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The Effects of Simulated Jet-lag and Running-Wheel Access on the Symptoms of Type-2 Diabetes in TallyHo/Jng J Mice

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1. Abstract:

Type 2 Diabetes mellitus (T2DM) is the most common form of human diabetes, accounting for about 90% of all cases and affecting about 27.85 million Americans. It is often accompanied by hyperglycemia and obesity. Diabetes has been linked to circadian rhythms (a 24-hour long daily biological clock) as it can affect critical clock genes and disruptions in the rhythm can lead to increased possibility of developing the disorder. Despite the connections between T2DM and the biological clock, there is still a gap of knowledge concerning how biological clock disruptions specifically affect body weight, blood glucose, and insulin levels in T2DM individuals or if there are ways to alleviate the clock stress that can lead to symptoms of T2DM. This study looked at the effects of voluntary exercise on common symptoms of diabetes, such as hyperglycemia and hyperinsulinemia, during jet lag. Two groups of five-week old C57BL/6J (B6; control) and TallyHo/JngJ (TH; diabetic) mice were kept in either a 12:12 LD cycle or a 6-hour advance simulated jet lag, and given standard chow and water *ad libitum*. Half of each strain received access to a running wheel while the other half were placed into a cage, which can monitor home cage locomotor activity, but without a running wheel. Weekly measurements of body weight, food and water consumption were recorded. In addition, a 12-hour fasting glucose tolerance test with 30, 60, and 120 min time-points was conducted every four weeks starting at age-week eight. Although diabetic animals seemed to benefit from access to a running wheel with increased insulin sensitivity, they did not show reduced severity of diabetic symptoms due to the exercise during the shifting conditions. Further research should explore other assets to alleviate circadian stressors.

2. Introduction

2.1 Type 2 Diabetes Mellitus

2.1.1 Understanding Type 2 Diabetes

Type 2 Diabetes mellitus (T2DM) is the most common form of human diabetes, accounting for about 90% of diabetes cases and affecting 27.85 million Americans (American Diabetes Association, 2014; Center for Disease Control and Prevention, 2014). According to the America Diabetes Association, T2DM costs the United States approximately 245 billion dollars a year in health care costs (2013). T2DM is a metabolic disorder that affects the way the body metabolizes sugar (glucose) by disrupting the production of insulin. In a normal human body, insulin is produced by the pancreas and aids in the utilization of glucose by the cells. In T2DM, the amount of insulin produced cannot keep up with the demands of the body due to decreased insulin sensitivity, thus blood glucose levels are elevated and eventually lead to other dysfunctions. This disorder is most often, but not always, accompanied by obesity and dyslipidemia (an abnormal amount of fat/cholesterol in the body), as well as long-term complications such as kidney damage and impaired cardiovascular function. T2DM is a polygenic trait in humans, meaning it is caused by a combination of genetics and risk factors like high calorie diets and reduced activity (Leahy, 2005).

The body has a tight regulation of blood glucose at a set point of about 90 mg/dL (5.0 mmol/L; Boyle & Zebriec, 2007). It keeps this regulation through an

elaborate negative feedback loop using the secretion of glucagon and insulin, secreted by the α and β-cells of the pancreas respectively. These hormones have complimentary functions: If blood glucose levels get too high, β cells release insulin until blood glucose is returned to a normal range; if blood glucose levels get too low, α cells then come into play, releasing glucagon which induces the breakdown of glycogen stores from the liver to glucose and replaces glucose in blood stream until returned to normal (Boyle & Zebriec, 2007). When the levels are returned to normal, secretion of the appropriate hormone ceases, connecting the negative feedback loop. This mechanism works to keep blood levels of glucose within a narrow range to avoid both hypoglycemia and hyperglycemia as both lead to adverse life threatening consequences (Knutson et al, 2007).

Both α and β-cells types lay within the islet of Langerhans, the hormone-producing tissue of the pancreas. In type 2 diabetes, the pancreatic β-cells have impairment in insulin secretion in response to glucose, a hallmark of the disease (Efendic & Ostenson, 1993). Research has shown that insulin response to a glucose challenge is biphasic: the acute phase happens during the first 10 min, followed by the late phase that persists until the glucose levels are returned to normal (Ostenson, 2001). In diabetic patients, the acute phase of insulin response is markedly reduced, while the late phase may be better preserved but is often delayed (Pigon et al., 1996; Ostenson, 2001). Additionally, diabetic patients present with decreased insulin sensitivity, a term used to refer to when higher amounts of insulin are needed to reduce blood glucose levels back to normoglycemia (Ostenson, 2001; Knutson et al., 2007). Therefore, doctors often use hyperinsulinaemia (or excessive

levels of insulin in the blood) as a marker for T2DM. This marks a big difference between type 1 and type 2 diabetes; insulin is present in TD2M but is rendered dysfunctional (Wojtaszewski & Richter, 1998) while in type 1 diabetes β-cells are attacked and destroyed as autoimmune response (Belle et al., 2011).

Type 2 diabetes can go untreated for a long time, as the hyperglycemia due to insulin resistance builds up gradually, rendering a 4-7 year delay in diagnosis (American Diabetes Association, 2014). During this delay period, patients are still at risk of developing complications from the initial hyperglycemia levels, although most classical symptoms of diabetes have not appeared yet. Classical symptoms of diabetes include: fatigue, increased thirst, headache, frequent urination, and blurred vision (American Diabetes Association, 2014). If hyperglycemia goes untreated, it can lead to the build up of ketone bodies as the body turns to fats as a source of energy instead of the carbohydrates; the high levels of ketone bodies produced by this switch can be toxic to the body, a condition called ketoacidosis. Ketoacidosis is usually hallmarked by a distinct fruity odor on breath, as acetone (a ketone) concentration rises. If left untreated, ketoacidosis leads to diabetic coma and can be fatal (American Diabetic Association, 2014).

2.1.2 Testing for Diabetic Symptoms

The prevalence of obesity and type 2 diabetes is increasing worldwide but particularly in the US (Mokdad et al., 2001). Given this rise in prevalence, medical professionals must have efficient ways to test for the disorder and develop effective

glycemic control programs for each patient. A few tests used to diagnose T2DM are: fasting plasma glucose (FPG), Random/Casual glucose test, and oral glucose tolerance test (OGTT). The FPG measures blood glucose levels after 8 hours of fasting. Typically, a number less than 100 mg/dL is considered within normal, while 100-125 mg/dL falls into the pre-diabetic range and anything above is considered full blown diabetes (American Diabetes Association, 2014). In addition, a random/casual glucose test may also be requested; this test involves a blood reading, taken at a non-specific time during the day, to assess for overall levels of glucose. If reading is above 200 mg/dl, the diagnosis is given as diabetes. In other cases, an OGTT can also be used to assess glucose tolerance, which refers to the ability to metabolize a particular amount of glucose and return blood sugar levels to baseline normoglycemia (Ostenson, 2001; Knutson et al., 2007). In clinical settings, glucose tolerance is assessed by the OGTT, which consists of ingesting a glucose solution and measuring glucose levels at frequent intervals for the next 2 hours. The glucose ingestion would be equivalent of eating a carbohydrate-rich meal, followed by a close monitor of the blood glucose levels, assessing for response over a pre-defined window of time. Thus, glucose tolerance depends on the ability of the pancreatic beta cells to release insulin both acutely (the first phase of insulin response, beta cell responsiveness) and in a sustained fashion (Ostenson, 2001; Boyle & Zebriec, 2007). It also depends on the ability of insulin to inhibit glucose production by the liver and promote glucose utilization by peripheral tissues, also known as insulin sensitivity (Knutson et al., 2007). The OGTT covers a variety of metabolic responses to a glucose challenge and can be efficiently used to diagnose

diabetes. The better the glucose clearance from the blood stream, the more efficient the response mechanism responsible for glucose homeostasis is. These tests are most commonly used and are vital in recognizing the needs of a patient when it comes to type 2 diabetes (American Diabetes Association, 2014).

2.1.3 Causes: Genetics or Environment?

If insulin secretion and sensitivity is impaired in type 2 diabetic patients, there must be underlying causes. There is a general consensus that the pathogenesis of type 2 diabetes has both strong genetic and environmental components (Hamman, 1992). So far, the genetic background has been characterized in only in a small number of patients. Velho and Froguel (1998) found at least five mutations in diabetic patients, which are autosomal dominant: mutations in the genes coding for glucokinase (MODY-2), hepatocyte nuclear factors (HNF)-4a, -1a and -1b (MODY-1, MODY-3 and MODY-5, respectively) and islet proliferation factor (IPF; MODY-4). The HNFs and IPF are transcription factors, which play a role in development and function of the pancreatic β cells, while glucokinase plays a role for cell as well as liver metabolism. Some other subgroups, about 5% of type 2 diabetes cases, consist of patients with latent autoimmune diabetes of the adult, or LADA (Zimmet, 1995), and diabetes secondary to rare genetic syndromes (Gerbitz et al. 1996). Together these identified genetic variants constitute about 15% of all type 2 diabetes cases; however, 70-85%, of the patients with TD2M appear to have a polygenic inheritance, meaning it entails many genes, which is then combined with

environmental factors, leading to the development of the disease via a stage of impaired glucose tolerance (Ostenson, 2001).

Environmental aspects that are often related to disease predisposition are obesity and low physical activity (Hamman 1992). Insulin resistance in obesity is manifested by an impaired ability of insulin to inhibit glucose output from the liver and to promote glucose uptake in fat and muscle (Saltiel and Kahn, 2001). These functional defects may result from impaired insulin signaling in target tissues as well as downregulation of the major insulin-responsive glucose transporter, GLUT4 (Ostenson, 2001). As part of a glucose transporter family, GLUT4 is found primarily in adipose tissues and striated muscle (skeletal and cardiac), while another glucose transporter GLUT2 is found on β-cells, vital for detection of blood glucose levels done by the β-cells (Stolarczyk et al., 2010). Exercise has been shown to upregulate expression of GLUT4 in skeletal muscle, but obesity is most often accompanied by low physical activity, another risk factor for the development of T2DM (Wojtaszewski & Richter, 1998).

As the research summarized above shows, a mix of genetic predisposition and lifestyle habits likely causes T2DM. The number of obese individuals worldwide has reached 2.1 billion, leading to an explosion of obesity-related health problems, often associated with increased morbidity and mortality (Li et al., 2005; Olshansky, 2005). As the epidemic continues, more and more factors are examined such as biological clock desynchrony and its effects on diabetes.

2.2 Circadian Rhythms

2.2.1 Description of Circadian Rhythms and the Master Biological Clock, the Suprachiasmatic Nucleus.

Biological clocks are the time keeping system in the body; they can regulate many daily biological rhythms even in the absence of environmental cues (Moore-Ede et al., 1982). Although these rhythms can last days, months, and even years, the term "circadian rhythm" is used for any rhythm that is set to 24 hours. The term circadian comes from the Latin terms: "circa", or about, and "diem" or day, to say circa diem, about a day. One may think that time is set by the earth's rotation around the sun, which lasts about a day but it is these biological structures that give our bodies a real sense of time. However, it is no surprise that the human body, among other organisms, have set themselves to respond to the light conditions and other aspects that make up their environment.

Jean-Jacques D'Ortous de Mairan, a very curious and bright French astronomer, intrigued by the daily opening and closing of the *Mimosa*, was the first one to observe that they could open and close at the same time every day in complete darkness, or without environmental cues (de Mairan, 1729). In 1935, Erwin Bünning, a German biologist, was the first to show that different circadian timing can be genetically inherited by crossing various strains of beans and demonstrating that the resulting period was an intermediate of the parental timing (as described in Bünning, 1973). Later on, considered one of the founders of the

modern field of chronobiology (the study of biological rhythms), Jürgen Aschoff promoted studies in humans where participants willingly spent days in caves or bunkers, isolated from the outside world to study the human circadian rhythm (Aschoff, 1965). In one experiment, Aschoff and Wever (1962) isolated individuals in an underground cavern below a Munich hospital, while in another, Siffre (1964) lived alone in a cavern for two months. Both experiments demonstrated that humans, much like other species, have the capability to express these "free-running" rhythms of about 24 hours. The field has since then expanded to even more species, which demonstrate these endogenous circadian systems (Pittendrigh and Minis, 1972).

To start off, a major pacemaker runs the biological clock. In mammals, the suprachiasmatic nucleus (SCN) was identified as the circadian pacemaker, while in other phyla the pineal gland is the major pacemaker; both are responsible for keeping time (Schulz and Steimer, 2009). The SCN was identified by lesion studies, which showed that without it, circadian rhythmicity was non-existent in a variety of physiological and behavioral assays such as hormone secretion, fluid drinking and locomotor activity (Moore and Eichler, 1972; Stephan and Zucker, 1972). This pacemaker is a small bilateral nucleus, separated by the third ventricle, located in the hypothalamus in the area above the optic chiasma, explaining its name (Shulz and Steimer, 2009). Explants of SCN, unlike other parts of the brain, have a rhythmicity that is autonomous and self-sustaining (Green & Gillette 1982, Groos & Hendriks 1982, Shibata et al. 1982). Furthermore, studies show that individual SCN neurons are autonomous and able to express their own circadian timing (Hastings

et al., 2003), therefore partial lesions of the SCN show little to no effect on the pacemaker. To further classify SCN as the master biological clock, neural grafts of SCN tissue can restore circadian rhythms to arrhythmic animals, whose own nucleus had been ablated or injured (DeCoursey, 1986). Once the SCN is replaced in the host, the restored rhythms always exhibited the period of the donor genotype regardless of the direction of the transplant or genotype of the new animal (Ralph et al., 1990).

The SCN extorts influence on many areas and as such it is a well-connected area of the brain. First, it receives information from photoreceptors (cones and rods), as well as from special non-image-forming photoreceptors in the retina that connect, via the retinohypothalamic tract (RHT), directly to the SCN, providing photic information (Dibner et al., 2010). The RHT is made up intrinsically photosensitive ganglion cells (ipRGC), which contain the photo pigment melanopsin. This pathway is essential as it perceives and input information on light-dark cycles in the environment, directly synchronizing the clock (Dibner et al., 2010). These outside environmental cues that can synchronize the clock are referred to as zeitgebers, a German term keyed by Aschoff meaning “time-givers”. The main zeitgebers considered on earth is the sun, although other non-photic zeitgebers also exist independent of lighting conditions, such as food intake (Stephan et al., 1979), social calendars (Wittman et al., 2006), and even magnetic fields (Bliss and Heppner, 1976). On a normal day, the clock can entrain to days and nights, given that behavioral and physiological events happen at the same time of the day. Like an old clock, it regularly resets to adapt to new cues (Schulz and Steimer, 2009).

