Development and Characterization of a Mouse Model for *in utero* Type 2 Diabetes Exposure

by

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Land acknowledgement

As an immigrant to Canada, my family and I arrived on these shores without causing harm to the people who initially called this land their home. Today, as a Canadian citizen, a resident of Manitoba, and a student at the University of Manitoba, I humbly recognize that my MSc research project and my current residence are situated on the ancestral lands of the Anishinaabe, Cree, Oji-Cree, Dakota, and Dene peoples, and the homeland of the Métis Nation.

I deeply acknowledge that I am a beneficiary of this land and the rich legacy left by those who have stewarded it for generations. I hold profound respect for the Treaties that were forged on these territories and acknowledge the injustices and errors of the past.

In this moment, I commit myself to building meaningful partnerships with Indigenous communities, guided by the principles of reconciliation and collaboration. It is my aspiration to honor the traditions and cultures of the original inhabitants of this land as I strive to be a responsible steward of this shared home.

Foreword

I am deeply grateful to my supervisor, Dr. Christine Doucette, for providing me with the invaluable opportunity to undertake this project and for her unwavering support, valuable advice, and guidance throughout its execution. Her expertise and encouragement were instrumental in shaping this research.

I would also like to extend my heartfelt appreciation to our dedicated lab technician, Kristin Hunt, whose assistance and expertise in conducting the lab work were essential to the success of this study.

Finally, I wish to express my sincere thanks to my family for their constant encouragement and unwavering support during this journey. Their love and understanding have been a constant source of strength, enabling me to persevere through challenges and achieve my goals.

Without the collective contributions of these individuals, this research would not have been possible, and for that, I am truly grateful.

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List of Abbreviations

BW – body weight

CHRIM – Children's Hospital Research Institute of Manitoba

DIO - diet-induced obesity

DREAM – Diabetes Research Envisioned and Accomplished in Manitoba

FBG - fasted blood glucose

GBW – gestational body weight

GFBG – gestational fasted blood glucose

GRBG –gestational random blood glucose

GDM – gestational diabetes mellitus

GTT – glucose tolerance test

HAPO FUS – Hyperglycemia and Adverse Pregnancy Outcome Follow-up Study

HF – high fat

HFS – High-Fat and Sucrose Diet

hs-CRP – high-sensitivity C-reactive protein

ITT – insulin tolerance test

NICU – neonatal intensive care unit

PHAC – Public Health Agency of Canada

PGBW – pre-gestational body weight

PGFBG – pre-gestational fasted blood glucose

PGRBG –pre-gestational random blood glucose

RBG – random blood glucose

SEARCH – Search for diabetes in youth study

STZ – streptozotocin

T1D – type 1 diabetes

T2D – type 2 diabetes

TODAY – Type 2 Diabetes in Adolescents and Youth

VCAM1 – vascular adhesion molecule 1

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Abstract

BACKGROUND: The prevalence of childhood type 2 diabetes (T2D) in Canada, and particularly in Manitoba, is amongst the highest worldwide, resulting in an increased incidence of pregnancies complicated by T2D as children approach their reproductive years. Exposure to T2D *in utero* has detrimental effects on the health and well-being of offspring, including various malformations, stillbirth, congenital defects, metabolic disorders, a significantly higher predisposition to diabetes, early kidney failure, chronic low-grade inflammation, cardiovascular issues, mental health issues, and cognitive deficits. However, the mechanisms responsible for poorer health outcomes in T2D-exposed offspring and greater intergenerational risk remain unclear. To investigate the effects of maternal T2D on offspring physiology and health, an appropriate T2D mouse model is required. Existing approaches to induce T2D in mice often result in hypoinsulinemia and severe hyperglycemia, which does not accurately resemble T2D in humans. These approaches to induce T2D typically lead to offspring microsomia, whereas human offspring of T2D mothers often have macrosomia. Genetic models of T2D models either produce infertile mice or induce diabetes in males only, making it impossible to study the effects of *in utero* T2D exposure on offspring. Therefore, the development and characterization of an appropriate rodent model of T2D that can be used during pregnancy and more accurately resembling T2D in humans, is needed to effectively investigate the mechanisms responsible for the intergenerational transmission of T2D and mechanistic contributors to adverse health outcomes in exposed offspring.

METHODS: To develop a model of T2D pre-pregnancy, female C57BL/6J mice were exposed to a high-fat and sucrose (HFS) diet for varying durations (4, 6, 8, and 10 weeks) to induce obesity and insulin resistance. Subsequently, a one-time administration of different concentrations of streptozotocin (STZ; 75 mg/kg, 100 mg/kg, or 150 mg/kg), a chemical that specifically targets and kills pancreatic β-cell, was used to impair adaptive beta cell function without completely eliminating the β-cell pool. Weekly body weight and biweekly fasted blood glucose measurements were performed on all female mice. Two weeks after the STZ injection, the mice underwent glucose metabolism assessments, including a glucose tolerance test (GTT), insulin tolerance test (ITT), and weekly fasted blood glucose and body weight measurements. Control mice were fed a chow diet and injected with citrate buffer (the vehicle for STZ) and underwent metabolic assessments alongside the T2D groups. After pre-gestational metabolic assessments were completed, female mice were mated with healthy chow-fed C57BL/6J male mice. Maternal glycemia and body weight were measured during each trimester of gestation (weekly). Additionally, an HFS-only control group (no STZ) was established to determine the impact of the HFS diet alone. These mice were placed on the HFS diet for 10 weeks and then injected with vehicle (citrate buffer) and metabolically assessed.

RESULTS: The study revealed that female mice fed an HFS diet for 10 weeks prior to a 150 mg/kg STZ injection developed mild-to-moderate fasted hyperglycemia, glucose intolerance, insulin resistance, and higher fasted glycemia, which was maintained throughout gestation compared to control mice, indicative of pre-existing T2D prior to pregnancy and T2D throughout pregnancy. In contrast, the HFS-only control group of mice, while showing increased body weight, developed less glucose intolerance and maintained

euglycemia, suggesting that the reduction of β-cell mass via STZ was needed for T2D development in C57B6 female mice.

CONCLUSIONS: These findings suggest that through a combination of an HFS diet (10 weeks) and STZ injection (150 mg/kg of STZ), T2D that includes key features of human T2D can be generated in female C57BL6 mice while maintaining fertility. This model can be used to study the effects of *in utero* exposure to T2D on offspring development and function, while also identifying pathophysiological mechanisms underlying structural and functional changes. This information gained from the use of this model has the potential to contribute to various therapeutic approaches, the development of new drugs, and the adjustment of current clinical protocols, among other potential outcomes.

Chapter 1. Introduction

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1.1. Global epidemiology of T2D

T2D is a serious global health threat. In 2021, the International Diabetes Federation (IDF) reported a staggering 536.6 million individuals (10.5%) aged 20 to 79 affected by T2D worldwide[1]. This prevalence is projected to escalate to 12.2% by 2045[1]. Notably, more urbanized and economically developed countries face a higher prevalence of T2D[1]. By 2045, the IDF anticipates T2D rates of 21.1% in middle-income countries and 12.2% in high-income nations[1]. The financial burden associated with diabetes reached $966 billion USD globally in 2021, with projections pointing towards a rise to $1,054 billion USD by 2045[1]. In Canada, T2D continues to grow as one of the most prevalent chronic diseases[2]. The Public Health Agency of Canada indicates that 8.9% of Canadians had diabetes in 2022, with 90% of those cases being T2D[2]. Furthermore, prediabetes affects 6.1% of Canadian residents aged 20 to 79, placing them at risk for T2D development[3]. In Canada, a higher prevalence of T2D is found amongst Indigenous (First Nations and Métis), African, East Asian and South Asian backgrounds[2]. Such differences are mainly attributable to social determinants of health originating from the effects of colonization that incorporated systemic racism, which has led to intergenerational trauma that echoes nowadays in various inequalities in many aspects of Canadians' lives[4]. Moreover, these inequalities negatively influence people's abilities to receive health care in full and in a timely manner, to apply preventive strategies, and to manage current conditions, placing them at higher risk for early and more severe complications of diabetes and reducing quality of life and life expectancy.

1.2. Epidemiology of T2D in Manitoba

In Manitoba, diabetes affects ~10% (151,000) of the general population, with 90-95% having T2D; however, the combined rate of type 1 diabetes (T1D), T2D and undiagnosed T2D is estimated to be ~15%[5]. Moreover, prediabetes prevails in ~196,000 (13%) of Manitobans[5]. By 2032, Diabetes Canada predicts that the combined rates of T1D and T2D will rise to 12%, the combined rate of T1D, T2D, and undiagnosed T2D to 18%, with prediabetes increasing to 213,000 (14%)[5]. Notably, Manitoba has the highest rates of youth-onset T2D in Canada, which places them amongst the highest the world. In 2006, a national surveillance study revealed that the incidence of T2D in Canadian children (<18 years old) was 1.54 per 100,000 children per year, while in Manitoba it was 12.45 per 100,000 children per year; ~8 times higher than the national average[6]. In 2020, the Manitoba Centre for Health Policy reported that the incidence of T2D in Manitoban children rose by 50% in the last decade[7]. First Nations children in Manitoba are the most affected, with diagnosis of T2D occurring approximately 25 times more often than in non-First Nations children[7,8], constituting a prevalence of over 1% of the pediatric population[9]. Furthermore, First Nations children have a higher risk of associated complications and comorbidities[10,11]. While it is recognized that the root cause of these health disparities in First Nations children is colonization and the ensuing intergenerational trauma and dietary transformation (from high protein and high unsaturated fat to high simple carbohydrates and high saturated fat content)[12,19], there is a lack of sufficient understanding of the underlying pathophysiological mechanisms that occur in childhood T2D and hence

our current clinical guidelines are insufficient for optimal control of glycemia in First Nations children living with T2D[13-15].

1.3. T2D and pregnancy

In adults, T2D more greatly affects males than females, while in youth, females are more prone to develop the disease, which can be partly explained by a higher degree of insulin resistance during childhood and puberty in females than males.[16] According to the SEARCH study, the incidence of T2D in people younger than 20 years old is increasing[17]. Such trends were observed from 2002 to 2017 and it was predicted that by 2060 the number of children with T2D will rise by 69%, if the same rate of incidence increase continues[17]. Importantly, higher rates of T2D among a younger population leads to a higher prevalence of T2D among women of childbearing age, thereby increasing the proportion of children exposed to T2D *in utero*.

Globally, it is estimated that 14% of all pregnancies are complicated by diabetes[52]. Alarmingly, ~50% of pregnant women with T2D in Manitoba have First Nations heritage[7]. Pregnant women with T2D are three times are more likely to be hospitalized during pregnancy and their newborns more often require longer hospitalizations and have four times more admissions to the neonatal intensive care unit[7]. Additionally, maternal hyperglycemia occurring around the time of conception has been shown to increase the risk of preterm birth, stillbirth, and birth defects[20].

1.4. Effects of maternal T2D exposure on the offspring

1.4.1. Early-life complications of T2D exposure in the offspring

Preterm birth (gestational age <37 weeks) occurs in 30-40% of women with diabetes mellitus[23,24] and there is 9-14% rate of miscarriage in mothers with preexisting T2D[21]. Higher risk is associated with suboptimal glycemic control and advanced stage of T2D: 44% miscarriage rate if HbA1C>11% and diabetes duration is longer than 10 years[22]. In normalized glycemia (HbA1C<7%), the rate of miscarriage is similar to general population[22]. The rate of birth defects has been shown to increase 4-8 times (from 1% up to 2%) in poorly controlled diabetes; however, it can be up to 18% in women with long-term preexisting diabetes[22]. Among malformations, two thirds are neurological and cardiovascular defects[22]. Neurodefects are seen 20 times more often in cases of maternal hyperglycemia compared to the general population[22]. Other malformations can involve skeletal (sacral agenesis is a pathognomonic sign of preexisting diabetes), genitourinary, and gastrointestinal systems[22].

In contrast to growth restriction that can be seen in children born to T1D mothers, fetal macrosomia (larger than 90th percentile for gestational age or greater than 4000g) is reported in 15-45% of newborns born to T2D mothers (up to a 3-fold increase in size compared to babies born in the general population), where hyperinsulinemia plays a significant role[22,23]. Macrosomia is a risk factor for complications during labor and delivery and postpartum in both mother and child[22,25]. Another acute consequence of maternal diabetes is neonatal hypoglycemia, which occurs in 15-25% of T2D-exposed

babies. Neonatal hypoglycemia requires proper management and may lead to seizers, coma, brain damage, and death[22]. Hypocalcemia due to immaturity of parathyroid glands is found in as many as 50% of neonates exposed to hyperglycemia *in utero*[22]. Polycytemia, which results from the overproduction of erythropoietin due to hypoxia and hyperinsulinemia, is another neonatal complication that has been shown to result from maternal hyperglycemia, which can cause ischemia and infarction of major vital organs such as kidneys or brain[22,53]. Hyperbilirubinemia can occur two times more often and may cause a toxic effect on the central nervous system[22]. Finally, among the short-term complications of maternal diabetes exposure is acute respiratory distress syndrome (ARDS)[22]. Due to maternal hyperglycemia, the full maturation of the lungs is delayed up to 38.5 weeks and higher rate of preterm birth causes incidence of ARDS in almost one-third of births[22]. In addition to various congenital malformations, long-term complications in children exposed to hyperglycemia *in utero* include a higher risk of T2D, metabolic syndrome, obesity, dyslipidemia, cardiovascular issues, low-grade latent inflammation, early kidney failure, neurocognitive development and mental health issues, as described in the following section[28].

1.4.1. Late complications of maternal T2D exposure in the offspring

1.4.1.1. Metabolic outcomes and fetal "programming"

Early life exposures can "program" a child's metabolic health by affecting various aspects of development as well as the functioning of pancreatic β-cells later in their lifecourse[28,29]. There are various mechanisms that can influence the development of offspring leading to T2D, including modification of stem cell differentiation, epigenetic alterations, variability in metabolome and microbiome, and dysregulation of the immune system[28]. The HAPO-FUS study demonstrated that higher maternal hyperglycemia is associated with poorer insulin sensitivity and a lower oral disposition index in the exposed child[34]. The TODAY study revealed that children exposed to maternal diabetes *in utero* were diagnosed with T2D at a younger age (-7 months), had higher baseline glycemia and HbA1C (+0.3%), and worse β-cell function (assessed by the C-peptide oral disposition index) compared to non-exposed children[30]. Clinical studies revealed that *in utero* exposure to diabetes mellitus among the Pima population was a major risk factor for diabetes and hyperglycemia in the offspring[26,27]. According to the SEARCH study, maternal diabetes is responsible for 47% of the risk of T2D in the offspring[31]. The DREAM team at CHRIM have also found that maternal T2D is associated with a 4-fold greater risk of childhood-onset T2D when compared to children who are exposed to gestational diabetes mellitus (GDM)[32]. Undoubtedly, maternal hyperglycemia influences future glucose metabolism and predisposes progeny to T2D; however, the exact mechanisms are unclear.

Youth T2D is also more aggressive and poorly controlled using current treatment protocols compared to adult T2D[28]. The TODAY study showed a treatment failure in almost 50% of participants because either their HbA1c level rose above 8% during study period or they could not titrate insulin to maintain glycemia within the target range[28,33]. Metformin with lifestyle modification did not show any difference in glycemia compared to metformin alone but metformin intensified with rosiglitazone did[33]. This suggests that the pathogenesis of T2D in children differs from adults and studies are needed to

investigate mechanistic contributors to youth T2D to adjust treatment protocols, possibly considering different treatment approaches and development of different preventative measures.

Exposure to hyperglycemia *in utero* stimulates adiposity risk in the offspring by the increase of fetal insulin production and is associated with metabolic disorders later in life[23,28,37]. In addition, maternal obesity, which often accompanies T2D and participates in its pathogenesis, also plays an important role in "adiposation" of the child acting via placental lipases that increase fetal-placental availability of free fatty acids[28,35,36]. Studies suggest that higher body weight is preserved and increased later in life in such children due to "malprogrammed" metabolism and higher leptin and lower adiponectin levels[23,37-39]. Larger BMI and abdominal circumference, increase in leptin by 34%, and decrease in adiponectin by 12% were found in children (6-13 years old) exposed to diabetes *in utero*[40]. Furthermore, there is a correlation between the risk of a childhood metabolic syndrome later in life that includes childhood obesity, hypertension, dyslipidemia, and glucose intolerance later in life[23,38].

Moreover, children born to women with diabetes showed increased biomarkers of endothelial damage and inflammation (E-selectin and VCAM1), and systolic blood pressure, suggesting signs of preclinical stages of atherosclerosis[23,40]. In combination with T2D and abdominal obesity, such findings place those children at risk for cardiovascular disorders. The Finnish study on children 4.4 – 9.7 years old demonstrated significantly higher levels of high-sensitivity C-reactive protein (hs-CRP) in the diabetes-exposed group compared to the control[41]. Low-grade latent inflammation (measured by hs-CRP) is an independent risk factor for vascular- and all-cause mortality and may be a considered as a treatment target in patients with T2D to lower risk for cardiovascular diseases[42].

1.4.1.2. Effects of maternal T2D on kidney development and function

In addition to various malformations, children exposed to maternal hyperglycemia show various developmental and functional issues. Murine models of diabetes have shown that compared to normoglycemic conditions, the offspring of mothers with diabetes develop renal hypertension, microalbuminuria, and glucose intolerance explained by the imbalance of renin-angiotensin-aldosterone system and accumulation of extracellular matrix protein within the renal cortex[44]. Children living with T2D, who were exposed to T2D *in utero,* are associated with a higher risk of having albuminuria at the time of T2D diagnosis[45], suggesting pre-existing risk. In humans, nephrogenesis starts at ~week 5 and lasts until weeks 34-36 of gestation with the formation of first nephrons occurring at ~week 9[49]. Therefore, exposure to maternal T2D throughout gestation is likely a critical determinant of nephron devlopment[50,51], and ultimately kidney function. However, the exact underlying mechanisms are unclear and the majority of animal studies on effects of maternal hyperglycemia on kidneys are run on T1D models but not T2D, which has a different pathogenesis.

1.4.1.3. Neurological and mental outcomes for offspring exposed to T2D *in utero*

Neurological defects are very common in children exposed to hyperglycemia *in utero*[22]. Studies on both humans and animals show connection between maternal diabetes status and motor deficits,

cognitive impairment, mental issues such as autism spectrum disorders, attention deficit hyperactivity disorder, learning abilities, and psychiatric disorders[53]. Fetal "programming" takes place during prenatal period and intrauterine stress increases children's susceptibility to psychopathology.[54]. Among possible mechanisms that lead for the hyperglycemia adverse effects on fetal nervous system are neuroinflammation, oxidative stress, iron deficiency, epigenetic influences, and metabolic dysregulation[53]. Therefore, exposure to maternal T2D throughout gestation is likely a critical determinant of organogenesis and, ultimately, physical and mental health. However, the underlying mechanisms are often unclear, and maternal T2D murine models will be helpful in studying them.

