# Outline

Early Time Restricted Feeding Does not Alter Food intake or Gestational Weight Gain

* There is a ~7-day adaptation period to eTRF
* Food intake is greater for eTRF dams at 6 hours, but not 24 hours
* eTRF does not cause weight loss during pregnancy

Insulin Responsiveness is Similar in eTRF Dams, but There is a More Robust Rebound from Hypoglycemia

* Insulin tolerance is similar but there is a more severe rebound of glucose after drop in eTRF dams
* Will do insulin elisa \*insulin levels are XXX in eTRF

Fecundity, birthweights and growth are similar between control and eTRF pregnancies

* similar latency/fertility
* No differences in GA or birthweight
* Significant reductions in litter size and 3-day survival
* Growth to PND 21

# Abstract

# Introduction

Dietary health during pregnancy has long been a topic of intense research interest. Since the early days of the developmental origins of health and disease (DOHaD) hypothesis when Dr. 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 (1). The most prominent of these studies children who were *in utero* during extreme famine during the “Dutch Hunger Winter” during the second world war. Finding that times of dramatically reduced food intake during pregnancy could impart higher risk for cardiometabolic risk in adulthood, even if risk ratios were adjusted for infant birthweights (2, 3). Since that time, studies seek to understand the role of adverse nutrition in the womb and its impacts on children once they are born and even well after having reached adulthood.

For ethical reasons, much work in DOHaD has been adapted to preclinical models of pregnancy. Poor nutrition in pregnancy is often accomplished in animal model through means of calorie restriction, protein restriction, or uterine artery ligation. MORE HERE Such studies often, but not always, find that pups born to dams who experienced restriction of some sort during pregnancy are smaller. When pups are followed to later stages of life, like adolescence and adulthood, there can be metabolic and body composition alterations, such as increased adipose tissue. glucose intolerance, or insulin resistance.

There is evidence to suggest that timing of food intake is an important, yet critically understudied aspect of nutrition during pregnancy. There are few models of time-restricted feeding in pregnant rodents in the scientific literature. These projects find that time-restricted feeding of high fat, high sucrose diets in rodents can reduce oxidative stress in placental tissues that results from overnutrition(4), and improve fetal lung development compared to *ad libitum* fed high fat, high sucrose dams (5). There is also evidence that estrus cyclicity and follicle development that can occur with poor nutrition are rescued with TRF of HFHS feeding compared to *ad libitum* HFHS(6). Two studies have found that TRF during pregnancy has impact for insulin homeostasis in adulthood. One finding that glucose intolerance on chow in adult offspring from eTRF dams (7), and another from our group finding that glucose intolerance only occurs in male offspring after long term high fat, high sucrose feeding (11). As the majority of the attention that has been paid to this dietary manipulation focuses on resultant offspring, scientist have failed to characterize the effects of TRF during the course of the pregnancy in the dam.

Although preclinical 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 about attitudes of trying time-restricted eating during the course of pregnancy, 24.7% of those polled said they would be open to trying a time-restricted regimen during the course of pregnancy to improve their health (9). There was also a qualitative response from one participant who stated they had practiced intermittent fasting during their pregnancy, after finding out they were 9-weeks pregnant while already following this diet. Recently, a case study of manipulation of the feeding window and reducing meal numbers to manage gestational diabetes reduced postprandial blood glucose when dietary quality manipulation and exercise was insufficient in gaining control of GDM (10). 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 (11). 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 (12, 13), does not impact birth weight (13, 14), and in some studies reduced odds of developing gestational diabetes(12, 13, 15). However, Ramadan is not an effective 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 is warranted. Recent epidemiological evidence suggests that eating overnight, although somewhat common, can be associated with poorer pregnancy birth outcomes (16). This 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 improving 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.

# Methods

## Animal Husbandry

Age-matched (age in weeks) male and female C57BL/6J mice were obtained from The Jackson Laboratories. 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.

## Animal Dietary Intervention

Dams were randomized to either 24-hour access *ad libitum* (AL), or 6-hour early-time restricted feeding (eTRF) of standard laboratory chow (24% Protein, 5% Fat, 35.7%Carbohydrate). the 6-hoour period mend that food was measured to the nearest 0.1 gram, then given in a hopper. We also measured the food in AL dam cages at ZT16. 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, and AL dams had their same hoppers replaced in their new cages. Food intake is determined in both 6-hour (ZT16-ZT20), and 24-hour intervals(ZT16-ZT16). Dams began dietary treatment

## 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 cohort 2. One hour before food was given (ZT13), 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 in (17, 18). We calculated the total number of days in each stage for each dam, then averages were taken for each maternal dietary regimen.

## Matin, 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.

## Intraperitoneal Insulin tolerance testing

Insulin tolerance was measured via an insulin tolerance test (ITT). 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 was 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 calculate the rate of rebound after hypoglycemia by limiting the data to data collected 75-120 minutes after injection, then modeling the linear rise in glucose a time:treatment interaction.

## 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).

## 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.

## 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 a equal distribution as between stages. For repeated measures, such as food intake, and body composition, linear mixed effect modeling was completed using lme4 (19). 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).

# Results

## 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 ZT16-ZT20 or *ad libitum* (AL) feeding of laboratory chow (**Figure 1A**)(8). 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**, pday: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**, pdiet: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**, pdiet = 0.66 and **Figure 2E**, pdiet = 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.

## Insulin Responsiveness is Similar in eTRF Dams, but There is a More Robust Rebound from Hypoglycemia

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 ~~0.35 ±0.07 mg/dL~~ 17.6±12.6 mg/dL greater glucose at each time point than AL dams during the course of the full 120 minutes (pdiet\*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 sensitivity. 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 hypoglycemia 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). INSERT INSULIN ELISA HERE. These data suggest that insulin sensitivity is similar to normal pregnancies in AL fed dams, but that there is a more robust response to hypoglycemia in dams who undergo chronic, prolonged overnight fasts during the perinatal period.

## Fecundity, birthweights and growth are similar between control 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.

## Pup growth to PND 21

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.

# Discussion

To our knowledge, this is the first report of the effects of chow-fed 6-hour eTRF on maternal food intake, insulin sensitivity. We find that despite the very narrow window of food availability, there is negligible effects on the dam during the course of pregnancy. There is

Hua and colleagues found increase pups per litter in both HFHS and CD

* Another limitation is that for estrus testing, we only have data for cohort 2. It is not possible to fully understand the effects of eTRF on fertility from the methods we employed. Hua and colleagues evaluated this more thoroughly, finding that TRF (# hours, which diet)
* Talk about successful mating in virgin dams and estrus/ limitation of the crudeness of the methods we used compared to Hua

Reduced litter size and reduced survival – could be secondary to pups being cannibalized before we count them?

This study has many stregnths, namely the replication of the results in 3 successive cohorts, the high sample size

# Figure Legends

Figure 1: Experimental Schema

A) Shows the light:dark cycle and availability of food for eTRF and AL dams during the perinatal period B)

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