The SCN also has many output pathways to the brain and parts of the body, transmitting information through neurochemicals via neuronal connections (Dibner et al., 2010) and allowing it to control many physiological and behavioral aspects of the rhythm. The circadian system's ability to synchronize, often referred to in chronobiology as entrain, to outside cues leads it to prepare the organism to respond to environmental changes, such as lighting conditions, thus giving it an evolutionary advantage; failure to do so has been implicated in disease disposition (Schulz and Steimer, 2009).

When environmental cues are not present, the clock is said to be free running and the rhythm established is the free-running rhythm. The free-running rhythm is usually slightly shorter or longer then the entrained 24-hour rhythm (Schulz and Steimer, 2009). The term "period" is usually used to describe how long one cycle is. Therefore, free from environmental clues, the circadian period of a fruit fly, for example, can range usually between 23.6 and 24.5 hours (Peschel & Helfrich-Förster, 2011). Most studies examining the free running rhythm are done in constant darkness (DD), as a good measure of how these clocks are endogenous rather than a passive response to their environment.

2.2.2 Molecular Aspects of the Circadian Clock

Although the inheritability of the circadian clock has been demonstrated in plants (Bünning, 1969), *Drosophila* (Rensing et al., 1968), and in mammals, it wasn't until recently that the molecular biology of the circadian clock was fully understood

(Reppert and Weaver, 2002; Schibler, 2009). The molecular clock is made up of a combination of interlocking positive and negative feedback loops, interacting at both transcriptional and translational levels. Members of the basic helix-loop-helix "Period Arnt Single-Minded" (PAS) transcription factor family make up the positive feedback loop: CLOCK (*Circadian Locomotor Output Cycles Kaput*) and Bmal1 (*Brain and muscle aryl hydrocarbon receptor nuclear translocator (ARNT)-like 1) (*Ko and Takahashi, 2006). Through PAS protein-protein interactions, CLOCK and BMAL1 heterodimerize and after entering the nucleus initiate transcription of target genes containing E-box *cis*-regulatory enhancer sequences which include *Period* (*per1*, *per2*, and *per3*) and *Cryptochrome (Cry1* and *Cry2;* Ko and Takahashi, 2006; Lowrey and Takahashi, 2004). The negative feedback loop is caused by the PER and CRY proteins, which after being translated, heterodimerize and enter back into the nucleus, accumulating to inhibit their own transcription by acting on the CLOCK:BMAL1 complex (Ko and Takahashi, 2006). The progressive degradation of the PER:CRY complex during the night eventually releases the CLOCK:BMAL1 complex by the morning, starting the cycle anew (Zhang and Kay, 2010).

Another regulatory transcriptional-translational feedback loop is also involved in the molecular clock mechanism. CLOCK:BMAL1 heterodimers also activate the transcription of nuclear receptors RORα and REV-ERBα, which subsequently bind to retinoic acid related orphan receptor response elements (ROREs), which regulate *Bmal1* expression (Ko and Takahashi, 2006). Studies show that RORα activate its expression, while REV-ERBα repress it, thus regulating the proper time for BMAL1 to heterodimerize with CLOCK and initiate transcription of

Per and *Cry* (Ko and Takahashi, 2006). Post-translation modifications by the enzymes Casein kinase 1 epsilon and Casein kinase 1 delta (CK ε and CK1δ), aid in protein turnover and circadian timing as mutations in CK ε and CK1δ alter kinase activity and lead to shorter circadian periods in mammals (Lowrey and Takahashi, 2004; Xu et al., 2005).

Together, these proteins and genes work to together to form a time-related homeostasis, translating time-of-day information into physiological signals via transcriptional control of clock target genes (Zhang and Kay 2010). A schematic review of these transcriptional-translational feedback loops is given in Figure 1.

2.2.3 Peripheral Oscillators

There is hierarchical nature to the mammalian circadian clock network. Genes responsible for the molecular clock are expressed in several tissues throughout the human body outside of the SCN, such as other brain areas (Abe et al., 2002), the retina (Tosini and Menaker, 1996), and many peripheral tissues (Balsalobre et al., 1998; Yamazaki et al., 2000; Damiola et al, 2000), suggesting the existence of circadian clocks in other tissues. Cultured retina exhibit a circadian rhythm in melatonin synthesis, which is kept even under constant darkness conditions, meeting the conditions of an autonomous clock. In fact, the circadian clock system within the retina was the first peripheral clock to be identified (Tosini and Menaker, 1996). Another research group used PERIOD2::LUCIFERASE to elucidate present and persistent circadian oscillations within mouse peripheral tissues (Yoo et al., 2004).

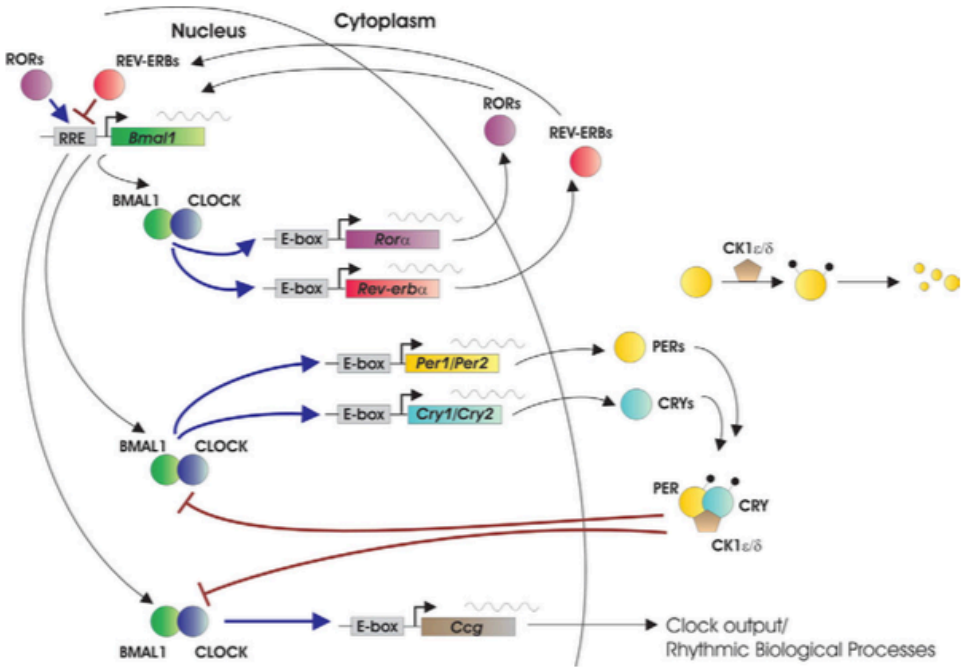

Figure 1. Schematic showing interlocking feedback loops of the molecular biological clock (From Ko and Takahashi, 2006).

Essentially, peripheral clocks have the same molecular clock make up as the SCN, generating self-sustaining, robust circadian rhythms.

These clocks can be coordinated from SCN by neuronal signals, hormones or metabolites, and even local peripheral clocks that are synchronized directly by the SCN (Dibner et al., 2010). Connections and inputs to and from SCN to peripheral clocks are shown in Figure 2. Research has shown that feeding cycles are the strongest Zeitgebers for peripheral clocks (Yamazaki et al, 2000). In addition, body temperature rhythms, influenced directly by the SCN, among other aspects directly controlled by the SCN, can play a role in synchronizing these clocks. Although peripheral clocks continue to run in SCN-lesioned mice, they are not longer synchronized and in-sync with one another (Yoo et al, 2004). So many areas of the body express these clocks, it is no surprise that deletion of these essential clock genes lead to changes in daily feeding, activity and other cycles, leading to detrimental effects to health (Lamia et al., 2008).

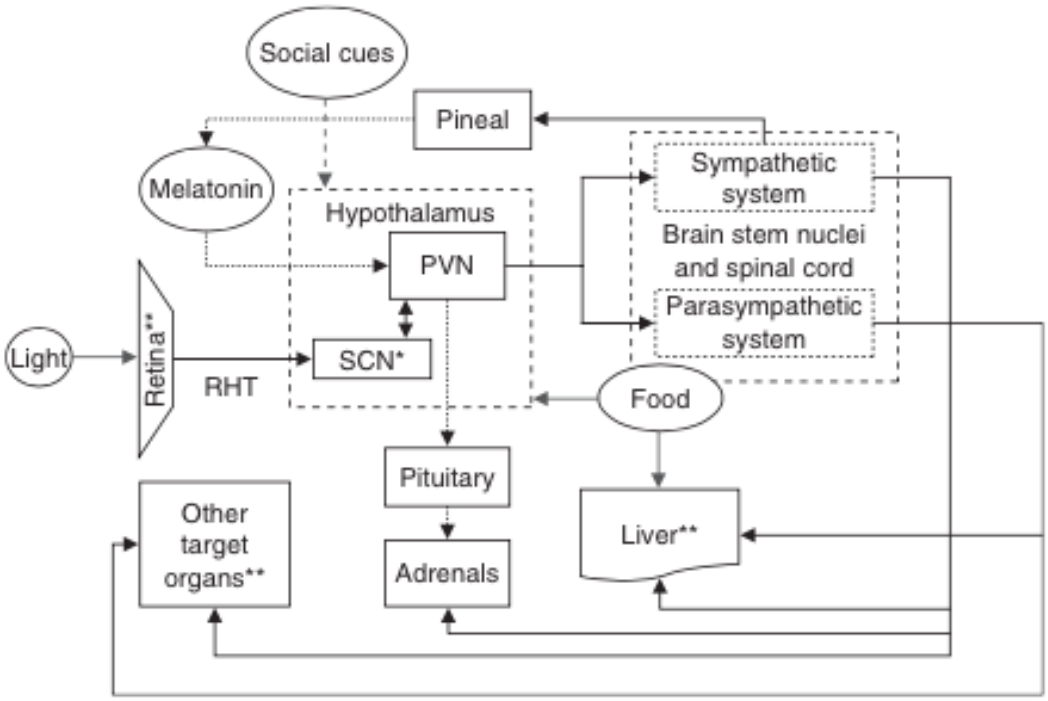

Figure 2. Schematic Diagram of SCN Inputs (from Schulz and Steimer, 2009). SCN is depicted with * and peripheral clocks with **.

2.3 Connecting the Dots: Circadian Disruptions and Diabetes

2.3.1 Sources of Circadian Disruptions

The endogenous circadian clock regulates 24-h rhythms that present themselves in activity patterns, heart rate, blood pressure, hormones and other biological activities. These rhythms respond to light and dark changes in the environment but are also entrainable by feeding patterns, among other factors (Schulz and Steimer, 2009). It is then easy to imagine that these rhythms can easily be disrupted. Main sources of circadian desynchrony are shift work, jet lag, and sleep disruption or sleep deprivation. Although, disruptions to the circadian rhythm can come in many forms, they have all been connected to health concerns as alterations have been shown to interrupt metabolic and endocrine functions, affecting body weight regulation as well as glucose/lipid homeostasis (Spiegel et al., 1999; Scheer et al., 2009).

Although adaptable, these biological clocks cannot stay in sync through ever changing patterns. In modern society, as individuals are continuously more connected, the pressure to be up on your feet and on the go is ever-present. In fact, studies show that Americans sleep less and less as the years go by. Starting in 1960, a survey study conducted by the American Cancer Society found that a typical night of sleep lasted about 8.0 to 8.9 hours (Kripke et al., 1979). However, a study conducted by the National Center for Health Statistics in 2005, reported that 30% of men and women between the ages of 30 and 64 say they sleep 6 hours or less per

night. It seems to be an on-going epidemic for not only American adults but all over the world, as sleep deprivation affects about 45% of adults worldwide (Bonnet and Arand, 1995). Interestingly, it does not take a long time for the effects of circadian misalignment to present itself physiologically. A human study demonstrated that even one-week of sleep deprivation (an average of 4 hours of sleep per night) in young, healthy subjects, can produce a pre-diabetic state (Van Cauter et al., 1997).

According to the 2004 data from the Bureau of Labor Statistics, almost 15 percent of Americans work full time on shift work, or jobs with irregular schedules. Shift workers constantly disturb their circadian rhythms by working odd hours and exhibit adverse health effects, such as poor sleeping quality, diabetes, and hypertension even in retired workers (Knutson et al., 2007; Guo et al, 2013). It also means that our most important personnel such as medical doctors and police officers are at risk of persistent poor health. Those involved in shiftwork routinely do worse on measures of cognition and work related fatigue (Machi et al., 2012). Although, the longer these workers had been out of work, the effects decreased as well indicating that shift workers could get rid of the effects caused by shift work and reduce the health damage after leaving a shift position (Guo et al, 2013). For retired shift-workers, the job mainly affected sleeping quality, but the impact of poor sleeping quality would last up to 20 years after terminating shift work (Guo et al, 2013). As studies have demonstrated that shift work can disrupt sleep patterns and thus other parts of the circadian rhythm, shift work can then be recognized as a risk factor for many health problems as it interrupts essential time-keeping system of the body (Knutson, 2007; Antunes et al., 2010).

Light-dark cycles are the most potent circadian zeitgeber. As part of a highly connected and modern society, it is completely normal to cross time zones and see a new piece of the world or to visit family. Individuals such as businessman and pilots who make constantly flying a part of their job, experiencing different time zones can be challenge. If their travel needs lead them far away from home, it would take them a couple of days for the internal clock to readjust - a phenomenon typically called jet lag. Some the symptoms of jet lag include increased fatigue, loss of concentration and motivation, and increased irritability (Herxheimer and Petrie, 2002). Due to the differences between the internal clock and the environment, the clock is slow to re-synchronize to the new schedule making jet lag also a source of circadian desynchrony. In fact, studies demonstrate that sleep deprivation or circadian desynchrony, regardless of the source, can lead to hypertension, cardiovascular disease, and a series of metabolic disorders such as type 2 diabetes (Spiegel et al., 2005).

