 6.44 mg/dL, 95% CI (-2.00, 14.69), p=0.14.

4.4.3 Infant Birth Weight is Inversely Associated with the Timing of Eating During Pregnancy

Directionality of associations for birth weight with eating variables in the 2nd trimester were similar between weekdays and weekends. Generally, later eating times, and later fasting midpoint were associated with lower infant birth weights (**Figure 14C; Tables 8 & 9**). During the second trimester on weekends, each hour later timing of the first meal was significantly associated a 125.28-gram reduction in infant birth weight (95% CI(-214.56, -36.00), p =0.0072). Later fasting midpoint was also associated with a 122.6-gram lower birth weight (95% CI(-230.0, -15.40), p =0.028). However, after adjustment for gestational age at delivery, both associations for timing of the first meal (-53.75 g CI (-120.6, 13.00), p=0.12) and fasting midpoint were partially attenuated (-65.12 CI (-142.9, 12.7), p=0.10), and remained similar with subsequent adjustment for infant sex, sleep duration, annual household income, race/ethnicity, and physical activity. There was no apparent relationship between fasting duration and infant birth weight during the second trimester.

The relationship between timing of eating and fasting variables were less consistent between weekdays and weekends in the 3rd trimester. The timing of the last meal was positively associated with infant birthweight on weekdays (**Figure 14D**, 68.69 grams CI (1.64,138.96), p =0.0603), but negatively associated on weekends, although neither of these associations were statistically significant. On weekdays in the 3rd trimester, each hour later eating midpoint was related to a 104.7-gram lower in infant birth weight (**Tables 8 &9; Figure 14D**, p=0.0001). This directionality was also observed for longer fasting durations, which were associated with 82.3 CI

(-151.0, -55.1) lower infant birth weights(p =0.022). Neither of these associations remained after adjustment for gestational age at delivery.

After observing that infant birth weight models were significantly attenuated in magnitude and significance by the addition of gestational age, we conducted a sensitivity analysis that excluded preterm deliveries. We employed this restricted analysis because we had so few individuals with preterm deliveries in our sample (**Tables 1 & 3**). In the full sample that included individuals who delivered preterm, longer fasting duration and later fasting midpoint was associated with significantly lower infant birthweights (**Figure 14E**; -82.3 CI(-151.0, -13.9) p=0.022 and -104.7 CI(-154.0, -55.1) p=0.0.00011, respectively). These associations were absent in the analysis that excluded individuals who delivered preterm for both fasting duration 8.3 CI (-80.4, 97.2), p=0.85 and fasting midpoint -81.7 CI(-232.0, 68.3) p=0.29.

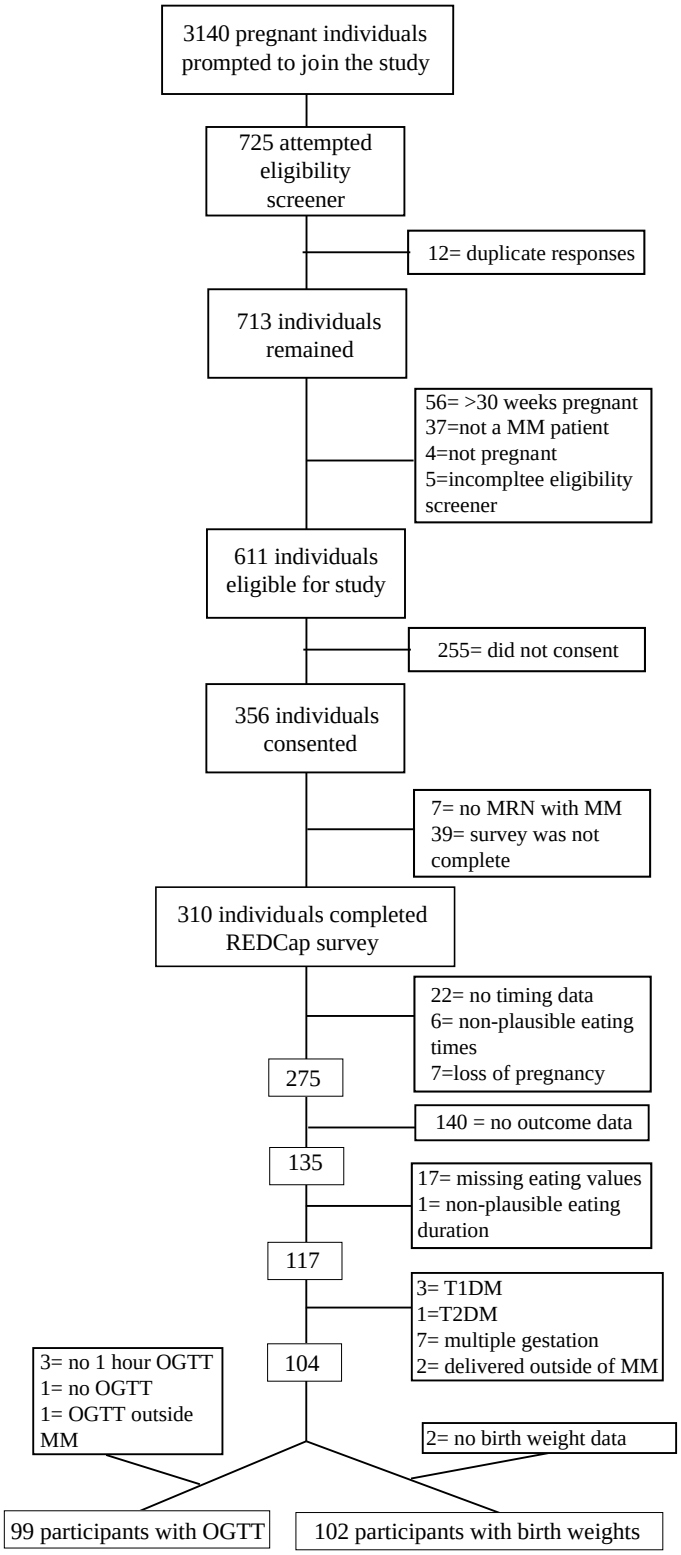


Figure 13: PRESS Participant Recruitment, Data Cleaning, and Sample Size

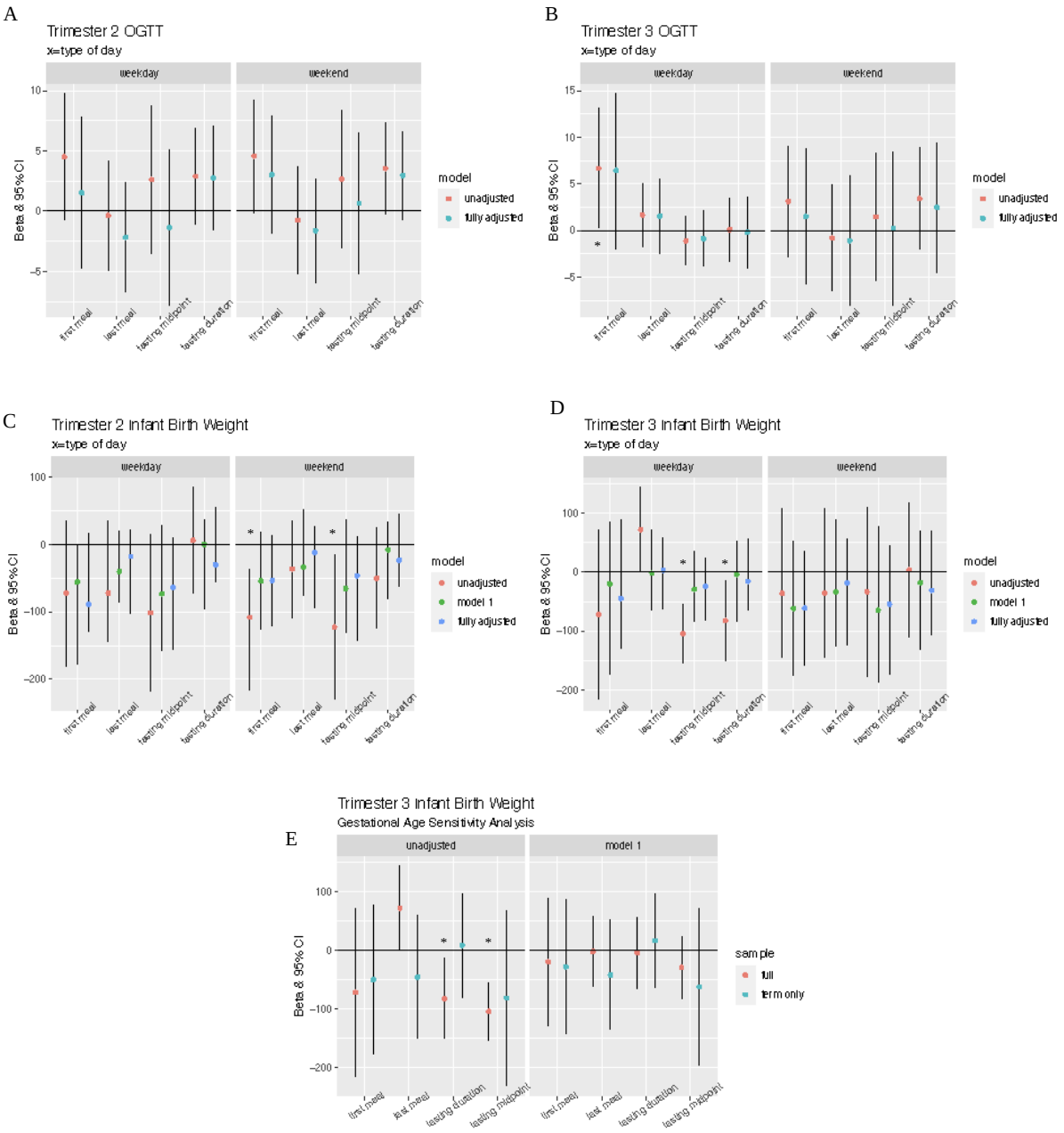


Figure 14: Associations of Timing of Eating and Fasting Duration with OGTT and Infant Birth Weight

A) Trimester 2 associations of the timing of eating with parent 1-hour oral glucose tolerance test on weekdays and weekends. Red models are adjusted for pre-pregnancy BMI, physical activity, sleep duration, household income, and self-report race/ethnicity. B) Trimester 3 associations (Beta and 95% CI) of timing of eating variables on weekdays and weekends related to with parent 1-hour oral glucose tolerance test on weekdays and weekends. Red models are adjusted for pre-pregnancy BMI, physical activity, sleep duration, household income, and self-report race/ethnicity. C) Trimester 2 associations (Beta and 95% CI) of timing of eating variables on weekdays and weekends related to infant birth weight in grams. Red models are unadjusted,

green model is adjusted for gestational age at delivery, and blue model is adjusted for gestational age at deliver, sex of infant, sleep duration, household income, parent self-reported race/ethnicity, and physical activity. D) Trimester 3 associations (Beta and 95% CI) of timing of eating variables on weekdays and weekends related to infant birth weight in grams. Red models are unadjusted, green model is adjusted for gestational age at delivery, and blue model is adjusted for gestational age at deliver, sex of infant, sleep duration, household income, parent self-reported race/ethnicity, and physical activity. E) Sensitivity analysis of Trimester 3 weekday associations with infant birthweight. Red models are in the full samples, green models exclude individuals who delivered preterm.

