**Title: Gestational Early-Time Restricted Feeding Results in Sex-Specific Glucose Intolerance in Adult Male Mice**

**Abstract**

The timing of food intake is an emerging dietary tool for management of nutrition-related diseases. One method of intermittent fasting that manipulates this is time-restricted feeding (TRF). Birthing parents experience disruptions to food intake for diverse reasons and therefore may experience periods of fasting similar to TRF protocols. These nutritional exposures during pregnancy are vastly understudied. Because interest in TRF is gaining popular interest and feeding may be interrupted in those who are expecting, it is important to study TRF during pregnancy for long term effects on the resultant offspring. Using a mouse model, the effects of gestational exposure to TRF using a chow diet are assessed over the life course in both male and female offspring. Offspring body composition was similar between experimental groups in both males and females from weaning (day 21) to adulthood (day 70), with minor increases in food intake in eTRF females and improved glucose tolerance in males. After 10 weeks of high fat diet feeding, male eTRF offspring tend to be more sensitive to insulin, develop glucose intolerance and have impaired insulin secretion. Gestational eTRF creates male-specific deleterious effects on glucose homeostasis after chronic high fat diet feeding in offspring. Further studies are needed to determine the effect gestational eTRF has on the male pancreas as well as elucidate the mechanisms that protect females from this metabolic dysfunction. **Introduction**

Recent research has highlighted that not only nutrient density and energy intake, but also that timing of intake in reference to circadian rhythms can play a part in health and disease. Robust rodent studies demonstrate that the timing of food intake is a strong zeitgeber, capable of programming metabolic systems for either poor health with models of chrono-disruption, or good health with models of dark-cycle timed feeding. The goal of time-restricted feeding (TRF), a method of intermittent fasting, is to align calorie intake with naturally occurring circadian rhythms in order to optimize health.

While prevalence of TRF, often called time-restricted eating in human studies, in human populations is currently unknown, research interest and lay materials detailing the diet are increasing, suggesting an increasing need to understand the consequences of this feeding paradigm. Birthing parents may have periods of time with limited food intake for many reasons; among them, food insecurity, disordered eating behaviors, nausea and vomiting of pregnancy/morning sickness, or intentional timing of eating for weight maintenance. The closest proxy that has the most available literature is fasting during the month of Ramadan while pregnant. The available literature rarely evaluates the effects of the practice past early infancy in the resultant children. Review of these studies find that children born to those who fasted during pregnancy have babies with similar birth weights (Daley et al., 2017; Hizli et al., 2012; Savitri et al., 2018; Ziaee et al., 2010), and are not at higher risk for pre-term birth (Awwad et al., 2012; Daley et al., 2017; Glazier et al., 2018; Hizli et al., 2012; Savitri et al., 2014). However, some studies find that longer periods of fasting(Savitri et al., 2014) or earlier timing of fasting in pregnancy (Ziaee et al., 2010) may increase risk of infants being classified as low birth weight .

Because research is limited to Ramadan fasting, more detailed modeling TRF in pregnancy is warranted, as it likely exists in human populations and effects are unknown.

Previous studies of maternal diet during pregnancy have focused on dietary restriction or macronutrient excess in pregnancy, with very little focus on temporality of food intake. To date, one study of TRF during pregnancy in animals exists. This was focused on fetal health and completed in the context of preventing complications from overnutrition (a high fat diet) during gestation. Upadhyay and colleagues found that 9-hour TRF was sufficient to improve fetal lung development by E18.5 compared to ad libitum fed dams (Upadhyay et al., 2020) and placental oxidative stress markers (Upadhyay et al., 2019). Still others have evaluated pre-conception use of TRF in promoting fertility and preventing loss of estrus cyclicity in females undergoing HFD feeding (Hua et al., 2020). These approaches do not evaluate the chronic, postnatal effects of TRF and the function of TRF is obscured by the use of a HFD.