2.3.2 Effects of Circadian Misalignment on Glucose Homeostasis

The body tightly regulates blood levels of glucose. Even during overnight sleep, glucose levels remain stable, especially when compared to glucose falls for individuals who are awake in a recumbent position (Van Cauter et al., 1997). This feat requires a very strict play between insulin and glucagon secretion to maintain blood glucose, or glucose homeostasis. Studies show that the SCN plays a role in daily rhythms of plasma glucose, insulin, and glucagon (Yamamoto et al., 1987).

Plasma glucose concentrations increase right before the beginning of the activity, a phenomenon known as the dawn phenomenon, and this bout in glucose also require higher insulin activity (Bolli et al., 1984). As insulin resistance is often considered a hallmark for T2DM, insulin sensitivity is often used as a measure of diabetes. Insulin resistance occurs when higher levels of insulin are needed to reduce blood glucose after the administration of the same amount of exogenous glucose (Spiegel et al., 2005; Knutson et al., 2007).

Studies show that both insulin sensitivity and β cell function are influenced by sleep (Spiegel et al., 2005). First, it could be due to the fact that insulin is released from the pancreas in a circadian rhythm (Peschke et al., 1998). The lack of a functioning circadian rhythm leads to altered insulin production, which is critical for glucose homeostasis (Sadacca et al., 2010). Clock $^{\Delta 19/\Delta 19}$ mutant mice exhibit decreased expression of genes involved in insulin signaling and glucose sensing (Marcheva et al., 2010), while ablation of suprachiasmatic nucleus, the master biological clock, impairs control of glucose homeostasis (la Fleur et al., 2001). In addition, *Bmal1* knockout mice show fasting hypoglycemia, reduced plasma insulin levels, as well as increased adiposity (Rudic et al., 2004; Marcheva et al., 2010). Both CLOCK and Bmal1 are essential components of the circadian clock, indicating the importance of rhythms in glucose homeostasis.

2.3 Closing the Gap: Daily Rhythms and Symptoms of Diabetes

Physiological and behavioral alterations to the circadian rhythm or the biological clock as a whole (e.g., shift-work or jet-lag) have been have been linked to increases in appetite and weight gain, as well as an increased possibility of developing T2DM (Boden et al., 1999; Kawakami et al., 2004; Morikawa et al., 2005; Shi et al., 2013). Evidence has shown that diabetes and the biological clock are linked as diabetes can affect sleep cycles and genes that regulate the circadian rhythm (Rudic et al., 2004; Marcheva et al., 2010). Conversely, having an altered or absent clock can lead to increased possibility of developing the disorder (Morikawa et al., 2005; Shi et al., 2013). As a polygenic trait, environmental factors play a major role in the development of T2DM (Birkeland & Berg, 2001). Recently, there has been an increase in prevalence of obesity and diabetes around the world, which can have negative consequences for longevity and quality of life for all (Knutson, 2007). It is estimated that by 2030, an estimated 4.4% of the world's population will be afflicted, corresponding to 370 million diabetics (Wild et al., 2004). This epidemic has then sent the world searching for answers that might lie within the connection between daily rhythms and T2DM.

It is has been long established that obesity is a major risk factor for T2DM. Studies have shown that the feeding/fasting cycle is the main synchronizer of peripheral clocks such as the pancreas (Damiola et al., 2000). One possible pathway is through decreased regulation of leptin (an appetite suppressant), as leptin reductions have been detected during sleep deprivation studies (Sridhar and

Madhu, 1994). Leptin is a hormone released by the adipocytes; leptin is the major player in food intake regulation, providing information about energy status to regulatory centers in the hypothalamus (Ahima et al., 2000). Sleep loss alters the ability of these vital hormones to function properly, leading to the excessive caloric intake seen in diabetic individuals (Shridhar and Madhu, 1994). It could be that obesity leads to dysfunctions of adipocytes. Adipose cells synthesize proteins, called adipocytkines that are responsible for chronic inflammatory process that precedes insulin resistance and plays a fundamental role in physiopathological mechanisms that lead to obesity in T2DM (Martins et al., 2007). Sleep loss and alterations in leptin are closely correlated with alterations in lipoproteins (VLDL and HDL) typically found in insulin-resistance states (Schafer et al., 2002). A combination of lower levels of leptin, higher levels of ghrelin (the appetite stimulant) and increased intake of high caloric foods could lead to the weight gain seen in T2DM. It would also explain why weight gain seems to be a precursor for the disorder. A short duration or partial sleep deprivation (4 h of sleep per night for one week) can lead to impaired glucose tolerance, higher secretion of cortisol, and reduced leptin secretion in healthy individuals (Spiegel et al., 1999). It could be that these disruptions to leptin are the starting signal for the symptoms that follow. In a glimmer of hope, a study found that sleep deprivation contributes to the development of an insulin resistance but it can be partially reversed by physical activity (VanHelder et al., 1993).

Collectively, these studies suggest a role of sleep loss in the epidemic of obesity and diabetes (Figure 3). There is accumulating evidence that our most

valuable jobs (physicians, nurses, police officers) are also the most vulnerable to health concerns such as hypertension and metabolic disorders. It must be emphasized that significant sleep loss exists in one-third or more of normal adults, the effects are large and replicable and that similar effects can be produced in the laboratory with both animal models and humans (Bonnet and Arand, 1995). Appropriate sleep not only not only restores brain function but also modulates the metabolic, endocrine and cardiovascular systems (Trenell et al., 2007). But, still, despite the connections between T2DM and the biological clock, there is still a gap of knowledge concerning how biological clock disruptions specifically affect body weight, blood glucose, and insulin levels in T2DM individuals or if there are ways to alleviate the clock stress that can lead to symptoms of T2DM.

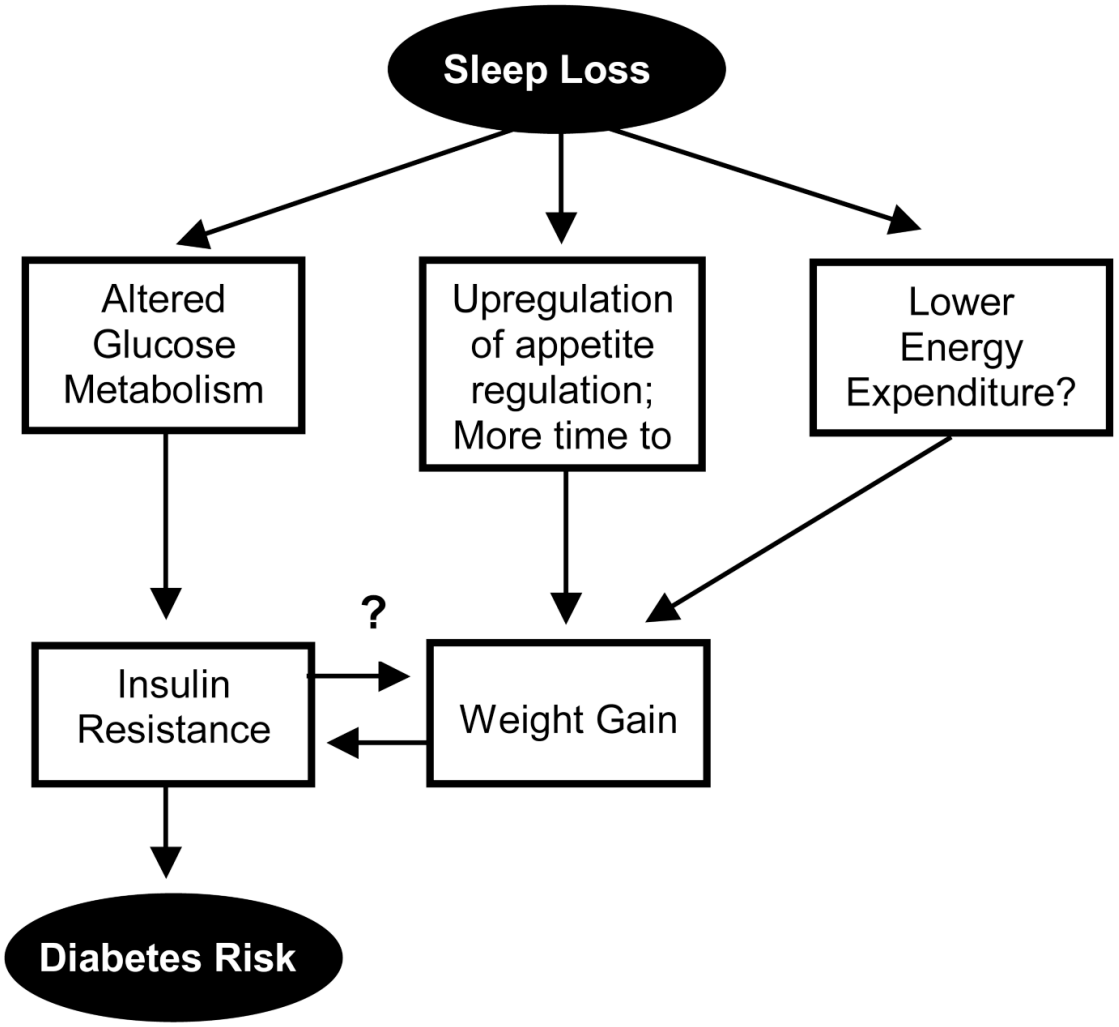

Figure 3. Summary of connections between sleep loss (regardless of source) to the increased risk of developing diabetes (from Knutson et al, 2008).

2.4 Study Design

This study will investigate the effects of voluntary exercise in the form of a running-wheel on diabetic symptoms (such as plasma glucose, glucose tolerance, insulin, and cholesterol levels) in a T2DM mouse model - the TALLYHO/Jngl (TH) and a control mouse (C57BL/6J (B6)). Male TH are genetically comparable to diabetic humans, developing diabetes at 10 weeks of age, showing increased levels of blood glucose and insulin (Figure 4; Kim et al., 2006; Stewart et al., 2010). The male TH line was selectively bred for 26 generations to develop hyperglycemia and in time exhibit extreme obesity, severe hyperinsulinema and high cholesterol; the first animal model to do so (Kim et al., 2001). The male TH mice show an enlargement of the Islets of Langerhans in the pancreas, the body part associated with insulin release, indicating the cause of hyperinsulinemia and decreased glucose tolerance (Kim et al., 2006). The symptoms described are unique to the male TH and do not hold true for the female TH, thus only male TH mice will be used for this study. In addition, traditionally only male mice are used in circadian studies as the estrous cycle may interfere with the observed locomotor activity rhythm. Thus, this mouse may be a good model for testing the effects of circadian disruption on obese T2DM.

The B6 mice are the most widely used inbred strain and was the first to have its genome sequenced (Chinwalla et al., 2002). They are also susceptible to obesity, type 2 diabetes and atherosclerosis, but not nearly to the degree of TH mice. In fact, studies have shown that B6 mice exhibit a mild T2DM, which is controlled if kept on a standard or low-fat chow (Parekh et al, 1998). Although not the background strain for the TH, B6 mice are frequently used as control in other studies (Sung et al., 2005; Kim et al., 2006; Stewart et al., 2010) and are included in this protocol.

Preliminary research done in our lab showed different glucose levels for the TH mice with access to running-wheels when compared to studies done in the past (Figure 5). We also saw a difference in body mass when compared to the other studies on TH mice (Figure 6; Kim et al., 2006; Stewart et al., 2010). The only differences between the studies performed in our lab and other studies using TH mice were that our study used single housing and access to a running-wheel. These results illustrate that individuals that are genetically predisposed to developing T2DM may be able to decrease symptoms such as hyperglycemia and obesity through moderate to intense voluntary exercise.

To test these effects, individual mice were housed in either running-wheel or non-running cages, both of which were connected to a computer to monitor their activity continuously. We then set lighting condition 12:12 with animals on and off the wheel, which is vital to establish how much of a change in diabetic symptoms occurs during running-wheel access. This set the stage for the interactions effects, which we then examined further by using another epoch in a simulated jet-lag condition. Circadian stressors such as jet lag and shift work are known to alter food cravings (increased intake of carbohydrates and preference; see section 2.4) so we simulated jet lag in the lab by giving a 6-hour advance to the animals. It is the equivalent of taking a flight from Boston to Munich, Germany, every four weeks, allowing us to not only look at the effects of running-wheel access on the diabetic symptoms during 12:12 light-dark conditions but also under stressful conditions such as a clock disruption through changing lighting conditions within the lab. The goal of this protocol is to uncover whether circadian stressors affect plasma glucose and insulin levels or produces increases in body weight or food consumption.