Table 6: Associations between mid-gestation 1-hour glucose tolerance test value and timing of eating in trimesters 2 and 3

	intercept	Unadjusted	Fully Adjusted
Trimester 2		Beta (95% CI)	Beta (95% CI)
Timing of First Meal	81.82±21.79	4.5 (-0.74, 9.76)	1.53(-4.75, 7.81)
P value		0.10	0.64
Timing of Last Meal	125.71±47.28	-0.37 (-4.90, 4.18)	-2.16 (-6.66, 2.35)
P value		0.87	0.35
Fasting Midpoint	112.32±7.58	2.62 (-3.48, 8.71)	-1.38 (-7.84, 5.07)
P value		0.40	0.68
Fasting Duration	84.42±23.98	2.90 (-1.11, 6.91)	2.76 (-1.58, 7.10)
P value		0.16	0.22
Trimester 3		Beta (95% CI)	Beta (95% CI)
Timing of First Meal	62.504±26.18	6.67 (0.30, 13.07)	6.44 (-2.00, 14.69)
P value		**0.045**	0.14
Timing of Last Meal	81.76±35.34	1.67 (-1.71, 5.04)	1.56 (-2.40, 5.51)
P value		0.34	0.44
Fasting Midpoint	118.68±4.75	-1.10 (-3.72, 1.52)	-0.85 (-3.84, 2.15)
P value		0.41	0.58
Fasting Duration	114.63±20.12	0.102 (-3.26, 3.46)	-0.17 (-3.98, 3.63)
P value		0.95	0.93

P value is from a Wald's test

unadj: OGTT (mg/dL) ~ exposure

Fully adjusted: unadj + Parent BMI, physical activity, sleep duration, household income, race/ethnicity

Table 7: Associations between mid-gestation 1-hour glucose tolerance test value and timing of eating on weekends in trimesters 2 and 3

	intercept	Unadjusted	Fully Adjusted
Trimester 2	intercept	Beta (95% CI)	Beta (95% CI)
Timing of First Meal	77.60±21.34	4.57 (-0.096,9.25)	3.02 (-1.84, 7.88)
P value		0.058	0.23
Timing of Last Meal	133.72±46.89	-0.75 (-5.15, 3.67)	-1.62 (-5.90, 2.67)
P value		0.74	0.46
Fasting Midpoint	110.73±8.71	2.64 (-3.04, 8.33)	0.65 (-5.15, 6.45)
P value		0.36	0.83
Fasting Duration	75.28±23.49	3.54 (-0.23, 7.32)	2.96 (-0.70, 6.63)
P value		0.07	0.12
Trimester 3		Beta (95% CI)	Beta (95% CI)
Timing of First Meal	88.45±26.69	3.12 (-2.80, 9.04)	1.49 (-5.76, 8.75)
P value		0.31	0.69
Timing of Last Meal	132.80±60.75	-0.81 (-6.48, 4.86)	-1.09 (-8.03, 5.83)
P value		0.78	0.76
Fasting Midpoint	111.6±10.53	1.47 (-5.37, 8.31)	0.19 (-8.05, 8.43)
P value		0.68	0.96
Fasting Duration	75.51±32.88	3.42 (-2.02, 8.87)	2.49 (-4.46, 9.43)
P value		0.22	0.49

P value is from a Wald's test

unadj:OGTT (mg/dL) ~ exposure

Fully adjusted: unadj + Parent BMI, physical activity, sleep duration, household income, race/ethnicity

Table 8: Associations between infant birth weight and timing of eating on weekdays during trimesters 2 and 3

	intercept	Unadjusted	Model 1	Fully Adjusted
Trimester 2		Beta (95% CI)	Beta (95% CI)	Beta (95% CI)
Timing of First Meal	3946.15±428.57	-71.64 (-174.96,31.64)	-55.44 (-128.52, 17.68)	-88.56 (-177.48, 0.65)
P value		0.18	0.14	0.055
Timing of Last Meal	4572.07±908.40	-59.04 (-145.80, 28.04)	-39.82 (-101.52, 21.89)	-17.42 (-84.60, 19.68)
P value		0.19	0.21	0.61
Fasting Midpoint	3595.6±145.7	-101.2 (-218.00, 16.00)	-72.95 (-156.00, 10.10)	-63.85 (-156.60, 28.88)
P value		0.094	0.089	0.18
Fasting Duration	3291.90±471.30	6.60 (-72.3, 85.5)	0.478 (-55.4, 56.3)	-29.7 (-95.7, 36.2)
P value		0.87	0.99	0.38
Trimester 3		Beta (95% CI)	Beta (95% CI)	Beta (95% CI)
Timing of First Meal	3857.87±572.17	-59.40 (-199.08, 80.28)	-19.87 (-128.52, 88.56)	-44.64 (-174.24, 84.96)
P value		0.41	0.72	0.05
Timing of Last Meal	1985.29±734.01	68.69 (1.64, 138.96)	-2.04 (-62.28, 57.96)	3.35 (-64.44, 71.28)
P value		0.0603	0.951	0.92
Fasting Midpoint	3652.10±88.0	-104.70 (-154.00, -55.1)	-29.13 (-82.3, 24.1)	-24.32 (-83.40, 34.80)
P value		**0.00011**	0.28	0.42
Fasting Duration	4339.9±410.2	-82.3 (-151.0, -13.9)	-4.15 (-65.0, 56.7)	-15.62 (-83.6, 52.3)
P value		**0.022**	0.89	0.65

P value is from a Wald's test

unadj: infant birth weight (g) ~ exposure

model 1: unadjusted model + gestational age at delivery

Fully Adjusted: Model 1+ sex of infant, sleep duration, household income, race/ethnicity, physical activity

Table 9: Associations between infant birth weight and timing of eating on weekends during trimester 2 and 3

	intercept	Unadjusted	Model 1	Fully Adjusted
Trimester 2		Beta (95% CI)	Beta (95% CI)	Beta (95% CI)
Timing of First Meal	4482.78±408.34	-125.28 (-214.56, -36)	-53.75 (-120.60, 13.00)	-53.28 (-125.64, 18.76)
P value		**0.0072**	0.12	0.15
Timing of Last Meal	4219.66±901.54	-41.04 (-125.64, 43.92)	-33.08 (-92.88, 26.82)	-11.84 (-75.60, 52.20)
P value		0.35	0.28	0.72
Fasting Midpoint	3717.7±165.5	-122.6 (-230, -15.4)	-65.12 (-142.9, 12.7)	-46.4 (-130.6, 37.8)
P value		**0.028**	0.10	0.28
Fasting Duration	3971.4±462.0	-49.8 (-124.0, 24.5)	-7.60 (-61.4, 46.2)	-23.10 (-80.0, 33.8)
P value		0.19	0.78	0.43
Trimester 3		Beta (95% CI)	Beta (95% CI)	Beta (95% CI)
Timing of First Meal	3593.58±568.15	-23.80 (-149.76, 102.24)	-61.92 (-158.04, 34.70)	-61.20 (-174.60, 52.56)
P value		0.71	0.21	0.30
Timing of Last Meal	3893.98±1248.83	-24.30 (-141.12, 92.52)	-33.48 (-123.12, 56.16)	-18.04 (-125.64, 89.28)
P value		0.68	0.47	0.74
Fasting Midpoint	3480.4±219.3	-33.4 (-177.0, 110.0)	-64.78 (-174.2, 44.7)	-54.68 (-186.9, 77.6)
P value		0.65	0.25	0.42
Fasting Duration	3341.13±689.70	3.69 (-110.0, 117.0)	-17.9 (-105.6, 69.7)	-30.97 (-131.2, 69.2)
P value		0.95	0.69	0.55

P value is from a Wald's test

unadjusted: infant birth weight (g) ~ exposure

model 1: unadjusted + gestational age at delivery

Fully Adjusted: Model 1+ sex of infant, sleep duration, household income, race/ethnicity, physical activity

4.5 Discussion

In this study of 102 parent-child dyads, we found that later timing of the first meal on weekends during the 2nd trimester was associated with a lower infant birth weights. There were also lower infant birthweights with later fasting midpoints and longer fasting duration during the 3rd trimester. These associations lost statistical significance after adjustment for gestational age at delivery, suggesting that preterm delivery could likely mediate this relationship. Third trimester later meal initiation and fasting midpoint were also related to modestly increased OGTT values in mid-gestation which lost significance but not magnitude after adjustment for covariates. To

our knowledge, this is the first report in humans that finds later meal timing is associated with reduction in birth weight. As stated previously, there is a dearth of studies that evaluate timing of eating and duration of fasting in pregnancy outside of the literature describing observance of Ramadan fasting. Our findings are consistent with many studies of women fasting while pregnant during Ramadan. Reductions in birth weight were common, but they often failed to meet statistical significance (Savitri et al., 2014, 2018; Ziaee et al., 2010). Rates of low-birthweight or small for gestational age have also found to be increased in pregnant women observing Ramadan (Cross et al., 1990; Daley et al., 2017; Opaneye et al., 1990; Ziaee et al., 2010). Although we did not evaluate this outcome, it should be considered as a future direction.