1.5 Studying underlying mechanisms of T2D exposure on the offspring

To investigate the mechanistic contributors and pathophysiological mechanisms underlying the detrimental effects of maternal T2D exposure and further determine the optimal intervention windows, a proper mouse model is required. Existing approaches to induce T2D in mice often result in uncontrolled and severe hyperglycemia, which does not accurately resemble T2D in humans during pregnancy[23, 25, 39, 55-58]. These models typically lead to offspring microsomia, whereas human offspring of T2D mothers often have macrosomia pregnancy[23, 25, 39, 55-58]. This suggests that the impact of maternal diabetes on offspring may be different from what is observed in current mouse models.

A maternal T2D mouse model that is suitable for use in such studies, should develop hyperinsulinemia due to hyperplasia of β-cells[76] with mild-to-moderate basal and moderate-to-high postprandial hyperglycemia as well as insulin resistance prior to gestation. Ideally, this should develop within 3-5 months of age to successfully complete mating by the age of 6-months-old to exclude aging as another potential confounder. Mouse offspring that result from such T2D exposure should have normal or increased weight. The goal of this thesis is to develop and characterize an appropriate model of T2D in mice that can be used to explore the impact of maternal T2D in utero exposure on the offspring.

1.6 Current models of T2D in mice

Jackson Labs, a world-leading supplier of experimental mice and rodent models, has produced T2D mouse models since the 1960s but they acknowledge that there is "no single diabetic mouse model that recapitulates all of the features or complications of human diabetes"[55]. The most common mouse models used in T2D research are diet-induced obesity (DIO) models such as the *Lepob/ob* and *LepRdb/db* mouse models, which result from homozygous mutations in the leptin and leptin receptor genes, respectively. These mutations in leptin signaling cause hyperphagia, morbid obesity, chronic hyperglycemia, and pancreatic β-cell atrophy[55]. Importantly, these mice are infertile as such they cannot be used in studies that examine T2D during pregnancy[56]. Interestingly, heterozygous mice ob/- and db/- do not develop significant hyperglycemia compared to controls[57]. Driven by hyperphagia, which ultimately leads to the exhaustion of β-cells, KK-Ay mice typically begin to exhibit signs of T2D around 4 to 6 months of age, with the disease becoming more pronounced as they age[55]. Polygenic mice such as NONcNZO10/LtJ, and TALLYHO/JngJ develop hyperinsulinemia due to β-cell hypertrophy but only by 6 months of age or later, which is too late for studying T2D in pregnancy, and the main disadvantage is

T2D develops only in males[55]. As such, the development of a model of T2D is needed to more appropriately examine how T2D exposure *in utero* influences offspring health.

1.7 Dietary exposure/diet-induce obesity and glucose metabolism in mice

Another approach is to drive metabolic dysfunction is to trigger diet-induced obesity. In this approach, animals are chronically fed a high fat (HF) diet or high fat and sucrose (HFS) diet, mimicking a modern/westernized diet in humans, which drives obesity and insulin resistance[66]. This is an effective method and successfully utilized for studying GDM because of the presence of insulin resistance in females prior to pregnancy, while gestation triggers development of GDM[60]; however, sole HFS diet treatment does not manifest into pregestational T2D. Heydemann's study showed that some mouse strains (Lrg, A/J, BXD77, BALB/cJ) are especially resistant to hyperglycemia and MRL female mice require longer than 6-8 months exposure to HFS diet to develop T2D, which does not align with the goals of the study because of the potential aging related changes that can affect offspring or cause fertility issues in dams[58]. Consequently, some stimulus to reduce beta cell functionalilty in addition to the diet-driven insulin resistance would be beneficial to generate T2D.

1.8 Streptozotocin use in mice

Researchers have successfully used streptozotocin (STZ), a chemotherapeutic agent for pancreatic insulin producing tumors, to induce T1D diabetes in mice[59,60]. STZ selectively targets β-cell, enters the cell via GLUT2 transporters, causing mitochondrial damage, alkylation of the DNA, and eventual cell death within 48 hours[59,74,75]. We speculate that a modest STZ injection administered intraperitoneally to the animal can selectively reduce the population of β-cells, without complete removal, thereby instigating the onset of T2D alongside dietary exposure. This combination ensures that compensatory β-cell mechanisms are insufficient to sustain normoglycemia in the face of diet-induced insulin resistance and the animal develops hyperglycemia.

There are different approaches to induce diabetes in mice using STZ, including a single large dose or multiple low doses of STZ. The C57BL/6 substrain of mice are considered to be highly sensitive to STZ (DBA/2 > C57BL/6 > MRL/MP > 129/SvEv > BALB/c)[69]; however, female C57BL/6 mice are significantly less sensitive to STZ than males[68, 69]. For instance, while a cumulative dose of 50-55 mg/kg of STZ during 3-5 consecutive days causes T1D (i.e. kills more than 70% of pancreatic β-cell) in 100% of males, only one-third female mice developed T1D[68, 69, 71]. Females were shown to be sensitive to a one time 150 mg/kg dose or 75 mg/kg for 5 consecutive days[69,70]. It is anticipated that an exposure to HFS diet and diet-induced obesity (DIO) in female C57BL/6J mice would develop β-cell hyperplasia, which would require a significantly higher dose of STZ than for mice on chow diet, as shown in previous studies[58,61].

A combination of DIO and STZ has been widely used in the past for modeling T2D in rodents. Zhuo et al. reported that a low dose of STZ can partially impair β cells in the pancreas, while prolonged

consumption of HFSD can elevate lipid metabolites, disrupt the insulin signaling pathway, and activate the p38 mitogen-activated protein kinase (MAPK) pathway[43]. Other studies have shown that HFS+STZ is effective in inducing T2D characteristics, such as hyperglycemia, hypertriglyceridemia, and other metabolic disturbances in rodents[46-48]. However, these studies were primarily conducted in males, or they indicated that females are less responsive to the treatment[43,46-48]. Additionally, none of these studies attempted to replicate maternal T2D in mice, which is the primary goal of the current project[43,46-48].

1.9 Selection of a mouse strain

Choosing a mouse model for metabolic research can be challenging. C57BL/6J mice are known for its susceptibility for developing DIO and commonly used for metabolic research due to a deletion of the nicotinamide nucleotide transhydrogenase (*Nnt*) gene responsible for a mitochondrial enzyme involved in NADPH synthesis[61,62]. The Nnt mutation is associated with reduced glucose-stimulated insulin secretion and impaired glucose tolerance[61,63]. C57BL/6J mice have been successfully used for a GDM model that develop GDM via HF or HFS diet and subsequent pregnancy[64,65].

1.10 Phenocopying human T2D physiology during pregnancy

Maternal type 2 diabetes (T2D) during pregnancy is characterized by insulin resistance, hyperglycemia, and alterations in glucose metabolism, which can lead to complications for both the mother and the offspring[20,23,25,30,86]. In humans, T2D during pregnancy is typically marked by moderate-to-high postprandial hyperglycemia, with fasting blood glucose levels exceeding 5.3 mmol/L and postprandial levels surpassing 11.1 mmol/L[86]. Between these values lies a grey area indicative of impaired glucose tolerance or impaired fasting glucose[86]. Insulin resistance progressively increases throughout gestation[23,30, 86]. During the first trimester, insulin resistance rises as pregnancy-related hormones increase, and in the second trimester, this intolerance worsens[23-25]. Glycemic targets for the first and second trimesters include fasting blood glucose (FBG) <5.3 mmol/L, 1-hour postprandial glucose <7.5 mmol/L, 2-hour postprandial glucose <6.7 mmol/L, and HbA1c <6.5%[86]. In the third trimester, in order to minimize risks such as macrosomia and other complications of diabetes, the glycemic targets are further reduced: FBG <4.5 mmol/L, 2-hour postprandial blood glucose between 5.6 and 6.1 mmol/L, and HbA1c ≤6.1%[86]. Since maternal insulin does not cross the placenta and fetal insulin crosses the hemato-placental barrier only minimally, the reduction in maternal glycemia during the third trimester can be partially attributed to the utilization of glucose by the fetus[23-25,30]. This explains the occurrence of fetal macrosomia in cases of poorly controlled diabetes.

In Canada, the majority of pregnant women with T2D receive prenatal care and diabetes management, although access to healthcare can be more limited in rural and remote areas[87-89]. In contrast, most mouse models of T2D during pregnancy involve uncontrolled diabetes, often accompanied with severe hyperglycemia, which leads to more pronounced fetal developmental issues in the short term[55-60]. These models do not accurately replicate the real-world scenario, where most women do not experience severe hyperglycemia during pregnancy[23,25,30,86-89]. As a result, it is crucial for these models to better

phenocopy human pregnancy, particularly focusing on mild-to-moderate fasting hyperglycemia. Additionally, rodents exhibit different feeding patterns, consuming food throughout the day without designated meal times[55-60]. Variability in data on non-gestational and gestational glycemia in mice has been reported, with significant discrepancies across studies[55-60]. Defining glycemic thresholds is essential for improving the phenocopying of human T2D during pregnancy in animal models and human's target glycemic ranges maybe used as reference.

1.11 Hypothesis

We hypothesize that a combination of HFS diet mimicking modern diet with a low-dose of STZ will induce T2D in C57BL/6J female mice which can serve as a model to study exposure to maternal T2D *in utero*. We anticipate that this mouse model will better phenocopy the clinical course of T2D in human pregnancy, which is characterized by mild-to-moderate basal and moderate-to-high postprandial hyperglycemia as well as insulin resistance and fetal normo- or macrosomia.

1.12 Specific Aims

1.12.1. Aim 1: To develop a protocol for pregestational T2D induction in female mice with a low dose STZ injection and HFS diet.

Maternal T2D was induced in female C57BL6J mice preconception using a high fat and sucrose (HFS) feeding approach (to induce insulin resistance) combined with a low-dose of STZ to induce β cell dysfunction and hyperglycemia[45]. As this is a novel model, various doses of STZ and duration of diet exposure were tested to determine the best combination to effectively induce T2D in female mice prior to mating. HFS diet (45% fat; 35% carbohydrate; Research Diets D12451) was fed to wild type C57BL6J female mice at weaning for 4, 6, 8, and 10 weeks. After 4, 6, 8, or 10 weeks, mice were injected with one of 3 dose concentrations of STZ (75, 100, and 150 mg/kg BW), administered intraperitoneally. After two weeks, while remaining on the HFS diet, mice underwent a glucose tolerance test (GTT) with blood collection for insulin quantification, an insulin tolerance test (ITT) and fasted blood glucose levels assessed to determine if the animals have developed T2D. Two control groups were established: one is on HFS diet and other one on a chow diet; both injecting citrate buffer (sham) instead of STZ.

1.12.2. Aim 2: To determine the severity of T2D during pregnancy.

Upon successfully inducing T2D in C57BL6J female mice using the approach described in Aim 1, T2D females were mated with healthy chow-fed males to produce offspring that were exposed to T2D *in utero*. Pregnant female mice were assessed weekly during gestation (three measurements in total, in each trimester) with fasted blood glucose and body weight measurements to ensure that the mothers had appropriate T2D during their pregnancies. A control cross was set up with female mice fed a chow diet and injected with vehicle (citrate buffer) at the same time point as the experimental group, maintaining normoglycemia and metabolic health entering and during pregnancy.

Chapter 2. Methodology

Dr. Christine Doucette contributed to the development and shaping of the study methodology.

Kristin Hunt provided expertise, training, and assistance in technical procedures, animal care, and data analysis.

Bo Xiang provided expertise and dedicated training in ultrasound techniques and animal mating.

Chapter 2. Methodology

2.1 Animals and Animal Housing

At 3 weeks of age, after weaning, female C57BL/6J mice were transferred to the animal care facility at the Children's Hospital Research Institute of Manitoba (CHRIM) from the University of Manitoba Central Animal Care colony. These mice were placed on a standard rodent chow diet for one week for acclimatization. All mice were maintained on a 12-hour light:12-hour dark schedule and all animal studies were approved by the University of Manitoba Animal Care Committee.

2.2 High-Fat and Sucrose Diet (HFS Diet)

The HFS diet has been successfully utilized in various research studies for diet-induced obesity (DIO) studies, development of insulin resistance, and glucose intolerance[58,60,61,64]. Furthermore, it resembles the modern dietary style that is also called the Western diet[66]. The selected HFS diet used in this study was composed of 35% kcal from fat and 45% from sucrose produced by Research Diets, D12451[67] (Table 1). Additional information on D12451 diet is in Table A1 and Table A2 (Appendix). During the experiment, the same type of diet was ordered twice in bulk. Note that te "HFS only" group was on the HFS diet from the second batch.

Table 1. Formulation of HFS diet with 45 kcal% Fat (D12451), according to the Research Diets website[67].

Class description	Ingredients	Grams
Protein	Casein, Lactic, 30 Mesh	200.00 g
Protein	Cystine, L	3.00 g
Carbohydrate	Sucrose, Fine Granulated	176.80 g
Carbohydrate	Lodex 10	100.00 g
Carbohydrate	Starch, Corn	72.80 g
Fiber	Solka Floc, FCC200	50.00 g
Fat	Lard	177.50 g
Fat	Soybean Oil, USP	25.00 g
Mineral	S10026B	50.00 g
Vitamin	Choline Bitartrate	2.00 g
Vitamin	V10001C	1.00 g
Dye	Dye, Red FD&C #40, Alum. Lake 35-42%	0.05 g
	Total:	858.15 g

2.3 Streptozotocin (STZ) Administration

STZ is a chemotherapeutic drug that eliminates β-cells causing their DNA and mitochondrial damage: it selectively targets β-cell, enters the cell via GLUT2 transporters, leading to production of reactive oxygen species causing mitochondrial damage, alkylation of the DNA, and eventual cell death in 48 hours[59,74,75] For this study, a single low dose injection of STZ was chosen in three different doses 75 mg/kg, 100 mg/kg, or 150 mg/kg to test various degrees of β-cell function and determine which is the best for the induction of T2D in a background of insulin resistance and glucose intolerance induced by the HFS diet (Table 2, Figure 1). Due to the exposure to HFS diet and diet-induced obesity, it was expected that female C57BL/6J mice would develop β-cell hyperplasia, which would require a significantly higher dose of STZ than for mice on chow diet in previous studies[76]. Thus, 3 different doses were chosen: 75 mg/kg, 100 mg/kg, and 150 mg/kg, in combination with various HFS diet lengths (Table 2, Figure 1). STZ (Sigma Aldrich, Oakville, ON) was diluted in citrate buffer (pH 4.5) in three different concentrations based on the required dose to maintain similar volumes of the solution, which was further injected intraperitoneally. Control mice received 0.1M citrate buffer without STZ: sodium citrate hydrate and citric acid dissolved in distilled water to achieve 4.5 pH (see Appendix section for the citrate buffer recipe).

2.4 Study design

The study workflow is schematically shown in Figure 1 and the number of animals for each group is summarized in table 2 below.

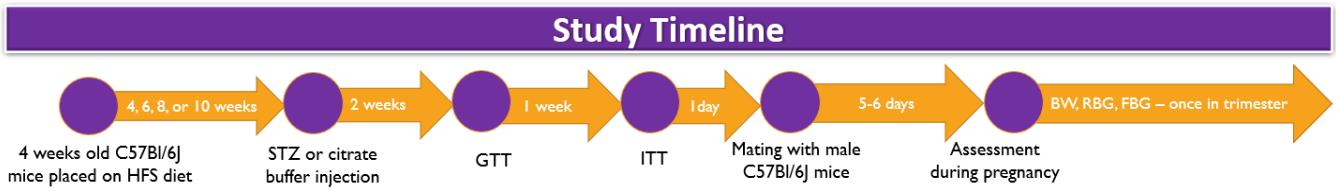

Figure 1. ***Experimental Workflow and Study Design.***

At 4 weeks of age, experimental animals were assigned to different dietary groups (4, 6, 8, or 10 weeks on HFS diet) while control groups remained on chow diet for 6, 8, or 10 weeks. Following the assigned dietary periods, mice were injected with either STZ or citrate buffer. Two weeks and three weeks post-STZ or citrate injection, glucose tolerance tests (GTT) and insulin tolerance tests (ITT) were conducted respectively. After complete metabolic assessment, female mice were mated with healthy C57BL/6J male mice and once pregnancy was established, the dams underwent gestational assessment of glucose metabolism (body weight, fasted, and random blood glucose) in each trimester.

The total number of animals participated in the study: 60 experimental and 16 control C57BL/J6 female mice; 8 C57BL/J6 chow fed males for the mating purposes (Table 2). The C57BL/J6 female mice were randomly divided into 2 dietary groups (HFS experimental and chow diet controls) for 4, 6, and 10 weeks of diet, 4 animals per cage (Table 2). A total of 20 female mice were included in the 10-week HFS diet and 150 mg/kg STZ group, with 16 mice remaining after the initial four were included to replicate

the results. Additionally, 8-week/100 mg/kg and 8-week/150 mg/kg STZ groups were included, along with an HFS control group, to assess the effects of a shorter diet duration and the influence of the diet alone, in order to identify the optimal dose for achieving the study's objectives.

Table 2. Number of female mice per group and groups assignment

Diet and length	STZ dose			Control
	75 mg/kg	100 mg/kg	150 mg/kg	Vehicle injection
6 weeks of chow diet				4
8 weeks of chow diet				3
10 weeks of chow diet				3
4 weeks of HFS diet	4	4	4	0
6 weeks of HFS diet	4	4	4	0
8 weeks of HFS diet	0	4	4	0
10 weeks of HFS diet	4	4	20	6

Once placed on diet (either HFS or chow), body weight and fasted blood glucose were assessed weekly until STZ injection. HFS-fed mice were randomly assigned a STZ dose: 75 mg/kg, 100 mg/kg, or 150 mg/kg. During the 2-3 weeks post-STZ injection, mice were assessed for body weight and fasted blood glucose. After the 2-week washout period, all mice were subjected to a glucose and insulin tolerance test prior to mating with a healthy C57BL6/J male mouse to determine degree of dysglycemia.

When all post-STZ tests were done, mice were mated with healthy C57BL/J6 chow fed males. Glucose metabolic assessment and BW measurements were done every trimester during pregnancy. At the end of the study, mice were euthanized.

2.5 Body Weight (BW) and Fasted Blood Glucose (FBG) Measurements:

All animals were weighed on a weekly basis before mating, and then every 5-6 days thereafter. Fasted glycemia was measured biweekly before the injection of STZ or citrate buffer, then weekly after the injection, and, finally, every 5-6 days after mating. Blood glucose measurements were taken using a sterile lancet to poke the tail vein and a drop of blood taken for use in a Contour Next glucometer. Mice were fasted overnight (average 10-14 hours) prior to blood sampling.

