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Sexual Dimorphism in Response to Repetitive Bouts of Acute Exercise in Rodents with Type 1 Diabetes Mellitus

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Abstract

The purpose of this study was to examine sex-specific differences in the blood glucose (BG) response to recurrent aerobic exercise in type 1 diabetes. Specifically, we examined the role of peak estrogen (E2) concentrations during proestrus on BG response to prolonged aerobic exercise. To do so, nineteen Sprague-Dawley rats were assigned to four exercised groups: control female (CXF; n = 5), control male (CXM; n = 5), diabetic female (DXF, n = 5) and diabetic male (DXM, n = 4). Diabetes was induced in DX groups via subcutaneous injection of low dose streptozotocin. After four days of exercise, liver glycogen and BG responses were compared. The final bout of exercise took place during proestrus when estrogen concentrations were at their highest in the female rats. No liver glycogen differences between DXF and DXM or CXF and CXM were evident. During days 1-3, DXF had lower BG concentrations during exercise, but quicker BG recovery than DXM. Therefore, fluctuating estrogen concentrations may have reduced the sex based differences in BG response and fuel selection during exercise.

Keywords

Type 1 diabetes mellitus, exercise, estrogen, blood glucose, sex differences

Summary for Lay Audience

Type 1 diabetes mellitus (T1DM) is a disease that results in the body being unable to produce a hormone called insulin, which helps to control blood sugar levels. There is no singular cause of T1DM, and there is no known cure. Treatment of this disease includes insulin administration either by pump or injection to maintain blood sugar near normal levels. Diet and exercise can assist in the management of this disease; however, aerobic exercise, which is moderate or high intensity in nature for a prolonged period of time, can result in low blood sugar, or hypoglycemia. During aerobic exercise the body uses a combination of sugar (glucose) and fat as fuel, and for a person with T1DM it would be more beneficial to utilize primarily fat as a fuel source in order to maintain blood sugar. The primary female sex hormone estrogen has been shown to increase the usage of fat and spare the usage of glucose. The level of estrogen fluctuates throughout the menstrual cycle and is considerably higher during the luteal phase of the cycle, which accounts for half of the cycle length. It is possible that due to the higher estrogen levels during this phase of menstruation females with T1DM may be more likely to use fat than sugar during aerobic exercise, protecting them against exercise induced hypoglycemia to a greater degree than males with T1DM. In this thesis we studied four rodent groups: diabetic males, diabetic females, nondiabetic males and nondiabetic females. All animals underwent four consecutive days of aerobic exercise training. Vaginal cytology was used to determine the cycle length and pattern in the rats, and the female rats were staged so that the final day of exercise took place during the phase with the highest estrogen levels (proestrus). Liver samples were taken on the final day of exercise to elucidate and glycogen sparing effect. The primary findings of this study was that the diabetic females recovered from exercise faster than the diabetic males. We did not find a glycogen sparing effect between the diabetic males and the diabetic females; however, both

the diabetic males and females had significantly less liver glycogen than their non-diabetic counterparts by the end of the four day protocol. These findings indicate that there is less risk of hypoglycemia to the T1DM female during recurrent aerobic exercise when estrogen levels are high, but no protective effect against liver glycogen depletion.

Co-Authorship Statement

Dr. Jamie Melling of Western University, London, Ontario, Canada assisted in the development of the project design, interpretation of results and revision of thesis.

Dedication

To Mom, Dad, Kelly, Dennis and Austin. Thank you from the bottom of my heart.

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List of Abbreviations

BG – blood glucose

CO_2 – carbon dioxide

CT – conventional insulin therapy

CVD – cardiovascular disease

DCCT – Diabetes Control and Complications Trial

E2 – estrogen or 17β estradiol

ER – estrogen receptor

FFA – free fatty acid

FP – follicular phase

FSH – follicle stimulating hormone

GH – growth hormone

GP-ER – G-protein coupled estrogen receptor

HbA_{1c} – glycated hemoglobin

IIT – intensive insulin therapy

IMCL – intramyocellular lipid content

LH – luteinizing hormone

LP – luteal phase

O_2 – oxygen

RER – respiratory exchange ratio

STZ – streptozotocin

T1DM – type 1 diabetes mellitus

T2DM – type 2 diabetes mellitus

VO_{2max} – maximal oxygen uptake

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Chapter 1

1.1 Overview of Type 1 Diabetes Mellitus

Diabetes is a category of diseases that is characterized by the body's inability to produce or utilize insulin efficiently. Type 1 and type 2 diabetes mellitus are the two main types of diabetes, with the incidence of type 1 staying slowly increasing overtime and accounting for approximately 10% of diabetes diagnoses in Canada[1]. In type 2 diabetes mellitus (T2DM) a combination of lifestyle, environmental and genetic factors result in chronic hyperglycemia leading to hyperinsulinemia and insulin resistance [2, 3]. As a result glucose metabolism is disrupted, and many cells are unable to use glucose from the blood stream. Chronic hyperglycemia and increased free fatty acids (FFA) cause oxidative stress to the β-cells of the pancreas, worsening the state of hyperglycemia [4,5]. T2DM accounts for approximately 90% of all cases of diabetes and is more common in developed or industrialized countries [6,7]. The global prevalence of diabetes is expected to rise to 10.5% by 2040, and the increase is expected to be due to an increase in T2DM cases[7]. Although there are severe comorbidities associated with T2DM, the symptoms can be largely managed through diet and exercise modifications if caught early, and several antihyperglycemic agents are available[8]. Majority of individuals diagnosed with T1DM are diagnosed prior to the age of 20, whereas T2DM is diagnosed more often in adults although diagnoses in younger individuals are becoming more common[5].

The pathophysiology of type 1 diabetes mellitus (T1DM) is an entirely separate process than that of T2DM, in that the β-cells of the pancreas are destroyed in an

autoimmune cytokine-mediated reaction such that the pancreas can no longer produce sufficient amounts of insulin [4,9]. The disruption of the pancreatic β-cells is accompanied by α-cell disruption, resulting in elevated glucagon levels which further disrupts glucose homeostasis [5,10]. Glucagon is an important metabolic hormone released by the α-cells of the pancreas in response to hypoglycemia onset (lowered blood glucose) to raise BG through glycogenolysis to increase BG, and in periods of hyperglycemia glucagon expression is suppressed. This suppression of glucagon is disrupted in T1DM, resulting in no reduction in glucagon levels during periods of hyperglycemia which can further increase BG levels[10]. The state of hyperglycemia is further worsened by the usage and availability of free fatty acids (FFAs), which are metabolized by the liver into ketone bodies[10]. When ketone body production exceeds the body's ability to utilize them a condition called ketoacidosis results and can be fatal, especially for children[10,11]. Prior to the development of insulin therapy, T1DM was fatal usually within 1-2 years, due to starvation, coma, and ketoacidosis[12,13]. The discovery of insulin meant the patient with T1DM could utilize exogenous insulin to normalize glycemia. The initial results of this new insulin treatment were temporary, and a continued dosage of insulin was required to keep symptoms from resurfacing[14]. It was later discovered that complications of both T1DM and the insulin treatment itself were common without precise control of dosage. Retinopathy, nephropathy and cardiovascular complications are all linked to poor glycemic control while taking insulin[14-16]. However, when administered properly the use of insulin to treat T1DM has reduced mortality and increased life expectancy overall; the common causes of mortality related to T1DM are now complications such as cardiovascular disease rather than the historical [13].

Further developments in the medical field culminated in the Diabetes Control and Complications Trial, an extensive cohort study that examined the benefits of intensive insulin therapy (IIT) compared to conventional therapy (CT)[17]. CT, a more traditional approach to treatment consisted of one to two injections of insulin per day, with the goal of preventing symptoms of hyperglycemia or causing hypoglycemia. IIT is a more involved process, with multiple smaller doses of insulin throughout the day, which were adjusted based on self-administered BG tests. Participants were also advised to alter their insulin dosage based on exercise and diet[17]. The purpose of IIT is to maintain a consistent BG (3.9 -6.7 mmol/L) level throughout the day, and a monthly hemoglobin A_{1c} (HbA_{1c}) of less than 6%. The HbA_{1c} is a measure of glycated hemoglobin, a process when a component of one of the hemoglobin dimers (HbA) is exposed to a hyperglycemic environment and this component becomes glycated[19]. The DCCT showed that participants undergoing IIT had tighter glycemic control and lower HbA_{1c} combined with reductions in complications associated with diabetes than those undergoing CT[17] Moreover, IIT decreased the risk and progression of retinopathy, reduced neuropathy and nephropathy, but also caused a threefold increase in the risk of hypoglycemia compared to the CT treatment[17,18,20,21]. Hypoglycemia is commonly described as a BG below 3.0mmol/L, however in diabetics a BG between 3.0-3.9 mmol/L is sufficient to require intervention to avoid visual disturbance, headaches, loss of consciousness or seizures[22]. Participants who experienced one bout of severe hypoglycemia were more likely to have subsequent hypoglycemic episode. Lower HbA_{1c} was also a predictor of hypoglycemia but prior hypoglycemia was the strongest predictor. Interestingly, the relative risk of

hypoglycemia between CT and IIT was greater for males than females, although this was not a main discussion point in the studies[18,20].

1.2 Cardiovascular benefits to aerobic exercise and Exercise-Induced Hypoglycemia in T1DM patients

As noted above, IIT is used to modify the insulin dosage when performing physical activity in order to avoid hypoglycemia by monitoring insulin dosage multiple times throughout the day. Indeed, the fear of exercise related hypoglycemia is one of the main non-physical barriers to exercise for type 1 diabetics[21]. Exercise is often prescribed to patients with T1DM due to the well-known associated cardiovascular benefits[22-24]. Therefore, a clear understanding of the potential risks that may be incurred during exercise is essential to safely prescribe an exercise program in order to prevent post-exercise or nocturnal hypoglycemia development[22-24]. For example, the type, duration, intensity and modality of the exercise can impact the risk of hypoglycemia as the BG response to the exercise is dependent upon multiple factors[22,23]. Aerobic exercise training has been observed to reduce many of the complications often associated with moderate hyperglycemia in insulin treated rodent models[25]. For example, insulin resistance is improved in rodents that have undergone a 10-week long aerobic training program, likely due to the insulin like effect of muscle contractions[26]. Muscle contraction causes GLUT4 transporters to move to the membrane of the muscle cell and increases the amount of glucose that is taken into the cell, similar to the effects of insulin[27]. Failing to account for this increase in glucose removal from the blood stream or failing to reduce insulin levels prior to exercise can increase the risk of hypoglycemia. High-intensity aerobic exercise

training has been shown to maintain parasympathetic function[27,28] and cardiac performance[25] in insulin treated T1DM rats. T1DM is known to reduce autonomic control of cardiac muscle, and aerobic exercise training may improve vagal control of heart rate[27]. Aerobic training may also decrease insulin dosage requirements[27,29]. By the end of a 10-week training program including high intensity or low intensity aerobic exercise, insulin requirements were significantly reduced, and the high intensity aerobic exercise resulted in the greatest reduction in insulin dosage[27,29]. Aerobic exercise also improves lipid levels and cardiovascular fitness is improved, and all-cause mortality risk is lowered[24.]

While the risk of hypoglycemia onset is evident across each mode of exercise likely due to contraction mediated glucose uptake, decreased counterregulatory hormonal response and failure or lack of knowledge to appropriately adjust insulin prior to prolonged exercise[22]. Resistance exercise is associated with less of a decline in BG response to exercise in comparison to aerobic exercise, likely due to the minimal effect on reducing insulin requirement[29]. Although a decreased BG during the late recovery period (four to six hours post exercise) or overnight, termed nocturnal hypoglycemia[30], might be more apparent with resistance exercise. Resistance exercise training has not definitively been shown to improve HbA_{1c} levels in T1DM but has shown improvement in these levels for T2DM; however, HbA_{1c} does not measure the other physical benefits of resistance exercise such as increased strength[29-31]. Resistance exercise ultimately presents lower risk of hypoglycemia, and benefits such as increased muscular strength, bone density and ability to perform functional tasks are similar to those seen in non-diabetics[24]. Overall, a combination of both exercise and resistance training is beneficial to

diabetics, and aerobic training may be the most beneficial in terms of reducing comorbidities associated with T1DM so long as the appropriate measures such as insulin reduction or carbohydrate loading are taken[22,32]. In fact, aerobic exercise training has been shown to lead to greater CVD improvements than IIT, the standard treatment for T1D[99]. These improvements in aerobic exercise-treatment animals were evident despite the use of CT to minimize the risk of hypoglycemia during exercise.

The issue of exercise becomes more complex when examining athletes or avid exercisers with T1DM who are required perform repetitive bouts of intense exercise two or more days in a row, and sometimes multiple times a day. The effects of a single bout of aerobic exercise may exacerbate the hypoglycemic onset during subsequent exercise, particularly if that exercise is partaken during the extended recovery period when BG levels may not be fully recovered[35]. During prolonged aerobic exercise there is a marked increase in muscle glucose uptake, increased hepatic glucose release and gluconeogenesis, rarely resulting in hypoglycemia under normal conditions in non-diabetic individuals[35]. In these healthy individuals, glucagon, catecholamines and cortisol are secreted in response to acute exercise, resulting in availability of glucose for the working muscle[39]. However, during subsequent exercise a decrease in metabolic and neuroendocrine responses is evident in both diabetic and healthy subjects of both sexes[33,34].

The neuroendocrine response to exercise had been observed to be greater in healthy males than healthy females, but only at moderate intensities[36,37]. Epinephrine, norepinephrine and growth hormone (GH) for example are lower in females than in males during hypoglycemia and periods of normal glycemia. It has been suggested that

women have greater sensitivity to the actions of these hormones, resulting in lower amount of hormone required to elicit the same response[38]. Antecedent periods of hypoglycemic stress, either due to exercise or exogenous insulin correction, results in a blunted neuroendocrine response in both sexes, but the blunting is more profound in males and therefore the effects of hypoglycemia are more apparent in males[38]. The glucagon response is lower following previous periods of hypoglycemia in both sexes but is more severely inhibited in males[34]. There are many potential mechanisms that may explain these sex mediated differences in the glycemic response to exercise; however, it is believed that differences in the lipolytic metabolism is responsible. It is plausible that women are protected against hypoglycemia development during acute or repetitive exercise through a stronger reliance on fat metabolism and a reduction in muscle uptake of glucose in comparison to men[33,39].

1.3 The Glucoregulatory response to exercise

Prolonged exercise causes a disruption of the normal state of metabolism and in turn the body takes actions against the potential development of hypoglycemia. While the threshold of hypoglycemia where symptoms occur is around 3.0 mmol/L, any further reduction in blood glucose below 3.8 mmol/L results in a counterregulatory response [41,42]. Epinephrine and glucagon are the first to be released, followed by growth hormone release shortly after. Epinephrine and glucagon are signal molecules that initiate a cascade of events to stimulate glycogenolysis in the muscle and liver. Below 3.5 mmol/L cortisol is released, stimulating gluconeogenesis and suppressing glycogen synthesis[43].

Muscle cells decrease glucose uptake and adipose tissue is stimulated to increase lipolysis, ensuring alternate forms of energy can supply the brain[43]. Each of these hormones become critical when BG drops below physiologically normal levels and although some studies have shown cortisol deficiency results in a significantly slower return to resting BG levels, the presence of all hormones are required to protect and correct hypoglycemia[42,44,45]. In response to aerobic exercise in non-diabetics, the glucoregulatory response is greater in males; however, the sensitivity to the hormones involved seems to be greater in females[30]. During exercise, hormonal release in females may be lower, but the effects such as lipolysis are greater in females; moreover, following prior hypoglycemia females are able to maintain a more adequate glucoregulatory response than males[33,34,38]. The mechanism behind why diabetic females maintain a glucoregulatory response to exercise is unclear, however it has been suggested that the differences in sex hormones plays a role[50,51].

Athletes or exercisers with T1DM show a different response to aerobic exercise than non-diabetics. Due to the utilization of exogenous insulin, circulating insulin levels are higher than non-diabetics[40]. The increased insulin level, even if reduced prior to exercise, results in greater muscle uptake of glucose such that there is a drop in BG during moderate aerobic exercise [22,32,46]. The hormone response to acute exercise is different in T1D than in non-diabetic males; however, these studies were conducted using male rats so it is not clear if the same hormone changes would be present in females[46].

1.4 The Menstrual and Estrous Cycles

Due to naturally occurring hormone fluctuations, fewer exercise studies have included the study of females. During the menstrual cycle, serum estradiol (E2), progesterone, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) fluctuate in a relatively predictable manner[52]. While the pattern of hormone cycling may be similar, there can be significant variation in the level of these hormones between individuals due to race, age or genetic variation[53-55]. Studies which do examine sex differences in response to exercise are typically performed during the follicular phase (FP) of the menstrual cycle as estrogen levels are at their lowest and most consistent values, making analysis between the sexes simpler[51]. Some studies are further complicated by the use of oral contraceptives which pharmaceutically inflate the estrogen and/or progesterone levels and seemingly demonstrate that higher estrogen levels are beneficial to training adaptations[56,57]. By limiting the influence of sex hormones on the outcome these studies neglect half of the lifespan of sexually mature females.

