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

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**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 intentional or unintentional fasting similar to TRF protocols. Because interest in TRF is gaining popular interest and feeding may be interrupted in those who are pregnant, it is important to study TRF during pregnancy for long term effects on the resultant offspring. Using a mouse model, we tested the effects of gestational exposure to TRF 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, high sucrose diet feeding, male eTRF offspring were more sensitive to insulin, but develop glucose intolerance with impaired insulin secretion. As such, gestational eTRF causes sex-specific deleterious effects on glucose homeostasis after chronic high fat diet feeding in male 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 and energy intake, but also that timing of intake in reference to circadian rhythms can play a part in health and disease(Manoogian & Panda, 2017). 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.

To our knowledge, only no estimate of the prevalence of TRF patterns in the general human population exists. However, according to one sample, up to ten percent of people surveyed who state that they follow a diet endorse using “intermittent fasting” in the year 2020, making it the most prevalent dietary intervention in their sample (International Food Information Council, 2020). 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, religious practice, food insecurity, disordered eating behaviors, nausea and vomiting of pregnancy/morning sickness, or intentional timing of eating for weight maintenance. The most available literature is for fasting during the month of Ramadan while pregnant. 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. The literature is most focused on the effects of the practice during infancy and early childhood in the resultant children(Glazier et al., 2018). 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 at 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 complicated 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., 2020), 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 shown that 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 trials 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). Similarly, insulin sensitization is shown in 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 have been many different ways that TRF is employed in human studies; with varying length of feeding window, timing of feeding window, control of caloric intake, inpatient observation or outpatient adherence monitoring. As such, the biological effects of this eating strategy are not clear, even in non-pregnant humans.

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.  **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% 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 (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 modifications to the pre-gestational and gestational diet. 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, Eppendorf microcentrifuge, model 5415R) for 20 minutes at 2000 g. 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 (Bates et al., 2015). 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 an interaction between the two. Those results with a significant interaction were then assessed separately by sex. 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.

**Results**

*Gestational eTRF increases 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 window (**Figure 1A**). 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 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 modification of maternal eTRF on body weight (**Figure 2A,** pdiet=0.47), lean mass (**Figure 2C,** pdiet=0.45), or fat mass (**Figure 2B**, pdiet=0.47). There was no significant interaction between sex and maternal group intervention in cumulative food intake (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. (**Figure 2D**, 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* *modestly improves glucose 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 averaged 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). As expected, males had a higher AUC than females (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) did not reach significance for 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 not driven 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 expected elevations of glucose levels in male mice . The summarized AUC for the GTT (**Figure 2H**) showed a significant interaction between sex and maternal dietary treatment (psex:diet=0.00082) wherein 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; **Figure 2I**) but did not differ significantly by maternal dietary treatment (pdiet=0.18). There are modest effects of gestational eTRF present in young, chow-fed male offspring that were not explained by differences in weight or body composition, which was comparable between groups.

*HFD 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 an 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 continued to be monitored weekly. Similar to the findings on chow, with HFD, there were no major 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. Cumulative HFD consumption was comparable between females and males (psex=0.72), and maternal restriction groups (**Figure 3D**, pdiet=0.72). 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 indistinguishable between eTRF and AL offspring (pdiet=0.93).

We repeated an ITT and GTT after 10 weeks of HFD feeding. During ITT, there was a significant interaction between sex and diet. in the mixed linear effect modeling (**Figure 3E**, psex:diet=0.03). 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) while eTRF males had 20.4% lower AUC (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 within the same sex (**Figure 3I,** pdiet=0.83). Glucose tolerance tests in **Figure 3G**, also showed significant effect of interaction (psex:diet=0.011), though now in the opposite direction. During GTT, eTRF males trended toward glucose intolerance with 53mg/dL higher glucose than AL males (pdiet=0.14). This was not observed in eTRF offspring females, which had similar blood glucose during the GTT compared to AL females (pdiet=0.61). The GTT AUC had a significant interaction between sex and treatment (**Figure 3H,** (psex:diet<0.0001). AUC was 5% lower in eTRF females (pdiet=0.07) but was 13.5% higher in eTRF male offspring compared to AL males (pdiet<0.0001). Taken together, these tests suggest eTRF male-specific glucose intolerance and insulin sensitivity. Given that we cannot explain glucose intolerance in males via reduced insulin sensitivity, we evaluated insulin secretion.

To test for insulin secretion defects we conducted an *in vivo* glucose stimulated insulin secretion (GSIS) assay (**Figure 3J**).Females had lower levels of insulin than males (as expected; psex<0.0001). There was a trend toward lower insulin levels in eTRF offspring of 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. The interaction between offspring sex and maternal restriction did not reach significance (psex:diet=0.064), but the GSIS lends support to a model that sex-specific defects in insulin secretion result in sex-specific glucose intolerance 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 the 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, we propose that this is attributable to impaired insulin secretion, as insulin secretion tended to be lower in eTRF males compared to their AL counterparts during GSIS. 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 not observed in our current study, likely due to our eTRF occurred in the context of chow feeding. 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., 2020). This could indicate a compensatory response in the female offspring as a results of eTRF during pregnancy. Interestingly this did not result in differing weight or body composition, suggesting that this increased food intake is matched by decreased caloric extraction, or increased energy expenditure in these mice.

When evaluating the results of our study, we noted that glucose intolerance after maternal exposure is observed in studies that model intrauterine or fetal growth restriction. Similar trends are 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. In rodent models of placental insufficiency resulting in intra-uterine growth restriction (IUGR) (Boehmer et al., 2017). It also 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 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 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 define the mechanism driving the worsening of glucose tolerance in adulthood. Finally, our model used healthy non-obese dams and our results cannot be extended to effects of eTRF in the context of metabolic syndrome or obesity during pregnancy.

There are many strengths to this study. Among them are the long follow up period for a gestational exposure, normalization of litter size, repeated 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 was demonstrated to have sex-specific impairments in male glucose tolerance in adulthood, likely due to impaired insulin secretion. 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 mechanisms that underlies this novel phenotype.

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