2.6 Glucose Tolerance Tests (GTT):

A glucose tolerance test (GTT) was performed two weeks after STZ injection following overnight fasting (10-14 hours). 10% freshly made glucose solution was used each time by dissolving anhydrous

dextrose in distilled water, further filter sterilized with 70 um filter. The solution was injected intraperitoneally at a concentration of l g per kg of body weight, with body weight being assessed immediately prior to the start of the GTT. Blood glucose measurements were performed at 0, 10, 20, 30, 60, and 120 minutes of the test after a tail vein puncture using a sterile lancet and using a Contour Next glucometer (Bayer). Additionally, tail vein blood collection into 300 uL capillary tubes was performed to assess plasma insulin levels at 0, 10, 20, and 60 minutes post-glucose administration, stored on ice until the end of the test. Aprotinin (a protease inhibitor) was added to each plasma sample in the concentration of 8.19 uL (500 KIU) of aprotinin per 1mL of blood (ThermoScientific). Blood samples were centrifuged prior to plasma collection at 1000g for 5 minutes at 4℃ using ThermoScientific Sorvall Legend micro 21 centrifuge. Plasma samples were snap frozen with liquid nitrogen and stored at -80℃ prior to measurement of insulin concentration using an ultrasensitive mouse insulin ELISA kit (ALPCO, Salem, NH, USA; 80-INSMSU-E10).

2.7 Insulin Tolerance Test (ITT)

An insulin tolerance test (ITT) was performed one week after the GTT. Following a morning 4-hour fast, insulin (0.625U/kg of body weight) was injected intraperitoneally. Freshly made insulin solution was made prior to each ITT test by dissolving 5 uL of Insulin (Sigma Aldrich, Oakville, ON) in 8 mL of 0.9% solution of NaCl (Sigma, Oakville, ON), further filter sterilized with 70 um filter. The solution was injected intraperitoneally at a concentration of l g per kg of body weight, with body weight being assessed immediately prior to the start of the GTT. A cut-off point for intervention to correct hypoglycemia with 10% glucose solution was established at 1.0 mmol/L. Glucose solution was made freshly before the ITT as described in the GTT section. Further, data from animals that underwent hypoglycemia correction during ITT was excluded. Blood glucose was measured using a Contour Next glucometer (Bayer) at 0, 15, 30, 45, 60, 90, and 120 minutes post-insulin injection.

2.8 Establishment of Pregnancy and Metabolic Assessment of Pregnant Female Mice:

After a complete glucose metabolism assessment following diet exposure and STZ injection, the female mice were mated with healthy C57BL/6J chow-fed males. One male was added to one cage with females – up to 5 animals in total per cage. As soon as pregnancy was established (determined by ultrasound and described below), males were removed to a separate cage or to a different cage with females for mating.

In the period between 7 to 14 postcoital days, on a weekly basis mice underwent high frequency ultrasound diagnostic of pregnancy and gestational staging until pregnancy is confirmed using VisualSonics Vevo 2100 Ultrasound machine in the Central Animal Care Services (CACS) facility. Prior to sonography, mice were weighed and sedated using inhalational anesthesia to immobilize them during the procedure. Mouse hair on the lower abdominal area was removed with the chemical provided (Nair Shower Power Hair Removal Cream) by the animal facility to reduce distortion of the echo-signal. Ultrasound gel was applied on the lower abdominal area to facilitate sound propagation. Each visualization

was recorded in the system to provide evidence of findings. After completion of the procedure, mouse skin was cleaned and the animal was returned to the cage for recovery.

Mouse gestational period is ~19-21 days[72,73], as such, the dams were assessed every 5-6 days during pregnancy: approximately once per trimester. First, random blood glucose (RBG) was measured by taking a randomly timed tail vein blood drop and measuring blood glucose concentration using a Contour Next glucometer. Body weight was also assessed. Mice were then fasted overnight (on average 10-14 hours), and fasted blood glucose and body weight were assessed. After the last assessment in the 3rd trimester, mice were euthanized.

2.9 Statistical Analyses:

All collected data was analyzed using Prism software. A two-way ANOVA, one-way ANOVA, and a student t-test were used to compare groups. A significance level of 0.05 was employed to establish statistical significance.

2.10 Ethical considerations:

The study was conducted under protocol #22-020, approved by the CACS and Ethical Committee at the University of Manitoba.

Chapter 3. Results

Chapter 3. Results

3.1 Pre-gestational Metabolic Assessment

3.1.1 Body Weight (BW):

10 weeks HFS diet drives sufficient weight gain and obesity development in C57BL6/J female mice:

To determine the length of diet that was most appropriate to induce sufficient obesity and subsequent metabolic stress, mice were placed on HFS diet for 4 different lengths of time: 4 weeks, 6 weeks, 8 weeks and 10 weeks. Body weight was then assessed weekly (Figure 2). All mice entered this study at 4 weeks of age and had a similar starting body weight (Figure 2A-D). Mice fed the HFS diet for 4, 6 and 8-week groups showed a very little or no significant changes in body weight compared to chow-fed control mice (Figure 2); however, 10 weeks of HFS diet resulted in significantly increased body weight compared to chow-fed controls (Figure 2).

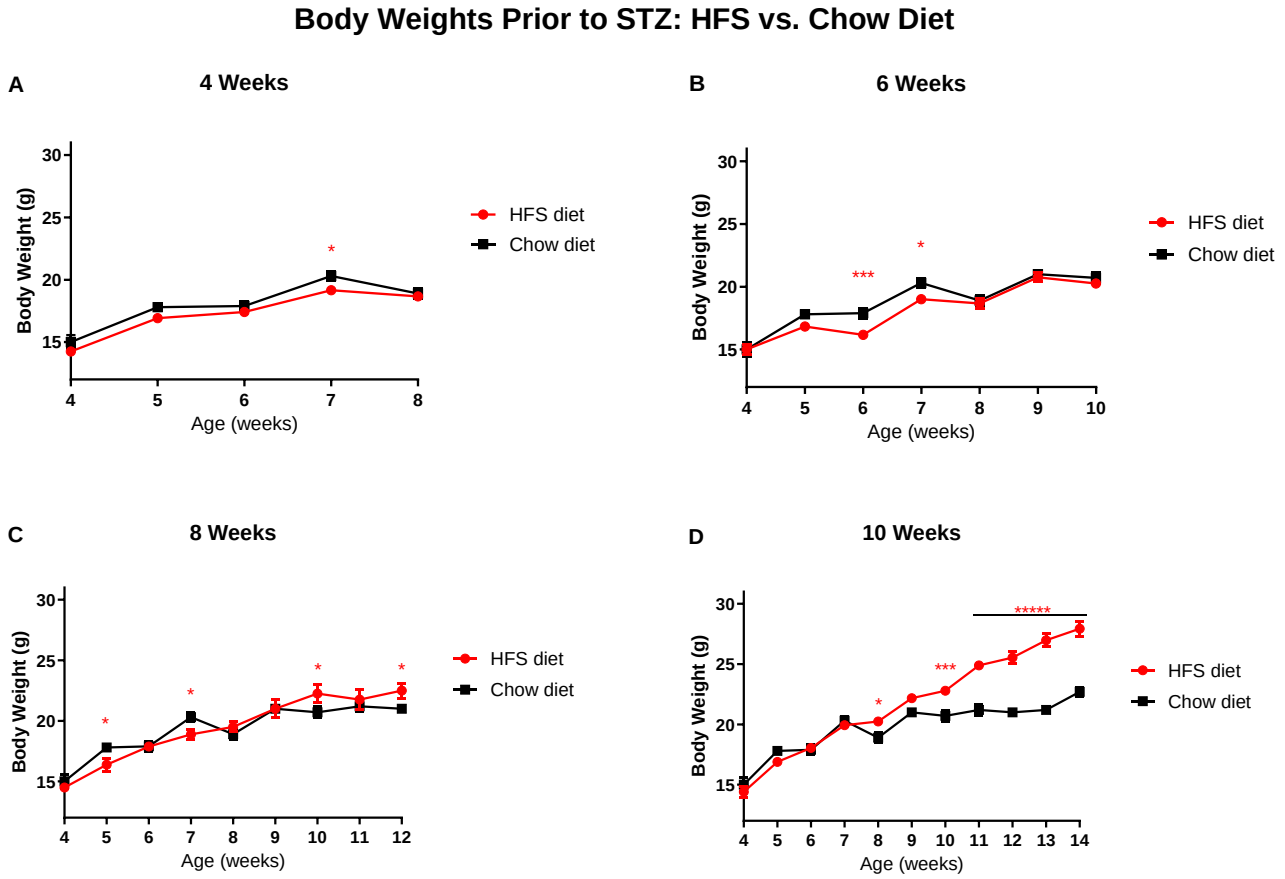

Figure 2. Comparison of body weight (g) between female mice consuming HFS diet and chow diet for different lengths of time. Weekly measurements were initiated at 4 weeks of age when the HFS diet started. Figures 2 A-D: body weight changes over time in 4-weeks HFS (**A**, n=12), 6-weeks HFS (**B**,

n=12), 8-weeks HFS (**C**, n=8), and 10-weeks HFS (**D**, n=20) on HFS diet groups combined regardless of their future STZ dose assignment vs. controls (n=10). Analysis performed on GraphPad Prism software using two-way ANOVA (A-D) and multiple unpaired t-tests (E-I). *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001.

150mg/kg body weight STZ reduces weight gain in mice fed HFS diet for 10 weeks, suggesting T2D development:

To determine the effect of HFS diet exposure without STZ, we calculated the change in body weight that occurred from the beginning of the HFS or chow diet and until STZ or citrate buffer injection. Figure 3 shows that among all groups, only 10-week HFS diet group had a significant increase in BW compared to controls. Notably, the HFS Control (HFS+Citrate) group had more pronounced increase in BW, which we attributed to a different dietary batch, although it was from the same manufacturer.

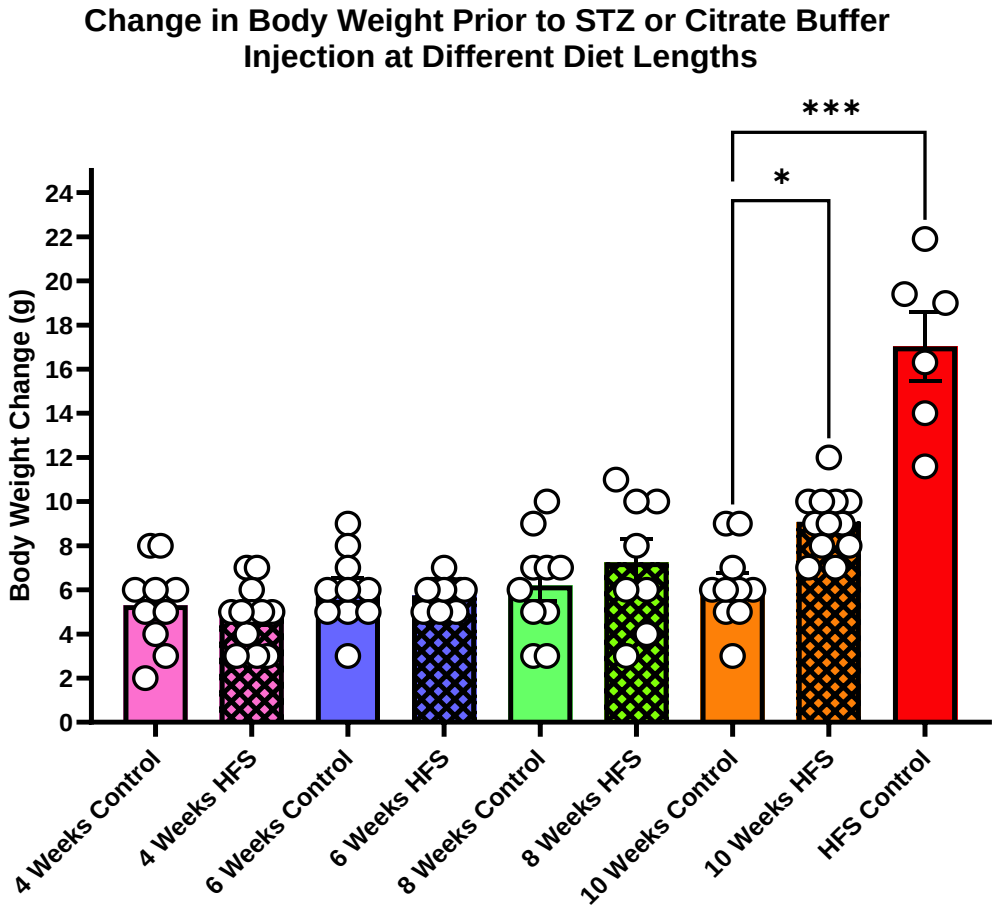

Figure 3. Comparison of changes in body weight related to HFS exposure of various lengths before the injection of STZ or citrate buffer. To calculate the change in body weight before injection of STZ, animals were weighed on their final week of HFS diet prior to STZ injection (at 4-, 6-, 8-, and 10 weeks of HFS diet) and their baseline weight at 4 weeks of age (when they entered the study) was subtracted. HFS Control is a group on 10 weeks of HFS diet (further injected with citrate buffer instead of STZ), similar

to 10 weeks HFS (further injected with STZ) at this evaluation point, but taken into the study later and fed HFS diet from the different batch. Statistical analysis using two-way ANOVA was performed using GraphPad Prism software. *=p<0.05, ***=p<0.001.

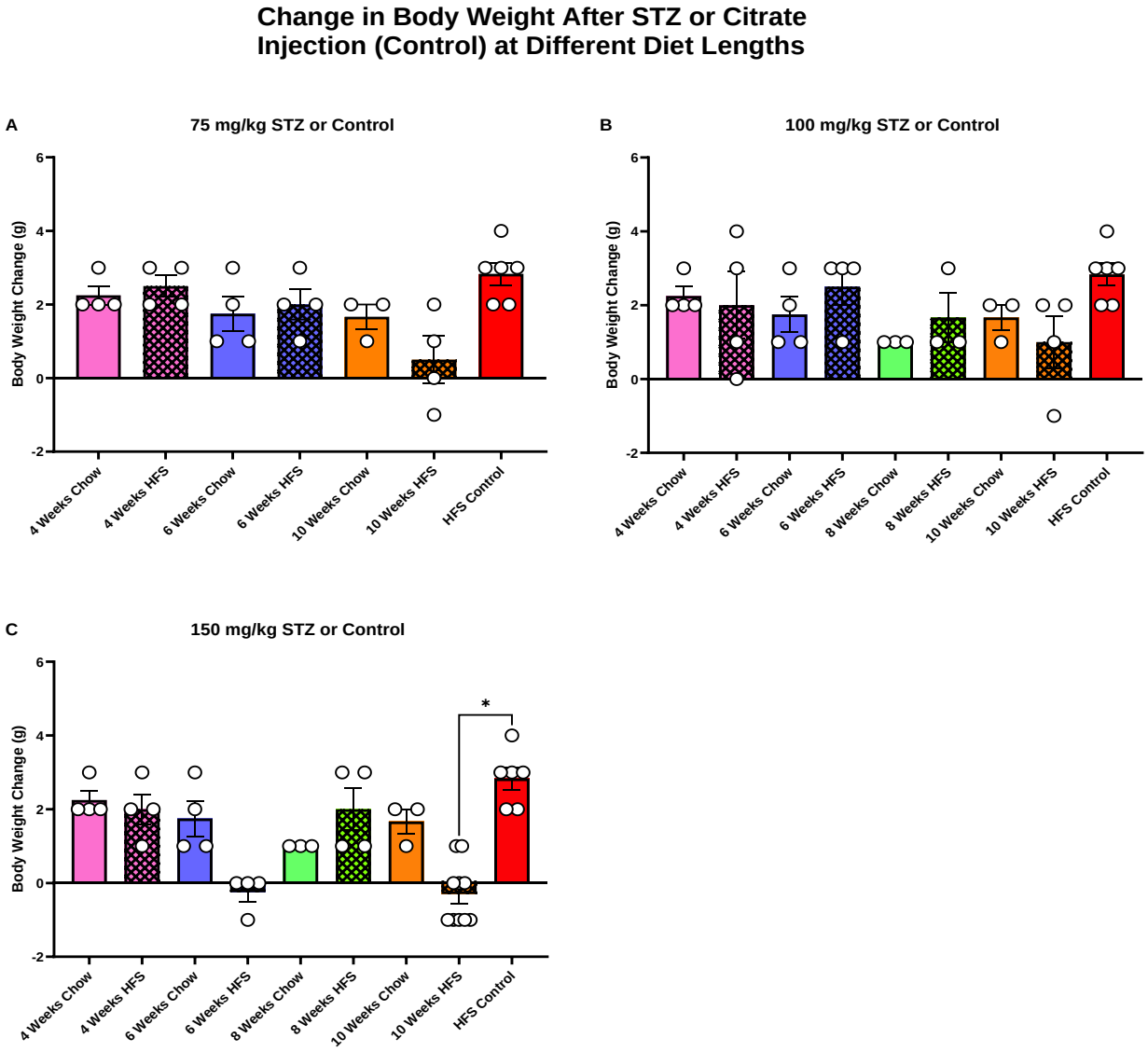

Figure 4. The change in body weight that occurs three weeks post-STZ injection in mice fed an HFS diet for varying lengths of time. To calculate the change in body weight after the injection of STZ, animals were weighed three weeks after STZ injection (**A:** 75mg/kg; **B:** 100mg/kg **C:**150mg/kg) or citrate buffer in the control group and this weight subtracted from their body weight immediately prior to STZ injection. HFS Control is a group on 10 weeks HFS diet injected with citrate buffer. Weight changes showed in 4 dietary lengths: 4-, 6-, 8-, and 10 weeks of HFS diet or chow diet in controls. Statistical analysis using two-way ANOVA was performed using GraphPad Prism software. *=p<0.05, **=p<0.01.

Figure 4 depicts the change in body weight among groups exposed to either STZ or citrate buffer (vehicle control) under the various dietary durations, calculated as the weight of the animal (g) three weeks after STZ injection minus the weight of the animal (g) the day STZ was injected. Results from the HFS only group, exposed to 10 weeks of HFS diet but injected with citrate, further underscore the impact of STZ on body weight (Figure 4C). Notably, minor weight loss is associated with new onset T2D[78], suggesting that the 10 weeks + 150mg/kg STZ group of mice are experiencing toxic effects of STZ.

In summary, 10 weeks exposure to HFS diet showed the most substantial weight increase prior to STZ injection (Figures 2 and 3). After STZ injection, the 10-weeks HFS in combination with 150 mg/kg STZ dose resulted in a more pronounced effect on body weight than smaller doses of STZ or no drug (Figure 4C). This post-STZ weight loss is thought to mimic the clinical features of relative insulin deficit.