The hormonal cycle which occurs in females can be described based on changes within the uterus or the ovary and occur within the same time period. The follicular (FP), ovulatory and luteal phases (LP) are part of the ovarian cycle, which is dictated by the maturation of the follicle, oocyte release (ovulation), and formation of the corpus luteum[58]. The FP is thirteen days long, ovulation is typically the 14th day, and the luteal phase lasts approximately fourteen days. The uterine cycle is comprised of menstruation (5 to 7 days), the proliferative phase, and the secretory phase (14 days). Menstruation aligns with the start of the follicular phase and the degradation of the corpus luteum[58]. For the purposes of this review, the FP and LP of the ovarian cycle were examined with

regard to the hormones that are released during these phases. The ovarian (estrogen and progesterone) and pituitary (LH and FSH) hormones are released in a cyclical manner and are modulated by both positive and negative feedback loops[52]. During the luteal phase (LP), E2 levels increase, and suppress the release of LH and to a lesser degree FSH. Progesterone is also released from the corpus luteum, causing a surge in progesterone levels as well. If there is no fertilization, the FP begins with the shedding of the uterine lining. E2 levels are decreased, and FSH is released from the pituitary to aid in the maturation of new follicles. As the follicle matures, E2 levels rise slowly, and positive regulation of LH results in high LH levels. This increase in LH marks the beginning of the LP[52,59]. While LH, FSH and progesterone levels influence multiple pathways in the body, E2 is considered the most important in terms of its influence on gene transcription and signaling cascades relating to metabolism. In particular, E2 has been shown to regulate adipocyte metabolism[52,59].

17β estradiol is the primary form of circulating E2 in humans. E2 exerts its influence by binding to estrogen receptor alpha (ERα), estrogen receptor beta (ERβ), or G-protein coupled estrogen receptor (GP-ER). The uterus is the main E2 target, and changes in the uterine lining can be observed and used to approximate E2 levels[60,61]. E2 is carried through the bloodstream into the cell, where it binds with ERα or ERβ, which exert effects on gene expression, notably in the liver and pancreas in addition to the uterus[61-63]. E2 binding to either ERα or ERβ cause the receptor to dissociate from HSP90, a stabilization and chaperone protein, in the cytosol and translocate into the nucleus where it binds to specific locations on the genome[61]. In the rat, the estrous cycle is a four stage cycle that occurs over the course of 4-5 days and is equivalent to the

menstrual cycle in humans[60,69,70]. Puberty occurs following the fourth post-natal week, when the ovaries mature in response to increases in LH, and maturation is achieved 60 days postnatally[60,71]. There are substantially higher levels of E2 and progesterone observed after 45 days, indicating maturity and that the reproductive cycle has commenced[71]. It is widely recognized that estrogen levels are the lowest during estrus[72-74] and typically highest during proestrus [70,72,73,75]. Metestrus and diestrus typically have the highest levels of progesterone[76] and are sometimes considered one phase or different stages of the same phase[60,77,78]. Metestrus is usually the shortest stage and therefore it is likely for this stages to be missed or to be in transition into diestrus[60,69]. Figure 1 presents the differences between the relative concentrations of estrogen during the various phases of the menstrual and estrous cycles. The highest estrogen concentrations occur over days 14-28 in the human, and within a 12 hour time frame in the rat.

In the liver, gene expression is altered over the four day estrus cycle in mice in accordance with the level of estrogen present, and these changes are predominantly in carbohydrate and lipid metabolism[61,63]. Elevated E2 levels during proestrus were correlated with high mRNA and differing patterns of ERα activation when compared to lower E2 phases; these changes were evident within 24 hours suggesting that throughout the four day cycle E2 changes exert both rapid and genetic effects[63]. GP-ERs are found throughout the body, in the liver, islet cells of the pancreas, ovaries and adipose tissue among others[62,64,65]. The binding of E2 to GP-ER results in an increase of cyclic AMP (cAMP) and Ca^{2+} [61]. E2 can have both rapid and genomic effects on the body, resulting in daily differences in gene products and metabolic homeostasis. One genomic effect of E2 occurs within the pancreas. Insulin gene expression varies with estrus, with the greatest

levels of insulin gene expression observed during proestrus when E2 levels are highest. It has been reported that clinically high E2 levels may induce insulin resistance due to the modulation of insulin gene expression leading to hyperinsulinemia and reduced effects of insulin [62] E2 levels stimulate ER expression, and ERs are also implicated in insulin-like growth factor promoters, progesterone receptor expression and tumor growth[66-68]. The effects of E2 on fuel selection processes (*Fig. 1)* and the sexual dimorphism in response to exercise are discussed further in section 1.6.

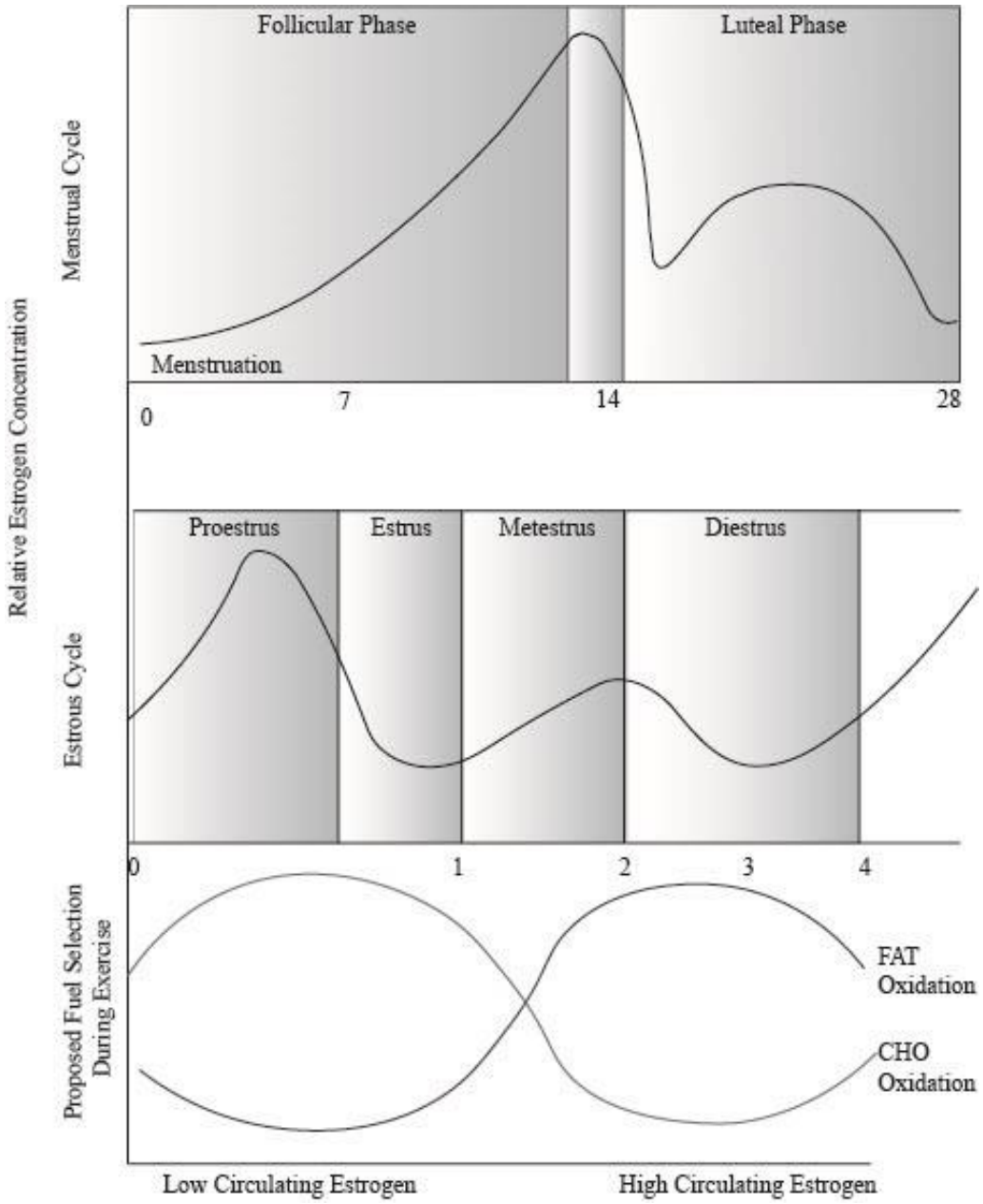


Figure 2 *A comparison of the relative estrogen concentration in the blood during the menstrual and estrous cycles. The level of circulating estrogen is higher during the luteal phase (LP) of the menstrual cycle despite the peak around day 14. In estrous, the estrogen concentration in the blood is highest during proestrus, an approximately 12 hour phase. Proposed fuel usage of lipids (FAT) and carbohydrates (CHO) in relation to estrogen concentration is also shown. High circulating estrogen would occur during LP in humans and proestrus in the rat.*

1.5 Methods to Study the Estrous Cycle and T1 Diabetes

Historically, the phases of the estrous cycle were determined through observed behavioural changes or through visual and microscopic examination of the uterine tissues or vaginal secretions[60]. Vaginal cytology either through lavage or swabbing has since been determined to be highly accurate in phase determination[60,69,74,79]. Multiple staining methods may be used to interpret samples, with Papanicolaou's stain recognized as the optimal choice for visualizing abnormalities and detailed structure in humans, but several other stains including standard hematoxylin and eosin stain sufficient for visualizing structure[60,77,79]. A minimum of 14 consecutive samples, one per day at the same time each day, are needed to determine the length of the cycle[60]. Collecting consecutive samples avoids blind reading, or the process of attempting to read one stain without context of the previous day's stain which can provide inconsistent results, particularly if the timing of the swab is during a transitional period[60,77]. Proestrus is characterized by round nucleated cells that may be clumped together or may appear in strands, however the clustering does not need to be present to recognize proestrus. Estrus shows predominantly keratinized anucleated epithelial cells often with jagged edges, with some nucleated epithelial cells possible. If the smear is not performed properly it is possible for the keratinized epithelial cells to be present in other stages, as these cells are present in the lower vaginal canal[60]. Neutrophils may be present in both metestrus and diestrus and are present only in low numbers in proestrus and estrus. The metestrus phase contains roughly equal proportions of neutrophils, nucleated and keratinized epithelial cells, with greater numbers of nucleated cells appearing towards the end of the stage. Diestrus contains fewer keratinized epithelial cells than metestrus, and neutrophils and nucleated epithelial cells

present but in lower numbers than metestrus. These two stages are sometimes combined into one due to these similarities, however they can often be distinguished by the lower cellularity during diestrus[60,76-78].

The low-dose STZ rodent model imitates the development of T1DM in humans[25], and due to the short estrous cycle, this model means that each phase of the cycle can be examined within a four to five day span, rather than the 28-day cycle in humans. The determination of the cycle phase through vaginal cytology is well established, and the phases of the estrus cycle directly related to levels of sex hormones during those phases. Therefore, staging the exercise based on these vaginal swabs should allow an examination of the response to recurrent exercise when the lowest and highest levels of estrogen are present.

1.6 Sexual Dimorphism in Response to Exercise

There are well established differences in hormone levels and the glucoregulatory between males and females during multiple types of exercise. There is support for the role of estrogen in these metabolic differences, as evidenced by studies which utilize E2 supplementation in men or postmenopausal women, or direct comparisons between LP (high E2) and FP (low E2)[51,56,57]. Indeed, supplementation oral E2 has resulted in higher lipid oxidation rates and greater carbohydrate preservation during endurance exercise, and that under normal conditions males have a greater reliance on carbohydrates compared to females in both LP and FP[80-83]. In studies using ovariectomized females, E2 supplementation spared heart, liver and muscle glycogen and increased fat oxidation

during prolonged submaximal and maximal treadmill exercise[82,83]. Prior administration of E2 to males causes a shift toward lipid utilization during an acute bout of exercise[81]. It was stated that these differences in fuel selection between sexes were due to differences in body composition and fitness levels; however, the participants were matched based on relative VO_{2max}. Further, the differences in glycogen sparing appear between females in LP and FP, with LP preserving more total and muscle glycogen than both females in FP and males, while no difference in muscle glycogen content was evident between females in FP and males[80]. Aerobic exercise during the LP results in greater release of estrogen than exercise during the FP[84]. The pattern of growth hormone (GH) release at rest and during exercise is also sexually dimorphic, with females in both phases of the menstrual cycle releasing greater GH in response to aerobic exercise and under resting conditions[84,85]. Others have found that the relative increase in GH from baseline is greater in males even though the peak level reached is greater in females, whereby this peak is also reached faster in females than males[86]. Others still have found the opposite to be true, with males having higher GH release during aerobic and resistance exercise[87,88]. There is some discrepancy in the literature, and this may be due to the baseline GH values being greater in females at baseline, or due to other differences in sex hormones.

GH, epinephrine and glucagon release during the glucose counterregulatory response to hypoglycemia may be influenced by estrogen[89-91]. As detailed above, there is a blunting effect of antecedent hypoglycemia on the counterregulatory response to subsequent hypoglycemia[41,42]. There is conflicting data as to whether or not this effect is different between the sexes, with some evidence demonstrating that estrogen reduces the counterregulatory hormone response in females in comparison to males[89]. Other studies

have demonstrated that the counterregulatory response following prior hypoglycemia is actually worse in males and that only deeper hypoglycemia elicits any reduction in the counterregulatory response in females[90,92]. Since the counterregulatory response may be preserved in females following prior hypoglycemia, males may be at a greater risk of subsequent hypoglycemia Exercise during the LP may also result in higher lipid oxidation than exercise of the same type and intensity during the FP, although in human studies these findings have been challenged as the measure to examine fuel selection in these studies is the respiratory exchange ratio (RER)[80,92]. RER relates CO_2 production to O_2 uptake which increases during intense exercise and is an indirect measure of fuel source during exercise[93,94]. Higher RER is associated with carbohydrates as the primary fuel source, and low RER is associated with fat as the primary fuel source. RER if used alongside other measures of fuel selection, such as rates of glucose appearance or disappearance, or euglycemic clamping, can be an accurate means to determine fuel selection; however, RER is influenced by training level, dietary fat intake, ketogenesis and muscle fiber composition[93,94,95]. Interestingly, RER during exercise is lower in females than in males, indicating that the female body relies more on lipid oxidation than carbohydrate[36,38,96]. When dietary intake differs significantly between the sexes, with fat intake higher in males than females, the difference was not apparent[95]. Higher body fat levels in females may be responsible for some of this effect, or higher intramyocellular lipid (IMCL) levels which is also reported to be elevated in females[97,98]. While these differences can be largely attributed to estrogen[97], the increased IMCL content does not necessarily translate into higher IMCL turnover[99]. The difference in RER, fuel selection and glycogen sparing between the sexes is greater during LP than FP, suggesting that the

higher E2 levels are partially responsible for this shift[80, 96]. This study proposes that the lipid and carbohydrate usage will shift as a result of high estrogen circulation during proestrus as shown in Figure 1. It has been suggested that intramuscular recovery is greater during LP due to the anti-oxidant effects of E2[101]. In this study it was shown that creatine kinase (CK) levels were significantly higher following aerobic exercise during the FP than exercise during the LP. CK is a marker of damage to the cell and is present in greater quantities following intense prolonged exercise. The properties of E2 as an anti-oxidant are suggested to either increase the healing process or decrease the damage incurred during prolonged exercise[101].

1.7 Rationale

This study serves to fill a gap in the literature regarding the sex dependent effects of repetitive bouts on hypoglycemia onset during and following exercise. The glucoregulatory response to exercise is different between the sexes, and it is possible that estrogen is responsible (directly or indirectly) for these differences. Studies have demonstrated that different phases of the menstrual cycle has an effect on glycogen usage during exercise in healthy patients but have not elucidated a mechanism for this phenomenon[80,84]. Females have higher lipid oxidation during exercise, less glycogen depletion and are less effected by blunting of the counterregulatory defense against hypoglycemia[61,80]. As such, females may be more protected against exercise-induced hypoglycemia due to an E2 mediated fuel selection shift towards lipid oxidation and quicker BG recovery. Moreover, it is possible that the protective effects may be greater during the luteal phase when estrogen levels are at the highest.

Aerobic exercise has been shown to decrease blood glucose and complications associated with T1DM[27,28,30,32]. The risk of hypoglycemia during and after exercise is a main factor in exercise avoidance by patients with T1DM[21]. Increased levels of estrogen in healthy subjects are correlated with increased fat usage and carbohydrate sparing[80-83]. The RER of exercising females in the LP and FP of the menstrual cycle is lower than the RER of exercising males, with LP during RER lower than RER during FP. It is unknown what role estrogen plays in female patients with T1DM and the mitigation or reduction in hypoglycemia risk. It is plausible that T1DM females may be at less risk for hypoglycemia during the LP phase of the menstrual cycle when estrogen levels are at their highest.

1.8 Purpose and Hypothesis

The purpose of this study was to examine the blood glucose response to recurrent aerobic exercise in male and female rodents with streptozotocin-induced T1DM treated with intensive insulin therapy. We examined whether consecutive days of aerobic exercise would result in differences in blood glucose (BG) response and liver glycogen usage between groups. We believe that the sex hormone estrogen may provide protection from hypoglycemic episodes in females, as estrogen shifts fuel selection toward lipid usage and is glycogen sparing (*Fig. 1)*. The changes in hormones throughout the reproductive cycle have been shown to alter fuel selection and the counterregulatory response to hypoglycemia. To do so, female animals (T1D and non-T1D) were exercised starting during the lowest estrogen period and finishing in the highest estrogen period to show the greatest protective effects possible and to encompass the entire estrus cycle. We

hypothesized that female T1DM rodents would have higher blood glucose during and post exercise than male T1DM rodents. Secondly, we hypothesized that females T1DM rodents would have fewer instances of exercise-induced hypoglycemia than their male diabetic counterparts and a fuel selection shift would result in liver glycogen sparing for the T1DM females even after four days of repetitive exercise.