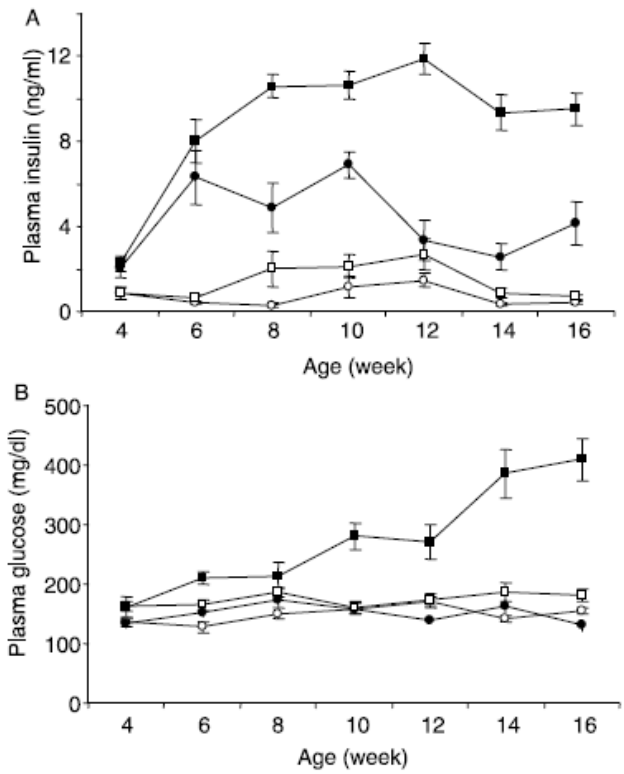

Figure 4. Changes in plasma levels of (A) insulin and (B) glucose in TallyHo and wild-type mice (B6) from 4 to 16 weeks of age. Male TallyHo mice show both increased insulin levels (A) starting at 8-weeks of age, and increased blood glucose (B) at 10-weeks old compared to wild-type mice. These symptoms mimic Type 2 diabetes. Open symbols represent wild-type (B6) and TallyHo, respectively. Squares and circles represent male (M) and female (F), respectively. (Kim et al., 2006

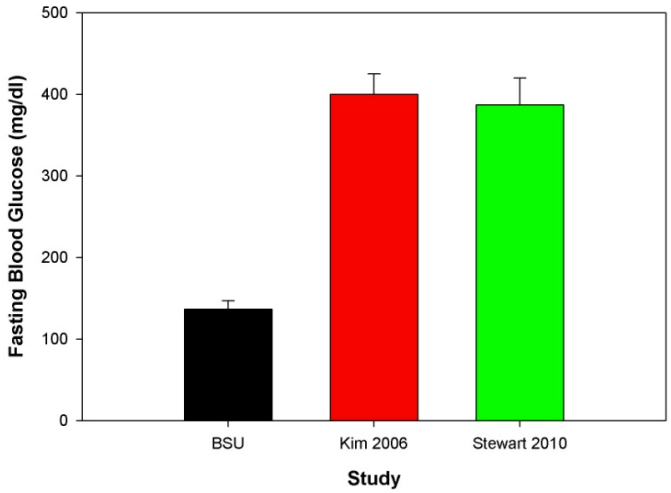

Figure 5. 4-hour fasting glucose level in 16-week old, male TH mice compared among two published studies and our study at BSU. TH mice given access to running-wheel exhibited a 200+ difference in blood glucose levels when compared to TH without access to running-wheels.

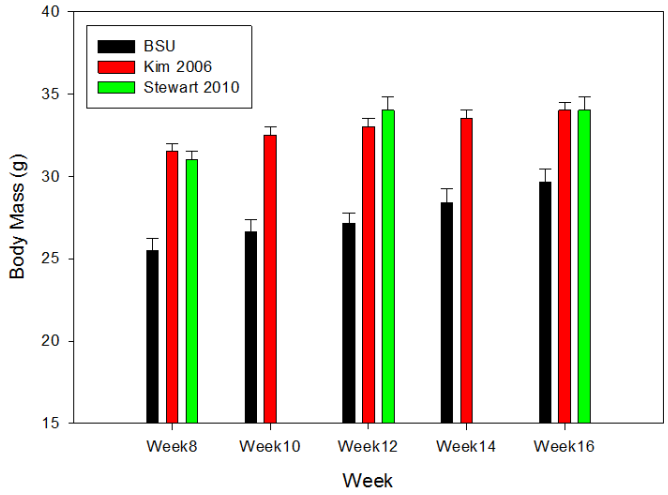

Figure 6. Body mass measure for 8-16 week old, TH male mice compared to two other studies: Kim et al., 2006 (gray) and Stewart 2010 (light) and our study at BSU (black). BSU TH mice given access to a running wheel exhibit a lower body mass when compared to other studies using TH mice.

2.4.1 Research Question & Experimental Predictions:

Our leading research questions were: how does jet lag affect the T2DM symptoms of the TH mice? And, can exercise alleviate the stress created by this clock desynchrony? Based on the preliminary results (Figures 5 and 6), we predicted the following: both diabetic and non-diabetic individuals with access to running-wheels will show a decrease in both blood sugar and insulin levels, as exercise is well known to alleviate the symptoms of diabetes; they would also demonstrate a better response to a glucose tolerance test through quicker uptake of glucose into cells, thereby decreasing blood glucose levels.

Our next question then assessed how exercise affects the way the mice respond to additional stress to their biological clock through simulated jet lag. We predicted that, as chronic stress produces increases in blood glucose and body mass, stress to the biological clock through simulated jet lag would produce an increase in diabetic symptoms. We also inferred that the T2DM individuals would show a larger increase in blood glucose and increased glucose intolerance than non-diabetic individuals in response to jet lag. And, since exercise is extremely beneficial in relieving stress, we predicted that all individuals given access to running-wheels most likely would be better able to cope with the stress of jet lag, being better able to resynchronize to a regular light-dark cycle.

3. Materials and Methods

3.1 Statement on Animal Care

All animal studies were carried out with the approval from the Bridgewater State University's Institutional Animal Care and Use Committee (IACUC; Appendix A).

3.2 Part 1: Circadian Assessment of TallyHo Mice

Eight five-week old male TH and B6 were individually housed in running-wheel cages (Starr Life Sciences, wheel diameter: 23 cm) and were fed standard chow (Lab Diet 5001) and water *ad libitum*. Running-wheel activity was monitored by the Vital View Data Acquisition System (Starr Life Sciences). Mice were initially maintained on a 12:12 light-dark (LD; ~50 lux) cycle for three weeks to assess stable, light-entrained rhythms. After the three-week entrainment period, animals were placed in constant darkness (DD) for the remainder of the experiment. Running-wheel activity data was then used to determine the free-running rhythm (using the chi-square periodogram in ClockLab (Actimetrics, Wilmette, IL)) pre- (aged-weeks 6-8) and post- (aged weeks 10-12) diabetes (the onset of diabetes was determined by an increase in blood glucose levels (see below) after week 10). The change in the free-running period and activity parameters were calculated by taking the difference between pre and post diabetes epoch. An activity bout analysis was conducted for

all four treatments to determine bout length, counts per bout, and bouts per day under each treatment in DD, as conducted by Nascimento et al., (2015). An activity bout was defined as being greater than or equal to the average size of activity counts throughout the activity phase, separated by at least 10 minutes of inactivity.

3.3 Part 2: The Effects of an Acute Circadian Disruption on the Symptoms of Type 2 Diabetes of the TallyHo/JngJ Mouse Model

Thirty-two male B6 and twenty-seven male TH mice, approximately five weeks of age, were house individually with standard chow and water provided *ad libitum*. Approximately half of each strain was placed individually in a cage with running wheel access (Starr Life Sciences, wheel diameter: 23 cm) and the other half was placed in a control housing which can track activity through infrared beam crosses (Starr Life Sciences); activity for both conditions was recorded using the Vital View Data Acquisition System. Then, half of each genotype was exposed to wheel or no wheel, were placed in two different lighting conditions: a 12:12 light-dark cycle (LD; 06:00-18:00) while the other half in was exposed to a simulated jet-lag condition (experimental) with six hour advances every four weeks, for a total of six shifts. These 6-hour advances were the equivalent to taking a flight from Boston to Munich, Germany, every four weeks. Thus, the eight groups were as follows: B6 wheel LD (N=9), B6 no-wheel LD (N=8), TH wheel LD (N=6), TH no-wheel LD (N=7), B6 wheel shift (N=7), B6 no-wheel shift (N=8), TH wheel shift (N=7), TH no-wheel

shift (N=7). Weekly measurements of food, water intake, and body mass were conducted for all mice in all conditions.

A 12-hour fasting glucose tolerance test (GTT) was conducted every four weeks starting at aged-week eight until aged-week 24. Blood glucose levels measured using One-Touch Ultra Glucose Meters (Rubenstein et al., 2013). Twelve hours prior to testing, food was removed, wheels were locked and animals were given new bedding to avoid possible influences from these sources to measurements. A baseline blood glucose measurement was taken prior to an intraperitoneal injection of 2-mg/g glucose per body weight (20% solution). After injection, blood glucose values were determined at 30, 60, and 120 minutes post-injection. Animals were then placed back into cages, wheels were unlocked and food was replaced.

At aged-week 32, 4-hour fasting serum insulin was measured using a mouse insulin sandwich Enzyme-Linked Immunosorbent Assay (ELISA; Ultra-Sensitive Mouse Insulin ELISA Kit, Crystal Chem, Downers Grove, IL). In addition, whole blood levels of total cholesterol, HDL cholesterol, and triglycerides were measured using the CardioChek system (Polymer Technology Systems Diagnostics, Indianapolis, IN).

Lipid panel, insulin ELISA, and GTT data were analyzed using a three-way ANOVA on Systat 12 (Systat, Chicago, IL), for a three-way interaction between genotype, exercise and lighting conditions. If three-way interaction was found, separate two way ANOVAs were used to further analyze two-way interactions between each variable. Differences were considered significant at $P < 0.05$.

4. Results

4.1 Part 1: Evaluation of Circadian and Activity Parameters of TallyHo mice

A paired t-test revealed that there is no significant change in the average free running period lengths pre- (23.72 h) and post- (23.75 h) the onset of diabetes (P=0.50). In addition, there are no significant differences (all p>0.10) between pre- and post-diabetic TH mice, regarding average wheel turns per day (Pre = 28.62, Post = 24.16), average counts per bout (Pre = 586.63, Post = 442.93), average time per bout (Pre = 46.49, Post = 40.36), and number of bouts per day (Pre = 8.30, Post = 8.67). TallyHo mice are able to successfully entrain to an LD cycle and show a robust circadian activity rhythm in DD (Figure 7).

When compared to B6 mice (49.36), TallyHo mice (28.62) exhibit significantly lower locomotor activity in terms of average of number of wheel turns per day (P=0.016). Additionally, TH mice, when compared to B6 mice, display more bouts per day (TH= 8.30, B6 = 5.11; P = 0.015), but significantly shorter average bout length (TH=46.49, B6 = 96.58; P<0.001) and reduced wheel turns per bout (TH = 586.63, B6 = 1402.72; P = 0.004; Figure 7). Thus, it appears that B6 mice have higher and more concentrated levels of activity compared to TH mice. There were no differences found between TH mice and B6 mice, regarding average free running circadian activity period in DD from weeks 8 through 10 (23.72 h, 23.72 h; P = 0.97).

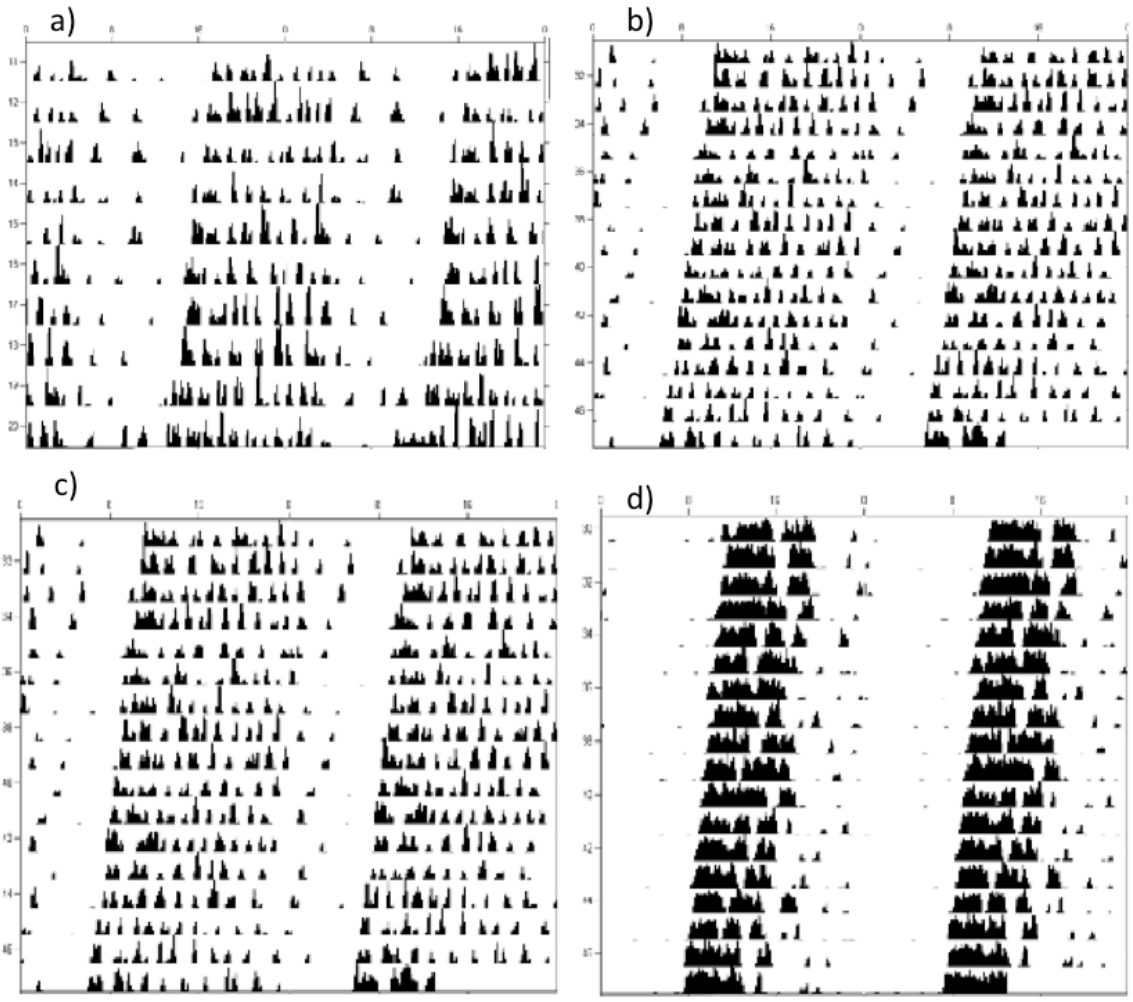

Figure 7 | Representative double-plotted actograms illustrating the free-running rhythms for TallyHo/JngJ mice. (a) Pre- and (b) post-diabetes behavioral circadian activity for TH mice. No differences were observed between in the behavioral circadian activity of the pre- and post- diabetes TH mice (P=0.5). (c) free -running activity of the diabetic TH and (d) B6. B6 mice show increased wheel turns per day (P=0.004) and increased bout length (P<0.001), but reduced bouts per day than TH mice (P=0.015). Y axis represents days and X axis represents time of day.