Although we were underpowered, our results suggest that preterm delivery could mediate the relationship between the timing of eating during gestation and infant birth weight. There have been studies that have linked chronobiological behaviors to risks of preterm birth, but these studies focused on sleep (Martin-Fairey et al., 2019) or the relationship between shortened sleep and eating during the night (Loy, Cheung, et al., 2020). Although we included measurements of sleep duration in our fully adjusted models for birth weight, we did so using a self-report measure that does not include sleep quality, architecture, or disturbances. It is possible that changes in sleep could underlie part of this association seen in our study, but we are limited by instrumentation error inherent in our measurement. Further independent examinations of the relationship between chronobiological behaviors, including eating and sleeping, should be conducted with respect to infant size for gestational age. This study had fewer than 10 individuals (<10% of the sample) that delivered preterm, so we were underpowered to examine this association any further.

The associations we found between the timing of eating during the 2nd or 3rd trimester and parent oral glucose tolerance test in mid-gestation were small in magnitude and failed to reach statistical significance after adjustment with covariates (although directions of the associations remained consistent). Based on our sample size and R-squared value for detecting changes in OGTT from fasting duration in trimester 3 on weekdays, we only achieved 68% power. This suggests that with a larger sample, we would have been more able to detect significant associations. Despite our more limited sample size, our findings are in line with another study in 1061 pregnancy people in the GUSTO cohort that demonstrated that longer duration of overnight fast (-0.03mmol/L), and greater meal frequency (-0.02mmol/L) were both related to a small but statistically significant decrease in fasting glucose during mid-gestation (Loy et al., 2017). The same study found that longer fasting duration and greater meal frequency was also positively associated with the 2-hour oral glucose tolerance test values. In relation to Ramadan, gestational glycemia was increased by observing fasting during pregnancy (Baynouna Al Ketbi et al., 2014), increased HOMA-IR (Ajabnoor et al., 2014). The mechanism for the associations in unknown, as few studies examine glucose response after fasting in pregnant populations. There is evidence that there is blunting of the cortisol diurnal rhythm (Bahijri et al., 2013) in pregnant populations who fast for Ramadan. As the fasting period is also paired with chronodisruption and sleep changes, it is difficult to compare this finding to the current study. One study in pregnant rats sought to understand the effect of pregnancy on the counterregulatory glucose response in rats and they found that pregnancy blunted hormonal responses to hypoglycemia (Rossi et al., 1993), but did not ask additionally what role fasting plays in this response. As such, blunting of the counterregulatory response does not explain the elevations in blood glucose in relation to longer durations of fasting during pregnancy from the current study. This could be related to differences

in glycemic homeostasis in rats compared to humans. There is also the concern that the strongest relationship with mid-gestation glycemia occurs in trimester 3, which is after the timing of the OGTT. This may be explained by reverse causality in those who had been diagnosed with gestational diabetes. This is because all patients who are diagnosed with GDM are referred to a dietitian and a diabetes educator, which would result in implementation of lifestyle changes including dietary quality and even pharmacological management. Nonetheless, we had low rates of GDM in our sample (12 cases total). It is also possible that dietary quality as an independent factor or in relation with timing of eating drove much of the association. This makes sense since earlier eating times has been shown to be associated with better dietary quality in pregnant populations(Gontijo et al., 2020). Taken together, the data suggest that lengthening the duration of the fast or manipulating timing of eating may not be a suitable dietary regimen in pregnant populations that are at risk for low-birth-weight infants or GDM.

The current study was impacted by several limitations. First, the sample size was relatively small, and few participants had repeated measurements, affecting our statistical power to detect associations in our outcomes, especially after adjustment for relevant covariates. There is also the possibility that residual confounding remains in our assessments, either based on measurement and instrumentation error, or effects from covariates that were not collected. The results are also difficult to generalize, as the PRESS cohort is not a racially, ethnically, or economically diverse population. Generalization to other pregnant people should be limited to populations that are similar in sociodemographic and lifestyle characteristics. There is likely an effect of these lived experiences on the timing of eating that we did not fully capture with our instruments, especially given that there were numerous associations between sociodemographic characteristics and eating timing and duration.

This study had several strengths that distinguish it from other studies assessing the timing of eating in pregnant populations. The exposures analyzed in the current work included multiple trimesters, which allows the possibility to detect distinct associations during specific periods of fetal development. We also evaluated weekday and weekend responses separately, as evidence from NHANES suggests that diet vary significantly in timing and quality between the two (An, 2016). The lack of shift work evident in responses also make assessing the relationship between timing of eating and perinatal health outcomes more straightforward than if there had been shift workers.

4.6 Conclusions

Later timing of eating during the second and third trimester of pregnancy may result in lower infant birth weights, potentially through preterm delivery. Longer fasting durations and later fmeal timing may also be related to a modest increase in mid-gestation OGTT. As such, timing of eating and duration of overnight fasts should be considered as both an area of further research and a factor to consider when counseling patients navigating pregnancy. These current study must be replicated in a more diverse and larger population to confirm these associations before formal recommendations can be made for clinical practitioners seeking to modulate circadian health of pregnant populations.

Chapter 5 Gestational Early Time-Restricted Feeding Results in Sex-Specific Glucose Intolerance in Adult Male Mice

5.1 Abstract

The timing of food intake is a novel dietary component that impacts health. Time-restricted feeding (TRF), a form of intermittent fasting, manipulates food timing. The timing of eating may be an important factor to consider during critical periods, like pregnancy. Nutrition during pregnancy, too, can have lasting impact on offspring health. The timing of food intake and has not been thoroughly investigated in models of pregnancy, despite evidence that interest in the practice exists. Therefore, using a mouse model, we tested the effects of gestational early TRF (eTRF) over the life course of male and female offspring and after high fat, high sucrose (HFHS) diet challenge. Body composition was similar between groups in both sexes from weaning to adulthood, with minor increases in food intake in eTRF females and slightly improved glucose tolerance in males. After 10 weeks of HFHS, male eTRF offspring developed glucose intolerance. Further studies should assess the susceptibility of males, and apparent resilience of females, to gestational eTRF related changes in islet physiology and HFHS diet in adulthood.

5.2 Introduction

All mammals have cell-autonomous clocks that coordinate the rhythm of metabolism. The molecular clock consists of the CLOCK:BMAL1 heterodimer that binds to regulatory elements in DNA (E boxes), among them are its own repressors cryptochrome (1 & 2) and period (1-3) (Lee et al., 2001). The nuclear hormone receptors ROR(α, β, and γ) and REV-ERB

(α and β) activate or repress expression of BMAL1 respectively (Panda, 2016; Takahashi, 2017). This highly coordinated transcription factor system entrains circadian rhythm in the central clock, the suprachiasmatic nucleus (SCN) of the brain, according to external cues. Peripheral tissues also possess internal clocks that can be entrained. This system imparts a rhythm of metabolism, programming predominance of melatonin during the night hours and cortisol/corticosterone during early waking hours (Panda, 2016). Factors capable of manipulating, or entraining, this system are called zeitgebers. One such potent zeitgeber is food intake (Pickel & Sung, 2020).

The timing of food intake in reference to circadian rhythms can impact propensity for health or disease (Manoogian & Panda, 2017). Time-restricted feeding/Eating (TRF/E), a method of intermittent fasting, is thought to align caloric intake with naturally occurring circadian rhythms of metabolism, acting as a zeitgeber. Timing of food intake is capable of programming metabolic systems for either poor health from chronodisruption, or good health with either diurnal or nocturnal feeding, depending on the species.

To our knowledge, no estimate of the prevalence of TRE in humans exists. However, according to one sample, up to ten percent of people surveyed that stated they followed a diet in the year 2020 had attempted "intermittent fasting," making it the most prevalent dietary intervention in that sample (International Food Information Council, 2020). There are critical periods of development in the lifespan where changes to dietary behaviors can impact current and future health status. One such critical period is pregnancy. During pregnancy, habitual timing of food intake may be altered for many reasons: religious practice, food insecurity, disordered eating behaviors, nausea and vomiting of pregnancy/morning sickness, changes in taste/food preferences, or intentional timing of eating for weight maintenance. Very little

research has evaluated the timing of eating during pregnancy and its impact on offspring health. One cross-sectional analysis found that extending the overnight fast during pregnancy was associated with lower blood glucose levels at mid gestation (Loy et al., 2017). Another recent work demonstrated that up to 23.7% of a human pregnant and recently post-partum cohort said they would be willing to try TRE during pregnancy (Flanagan et al., 2022). However, there is currently no information on the long-term implications of this dietary strategy for progeny. The most available literature examines fasting during the month of Ramadan while pregnant. Review of these studies found that children born to those who fasted during pregnancy have similar birth weights and rates of pre-term birth as those who did not fast (Glazier et al., 2018). In a recent review, Ramadan exposure *in utero* was associated with smaller body size and stature in later periods of life (Oosterwijk et al., 2021). However, these studies are limited and Ramadan fasting is an imperfect model for TRF, as food intake is not only limited in duration but also not permitted during the normal active phase for humans.

There is much interest in the TRE diet and interruptions in food intake are known to occur during pregnancy; however, research about the effects of intentional fasting during pregnancy is limited to the observance of Ramadan, a cross-sectional study about attitudes toward the practice (Flanagan et al., 2022), and one case report of fasting to improve gestational diabetes (Ali & Kunugi, 2020). Detailed modeling of TRF in pregnancy is warranted, as TRE is currently thought to exist in human populations (Ali & Kunugi, 2020; Flanagan et al., 2022) yet, long-term effects are unknown.

Previous studies of maternal diet during pregnancy have focused on dietary restriction or macronutrient excess in pregnancy, with little-to-no attention directed toward temporality of food intake. At the time of this manuscript, two studies of TRF during pregnancy in rodents exist. The

first emphasized fetal health and was completed in the context of preventing complications from a high fat, high sucrose diet (HFHS) during gestation in a rat model. Upadhyay and colleagues found that 9-hour TRF improved fetal lung development (Upadhyay et al., 2020) and placental oxidative stress markers (Upadhyay et al., 2019) at embryonic day (E)18.5 compared to ad libitum fed dams. This approach did not evaluate the long-term, postnatal effects of TRF and the independent effects of TRF are complicated by the use of a high fat, high sucrose diet. The second, also in rats, evaluated 12 hour access in light and dark cycles to a chow diet during pregnancy and followed male and female resultant offspring until 150 days of age (Prates et al., 2022). Adult female offspring of dams fed in the dark cycle with TRF were found to be glucose intolerant *in vivo*, and reduced glucose stimulated glucose secretion *in vitro* in both male and female offspring islets. altered glucose metabolism in adult offspring of TRF fed dams (Prates et al., 2022). However, this study compared 12 hour feeding to ad libitum feeding in pregnancy, leaving more restrictive windows unexamined.