Bibliography

1. Diabetes Canada. Diabetes in Canada: Backgrounder. Ottawa: *Diabetes Canada*; 2020.
2. Reaven, G. M. (1980). Insulin-independent diabetes mellitus: Metabolic characteristics. *Metabolism*, *29*(5), 445–454. https://doi.org/https://doi.org/10.1016/0026-0495(80)90170-5
3. Chatterjee, S., Khunti, K., & Davies, M. J. (2017). Type 2 diabetes. *The Lancet*, *389*(10085), 2239–2251. https://doi.org/10.1016/S0140-6736(17)30058-2
4. Cerf, M. E. (2013). Beta cell dysfunction and insulin resistance. *Frontiers in Endocrinology*, *4*(MAR), 1–12. https://doi.org/10.3389/fendo.2013.00037
5. Guyton AC, Hall JE (2006). Textbook of Medical physiology. 11th Edition. Elsevier Inc, New Delhi.
6. Xu, G., Liu, B., Sun, Y., Du, Y., Snetselaar, L. G., Hu, F. B., & Bao, W. (2018). Prevalence of diagnosed type 1 and type 2 diabetes among US adults in 2016 and 2017: Population based study. *BMJ (Online)*, *362*. doi:10.1136/bmj.k1497
7. Ogurtsova, K., da Rocha Fernandes, J. D., Huang, Y., Linnenkamp, U., Guariguata, L., Cho, N. H., ... & Makaroff, L. E. (2017). IDF Diabetes Atlas: Global estimates for the prevalence of diabetes for 2015 and 2040. *Diabetes Research and Clinical Practice*, *128*, 40-50. doi: 10.1016/j.diabres.2017.03.024
8. Lipscombe, L., Booth, G., Butalia, S., Eurich Bsp, D. T., Goldenberg, R., Khan, N., … Simpson Bsp, S. (2018). Diabetes Canada 2018 clinical practice guidelines for the prevention and management of diabetes in Canada: Pharmacologic

glycemic management of type 2 diabetes in adults. *Canadian Journal of Diabetes*, *42*(Suppl. 1), S88–S103. doi: 10.1016/j.jcjd.2017.10.034

9. Lu, J., Liu, J., Li, L., Lan, Y., & Liang, Y. (2020). Cytokines in type 1 diabetes: mechanisms of action and immunotherapeutic targets. *Clinical and Translational Immunology*, *9*(3), 1–17. doi: 10.1002/cti2.1122
10. Ozougwu, O. (2013). The pathogenesis and pathophysiology of type 1 and type 2 diabetes mellitus. *Journal of Physiology and Pathophysiology*, *4*(4), 46–57. doi: 10.5897/jpap2013.0001
11. Wolfsdorf, J., Craig, M. E., Daneman, D., Dunger, D., Edge, J., Lee, W., ... & Hanas, R. (2009). Diabetic ketoacidosis in children and adolescents with diabetes. *Pediatric Diabetes*, *10*(Suppl 12), 118-133. doi: 10.1111/j.1399-5448.2009.00569.x
12. Rosenfeld, L. (2002). Insulin: Discovery and Controversy. *Clinical Chemistry*, *48*(12), 2270–2288. https://doi.org/10.1093/clinchem/48.12.2270
13. Secrest, A. M., Washington, R. E., & Orchard, T. J. (2014). Chapter 35: Mortality in Type 1 Diabetes. *Diabetes in America*, 1–16.
14. Banting, F. G., Best, C. H., Collip, J. B., Campbell, W. R., & Fletcher, A. A. (1922). Pancreatic extracts in the treatment of diabetes mellitus: preliminary report. *Canadian Medical Association Journal*, *145*(10), 1281–1286.
15. Iribarren, C., Karter, A. J., Go, A. S., Ferrara, A., Liu, J. Y., Sidney, S., & Selby, J. V. (2001). Glycemic control and heart failure among adult patients with diabetes. *Circulation*, *103*(22), 2668–2673. https://doi.org/10.1161/01.CIR.103.22.2668

16. Thorn, L. M., Forsblom, C., Fagerudd, J., Thomas, M. C., Pettersson-Fernholm, K., Saraheimo, M., … Groop, P. H. (2005). Metabolic syndrome in type 1 diabetes: Association with diabetic nephropathy and glycemic control (the FinnDiane study). *Diabetes Care*, *28*(8), 2019–2024. https://doi.org/10.2337/diacare.28.8.2019
17. DCCT Research Group. (1993). The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. *New England Journal of Medicine, 329*(14), 977–986. https://doi.org/10.1056/NEJM199309303291401
18. DCCT Research Group. (1997). Hypoglycemia in the diabetes control and complications trial. (1997). *Diabetes*, *46*(2), 271 LP-286. https://doi.org/10.2337/diab.46.2.271
19. Gallagher, E. J., Le Roith, D., & Bloomgarden, Z. (2009). Review of hemoglobin A(1c) in the management of diabetes. *Journal of Diabetes*, *1*(1), 9–17. https://doi.org/10.1111/j.1753-0407.2009.00009.x
20. DCCT Research Group. (1991). Epidemiology of severe hypoglycemia in the diabetes control and complications trial. The DCCT Research Group. *The American Journal of Medicine, 90*(4), 450–459. doi: 10.1016/0002-9343(91)80085-Z
21. Brazeau, A.-S., Rabasa-Lhoret, R., Strychar, I., & Mircescu, H. (2008). Barriers to physical activity among patients with type 1 diabetes. *Diabetes Care*, *31*(11), 2108 LP-2109. doi: 10.2337/dc08-0720

22. Yardley, J. E., & Sigal, R. J. (2015). Exercise strategies for hypoglycemia prevention in individuals with type 1 diabetes. *Diabetes Spectrum*, *28*(1), 32–38. doi: 10.2337/diaspect.28.1.32

23. Pinsker, J. E., Kraus, A., Gianferante, D., Schoenberg, B. E., Singh, S. K., Ortiz, H., … Kerr, D. (2016). Techniques for exercise preparation and management in adults with type 1 diabetes. *Canadian Journal of Diabetes*, *40*(6), 503–508. doi: 10.1016/j.jcjd.2016.04.010

24. Colberg, S. R., Sigal, R. J., Yardley, J. E., Riddell, M. C., Dunstan, D. W., Dempsey, P. C., … Tate, D. F. (2016). Physical activity/exercise and diabetes: A position statement of the American Diabetes Association. *Diabetes Care*, *39*(11), 2065–2079. doi: 10.2337/dc16-1728

25. Melling, C. W. J., Grisé, K. N., Hasilo, C. P., Fier, B., Milne, K. J., Karmazyn, M., & Noble, E. G. (2013). A model of poorly controlled type 1 diabetes mellitus and its treatment with aerobic exercise training. *Diabetes and Metabolism*, *39*(3), 226–235. doi: 10.1016/j.diabet.2013.02.004

26. Dotzert, M. S., Murray, M. R., McDonald, M. W., Olver, T. D., Velenosi, T. J., Hennop, A., … James Melling, C. W. (2016). Metabolomic response of skeletal muscle to aerobic exercise training in insulin resistant type 1 diabetic rats. *Scientific Reports*, *6*(April), 1–10. doi: 10.1038/srep26379

27. Grisé, K. N., Olver, T. D., McDonald, M. W., Dey, A., Jiang, M., Lacefield, J. C., … Melling, C. W. J. (2016). High intensity aerobic exercise training improves deficits of cardiovascular autonomic function in a rat model of type 1 diabetes

mellitus with moderate hyperglycemia. *Journal of Diabetes Research, 2016*. doi:10.1155/2016/8164518

28. Grise, Kenneth N. (2012). The effects of exercise training on indices of cardiovascular autonomic neuropathy in STZ-induced type 1 diabetic rats treated with insulin. *Electronic Thesis and Dissertation Repository*. 822.
29. Hall, K. E., McDonald, M. W., Grisé, K. N., Campos, O. A., Noble, E. G., & Melling, C. W. J. (2013). The role of resistance and aerobic exercise training on insulin sensitivity measures in STZ-induced type 1 diabetic rodents. *Metabolism: Clinical and Experimental*, *62*(10), 1485–1494. doi:10.1016/j.metabol.2013.05.012
30. Brockman, N. K., & Yardley, J. E. (2018). Sex-related differences in fuel utilization and hormonal response to exercise: implications for individuals with type 1 diabetes. *Applied Physiology, Nutrition, and Metabolism*, *43*(6), 541-552.
31. Kennedy, A., Nirantharakumar, K., Chimen, M., Pang, T. T., Hemming, K., Andrews, R. C., & Narendran, P. (2013). Does exercise improve glycaemic control in type 1 diabetes? A systematic review and meta-analysis. *PloS one*, *8*(3), e58861. doi:10.1371/journal.pone.0058861
32. McDonald, M. W., Hall, K. E., Jiang, M., Noble, E. G., & Melling, C. W. J. (2014). Ischemia-reperfusion injury and hypoglycemia risk in insulin-treated T1DM rats following different modalities of regular exercise. *Physiological Reports*, *2*(11), 1–12. doi:10.14814/phy2.12201

33. Galassetti, P., Mann, S., Tate, D., Neill, R., Wasserman, D. H., & Davis, S. N. (2001). Effect of morning exercise on counterregulatory repsonses to subsequent, afternoon exercise. *J Appl Physiol, 91, 91-99.* doi: 1 0.1152/jappl.2001.91.1.91
34. Galassetti, P., Tata, D., Neill, R., Morrey, S., Wasserman, D. H., & Davis, S. N. (2003). Effect of antecedent hypoglycemia on counterregulatory responses to subsequent euglycemic exercise in type 1 diabetes. *Diabetes, 52*, 1761–1769.
35. Camacho, R. C., Galassetti, P., Davis, S. N., & Wasserman, D. H. (2005). ew. *Exercise and Sport Sciences Reviews*, *33*(1), 17–23.
36. Tarnopolsky, L. J., MacDougall, J. D., Atkinson, S. A., Tarnopolsky, M. A., & Sutton, J. R. (1990). Gender differences in substrate for endurance exercise. *Journal of applied physiology*, *68*(1), 302–308. doi:10.1152/jappl.1990.68.1.302
37. Marliss, E. B., Kreisman, S. H., Manzon, A., Halter, J. B., Vranic, M., & Nessim, S. J. (2000). Gender differences in glucoregulatory responses to intense exercise. *Journal of Applied Physiology*, *88*(2), 457–466. doi:10.1152/jappl.2000.88.2.457
38. Hedrington, M. S., & Davis, S. N. (2015). Sexual dimorphism in glucose and lipid metabolism during fasting, hypoglycemia, and exercise. *Frontiers in endocrinology*, *6*, 61. Doi:10.3389/fendo.2015.00061
39. Bahr, R., Høstmark, A.T., Newsholme, E.A., Grønnerød, O. And Sejersted, O.M. (1991), Effect of exercise on recovery changes in plasma levels of FFA, glycerol, glucose and catecholamines. Acta Physiologica Scandinavica, 143: 105-115. doi:10.1111/j.1748-1716.1991.tb09205.x

40. Liu, H. Y., Cao, S. Y., Hong, T., Han, J., Liu, Z., & Cao, W. (2009). Insulin is a stronger inducer of insulin resistance than hyperglycemia in mice with type 1 diabetes mellitus (T1DM). *Journal of Biological Chemistry*, *284*(40), 27090–27100. doi:10.1074/jbc.M109.016675
41. Cryer P. E. (1993). Glucose counterregulation: prevention and correction of hypoglycemia in humans. *The American Journal of Physiology*, *264*(2 Pt 1), E149–E155. doi:10.1152/ajpendo.1993.264.2.E149
42. Schwartz, N. S., Clutter, W. E., Shah, S. D., & Cryer, P. E. (1987). Glycemic thresholds for activation of glucose counterregulatory systems are higher than the threshold for symptoms. *Journal of Clinical Investigation*, *79*(3), 777–781. doi:10.1172/JCI112884
43. Kuo, T., McQueen, A., Chen, T. C., & Wang, J. C. (2015). Regulation of glucose homeostasis by glucocorticoids. *Advances in experimental medicine and biology*, *872*, 99–126. doi:10.1007/978-1-4939-2895-8_5
44. Sprague J. E. & Arbeláez A. M. (2011). Glucose counterregulatory responses to hypoglycemia. *Pediatric Endocrinology Review*. 2011;9(1):463-475.
45. Rizza, R. A., Cryer, P. E., & Gerich, J. E. (1979). Role of glucagon, epinephrine and growth hormone in human glucose counterregulation. *Journal of Clinical Investigation*. 64:62-71. doi: 10.1172/JCI109464
46. McDonald, M. W., Murray, M. R., Grise, K. N., Olver, T. D., Dey, A., Shoemaker, J. K., … Melling, C. W. J. (2016). The glucoregulatory response to high-intensity aerobic exercise following training in rats with insulin-treated type

1 diabetes mellitus. *Applied Physiology, Nutrition, and Metabolism*, *41*(6), 631–639. doi: 10.1139/apnm-2015-0558

47. Bussau, V. A., Ferreira, L. D., Jones, T. W., & Fournier, P. A. (2007). A 10-s sprint performed prior to moderate-intensity exercise prevents early post-exercise fall in glycaemia in individuals with type 1 diabetes. *Diabetologia*, *50*(9), 1815–1818. doi:10.1007/s00125-007-0727-8
48. Farinha, J. B., Krause, M., Rodrigues-Krause, J., & Reischak-Oliveira, A. (2017). Exercise for type 1 diabetes mellitus management: General considerations and new directions. *Medical Hypotheses*, *104*(June), 147–153. doi:10.1016/j.mehy.2017.05.033
49. Gallen, I. W., Hume, C., & Lumb, A. (2011). Fuelling the athlete with type 1 diabetes. *Diabetes, Obesity and Metabolism*, *13*(2), 130-136. doi:10.1111/j.1463-1326.2010.01319.x
50. Galassetti, P., Tate, D., Neill, R. A., Morrey, S., Wasserman, D. H., & Davis, S. N. (2004). Effect of sex on counterregulatory responses to exercise after antecedent hypoglycemia in type 1 diabetes. *American Journal of Physiology-Endocrinology and Metabolism*, *287*(1), E16-E24.
51. Brockman, N. K., Sigal, R. J., Kenny, G. P., Riddell, M. C., Perkins, B. A., & Yardley, J. E. (2020). Sex-related differences in blood glucose responses to resistance exercise in adults with type 1 diabetes: A secondary data analysis. *Canadian Journal of Diabetes, 44*(3), 267–273.e1. doi:10.1016/j.jcjd.2019.08.006

52. Van Look, P. F. A., & Baird, D. T. (1980). Regulatory mechanisms during the menstrual cycle. *European Journal of Obstetrics Gynecology and Reproductive Biology, 11*(2), 121–144. doi:10.1016/0028-2243(80)90018-0

53. Nyholm, H., Djursing, H., Hagen, C., Agner, T., Bennett, P., & Svenstrup, B. (1989). Androgens and estrogens in postmenopausal insulin-treated diabetic women. *Journal of Clinical Endocrinology and Metabolism*, *69*(5), 946–949. doi:10.1210/jcem-69-5-946

54. Marsh, E. E., Shaw, N. D., Klingman, K. M., Tiamfook-Morgan, T. O., Yialamas, M. A., Sluss, P. M., & Hall, J. E. (2011). Estrogen levels are higher across the menstrual cycle in African-American women compared with Caucasian women. *Journal of Clinical Endocrinology and Metabolism*, *96*(10), 3199–3206. doi:10.1210/jc.2011-1314

55. Haiman, C. A., Dossus, L., Setiawan, V. W., Stram, D. O., Dunning, A. M., Thomas, G., … Ziegler, R. G. (2007). Genetic variation at the CYP19A1 locus predicts circulating estrogen levels but not breast cancer risk in postmenopausal women. *Cancer Research*, *67*(5), 1893–1897. doi: 10.1158/0008-5472.CAN-06-4123

56. Bernardes, R. P., & Radomski, M. W. (1998). Growth hormone responses to continuous and intermittent exercise in females under oral contraceptive therapy. *European Journal of Applied Physiology and Occupational Physiology*, *79*(1), 24–29. doi: 10.1007/s004210050468

57. Bonen, A., Haynes, F. W., & Graham, T. E. (1991). Substrate and hormonal responses to exercise in women using oral contraceptives. *Journal of Applied Physiology, 70*(5), 1917–1927. doi: 10.1152/jappl.1991.70.5.1917

58. Baker, F. C., & Driver, H. S. (2007). Circadian rhythms, sleep, and the menstrual cycle. *Sleep Medicine, 8*(6), 613–622. doi:10.1016/j.sleep.2006.09.011

59. Tena-Sempere, M. (2015). Chapter 35: Physiological mechanisms for the metabolic control of reproduction. In T. M. Plant, E. Knobil, & J. D. Neill (Eds.), *Knobil and Neill's physiology of reproduction* (Fourth ed., Vol. 2, pp. 1605-1630). Academic Press.

60. Cora, M. C., Kooistra, L., & Travlos, G. (2015). Vaginal cytology of the laboratory rat and mouse:review and criteria for the staging of the estrous cycle using stained vaginal smears. *Toxicologic Pathology, 43*(6), 776–793. doi:10.1177/0192623315570339

61. Palmisano, B. T., Zhu, L., & Stafford, J. M. (2017). Role of estrogens in the regulation of liver lipid metabolism. In *Sex and Gender Factors Affecting Metabolic Homeostasis, Diabetes And Obesity* (pp. 227-256). Springer, Cham.