Many investigations of TRF exist in adult rodent models outside of the context of pregnancy. These have found TRF of a high fat diet 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; Wang et al., n.d.), can improve 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 high fat diet feeding. Other models have evaluated if TRF is sufficient to entrain peripheral clocks in mice with genetic clock knockout manipulation (Chaix et al., 2019) or lesion to the central clock region of the brain (Hara et al., 2001).

Literature of TRF in human populations is less consistent with some TRF experiments yielding significant weight loss (Cienfuegos et al., 2020; Gabel et al., 2018; Gill & Panda, 2015; Karras et al., 2021; Moro et al., 2016) while others do not (Antoni et al., 2018; Lowe et al., 2020; Sutton et al., 2018). There have been studies that find that insulin sensitization can happen 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 studies of TRF (Gabel et al., 2018; Lowe et al., 2020; McAllister et al., 2019). There has been many different ways that TRF is employed in human studies; with varying length of feeding window, timing of feeding window, inpatient observation or outpatient adherence monitoring. This makes these studies all the more difficult to interpret when attempting to understand the mechanistic effects of this eating strategy.

Taking together the likelihood that food intake can be time-disrupted in pregnancy and the evidence of TRF being sufficient to alter 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.  **Methods**

*Animal care and use*

Virgin female C57BL/6J mice were obtained from Jackson Laboratory (RRID IMSR\_JAX:000664). All animals were maintained on a 12-hour (12 dark (ZT12):12 light (ZT0); ZT = zeitgeber time) dark cycle in a temperature and humidity controlled room. After one week of acclimatization, they were single housed and dietary treatment began (either eTRF or AL feeding). Dams were randomized to either early time-restricted feeding (eTRF) or *ad libitum* (AL) feeding during gestation (n 8= eTRF, 9=AL). Dams fed AL had 24 hour access to a chow laboratory diet (NCD, Picolab Laboratory Rodent diet, 5L0D; 5% Fat / 24% Protein/ 71% Carbohydrate). Dams fed eTRF had 6 hours of NCD food access during the early dark cycle (zeitgeber time , ZT 14-ZT 20. Water was provided *ad libitum* throughout the study. 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 female cage until copulatory plug appeared. Each day, dams were transferred to a clean cage at ZT20, allowing for a clean cage free of food for eTRF animals and similar levels of handling between experimental groups. After birth, all dams were switched to AL unrestricted feeding of NCD and maintained on this diet until PND 21.5. This meant that any phenotype in the offspring could be attributable to the gestational diet exclusively. All experimental protocols were reviewed and approved by The University of Michigan Institutional Animal Care and Use Committee.

*Offspring growth and food intake monitoring*

Pups born to either eTRF or AL dams 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 among 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 throughout the course of the experiment. Food intake is represented as a per animal per day average. After PND 70, all animals were switched to 45% High Fat Diet (HFD; Research Diets D12451; 45% Fat/ 20% Protein/ 35% Carbohydrate).

*Insulin Tolerance and Glucose Tolerance Testing*

Baseline glucose and insulin tolerance were assessed at young adulthood towards the end of the NCD diet period (PND 60-70). 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 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 high fat diet feeding (PND 140-160) (insulin dose 2.5U/kg lean mass, glucose dose 1.0g/kg lean mass). Area under curve was calculated by taking the sum of glucose at each time point for each animal, 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.

*Glucose Stimulated-Insulin Secretion testing in vivo*

One week after GTT and ITT, animals underwent GSIS (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 anaesthetized with isofluorane via drop jar and a baseline blood sample was collected via retro-orbital bleed with heparinized capillary tube. Following baseline blood collection, an intra-peritoneal injection of D-glucose (1.0g/kg lean mass) was given. After 15 minutes had elapsed from injection, animals were again lightly anaesthetized in the same manner and another blood sample was collected via retro-orbital bleed. Blood samples were allowed to clot on wet ice (~20 minutes), then were spun down in a cold centrifuge (4 degrees C, MODEL # HERE) for 20 minutes at 5000 RPM. Serum was pipetted off and stored at -80 degrees C until analysis. Serum insulin was assessed via commercially available ELISA kit (AIPCO 80-INSMSU-E10) Insulin was assessed in 5uL of serum and read via colorimetric assay.