The effects of TRF in non-pregnant human populations are inconsistent. Some TRF trials result in significant weight loss (Cienfuegos et al., 2020; Gabel, Hoddy, Haggerty, et al., 2018; Gill & Panda, 2015; Moro et al., 2016) while others do not (Antoni et al., 2018; Lowe et al., 2020; Sutton et al., 2018). Similarly, insulin sensitization results in some (Cienfuegos et al., 2020; Hutchison et al., 2019; Jamshed et al., 2019; Sutton et al., 2018; Wilkinson et al., 2020), but not all trials of TRF (Gabel, Hoddy, Haggerty, et al., 2018; Lowe et al., 2020). The way TRF is employed in human studies is rarely consistent, with varying lengths of feeding window (4-12 hours), timing of feeding window (early vs late), control of caloric intake (isocaloric vs ad libitum feeding), and inpatient observation or outpatient adherence monitoring. As such, the biological effects of this eating strategy are not clear, even in non-pregnant humans.

Results from rodent models of TRF are more consistent than human trials. These have found TRF of a HFHS reduces body weight compared to ad libitum feeding (Boucsein et al., 2019; Chaix et al., 2014; Chung et al., 2016; Das et al., 2021; Hatori et al., 2012; Sherman et al., 2012), can improve Homeostatic Model Assessment for Insulin Resistance (HOMA-IR) (Chung et al., 2016; She et al., 2021; Sherman et al., 2012), and may limit complications like insulin resistance (Das et al., 2021; Hatori et al., 2012) from HFHS feeding.

Taking together the likelihood that food intake can be time-disrupted in pregnancy and the evidence of TRF being a potent method to improve body composition and glycemic health in adult mice, we sought to evaluate the impact of TRF of normal laboratory chow (6-hour, early dark-cycle) before and during pregnancy on resulting offspring body composition and glycemic health through adulthood.

5.3 Methods

5.3.1 Animal Husbandry

Female C57BL/6J mice were obtained from Jackson Laboratory (RRID IMSR_JAX:000664). All animals were maintained on a, 12-hour light/dark (12 dark (ZT12, 6pm):12 light (ZT0, 6am); ZT = zeitgeber time) cycle in a temperature and humidity-controlled room. After one week of acclimatization, they were single-housed and were assigned to feeding groups. Dams were randomized to either early time-restricted feeding (eTRF) or *ad libitum* (AL) feeding during gestation (n 8= eTRF, 9=AL). This study was completed in two independent cohorts of animals. The phenotypes noted in offspring were highly consistent between cohorts. Therefore, data shown is the combined total from cohorts one and two and statistical tests do not include effects of cohort in the model. Dams fed AL had 24-hour access to a chow diet (NCD, Picolab Laboratory Rodent diet, 5L0D; 5% of Calories from fat, 24% from protein, 71% from

carbohydrates). Dams fed eTRF had 6 hours of NCD food access during the early dark cycle (ZT 14-ZT 20). Water was provided *ad libitum* throughout the study to both experimental groups. After one week of either AL or eTRF feeding (beginning age 120 days), age-matched males were introduced into cages for breeding. Males were kept in the cage until a copulatory plug was detected. Daily, dams were transferred to a clean cage at ZT20, allowing for a cage free of food for eTRF animals and similar levels of handling between experimental groups. After birth, all dams switched to AL and were maintained on this diet until weaning at postnatal day (PND) 21.5. Therefore, any phenotype in the offspring is attributable to modifications to the pre-gestational/gestational diet. All experimental protocols were reviewed and approved by The University of Michigan Institutional Animal Care and Use Committee.

5.3.2 Offspring Growth and Food Intake Monitoring

Pups born were weighed and counted within 24 hours of birth. Litters were reduced to 4 pups (2 male, 2 female, when possible) at PND 3.5 to standardize milk supply between litters. At PND 21.5, offspring were weighed and body composition was assessed using EchoMRI 2100 (EchoMRI) before being weaned by sex and maternal feeding regimen and housed 4-5 per cage (eTRF males = 11, eTRF females = 19, AL males = 16, AL females =17). Offspring were given AL access to NCD until PND 70. Food intake and body composition were assessed weekly. Food intake is represented as an average per animal per day. After PND 70, all animals began AL 45% High Fat, High Sucrose Diet (HFHS; Research Diets D12451; 45% Fat/ 20% Protein/ 35% Carbohydrate). Weekly body composition and food intake measurement continued during HFHS feeding.

5.3.3 Intraperitoneal Insulin Tolerance and Glucose Tolerance Testing

Baseline intraperitoneal insulin (ITT) and glucose tolerance tests (GTT) were assessed at young adulthood towards the end of the NCD diet period (PND 60-70, in that order). Animals were transferred into a cage with no food during the early light cycle (ZT 2), with water freely available. After 6 hours, fasting blood glucose was assessed using a tail clip and a handheld glucometer (OneTouch Ultra). Shortly thereafter, an intraperitoneal injection of insulin was administered (Humulin, u-100; 0.75U/kg lean mass). Blood glucose was assessed by glucometer every 15 minutes for 2 hours. One week later, glucose tolerance was assessed in a similar way (D-Glucose,1.5g/kg lean mass). Insulin and glucose tolerance were then re-assessed after HFHS feeding (PND 140-160) (insulin dose 2.5U/kg lean mass, glucose dose 1.0g/kg lean mass). Area under curve was calculated for each animal by taking the sum of glucose at each time point, and then was averaged by sex and maternal feeding regimen. Rates of drop for ITT were calculated by limiting the dataset to the initial period after insulin administration (<60 minutes), taking the log of the glucose values and generating a slope for each animal. After each animal's rate of drop was calculated, values were averaged by sex and treatment.

5.3.4 Glucose Stimulated Insulin Secretion

One week after GTT and ITT, animals underwent intraperitoneal glucose stimulated insulin-secretion (GSIS) testing (PND 160-170). At ZT2, animals were placed in a clean cage without food and with unrestricted access to water. After a 6-hour fast, animals were lightly anesthetized with isoflurane via drop jar and a baseline blood sample was collected via retro-orbital bleed with a heparinized capillary. Following baseline blood collection, an intraperitoneal injection of D-glucose (1.0g/kg lean mass) was given. After 15 minutes, animals were lightly anesthetized in the same manner and another blood sample was collected. Blood samples were allowed to clot on wet ice (~20 minutes), then were spun down in a cold centrifuge (4° C, Eppendorf

microcentrifuge, model 5415R) for 20 minutes at 2000 g. Serum was pipetted off and stored at -80 °C until analysis. Serum insulin was assessed via a commercially available ELISA kit (ALPCO 80-INSMSU-E10). Insulin was assessed in 5uL of serum and read via colorimetric assay.

5.3.5 Statistical Analyses

All measures with p-values <0.05 were considered statistically significant. Data are presented as mean +/- standard error throughout. All statistical analyses were performed using R version 4.0.2 (R Core Team, 2021). Repeated measures, such as body composition, cumulative food intake, and responses to GTT or ITT were assessed via mixed linear effects modeling with random effects of mouse ID and dam and fixed effects of maternal dietary treatment, age, and sex using lme4 version 1.1-26 (Bates et al., 2015). Body composition and food intake were measured separately in 2 phases: during NCD feeding, and after being switched to HFHS. Analyses were tested for significant interactions between sex and maternal dietary treatment. Models were assessed using a two-way ANOVA for sex and maternal dietary treatment, with an interaction between the two. If a significant interaction was observed, sex-stratified models were then used and the p-value for the interaction was reported. Otherwise, sex was used as a covariate in a non-interacting model. Observations were tested for normality by Shapiro-Wilk test and equivalence of variance by Levene's test. Pairwise measures that were normal and of equal variance utilized Student's *t*-tests. Measures that were not normally distributed used non-parametric Mann-Whitney tests.

5.4 Results

5.4.1 Gestational eTRF Increases Food Intake, but Not Body Weight in Early Life

To model gestational early time restricted feeding (eTRF), we used a normal chow diet (NCD) and assigned female mice to either unrestricted (*ad libitum,* AL) or 6 hours of restricted food availability between ZT14-20 (eTRF) (**Figure 15A**). This period represents the active phase of both pregnant and non-pregnant mice(Ladyman et al., 2018). This approach limits potential sleep disruptions and is more translationally relevant to human dietary restriction. This treatment started a week before mating and continued through delivery (**Figure 15B**). We find no evidence of maternal eTRF causing significantly lower daily food intake during pregnancy nor are there changes in body weight (**Supplementary Figure 1A&B**). Litters were normalized to equal sizes to reduce variability and effects of lactation.