62. Morimoto, S., Cerbón, M. A., Alvarez-Alvarez, A., Romero-Navarro, G., & Díaz-Sánchez, V. (2001). Insulin gene expression pattern in rat pancreas during the estrous cycle. *Life Sciences, 68*(26), 2979–2985. doi: 10.1016/S0024-3205(01)01100-6

63. Villa, A., Della Torre, S., Stell, A., Cook, J., Brown, M., & Maggi, A. (2012). Tetradian oscillation of estrogen receptor α is necessary to prevent liver lipid

deposition. *Proceedings of the National Academy of Sciences of the United States of America*, *109*(29), 11806–11811. doi:10.1073/pnas.1205797109

64. Sharma, G., Mauvais-Jarvis, F., & Prossnitz, E. R. (2018). Roles of G protein-coupled estrogen receptor GPER in metabolic regulation. *The Journal of Steroid Biochemistry And Molecular Biology*, *176*, 31-37. doi:10.1016/j.jsbmb.2017.02.012
65. Shi, H., Kumar, S. P. D. S., & Liu, X. (2013). *G protein-coupled estrogen receptor in energy homeostasis and obesity pathogenesis*. *Progress in Molecular Biology and Translational Science* (Vol. 114). doi: 10.1016/B978-0-12-386933-3.00006-6
66. Petrie, W. K., Dennis, M. K., Hu, C., Dai, D., Arterburn, J. B., Smith, H. O., … Prossnitz, E. R. (2013). G protein-coupled estrogen receptor-selective ligands modulate endometrial tumor growth. *Obstetrics and Gynecology International*, *2013*, 1–17. doi: 10.1155/2013/472720
67. Maor, S., Mayer, D., Yarden, R., Lee, A., Sarfstein, R., Werner, H., & Papa, M. (2006). Estrogen receptor regulates insulin-like growth factor-I receptor gene expression in breast tumor cells: involvement of transcription factor Sp1. *Journal of Endocrinology*, 191(3), 605-612. doi: 10.1677/joe.1.07016
68. Schultz, J. R., Petz, L. N., & Nardulli, A. M. (2003). Estrogen receptor α and Sp1 regulate progesterone receptor gene expression. *Molecular and Cellular Endocrinology*, *201*(1-2), 165-175. doi: 10.1016/S0303-7207(02)00415-X
69. Long, J. A., & Evans, H. M. (1922). *The oestrous cycle in the rat and its associated phenomena* (Vol. 6). University of California Press.

70. Marcondes, FK, Bianchi, FJ and Tanno, A. (2002). Determination of the estrous cycle phases of rats: Some helpful considerations. *Brazilian Journal of Biology*, *62*(4). doi:10.1590/s1519-69842002000400008

71. Leibowitz SF, Akabayashi A, Alexander J, Karatayev O, Chang GQ. Puberty onset in female rats: relationship with fat intake, ovarian steroids and the peptides, galanin and enkephalin, in the paraventricular and medial preoptic nuclei. *Journak of Neuroendocrinology*. 2009;21(6):538-549. doi:10.1111/j.1365-2826.2009.01870.x

72. Shors, T. J., Pickett, J., Wood, G., & Paczynski, M. (1999). Acute stress persistently enhances estrogen levels in the female rat. *Stress*, *3*(2), 163–171. doi:10.3109/10253899909001120

73. Faccio, L., Da Silva, A. S., Tonin, A. A., França, R. T., Gressler, L. T., Copetti, M. M., … Monteiro, S. G. (2013). Serum levels of LH, FSH, estradiol and progesterone in female rats experimentally infected by Trypanosoma evansi. *Experimental Parasitology*, *135*(1), 110–115. doi:10.1016/J.EXPPARA.2013.06.008

74. Zenclussen, M. L., Casalis, P. A., Jensen, F., Woidacki, K., & Zenclussen, A. C. (2014). Hormonal fluctuations during the estrous cycle modulate heme oxygenase-1 expression in the uterus. *Frontiers in Endocrinology*, *5*(MAR), 1–6. doi:10.3389/fendo.2014.00032

75. Sato, J., Nasu, M., & Tsuchitani, M. (2016). Comparative histopathology of the estrous or menstrual cycle in laboratory animals. *Journal of Toxicologic Pathology*, *29*(3), 155–162. doi: 10.1293/tox.2016-0021

76. Ajayi, A. F., & Akhigbe, R. E. (2020). Staging of the estrous cycle and induction of estrus in experimental rodents: an update. *Fertility Research and Practice*, *6*(1), 1–15. doi: 10.1186/s40738-020-00074-3

77. Yuan Y. & Carlson R. G. (1987). Structure, cyclic change, and function, vagina and vulva, rat. In: Jones T. C., Mohr U., & Hunt R. D., (eds). *Genital System*. Berlin (Germany): Springer-Verlag;. p. 161-8.

78. Goldman, J. M., Murr, A. S., & Cooper, R. L. (2007). The rodent estrous cycle: characterization of vaginal cytology and its utility in toxicological studies. *Birth Defects Research. Part B, Developmental and Reproductive Toxicology*, *80*(2), 84–97. doi: 10.1002/bdrb.20106

79. Chantziantoniou, N., Donnelly, A. D., Mukherjee, M., Boon, M. E., & Austin, R. M. (2017). Inception and development of the papanicolaou stain method. *Acta Cytologica*, *61*(4-5), 266–280. doi: 10.1159/000457827

80. Devries, M. C., Hamadeh, M. J., Phillips, S. M., & Tarnopolsky, M. A. (2006). Menstrual cycle phase and sex influence muscle glycogen utilization and glucose turnover during moderate-intensity endurance exercise. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *291*(4), R1120–R1128. doi:10.1152/ajpregu.00700.2005

81. Hamadeh, M.J., Devries, M.C., & Tarnopolsky, M.A. (2005). Estrogen supplementation reduces whole body leucine and carbohydrate oxidation and increases lipid oxidation in men during endurance exercise. *The Journal of Clinical Endocrinol*ogy & *Metabolism*, 90(6): 3592-9. doi:10.1210/jc.2004-1743.

82. Kendrick, Z. V., Steffen, C. A., Rumsey, W. L., & Goldberg, D. I. (1987). Effect of estradiol on tissue glycogen metabolism in exercised oophorectomized rats. *Journal of Applied Physiology*, *63*(2), 492-496. doi:10.1152/jappl.1987.63.2.492

83. Hatta, H., Atomi, Y., Shinohara, S., Yamamoto, Y., & Yamada, S. (1988). The effects of ovarian hormones on glucose and fatty acid oxidation during exercise in female ovariectomized rats. *Hormone and Metabolic Research*, *20*(10), 609–611. doi:10.1055/s-2007-1010897

84. Kraemer, R. R., Heleniak, R. J., Tryniecki, J. L., Kraemer, G. R., Okazaki, N. J., & Castracane, V. D. (1995). Follicular and luteal phase hormonal responses to low-volume resistive exercise. *Medicine and Science in Sports and Exercise*, *27*(6), 809–817. doi: 10.1249/00005768-199506000-00004

85. Wideman, L., Weltman, J. Y., Shah, N., Story, S., Veldhuis, J. D., & Weltman, A. (1999). Effects of gender on exercise-induced growth hormone release. *Journal of Applied Physiology*, *87*(3), 1154-1162. doi: 10.1152/jappl.1999.87.3.1154

86. Pritzlaff-Roy, C. J., Widemen, L., Weltman, J. Y., Abbott, R., Gutgesell, M., Hartman, M. L., ... & Weltman, A. (2002). Gender governs the relationship between exercise intensity and growth hormone release in young adults. *Journal of Applied Physiology*, *92*(5), 2053-2060. doi: 10.1152/japplphysiol.01018.2001

87. Bunt, J. C., Boileau, R. A., Bahr, J. M., & Nelson, R. A. (1986). Sex and training differences in human growth hormone levels during prolonged exercise. *Journal of Applied Physiology*, *61*(5), 1796-1801. doi: 10.1152/jappl.1986.61.5.1796

88. Kraemer, W. J., Gordon, S. E., Fleck, S. J., Marchitelli, L. J., Melloo, R., Dziados, J. E., … Fry, A. C. (1991). Endogenous anabolic hormonal and growth factor responses to heavy resistance exercise in males and females. *International Journal of Sports Medicine*, *12*(2), 228–235. doi: 10.1055/s-2007-1024673
89. Sandoval, D. A., Ertl, A. C., Richardson, M. A., Tate, D. B., & Davis, S. N. (2003). Estrogen blunts neuroendocrine and metabolic responses to hypoglycemia. *Diabetes*, *52*(7), 1749–1755. doi: 10.2337/diabetes.52.7.1749
90. Davis, S. N., Shavers, C., & Costa, F. (2000). Gender-related differences in counterregulatory responses to antecedent hypoglycemia in normal humans. *Journal of Clinical Endocrinology and Metabolism*, *85*(6), 2148–2157. doi:10.1210/jcem.85.6.6641
91. Galassetti, P., Tate, D., Neill, R. A., Morrey, S., Wasserman, D. H., & Davis, S. N. (2004). Effect of sex on counterregulatory responses to exercise after antecedent hypoglycemia in type 1 diabetes. *American journal of physiology. Endocrinology and metabolism*, *287*(1), E16–E24. doi: 10.1152/ajpendo.00480.2002
92. Tarnopolsky M.A., Atkinson S.A., Phillips S.M. & MacDougall J.D. (1995). Carbohydrate loading and metabolism during exercise in men and women. *Journal of Applied Physiology* 78, 1360–1368. doi: 10.1152/jappl.1995.78.4.1360
93. Simonson, D. C., & DeFronzo, R. A. (1990). Indirect calorimetry: methodological and interpretative problems. *American Journal of Physiology, Endocrinology and Metabolism*, *258*(3), 399-412. doi: 10.1152/ajpendo.1990.258.3.399

94. Ramos-Jiménez, A., Hernández-Torres, R. P., Torres-Durán, P. V., Romero-Gonzalez, J., Mascher, D., Posadas-Romero, C., & Juárez-Oropeza, M. A. (2008). The respiratory exchange ratio is associated with fitness indicators both in trained and untrained men: A possible application for people with reduced exercise tolerance. *Clinical Medicine Insights: Circulatory, Respiratory and Pulmonary Medicine*, *2*, 1-9. doi: 10.4137/ccrpm.s449

95. Goedecke, J. H., Gibson, A. S. C., Grobler, L., Collins, M., Noakes, T. D., & Lambert, E. V. (2000). Determinants of the variability in respiratory exchange ratio at rest and during exercise in trained athletes. *American Journal of Physiology, Endocrinology and Metabolism*, *279*(6 42-6), 1325–1334. doi: 10.1152/ajpendo.2000.279.6.e1325

96. Devries, M. C. (2016). Sex-based differences in endurance exercise muscle metabolism: Impact on exercise and nutritional strategies to optimize health and performance in women. *Experimental Physiology*, *101*(2), 243–249. doi:10.1113/EP085369

97. Ellis, G. S., Lanza-Jacoby, S., Gow, A., & Kendrick, Z. V. (1994). Effects of estradiol on lipoprotein lipase activity and lipid availability in exercised male rats. *Journal of Applied Physiology*, *77*(1), 209–215. doi:10.1152/jappl.1994.77.1.209

98. Tarnopolsky, M. A. (2000). Gender differences in substrate metabolism during endurance exercise. *Canadian Journal of Applied Physiology*, *25*(4), 312-327. doi:10.1139/h00-024

99. McDonald, M. W., Olver, T. D., Dotzert, M. S., Jurrissen, T. J., Noble, E. G., Padilla, J., & Melling, C. J. (2019). Aerobic exercise training improves insulin-induced vasorelaxation in a vessel-specific manner in rats with insulin-treated experimental diabetes. *Diabetes and Vascular Disease Research*, *16*(1), 77–86. https://doi.org/10.1177/1479164118815279

100. Tremblay, J., Peronnet, F., Massicotte, D., & Lavoie, C. (2010). Carbohydrate supplementation and sex differences in fuel selection during exercise. *Medicine and Science in Sports and Exercise*, *42*(7), 1314–1323. doi:10.1249/MSS.0b013e3181cbba0b

101. Hackney AC, Kallman AL, Ağgön E. Female sex hormones and the recovery from exercise: Menstrual cycle phase affects responses. *Biomed Hum Kinet*. 2019;11(1):87-89. doi:10.2478/bhk-2019-0011

Chapter 2

2.1 Introduction

Type 1 diabetes mellitus (T1DM) is a disorder resulting from the autoimmune mediated destruction of the beta-cells in the pancreas. These cells are insulin producing, and their destruction results in sub-optimal or no circulating endogenous insulin. The failure of the body to produce optimal insulin levels in the blood results in increased blood glucose levels, leading to chronic hyperglycemia in the absence of insulin therapy treatment. As a result, several health complications are associated with chronic hyperglycemia including retinopathy, nephropathy, and cardiovascular disease [1-3]. A large cohort trial, The Diabetes Control and Complications Trial (DCCT) demonstrated the importance of intensive insulin therapy in the reduction of many of these diabetes related complications, in contrast to the more traditional conventional insulin therapy treatment. However, intensive insulin therapy increases the risk for hypoglycemia onset three-fold in these patients[4-6]. The fear of exercise-related hypoglycemia is one of the most common barriers to attaining the recommended physical activity guidelines among the T1DM population [7].

In studies on hypoglycemia in non-diabetics, antecedent bouts of hypoglycemia, induced by either exercise or administering exogenous insulin, have been shown to blunt or reduce the neuroendocrine and glucoregulatory responses to subsequent bouts of exercise[9, 10]. There is a sexual dimorphism in these hormonal responses to repetitive exercise, whereby the blunted hormonal response to subsequent exercise is more pronounced in males[9-11]. These sex related differences are apparent during moderate

intensity aerobic exercise and are not evident during more intense forms of exercise [12]. Importantly, little information is available as to the impact of antecedent or successive bouts of exercise in females with T1DM. Indeed, it has been shown that similar hormonal blunting effects of antecedent exercise are evident in both sexes; however, female patients with T1DM experience a less pronounced effect than male patients. The impact of this altered glucose regulatory hormonal response on hypoglycemia risk is not known. Many studies investigating the sex differences in glucose hormonal responses in patients with T1DM often maintain normal BG throughout the exercise and recovery periods; thus, alterations in blood glucose levels in response to repetitive exercise were not determined [9,10].

Estrogen (E2) plays a key role in maintaining glucose homeostasis in the body as higher levels of E2 are linked with lower respiratory exchange ratio (RER) and higher lipid oxidation [13-16]. In healthy women, circulating E2 levels during the luteal phase (LP) are highest and can be potentiated by exercise during this menstrual cycle phase [16-18]. Further, the use of oral contraceptives in males and postmenopausal females resulting in elevated estrogen levels has also been shown to increase Free Fatty Acid (FFA) levels during heavy aerobic exercise, suggesting an E2-mediated shift towards fat oxidation during the exercise bout, while carbohydrate metabolism was not impaired [17,18]. The increased levels of E2 also have an impact on exercise-mediated secretion of growth hormone (GH), a hormone that is well established to elevate hepatic glucose release during strenuous exercise[16,17,19]. Further, females have higher resting GH levels than males, and larger GH response to exercise is evident when E2 levels are highest [16,18]. Due to the lower and relatively stable E2 levels during FP compared to LP and the potential

metabolic influence of this hormone, studies [9,10] are often conducted during the FP when E2 levels are the most stable and closer to (but still higher than) levels in males [9,10,12,19]. In female patients with T1DM, these apparent fuel selection differences as a result of elevated E2 during the LP could have an impact on counterregulatory measures against rapid reductions in blood glucose, as a shift towards lipid oxidation could be glycogen sparing; ultimately, leading to reduced risk of hypoglycemia in female T1D pateints[16,19].

The purpose of this study was to examine the sexual dimorphism in the blood glucose response to recurrent bouts of prolonged aerobic exercise in T1D and non-T1D rats. The menstrual cycle in humans is comprised of LP and the follicular (FP) phases, with LP characterized by high levels of E2 and greater fluctuations of E2 than FP. To observe the role of E2 we compared the blood glucose response to aerobic exercise over the four day estrous cycle of the rat. Moreover, to examine the glycogen sparing effect of E2 during recurrent exercise in female T1DM rats we examined hepatic glycogen levels immediately after the fourth bout of exercise during the proestrus phase of the estrous cycle, the phase shown to have the highest E2 levels. We hypothesized that fuel selection differences would result in female T1DM and non-diabetic rats utilizing fat oxidation over carbohydrates, resulting in a minimal change in blood glucose levels during and following aerobic exercise that was concomitant with higher post-exercise liver glycogen content in comparison to their male counterparts.

2.2 Materials and Methods

2.2.1 Ethics Approval

This study was approved by the University Council of Animal Care of Western University (London, Ontario, Canada) and conducted in accordance with the standards of the Canadian Council on Animal Care.

2.2.2 Animals

Twenty Sprague Dawley rats (10 males, 10 females) were obtained from Charles River Laboratories (St. Constant, Que., Canada) at 8 weeks of age. The rats were housed in standard cages in same sex pairs until assignment to groups at which point animals were housed in pairs with the fifth rat in each group housed singly. Rats were maintained on a light-dark cycle of 12 h, with temperature held at 20.5°C and relative humidity at 40%. Standard rat chow and water were provided ad libitum for the duration of the study.

2.2.3 Experimental Groups

Rats were randomly assigned into one of four groups: control (non-T1DM) exercise male (CXM, $n = 5$), control exercise female (CSF, $n = 5$), T1DM exercise male (DXM, $n = 5$), and T1D exercise female (DXF, $n = 5$). Animals in DXM and DXF underwent a diabetes induction protocol in the second week of the study.

2.2.4 Experimental Design

2.2.4.1 Diabetes Induction

Animals were acclimatized for five days following transportation to the animal facility. Following this period, T1DM groups (DXF and DXM) underwent a 5-day protocol of intraperitoneal low dose injections of streptozotocin (STZ; Sigma-Aldrich) to induce T1DM (week two, *Fig. 3*). For seven consecutive days, 20mg/kg of STZ (dissolved in a citrate buffer; 0.1M, pH 4.5) was injected each day and was administered within fifteen minutes of drug preparation. Diabetes was confirmed by two consecutive days of non-fasted blood glucose concentrations greater than 18mmol/L. Once diabetes was confirmed, insulin pellets were implanted (week three, *Fig. 3)* subcutaneously via surgical incision in the abdomen of all rats (1 pellet = 2 U insulin/per day). All animals in the diabetic groups were given one insulin pellet to begin with regardless of weight. Insulin therapy was adjusted throughout the study by addition or removal of pellets as needed. Blood glucose was maintained in this manner at an intended range of 4-8 mmol/L.