3.1.2. Fasted Blood Glucose (FBG)

HFS diet significantly increased FBG in the 8- and 10-week HFS diet groups, prior to STZ injection:

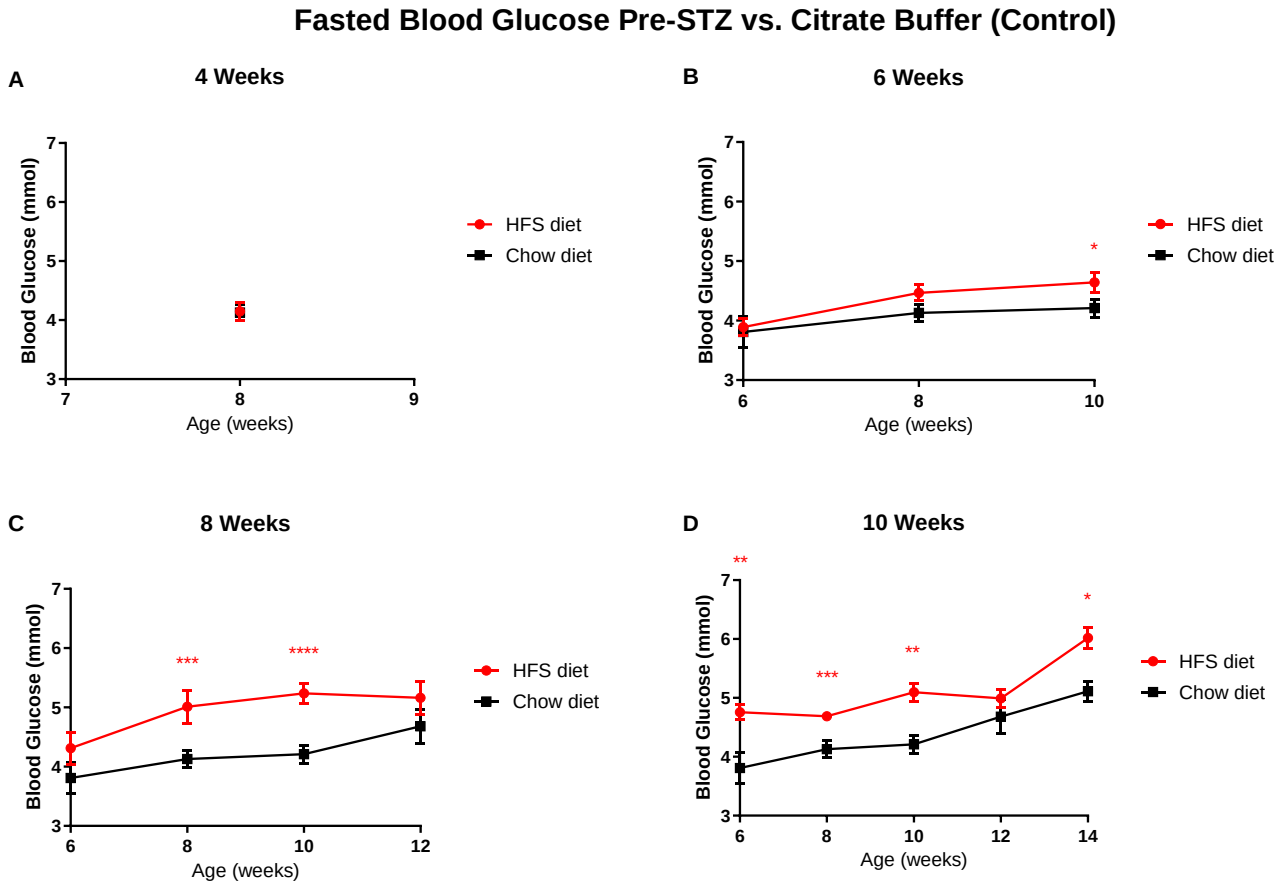

Figure 5. Fasted blood glucose in female mice consuming HFS diet compared to chow diet. Bi-weekly measurements were initiated at 6 weeks of age when the HFS diet started. Figures 5A-D: fasted blood

glucose changes over time in 4 weeks (**A**, n=12), 6 weeks (**B**, n=12), 8 weeks (**C**, n=8), and 10 weeks (**D**, n=20) on HFS diet groups combined regardless of their future STZ dose assignment vs. controls (n=10). Analysis performed using GraphPad Prism software using multiple unpaired t-tests. *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001.

FBG was measured every two weeks prior to injection of STZ and every week after STZ or citrate injection. To demonstrate the impact of the HFS diet prior to STZ injection on fasted blood glucose, all mice on HFS diet (prior to STZ dosing) were compared to control chow-fed mice (Figure 5). Note that in Figure 5A, only one data point is shown because we had technical issues with the first reading (it was excluded) leaving only a second reading.

After 4- and 6-weeks on the HFS diet, no significant difference in FBG was observed prior to STZ injection (Figures 5A-B). In the 8- and 10-weeks HFS groups, a significant difference in FBG was observed compared to controls (Figures 5C-D), which can be attributed to the length of the diet and its effect on glucose metabolism. Notably, 10 weeks on HFS diet group had the highest increase in FBG prior to injection of STZ (Figure 5D).

STZ injection increased FBG in the 8-week HFS group:

To determine the influence of STZ on FBG in each diet group, we specifically assessed FBG post-STZ injection (Figure 6). The analysis indicates no significant difference in most groups (Figure 6). In 4-weeks HFS group, 75 mg/kg subgroup showed difference at only one point (week 11), while 6-weeks HFS group showed no difference in all STZ subgroups (Figure 6A-B). Groups of 8- and 10-weeks on diet had more pronounced results compared to other groups (Figure 6C-D).

Additionally, the 8 weeks HFS group had significantly higher FBG during the third week post-STZ injection in both 8-weeks HFS+100mg/kg STZ and 8-weeks HFS+150 mg/kg STZ (Figure 6C). In 10 weeks HFS only subgroup baseline FBG was notably higher than controls, while 10-weeks HFS+150 mg/kg STZ subgroup showed higher glycemia at 4 weeks after STZ injection compared to controls (Figure 6D).

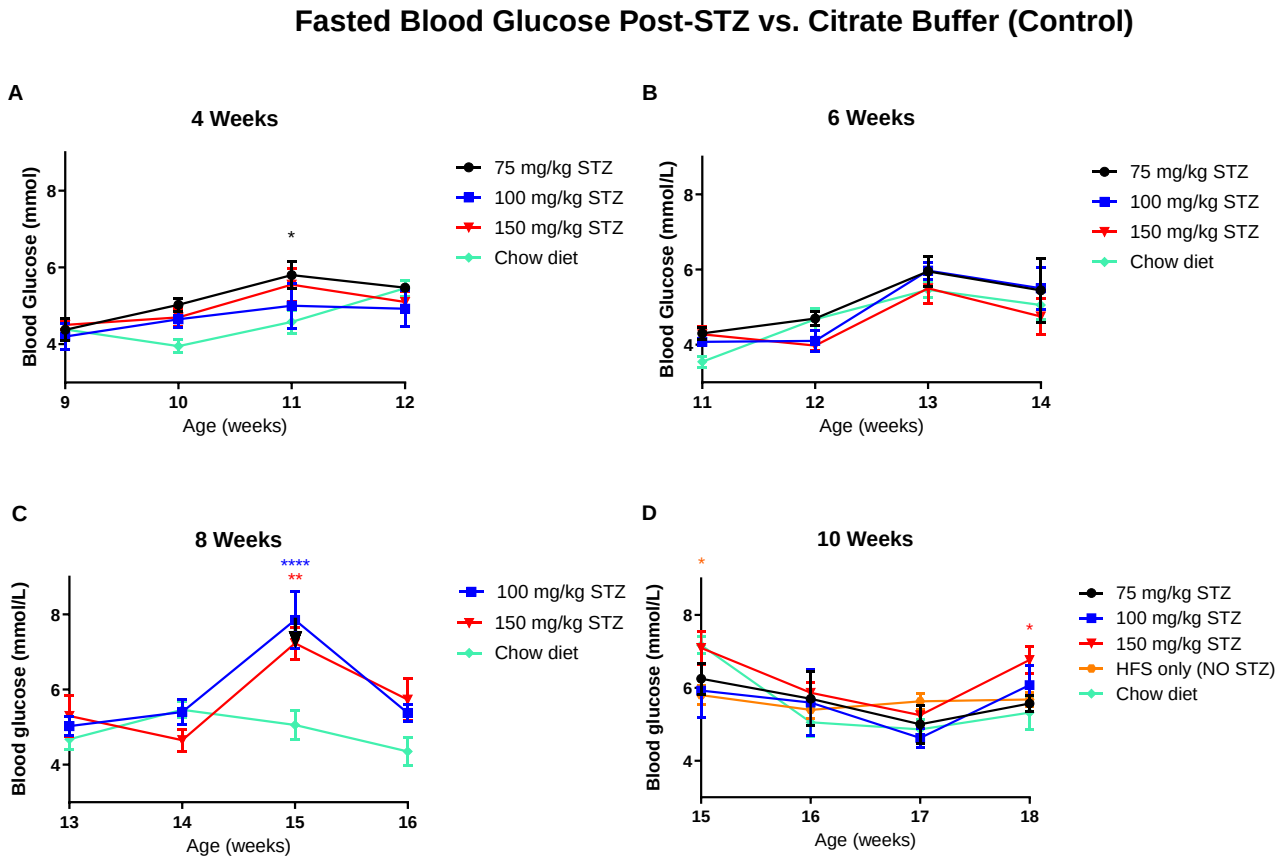

Figure 6. Comparison of fasted blood glucose between female mice consuming HFS diet for different lengths of time and control groups (chow diet) during one-four weeks period after the injection of different doses of STZ or citrate buffer (control). Weekly measurements were initiated after the injection of STZ at 4-weeks HFS (**A**, n=12), 6-weeks HFS (**B**, n=12), 8-weeks HFS (**C**, n=8), and 10-weeks HFS (**D**, n=20) groups. Controls were on chow diet group and injected with citrate buffer (n=10). HFS only subgroup (n=6) was placed on HFS diet for 10 weeks prior to injection of citrate buffer. After the injection, mice continued consumption of HFS or chow diet. Analysis performed using GraphPad Prism software using two-way ANOVA. *=p<0.05, **=p<0.01, ****=p<0.0001.

In summary, both the 4-week and 6-week groups exhibited almost no difference from the control group during the pre-injectional period. Both, 8-week and 10-week groups demonstrated higher FBG when compared to controls in both the 100 mg/kg HFS and 150 mg/kg HFS subgroups and 150 mg/kg and HFS control subgroups respectively (Figures 5C-D, 6C-D). The FBG values in the 100 mg/kg and 150 mg/kg subgroups in the 8-week and 10-week groups fell within the study's objectives of achieving mild-to-moderate fasted hyperglycemia.

3.1.3. Glucose Tolerance Tests

STZ injection causes impaired glucose tolerance in a dose-dependent manner that is dependent on length of HFS diet:

Two weeks after the administration of STZ or citrate buffer, mice underwent an intraperitoneal glucose tolerance test (GTT) to assess how the animals handled a glucose load. The impact of higher STZ doses was evident by a more robust rise of glycemia and a prolonged restoration period, while the duration of dietary exposure demonstrated its influence by affecting basal glycemia at the test's onset (Figure 7). Additionally, both 4- and 6-weeks HFS groups showed narrower and lower glycemic excursion comparing to 8 and 10 weeks HFS groups (Figure 7 A-D).

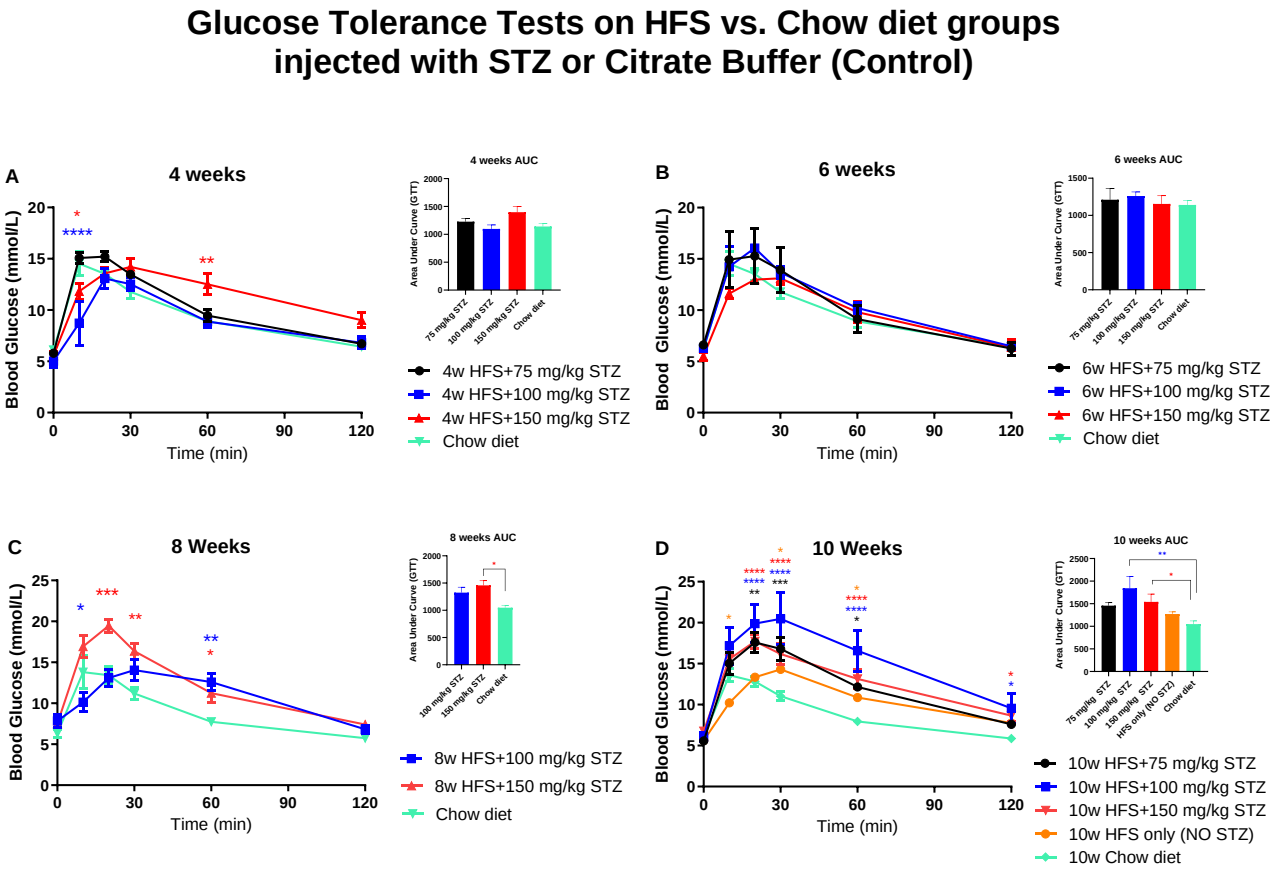

Figure 7. Comparison of responses to the glucose tolerance test between various lengths of HFS diet group and control groups. Tests were conducted two weeks after the STZ or citrate buffer injection at 4-weeks HFS (**A**, n=12), 6-weeks HFS (**B**, n=12), 8-weeks HFS (**C**, n=8), and 10-weeks HFS (**D**, n=20) groups. Controls were on chow diet group and injected with citrate buffer (n=10). HFS only subgroup (n=6) was placed on HFS diet for 10 weeks prior to injection of citrate buffer. After the injection, mice continued consumption of HFS or chow diet. Analysis performed using GraphPad Prism software using

two-way ANOVA (Blood Glucose during GTT) and one-way ANOVA (AUC). *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001.

Figure 7A shows the results of the GTT in the 4-weeks HFS diet group. At the 10-minute point, the 4-weeks HFS+100 mg/kg STZ and 4-weeks HFS+150 mg/kg STZ subgroups displayed significantly lower glycemia compared to the control group (Figure 7A). At the 60-minute point, the 4-weeks HFS+150 mg/kg STZ subgroup exhibited significantly higher glycemia compared to the control (Figure 7A). However, the analysis of areas under the curve did not show any significant differences (Figure 7A inset).

The GTT results for the 6-weeks HFS group are illustrated in Figure 7B; no statistically significant differences were observed between the subgroups (Figure 7B). Similarly, no differences found between areas under the curve (Figure 7B inset).

In the 8-week HFS group, both 8-weeks HFS+100mg/kg STZ and 8-weeks HFS+150mg/kg STZ caused significant glucose intolerance as demonstrated by significantly higher blood glucose levels at the 10-, 20-, 30- and 60-minute time points in the GTT (Figure 7C) and greater areas under curve (Figure 7C inset) compared to the controls. Notably, 8-weeks HFS+150mg/kg STZ group showed more prominent rise in glycemia compared to 8-weeks HFS+100mg/kg STZ (Figure 7C), which is additionally supported by the significantly larger area under the curve (Figure 7C inset).

Figure 7D depicts the results of the GTT in the 10-week HFS diet group. 10-weeks HFS+150 mg/kg STZ showed significantly higher glycemia compared to the controls at 10, 20, 30, 60, and 120 minutes (Figure 7D), while 10-weeks HFS+100 mg/kg STZ demonstrated significantly higher difference in a fewer time points compared to controls (Figure 7D). Furthermore, 10-weeks HFS+100 mg/kg STZ had a higher variability in results compared to 10-weeks HFS+150 mg/kg STZ group (Figure 7D).

An HFS only group was included to isolate the effects of HFS diet and STZ on glucose metabolism. The HFS only subgroup showed impaired glucose tolerance at 30-, 60- minutes and slower return to the baseline at the end of the test compared to controls on chow diet (Figure 7D); however, this group showed smaller glucose intolerance in contrast to all 10-week HFS+STZ groups (Figure 7D). Both 10-weeks HFS+100 mg/kg STZ and 10-weeks HFS+150 mg/kg STZ were statistically larger than chow+citrate buffer controls areas under the curves (Figure 7D inset).

100mg/kg STZ and 150mg/kg STZ had the greatest impact on glucose tolerance after 8- and 10-weeks of HFS diet:

Based on the results in the previous section, it appeared that the 100mg/kg and 150mg/kg STZ does had the greatest impact in the 8- and 10-week HFS diet groups. To better visualize this, the glucose tolerance curves (and corresponding AUCs) for all the groups that received 100mg/kg STZ (Figure 8A) or 150mg/kg STZ (Figure 8B) are graphed together.