4.2 Part 2: Effects of Circadian Disruptions on the Diabetic Symptoms of the TH mice

4.2.1 Activity Parameters & Body Weight under LD & 6-hour Advance Conditions

Five-week old diabetic TH and B6 mice either on or off the wheel were exposed to either a control 12:12 LD or a 6-hour advance of the light cycle every four weeks, and activity was monitored under each condition (Figure 8; Figure 9). Both B6 and TH exhibited robust and stable rhythms under LD (Figure 8) and jet-lag (Figure 9), displaying an ability to entrain to their zeitgeber as previously shown in the preliminary assessment. We then assessed cage activity and a bout analysis revealed that under normal conditions, there are no differences in overall activity for between the B6 and TH mice ($F_{1,49}$=0.05, P=0.83; Table 1). As expected, all animals with access to a running wheel are significantly more active ($F_{1,49}$= 76.62, P<0.001). Examining light phase activity, we found no differences in LD ($F_{1,26}$=0.21, P=0.65) but during 6-h advance conditions, animals off the wheel are more active during the light regardless of exercise condition ($F_{1,23}$=21.04, P<0.001), a time when these nocturnal animals are supposed to be resting. With running-wheel access in LD, both B6 and TH mice have a higher proportion of light activity to total activity when compared to their counterparts off the wheel ($F_{1,26}$=6.40, P<0.001). In shifting, the wheel by type interaction is erased but B6 have a higher light activity to total activity proportion when compared to TH ($F_{1,23}$=4.63, P=0.042).

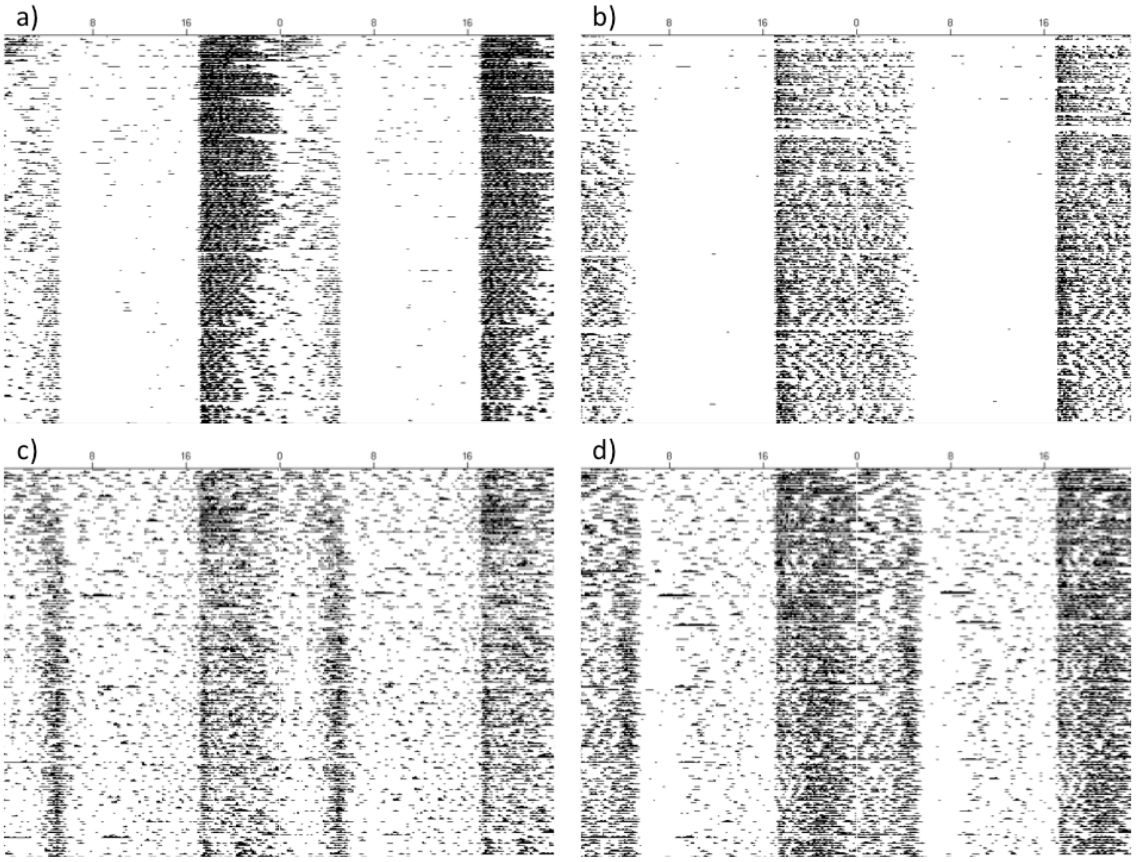

Figure 8 | Representative double-plotted actograms for B6-wheel (a), TH-wheel (b), B6-no wheel (c) and (d) TH-no wheel under a 12:12 Light-Dark cycle. All animals display a robust, zeitgeber-entrained circadian rhythm both on and off the wheel

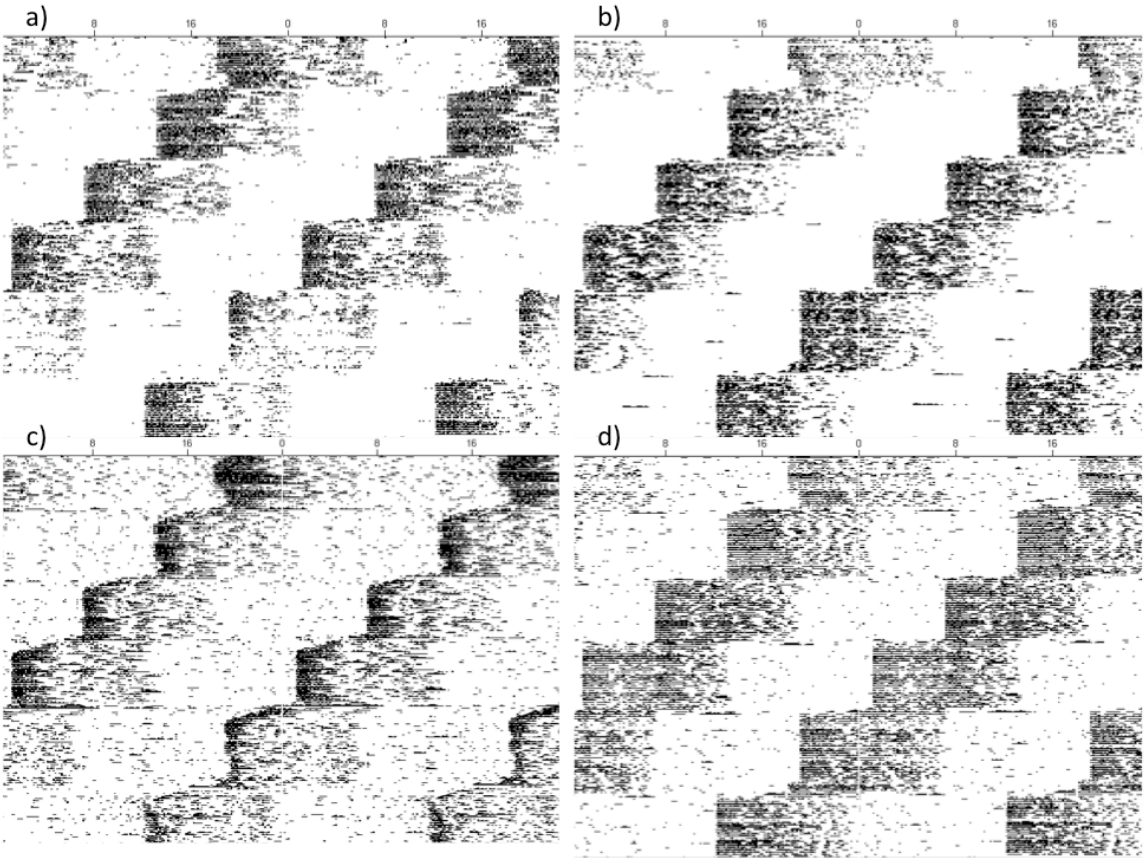

Figure 9 | Representative double-plotted actograms for animals in a 6-hour advance of the light cycle every four weeks. (a) B6 wheel, (b) TH-wheel, (c) B6- no wheel, (d) TH-no wheel. All animals display a robust, zeitgeber-entrained circadian rhythm, being able to follow the 6-hour advance introduced every 4 weeks, both on and off the wheel.

Genotype	Wheel Access	Lighting Condition	N	Activity per 10 min bin	Light Activity	Dark Activity	Light to Dark Activity Ratio	Activity Bout Length (min)	Counts per Bout	Bouts per Day
B6	Y	LD	9	43.45 ± 2.57^{a}	10.87 ± 0.77$_{a}$	76.87 ± 4.59^{a}	0.14 ± 0.01$_{a}$	67.86 ± 5.27^{a}	1072.66 ± 108.35^{a}	5.79 ± 0.29$_{a}$
TH	Y	LD	6	40.73 ± 4.82^{a}	9.53 ± 0.92$_{a}$	69.41 ± 10.06^{a}	0.15 ± 0.01$_{a}$	49.05 ± 4.02^{b}	703.60 ± 107.25^{b}	8.25 ± 0.56$_{b}$
B6	N	LD	8	19.03 ± 1.12^{b}	8.71 ± 0.73$_{a}$	29.84 ± 1.82^{b}	0.29 ± 0.02$_{b}$	40.38 ± 2.24^{c}	218.95 ± 17.09^{c}	11.49 ± 0.41$_{c}$
TH	N	LD	7	19.65 ± 3.60^{b}	10.74 ± 1.57$_{a}$	28.64 ± 6.00^{b}	0.41 ± 0.04$_{c}$	40.18 ± 4.63^{c}	246.30 ± 53.99^{c}	10.37 ± 0.51$_{c}$
B6	Y	Shift	7	38.55 ± 2.57^{a}	4.14 ± 0.37$_{b}$	65.02 ± 6.50^{a}	0.08 ± 0.01$_{d}$	47.20 ± 5.96^{b}	708.17 ± 123.05^{a}	7.47 ± 0.47$_{b}$
TH	Y	Shift	7	38.81 ± 5.08^{a}	2.54 ± 0.38$_{b}$	65.78 ± 7.81^{a}	0.05 ± 0.01$_{d}$	36.91 ± 3.60^{c}	566.25 ± 102.65^{b}	8.99 ± 0.41$_{b}$
B6	N	Shift	8	22.00 ± 2.12^{b}	8.31 ± 0.69$_{a}$	37.03 ± 3.83^{b}	0.25 ± 0.03$_{b}$	38.55 ± 3.58^{c}	267.22 ± 39.75^{c}	11.37 ± 0.37$_{c}$
TH	N	Shift	7	16.97 ± 1.36^{b}	4.65 ± 1.03$_{b}$	28.77 ± 2.80^{b}	0.17 ± 0.04$_{a}$	43.91 ± 4.13^{c}	218.12 ± 29.91^{c}	11.02 ± 0.81$_{c}$

Table 1 | Summary of activity profiles for all exercise and lighting conditions. Data given as mean (mean ± SEM). In LD, there are no differences in over all activity of the animals, although TH-wheel mice display a significantly higher number of bouts per day while B6 have longer activity bouts and counts per bout. During LD, there are no differences between genotypes off the wheel. However, in shifting conditions, animals off the wheel are more active during the light.

TH mice's characteristic "choppy" activity previously described as having significantly more bouts per day, decreased bout length and counts per bout, was still present in LD (Table 1). Not surprisingly, all animals on the running wheel have significantly longer bouts and counts per bout but less bouts per day (all $P<0.004$). However, bout length and counts per bout were higher in LD conditions (both $P<0.005$). Off the wheel, there was no interaction between light and exercise condition. All displayed lower activity parameters off the wheel that is most likely due to the exercise condition.

Although we did not expect TH mice to lose weight throughout the course of the experiment, after prolonged exposure to running wheel, TH-wheel in LD started to exhibit body weight changes due to voluntary exercise at aged week 24 (Figure 10a). B6 mice did not show the same trend, but were significantly lighter than TH mice. However, once exposed to shifting conditions, TH showed no benefit from the running-wheel access, as there was no significant weight difference between exercise conditions (Figure 10b).

4.2.2 Impact of LD and 6-hour Advances on Food & Water Intake

A three-way ANOVA followed by Tukey's pairwise comparison revealed that all animals with access to a running wheel drank significantly more water then their

counterparts off the wheel compared to body weight ($F_{1,52}$= 10.57, *P*=.002), with the diabetic TH mice drinking significantly more than the non-diabetic B6 mice ($F_{1, 52}$=

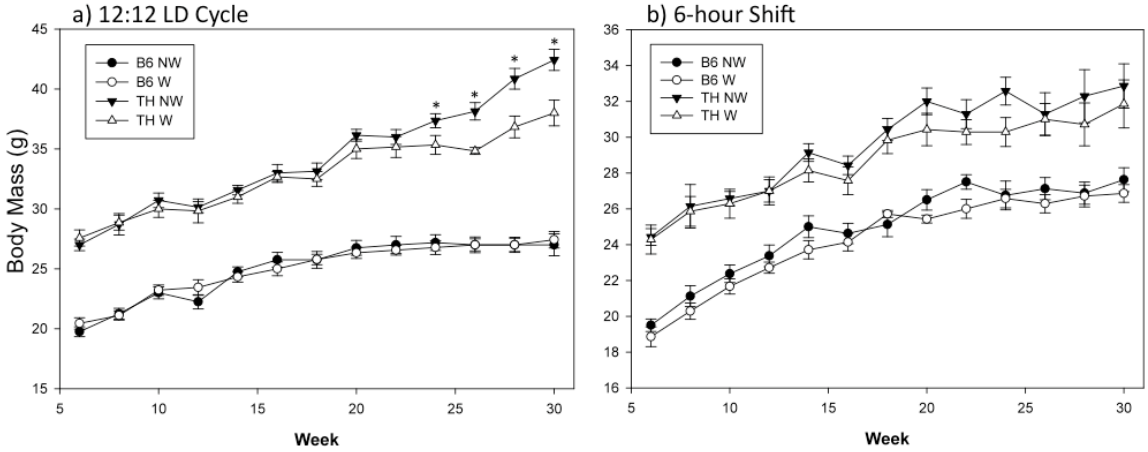

Figure 10 | Changes in Body Weight for C57BL/6J (B6) and TallyHo/JngJ (TH) from 6 to 30 weeks of age. Triangles and circles represent TH and B6, respectively. Open and filled symbols represent with access to a running wheel and no wheel, respectively. Data are means +/- SEM. **P*<.05 statistical significance for changes in body weight. Under normal LD conditions, TH mice exhibit reduced body weight at aged-week 24. No differences are detected under shifting conditions.