2.2.4.2 Exercise Protocol

One week prior to exercise (week 7) all animals underwent familiarization with the treadmill. The familiarization process was designed to allow animals to become acquainted with the speed of the treadmill and consisted of two days of 15 min of running up to 27m/min for females and 20m/min for males. During the week of exercise (week 8), all animals participated in 1 hour of aerobic exercise at 9am for four consecutive days

at approximately 75% of their maximal oxygen consumption. To account for differences in maximal oxygen uptake and weight, the running speed for female groups was 25m/min at 6% grade and 18m/min at 6% grade for males. Treadmill speeds were based on studies reporting oxygen consumption values in exercising male and female rats[39]. While males have higher VO2max in most situations, the weight discrepancy resulted in males needing to exercise at a lower speed which still reflected the same percentage of absolute VO2max. Animals were monitored during a recovery period of 60 minutes postexercise (120 minutes from the initiation of exercise) (*Fig. 2*). Additionally, DXF and CXF were staged to ensure that the final day of exercise would be during proestrus. Due to the differing treadmill speeds DXM and CXM were exercised immediately following DXF and CXF exercise was complete for that day. DXM and CXM were not staged based on any hormonal fluctuations,

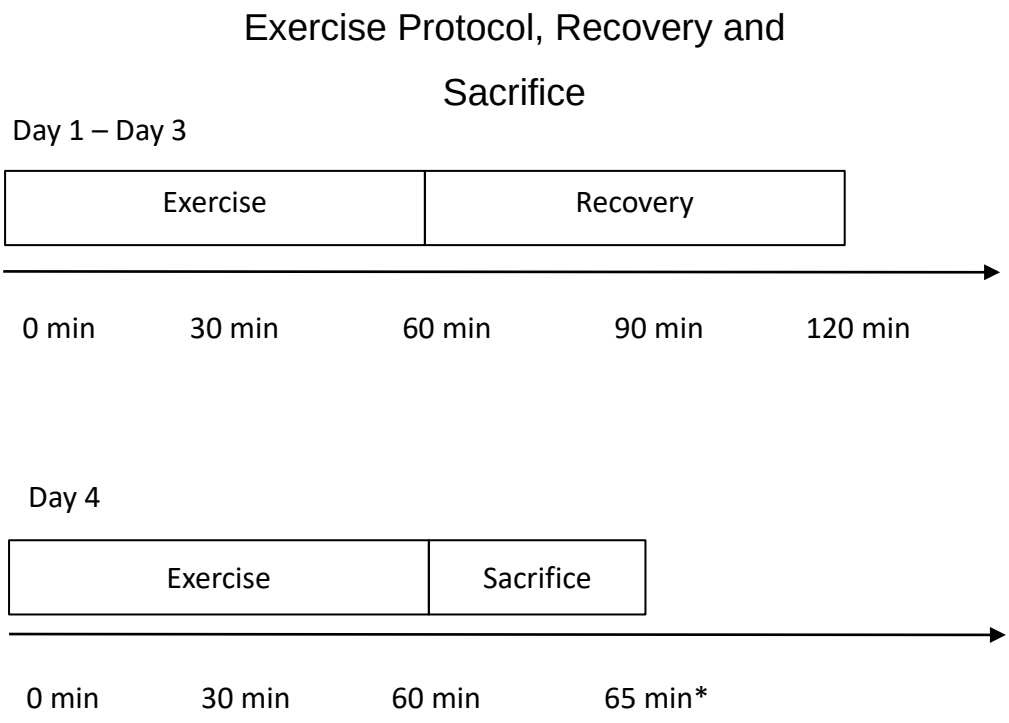


Figure 2 *Exercise protocol for days 1 through 3, and day 4, respectively. Saphenous vein blood samples were taken at all timepoints unless otherwise indicated (*). Sacrifice was performed immediately following exercise and tissue samples used in this study were taken within five minutes post exercise.*

Weekly Timeline

Week 1	• Acclimatization
Week 2	• STZ protocol for DX groups • Daily weigh-ins for CX groups.
Week 3-6	• Insulin pellet adjustment for DX groups • Vaginal swab protocol for DXF and CXF • Twice weekly weigh-ins and blood draws for blood glucose concentrations
Week 7	• Treadmill familiarization in two separate bouts of 15 min each
Week 8	• Four daily bouts of one hour of treadmill exercise at 80% VO_{2max} • DXF and CXF staged such that the final bout of exercise occurs during proestrus • Blood draws at 0 min, 30 min, 60 min, 90 min and 120 min • Day 4 sacrifice at 60 min via cardiac puncture under isoflurane

Figure 3 *Timeline of events from week 1 through week 8. Week 1 consisted of acclimation to environment. Week 2 consisted of streptozotocin (STZ) injections to induce type 1 diabetes mellitus in animals assigned to diabetic female (DXF) or diabetic male (DXM). No procedures other than weighing and blood draws occurred for control female or control male (CXF and CXM, respectively). During week 3- week 6 insulin pellets were implanted surgically in DXF and DXM and adjusted as needed throughout the remaining weeks. Week 7 all animals underwent two days of familiarization with the treadmill protocol. Week 8 all animals underwent four days of consecutive aerobic exercise. On the final day of week 8 all animals were euthanized, and tissue samples collected.*

2.2.5 Experimental Measures

2.2.5.1 Body Weight and Blood Glucose

Body weights and blood glucose levels were recorded weekly throughout the study. Weekly blood samples (~50 μL) were obtained via saphenous vein puncture in the hind leg. Blood glucose was measured using the Freestyle Lite Blood Glucose Monitoring System (Abbot Diabetes Care, INC.). During the week of exercise (week 8), blood samples were collected prior to commencing exercise, at 30min intervals during exercise until 60 min post-exercise via saphenous vein puncture on alternating hind legs. Blood was collected in non-coated tubes then centrifuged at 3000rpm for 20 min at 4°C. Supernatant was obtained and stored at -80°C until analysis.

2.2.5.2 Vaginal Swabs

In Week 2 and 3 of the experiment, DXF and DSF underwent daily vaginal swabs for twelve days to determine the length of the estrus cycle. A sterile cotton swab was dipped in saline (0.9 M) and inserted into the vagina at a 45° angle, half turned, then remove. The swabs were then rolled horizontally onto a clean slide with firm pressure. Animals underwent additional swabbing once per week to confirm estrus stage prediction, including the week prior to exercise. Upon determination of the length of the estrus cycle, females were staged such that the last day of exercise occurred during proestrus.

2.2.5.3 Tissue Collection

Rats were euthanized within five minutes of the final bout of exercise to allow hepatic tissues to be examined for glycogen levels (day 4). Animals were placed under 5% isoflurane until all reflexes were absent and euthanized via cardiac puncture. Liver tissue was obtained and frozen in liquid nitrogen, then stored at -80°C until analysis.

2.2.6 Hematoxylin and Eosin Staining

Vaginal slides were dried for at least five minutes, then placed in a fixing solution (Rapid Fixx, Shandol) for one minute (Appendix D). Slides were placed in Harris Haematoxylin for 1 minute, then rinsed in tap water for 1 minute. Excess stain was removed with minimal amounts of water and dehydrated in 70% ethanol for 1 minute. Slides were submerged in 5% Eosin solution for 1 minute. Slides were rinsed again in tap water to remove excess stain and dehydrated in ascending alcohol. Finally, slides were cleared using xylenes and mounted using toluene-based media. Following staining slides were examined under a Zeiss AxioVert S100 microscope at 10x magnification to determine the phase of estrus (see *Fig. 5*).

2.2.7 Estrogen Quantification

Serum estrogen concentration was determined via enzyme-linked immunosorbent assay (17 beta Estradiol ELISA Kit, Abcam). Samples were obtained from the day 1 and day 4 pre-exercise saphenous vein blood draw (t = 0). Samples, standards, and blanks were added to a 96-well plate precoated with anti-Estradiol IgG. 17 beta Estradiol-HRP conjugate was added to each well. The plate was incubated for 2 hours at 37°C followed

by aspiration and washed three times. Substrate solution was added and incubated in the dark at room temperature for 30 minutes. Immediately following the incubation period, stop solution was added to each well in the same order as substrate solution. Absorbance was measured at 450nM within ten minutes of adding the stop solution.

The absorbance of the sample was adjusted based on the absorbance of a blank, and a standard curve (R = 0.97) was developed using prepared standards. The relationship between absorbance and concentration is logarithmic, therefore an equation was developed based on the standard curve to reflect the logarithmic relationship between absorbance and concentration.

2.2.8 Liver Glycogen Content

2.2.8.1 Liver Homogenization

Liver samples were removed from storage at -80°C and kept on ice. Approximately 20 mg of tissue was excised from the sample and placed into a 2.0 mL Eppendorf tube (Appendix A). Samples were submerged in 30% potassium hydroxide solution saturated with sodium sulfate, and placed in boiling water for 30 min. Samples were precipitated in 95% ethanol for 30 min in an ice bath, followed by centrifugation at 3000 rpm for 30 min. The supernatant was discarded, and the glycogen pellet immediately dissolved in ddH_2O.

2.2.8.2 Glycogen Quantification

Homogenized liver samples were added to a 96 well uncoated plate. A colour reaction was developed by rapid addition of 5% phenol and 98% sulfuric acid to the

sample. Samples were placed in a water bath (37°C) for 20 min. Samples were analyzed in triplicate at a 490 nm on a microplate reader and compared to known glycogen standards (Appendix A).

2.2.9 Data Analysis

Liver glycogen content and blood glucose levels were compared using a three-way analysis of variance (ANOVA) with sex, time and diabetes as factors using GraphPad Prism 8 (GraphPad Software, Inc.). Post-hoc analysis was performed using Tukey's multiple comparisons test when significant differences were found. Estradiol concentration on day 1 (diestrus) and day 4 (proestrus) between the sexes was analyzed using a two-way ANOVA and post-hoc analysis was performed using a Sidak test. Significance was accepted at an alpha value of 0.05.

2.3 Results

2.3.1 Animal Characteristics

Results of both body weight and blood glucose were analyzed to examine the influence of diabetes (T1DM vs. non-T1DM), time (week of study), and sex (male vs. female). Body weight (*Fig. 4a*) and blood glucose (*Fig. 4b*) data were collected for 19 animals (DXF $n = 5$, DXM $n = 4$, CXF $n = 5$, CXM $n = 5$). One animal from DXM was excluded as they were unable to complete the full hour of exercise on day three. There was a significant interaction between time and diabetes ($P < 0.0001$), and time, diabetes and sex ($P < 0.05$). In week two of the study, DXF and DXM had higher blood glucose levels than CXM and CXF ($P < 0.0001$). There was no difference in week two blood glucose levels between DXF and DXM ($P > 0.999$). At week six, blood glucose levels were significantly elevated in DXM and CXM ($P < 0.05$). At week eight, blood glucose was significantly higher in DXM compared to both CXM and CXF ($P < 0.05$). At no point was there a statistically significant difference in blood glucose between DXF and DXM. The initial dosage (week 2) of insulin (ug/g) was higher in DXF ($P < 0.05$) primarily due to the smaller size of the females (*Fig. 6*). There was no difference in insulin dosage/gram between the groups in week 8 of the study ($P > 0.99$).

The body mass of DXF and CXF were significantly lower than that of DXM and CXM ($P < 0.0001$). No differences in final weight (week 8) between DXM and CXM was evident ($P > 0.05$) and DXF and CXF ($P > 0.05$). The effects of sex and week were statistically significant on weight ($P < 0.0001$) while diabetes was not ($P > 0.05$) statistically different. There was an interaction between time and diabetes ($P < 0.01$), time

and sex ($P < 0.0001$), diabetes and sex ($P < 0.0001$), and time, diabetes and sex. ($P < 0.0001$).

2.3.2 Vaginal Swabs

Representative vaginal images of each estrous stage are shown in Figure 5. Proestrus (*Fig. 5a*) shows nucleated cells that present in clumps or sheets. Estrus (*Fig. 5b)* shows an abundance of keratinized, denucleated epithelial cells. Metestrus (*Fig. 5c)* and diestrus *(Fig. 5d)* both show some nucleated cells and neutrophils, with metestrus showing higher cellularity than diestrus. Slides were stained using a standard hematoxylin and eosin protocol and examined against known examples of the stages of estrous for neutrophil content, the presence and number of keratinized cells, and the presence of mucus among other indicators.

2.3.3 Blood Glucose Response to Exercise

Results from the first three days (estrus, metestrus and diestrus) of exercise are presented together due to the lower E2 levels observed during these days (*Fig. 7*). There was a significant effect of time ($P < 0.05$) and a significant interaction between time and diabetes ($P < 0.0001$). Pre-exercise blood glucose (BG) levels in CXM were significantly lower in comparison to 30, 60, and 90 minutes ($P < 0.05$), but BG returned to normal resting BG by 120 minutes. A slightly different pattern was observed in the CXF group. CXF BG levels were higher ($P < 0.05$) during exercise (at 30 and 60 minutes) than pre-exercise blood glucose levels; however, the BG was normalized by 90 minutes and maintained until 120 minutes post exercise. There was a trend towards a decrease in BG

values from the pre-exercise levels to 30 minutes of exercise until the completion of exercise (60 minute) but this change did not reach the level of significance. ($P > 0.05$ and $P > 0.05$ respectively). At 30 and 60 minutes of exercise, blood glucose in DXF was significantly lower ($P < 0.05$) than the CXF counterparts but no difference in BG levels between DXF and CXF were detected at 90 minutes ($P > 0.05$). The DXF blood glucose levels at 90 minutes were significantly lower ($P < 0.05$) than the final blood glucose at 120 minutes (60 minutes post exercise). Additionally, the final recovery blood glucose at 120 minutes in DXF was significantly higher ($P < 0.05$) than the 120 minutes CXF blood glucose. There was a main effect of time ($P < 0.0001$) and interactions between time and diabetes ($P < 0.0001$) and time, diabetes and sex ($P < 0.0001$)

Day 4 BG levels were obtained at pre-exercise (0 min) and 30 and 60 minutes of exercise (*Fig. 8*). After 30 minutes of exercise, both DXF and DXM were significantly lower ($P < 0.05$) than their control counterparts (CXF and CXM). However, these values returned to baseline by the end of the exercise (60 minutes). There were no differences in blood glucose between groups at 60 minutes ($P > 0.05$). There was a main effect of time ($P < 0.05$) and an interaction between time and diabetes ($P < 0.0001$).

2.3.4 Estrogen Concentration

Estrogen concentrations measured using ELISA are shown in Figure 9. There was a significant effect of time and an interaction between time and sex ($P < 0.05$). Blood serum was obtained from day 1 and day 4 of exercise prior to exercise ($t = 0$). As confirmed by vaginal cytology, day 1 serum was obtained during estrus, and day 4 serum was obtained during proestrus. Day 4 DXF had significantly higher estrogen than both

day 1 DXF and day 4 DXM. There was no difference in estrogen concentration on day 1 between DXF and DXM ($P > 0.5$). Day 1 and day 4 estrogen concentration was not different in DXM ($P > 0.5$).

2.3.5 Liver Glycogen Content

Liver samples were taken immediately following the final bout of exercise on the fourth day. There was a main effect of diabetes ($P < 000.1$). There was no interaction between sex and diabetes ($P > 0.05$) (*Fig. 10*). There was no difference in liver glycogen between CXF and CXM ($P > 0.05$) or between DXF and DXM ($P > 0.999$). Liver glycogen was significantly lower in DXF compared to CXF ($P < 0.0001$). DXM also had significantly lower liver glycogen content than CXM ($P < 0.0038$).