*Statistical analysis*

All measures whose 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. Body composition and food intake were measured separately in 2 phases; one during NCD feeding, and another after being switched to HFD. Analyses were tested for significant interactions between sex and maternal dietary treatment. If a significant interaction was observed, sex-stratified models were then used and the p-value for the interaction was reported. Models were assessed using a two-way ANOVA with for sex and maternal dietary treatment, with interaction. Those with interaction present were then assessed separately by sex; observations were tested for normality by Shapiro-wilk test and equivalence of variance by Levene’s test. Measures that were normal and of equal variance utilized Student’s t-tests. Measures that were not normal used non-parametric Mann-Whitney tests.

**Results**

*Gestational eTRF affects food intake, but not body composition in early life*

To model 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), or 50% of their active nocturnal eating window (**Figure 1A**). This treatment started a week before mating and continued through delivery (**Figure 1B**). Litters were normalized to equal sizes to reduce variability.

The pups 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 of maternal restriction on body weight (**Figure 2A,** pdiet=0.47), lean mass (**Figure 2C,** pdiet=0.45), or fat mass (**Figure 2B**, pdiet=0.47). Interestingly, food intake over the first 70 days of life demonstrated a significant reduction in food intake for offspring exposed to eTRF during pregnancy (17.8% lower in females; p=0.00068; 9.4% lower in males, p=XXX, **Figure 2D**). Although food intake is higher in males than females, as is expected, it failed to reach statistical significance (psex = 0.054). There is no interaction between sex and maternal restriction group present (pdiet\*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. (pdiet = 0.016). By comparing the efficiency by which food is converted into stored mass, this resulted in a 12% reduced feeding efficiency in eTRF female offspring (psex<0.00001) that failed to reach significance in males (**Supplementary** **Figure 1A**).

*Gestational eTRF* *improves insulin tolerance in young adult males*

To assess glucose homeostasis in the offspring, we conducted insulin tolerance (ITT) and glucose tolerance (GTT) tests between PND 60 and 70. Male offspring had 15mg/dL higher blood glucose during insulin tolerance testing compared to females (psex=0.0018), but no effect of maternal dietary restriction was evident through linear mixed effect modeling (**Figure 2E**, pdiet=0.73). Summarizing the ITT by calculating the area under the curve (AUC) demonstrated a significant effect of both maternal restriction where eTRF offspring had lower AUC compared to AL offspring, 8.5% and 2.2% lower in females and males respectively (pdiet=0.013) and of sex where males had a higher AUC than females, which is expected in adulthood (psex<0.0001, **Figure 2F**). There was no significant diet:sex interaction. The initial response to insulin (the rate of glucose decline over the first 45 minutes) was not significantly different by sex (psex=0.10) or treatment (pdiet=0.83). This suggest that gestational eTRF slightly impairs the response to insulin challenge in adult mice, and that this is largely driven by baseline fasting glucose levels and not by increased fat mass.

Glucose tolerance was similar in young adulthood between groups in both males and females (**Figure 2G**). We found no significant effect of diet (pdiet=0.53) on the rise in blood glucose during GTT, but there was an effect of sex (psex=0.0093) on glucose tolerance, again with greater expected elevations of glucose levels in male mice . The summarized AUC for the GTT (**Figure** ) showed a significant interaction between sex and maternal dietary treatment (psex:diet=0.00082) where eTRF males had a lower 8.2% AUC than their AL counterparts (pdiet<0.0001), this effect was absent in females (pdiet=0.99). Fasting blood glucose, assessed before ITT and GTT, was 10.4% higher in males than in females (psex=0.0054) but did not differ by maternal dietary treatment (pdiet=0.18). This suggests there are modest early effects of gestational eTRF present in male offspring that were not explained by body composition, which was comparable between groups, or food intake, which was higher in eTRF animals.