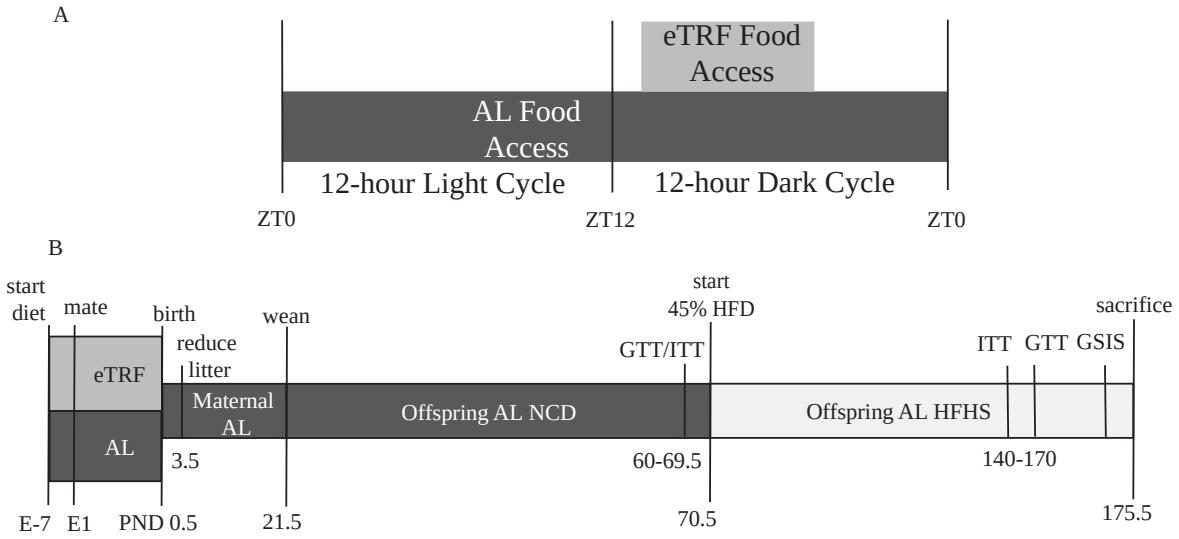
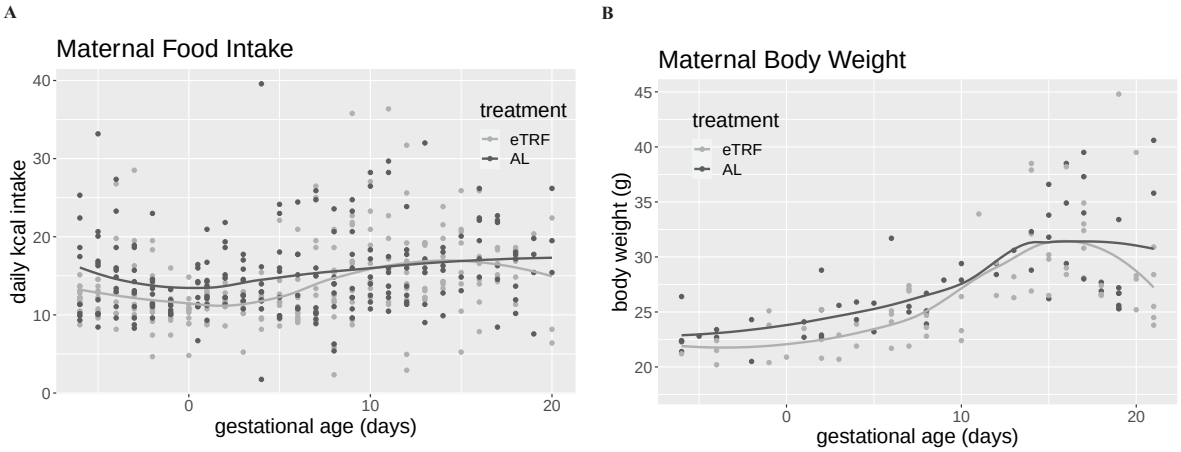


Figure 15: Early Time-Restricted Feeding Protocol

A) Food availability and timing for dams during pregnancy. Food access began at ZT13 for early Time-Restricted Feeding dams (eTRF, light gray, n=8) and continued until ZT129, total of 6 hours. Food was available 24 hours a day for ad libitum dams (AL, dark gray, n=9). **B)** Offspring experimental protocol. After birth, all dams had AL access to laboratory chow (NCD). Litters were reduced to 4 (2 males, 2 females when possible) on post-natal day (PND) 3. Offspring were weaned by maternal feeding regimen at PND 21 and maintained on AL NCD for 70 days. Weekly body composition and food intake measurements were taken throughout the experiment. At 70 days of age, insulin tolerance tests (ITT) and glucose tolerance tests (GTT) were conducted before switching all animals to a 45% high fat, high sucrose diet (HFHS) with sucrose. Animals were on HFHS for 10 weeks before repeating ITT and GTT, and an in vivo glucose stimulated insulin secretion test (GSIS). Animals were euthanized after these tests. Abbreviations: zeitgeber time (ZT), ZT0 = lights on, ZT12 = lights off.

The pups resulting from this experiment were weighed and their body composition was assessed weekly, then analyzed using linear mixed effect modeling. We found significant and expected effects of age and sex (older mice weigh more than younger mice and male pups weigh more than females), but no effect modification of maternal eTRF on body weight (**Figure 16A,** p_{diet}=0.47), lean mass (**Figure 16C,** p_{diet}=0.45), or fat mass (**Figure 16B**, p_{diet}=0.47). There was no interaction between sex and maternal feeding regimen in cumulative food intake ($p_{diet*sex}$=0.38). However, cumulative food intake in the NCD period is 22% higher in eTRF females than AL females and 10% higher in eTRF males than AL males (**Figure 16D**, p_{diet} = 0.016). Assessing the efficiency by which food is converted into stored mass resulted in a 12% reduced feeding efficiency in eTRF female offspring (p_{sex}<0.00001) which is not present in males (**Supplementary Figure 2A**).



Supplemental Figure 1: Maternal Food Intake and Body Weight during Gestation

A) Maternal food intake from one week before pregnancy until delivery B) Maternal body weight from one week before pregnancy until delivery. Dams in analysis n 8= eTRF, 9=AL.

5.4.2 Gestational eTRF Modestly Improves Glucose Tolerance in Young Adult Males

To assess glucose homeostasis in the offspring, we conducted ITTs and GTTs between PND 60 and 70. Male offspring averaged 15mg/dL higher blood glucose during insulin tolerance testing compared to females (p_{sex}=0.0018), but no effect of maternal dietary restriction was evident through linear mixed effect modeling (**Figure 16E**, p_{diet}=0.73). Summarizing the ITT by calculating the area under the curve (AUC) demonstrated there was no diet:sex interaction ($p_{diet:sex}$=0.069), but an effect of maternal restriction where eTRF offspring had lower AUC compared to AL offspring, 8.5% and 2.2% lower in females and males respectively (p_{diet}=0.013), and a significant effect of sex (p_{sex}<0.0001). As expected, males had a higher AUC than females (**Figure 16F**). The initial response to insulin (the rate of glucose decline over the first 60 minutes, not pictured) was not significant for sex (p_{sex}=0.10) or treatment (p_{diet}=0.83). These data suggest that gestational eTRF slightly improves the response to insulin challenge in adult mice, and that this is not driven by increased fat mass.

Glucose tolerance was similar in young adulthood between groups in both males and females (**Figure 16G**). We found no significant effect of diet (p_{diet}=0.53) on the rise in blood glucose during GTT, but there was an effect of sex (p_{sex}=0.0093) on glucose tolerance, again with expected higher glucose levels in male mice. The summarized AUC for the GTT (**Figure 16H**) shows a significant interaction between sex and maternal dietary treatment ($p_{sex:diet}$=0.00082). eTRF males had an 8.2% lower AUC than their AL counterparts (p_{diet}<0.0001) while this was absent in females (p_{diet}=0.99). Fasting blood glucose, assessed before ITT and GTT, was 10.4% higher in males than in females (p_{sex}=0.0054; **Figure 16I**), but did not differ significantly by maternal dietary treatment (p_{diet}=0.18). Taken together these data suggest that gestational eTRF

has very a mild effect on adult offspring, despite the narrow feeding window. Offspring whose mothers were fed eTRF had slightly improved responses to insulin and glucose challenge but no differences in body weight or in fat mass.

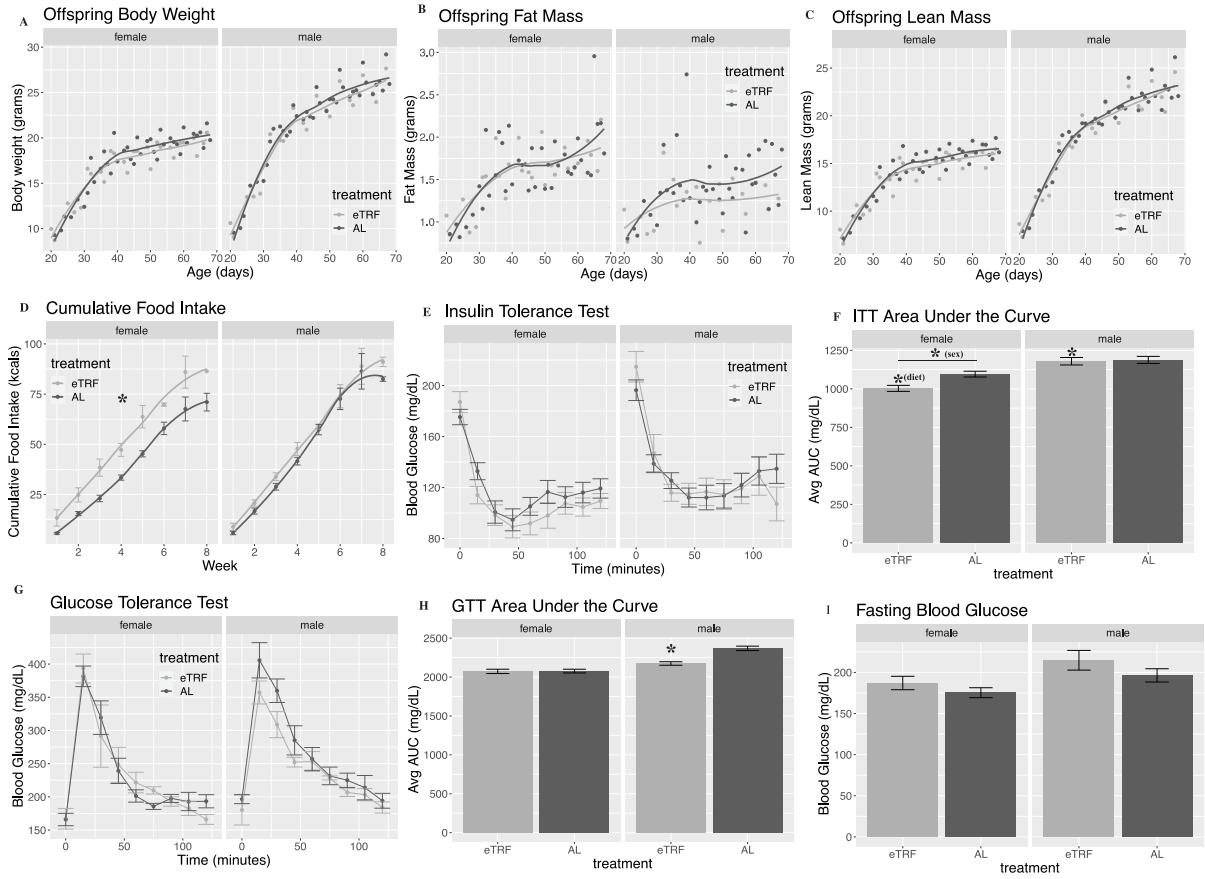
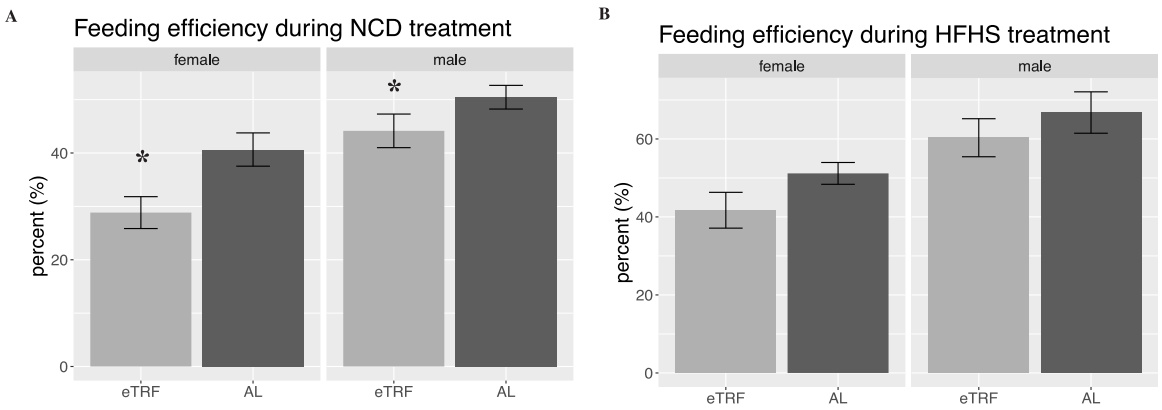