8.05, *P*= .006; Figure 11c & 11d). While TH mice drank significantly less during normal conditions when compared to being under a circadian stress ($F_{1, 52}$ = 3.86; *P*=0.01; Figure 11), the same was not true for the B6 ($F_{1, 52}$ = 3.86; *P*=0.98; Figure 11a & 11b). However, as expected, B6 mice with access to a running wheel had higher food consumption then B6 without a running wheel in LD ($F_{1, 27}$= 14.61, *P*=0.001; Figure 11), although the same did not stay true during shifting conditions (*P*=0.36). Unlike the B6, the wheel-TH mice had no difference in food consumption compared to the TH off the wheel in LD ($F_{1,24}$= 3.85; *P*=0.22). Both TH and B6 mice had lower food consumption per body weight during the LD cycle when compared to shifting ($F_{1,24}$=17.04; *P*<0.001; $F_{1,27}$=12.96; *P*=0.001, respectively).

4.2.3 Effects of Circadian Stress on the Blood Assays of the TallyHo mice

Glucose Tolerance Test (GTT)

Glucose tolerances was tested at weeks 8, 12, 16, 20 and 24, we analyzed outcomes using a three-way ANOVA and found that the TH mice with access to a running wheel exhibited better glucose clearance at least one time point in all but one GTT (Figure 12). All the animals followed a general trend, exhibiting high levels of glucose at the 30 minutes time point immediately after the glucose injection. At 60 minutes, there was a significant difference between the non-diabetic and the

diabetic mice with the TH mice still showing very high levels of glucose in the blood stream. At the two-hour time point, there was a significant difference between the TH-wheel and

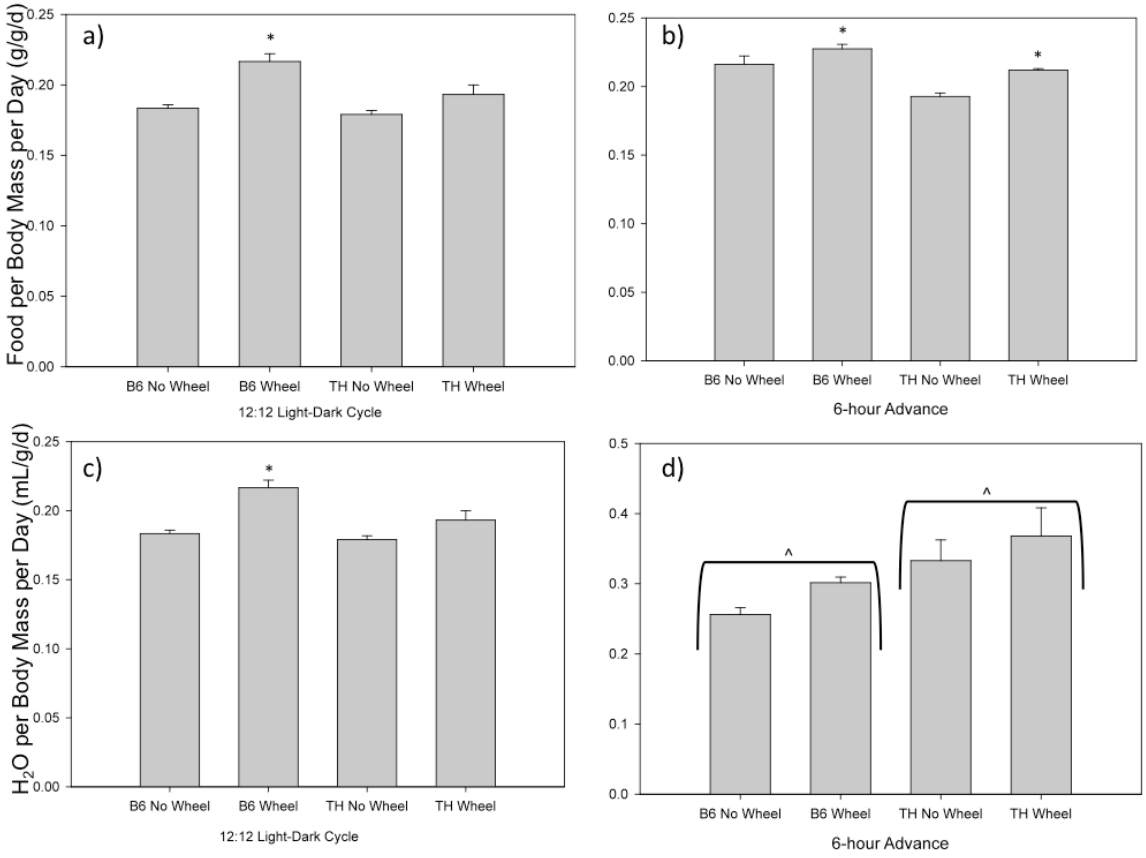

Figure 11 | Weekly fluid and water intake per body weight across all eight groups (mean ± SEM). B6 animals in normal conditions exhibited higher food and water intake when given access to a running wheel. In shifting conditions, TH mice also exhibit higher food consumption on the running-wheel (* P<.05). In LD, both genotypes drank significantly more when exposed to a running-wheel. In shifting conditions, TH ate significantly more than the non-diabetic controls (^ P<0.001).

the TH no wheel. The TH with no access to a running wheel were significantly higher than all other animals at weeks 8, 16, and 20 demonstrating that exercise was beneficial for glucose clearance in the TH mice (Figure 12). The B6 mice showed no difference in glucose tolerance at any time point if given a running wheel. When analyzed further to assess the GTT as whole, TH-wheel did significantly better than TH off the wheel at week 24, demonstrated by a smaller area under the curve (Figure 13). In the shifting conditions, we saw that the 6-hour advance closed the gap between the diabetic and non-diabetic animals (i.e. 8-weeks LD $P<0$; 6hr advanced $P=.06$). Although the general trend of elevations and drops in blood glucose are still there, there are no significant differences between the genotypes or exercise groups (Figure 13).

Insulin ELISA & Lipid Profiles

We further analyzed diabetic symptoms of the TH mouse with an insulin ELISA. We observed a three-way interaction between light condition, exercise groups and genotype (Figure 14). As expected, there was a significant gap between the non-diabetic B6 and diabetic TH mice, with B6 exhibiting lower levels of insulin overall, regardless of condition (all $P<0.001$). However, both B6 and TH had differences between LD and jet lag conditions. B6 off the wheel exhibited higher insulin levels in a simulated jet-lag when compared to a stable LD cycle ($F_{1,20}=0.57$; $P<0.002$), while TH show a dramatic decrease in insulin levels in the shift condition

($F_{1,20}$=27.37; *P*<0.009; Figure 14). There was a significant difference in insulin levels between non-running wheel TH mice and running-wheel TH in LD ($F_{1, 20}$=25.91, *P*<0.001). Interestingly, on running wheel, TH LD mice show the same insulin levels as TH in shift condition (*P*=0.63). Once again, B6 did not show any benefit or differences when given access to a running wheel, insulin levels were similar for both conditions (*P*=0.46).

We then assessed lipid profiles of each group, testing for total cholesterol, HDL, and triglycerides (Table 2). Similar to the insulin and glucose tolerance outcomes, TH mice with access to a running wheel also exhibited overall improved lipid profile in LD with reductions in total cholesterol ($F_{1, 21}$=8.54, *P*<0.008) and triglycerides (marginally significant at *P*=.077). However, the effect disappears during the 6-hour advance, with no differences in the exercise conditions. The B6 mice did not seem to benefit from the running wheels, once again showing no difference in lipid profiles between the running wheel and non-running wheel mice. B6 also did not seem to be affected by the exposure to the 6-hour advance condition.

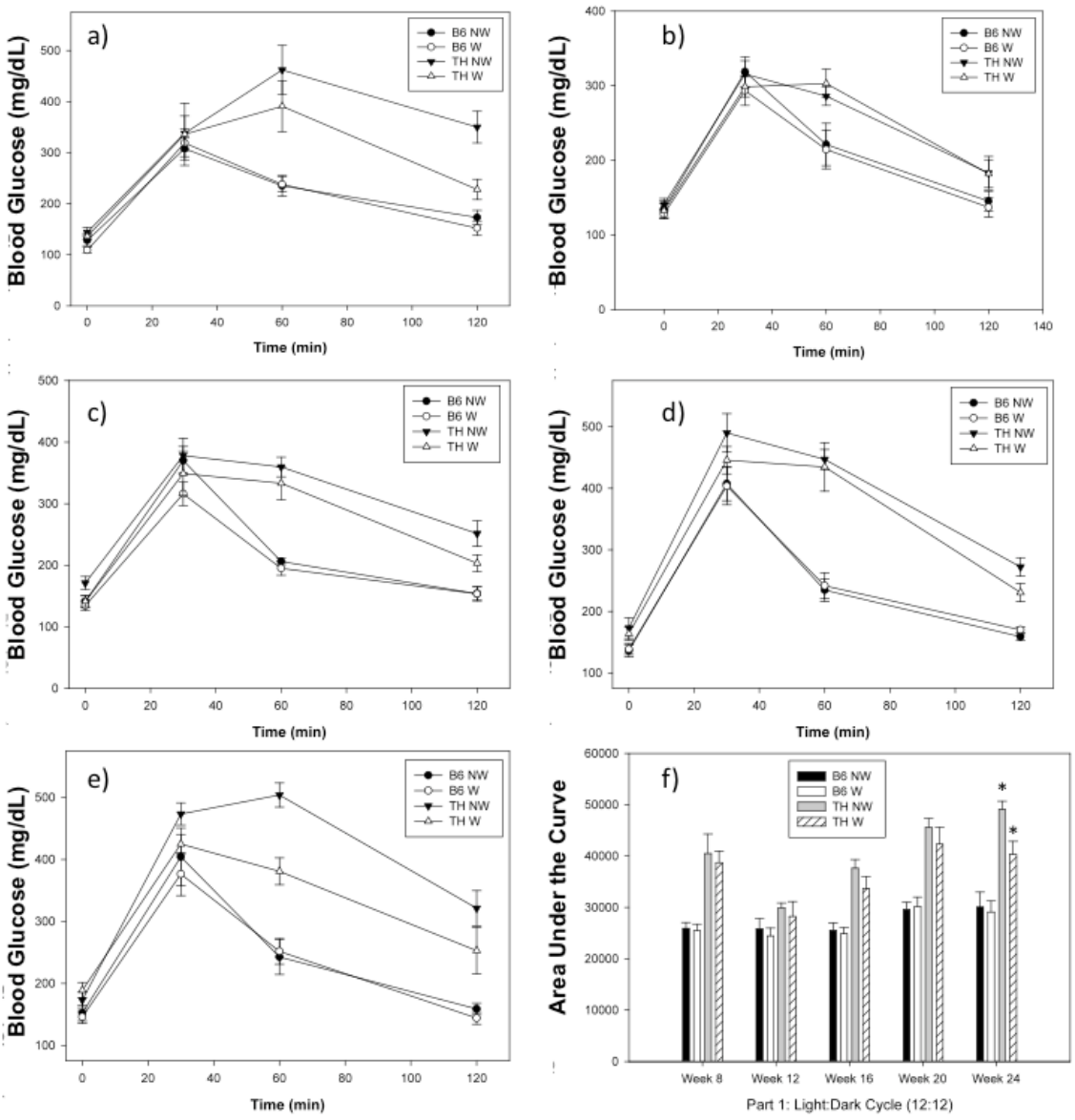

Figure 12 | The effects of exercise on Glucose Tolerance Test (GTT) in a stable 12:12 Light:Dark Cycle at aged-week 8 (a), 12 (b), 16 (c), 20 (d), and 24 (e) for both B6 and TH mice. Area under the curve (AUC) for each group was determined and analyzed using a thee-way analysis of variance (ANOVA). At all weeks, excepting Week 12, TH had significantly higher AUC and thus poorer glucose tolerance than B6, regardless of housing condition. Data are means +/- SEM. **P*<0.05 for the pairwise comparison between TH NW and TH W at Week 24 LD.