*HFD Feeding in adult offspring exposed to eTRF durinig gestation generates sex-specific glucose intolerance*

Given that adult offspring were minimally affected by gestational eTRF exposure, we administered an overnutrition challenge; AL access to 45% of energy from fat after PND 70. Food intake and body composition continued to be monitored weekly. Even with HFD, there were no distinct differences between eTRF and AL offspring in body weight (**Figure 3A**, pdiet=0.99), fat mass (**Figure 3B,** pdiet=0.65),or lean mass (**Figure 3C,** pdiet=0.47). Therefore, offspring of eTRF and AL experienced a similar transition in body composition to overnutrition challenge. Females and males consumed similar amount of HFD (psex=0.088), but AL offspring consumed 4.5% less HFD over the course of the feeding period compared to eTRF offspring (**Figure 3D**, pdiet=0.00068). Feeding efficiency was greater in males than in females, which is expected and consistent with the NCD period (**Supplemental Figure 1B,** psex = 0.00023). Efficiency continued to be lower in eTRF animals (19% and 10% in females and males, respectively), although this failed to reach statistical significance (pdiet=0.93).

We repeated an ITT and GTT after 10 weeks of HFD feeding. During ITT, there was a significant interaction in the mixed linear effect modeling (**Figure 3E**, psex:diet=0.03), necessitating sex-stratified analysis. Female eTRF were similar in response to insulin, with less than a 1 mg/dL difference from their AL counterparts (pdiet=0.85), but male eTRF offspring tended to be more insulin sensitive and had 25mg/dL lower glucose than AL males (pdiet=0.17). These findings were confirmed by calculating the AUC where eTRF females had 7% greater AUC than AL females (**Figure 3F**, pdiet=0.20) and eTRF males had 20.4% lower AUC during the insulin tolerance test than AL males (pdiet<0.0001). The initial rate of glucose decline was greater in females compared to males (psex=0.029) but there was no differences between eTRF and AL offspring (pdiet=0.23). The trend toward insulin sensitivity from the ITT was not explained by fasting blood glucose, as females had was 23% lower FBG than males (psex<0.0001) but were similar between eTRF and AL offspring of the same sex (**Figure 3I,** pdiet=0.83). GTT (**Figure 3G**), also showed significant effect of interaction (psex:diet=0.011), so sex-specfic analyses were completed. During GTT, eTRF males trended toward glucose intolerance with 53mg/dL higher glucose than AL males (pdiet=0.14). This was not present in eTRF females, who had 11mg/dL higher blood glucose during GTT compared to AL females (pdiet=0.61). The GTT AUC had a significant interaction effect (**Figure 3H,** (psex:diet<0.0001), so sex-stratified analyses were conducted. AUC trended 5% lower in eTRF females (pdiet=0.07) but was 13.5% higher in eTRF males compared to AL males (pdiet<0.0001). Taken together, these tests suggest eTRF male-specific glucose intolerance and insulin sensitivity. To further understand this eTRF male-specific phenotype, we sought to assess for insulin secretion defects by conducting an *in vivo* glucose stimulated insulin secretion (GSIS) assay (**Figure 3J**).Females had lower levels of insulin than males, which is expected (psex<0.0001). There was a trend toward lower insulin levels in eTRF offspring oof both sexes (pdiet=0.071). Females had similar increases in insulin in response to glucose injection, 139% in AL versus 137% eTRF. Male AL offspring had 48% increase in insulin whereas this was reduced to an 18% increase for eTRF males. Despite these differences between sexes in effect sizes, the interaction between offspring sex and maternal restriction was not significant (psex:diet=0.064). The GSIS lends support to the theory that there could be a defect in insulin secretion after HFD challenge in males exposed to eTRF *in utero.*