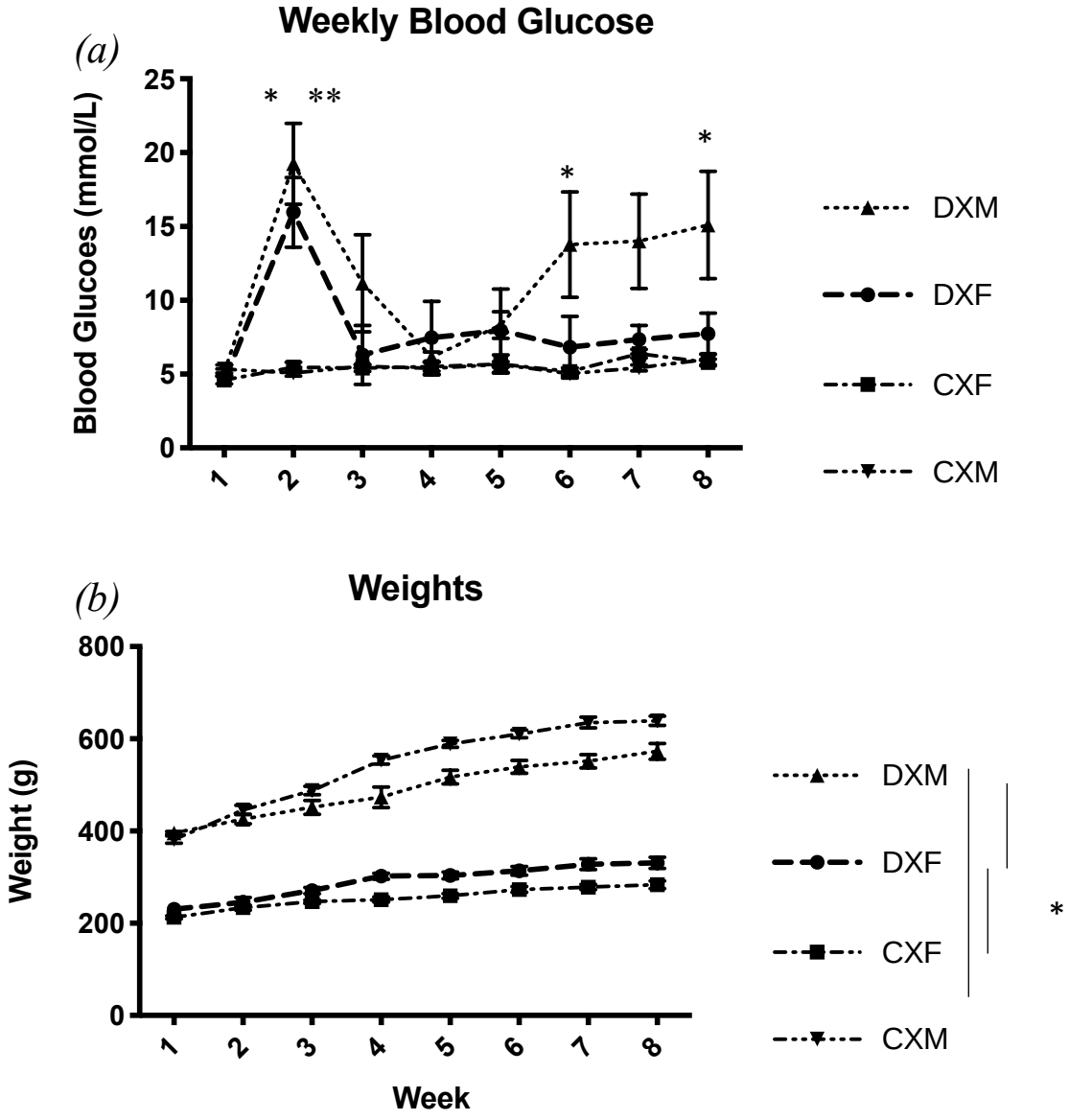


Figure 4 (a)*Mean non-fasted blood glucose (mM). All data are presented as mean ± SEM. * denotes significant difference between diabetic males (DXM) and control males (CXM) (P < 0.05), ** denotes diabetic females (DXF) significantly different than control females (CXF) (P < 0.05).*

(b) *Mean body mass (g). All data are presented as mean ± SEM. * denotes P < 0.05.*

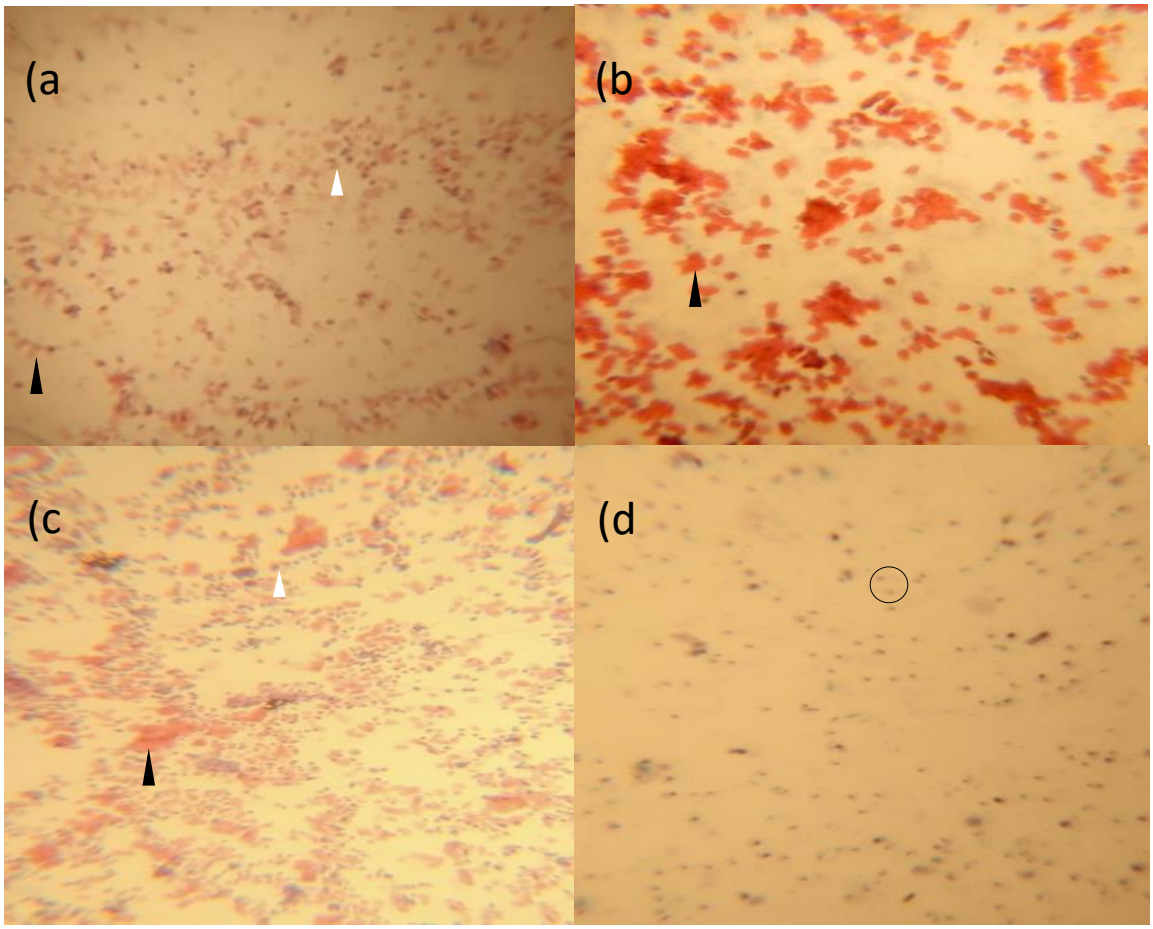


Figure 5 *Vaginal smears of (a) proestrus, (b) estrus, (c)metestrus and (d) diestrus. Nucleated epithelial cells (white triangle) and clumping are present in proestrus. Keratinized epithelial cells (black triangles) are predominant in estrus. Neutrophils (circled) are highly present in diestrus.*

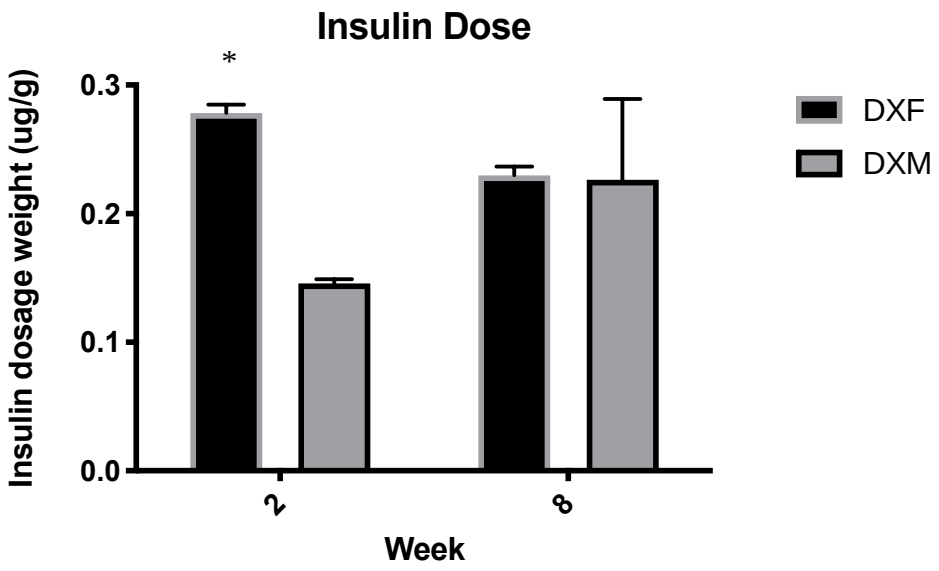


Figure 6 *Weekly insulin dose comparison between week 2 (diabetes induction) and week 8 (exercise and sacrifice). Initial insulin dose was significantly greater in the diabetic female group (DXF) (* denotes significance at $P < 0.05$) due to the weight difference between DXF and diabetic males (DXM). By week 8 insulin dose was comparable between the two groups.*

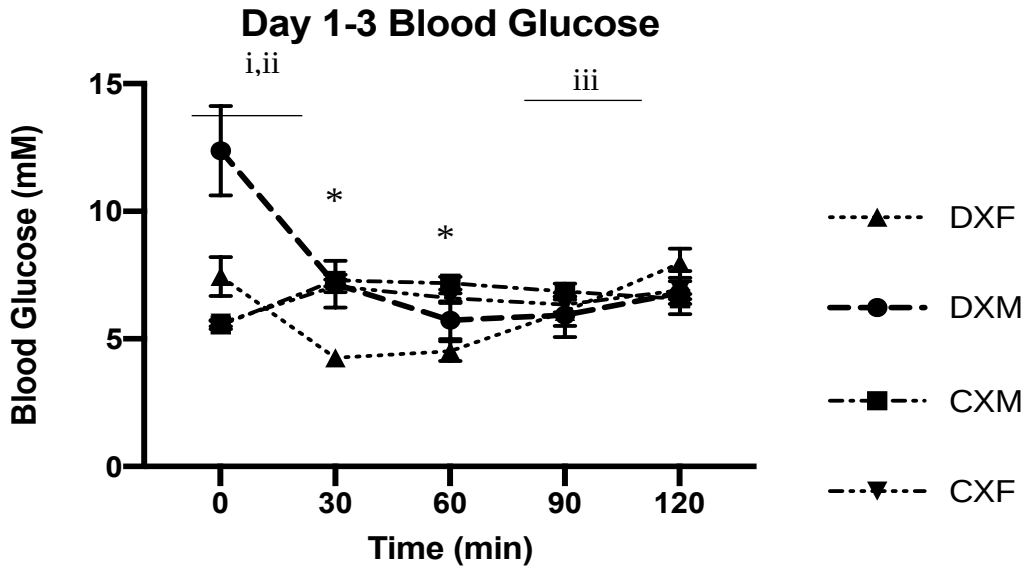


Figure 7 *Mean blood glucose data (mmol/L) from days 1-3 are presented together. All data are presented as mean ± SEM. * denotes significance. (P < 0.0001) between diabetic and control females (DXF and CXF, respectively). i denotes significance within control males (CXM) (P < 0.0001) and ii denotes significance within CXF (P < 0.01). iii denotes significance within DXF (P < 0.05). DXM (diabetic males) had no significant changes or differences.*

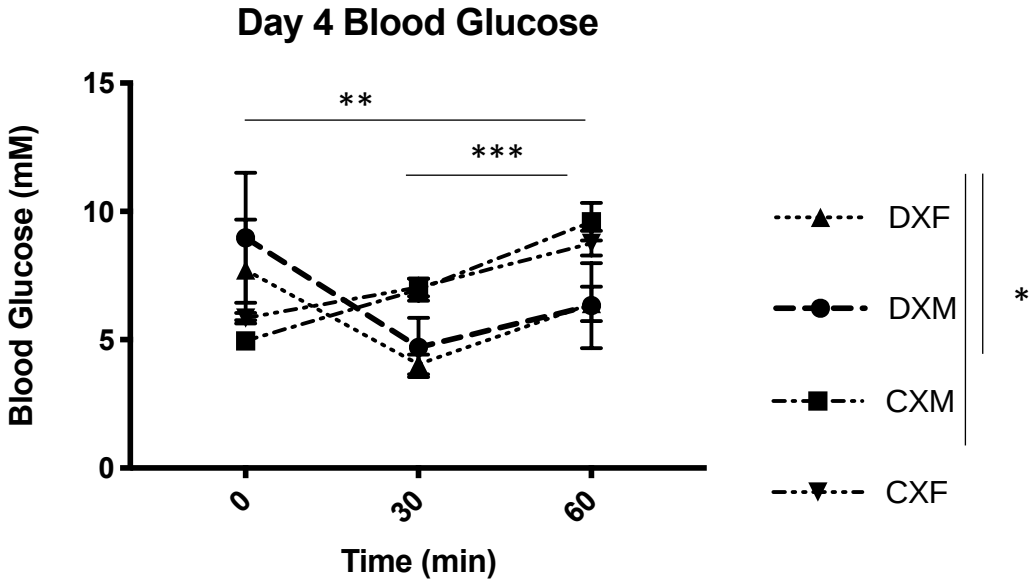


Figure 8 *Mean blood glucose (mM) from day 4 exercise. All data are presented as mean ± SEM. * denoted significance at t = 30 minutes (P < 0.05), ** denotes significance (P < 0.05) between starting and ending blood glucose in control females and males (CXF and CXM, respectively) *** denotes significance (P < 0.05) between 30 to 60 minutes in diabetic females (DXF). There were no differences in blood glucose between CXM and diabetic males (DXM).*

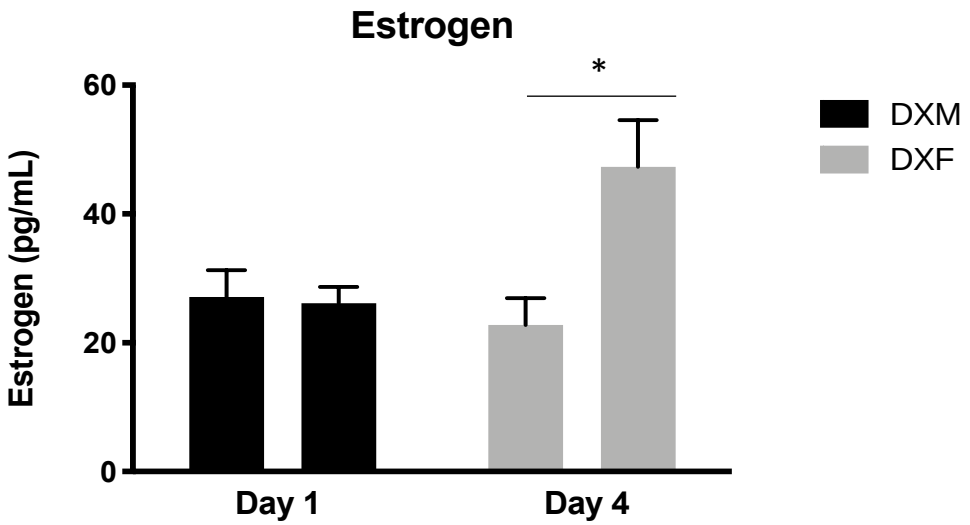


Figure 9 *Serum estrogen (pg/mL) from pre and post exercise protocol between DXM (diabetic exercised males) and DXF (diabetic exercised females). All data are presented as mean ± SEM. * denotes P < 0.05.*

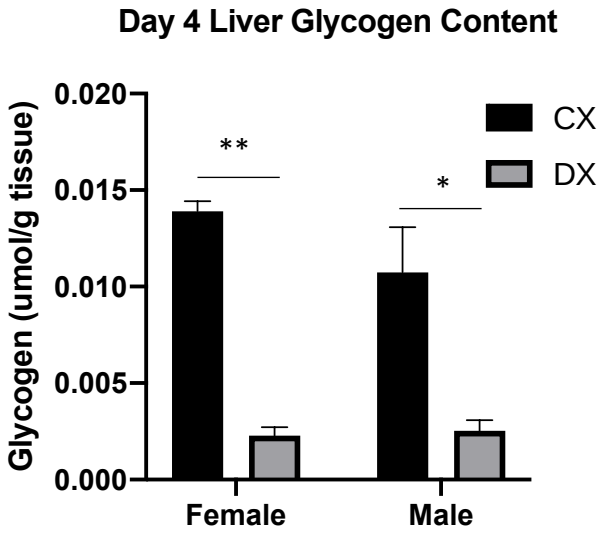


Figure 10 *Liver glycogen content (umol/g tissue) from tissue collection on day four following the final bout of exercise for control exercise and diabetic exercise (CX and DX, respectively) males and females. All data are presented as mean ± SEM. * denotes P < 0.05, ** denotes P < 0.0001.*

2.4 Discussion

Diabetes research has evolved since the DCCT to recognize the benefits of aerobic exercise for the individual with type 1 diabetes. However, much of the research continues to focus on male subjects, and studies that do utilize female subjects, typically use physiological states in which these individuals demonstrate low hormonal levels (FP) or are experimentally regulated[9,10,12,17-18]. Estrogen is known to impact the progression and risk factors associated with many other diseases, yet few studies have investigated the effect of estrogen on the metabolic complications associated with T1DM. Indeed, estrogen levels are correlated with a shift in fuel usage towards higher fat oxidation, and therefore may be protective to female athletes with T1DM. As such, this study aimed to examine if the blood glucose response to repetitive exercise would differ between male and female non-T1DM and T1DM rodents, and if this response would differ with respect to the different phases of the estrous cycle whereby estrogen levels fluctuate throughout. This study also resulted in a model for staging female rats for exercise studies to examine the role of estrogen in multiple pathways and disease models.