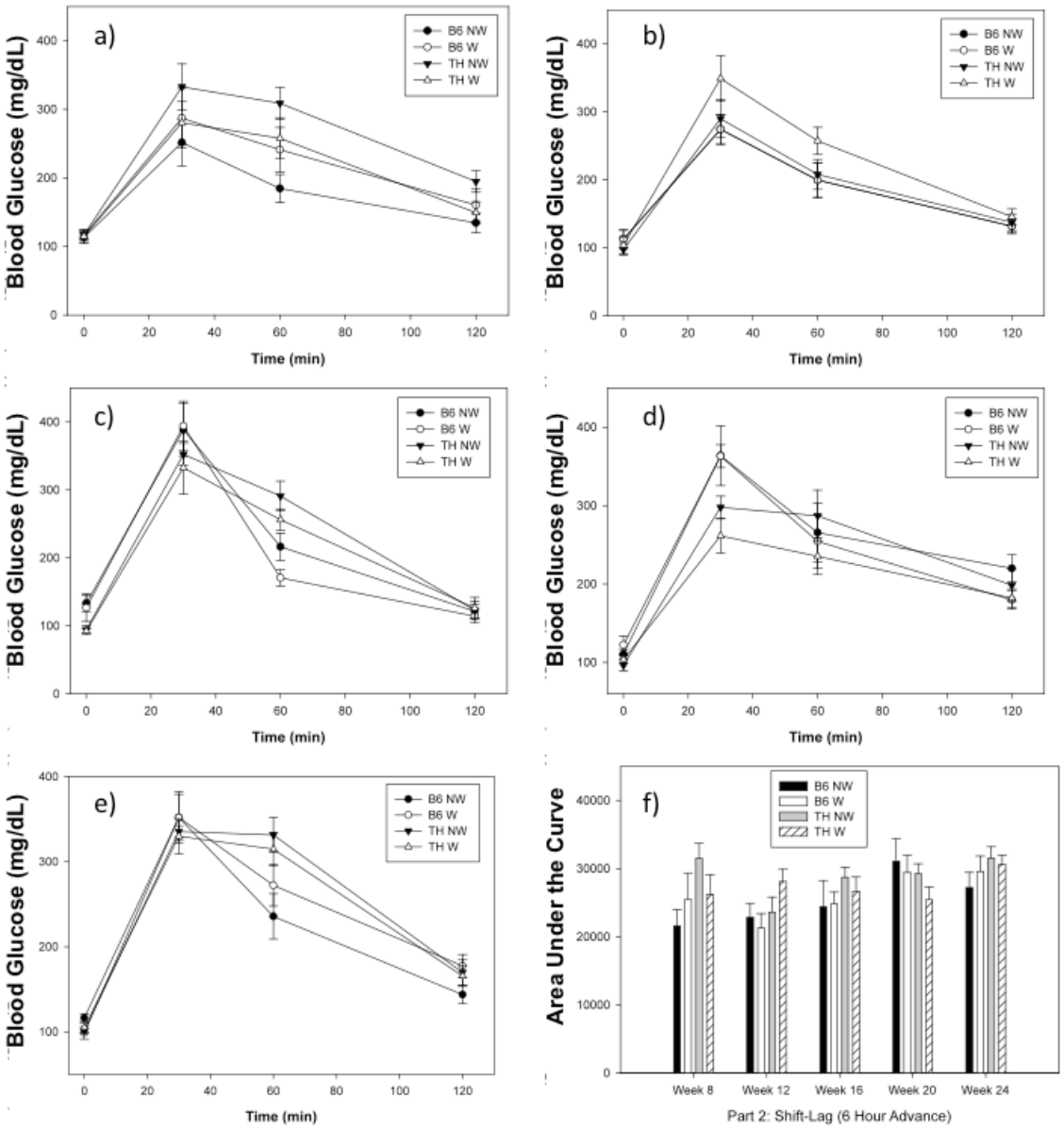

Figure 13 | The effects of a 6-hour light cycle advance every four weeks on the symptoms of diabetes of the TH mice. GTT at aged-week 8 (a), 12 (b), 16 (c), 20 (d) and 24 (e). TH mice under shifting conditions had reduced AUC, partly due to significantly lower Time 0 measurement, compared to TH under a 12:12 LD cycle. No differences were found between B6 in shifted conditions vs. LD. No significant differences were found among any of the Genotypes and Housing conditions during shifting conditions.

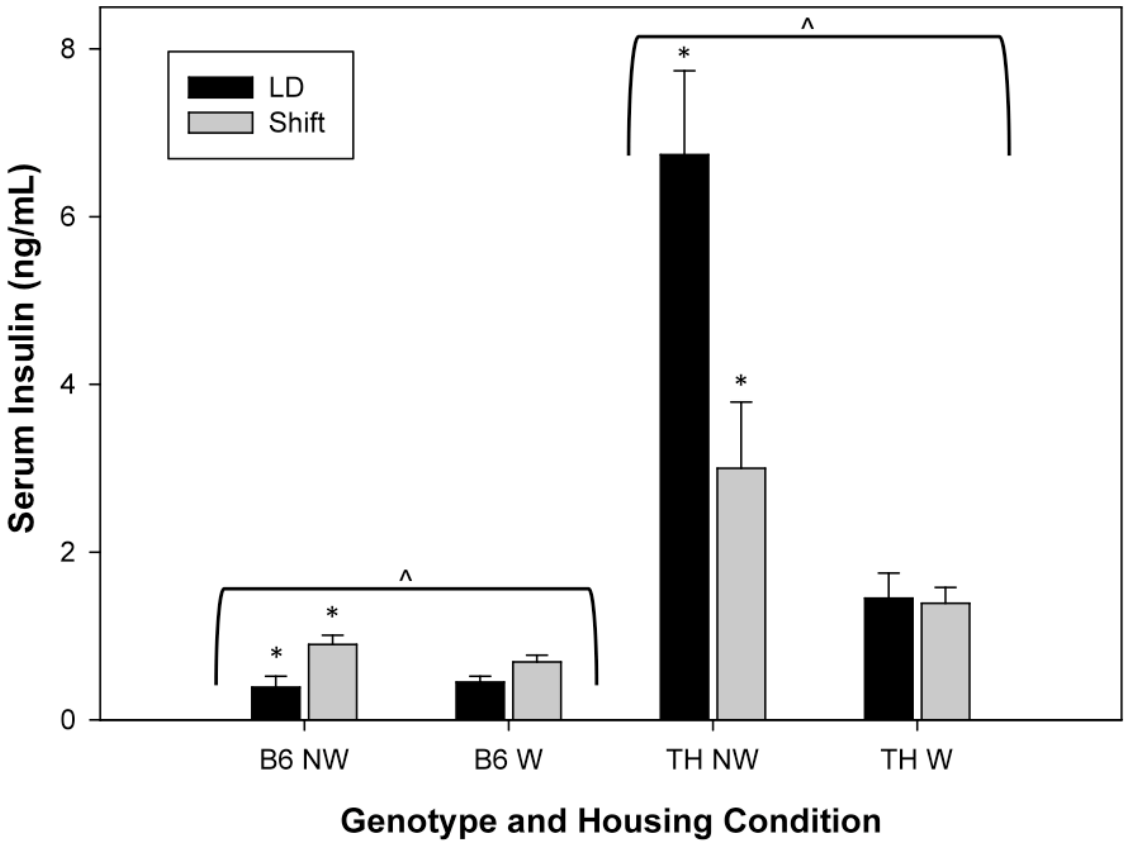

Figure 14 | Insulin Enzyme-Linked Immunosorbent Assay (ELISA) for insulin levels of TallyHo/JngJ (TH) and C57B6/J (B6). TH mice exhibited increased serum insulin levels compared to B6. In LD, TH mice on a wheel showed decreased insulin levels compared to TH housed without a wheel in LD, but not under shifted conditions. TH mice under shifted conditions exhibited significantly lower insulin levels compared to TH NW in LD, but not TH W LD. Lastly, in shifted B6 mice without a wheel increased insulin levels were found, but not in shifted B6 with a wheel. Data are means +/- SEM. * indicates significant difference in the pairwise comparison, ^ indicates a significant genotype difference, both $P < 0.05$

Genotype	Wheel Access	Lighting Condition	Total Cholesterol	HDL Cholesterol	Triglyceride
B6	N	LD	<100[a]	63 ± 5[a]	120 ± 10[a]
B6	Y	LD	<100[a]	55 ± 6[a]	123 ± 13[a]
TH	N	LD	175 ± 6[b]	>85[b]	469 ± 19[b]
TH	Y	LD	135 ± 7[c]	>85[b]	381 ± 21[c]
B6	N	Shift	<100[a]	48 ± 5[a]	116 ± 9[a]
B6	Y	Shift	<100[a]	56 ± 3[a]	145 ± 14[a]
TH	N	Shift	107 ± 5[c]	>85[b]	347 ± 39[c]
TH	Y	Shift	116 ± 4[c]	>85[b]	293 ± 53[c]

Table 2 | Lipid profile for 32-weeks old TallyHo/JngJ (TH) and C57B6/J (B6) mice under different exercise and lighting conditions. In LD, TH mice with access to a running wheel exhibit improved lipid profiles when compared to TH with no wheels, a result that disappears when TH mice are under simulated jet lag conditions. Different letters indicate a significance $P<0.05$.

5. Discussion

There are many studies, which have linked diabetes to the biological clock (Boden et al., 1999; Kawakami et al., 2004; Morikawa et al., 2005; Sadacca et al., 2010; Shi et al., 2013). In fact, a bidirectional relationship has been shown between diabetes and the clock, as having a dysfunctional clock or no clock at all can lead to an increased possibility of developing T2DM (Boden et al., 1999; Kawakami et al., 2004; Morikawa et al., 2005; Shi et al., 2013) and having diabetes can affect critical genes that regulate the circadian rhythm within the pancreas, such as "*clock*", "*bmall*" and "*period*" (Rudic et al., 2004; Marcheva et al., 2010). However, there is still a knowledge gap between the two. By using a T2DM mouse model (the TallyHo/JngJ mice (TH)), we sought to gain insight into how biological clock disruptions specifically affect body weight, blood glucose, and insulin levels in T2DM individuals or if there are ways to alleviate the clock stress that can lead to symptoms of T2DM. We report that although TH mice benefited from running-wheel access, by increasing glucose tolerance and reducing hyperinsulinemia, the same was not true under jet-lag conditions. Mice exposed to a 6-hour light cycle advance did not benefit from the access to a running-wheel. Furthermore, when a glucose tolerance test was performed one day after was implemented, the gap between the diabetic and non-diabetic mice was closed, indicating that both genotypes felt the effects of the circadian disruption.

It is the first time, to our knowledge, that the TH is utilized in a circadian study. We first characterized the behavioral circadian activity of the TH mice, which show robust and stable rhythms. Unlike the TH, the B6 is readily used in studies that

examine the biological clock (Schwartz & Zimmerman, 1990; Nascimento et al., 2015) but can also develop T2DM if fed a high fat diet (Parekh et al., 1998). However, despite having much different phenotypes for diabetic symptoms, there are no differences in behavioral free-running period and entrainment of the strains.

After the circadian assessment, it was not surprising to find that the TH mice were just as active on the running-wheel when compared to the B6 in terms of number of wheel-turns or home-cage activity per 10 min bin throughout the entire day. However, TH mice had significantly more bouts per day, each having decreased bout length and counts per bout, creating a "choppy" activity profile. On the other hand, B6 mice have significantly less bouts per day but bouts lasted longer, especially right in the beginning of the dark or activity phase for these nocturnal animals. It is possibly due to body mass differences, as TH mice are consistently heavier than B6 mice throughout the course of this experiment and others (Kim et al., 2006; Stewart et al., 2010), providing an extra physical challenge for the TH mice. Still, the TH mouse was developed on a different background strain (Swiss - SWR/J) than the B6 mouse (Kim et al., 2006); as the B6 mouse is considered a "high-locomotor activity" mouse strain (Chinwalla et al., 2002), differences in background strain cannot be ruled out as a possibility for the source of the activity level differences found between the two strains.

TH mice, after being exposed to housing with a wheel, started to show a significant decrease in body weight after 24 weeks, indicating prolonged exposure to voluntary exercise can help manage body weight for this mouse strain. The result came as surprise, since previous studies show increase in food consumption for

mice when they are exposed to a running-wheel, thus replacing calories lost (Swallow et al, 2001; Jung et al, 2010). When we examine food per body weight, we expected all mice with access to a running wheel to exhibit higher food per body weight. While B6 mice had both increased food and water intake on the wheel, the TH mice did not significantly increase their food intake, suggesting a possible explanation as to why we started to observe a difference between the exercise groups for the TH. Although that has been shown to be true in humans (Hamman, 2006), the same is not seen in most mouse strains (Swallow et al, 2001; Jung et al, 2010). B6 are notoriously good at balancing their energy expenditure and caloric intake, thus there seems to be something that is affecting the TH mice specifically. Increased fat deposits, a characteristic of the T2DM phenotype displayed by the TH mice, have been linked to decreases in dopamine signaling within the brain in humans (Wang et al., 2001) and dopaminergic system has also been liked to wheel running. Wheel-running for mice is seen as a rewarding activity, activating and increasing the dopaminergic system pathway (O'Dell et al., 2007) and is even used by mice in the wild (Meijer & Robbers, 2014). As TH mice show increased fat deposits as well as both insulin and leptin (Kim et al., 2006), which are inhibitors of dopamine (Palmiter, 2007), it could be that the body weight reduction observed in the current experiment can also be due to increased dopamine signaling in the brain, or a combination of low food consumption and dopamine signaling. Even with the decrease in body weight, TH mice are still considered obese (Kim et al., 2006), with a body weight significantly above the non-diabetic controls, but they seem to benefit from the wheel the most.

Another parameter often used to diagnose human diabetics is an oral glucose tolerance test (OGTT). We used the same approach to assess the severity of diabetic symptoms in each exercise group. We observed that under normal conditions, prolonged accessibility to a running-wheel may reduce diabetes symptoms in TH mice, as TH mice exhibited an improved glucose tolerance as indicated by a significantly smaller area under the curve at week 24. It is then important to point out that wheels are locked during the GTT, results are not due to wheel running during the extent of the test, but an accumulation of exercise prior to the GTT. An increase in insulin is commonly found in insulin resistant individuals, as the body makes failed attempts to restore blood glucose to normoglycemia (Weir et al., 2004; Leahy, 2005). As such, a decrease in insulin levels is a sign of decreased insulin resistance, as the body feels less compelled to make up for high levels of blood glucose. To test for insulin, we then also measured the levels of insulin between mice with and without access to a running-wheel, finding that mice on a wheel had decreased levels of insulin, suggesting an increase in insulin sensitivity or better overall functioning of insulin. Additionally, exercise has been shown to upregulate GLUT4, the major insulin-responsive glucose transporter of the skeletal muscle (Wojtaszewski et al., 2002), thus current results could indicate an increase in glucose transporters as a mediator for increased glucose sensitivity of the TH mice and reduced insulin levels.

When a lipid profile was built for all exercise and non-exercise forms for the LD conditions, we once again report improved profiles for TH with access to a running-wheel. Although dyslipidemia is common in T2DM individuals (Weir et al.,

2004), that was not seen with the TH-wheel thus indicating an improved functioning of fat metabolism. However, the same was not true for the B6 mice, which at no point seemed to benefit from access to a running-wheel in any of the blood assays. These results indicate that a prolonged exercise regimen, without increasing food intake, can provide alleviation of many parameters of obesity and type-2 diabetes (Hamman, 2006; Van Dijk, 2013).