**Discussion**

This study is the first to describe the long-term effects of gestational eTRF on offspring and to assess response to an overnutrition challenge. We find that deleterious effects of gestational eTRF on glucose intolerance are present only in male adults who have been exposed to long term HFD feeding. Based on glucose stimulated insulin secretion testing, this may be attributable to either insulin secretion or beta cell mass of the pancreas, as insulin secretion tended to be lower in eTRF males compared to their AL counterparts during GSIS. C-peptide, a measure of insulin secretion, has been lowered in MC4R knockout mice fed TRF (Wang et al., n.d.) but this model alleviated impaired glucose tolerance, unlike the present study. Other studies of TRF in mice, although not during gestation, have found that the fasting insulin is lowered when employed alongside high fat diet feeding (Chung et al., 2016; Das et al., 2021; Hatori et al., 2012; Sherman et al., 2012; Woodie et al., 2018) and resulting HOMA-IR is improved (Chaix et al., 2019; Hatori et al., 2012; Woodie et al., 2018). Our finding that fasting blood glucose is unchanged compared to AL exposed mice has also been reported in other models using both a high fat diet and (Chaix et al., 2019; Chung et al., 2016; Woodie et al., 2018). However, feeding HFD with time-restriction oftentimes is associated with lower bodyweights compared to the AL group (Chung et al., 2016; Hatori et al., 2012; Sherman et al., 2012), which is absent in the current study. The findings of elevated food intake in females exposed to eTRF *in utero* is also novel, as most studies find that TRF results in lower food intake when paired with high fat diet (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; Wang et al., n.d.).

Similar trends can be found in descriptions of exposure that result in animal models of intrauterine growth restriction. It is possible that time-limiting the availability of nutrients to the fetus through eTRF programmed the offspring pancreas for nutrient scarcity and resulted in impaired beta cell development or smaller sized islets leading to less insulin secretion, which has been seen in rodent models of placental insufficiency resulting in intra-uterine growth restriction (IUGR) (Boehmer et al., 2017). It may be that the smaller islets were able to compensate in young male offspring during a lower-calorie diet and therefore the effect did not become apparent until an overnutrition challenge later on. One study of developmental exposure to TRF found that adolescent males who were fed TRF the first 4 weeks after weaning developed had smaller islets of Langerhans, and higher blood glucose compared to those fed AL (Hu et al., 2019). Similar studies that expose offspring who were IUGR to overnutrition in post-natal life find that females that had IUGR exposed to a high fat, high sucrose diet have worsened glucose tolerance, not males (Intapad et al., 2019). Still others see that in utero insults like uterine artery ligation is sufficient to impair glucose tolerance and may be attributable to reduced beta cell mass over the course of life (Simmons et al., 2001).

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 perceived by the study team, and therefore could play a part in the effects seen in the offspring. Second, although we see a robust effect in glucose intolerance and trends of lower insulin secretion in male eTRF offspring in adulthood, we were not able to collect the pancreas and evaluate islet size or beta cell mass to decipher the mechanism driving the worsening of glucose tolerance in adulthood.

There are many strengths to this study. Among them are the long follow up period for a gestational exposure, repeated and compartment specific measurement of body composition, and food intake measurements over the life course in the resultant offspring. Another is 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 male (Hatori et al., 2012; Sherman et al., 2012) or female mice(Chung et al., 2016; Das et al., 2021).  **Conclusion**

Offspring who are exposed to TRF of NCD *in utero* have similar body composition, glucose tolerance, and insulin tolerance in early adulthood in both males and females. Gestational eTRF has sex-specific deleterious effects on male glucose tolerance in adulthood. Insulin sensitivity and glucose intolerance develop in males after chronic HFD feeding and occurs without increase in body weight, fat mass, or food intake compared to age matched AL males. More research is warranted to understand the mechanism that underlies this male-specific phenotype.

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