Figure 16: Early Life Body Composition, Food Intake, and Glycemic Homeostasis

A) Body weight in grams from PND21-PND70 in males and females, averaged by age, maternal feeding regimen, and sex. **B)** Fat mass in grams from PND21-PND70 in males and females, averaged by age, maternal feeding regimen, and sex. **C)** Lean mass in grams from PND21-PND70 in males and females, averaged by age, maternal feeding regimen, and sex. **D)** Food intake in kcals per mouse per day, averaged by week, maternal feeding regimen, and sex. *p-value <0.05 for diet. **E)** Insulin tolerance test (ITT) ~PND 70, averaged by maternal feeding regimen, sex, and time in minutes. **F)** Area under the curve (AUC) for ITT, averaged by maternal feeding regimen, and sex. * indicates p-value <0.05 for effect of diet in males. **G)** Glucose tolerance test (GTT) ~PNG 70, averaged by maternal feeding regimen, sex, and time in minutes. **H)** AUC for GTT, averaged by maternal feeding regimen, and sex. * indicates p-value <0.05 for effect of diet in males. **I)** Fasting blood glucose (FBG) PND 70, averaged by maternal feeding regimen and sex. Animals included in body composition measurements, FBG, ITT, and GTT, n=11 eTRF males, 16 AL males, 19 eTRF females, 17 AL females. Number of cages in food intake analysis n=4 eTRF males, 5 AL males, 4 eTRF females, 5 AL females.

5.4.3 HFHS Feeding in Adult Offspring Exposed to eTRF During Gestation Generates Sex-Specific Glucose Intolerance

Given that adult offspring were minimally affected by gestational eTRF exposure, we administered a high fat, high sucrose (HFHS) overnutrition challenge; *ad libitum* access to 45% of energy from fat and 17% of energy from sucrose after PND 70. Food intake and body composition measurements continued weekly. The average weekly food intake increased by 67.6% in AL offspring and by 31.8% in eTRF offspring after switching to HFHS, both of which exceed energy needs for adult mice (Nutrition, 1995). Similar to the findings on chow, with HFHS, there were no major differences between eTRF and AL offspring in body weight (**Figure 17A**, p_{diet}=0.99), fat mass (**Figure 17B,** p_{diet}=0.65), or lean mass (**Figure 17C,** p_{diet}=0.47). Therefore, offspring of eTRF and AL experienced similar changes in body composition in response to overnutrition. Cumulative HFHS consumption was comparable between females and males (p_{sex}=0.72), and maternal restriction groups (**Figure 17D**, p_{diet}=0.72). Feeding efficiency was greater in males than in females, which is consistent with the NCD period (**Supplemental Figure 2B,** p_{sex} = 0.00023). However, unlike the NCD period, efficiency was indistinguishable between eTRF and AL offspring (p_{diet}=0.93).



Supplemental Figure 2: Feeding Efficiency Throughout Adulthood

A) Feeding efficiency (%) in males and females, calculated based on food intake and body composition changes during the NCD period (before PND 70). (p_{sex}<0.001, p_{diet}=0.002). **B)** Feeding efficiency in males and females during the HFHS period (after PND 70). (p_{sex} = 0.00023, p_{diet} = 0.093).

We repeated an ITT and GTT after 10 weeks of HFHS feeding. During the ITT, there was a significant interaction between sex and diet using mixed linear effect modeling (**Figure 17E**, $p_{sex:diet}$=0.03). Female eTRF had a similar response to insulin, with less than a 1 mg/dL difference from their AL counterparts (p_{diet}=0.85), but male eTRF offspring tended to be more insulin sensitive with 25mg/dL lower glucose compared to AL males (p_{diet}=0.17). It could also be true that females were more resilient to impairments from the HFHS diet. These findings were confirmed by calculating the AUC where eTRF females showed no difference in AUC compared to AL females (**Figure 17F**, p_{diet}=0.20) while eTRF males had 20.4% lower AUC than AL males (p_{diet}<0.0001). The initial rate of glucose decline (not pictured) was greater in females compared to males (p_{sex}=0.029) but there were no differences between eTRF and AL offspring (p_{diet}=0.23). The trend toward insulin sensitivity from the ITT was not explained by fasting blood glucose, as females had 23% lower fasting blood glucose than males (p_{sex}<0.0001) but were similar between

eTRF and AL offspring within the same sex (**Figure 17I,** p_{diet}=0.83). Glucose tolerance tests in **Figure 17G**, also showed significant effect of interaction ($p_{sex:diet}$=0.011), although now in the opposite direction. During GTT, eTRF males trended toward glucose intolerance with an average of 53mg/dL higher glucose than AL males during the course of the experiment (p_{diet}=0.14). This was not observed in female eTRF offspring, which had similar blood glucose during the GTT compared to AL females (p_{diet}=0.61). The GTT AUC showed interaction between effects of sex and treatment (**Figure 17H,** ($p_{sex:diet}$<0.0001)). AUC was 5% lower in eTRF females (p_{diet}=0.07) but was 13.5% higher in eTRF male offspring compared to AL (p_{diet}<0.0001). Taken together, these tests suggest eTRF results in males who experience glucose intolerance and insulin sensitivity whereas females are more resilient to glycemic changes after gestational eTRF. Given that we cannot explain glucose intolerance in males via reduced insulin sensitivity, we next evaluated insulin secretion.

After noticing a consistent trend in both cohorts of eTRF males developing glucose intolerance after HFHS diet exposure, we sought to explore cohort 2 more closely for insulin secretion defects, via an *in vivo* glucose stimulated insulin secretion (GSIS) assay (**Figure 17J**). Females had lower levels of insulin than males (p_{sex}<0.0001). There was a non-significant trend toward lower insulin levels in eTRF compared to AL offspring of both sexes (p_{diet}=0.071). Females had similar increases in insulin in response to glucose injection, 139% in AL versus 137% eTRF. Male AL offspring had a 48% increase in insulin whereas this was just an 18% increase for eTRF males. There was no interaction between sex and maternal restriction ($p_{sex:diet}$=0.064). Females have 94% greater fold-change insulin secretion in response to glucose challenge than male offspring (p_{sex}=0.0027) but there was no impact of maternal restriction on fold change secretion (p=0.85, **Figure 17K**). Male and female offspring of eTRF dams had lower

baseline insulin values compared to AL dams, which we believe resulted in the similarity of fold change insulin secretion between maternal restriction groups. This study was not conclusive as it had a lower sample size and failed to reach statistical significance, but could indicate that insulin secretion is more impaired in eTRF offspring than in AL, especially after HFHS challenge in males.

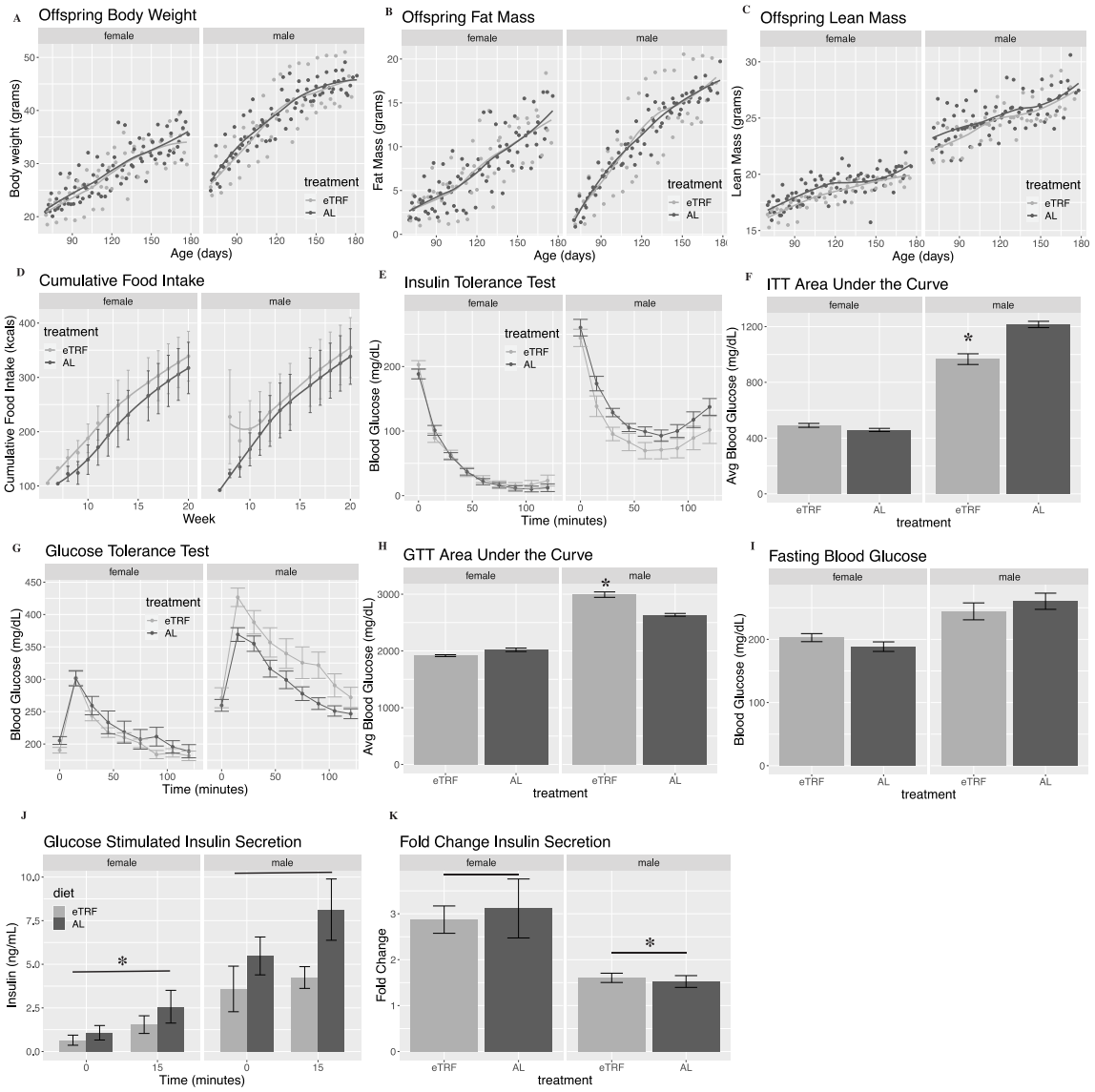


Figure 17: Body Composition, Food Intake, and Glycemic Response to High-Fat, High-Sucrose Diet Feeding in Adulthood