The DXF response to exercise during days 1-3 differed in comparison to the CXF group (*Fig. 7)*. During exercise in CXF, the glucose counterregulatory response to exercise was evident and blood glucose was increased in comparison to pre-exercise levels. During the recovery period, the blood glucose levels returned to baseline levels by 120 minutes. DXF on the other hand had a drop in blood glucose from pre-exercise to 30 minutes of exercise, and a gradual recovery from 60 to 120 minutes during the post exercise recovery. The change in DXF BG from 0 to 30 minutes was not significantly

different; however, by 30 minutes and again at 60 minutes the difference in BG between DXF and CXF group was statistically significant. Interestingly, the return to pre-exercise blood glucose was faster in DXF than that observed in the CXF group. CXM had a significant change in BG from pre exercise to 30 minutes, with an increase in BG levels. This response was not evident in the DXM group, with there being no significant change from 0 to 30 minutes in BG. There were no significant differences at any of the timepoints between DXM and CXM; however, the pattern of BG response appeared different when graphed (*Fig. 7).* There were no differences between the DXF and DXM or CXF and CXM at any timepoint within the first three days of exercise. The BG response was the same between the sexes, and the variation was primarily caused due to time and the interaction between time and diabetes. It is possible that the BG levels and response were similar due to fuel selection similarities between the sexes due to similar, stable E2 levels[16-19]. Previous studies from our lab have shown that aerobic exercise causes a decrease in BG in male diabetic rats as would be observed in an insulin treated type 1 diabetic male athlete undergoing exercise [20,21].

We hypothesized that BG levels in female T1DM rats during the final bout of exercise (day 4) would remain optimal due to increased levels of estrogen in the proestrus stage of estrous. This would be evident through an increased level of liver glycogen content due to the glycogen sparing effects of estrogen and the preferred utilization of fat. The DXF had significantly lower BG response at 30 minutes compared to CXF, which was similar to the response in days 1-3, suggesting there was still high glucose use despite higher E2 levels than the day before. The DXF BG was not different at any timepoint than DXM. CXM and CXF also did not differ in BG response at any timepoint

during day 4 *(Fig. 8).* Both CXM and CXF saw a significant increase in BG from starting to ending BG which may have been consistent with results from day 1-3 and representative of normal BG response to aerobic exercise[36, 37]. As show in Figure 7, the, BG during the first three days of exercise in the DXF and DXM groups dropped significantly from 0 to 30 minutes, while BG in the CXF and CXM groups increased significantly from baseline. While these data do not support our hypothesis suggesting a glycogen sparing role for estrogen during day 4 of exercise, it is plausible that the previous bouts of exercise stressed the glucoregulatory response system of DXF rodents such that the glucose sparing effects of estrogen were mitigated. The DXF group was the only group that had a post-exercise day 1-3 BG higher than that during exercise (t = 30min, 60min). DXF BG was also higher at 120 minutes than it was at 90 minutes (*Fig. 7).* It is possible that the hormonal differences between DXM and DXF resulted in greater fat oxidation during the exercise period, allowing the DXF group to return to resting BG levels faster than their male counterparts. E2 mediated shift in fuel selection (*Fig. 1*) towards fat during aerobic exercise protects against liver glycogen depletion and therefore smaller post exercise glucose uptake, resulting in BG leveling out faster in females[19,38]. The exercise intensity (approximately 75%) chosen for this study taxes glucose homeostasis as higher intensities primarily utilize stored carbohydrate for energy. The post-exercise period following exercise of such an intensity is when people with T1DM face the greatest risk of hypoglycemia as the body attempts to reestablish glucose homeostasis, and in males the greater reliance on carbohydrates during exercise and during recovery relative to females puts them at greater risk for post-exercise hypoglycemia [34].

Comparisons of day 1-3 and day 4 were not directly performed due to the differences in blood collection due to the sacrifice procedure, however we observed that there appeared to be an increase in all groups of BG at 60 minutes on day 4 (*Fig. 8*). This pattern is not typical of the recovery to aerobic exercise, nor is what was observed on day1-3 [21]. It is possible that this increase in BG during the final blood draw was due to the sacrifice procedure itself. The use of isoflurane as an anesthetic has been linked to increases in BG in human surgical patients, but it is unclear if the amount of isoflurane administered could have had such an immediate effect on BG levels [22]. Blood draw techniques which are animal sparing such as tail or saphenous vein draw are considered less stressful to the animal, however serial draws may be more stressful resulting in systemic changes in GH, BG, and epinephrine levels [22, 23]. It is possible that the serial blood draws coupled with the level of anesthesia administered to the animal the final blood glucose reading is inflated, but not to a level of significance. Exercise type and reinforcement methods have been noted to influence the stress and estrogen response to exercise; however, the methods used in this study (i.e. treadmill running and air pressure) have the smallest effect on these measures [24].

Weekly blood glucose levels (*Fig. 4a)* were as expected for control groups and remained stable throughout the 8 week period leading up to exercise. During week two of the study there was an increase in blood glucose concomitant with the low dose STZ protocol for diabetes induction, and a subsequent decrease in blood glucose following insulin pellet implantation (*Fig. 6*). In the final weeks of the study there was an increase in blood glucose in DXM relative to the other groups, and this increased blood glucose was not responsive to additional exogenous insulin. This change in blood glucose did not

reach the level of significance likely due to the small group size of DXM (n = 4) due to the exclusion of one animal from the study. The animal was excluded as it was unable to complete the full hour of exercise at the required intensity on day 3 of the study. Despite not reaching the level of significance, the elevated blood glucose is worth discussing further and may highlight some sex differences in glucose control during long term intensive insulin therapy. While there were no direct measures of insulin resistance, it is reasonable to propose that the DXM cohort became resistant to insulin as evidenced by daily glucose levels outside of the target range (4-7 mM). Hyperinsulinemia may have elicited insulin resistance development through a mechanism of oxidative stress and may further impair the body's insulin sensitivity response during exercise [25, 26]. The level of insulin in the DXM group was appropriate for the weight and size of the animals, and no such effect was seen in the female cohort who received the same amount of insulin relative to body size (*Fig. 6*).

Sample vaginal smears taken during the fourteen day swabbing protocol are shown in Figure 5. We were able to successfully identify the four stages of estrous in all female rats by examining the smear in its entirety and comparing the presence of neutrophils, keratinized epithelial cells, and nucleated epithelial cells [23]. Serum estrogen levels confirmed that the prediction method used resulted in accurate staging of the exercise. Day 4 estrogen levels were significantly higher than day 1 levels in females, and day 1 levels were not significantly different than male levels. Therefore, we believe that this method can be used to stage animals based on corresponding estrogen levels. This method involved two weeks of daily swabs to establish cycle length for each rodent, and confirmation of the predicted stage prior to experimentation. This method avoids blind

swabbing by establishing the initial cycle length, which prevented erroneous staging [27]. Experimental protocols were carried out in the morning as the changes throughout the cycle tend to occur at the same time each day, and it has been suggested that the swabbing in the morning presents the best chance of seeing all four stages[27, 28]. While various external factors including housing male and females rats in the same room have been said to induce synchronization of the estrous cycle in rodents [29] we did not find this to be the case. Melatonin release and light-dark cycles may also affect the cycle; however, the rats in question were on a controlled light dark schedule which has been shown to mitigate fluctuations in the estrus cycle[30]. The estrous cycles of all female rats in this study were four days in length, consisted of all four phases (*Fig. 5)*, and did not synchronize or appear to change based on environmental or experimental conditions.

We hypothesized that by performing the final bout of exercise during proestrus stage liver glycogen would be spared in both DXF and CXF compared to DXM and CXM; however, we found no differences between the sexes in liver glycogen content (*Fig. 10).* The only significant factor in liver glycogen content was diabetes, which was to be expected in males based on previous literature from our laboratory[20,21,37]. The proposed fuel usage shift in Figure 1 would suggest that the higher lipid usage would spare liver glycogen and therefore better protect DXF and CXF against exercise induced hypoglycemia. The absence of a glycogen sparing effect in females is in contrast to data showing lower glycogen usage in healthy exercising women in the LP [16] due to the higher estrogen levels. Further, it has been shown that females have a greater FFA level than males due to the fuel selection shift towards fat oxidation that is linked to estrogen [16, 19]. It is possible that due to the previous days of exercise the cumulative blunting effect on

DXF during low estrogen levels (metestrus, diestrus and estrus) created a reduced lipolytic response. The BG response between males and females in this study were the same in both diabetic and non-diabetic groups in spite of the similar reduction in liver glycogen, suggesting that the blunting effects on the counterregulatory system seen in females in other studies were mitigated in this group, potentially due to the higher presence of estrogen.[8, 10, 32.] Potential differences in muscle glycogen usage were not examined in this study and should be investigated in the future.

2.5 Conclusion

In conclusion, this study contributes to addressing the lack of literature regarding the effect of repetitive aerobic exercise on the glucoregulatory response to exercise in a male and female type 1 diabetic rat model. We hypothesized that female diabetic rodents would have higher BG throughout the four days of exercise than their male diabetic counterparts, and that this response in the female diabetic rodents would closely resemble the female controls. We also hypothesized that staging the exercise such that the final bout would occur during the period with the highest estrogen levels would contribute to maintaining BG throughout the prolonged exercise. Finally, we expected the liver glycogen content to be preserved in the female diabetic rodents due to the fuel selection differences that are observed between the sexes.

The current study presents a model for future research into the sexual dimorphism in healthy or diseased rodent models for studies that may be affected by fluctuations in female sex hormones. The use of vaginal cytology to determine the estrous phase is not a new technique; however, the application of this technique to provide a schedule for an

experimental protocol dependent upon estrogen level is to our knowledge a new technique that spares blood volume that can otherwise be used for other testing. The ability to detect the presence and quantity of cells in the smear is paramount to the accuracy of the staging, and therefore consecutive swabs should be taken for a sufficient period before making staging predictions.

There were no apparent differences in liver glycogen content between the male and female T1DM rats. However, there was a greater post-exercise blood glucose recovery in the DXF, suggesting that while there was a decrease in blood glucose during exercise the DXF group was able to counteract this effect more successfully than DXM. The DXM group may have needed longer to reach the resting blood glucose levels. It is possible that the increase in estrogen due to the staging of the exercise had an effect on the fuel selection during exercise or recovery although the mechanism of this cannot be elucidated from this study.

Bibliography

1. Iribarren, C., Karter, A. J., Go, A. S., Ferrara, A., Liu, J. Y., Sidney, S., & Selby, J. V. (2001). Glycemic control and heart failure among adult patients with diabetes. *Circulation*, *103*(22), 2668–2673. https://doi.org/10.1161/01.CIR.103.22.2668
2. Thorn, L. M., Forsblom, C., Fagerudd, J., Thomas, M. C., Pettersson-Fernholm, K., Saraheimo, M., … Groop, P. H. (2005). Metabolic syndrome in type 1 diabetes: Association with diabetic nephropathy and glycemic control (the FinnDiane study). *Diabetes Care*, *28*(8), 2019–2024. https://doi.org/10.2337/diacare.28.8.2019
3. Gubitosi-Klug, R. A., Lachin, J. M., Backlund, J. Y. C., Lorenzi, G. M., Brillon, D. J., & Orchard, T. J. (2016). Intensive diabetes treatment and cardiovascular outcomes in type1 diabetes: The DCCT/EDIC study 30-year follow-up. *Diabetes Care*, *39*(5), 686–693. https://doi.org/10.2337/dc15-1990
4. DCCT Research Group. (1993). The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. *New England Journal of Medicine, 329*(14), 977-986. DOI: 10.1056/NEJM199309303291401
5. Lachin, J. M., White, N. H., Hainsworth, D. P., Sun, W., Cleary, P. A., & Nathan, D. M. (2015). Effect of intensive Diabetes therapy on the progression of diabetic retinopathy in patients with type 1 diabetes: 18 years of follow-up in the DCCT/EDIC. *Diabetes*, *64*(2), 631–642. https://doi.org/10.2337/db14-0930

6. DCCT . (1997). Hypoglycemia in the diabetes control and complications trial. *Diabetes*, *46*(2), 271–286. https://doi.org/10.2337/diab.46.2.271
7. Brazeau, A.-S., Rabasa-Lhoret, R., Strychar, I., & Mircescu, H. (2008). Barriers to Physical Activity Among Patients With Type 1 Diabetes. *Diabetes Care*, *31*(11), 2108 LP-2109. https://doi.org/10.2337/dc08-0720
8. Davis SN, Shavers C, Mosqueda-Garcia R, Costa F. (1997). Effects of differing antecedent hypoglycemia on subsequent counterregulation in normal humans. *Diabetes*. 1997 Aug;46(8):1328-35. doi: 10.2337/diab.46.8.1328. PMID: 9231658.
9. Galassetti, P., Mann, S., Tate, D., Neill, R., Wasserman, D. H., & Davis, S. N. (2001). Effect of morning exercise on counterregulatory repsonses to subsequent, afternoon exercise. *J Appl Physiol, 91, 91-99.*
10. Galassetti, P., Tata, D., Neill, R., Morrey, S., Wasserman, D. H., & Davis, S. N. (2003). Effect of antecedent hypoglycemia on counterregulatory responses to subsequent euglycemic exercise in type 1 diabetes. *Diabetes*, *52*, 1761–1769.
11. Bahr, R., Høstmark, A.T., Newsholme, E.A., Grønnerød, O. And Sejersted, O.M. (1991), Effect of exercise on recovery changes in plasma levels of FFA, glycerol, glucose and catecholamines. Acta Physiologica Scandinavica, 143: 105-115. doi:10.1111/j.1748-1716.1991.tb09205.x
12. Marliss, E. B., Kreisman, S. H., Manzon, A., Halter, J. B., Vranic, M., & Nessim, S. J. (2000). Gender differences in glucoregulatory responses to intense exercise. *Journal of Applied Physiology*, *88*(2), 457–466. https://doi.org/10.1152/jappl.2000.88.2.457

13. Hamadeh, M.J., Devries, M.C., and Tarnopolsky, M.A. 2005. Estrogen supplementation reduces whole body leucine and carbohydrate oxidation and increases lipid oxidation in men during endurance exercise. J. Clin. Endocrinol. Metab. 90(6): 3592-9. doi:10.1210/jc.2004-1743. PMID:15755861.
14. Hatta H, Atomi Y, Shinohara S, Yamamoto Y, Yamada S. The effects of ovarian hormones on glucose and fatty acid oxidation during exercise in female ovariectomized rats. Horm Metab Res. 1988 Oct;20(10):609-11. doi: 10.1055/s-2007-1010897. PMID: 3220443
15. Kendrick, Z. V., C. A. Steffen, W. L. Rumsey, and D. I. Goldberg. Effect of estradiol on tissue glycogen metabolism in exercised oophorectomized rats. J. Appl. Physiol. 63: 492–496, 1987.
16. Devries, M. C., Hamadeh, M. J., Phillips, S. M., & Tarnopolsky, M. A. (2006). Menstrual cycle phase and sex influence muscle glycogen utilization and glucose turnover during moderate-intensity endurance exercise. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *291*(4), R1120–R1128. https://doi.org/10.1152/ajpregu.00700.2005
17. Kraemer, R. R., Heleniak, R. J., Tryniecki, J. L., Kraemer, G. R., Okazaki, N. J., & Castracane, V. D. (1995). Follicular and luteal phase hormonal responses to low-volume resistive exercise. *Medicine and Science in Sports and Exercise*, *27*(6), 809–817. https://doi.org/10.1249/00005768-199506000-00004
18. Bonen, A., Haynes, F. W., & Graham, T. E. (1991). Substrate and hormonal responses to exercise in women using oral contraceptives. *Journal of Applied Physiology*, *70*(5), 1917–1927. https://doi.org/10.1152/jappl.1991.70.5.1917

19. Brockman, N. K., & Yardley, J. E. (2018). Sex-related differences in fuel utilization and hormonal response to exercise: implications for individuals with type 1 diabetes. *Applied Physiology, Nutrition, and Metabolism*, *43*(6), 541-552.
20. McDonald, M. W., Hall, K. E., Jiang, M., Noble, E. G., & Melling, C. W. J. (2014). Ischemia-reperfusion injury and hypoglycemia risk in insulin-treated T1DM rats following different modalities of regular exercise. *Physiological Reports*, *2*(11), 1–12. https://doi.org/10.14814/phy2.12201
21. McDonald, M. W., Murray, M. R., Grise, K. N., Olver, T. D., Dey, A., Shoemaker, J. K., … Melling, C. W. J. (2016). The glucoregulatory response to high-intensity aerobic exercise following training in rats with insulin-treated type 1 diabetes mellitus. *Applied Physiology, Nutrition, and Metabolism*, *41*(6), 631–639. https://doi.org/10.1139/apnm-2015-0558
22. Behdad, S., Mortazavizadeh, A., Ayatollahi, V., Khadiv, Z., & Khalilzadeh, S. (2014). The Effects of Propofol and Isoflurane on Blood Glucose during Abdominal Hysterectomy in Diabetic Patients. *Dmj*, *38*(4), 311–316. https://doi.org/10.4093/dmj.2014.38.4.311
23. Bellinger, L. L., & Mendel, V. E. (1975). Hormone and glucose responses to serial cardiac puncture in rats (38465). *Proceedings of the Society for Experimental Biology and Medicine*, *148*(1), 5–8. https://doi.org/10.3181/00379727-148-38465
24. Shors, T. J., Pickett, J., Wood, G., & Paczynski, M. (1999). Acute stress persistently enhances estrogen levels in the female rat. *Stress*, *3*(2), 163–171. https://doi.org/10.3109/10253899909001120

25. Liu, H.-Y., Cao, S. Y., Hong, T., Han, J., Liu, Z., & Cao, W. (2009). Insulin is a stronger inducer of insulin resistance than hyperglycemia in mice with type 1 diabetes mellitus (T1DM). *Journal of Biological Chemistry*, *284*(40), 27090–27100. https://doi.org/10.1074/jbc.M109.016675

26. Peltoniemi, P., Yki-Järvinen, H., Oikonen, V., Oksanen, A., Takala, T. O., Rönnemaa, T., ... & Nuutila, P. (2001). Resistance to exercise-induced increase in glucose uptake during hyperinsulinemia in insulin-resistant skeletal muscle of patients with type 1 diabetes. *Diabetes*, *50*(6), 1371-1377.