Comparison of Glucose Tolerance Tests Among All Dietary Groups Injected with 100mg/kg, 150mg/kg of STZ, or Citrate Buffer

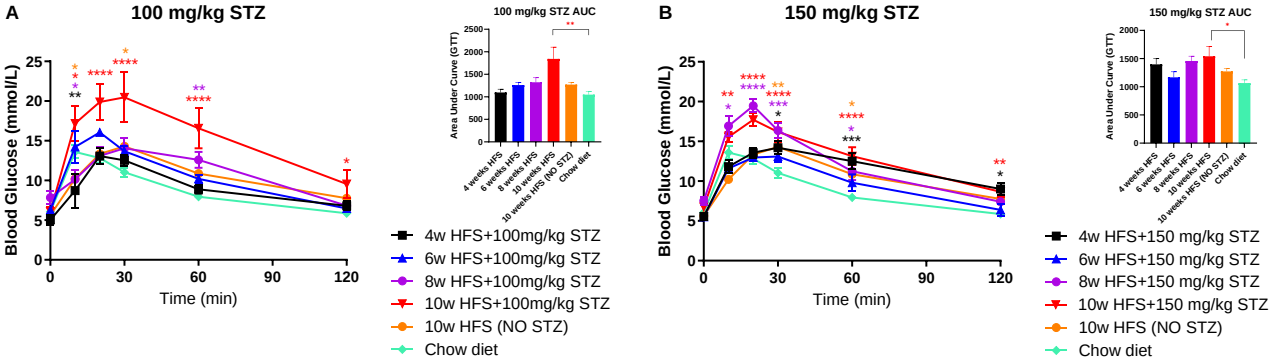

Figure 8. Comparison of responses to the glucose tolerance test among all dietary groups injected with 100 mg/kg, 150 mg/kg of STZ, or citrate buffer. Tests were conducted two weeks after the 100 mg/kg of STZ or citrate buffer (**A**) and 150 mg/kg of STZ or citrate buffer (**B**) injection at 4 weeks of diet, 6 weeks, 8 weeks, and 10 weeks. Areas under the curves calculated for each graph and included as insets. After the injection, mice continued consumption of HFS or chow diet. Analysis performed using GraphPad Prism software using two-way ANOVA (Blood Glucose during GTT) and one-way ANOVA (AUC). *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001.

Figure 8 shows GTT comparison across the subgroups injected with 100 mg/kg of STZ, 150 mg/kg of STZ vs. chow+citrate buffer and HFS only groups. The 10-weeks HFS+100 mg/kg STZ subgroup showcased significantly higher post-load glycemia at 10th, 20th, and 30th, and 60 minutes and a prolonged restoration at the 120th minute of the test, in comparison to chow+citrate buffer (Figure 8A). Longer exposure to the HFS diet showed higher rise in glycemia in response to the GTT (Figure 8A), which is additionally supported by the significantly higher area under the curve (Figure 8A inset).

Figure 8B compares GTT among various HFS lengths groups injected with 150 mg/kg of STZ or citrate buffer. Both the 8-weeks HFS+150 mg/kg and 10-weeks HFS+150 mg/kg STZ displayed the most significant elevation in blood glucose compared to controls and to all other groups (Figure 8B). It's worth noting that despite the 8-weeks HFS+150 mg/kg STZ had a higher peak increase in glycemia, the 8-weeks HFS+150 mg/kg STZ displayed a slower recovery of blood glucose levels (Figure 8B). Furthermore, the 10-weeks HFS+150 mg/kg STZ showed statistically larger than chow+citrate buffer controls areas under the curves (Figure 8B inset).

In summary, the analysis of glucose tolerance revealed that the combination of longer dietary exposure to high fat and sucrose (8 to 10 weeks) with higher doses of STZ (100-150mg/kg) are associated with glucose intolerance, suggesting that these conditions more appropriately drive the development of T2D.

3.1.4. Plasma Insulin Levels

During the GTT, tail vein blood samples were taken at specific intervals to quantify the *in vivo* insulin response to the glucose challenge. Graphical representation of the results and brief description is provided in the Appendix (Figures A5-A7), but was not included in the main thesis body a result of many missing points and technical difficulties. The results presented in Figures A5-A7 did not yield conclusive findings (see Appendix).

3.1.5. Insulin Tolerance Test

A higher dose of STZ and extended exposure to the HFS diet led to elevated baseline glycemia and reduced insulin responsiveness:

One week following the glucose tolerance test (GTT), an insulin tolerance test (ITT) was conducted to evaluate the sensitivity of the animals to insulin. Table A4 summarizes tests results (see Appendix).

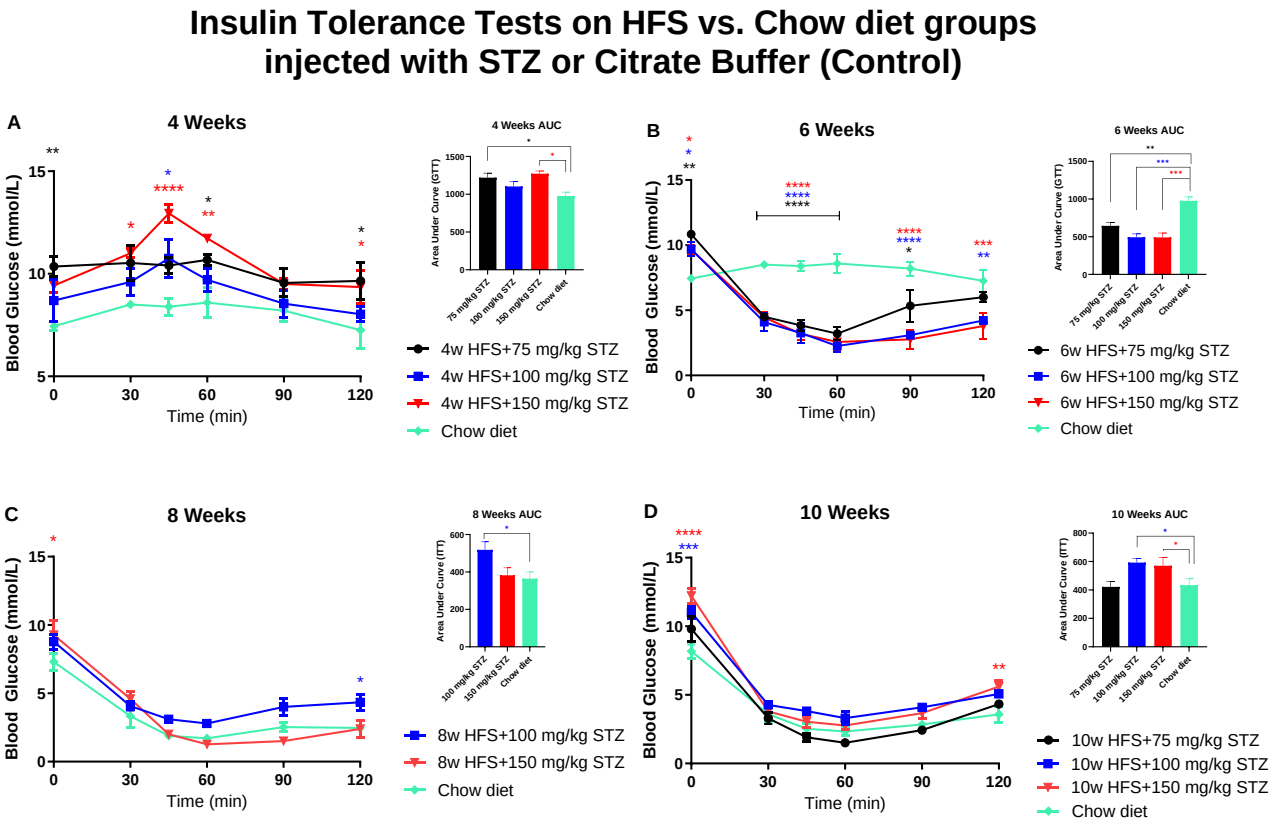

Figure 9. Comparison of responses to the insulin tolerance test between groups of various lengths of HFS diet and controls. Tests were conducted three weeks after the STZ or citrate buffer injection at Tests were

conducted two weeks after the STZ or citrate buffer injection at 4-weeks HFS (**A**, n=12), 6-weeks HFS (**B**, n=12), 8-weeks HFS (**C**, n=8), and 10-weeks HFS (**D**, n=20) groups. Controls were on chow diet group and injected with citrate buffer (n=10). After the injection, mice continued consumption of HFS or chow diet. Analysis performed using GraphPad Prism software using two-way ANOVA (Blood Glucose during GTT) and one-way ANOVA (AUC). *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001.

The 4-weeks HFS diet group and the 6-weeks chow group displayed unconventional reactions to insulin injection (Figure 9A-D). Specifically, the 4-weeks HFS+75 mg/kg STZ subgroup and 4-week chow+citrate buffer controls showed no change in blood glucose after injection with insulin, while the 4-week HFS+100 mg/kg STZ and 4-weeks HFS+150 mg/kg STZ subgroups exhibited an "inverted" reaction by increasing glycemia instead of decreasing it in response to insulin (Figure 9A). Considering the inverted reaction, the results of the ITT in 4 weeks group are not included in this study for further discussion.

In contrast, the 6-week experimental subgroups demonstrated a proper response to insulin, with a reduction in glucose levels from baseline as the tests progressed, while the control group showed no response (Figure 9B). This aligns with higher control levels of glycemia throughout the test in a dose-dependent manner: at all points, control vs. 6 weeks HFS+150 mg/kg STZ, 0-90 minutes – control vs. 6 weeks HFS+100 mg/kg STZ, and 0-60 minutes control over 6 weeks HFS+75 mg/kg STZ. Notably, at time 0, controls exhibited lower baseline glycemia than all experimental groups. The recovery from insulin injection was faster in the 6 weeks HFS+75 mg/kg subgroup than in the 6 weeks HFS+100 mg/kg STZ and 6 weeks HFS+150 mg/kg STZ subgroups (Figure 9B). However, because the control group showed no response (Figure 9B), the results should be excluded from the consideration.

Figure 9C illustrates the response to ITT in the 8-week HFS diet group. At the baseline 8 weeks HFS+150 mg/kg subgroup showed significantly higher glycemia, although, no difference throughout the test was found (Figure 9C). At the end of the test, 8 weeks HFS+100 mg/kg subgroup showed longer restoration time than controls (Figure 9C). The area under the curve supports this finding as well (Figure 9C inset).

The 10-week HFS diet group (10-week HFS+100 mg/kg STZ and 10-week HFS+150 mg/kg STZ) exhibited notably elevated baseline blood glucose levels compared to controls (Figure 9D). By the 120-minute mark, the 10-week HFS+150 mg/kg STZ subgroup demonstrated higher blood glucose than controls (Figure 9D). Both 10-week HFS+100 mg/kg STZ and 10-week HFS+150 mg/kg STZ showed significantly larger areas under the curves compared to controls (Figure 9D inset). HFS control group did not participate in the ITT test because it was included to separate the effect of HFS diet from STZ. FBG levels and GTT showed that despite of a more pronounced weight gain, dysglycemia was less profound comparing to HFS+STZ groups.

In summary, a higher dose of STZ coupled with prolonged dietary exposure to high fat and sucrose correlated with elevated baseline glycemia. Additionally, the 10 weeks on HFS diet group exhibited higher baseline glycemia and a less responsive reaction to insulin. Notably, the insulin tolerance tests for the 4

weeks group and 6 weeks control subgroup may be deemed inconclusive due to their insufficient response, possibly attributable to technical specificities as they were the initial insulin tolerance tests in the study.

3.2 Gestational Metabolic Assessment

In addition to performing a metabolic assessment of the mice before mating and pregnancy to ensure they had dysglycemia, we also wanted to assess metabolic health during pregnancy to ensure that dysglycemia and diabetes persisted throughout pregnancy. We assessed body weight, fasted and random blood glucose during each trimester (weekly).

3.2.1. Gestational Weight Gain

Longer exposure to the HFS diet correlates with higher pre-gestational and gestational body weights:

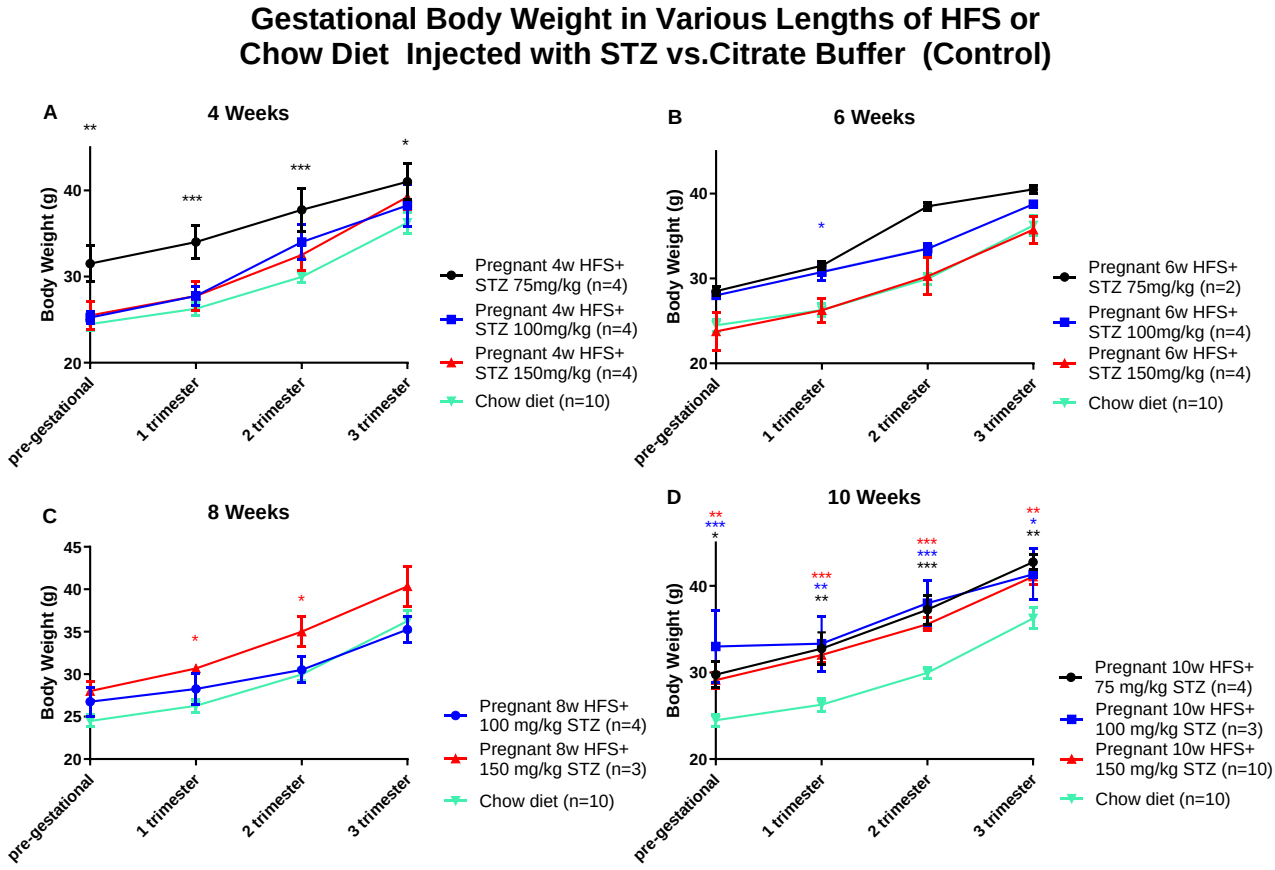

Figure 10. Comparison of pre-gestational and gestational body weights between females on various lengths of HFS diet group receiving different doses of STZ and the chow-fed, citrate-buffer-injected control group. Tests were conducted every 5-6 days before and after mating. A baseline FBG measurement was taken before pregnancy (pre-gestational measurement). Groups of animals assigned for 4 weeks (**A**),

6 weeks (**B**), 8 weeks (**C**), 10 weeks (**D**) on HFS or chow diet (control) and injected with 75 mg/kg, 100 mg/kg, 150 mg/kg of STZ or equivalent volumes of citrate buffer (control). Analysis performed using GraphPad Prism software using two-way ANOVA. *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001.

During pregnancy, mice were weighed weekly, and the data from Table A6 (see Appendix) provides insights into their pre-gestational body weight and third-trimester gestational body weight. Initially, larger gestational body weight gain was expected in experimental groups due to the nature of HFS diet. Although, the effect of the STZ dose effect on gestational body weight was unknown. At the end of the gestational part of the study, distinct trends emerged among the various experimental groups shown on the figure 10A-D.

Figure 10A compares gestational body weights between the pregnant 4-week HFS diet group and the control group. Pre-gestational body weight and throughout pregnancy, gestational body weight gain was significantly higher in the pregnant 4-week HFS+75 mg/kg STZ subgroup compared to pregnant controls (Figure 10A). However, during later stages of pregnancy, this difference had slightly narrowed, particularly 4-week HFS+100 mg/kg STZ and 4-week HFS+150 mg/kg STZ subgroups (Figure 10A).

Figure 10B illustrates a comparison of gestational body weights between the 6-week HFS diet group and the control group. In the 1st trimester, gestational body weight gain was significantly higher in the 6-week HFS+75 mg/kg and 6-week HFS+100 mg/kg STZ subgroups compared to controls (Figure 10B). During the 2nd trimester, the 6-week HFS+75 mg/kg STZ subgroup exhibited higher gestational body weight compared to control group (Figure 10B).

Figure 10C depicts changes in gestational body weight among the 8-week HFS diet group. The 8-week HFS+150 mg/kg subgroup showed significant differences in the 1st and 2nd trimesters gestational body weight compared to controls (Figure 10C).

All 10-week HFS diet experimental subgroups exhibited significantly higher pre-gestational body weight and gestational body weight than controls, suggesting that longer exposure to the diet is associated with baseline obesity and higher pregestational body weight and gestational body weight (Figure 10D).

In summary, the varied experimental conditions, encompassing HFS diet exposure and STZ treatment, resulted in notable variations in pre-gestational and gestational body weight. The pre-gestational body weight in controls, the 4-week HFS groups, and the 6-weeks HFS+150 mg/kg STZ subgroup was smaller than in other groups; however, they exhibited a greater weight gain during pregnancy than other subgroups (Figure 10A-D). The results suggest that extended exposure to the HFS diet correlates with higher pre-gestational and gestational body weights, highlighting the significant impact of dietary factors on gestational weight gain and its implications for obesity and related complications. The effect of STZ dose on gestational body weight is less pronounced with shorter dietary exposure compared to the 8- and 10-week groups (Figure 10A-D). Therefore, a combination of longer

HFS diet exposure and higher STZ doses, such as 100 mg/kg and 150 mg/kg, is more appropriate for developing a maternal T2D mouse model (Figure 10A-D).

3.2.2 Gestational Fasted Blood Glucose

10 weeks HFS+150 mg/kg STZ subgroup exhibited a direct association with higher maternal pre-gestational and gestational glycemia levels:

Gestational fasted blood glucose levels were tracked at each trimester of pregnancy. The dataset outlined in Table A7 (see Appendix) offers insights into the range and average values for both pre-gestational fasted blood glucose and gestational fasted blood glucose within each distinct group.