A) Body weight in grams from PND 70-175 in males and females, averaged by age, maternal feeding regimen, and sex. **B)** Fat mass in grams from PND 70-175 in males and females, averaged by age, maternal feeding regimen, and sex. **C)** Lean mass in grams from PND 70-175 in males and females, averaged by age, maternal feeding regimen, and sex. **D)** High fat, high sucrose diet (HFHS) intake in kcals per mouse per day averaged by week, maternal feeding regimen, and sex. **E)** Insulin tolerance test (ITT) after 10 week of HFHS, averaged by age, maternal feeding regimen, sex, and time in minutes. **F)** Area under the curve (AUC) for insulin tolerance test, averaged by maternal feeding regimen, and sex. * indicates, p-value <0.05 for diet

in males. **G)** Glucose tolerance test (GTT) after 10 weeks of HFHS, averaged by maternal feeding regimen, sex and time in minutes. **H)** Area under the curve (AUC) for GTT after 10 weeks of HFHS, averaged by maternal feeding regimen and sex. * indicates p-value <0.05 for effect of diet in males. **I)** Fasting blood glucose (FBG) after 10 weeks HFHS, averaged by maternal feeding regimen, and sex. **J)** Glucose stimulated insulin secretion (GSIS), averaged by maternal feeding regiment, sex, and time. * indicates p-value <0.05 for effect of sex. Animals included in body composition, FBG, ITT, GTT, and GSIS: n=11 eTRF males, 16 AL males, 19 eTRF females, 17 AL females. Cages in food intake analysis: n=4 eTRF males, 5 AL males, 4 eTRF females, 5 AL females.

5.5 Discussion

This study is the second to describe the long-term effects of gestational eTRF on offspring health and the first to describe their response to a high fat, high sucrose diet challenge. We find minimal effects associated with gestational eTRF while male and female offspring are consuming a chow diet through early adulthood. However, after prolonged HFHS diet feeding, there are deleterious effects on glucose tolerance only in adult male progeny. Taken together, results from insulin and glucose tolerance testing, and exploratory GSIS after HFHS feeding strongly implicates differences in insulin secretion between eTRF males. However, the latter was preliminary and did not reach statistical significance. The other study of gestational (12-hour) TRF of chow diet in rats also found evidence of glucose intolerance and insulin sensitivity in the offspring of TRF dams (Prates et al., 2022), which is similar to the phenotype we note in male eTRF offspring after prolonged HFHS feeding. However, there are some differences compared to the current study. Most notably, they found impaired glucose stimulated insulin secretion in both male and female offspring who had not been exposed to high fat diet. These glycemic effects *in vivo* were apparent in female offspring, but were present in both male and female offspring *in vitro.* Furthermore, this group found further impairments in eTRF offspring *in vivo* when timed feeding was during the light cycle. The modest reduction of insulin at baseline during GSIS in eTRF offspring may contribute to the modest insulin sensitivity seen after HFHS

feeding in the current study, and insulin sensitivity *in vivo* was evident in females in Prates and colleagues (Prates et al., 2022). There were reductions in insulin secretion in response to high glucose in male and female dark-cycle fed islets after gestational TRF, suggesting this may be a contributing mechanism for metabolic disruption in our model of gestational TRF.

Comparing the current study with other studies utilizing HFHS diets and TRF demonstrates some consistencies in glycemic outcomes. Fasting insulin can be lowered (Chung et al., 2016; Das et al., 2021; Hatori et al., 2012; Sherman et al., 2012; Woodie et al., 2018), similar to our findings, and resulting HOMA-IR can be improved with TRF (Chaix et al., 2019; Hatori et al., 2012; Woodie et al., 2018). Our finding that fasting blood glucose is unchanged in eTRF compared to AL exposed mice is consistent with other groups examining TRF with HFHS (Chaix et al., 2019; Chung et al., 2016; Woodie et al., 2018). Some differences in the current studies are not reflected in the literature; for example, elevated food intake while on NCD in female offspring exposed to eTRF *in utero* is novel and was not seen in the other longitudinal analysis of offspring health following gestational TRF (Prates et al., 2022). Studies of adult mice pairing TRF and HFHS report reduced food intake (García-Gaytán et al., 2020; She et al., 2021) or equivalent caloric intake when matched by diet (Das et al., 2021; Hatori et al., 2012; Hu et al., 2019; Sherman et al., 2012). This could indicate a compensatory response in the female offspring resulting from eTRF *in utero*. Interestingly, this did not result in differing body weight or composition, suggesting that this increased food intake is matched by decreased caloric extraction or increased energy expenditure in these mice.

The phenotype in male offspring from time-restricted feeding bears resemblance to animal models of mild intrauterine nutrient restriction, where glucose intolerance in resultant offspring can be a common phenotype. First described by Barker and colleagues, offspring who

were deprived of nutrition *in utero* were more likely to develop chronic, nutrition-related disease in adulthood (Barker et al., 1993). Since that time, multiple animal models for gestational nutrient restriction were developed; maternal overnutrition during pregnancy, maternal caloric restriction, maternal protein restriction, and surgically induced placental insufficiency through late gestation uterine artery ligation. Undernutrition in pregnancy can often result in offspring development of glucose intolerance (Alejandro et al., 2020; Shahkhalili et al., 2010; Yuan et al., 2011). The extent to which the effect is male-predominate is difficult to deduce as many groups either study male offspring exclusively (Radford & Han, 2019; Yuan et al., 2011) or analyze males and females together (Shahkhalili et al., 2010; J. Wang et al., 2016). Male offspring who had placental insufficiency can develop glucose intolerance in adulthood (Intapad et al., 2017, 2019), as can females (Jahandideh et al., 2020; Jansson & Lambert, 1999). Maternal overnutrition can also result in males with glucose intolerance (Zhang et al., 2019; Zheng et al., 2020). Therefore, metabolic effects being limited in the current study to male offspring is not unprecedented in the literature. Of note, these studies routinely find reductions in body weight as early as day 1 of postnatal life. This is inconsistent with the current study where we see no statistical reductions in body weight on either NCD or HFHS.

Although we did not evaluate insulin conclusively in the current study, glucose intolerance in nutrient restriction models has been found to co-occur with insulin-related abnormalities in the offspring, including lower insulin content in the pancreas (Shahkhalili et al., 2010), lower basal insulin levels (J. Wang et al., 2016), impaired insulin secretion (Jansson & Lambert, 1999; Yuan et al., 2011), increased pancreatic islet size (Zheng et al., 2020), altered vascularity of islets (Boehmer et al., 2017), or reduced beta cell mass (Simmons et al., 2001). These abnormalities are also accompanied by abnormal glucose tolerance in adulthood

(Alejandro et al., 2020; Zheng et al., 2020). However, in the present study we find modest improvement in male insulin sensitivity in adulthood in male offspring exposed to gestational eTRF. This finding is similar to the previous study where females exposed to gestational TRF had greater rates of glucose disappearance during insulin tolerance testing (Prates et al., 2022). We believe that the insulin sensitivity during high fat, high sucrose diet feeding in eTRF males is related to having lower basal levels of insulin compared to AL males in our model. This could result in peripheral tissues being more sensitive to insulin action despite an apparent insulin secretion impairment at the level of the pancreas.

In contrast to the previous study and some models of nutrient restriction in pregnancy, we did not observe major metabolic differences between restricted and unrestricted offspring until a HFHS diet challenge occurred in adulthood. This could suggest that gestational eTRF may be relatively safe to practice in the context of a healthful diet or absent a second challenge. However, it also suggests that in the context of unhealthy diet patterns, adult offspring may be ill-equipped to adapt to high-calorie food environments, leading to metabolic dysfunction. These studies differ both in the age of onset and duration of food restriction that are required to initiate glucose intolerance in offspring of TRF dams which also may explain these differences. Modeling of this dietary strategy remains incomplete, so translation to human clinical populations is not possible at this time. The similarity of the present study to those using diverse gestational stressors suggests that restriction of the total time pregnant dams is a novel dietary component that can have lasting impact on the spent eating in metabolic health of offspring and recommends further research on this novel component in the diet as a modulator of maternal and child metabolic health outcomes.

Although we have not investigated offspring pancreatic tissues, we hypothesize that alterations in the development of the pancreas may underlie the susceptibility of males for glucose intolerance and modest insulin sensitivity in eTRF offspring after HFHS feeding. There is recent evidence that this could be at least partially related to pancreas response to glucose, as *in vitro* and *in vivo* assessment of glycemic health were in line with our current findings, though more robust than the GSIS completed in the current study (Prates et al., 2022). Intrinsic changes in islet function are also possible. Studies done in adult male animals undergoing TRF with chronodisruption have also found that time-restricting food access reduced insulin production with secretion most affected (enhanced compared to controls) and found no effect of insulin tolerance (Brown et al., 2021). This is confirmed by one study of early post-natal exposure to TRF, which found that adolescent males who were fed TRF the first 4 weeks after weaning developed smaller islets of Langerhans and higher blood glucose compared to those fed AL (Hu et al., 2019). Therefore, future studies of gestational or developmental eTRF should examine islet size, pancreatic beta cell mass, and insulin secretion to investigate the mechanism of offspring glucose intolerance more conclusively.