27. Cora, M. C., Kooistra, L., & Travlos, G. (2015). Vaginal Cytology of the Laboratory Rat and Mouse:Review and Criteria for the Staging of the Estrous Cycle Using Stained Vaginal Smears. *Toxicologic Pathology*, *43*(6), 776–793. https://doi.org/10.1177/0192623315570339

28. Yuan Y, Carlson RG. Structure, cyclic change, and function, vagina and vulva, rat. In: Jones TC, Mohr U, Hunt RD, editors. *Genital System*. Berlin (Germany): Springer-Verlag; 1987. p. 161-8.

29. Priddy, B. M., Carmack, S. A., Thomas, L. C., Vendruscolo, J. C., Koob, G. F., & Vendruscolo, L. F. (2017). Sex, strain, and estrous cycle influences on alcohol drinking in rats. *Pharmacology Biochemistry and Behavior*, *152*, 61-67.

30. Rivest, R. W., Lang, U., Aubert, M. L., & Sizonenko, P. C. (1985). Daily administration of melatonin delays rat vaginal opening and disrupts the first estrous cycles: evidence that these effects are synchronized by the onset of light. *Endocrinology*, *116*(2), 779-787.

31. Rennie, M. J., Winder, W. W., & Holloszy, J. O. (1976). A sparing effect of increased plasma fatty acids on muscle and liver glycogen content in the exercising rat. *Biochemical Journal*, *156*(3), 647–655. https://doi.org/10.1042/bj1560647
32. Davis, S. N., Shavers, C., & Costa, F. (2000). Gender-related differences in counterregulatory responses to antecedent hypoglycemia in normal humans. *Journal of Clinical Endocrinology and Metabolism*, *85*(6), 2148–2157. https://doi.org/10.1210/jc.85.6.2148
33. Tremblay, J., Peronnet, F., Massicotte, D., & Lavoie, C. (2010). Carbohydrate supplementation and sex differences in fuel selection during exercise. *Medicine and Science in Sports and Exercise*, *42*(7), 1314–1323. https://doi.org/10.1249/MSS.0b013e3181cbba0b
34. Ridell, M. C., Partington, S. L., Stupka, N., Armstrong, D., Rennie, C., & Tarnopolsky, M. A. (2003). Substrate utilization during exercise performed with and without glucose ingestion in female and male endurance trained athletes. *International journal of sport nutrition and exercise metabolism*, *13*(4), 407-421.
35. Riddell, M. C., Scott, S. N., Fournier, P. A., Colberg, S. R., Gallen, I. W., Moser, O., … Bracken, R. M. (2020). The competitive athlete with type 1 diabetes. *Diabetologia*, *63*(8), 1475–1490. https://doi.org/10.1007/s00125-020-05183-8
36. Melling, C. W. J., Grisé, K. N., Hasilo, C. P., Fier, B., Milne, K. J., Karmazyn, M., & Noble, E. G. (2013). A model of poorly controlled type 1 diabetes mellitus

and its treatment with aerobic exercise training. *Diabetes and Metabolism*, *39*(3), 226–235. https://doi.org/10.1016/j.diabet.2013.02.004

37. McDonald, M. W., Murray, M. R., Grise, K. N., Olver, T. D., Dey, A., Shoemaker, J. K., … Melling, C. W. J. (2016). The glucoregulatory response to high-intensity aerobic exercise following training in rats with insulin-treated type 1 diabetes mellitus. *Applied Physiology, Nutrition, and Metabolism*, *41*(6), 631–639. doi: 10.1139/apnm-2015-0558
38. Henderson, G.C., Fattor, J.A., Horning, M.A., Faghihnia, N., Johnson, M.L., Luke-Zeitoun, M., et al. 2008. Glucoregulation is more precise in women than in men during postexercise recovery. *Am. J. Clin. Nutr*. 87(6): 1686-94. PMID:18541557.
39. Bedford, T. G., Tipton, C. M., Wilson, N. C., Oppliger, R. A., & Gisolfi, C. V. (1979). Maximum oxygen consumption of rats and its changes with various experimental procedures. *Journal of Applied Physiology*, *47*(6), 1278–1283. https://doi.org/10.1152/jappl.1979.47.6.1278.

Appendices

Appendix A: Phenol-Sulfuric Acid Assay for Glycogen Quantification

Part A: Solutions

30% KOH (w/v)

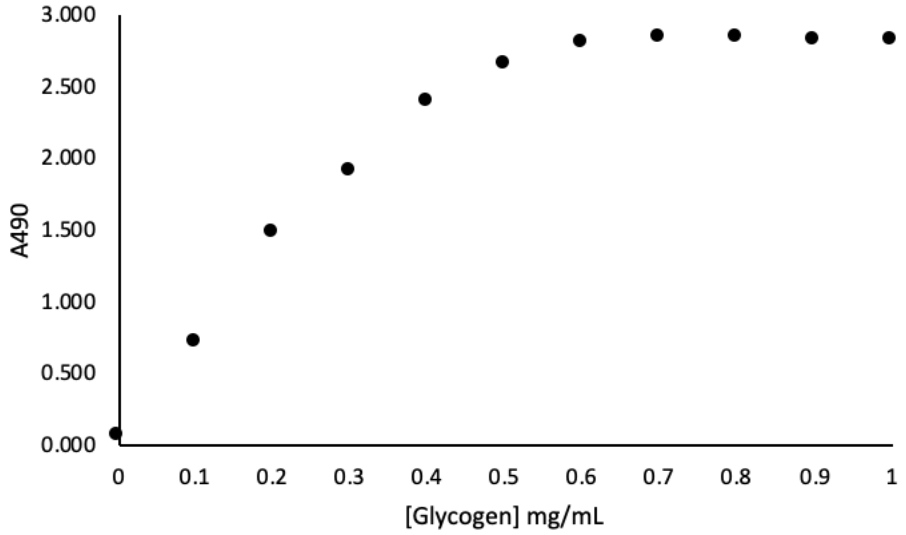
- 30 g of KOH pellets
- 100 mL of ddH_2O
- EXOTHERMIC REACTION — combine in an Erlenmeyer flask placed in an ice bath

95% Ethanol (v/v)

- 95 mL of 100% Ethanol
- 5 mL of ddH_2O

Glycogen Standards: 0.1, 0.2, 0.3, 0.4, 0.5 and 0.6 mg/mL

- Prepare large volumes of standards and store in 15 mL Falcon Tubes
- Standard curve flattens out after 0.6 mg/mL (see below)[1]
    - Linear portion spans from 0 to 0.5 or 0.6 mg/mL



[1] With a 5% (w/v) Phenol solution, the standard curve was linear from 0.0 – 1.0 mg/mL glycogen concentrations with an $R^2 = 0.99$.

100% Sulfuric Acid (Stock)

5% Phenol (v/v)

- 5 mL of 100% phenol (stock)
- 95 mL of ddH_2O

Part B: Tissue Homogenization and Glycogen Isolation

1. Cut and weigh approximately 20 mg of tissue, place into a 2.0 mL Eppendorf tube and keep on ice until the next step.
2. For 12 samples, saturated ~10 mL of 30% (w/v) KOH solution with Na_2SO_4.
3. Boil approximately 0.75 L of water and pour into the metal tray on the hot plate. Set the hot plate to MAX.
4. Add 500 µL of the Na_2SO_4-saturated KOH solution to each Eppendorf tube, ensuring that each sample is completely submerged.
5. Place samples in a boiling water bath for 30 minutes. Half way through, agitate the tubes until no pieces of tissue are visible.
6. Place tubes on ice and precipitate glycogen by adding 1 mL of 95% ethanol for 30 minutes.
7. Centrifuge tubes at 3000 rpm for 30 minutes.
8. Discard the supernatant and immediately dissolve the pellet in 1 mL of ddH_2O.
    a. Do NOT allow the pellet to dry

Part C: Glycogen Quantification

1. Turn on the hot water bath and set it to ~70 °C.
2. In a flat-bottom polystyrene 96-well microplate, pipette 50 uL aliquots of ddH_2O, glycogen standards and sample glycogen solutions in triplicate.
3. Add 150 µL of sulfuric acid to each well.
4. Quickly add 30 µL of 5% phenol to each well.
    a. Works best with a repeater pipette. Use a 5 mL CombiTip set to 150 µL for the sulfuric acid, and a 0.5 or 1.0 mL CombiTip set to 30 µL for phenol.
5. Cover plate in ParaFilm and place in a static hot water bath for 10 minutes.
6. Dry the microplate with KimWipes and measure absorbance using a microplate reader at 490 nm (1.0 seconds).
7. Calculate glycogen concentration (units: µmol of glycogen/g of tissue):

$$\frac{\mu\text{mol of glycogen}}{\text{g of tissue}} = \frac{A490}{k \times W \times MM_{Glycogen}}$$

Where:
A490 = Adjusted Absorbance at 490 nm (Sample Absorbance – Blank Absorbance)
k = slope of the standard curve (units μg^{-1})
W = mass of tissue sample used

$MM_{Glycogen}$ = molar mass of glycogen (666.5777 g/mol)

Materials

- Microplate reader
- Centrifuge
- 2.0 mL microcentrifuge tubes
- Flat bottom polystyrene 96-well microplate
- Glass plate, razor blade, tweezers
- Eppendorf tube rack
- Metal tray and hot plate
- Kettle
- Repeater pipette with 5.0 mL and 0.5 or 1.0 mL CombiTips (optional, but recommended)
- Static hot water bath

References

Masuko, T., Minami, A., Iwasaki, N., Majima, T., Nishimura, S.-I., & Lee, Y. C. (2005). Carbohydrate analysis by a phenol-sulfuric acid method in microplate format. *Analytical Biochemistry*, *339*(1), 69–72. https://doi.org/10.1016/j.ab.2004.12.001

Murray, Michael, "The Effect of Aerobic Exercise Training on Hepatic Glycogen Metabolism in Type 1 Diabetic Rats" (2015). *Electronic Thesis and Dissertation Repository*. 2726. https://ir.lib.uwo.ca/etd/2726

Appendix B: Multiple Low-dose Streptozotocin Induction

PURPOSE: To induce Type I diabetes in rats

MATERIALS: Gloves Lab Coat Streptozotocin (STZ) 5X Stock Citric Acid/Citrate Buffer - Anhydrous Citric Acid - Sodium Citrate Dihydrate - MilliQ Deionized Water 13M HCl 3 Falcon Tubes Sterile Filter

EQUIPMENT: Biological Safety Cabinet Weigh Scale pH Meter

PROCEDURE:

Preparing 5X Citric Acid/Citrate Buffer

1. For a pH 4.6 buffer at 765 mM (5X stock solution), in a beaker, Add:

   a. 13.8g Anhydrous Citric Acid (Sigma) or 15.1g Citric Acid Monohydrate b. 23.8g Sodium Citrate Dihydrate (Sigma), Mix into...

   c. 175mL of MilliQ water The pH should be at 4.6, Add HCl or NaOH to adjust (do not over-shoot pH)
2. Once the proper pH is obtained, add MilliQ water until you are close to the 200 ml mark (pH will move slightly). If satisfied with the pH, adjust volume in a 250 ml graduated cylinder and filter in a 0.2μm filter.
3. Store at room temperature. This is your 5X stock solution.

Making up Streptozotocin (STZ) for Injection *Animals should be pre-weighed prior to making up STZ to ensure accurate amounts of STZ to be prepared.

1. Using pre-made buffer, put 1 mL of buffer in a 50 mL Falcon Tube and add 4 mL of distilled water filtered through a 0.2μm syringe filter. Check the pH. This gives you a working concentration of 153 mM

2. The desired pH is between 4.5-4.7. Under the fume hood, add 1 drop at a time of concentrated HCl to the buffer, checking pH in between until desired pH is reached. 3. Once pH is reached, add 1 mL distilled water (sterile filtered through a 0.2μm syringe filter as before). If pH is below 4.5, restart. 4. Weigh out an appropriate amount of STZ for the number of animals (see calculations below) that will be injected in a 15-minute time frame. Ex. Rats will be injected at 20mg/kg, so for 10 animals at an ideal weight of 200g (avg. weight of rats to be injected), you will require a minimum of 40mg. 20mg/kg X 0.2kg = 4mg per animal
3. The amount of STZ weighed out should be more than the minimum as some solution will be lost in filtering. (4mg (per animal) X 12 rats = 48mg total (0.048g) 5. Dissolve the STZ into buffer (keeping in mind a comfortable injection volume). Shake to dissolve powder (approx. 1min). Sterile filter using a 0.2μm syringe filter. Ex. 48mg STZ ÷ 3 mL buffer = 16mg/mL solution 4mg ÷ 16mg/mL solution = 0.25mL 6. STZ is time dependent and must be used within 15 minutes
4. Injecting and Follow-Up of the Animals 1. Promptly inject each rat with the solution (intraperitoneal) at a dosage rate of 20mg/mL (in this example, 0.25mL). Do not use anymore STZ solution more than 15 minutes after it has been dissolved in the sodium citrate buffer. 2. Dispose of any container having come into contact with the STZ (in either powder or dissolved form) into a biohazardous waste receptacle. Dispose of needles into a sharps container. 3. Return injected rats to their cage. Record the date of STZ injection and add a biohazard label to the cage (leave biohazard label on cage for at least 3 days

following the last injection). 4. Repeat this procedure the following day. 5. Check blood glucose daily. Diabetes is achieved with two non-fasting blood glucose readings of >18 mmol Diabetes should be achieved after 5-8 injections (i.p. 20mg/kg).

REFERENCES: Low dose STZ induction protocol. Animal Models of Diabetic Complications Consortium AMDCC Protocols. 2003

O'Brien BA, Harmon B V, Cameron DP, Allan DJ. Beta-cell apoptosis is responsible for the development of IDDM in the multiple low-dose streptozotocin model. J Pathol 178: 176–181, 1996.

Melling CWJ, Grisé KN, Hasilo CP, Fier B, Milne KJ, Karmazyn M, Noble EG. A model of poorly controlled type 1 diabetes mellitus and its treatment with aerobic exercise training. Diabetes Metab 39: 226–235, 2013.

Appendix C: Insulin Pellet Implantation

Pellet implantation (for a rat):

1. Anesthetize the animal using the isoflurane machine by placing it in the induction chamber. Set isoflurane to 4-5% with an O2 flow rate of 1L/min. Open the stopcock valve so gas reaches the chamber. Keep in chamber until the animal is unconscious.
2. Remove the animal and place its nose in the nose cone, reduce the isoflurane to 3% to maintain the plane of anesthesia.
3. Shave the area where the pellet is to be implanted.
4. Using gauze (or a swab), apply 10% povidone-iodine solution to the skin, followed by 70% ethanol, to disinfect the site of insertion.
5. Hold the skin with forceps and make a subcutaneous incision.
6. Cleanse a 12g trocar with 10% povidone-iodine solution and insert it through the

puncture site at least 2 cm horizontally from the incision site.

7. Using forceps, briefly immerse the pellet in 10% povidone-iodine solution, rinse with saline and insert into the subcutaneous region.
8. Use 1 pellet for up to qthe first 350g of body weight.
9. Pinch the skin closed after the last pellet is inserted. Place a drop of 10 % povidone-iodine solution over the opening.
10. Close the incision by suturing.
11. Place the animal under a heat lamp and monitor until it recovers from anesthesia.
12. Record on the cage card that insulin pellets have been implanted.

Pellet removal:

1. Anesthetize the animal as described above for implantation.
2. Shave and palpate the area of implantation to locate pellets. Sterilize this area by applying 10% povidone-iodine solution followed by 70% ethanol.
3. Using a scalpel (or scissors), make an incision through the skin superficial to the location of the pellets.
4. Using forceps, remove the pellet. Some connective tissue may need to be cut away using scissors. Discard the pellet.
5. Close the incision by suturing.
6. Place the animal under a heat lamp and monitor until it recovers from anesthesia.
7. Record on the cage card that the pellets have been removed.

Appendix D: Hematoxylin & Eosin

- Alkaline Tap water substitute
  - 0.35g sodium bicarbonate
  - 2.0g magnesium sulphate
  - 100mL distilled water
- Harris Haematoxylin (prepared commercially)
- Acid Alcohol
  - 1% HCL – 5mL
  - 70% EtOH
    - 346.5 mL EtOH
    - 148.5 mL H_2O
- 1% Eosin Solution - stock

Procedure

1. Allow samples to dry for > 5 min
2. Place samples in Rapid Fixx for 1 minute
3. Hematoxylin for 20 min
4. Scott's Tap water substitute – sections should end up purple blue
   a. Rinse 5x
   b. Fill for 1 min
   c. Rinse 5x
5. Acid Alcohol 10 sec – sections should be reddish colour
6. Scott's Tap water - sections should end up purple blue
   a. Rinse 5x
   b. Fill for 1 min
   c. Rinse 5x
7. Eosin 3 min
8. Rinse with tap water 10-15 times to wash off surplus stain
9. Dehydrate
   a. 65% EtOH 2 min
   b. 80% EtOH 2 min
   c. 95% EtOH 2 min
   d. Anhydrous EtOH 2 min
   e. Xylenes 1 min
10. Mount with toluene-based mounting media

Appendix E: Animal Use Protocol Approval

PI :	Melling, Jamie
Protocol #	2018-063
Status :	Approved (w/o Stipulation)
Approved :	07/01/2018
Expires :	07/01/2022
Title :	The role of exercise on ameliorating the negative metabolic and cardiovascular effects of Type 1 diabetes.

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Protocol Introduction

The questions on this page activate specific sections within the AUP form.

Note that species selection is part of this introductory page

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Related Work Experience	Teaching Assistant Western University

Publications:

Santarossa, S., Lacasse, J., Larocque, J. *et al. #Orthorexia* on Instagram: a descriptive study exploring the online conversation and community using the Netlytic software. *Eat Weight Disord* **24,** 283–290 (2019).