Notably, a consistent general pattern emerged across all groups, characterized by an initial elevation of gestational fasted blood glucose during the first and second trimesters, followed by a subsequent decline in the third trimester (Figure 11A-D). This pattern suggests a fetal impact on maternal glycemic regulation similar to human pregnancy, when the fetus lowers maternal glycemia by utilizing her glucose with the increased production of insulin[23-25].

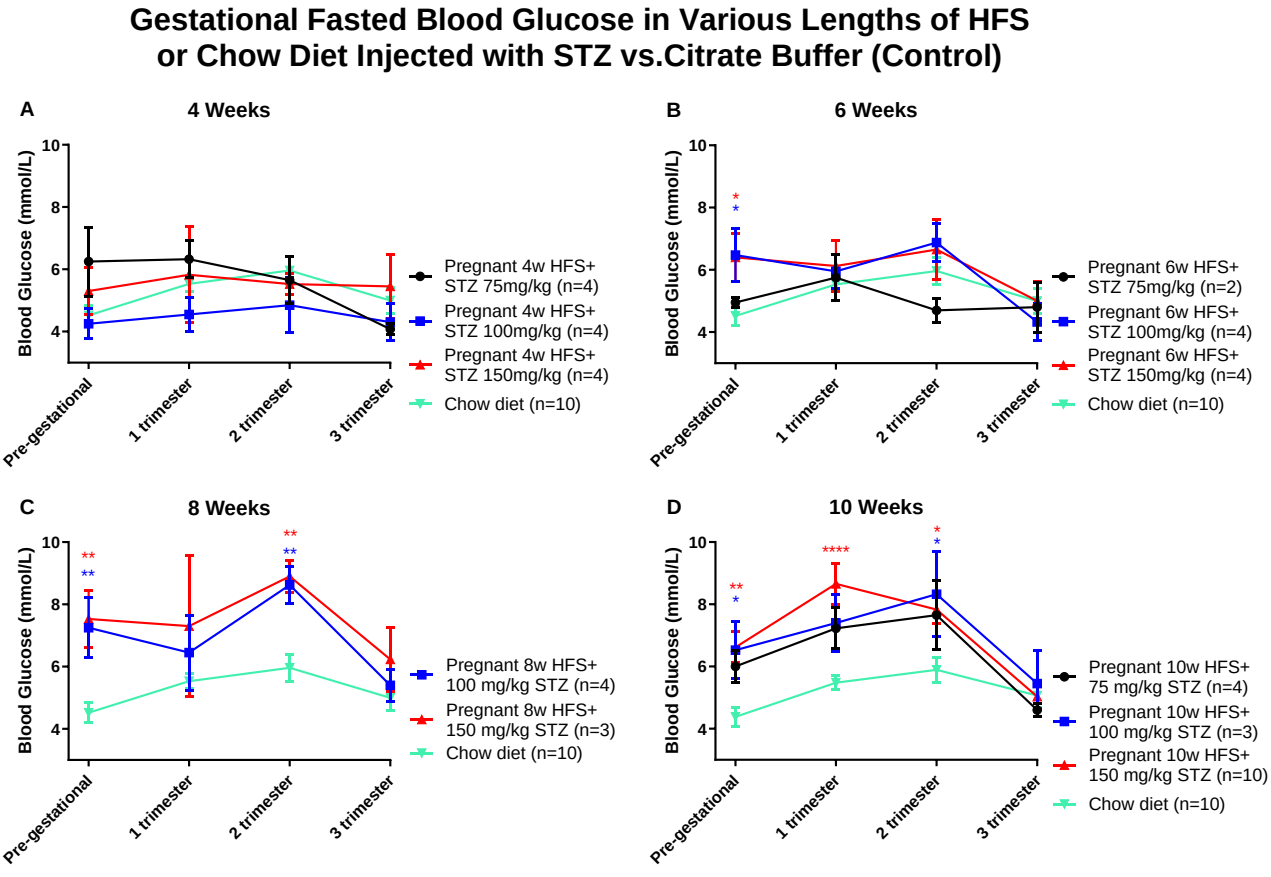

Figure 11. Comparison of pre-gestational and gestational fasted blood glucose between females on

various lengths of HFS diet group receiving different doses of STZ and the chow-fed, citrate-buffer-injected control group. During each trimester, mice were fasted for 10-14 hours and blood glucose measured using a glucometer. A baseline FBG measurement was taken before preganancy (pre-gestational measurement). Data was analyzed using GraphPad Prism software (2 Way ANOVA). Tests were conducted every 5-6 days before and after mating. Groups of animals assigned for 4 weeks (**A**), 6 weeks (**B**), 8 weeks (**C**), 10 weeks (**D**) on HFS or chow diet (control) and injected with 75 mg/kg, 100 mg/kg, 150 mg/kg of STZ or equivalent volumes of citrate buffer (control). Analysis performed using GraphPad Prism software using two-way ANOVA. *=$p<0.05$, **=$p<0.01$, ***=$p<0.001$, ****=$p<0.0001$.

Figure 11A-B shows the gestational fasted blood glucose of the 4- and 6-week HFS groups, respectively. Statistical analysis revealed no significant impact of STZ dose on FBG before or during pregnancy compared to the control group in the 4-week HFS group. Higher baseline glycemia was observed in the 6 weeks HFS+100 mg/kg STZ and 6 weeks HFS+150 mg/kg STZ subgroups (Figure 11B). Both groups show similar pattern of decreasing fasted maternal glycemia at the end of pregnancy (Figure 11A-B) suggesting fetal influence.

Figure 11C shows gestational fasted glycemia in the 8-week HFS group. After 8 weeks of HFS diet, 100 mg/kg and 150 mg/kg STZ induced higher fasted blood glucose compared to controls. Furthermore, average glycemia among longer exposed female mice to the HFS diet is higher than in 4- and 6-weeks dietary groups (Figures 11). During the first and second trimesters, the 8-week group demonstrated higher gestational fasted blood glucose levels than in 4-, 6 week, and control groups, particularly noticeable in the 8-week HFS+150 mg/kg STZ subgroup (Figure 11A-C).

Shifting the focus to the 10-week group, it is evident that 10 weeks HFS+150 mg/kg STZ subgroup fasted glucose levels were notably higher than those of the control group in pre-gestational measurements and during the first and second trimesters (Figure 11D). Notably, during the third trimester of the 10 weeks HFS groups, gestational FBG levels were similar to those observed in the control group (Figure 11D). This observation suggests a more substantial influence of the fetus on maternal glycemic reduction in mouse that are experiencing type 2 diabetes during pregnancy, especially in comparison to other experimental groups.

In summation, the combination of extended dietary exposure and higher STZ dosages exhibited a direct association with heightened maternal pre-gestational and gestational glycemia levels. Importantly, these changes suggest an interplay between maternal and fetal glucose metabolism, as is evident from the most pronounced maternal glycemic decline observed during the third trimester. Finally, all groups demonstrated a consistent pattern of initial elevation of gestational fasted blood glucose, followed by a subsequent decline in the third trimester, mimicking a typical course of human pregnancy in mothers with diabetes[23-25].

3.2.3 *Ad libitum* (Random) Gestational Blood Glucose

In addition to tracking gestational weight and FBG levels, random blood glucose (RBG) levels were also monitored in *Ad libitum* fed mice prior to pregnancy and in each trimester further.

Highest STZ doses in combination with 10 weeks on HFS diet showed elevated gestational random blood glycemia:

While it is important to acknowledge that this approach offers insights into glucose fluctuations, it is impacted by the divergent feeding habits of mice compared to humans. Mice partake in frequent, small meals, contrasting with humans' regulated consumption occurring 3-5 times daily and in larger portions. This distinction affects pancreatic function and can potentially lead to considerably higher glycemic spikes in humans. Despite these differences, an evaluation of gestational random blood glucose levels was conducted. Consistent with the trend observed in gestational fasted glycemia, gestational random blood glucose levels displayed an upward trajectory during the first and second trimesters, followed by a decline in the third trimester (Figure 12). This pattern, elucidated in Table A8 and visually represented in Figure 12A-D, aligns with the broader gestational glucose dynamics.

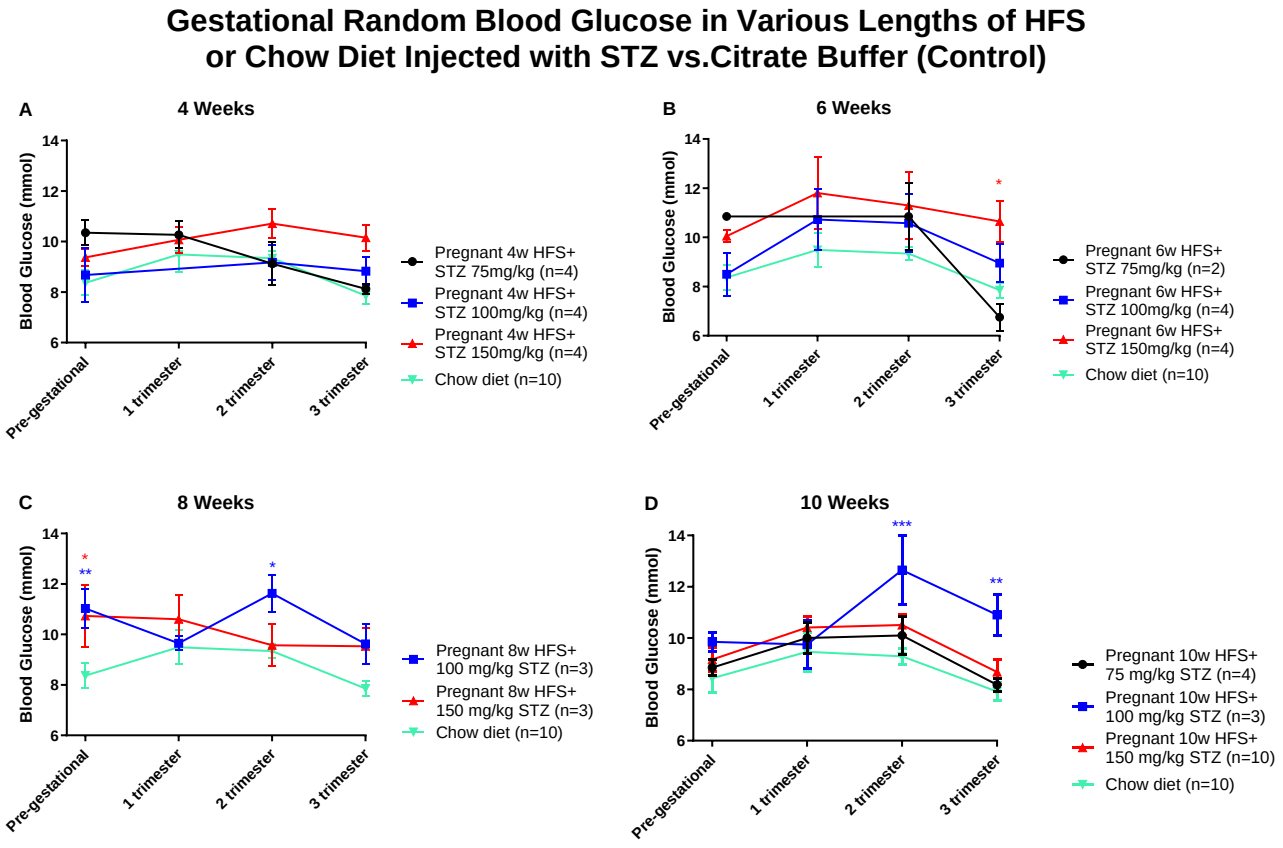

Figure 12. Comparison of pre-gestational and gestational random blood glucose between females on various lengths of HFS diet group receiving different doses of STZ and the chow-fed, citrate-buffer-

injected control group. During each trimester, blood glucose measured using a glucometer. A baseline random blood glucose measurement was taken before preganancy (pre-gestational measurement). Data was analyzed using GraphPad Prism software (2 Way ANOVA). Tests were conducted every 5-6 days before and after mating. Groups of animals assigned for 4 weeks (**A**), 6 weeks (**B**), 8 weeks (**C**), 10 weeks (**D**) on HFS or chow diet (control) and injected with 75 mg/kg, 100 mg/kg, 150 mg/kg of STZ or equivalent volumes of citrate buffer (control). Analysis performed using GraphPad Prism software using two-way ANOVA. *=$p<0.05$, **=$p<0.01$, ***=$p<0.001$, ****=$p<0.0001$.

Figure 12A presents a comparison of the 4-week group on gestational random blood glycemia, where no statistically significant differences were found. However, the 4-weeks HFS+150 mg/kg STZ subgroup demonstrated higher gestational random blood glycemia levels during the third trimester, aligning with the study goal of mild to moderate postprandial glycemia (Figure 12A).

Within the 6-week group, exhibited an association of higher doses of STZ with elevated random glycemia (Figure 12B). Notably, the 6-weeks HFS+75 mg/kg subgroup exhibited the highest pre-gestational random blood glucose and the lowest during the third trimester (Figure 12B). Nonetheless, this subgroup comprised only two remaining animals at this juncture, warranting result exclusion. The 6-weeks HFS+150 mg/kg STZ subgroup demonstrated a significantly higher glycemia at the end of gestation comparing to controls (Figure 12B).

The 8-weeks HFS+100 mg/kg STZ and 8 weeks HFS+150 mg/kg STZ subgroups showcased elevated blood glycemia pre-conceptionally and 8 weeks HFS+100 mg/kg STZ subgroup demonstrated a significantly higher glycemia during the second trimester comparing to controls (Figure 12C). Similarly, to fasted glycemia, this group observed a decline in glucose levels towards gestation's end, though remaining higher than control levels (Figure 12C).

The 10-week group displayed significantly higher gestational blood glycemia in 10-week HFS+100 mg/kg STZ comparing to controls in second and third trimesters (Figure 12D). Additionally, the 10-weeks HFS+100 mg/kg STZ subgroup manifested highly variable blood glucose elevations during pregnancy, deviating from other subgroups glycemic trends (Figure 12D).

Comparatively, the 8-week group exhibited higher random glucose levels than the 10-week group, yet variability was more pronounced (Figure 12C-D). Furthermore, the 10-weeks HFS+150 mg/kg STZ subgroup had a notably larger sample size than the 10 weeks HFS+100 mg/kg STZ and the 8-week group. Furthermore, the 10-weeks HFS+150 mg/kg STZ subgroup exhibited consistent results throughout the study. Analogous to fasted glycemia, this group observed declining glucose levels by gestation's culmination, though still higher than control levels (Figure 12). Coupled with the results of 10-weeks HFS+150 mg/kg STZ gestational random glycemia and other study tests, considering subgroup animal numbers and result reproducibility, this subgroup's outcomes were validated and reliable (Figure 12).

In sum, the pattern of random blood glucose during pregnancy echoed that of fasted glycemia, with an initial surge followed by a decrease in the third trimester. Elevated glycemia was most pronounced in the highest STZ doses coupled with extended HFS exposure, aligning with the study's objectives.

3.3 Conclusion:

The primary objective of this study was to establish and characterize a C57BL/6J mouse model of pre-existing type 2 diabetes (T2D) during pregnancy. By combining a high-fat and sucrose (HFS) diet with streptozotocin (STZ) injections, we aimed to induce glucose impairment resembling T2D, mimicking key clinical features of the disease. Our findings reveal that a 10-week exposure to the HFS diet, especially when combined with higher STZ doses (100 mg/kg and 150 mg/kg), effectively induced significant weight gain, elevated fasted and random blood glucose levels, impaired glucose tolerance, and reduced insulin responsiveness. These outcomes align with the study's objective to create a mouse model that closely mirrors the trajectory of T2D during pregnancy as observed in women.

Furthermore, the comprehensive analysis of pre-gestational and gestational glucose metabolism, alongside changes in body weight, highlights the critical role of prolonged dietary exposure and STZ dosage in developing T2D-like conditions. The 10-week HFS diet combined with 150 mg/kg STZ was identified as the most effective protocol for achieving consistent and reproducible results, demonstrating significant elevations in maternal glycemia and impaired glucose tolerance. This model not only replicates the dynamics of T2D during pregnancy but also provides a valuable platform for investigating the effects of maternal T2D on offspring health. The results of this study lay the groundwork for future research aimed at understanding the underlying mechanisms of T2D and its implications for maternal and fetal outcomes, offering potential avenues for therapeutic interventions.

Chapter 4. Conclusions and Discussion

Chapter 4. Conclusions and Discussion

In this study, we aimed to develop a robust mouse model of T2D that closely resembles the course of pre-existing T2D during human pregnancy. We evaluated several conditions of diet manipulation and beta cell destruction to drive development of T2D and we subsequently metabolically characterized the mice before and during pregnancy.

This chapter will provide a comprehensive interpretation of these findings and their implications, offering a synthesized perspective on the intricate relationships between maternal and fetal metabolic responses within the framework of this study. This in-depth comprehension is pivotal for advancing our understanding of the underlying mechanisms at play and has the potential to broaden the horizons for refined strategies in exploring how maternal T2D influences offspring. This knowledge could pave the way for enhanced interventions and therapeutic approaches, ultimately benefiting the health outcomes of the offspring.

4.1 Pre-gestational Patterns in Body Weight and Glycemic Dynamics Assessment

Both the HFS diet and STZ contributed to the development of the model, with each component playing a distinct pathogenetic role. For instance, the HFS diet exerts a dual influence by overloading pancreatic β-cells, thereby inducing their hyperplasia[58,61,76]. Additionally, the HFS diet contributes to the development of obesity, and insulin resistance, a hallmark of T2D that significantly influences disease onset and progression[84]. On the other hand, the inclusion of STZ aids in diminishing the β-cells pool, effectively challenging glucose metabolism and promoting the development of diabetes[59,74,75].

In this series of experiments, a notable discrepancy in weight gain was observed between the HFS-only control group and the HFS groups prior to STZ injection. Specifically, the issue arose when the mice in the HFS-only group (HFS Control), which were fed from a second batch of the same diet (D12451 by Research Diets), exhibited significantly different weight gain compared to their counterparts. The expectation was that all mice on the HFS diet would gain weight at a similar rate before the introduction of STZ. Another set of mice was fed from the second batch of the diet and they showed similar results to the HFS only group suggesting that the differences in weight gain could be attributed to dietary inconsistency between the batches of diet. However, regardless of the differences in body weight in the HFS only group and the 10-weeks HFS+STZ group, a less pronounced change in glycemia levels was identified during FBG and GTT tests in the HFS only group. This finding underscores the critical role of STZ treatment in the development of the T2D model, as the glycemic response was more consistent and pronounced in the groups that received STZ, regardless of the initial weight differences.