This study and the conclusions to be made from it have some limitations. First, the model of gestational eTRF may have resulted in differences in maternal behaviors that were not noted by the study team, and therefore could play a part in the effects seen in the offspring. Second, although we see a robust effect on glucose intolerance, we were not powered to conclusively establish lower insulin secretion in male eTRF offspring in adulthood, and have not yet evaluated islet size or beta cell mass to determine the mechanisms driving the worsening of glucose tolerance in adulthood in male mice or the resilience of female mice. We hope that future studies

will describe these effects in larger samples and with higher resolution so that more in-depth conclusions can be drawn.

There are many strengths to this study. Among them are the use of a animal model which facilitates consistency when compared to existing literature and allows for careful control of diet, genetics, and environment throughout gestation, which would be impossible at this point in a human trial. Further strengths include the long follow up period for a gestational exposure, controlling for the effect of litter size, repeated measurement of body composition, and food intake measurements over the life course in the resultant offspring. Finally, the inclusion of both male and female offspring in the study, as many metabolic assessments of TRF either focus exclusively on the effects of the regimen in males (Hatori et al., 2012; Sherman et al., 2012) or female mice (Chung et al., 2016; Das et al., 2021) is a strength. Finally, our model used healthy non-obese dams and our results cannot be extended to effects of eTRF in the context of metabolic syndrome, diabetes, or obesity during pregnancy.

5.6 Conclusion

Offspring who are exposed to eTRF of NCD *in utero* have similar body composition, glucose tolerance, and insulin tolerance in early adulthood in both males and females. Gestational eTRF resulted in male impairments in glucose tolerance in adulthood only after chronic HFHS feeding, whereas females appeared resilient to and did not develop differences. This occurs without increase in body weight, fat mass, or food intake compared to age matched AL males. More research is warranted to understand the mechanisms that underlie this novel phenotype.

Chapter 6 Conclusions and Public Health Significance

6.1 Summary of Findings

In summary, these data find that gestational TRF in a mouse model can negatively impact late gestation glycemic health in dams, reduce postnatal survival in pups, and may program male offspring for increased susceptibility to HFHS diet-related perturbations to glycemic homeostasis regulation in adulthood. The findings from human observational work are not in total agreement with the animal work; longer fasting duration during the third trimester was related to higher mid-gestation oral glucose tolerance test values and longer fasts and later meals in the second trimester of pregnancy were related to reductions in infant birth weight. It also provided evidence that despite the association between GDF15 levels and pregnancy complications in humans and the ability of exogenous GDF15 administration to impact body weight regulation and glycemic health in non-pregnant rodent models, ablation of *Gdf15* in mice during gestation does not affect body weight regulation, food intake, or glycemic health. All of these findings are novel in pregnant populations and elucidate the gap in knowledge around chrononutrition and its impact on pregnancy related health for both parent and offspring.

6.2 Implications of Findings

The findings of this work support the conclusion that the timing of eating and duration of overnight fasts are important contributors to nutrition status that merit further study in pregnant populations. Studies completed in this work seek to illuminate the role of TRE/TRF in pregnant individuals who receive limited Chrononutrition research attention because of their classification

as a vulnerable population. Although the work of this project elucidates previously unknown impacts of TRE/TRF, there are many unanswered questions about this modality. As such, this section will highlight major impacts from this work and discuss critical future directions that must be considered to completely understand the effect of this dietary regimen in pregnant human populations.

6.2.1 Safety and Readiness to Use TRE/TRF in Pregnancy as a Diet Modality

Although both the animal and human observational methods were undertaken in the current project in an effort to make translation of findings possible, the results make the full phenotype difficult to interpret. The bulk of the evidence from chapters 2 and 5 in mice suggest that the condensation of feeding windows during gestation has little to no effect on the dam, as long as caloric sufficiency is achieved in that eating window. In direct opposition to these results is the evidence from chapter 4, which finds that longer fasting duration in a human observational study is associated with greater glycemia in mid-gestation. For this dietary regimen to be translated to clinical practice, there would need to be more robust evidence in pregnant humans that the increases in glycemia do not relate to higher rates of impaired fasting glucose or gestational diabetes. Although rodent models are convenient and are good quality analogs for human cardiometabolic disease, the physiology of rodent pregnancy is not the same as human pregnancy. This is especially true when attempting to translate reductions in litter size and pup survival to humans, as there is no similar measure in human pregnancy. If an individual wanted to use the findings from this work to determine the safety of their own use of TRE during pregnancy, it must be emphasized that it is a largely unknown field for humans. Other groups' evidence in non-pregnant humans finds that it is similarly effective as traditional methods for reducing calorie intake and body weight. The ability of TRE to aid in regulation of calorie intake

could mean further research is merited in high-risk populations who may encounter metabolic illness concurrent with pregnancy, but this was outside of the scope of the current work and could be a key future direction. While we do not know what the effects are in pregnant humans in the long term, evidence from this dissertation show that longer fasting duration affected glycemia, and as a result may impact future risk of diabetes. We were not able to follow long-term growth of the infants from chapter 4, but doing so would give key insight into the safety concerns of this modality. We were underpowered to evaluate if these findings related to higher rates of metabolic illness in pregnancy for the parent or small-for-gestational age infants. This should be prioritized before recommending TRE as intervention for pregnant populations at high risk for delivering a child with macrosomia. These data are not complete or in enough agreement to say that deliberate fasting during pregnancy is healthy or advisable but makes a strong case that further analyses in both animal and human observational models are warranted, especially for high-risk individuals.

6.2.2 Implications for Glycemia in Healthy Individuals

Further synthesizing the data from chapter 2 in this dissertation, we saw pregnant female mice who had prolonged fasting periods were more insulin resistant after reaching glucose nadir late in gestation. Although it is doubtful that this intervention as designed results in gestational diabetes in mice, there is insufficient evidence to suggest that engaging in this practice creates clinically meaningful rises in glucose in pregnant people. More explicitly, this work was not a study in diabetic animals and excluded individuals with pre-existing diabetes from our analysis in chapter 4. We also limited studies of glycemia to one timepoint in both chapters. Therefore, the generalizability of the study findings is limited to healthy, lean populations who may be less inclined to attempt dietary modifications during pregnancy anyway.

6.2.3 Implications for Adverse Mental Health from TRE

A key consideration for the effects of TRE with humans working to improve their health through dietary modification is the possible unintended impacts on mental wellbeing. It is possible that the stringent restriction guidelines of TRE in the long term may result in overly restrictive food rituals, pathological eating behaviors, or even eating disorders. Although this is a common question that arises among practitioners who serve patient populations, there are few investigation that assess these outcomes after following a time-restricted eating pattern. One study specifically examined the impact of 12 weeks of TRE in adults with obesity and they found no increase in eating disorder symptoms or weight concern (Gabel et al., 2019, p. 201; Gabel, Hoddy, & Varady, 2018). Those with who had high baseline risk for eating disorders at baseline were not eligible for the study, so findings cannot be generalized to individuals with history of eating disorders or at elevated risk of disordered eating. Other studies find higher eating disorder symptomatology scores in adults engaged in intermittent fasting, even nearing clinical significance cut offs (Cuccolo et al., 2022). Because these studies are not consistent in directionality of the association between fasting and disordered eating, it is difficult to disentangle. Especially when one considers that dietary restraint can be both hallmark of moderation and healthful weight management and of disordered eating. It is logical that a highly restrictive regimen, like TRE, would not be advisable for individuals at increased risk for disordered eating behaviors. Indeed, it also seems likely that someone who is engaged in unhealthful abstention from eating could choose to do so under the guise of improving health through adopting a fasting diet. Refocusing on the pregnant population that was central to this dissertation, being pregnant might alleviate some of the eating disorder pathology associated with TRE, as individuals anticipate increases in body weight. Therefore, future work should

prioritize examination of the effect of prolonged fasts on eating disorder symptomatology in a pregnant population one could confidently determine if this practice does or does not increase risk of psychopathological eating behaviors.

6.2.4 Implications for Further Related Studies During Gestation

The interpretation of the results from the animal model of GDF15 ablation during pregnancy brings to mind important conclusions about the difference between causation and causality in human and murine disease. Despite the storied evidence of elevations in GDF15 being related to metabolic illness, systemic stressors, and most importantly, in health complications that arise during pregnancy results from chapter 3 find there is no meaningful differences in in genetic knockout pregnancies. If there were noticeable effects of GDF15 ablation in this model, it would merit further evaluation as a causal link to complications in pregnancy. However, it is likely is that GDF15 in circulation is only correlated with adverse pregnancy effects. The incorrect assumption of causality in human illness are not novel in nutritional science. In fact, one such association that successfully invaded clinical management of patients is that of dietary cholesterol and atherosclerosis. For decades, high levels of LDL cholesterol and elevated risk of atherosclerotic lesions were thought to be caused by excessive dietary intake of cholesterol. Since that time, countless studies have demonstrated that this was correlation, not causation (McNamara, 2000), and the recommendation to reduced dietary cholesterol in order to improve blood lipid levels is no longer in use (*Dietary Guidelines for Americans, 2020-2025*, 2020). Furthermore, the evidence from this dissertation provides more compelling evidence to evaluate GDF15 with more emphasis on supraphysiological levels of GDF15 and the genetic SNPs that are more convincingly related to hyperemesis gravidarum. The evidence that elevations in GDF15 during pregnancy causes real harm and difficulty for parents and increases risk of being

diagnosed with hyperemesis gravidarum is where research attention in this population should be focused. In fact, screening based on known SNPs, or using GDF15 levels as an indicator of emesis makes more sense as a clinical tool than does GDF15 as either a biomarker or pharmacological intervention for metabolic illness in pregnant individuals.

6.3 Closing thoughts and Importance to Future Work

As a whole the data from this dissertation supports that chrononutrition impacts pregnant populations by modulating parent glycemic response and infant growth. Although the evidence from animal models and human observational studies are not in total agreement, there is still one tangible conclusion that can be made from this work. This conclusion is that the timing of eating and duration of fasting during pregnancy is a modifiable component of the diet with limited research attention, despite our evidence that attempting this diet may have current and future impacts long after delivery. Further research is needed to better characterize the safety and long-term effects of manipulating dietary windows during pregnancy, especially in larger and more diverse human population

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