This study examined the effects of different dietary regimes and STZ dosages on body weight dynamics in a mouse model. The 10-week HFS diet group, regardless of the STZ combination, displayed a remarkable increase in body weight compared to other groups, underscoring the significant influence of extended dietary exposure on metabolic dynamics (Figures 2-4). The highest average FBG levels and the

most significant glucose intolerance during the pre-gestational period were observed in both the 8-week and 10-week exposures to the HFS diet combined with 150 mg/kg STZ, which aligned with the study's objective of achieving mild-to-moderate fasting hyperglycemia (Figures 5-6). Notably, the administration of STZ induced a transient general decline in FBG levels across all groups, followed by an increase in glycemia in various dietary groups, revealing a dose-dependent pattern (Figures 5-6). This suggests a toxic effect of STZ and a potential transient decrease in appetite.

In this study, due to inconsistencies and uncertainties in the literature, we did not establish strict glycemic ranges to define fasting mild-to-moderate glycemia or postprandial moderate-to-high hyperglycemia initially. Instead, experimental mice were compared to controls, and based on the "normal" control levels, thus, it can be approximated that a mild-to-moderate fasting blood glucose level is between 6.5 mmol/L and 9 mmol/L, and post-glucose load hyperglycemia is considered above 15 mmol/L during the glucose tolerance test for non-pregnant animals. This approximation comes from the figures achieved during the study and Diabetes Canada guidelines[85].

Summarizing the GTT outcomes after STZ or citrate buffer administration, higher STZ doses led to more robust glycemic reactions and extended restoration periods (Figures 7-8). The 8 weeks on HFS diet with 150 mg/kg STZ showed heightened initial and post-load glycemia, as well as an extended restoration period (Figures 7-8). On the other hand, the group exposed to the 10-week HFS only, coupled with citrate buffer injection, displayed elevated glycemia levels comparing to controls on chow diet (Figures 7-8). However, it is important to note that these levels remained lower than those observed in the 10-weeks HFS+150 mg/kg STZ subgroup, thereby reaffirming the inadequacy of a sole dietary intervention and further validating the model's efficacy (Figures 7-8). Notably, the 10-weeks HFS+100 mg/kg STZ subgroup showed heightened post-load glycemia and prolonged restoration but high variability in results, while the 10-weeks HFS+150 mg/kg STZ subgroup consistently exhibited substantial glycemic elevation (Figures 7-8). In summary, the GTT revealed that combination of longer dietary exposure (8 and 10 weeks) to high fat and sucrose with 100 mg/kg and 150 mg/kg of STZ are associated with higher glycemic response and longer recovery period compared to chow diet and citrate buffer injection (Figures 7-8).

Almost all experimental groups exposed to HFS diet group exhibited higher baseline insulin concentrations during GTT compared to the control group, with lower STZ doses associated with higher insulin levels, however, the validity of these results is compromised by the scarcity of reliable data points due to the minute concentration of insulin and the presence of missing data (Figure A1). Further refinements in methodology are necessary to enhance the quantification of insulin concentration in forthcoming studies.

The ITT was conducted to assess insulin sensitivity across all groups, however, the results for the 4 weeks group and 6 weeks control subgroups may be inconclusive due to insufficient response, possibly attributed to technical specifics, as these were the initial insulin tolerance tests in the study. Conversely, the 6-, 8-, and 10-weeks experimental groups demonstrated a proper insulin response, effectively lowering

glucose levels from baseline (Figure 9). The 10-weeks on HFS diet group displayed notably elevated baseline blood glucose levels compared to controls, with both STZ dose and dietary exposure duration influencing the glycemic response (Figure 9).

In conclusion, the results of pre-gestational metabolic assessments among the groups indicate that prolonged exposure to the HFS diet combined with a higher dose of STZ leads to significant alterations in glucose metabolism, effectively resembling the clinical trajectory of T2D. Notably, the most substantial response was observed in the group subjected to a 10-week diet alongside 150 mg/kg of STZ, although the 8-week exposure with 100 mg/kg of STZ could also be deemed influential. It is worth highlighting that a sole dietary exposure results in a notable increase in body weight (BW); however, it falls short of inducing a mild-to-moderate elevation in fasted blood glucose (FBG) or a moderate-to-severe response to a glucose load, as compared to the combination of the HFS diet and STZ.

4.2 Gestational Body Weight and Blood Glycemia Changes

Since the study aims to develop a maternal model with pre-existing T2D, successful mating and establishment of pregnancy was crucial. Moreover, pregnancy imposes significant metabolic challenges on the maternal body, allowing for a thorough examination and comparison of glucose metabolism changes across the groups. Therefore, the final pre-gestational measurements of BW, FBG, and RBG were compared to the gestational ones.

Comparing the last pre-gestational body weight and gestational weight gains across different experimental and control conditions has revealed that the 10 weeks on HFS diet group demonstrated considerably higher pre-gestational and gestational body weights compared to controls and other groups, suggesting a strong association between prolonged diet exposure and baseline obesity (Figure 10). Gestational fasted blood glycemia revealed that 4- and 6 weeks dietary exposure was inadequate in establishing T2D, while 8-week HFS+150 mg/kg STZ subgroup and 10-weeks group exhibited notably higher fasted glucose levels compared to the control group in pre-gestational measurements and throughout the initial and subsequent trimesters, which further emphasizes a robust correlation between higher STZ dosages and elevated glucose levels (Figure 11).

The comprehensive investigation into gestational fasted blood glycemia across trimesters has provided precise insights into the complex interplay of maternal glycemic control under varying experimental conditions. A recurring trend has surfaced, characterized by an initial rise in gestational fasted blood glucose during the first and second trimesters, succeeded by a subsequent decline in the third trimester. Studies on normal and diabetic mice with constant glucose monitoring showed similar pattern[79,80]. This intriguing pattern echoes the concept of fetal influence on maternal glycemic regulation, mirroring phenomena observed in human pregnancies[23-25].

Importantly, during the third trimester of the 10-weeks group, gestational fasted blood glucose levels either equaled or remained lower than those recorded in the control group (Figure 11). This

noteworthy observation suggests a more substantial influence of the fetus on maternal glycemic reduction, especially when compared to other experimental groups. Similarly to humans, gestational fasting glycemia levels are expected to be lower during the third trimester[86]. It is assumed that higher intrauterine glycemia stimulates fetal insulin production to utilize maternal hyperglycemia in diabetic animals, which is congruent with the previous studies about GLUT-3 expression on trophoblasts and placenta in normal and diabetic mice and explains partially the mechanisms behind fetal macrosomia in maternal diabetes[22,24,25,81]. This resemblance suggests that the model phenocopies human maternal course of T2D and based on the glycemia range, it can be roughly approximated that mild-to-moderate gestational glycemia lies within the range of 5.3-7.8 mmol/L.

The assessment of gestational random blood glucose levels revealed a pattern akin to that observed in fasted glycemia, characterized by an initial rise followed by a decline in the third trimester (Figure 12). It is important to acknowledge that while the dietary behaviors of mice differ from those of humans, the comprehensive evaluation of gestational random blood glucose levels offers a description of patterns and variations in gestational glucose dynamics within the experimental context. In the 8-week and 10-week groups demonstrated higher pre-gestational and gestational blood glycemia than controls (Figure 12). The 10-weeks HFS+100 mg/kg STZ subgroup showed increased but highly variable blood glucose elevations during pregnancy, deviating from other subgroup glycemic trends (Figure 12). Despite greater variability, the 10-week group, especially the 10-weeks HFS+150 mg/kg STZ subgroup, exhibited notably elevated pre-gestational and gestational blood glycemia compared to controls (Figure 12). Due to the differences in rodents feeding patterns from human's, their postprandial levels are difficult to connect to human's however, based on the figures from the study, it can be roughly approximated that gestational postprandial moderate glycemia is above 6.7 mmol/L.

In summary, the comprehensive assessment of gestational metabolic dynamics among the experimental groups reinforces the pre-gestational observations, highlighting the significant impact of prolonged exposure to the HFS diet coupled with higher STZ dosages in inducing substantial alterations in glucose metabolism. This emulation closely parallels the clinical progression of T2D. Upon meticulous analysis of the results, the most prominent metabolic response emerged within the group subjected to a 10-week dietary regimen along with 150 mg/kg of STZ. This particular group can confidently serve as a benchmark reference for establishing the protocol of a maternal mouse model with pre-existing T2D in the C57BL/6J mice strain.

Based on the study findings, the protocol is proposed for establishing a maternal mouse model with pre-existing T2D and described in the Appendix Table A9. This protocol integrates the observed study outcomes to establish a robust maternal mouse model of pre-existing T2D, enhancing the understanding of gestational metabolic dynamics and their potential implications for offspring health.

4.3 Significance of the Developed Mouse Model

The mouse model of T2D defined in this thesis holds significant relevance in the field of biomedical research, particularly in the study of how maternal T2D influences the metabolic health outcomes of the offspring. The described model, which combines extended HFS diet exposure with varying doses of STZ administration, mirrors certain clinical aspects of T2D and offers several noteworthy contributions.

The described mouse model closely emulates key metabolic features of T2D observed in humans, including elevated blood glucose levels and altered gestational metabolic patterns [23, 25, 28, 30-39]. Current mouse models of T2D have been instrumental in advancing our understanding of the disease; however, they have limitations, particularly when studying the effects of T2D during pregnancy. For instance, female mice exhibit hyperphagia, obesity, and chronic hyperglycemia but are infertile, making them unsuitable for studying T2D during pregnancy[55-57]. Additionally, heterozygous ob/- and db/- mice do not develop significant hyperglycemia compared to controls[57]. Models based solely on dietary exposure to high-fat or high-fat and sucrose diets are effective in generating insulin resistance; however, these diets require prolonged exposure (6-8 months) to develop T2D in females, which may lead to aging-related changes affecting offspring or causing fertility issues in dams[58,66,68]. Other studies that used a combined HFS+STZ approach either involved males or showed that females are less sensitive to the treatment and remain non-diabetic[43,46-48].

This study demonstrates that the administration of a single 150 mg/kg STZ injection combined with 10 weeks of dietary exposure in C57BL6J female mice effectively induces non-genetic T2D while preserving fertility. Thus, this model has the potential to facilitate the examination of the complex interplay between maternal glucose metabolism and fetal development, including how maternal hyperglycemia influences fetal growth, placental function, and the potential long-term consequences for offspring health.

Our study concluded at the end of the third trimester; however, weight assessment of the offspring could be conducted to evaluate for macrosomia or microsomia. This would provide further insights into the replication of a T2D environment, as opposed to a T1D environment, offering a more comprehensive model of T2D during pregnancy

This model enables researchers to investigate the mechanisms underlying detrimental effects of pre-existing T2D on the offspring within a controlled experimental environment. For instance, to study early complications of T2D in offspring, including preterm birth, miscarriage, birth defects, fetal macrosomia, neonatal hypoglycemia, hypocalcemia, polycythemia, hyperbilirubinemia, acute respiratory distress syndrome, etc.[22,23,25,28]. Moreover, the long-term metabolic outcomes and "programming" effects of maternal T2D exposure on offspring can be investigated, such as increased risk of T2D, metabolic syndrome, obesity, dyslipidemia, cardiovascular issues, low-grade inflammation, early kidney failure, neurocognitive development, and mental health issues[23,26-31,3-39]. Additionally, the model may provide insights into the underlying mechanisms contributing to these outcomes, including stem cell differentiation, epigenetic alterations, metabolomic changes, immune dysregulation, and neuroinflammation[22,53,54]. Overall, this model offers a comprehensive approach to studying the impact of

maternal T2D on offspring health, facilitating the development of preventive and therapeutic strategies to mitigate these adverse effects.

Additionally, this model may serve as a valuable tool to evaluate the effectiveness of potential interventions aimed at managing or preventing various health outcomes in the progeny [24-32, 34-44]. Researchers can scrutinize diverse therapeutic strategies, such as dietary adjustments, pharmacological interventions, or lifestyle modifications, to ascertain their impact on offspring health and well-being. Insights gained from the mouse model may inform clinical practice by enhancing our understanding of how maternal T2D impacts pregnancy outcomes.

4.4 Strengths of the Study

The study introduces a comprehensive mouse model that combines an HFS diet with varying doses of STZ to simulate key aspects of T2D and its gestational implications. This model offers a controlled environment for investigating complex metabolic interactions, yielding valuable insights into the pathophysiology of T2D and its effects on offspring health. Moreover, the study demonstrates a cost-effective approach to establishing a maternal mouse model for pre-existing T2D.

The study's thorough and regular assessments of various metabolic parameters enhance the reliability and robustness of the findings, allowing for an accurate diagnosis of maternal diabetes severity, which aligns with the study's objectives. By directly addressing a relevant clinical scenario and examining gestational effects, the model has the potential to improve our understanding of how maternal T2D impacts offspring health. This model enables researchers to explore the effects of maternal hyperglycemia on fetal growth and long-term outcomes as discussed above.

By replicating hallmark features of T2D and accounting for the combined effects of diet and STZ dosage, the study reflects the multifactorial nature of T2D development, mirroring real-world complexities. This approach provides a platform for testing various interventions, including dietary modifications and pharmacological treatments, to manage or prevent adverse health outcomes in offspring.

Through careful monitoring and analysis, the study offers insights into the mechanisms driving alterations in glucose metabolism, contributing to a deeper understanding of disease processes and potential therapeutic targets. The findings from this mouse model have the potential to guide clinical practice by enhancing our understanding of how maternal T2D affects pregnancy outcomes, ultimately aiding healthcare providers in designing more effective management strategies.

4.5 Limitations of the Study

While the study offers valuable insights into the gestational implications of T2D using a mouse model, several limitations should be acknowledged. First, although the mouse model successfully mimics certain aspects of T2D, it remains a simplified representation of the complex human condition. The biological differences between mice and humans may limit the direct applicability of these results to clinical scenarios. Additionally, the high-fat and high-sugar (HFS) diet used in the study, while relevant to typical Western diets, may not fully encompass the dietary complexities associated with human T2D.

Additionally, beyond dietary factors, various social determinants of health influence gestation and fetal development in the context of *in utero* T2D exposure. These include the management of T2D during pregnancy and preconception, as well as psychosocial factors such as stress, socioeconomic status, social support systems, employment, education, food security, ethnicity, cultural practices, and intergenerational trauma. While many of these determinants cannot be adequately modeled in rodents, they are critical to acknowledge as potential confounders when interpreting the effects of maternal T2D on fetal outcomes.

A notable limitation is the observed discrepancy in weight gain between the HFS-only control group and other HFS groups, which may have resulted from dietary inconsistencies between batches. This inconsistency could potentially lead to an underestimation of the effects of dietary duration and STZ treatment on metabolic outcomes.

Although some subgroups had limited sample sizes, the validation of results in a subsequent round of the study strengthens the reliability and validity of the findings. Lastly, external factors such as housing conditions and stress could have influenced the results, introducing potential confounding variables[82,83].

4.8. Conclusions and Summary

In conclusion, this study achieved its primary objective of developing a C57BL/6J mouse model with pre-existing T2D during pregnancy. Through analysis of various dietary durations and STZ dosages, it has been determined that a 10-week exposure to the HFS diet coupled with 150 mg/kg of STZ administration constitutes the optimal approach to induce T2D in female mice. The strengths of this model, encompassing its relative cost-effectiveness and efficient offspring production, underscore its practicality and its potential to advance our understanding of gestational T2D implications. With the capacity to replicate crucial metabolic features of human T2D, this model opens up pathways for further investigation into maternal-fetal interactions, risk assessment, intervention strategies, and even the broader implications of fetal programming. In summary, the *in utero* T2D mouse model described in this thesis holds the promise of deepening our understanding of gestational metabolic dynamics and ultimately improving the health outcomes for future generations.

Appendix

Citrate Buffer Recipe[77]

The instruction to prepare a citrate buffer at pH 4.5:

1. Take 800 mL of distilled water in a suitable container.
2. Add 25.703 g of sodium citrate dihydrate to the solution.
3. Add 2.421 g of citric acid to the solution.
4. Adjust the solution to the final desired pH of 4.5 using either HCl (hydrochloric acid) or NaOH (sodium hydroxide) using pH meter machine.
5. Finally, add distilled water until the volume reaches 1 L.

This citrate buffer can be useful for various applications, and it has a shelf life of up to 3 months at room temperature.

Table A1. Formulation of Mineral Mix S10026B in the Experimental Diet D12451. The picture is taken from the Research Diet website[67].

Class description	Ingredients	Grams
Carbohydrate	Sucrose, Fine Granulated	179.82 g
Mineral	Potassium Citrate, Monohydrate	330.00 g
Mineral	Calcium Phosphate, Dibasic	260.00 g
Mineral	Calcium Carbonate, Light, USP	110.00 g
Mineral	Sodium Chloride	51.80 g
Mineral	Magnesium Sulfate, Heptahydrate	51.52 g
Mineral	Magnesium Oxide, Heavy, DC USP	8.38 g
Mineral	Ferric Citrate	4.20 g
Mineral	Manganese Carbonate Hydrate	2.45 g
Mineral	Zinc Carbonate	1.12 g
Mineral	Chromium Potassium Sulfate	0.39 g
Mineral	Copper Carbonate	0.21 g
Mineral	Ammonium Molybdate Tetrahydrate	0.06 g
Mineral	Sodium Fluoride	0.04 g
Mineral	Sodium Selenite	0.01 g
Mineral	Potassium Iodate	0.01 g
	Total:	1000.00 g

Table A2. Formulation of Vitamin Mix V10001C in the Experimental Diet D12451. The picture is taken from the Research Diet website[67].

Class description	Ingredients	Grams
Carbohydrate	Sucrose, Fine Granulated	78.42 g
Vitamin	Vitamin E Acetate, 50%	10.00 g
Vitamin	Niacin (a.k.a. B3, Nicotinic Acid)	3.00 g
Vitamin	Biotin, 1%	2.00 g
Vitamin	Pantothenic Acid, d, Calcium (a.k.a. B5)	1.60 g
Vitamin	Vitamin D3, 100,000 IU/gm	1.00 g
Vitamin	Vitamin B12, Cyanocobalamin, 0.1%	1.00 g
Vitamin	Vitamin A Acetate, 500,000 IU/gm	0.80 g
Vitamin	Pyridoxine HCl (a.k.a. B6)	0.70 g
Vitamin	Riboflavin (a.k.a. B2)	0.60 g
Vitamin	Thiamine HCl (a.k.a. B1)	0.60 g
Vitamin	Folic Acid	0.20 g
Vitamin	Menadione Sodium Bisulfite	0.08 g
	Total:	100.00 g

Table A3. Pre-gestational Fasted Blood Glucose measurements in each group.

Group	Fasted Blood Glucose Range, mmol/L	Average Fasted Blood Glucose, mmol/L	95% Confidence Interval
4 weeks on HFS diet + 75 mg/kg of STZ	3.6-6.4	5.0	5.0±0.4
4 weeks on HFS diet + 100 mg/kg of STZ	3.4-6.8	4.7	4.7±0.6
4 weeks on HFS diet + 150 mg/kg of STZ	4.0-6.8	4.9	4.9±0.4
6 weeks on HFS diet + 75 mg/kg of STZ	4.0-6.9	5.0	5.0±0.6
6 weeks on HFS diet + 100 mg/kg of STZ	3.6-6.5	4.7	4.7±0.6
6 weeks on HFS diet + 150 mg/kg of STZ	3.7-6.7	4.6	4.6±0.6
8 weeks on HFS diet + 100 mg/kg of STZ	4.8-8.4	6.4	6.4±0.5
8 weeks on HFS diet + 150 mg/kg of STZ	3.9-8.9	5.9	5.9±0.6
10 weeks on HFS diet + 75 mg/kg of STZ	4.1-7.8	5.4	5.4±0.5
10 weeks on HFS diet + 100 mg/kg of STZ	4.3-8.2	5.6	5.6±0.5
10 weeks on HFS diet + 150 mg/kg of STZ	4.0-11.2	6.3	6.3±0.5
10 weeks on HFS diet + citrate buffer	4.8-6.5	5.4	5.4±0.4
6 weeks on chow diet + citrate buffer	3.3-4.4	3.7	3.7±0.4
8 weeks on chow diet + citrate buffer	4.4-5.7	5.0	5.0±0.4
10 weeks on chow diet + citrate buffer	4.2-6.2	5.1	5.1±0.5

Table A4. Comparison of the Glycemia During the GTT in Each Group.

Group	Glycemic range at the 0^{th} minute, mmol/L	Average Blood Glucose at the 0th minute, mmol/L (95% CI)	Glycemic range at the $10^{th,}$ 20^{th}, and 30^{th} minutes, mmol/L	Average Blood Glucose at the $10^{th,}$ 20^{th}, and 30^{th} minutes, mmol/L (95% CI)	Glycemic range at the 120^{th} minute, mmol/L	Average Blood Glucose at the 120th minute, mmol/L (95% CI)
4 weeks on HFS diet + 75 mg/kg of STZ	5.1-6.2	5.7	12.5-16.6	14.6	5.9-7.3	6.7
4 weeks on HFS diet + 100 mg/kg of STZ	4.0-6.7	5.0	10.3-15.2	12.3	5.7-8.4	6.8
4 weeks on HFS diet + 150 mg/kg of STZ	4.8-6.6	5.5	10.7-16.7	13.2	7.2-10.4	9.0
6 weeks on HFS diet + 75 mg/kg of STZ	6.3-6.6	6.5	6.8-20.1	14.7	4.8-6.2	6.3
6 weeks on HFS diet + 100 mg/kg of STZ	5.7-6.9	6.2	10.2-18.7	14.7	5.7-8.4	6.8
6 weeks on HFS diet + 150 mg/kg of STZ	4.8-6.7	5.5	10.4-15.0	12.6	4.8-8.5	6.4
8 weeks on HFS diet + 100 mg/kg of STZ	6.3-6.6	6.5	7.8-17.8	12.4	6.2-8.3	6.8
8 weeks on HFS diet + 150 mg/kg of STZ	6.5-8.2	7.5	14.4-20.9	17.6	7.0-8.3	7.4
10 weeks on HFS diet + 75 mg/kg of STZ	5.2-6.2	5.6	11.6-20.8	16.5	5.9-7.3	6.7
10 weeks on HFS diet + 100 mg/kg of STZ	4.7-7.3	6.1	12.2-29.5	19.1	6.2-14.5	9.5
10 weeks on HFS diet + 150 mg/kg of STZ	5.2-8.9	6.8	12.0-23.6	17.3	7.7-10.0	8.6
10 weeks on HFS diet + citrate buffer	5.2-6.5	5.6	9.0-15.8	12.6	7.0-8.8	7.8
6 weeks on chow diet + citrate buffer	5.6-7.3	6.3	10.0-16.9	12.8	5.6-5.9	5.7
8 weeks on chow diet + citrate buffer	5.3-6.1	5.8	7.1-14.5	11.1	4.5-5.7	5.3
10 weeks on chow diet + citrate buffer	5.6-6.4	6.1	10.6-17.2	13.3	5.9-7.0	6.4

Table A5. Comparison of the Glycemia During the ITT in Each Group.

Group	Glycemic range at the 0th minute, mmol/L	Average Blood Glucose at the 0th minute, mmol/L	Glycemic range at the 30th, 45th, and 60th minutes, mmol/L	Average Blood Glucose at the 30th, 45th, and 60th minutes, mmol/L	Glycemic range at the 120th minute, mmol/L	Average Blood Glucose at the 120th minute, mmol/L
4 weeks on HFS diet + 75 mg/kg of STZ	9.7-11.8	10.4	8.1-12.1	10.5	7.0-10.9	9.7
4 weeks on HFS diet + 100 mg/kg of STZ	6.0-10.4	8.7	8.0-11.9	10	7.1-8.7	7.6
4 weeks on HFS diet + 150 mg/kg of STZ	9.0-10.4	9.4	10.5-14.1	11.9	6.9-10.4	9.4
6 weeks on HFS diet + 75 mg/kg of STZ	10.7-11.0	10.9	2.7-4.6	3.9	5.6-6.4	6.0
6 weeks on HFS diet + 100 mg/kg of STZ	8.5-10.7	9.7	1.1-4.9	3.2	3.4-4.7	4.2
6 weeks on HFS diet + 150 mg/kg of STZ	8.9-10.5	9.7	1.4-5.4	3.4	6.9-10.4	9.4
8 weeks on HFS diet + 100 mg/kg of STZ	7.6-9.9	8.8	2.3-4.7	3.3	2.8-5.6	4.4
8 weeks on HFS diet + 150 mg/kg of STZ	7.2-10.9	9.3	1.1-5.6	2.6	1.8-3.0	2.4
10 weeks on HFS diet + 75 mg/kg of STZ	7.4-11.8	9.8	1.1-4.1	2.2	3.8-4.7	4.3
10 weeks on HFS diet + 100 mg/kg of STZ	10.4-12.3	11.0	2.5-4.8	3.8	4.4-5.5	5.1
10 weeks on HFS diet + 150 mg/kg of STZ	9.5-14.9	12.2	1.1-5.9	3.2	2.7-7.5	5.6
6 weeks on chow diet + citrate buffer	6.9-7.8	7.5	7.0-10.5	8.3	5.8-9.8	7.3
8 weeks on chow diet + citrate buffer	6.2-8.3	7.3	1.4-4.7	2.3	4.4-5.5	5.1
10 weeks on chow diet + citrate buffer	8.9-10.5	9.1	8.6-9.7	3.3	3.8-5.7	4.7

Table A6. Last Pre-gestational and Gestational Body Weights in Each Group.

Group	Pre-gestational Body Weight Range, g	Average Pre-gestational Body Weight, g	3rd Trimester Gestational Body Weight Range, g	Average 3rd Trimester Gestational Body Weight, g	Body weight gain, %
4 weeks on HFS diet + 75 mg/kg of STZ	27-35	31.5	37-47	41.0	30.2
4 weeks on HFS diet + 100 mg/kg of STZ	24-27	25.0	31-41	38.3	53.2
4 weeks on HFS diet + 150 mg/kg of STZ	22-30	25.5	35-42	39.3	54.1
6 weeks on HFS diet + 75 mg/kg of STZ	28-29	28.5	40-41	40.5	42.1
6 weeks on HFS diet + 100 mg/kg of STZ	27-29	28.0	38-39	38.8	38.6
6 weeks on HFS diet + 150 mg/kg of STZ	20-30	23.8	33-39	35.6	49.6
8 weeks on HFS diet + 100 mg/kg of STZ	24-31	26.8	31-38	35.6	32.8
8 weeks on HFS diet + 150 mg/kg of STZ	26-30	28.0	36-44	40.3	43.9
10 weeks on HFS diet + 75 mg/kg of STZ	27-34	29.8	41-45	42.8	43.6
10 weeks on HFS diet + 100 mg/kg of STZ	27-41	33.0	37-47	41.3	25.2
10 weeks on HFS diet + 150 mg/kg of STZ	26-35	29.1	36-45	41.0	40.9
Chow diet + citrate buffer	21-27	24.5	31-42	36.3	48.2

Table A7. Last Pre-gestational and Gestational Fasted Blood Glucose in Each Group.

Group	PGFBG Range, mmol/L	Average PGFBG, mmol/L	1st Trimester GFBG Range, mmol/L	Average 1st Trimester GFBG, mmol/L	2nd Trimester GFBG Range, mmol/L	Average 2nd Trimester GFBG, mmol/L	Average 3rd Trimester GFBG, mmol/L	3st Trimester GFBG, mmol/L
4 weeks on HFS diet + 75 mg/kg of STZ	4.3-9.1	6.3	5.3-8.0	6.3	3.8-7.5	5.7	3.6-4.4	4.1
4 weeks on HFS diet + 100 mg/kg of STZ	3.4-5.6	4.3	3.0-5.5	4.6	3.6-7.4	4.9	3.2-5.7	4.3
4 weeks on HFS diet + 150 mg/kg of STZ	4.2-7.5	5.3	3.7-10.4	5.8	5.1-6.5	5.5	4.3-8.5	5.5
6 weeks on HFS diet + 75 mg/kg of STZ	4.8-5.1	5.0	5.0-6.5	5.8	4.3-5.1	4.7	4.0-5.6	4.8
6 weeks on HFS diet + 100 mg/kg of STZ	5.1-8.8	6.5	4.4-6.6	6.0	5.8-8.1	6.9	3.0-5.9	4.3
6 weeks on HFS diet + 150 mg/kg of STZ	5.4-8.7	6.4	3.9-7.6	6.1	4.9-9.4	6.7	3.5-6.5	5.0
8 weeks on HFS diet + 100 mg/kg of STZ	4.6-9.2	7.3	4.5-9.9	6.5	7.2-10.1	8.6	4.3-6.8	5.4
8 weeks on HFS diet + 150 mg/kg of STZ	5.7-8.7	7.5	4.5-11.8	7.3	8.0-9.8	8.9	4.6-8.1	6.2
10 weeks on HFS diet + 75 mg/kg of STZ	5.2-7.4	6.0	6.0-8.6	7.2	5.7-10.3	7.7	4.2-5.1	4.6
10 weeks on HFS diet + 100 mg/kg of STZ	4.7-8.5	6.5	5.5-9.7	7.4	4.8-11.1	8.3	3.5-7.8	5.5
10 weeks on HFS diet + 150 mg/kg of STZ	4.8-9.3	6.6	5.5-11.5	8.7	5.8-10.3	7.8	3.3-7.4	5.0
Chow diet + citrate buffer	3.0-5.7	4.5	4.2-6.6	5.5	3.7-7.7	6.0	3.4-7.7	5.0

Table A8. Last Pre-gestational and Gestational Random Blood Glucose in Each Group

Group	PGRBG Range, mmol/L	Average PRBG, mmol/L	1st Trimester GRBG Range, mmol/L	Average 1st Trimester GRBG, mmol/L	2nd Trimester GRBG Range, mmol/L	Average 2nd Trimester GRBG, mmol/L	Average 3rd Trimester GRBG, mmol/L	3rd Trimester GRBG, mmol/L
4 weeks on HFS diet + 75 mg/kg of STZ	9.7-11.8	10.4	9.2-10.9	10.3	6.8-10.5	9.1	7.9-8.5	8.1
4 weeks on HFS diet + 100 mg/kg of STZ	6.0-10.4	8.7	no data	no data	7.7-10.4	9.2	7.3-10.0	8.8
4 weeks on HFS diet + 150 mg/kg of STZ	8.5-9.6	9.2	8.3-9.7	9.0	8.5-13.5	11.0	10.6-11.7	11.0
6 weeks on HFS diet + 75 mg/kg of STZ	10.7-11.0	10.6	no data	no data	9.5-12.2	10.9	6.2-7.3	6.8
6 weeks on HFS diet + 100 mg/kg of STZ	6.8-10.7	8.5	8.2-14.2	10.7	7.3-12.8	10.6	7.4-10.8	9.0
6 weeks on HFS diet + 150 mg/kg of STZ	9.4-10.5	10.1	9.3-15.8	11.8	7.5-13.9	11.3	8.5-12.5	10.7
8 weeks on HFS diet + 100 mg/kg of STZ	9.2-13.0	11.0	9.3-10.5	9.7	9.7-13.1	11.6	7.6-11.4	9.6
8 weeks on HFS diet + 150 mg/kg of STZ	8.4-12.5	10.7	9.5-12.5	10.6	8.1-11.0	9.6	8.5-10.9	9.5
10 weeks on HFS diet + 75 mg/kg of STZ	8.4-9.8	8.9	8.7-11.4	10.0	8.3-11.7	10.1	7.7-8.7	8.2
10 weeks on HFS diet + 100 mg/kg of STZ	9.0-10.5	9.9	7.4-11.5	9.8	10.4-16.3	12.7	9.3-12.7	10.9
10 weeks on HFS diet + 150 mg/kg of STZ	7.4-11.9	9.2	8.6-12.4	10.4	8.5-13.0	10.5	6.8-11.5	8.7
Chow diet + citrate buffer	6.3-10.4	8.4	5.9-12.9	9.5	8.1-10.6	9.3	6.2-9.3	7.8

Table A9. Protocol for establishing a maternal mouse model with pre-existing T2D.

Animal Acclimatization	Female C57BL/J6 mice should be transferred to the animal care facility at 3 weeks of age after weaning. They are to be placed on a standard chow diet for one week to ensure acclimatization.
Dietary Regimen	Upon reaching 4 weeks of age, experimental animals should be transitioned to a high-fat and high-sucrose (HFS) diet containing 35% kcal from fat and 45% from sucrose. This dietary composition should be maintained throughout the course of the study.
STZ Administration	At 14 weeks of age, administer a single intraperitoneal injection of 150 mg/kg of STZ dissolved in a 4.5 pH citrate buffer.
Mating Process	After a two-week interval from STZ treatment, introduce the treated female mice to healthy C57BL/J6 male mice for mating purposes.
Monitoring Parameters	To ensure comprehensive metabolic control and mitigate potential outliers that could impact the metabolic health of offspring, implement regular monitoring measures. These should include biweekly or weekly measurements of BW and FBG before the onset of pregnancy and confirmation of the development of T2D in mice through glucose tolerance testing (GTT). To minimize stress during pregnancy and the risk of litter cannibalization, which C57BL/6 mice are susceptible to, it is advisable to avoid invasive assessments during gestation.
Postpartum Care	Following successful litter delivery, it is recommended to maintain the pups within the maternal cage until they reach 21 days of age. At this point, separate the pups for subsequent investigations.

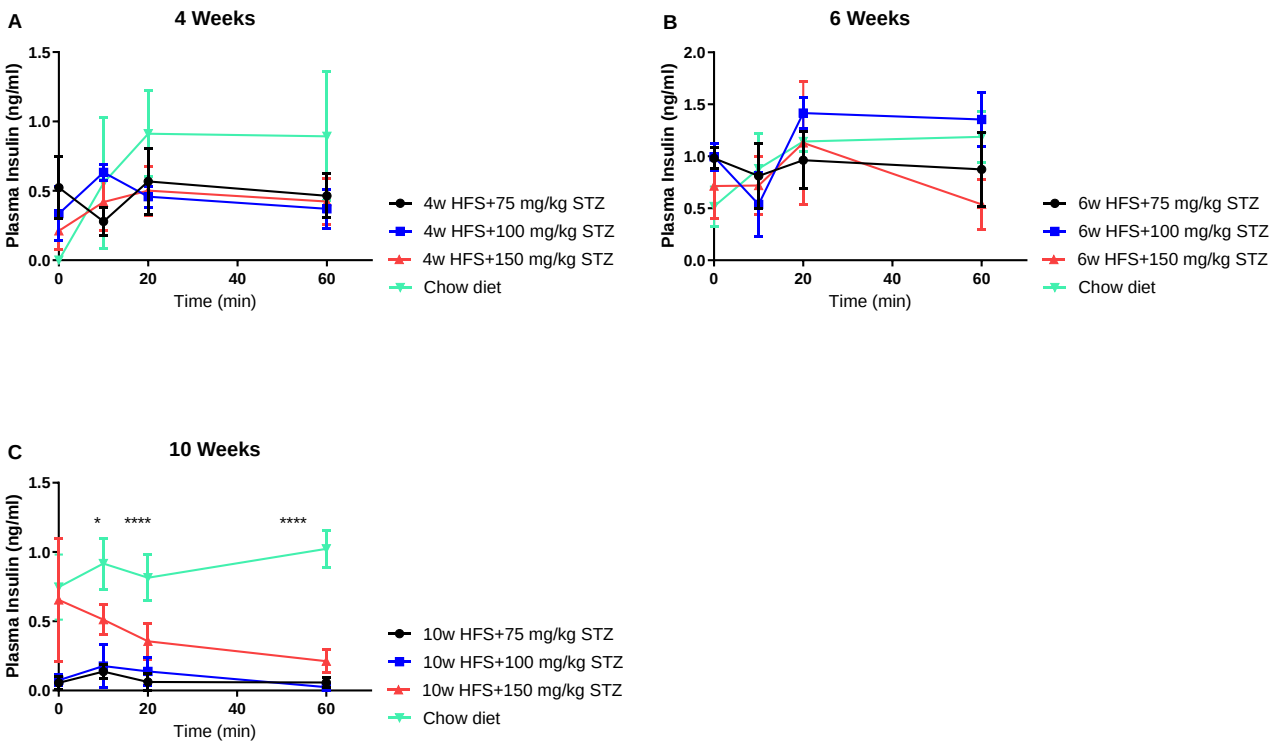

Figure A1. Comparison of plasma insulin concentrations in response to the glucose tolerance test between the various lengths of HFS diet injected with different STZ doses or chow diet and citrate buffer. Groups of animals assigned for 4 weeks (**A**), 6 weeks (**B**), 10 weeks (**C**) on HFS or chow diet (control) and injected with 75 mg/kg, 100 mg/kg, 150 mg/kg of STZ or equivalent volumes of citrate buffer (control). Analysis performed using GraphPad Prism software using two-way ANOVA. *=p<0.05, ****=p<0.0001. The lower dose of STZ was associated with higher insulin concentration. Notably, the 4-weeks HFS+75 mg/kg STZ subgroup showed a paradoxical decrease in insulin concentration at the 10-minute point. The control group demonstrated rapid increase in insulin concentration after glucose was injected (A). All 6 weeks groups after glucose injection exhibited a decrease in insulin concentration at the 10-minute point, followed by a rise at the 20-minute point (B). The 6-weeks HFS+100 mg/kg STZ subgroup showed slightly higher insulin levels than the control group, while the 6-weeks HFS+150 mg/kg STZ subgroup demonstrated a rapid decline by the 60th minute of the test (B). The 10-weeks HFS+150 mg/kg STZ demonstrated high variability in insulin concentration at the baseline (C). Due to many missing points, results for 10-weeks HFS+75 mg/kg STZ and 10-weeks HFS+100 mg/kg STZ were inconclusive (C).

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