When animals were put under a shifting condition, the data reported told a very different story. In all but one GTT, there was a type by light interaction driven entirely by the TH strain. To our surprise, TH mice under shifting conditions exhibited a significantly lower GTT, indicating better clearance during shifting conditions. This surprise was even more shocking in the context of human studies, which show that stress typically increases blood glucose levels in humans by increasing the stress hormone, epinephrine, which both increases glucose production and decreases glucose clearance (Rizza et al., 1979; Sham'oon et al., 1979; Rizza et al., 1980). One thought, is that TH mice could be clearing glucose better while under circadian stress but one day after the shift, TH mice show significantly lower baseline blood glucose levels indicating they start off lower then when in normal conditions. There are a few possibilities that come into play when considering these results, such as peripheral clock desynchrony and crashing of the animals.

First, there could desynchrony between the peripheral clock in the pancreas and the suprachiasmatic nucleus, the master biological clock. Aspects such as changes in feeding can lead to changes of the circadian pattern of gene expression in

the liver, but not in the master clock (Damiola et al. 2000), and thus have been shown to be a strong desynchronizer of peripheral clock. While TH mice also seemed to jump 'head first' into the new rhythm, as evident in the activity profile with most TH synchronizing within a day, we found that TH mice were more active in the light, a time of rest for these nocturnal animals. It could be that these animals were feeding during odd hours, desynchronizing peripheral clocks in the liver while still having a behavioral output from the SCN, which seemed to indicate a fast resynchronization (Damiola et al., 2000). In addition, the sudden jump in circadian synchronization could mean a very large difference in glucose homeostasis. Studies have shown that Bmal1 and Clock, core components of the molecular clock, contribute to regulation of glucose and hypoglycemic response to insulin (Rudic et al., 2004), and a circadian stressor such as a 6-hour advance could be significantly disrupting this homeostasis. If homeostasis is thrown off, our timing of tests could be exposing a part of the cycle that is expressed different during shift then when under normal conditions.

During GTT, insulin and lipid assays, the benefit of the running-wheel for the TH was erased. There were no significant differences when comparing exercise conditions, even after prolonged exposure to exercise. TH mice in shifted conditions exhibited lower insulin levels and lower glucose than TH in LD. However, another possibility is that we may have hit them at a time point during which their glucose levels were waning. Since we are catching these animals a day after the sudden shift in light-dark cycles, it could also be that there is increase in glucose during the day of the shift, as a stress response, and by the time we get to them, they are

experiencing a low point for glucose after the spike of insulin to clear the glucose and not way of replenishing glucose due to food being removed from cage.

Another surprising find was that the B6, which had almost been unaffected at this point, exhibited lower levels of insulin during the 6-hour shift condition when compared to the stable 12:12 LD cycle. This could indicate an increase in insulin resistance or the beginning of, for a mouse that is slightly diabetic. Other studies have shown that disrupting the clock by knocking out Bmal1 and Clock for example (Rudic et al., 2004), can lead to symptoms of diabetes. Insulin resistance is one of the hallmarks of and it could give us an insight as to why the 6-hour advance was able to close the gap between diabetic and non-diabetics.

Overall, the results indicate a benefit for the voluntary exercise for the TH mice, although that is less so under the effects of a circadian stressor such as jet lag. Although exercise has been shown to increase GLUT4, we were unable to measure this parameter in our current study. Further studies are warranted to look further into the molecular mechanisms of which exercise can alleviate diabetic symptoms. It would also be interesting to look at the effects of exercise on glucose tolerance past 24 weeks, which was when we truly starting seeing the effects of the accumulated time and access to the running-wheel, but were unable to continue due to diminished resources and man power. Furthermore, here we report that exercise can be beneficial under normal conditions but further research is warranted to examine strategies, which will elucidate how diabetic symptoms can be alleviated during circadian stressors as other studies have shown that shift-workers and consequent sleep deprivation can lead to a higher chance of developing diabetes.

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8. Appendix A

The Bridgewater State University IACUC reviews all requests to conduct research involving live vertebrate animals. This application must be completed, signed by the applicant and department chair, submitted to OGSP (Maxwell Library, Room 200), and approved by the IACUC before animal research can be conducted.

Title of Research Project

The Effects of Running Wheel Access, Ethanol, Diet, and Simulated Jet Lag on the Diabetic and Immune Phenotype of Mice Selected for Type II Diabetes or Diabetes Resistance

Contact Information

Principal Investigator (PI) and Primary Animal Care Giver:

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Additional Faculty and Staff Involved in Research:

Faculty: Dr. Kenneth Adams, Department of Biological Sciences, BSU employee
Dr. Karyn O'Connell, DVM, Visiting Lecturer Biological Sciences, BSU employee
BIOL 396/497 Research Students (2013-2015): Danielle Amaral (Holt), Karen Carlson, Nara Nascimento, Gina Nash, Dylon Pyne.

The above individuals will perform all the tasks described this protocol, have the necessary CITI IACUC training, and were taught by the PI to conduct all aspects of the protocol listed, without the necessary presence of the PI.

Students in BIOL 360 (Biological Clocks) and BIOL 482 (Neurobiology) will be able to perform these tasks provided they pass CITI Training for IACUC student, and will conduct these procedures only with the presence of the PI (Dr. Joseph Seggio). These students will not have access to the animal room (unless PI is present and lets them in, with proper training), and will not perform euthanasia on the mice.

Purpose of Application

Type II Diabetes mellitus (T2D) is a metabolic disorder that is characterized by high blood glucose in caused by insulin resistance, in contrast to Type I Diabetes Mellitus, where there is an absolute insulin deficiency due to destruction of Islet of Langerhans cells in the pancreas. T2D makes up about 90% of cases of diabetes and as of 2010 there are approximately 285 million people worldwide with the disease compared to around 30 million in 1985.

The purpose of this application is to test the effects of exercise (through running-wheel access), alcohol drinking, and circadian stressors (simulated jet-lag) on the plasma glucose and insulin levels in mice selected for T2D (TallyHo – TH) and other strains which are T2D resistant or controls. A preliminary study (IACUC Application: 2013-08) conducted in my lab has produced results that warrant future studies involving physiological processes in TH mice. First, we discovered that TH mice employed during our summer study have a significantly reduced body mass (3-5 grams) compared to mice from previous studies. The only differences are our mice are singly housed and have access to a running wheel. This study will first determine if TH mice housed singly in non-running-wheel cages, which can monitor home cage activity, do not show the reduced body mass as their running-wheel counterparts. Second, the decrease in body weight might correspond to a reduction in both plasma glucose and insulin levels, which are extremely high in the TH mouse strain. Third, we would like to determine if diabetic mice are more prone to the stress of chronobiological dysregulation (simulated Jet-Lag) in terms of higher glucose levels compared to control mice or mice who are T2D resistant. In addition, does access to a running wheel allow for the reduction of stressors and symptoms associated with jet-lag. Lastly, there are no studies available investigating the alcohol-drinking preference of TH mice; we would like to determine the effects of alcohol drinking on diabetes symptoms in these mice.

Test Organisms

1. Species: ***Mus musculus***
    - **a.** Common name (if applicable): **The following mouse strains will be used:**
        - **i. TallyHo/JngJ**

   ii. **A/J**
   iii. **C57BL6/J**
   iv. **Balb/cJ**
   b. Rationale for this species: **These mice are diabetes resistant or develop diabetes, or are control animals (see description below).**
2. Total number of animals: **8-12 per experimental group**
   a. Rationale for numbers of animals used: **8-12 can be considered a standard experimental group size, numbers needed to prevent loss of sample during the longitudinal study over the course of many months**
   b. Total number of animals on-campus at any one given time: **72**
3. Organism source: **Jackson Laboratories, Bar Harbor, ME, USA**
4. Are the animals on Bridgewater State College campus: **[X] yes** [ ] no
   a. Location of animals: **Conant Sci 160**
5. Dates of animal usage: **November 1, 2013 to October 31, 2015**

Housing

No juvenile rearing will be necessary, as all mice can be purchased with moderate funds. All mice employed in this study will be male. All mice will receive standard rodent chow (Lab Diet: Rodent 5001) unless noted below, and their drinking solution(s) ad libitum. If there is an experimental fluid condition, such ethanol, it can be given in a free-choice condition, where a second bottle of plain water will also be provided, or forced, where there will be only one bottle available (see Procedures). There will be two possible drinking solutions employed in this study: a) Plain Water or b) 10%-ethanol solution, presented in three possible conditions, i) water-only, ii) free-choice ethanol, or iii) forced ethanol.

Listed here are the 4 strains of mice employed in this study:

1. TALLYHO/JngJ (005314): TALLYHO mimics many characteristics of human non-insulin-dependent type 2 diabetes mellitus. Male TALLYHO mice can develop hyperglycemia, hyperinsulinemia, hyperlipidemia, moderate obesity, and enlargement of the islets of Langerhans. Onset of hyperglycemia is delayed compared to *ob*/*ob* (B6.V-*Lep*^{ob}) and *db*/*db* (BKS.Cg-*m* +/+ *Lepr*^{db}) mice beginning between 10 and 14 weeks of age. This mouse will be used as the type II diabetic mouse in this study.

2. A/J (000646): A/J inbred mice are commonly used to study resistances to metabolic and cardiovascular diseases. A/J mice fed an atherogenic diet (1.25% cholesterol, 0.5% cholic acid, and 15% fat) fail to develop atherosclerotic aortic lesions in contrast to several highly susceptible strains of mice (*e.g.* C57BL/6J). In addition to atherosclerosis resistance, A/J mice are resistant to diabetes, obesity, insulin resistance and glucose intolerance. On either chow or high fat diet, A/J mice maintain low glucose and insulin levels. This strain will be used to uncover how diabetes resistance mice respond to circadian stressors regarding their blood

glucose and insulin levels.

3. C57BL/6J (000664): C57BL/6J is the most widely used inbred strain and the first to have its genome sequenced. They are also susceptible to obesity, type 2 diabetes, and atherosclerosis, but not nearly to the degree exhibited to TallyHo mice. These mice also show high ethanol preference and high locomotor activity. This mouse will be used as a "moderate" phenotype, between the control mouse (Balb/cJ) and diabetic mouse (TallyHo).

4. BALB/C (000651 or 001026): BALB/C is a commonly used inbred mouse strain, normally used to study neurological disorders involving learning and memory and cancer research. This mouse is neither diabetic prone or diabetic resistant and will be used as the control for this protocol.

Individual mice will be housed in either running-wheel cages or non-running cages, both of which will be connected to a computer to monitor their activity continuously 24/7. The running wheel cage will monitor the number of wheel turns per day and act as a form of rewarding exercise. Mice placed into non-running wheel cages will have their home cage activity monitored via an infra-red (IR) beam. These mice will not have access to a running wheel or forms of exercise outside of movement in their cage. All mice will be placed onto Enrich-o-cob or Shepheard's Blend bedding. This bedding is a combination of 100% natural Bed-o'Cobs® corn cob bedding and twisted paper rolls (Alpha Twists). This bedding product provides enrichment as well as bottom-up absorption and superior odor control. Rodents unfurl and fluff the paper rolls then use them for burrowing and nesting. It also provides enrichment as the animals work diligently to separate the two types of bedding in the mix. It is non-dusty and free of paper fiber strands. Bedding will be changed every 6-7 days at random intervals so that the mice do not synchronize to the bed changing time. All mice will be placed into a 12 hour light and 12 hour dark cycle from 0600 to 1800. All mice will be purchased starting at 5- or 6-weeks of age.

Animal Welfare

Animals will be monitored for pain and distress through visual inspection of the mice and through monitoring their eating, drinking, and locomotor activity habits. Clear indicators of distress in mice can include: a reduction in eating (at least 4 grams per day) and drinking levels (3-6 ml per day), constituted by severe weight loss; premature hair loss; a reduction of wheel running activity; lethargy. Food and drinking solution consumption will be specifically measured approximately every 5-7 days, but a visual inspection of the bottles and food trays will be conducted daily. Weight and hair loss will be determined by visual inspection of the mice. Locomotor activity will be monitored continuously as a measure for the experiment – any reduction in activity will be quickly noticed. If an animal is determined to be in distress, he will be removed from the experiment immediately, placed into a clean cage with ample food/water, and monitored for several days. If the animal shows signs of improvement, he will be monitored more to determine if he has gained full

health. If the animal regains full health, he will remain alive in a separate cage in a separate room, especially if the animal was an experimental animal. If the animal was a water-only animal, he may or may not reenter the study, depending upon the lighting protocol being used and/or other parameters of the experiment at that particular time. If the animal does not recover, he will be euthanized as described below.

Enrichment will be provided in the form of a running wheel to some mice– rodents love running wheels. Social isolation is commonly used in circadian studies, as the individual locomotor activity is being monitored, and with two mice in one cage, individual monitoring cannot occur. All mice will be placed onto Enrich-o-cob or Shepheard's Blend bedding. This bedding is a combination of 100% natural Bed-o'Cobs® corn cob bedding and twisted paper rolls. This bedding product provides enrichment as well as bottom-up absorption and superior odor control. Rodents unfurl and fluff the paper rolls then use them for burrowing and nesting. It also provides enrichment as the animals work diligently to separate the two types of bedding in the mix. It is non-dusty and free of paper fiber strands.

Male mice can live for years in these isolated conditions, and have in some long term circadian studies. This monitoring will be especially vigilant upon the introduction of the alcohol or other solutions after approximately 6 weeks into the experiment (see below). After the induction of the increased carbohydrate solutions, we will monitor the mice for any shaking as well as a sharp increase or decrease in body weight (past the moderate obesity normally found in these mice). On rare occasions, some mice might develop pathogen based illnesses. Some of the most noticeable symptoms of pathogen based illness in mice include: nasal discharge, diareahea, blood in the bedding and/or skin rash or lesions. These animals will be immediately quarantined, removed from the study permanently, and will be euthanized as described below.

Proper blood collection methods will be utilized (see Procedures) and the investigators will limit the daily collection to no more than 1% of total blood (i.e., 0.25 ml or 250 µl from a 25 gram mouse) in a given day. The full allotment of 1% blood drawn will be used for the CardioChek and ELISA tests only. These procedures will be conducted in mice at 8-weeks old at least. A minimum 2-week recovery period is needed for the average healthy adult animal to recover from this blood loss. Although the blood volume is restored within 24 hours after blood withdrawal, a minimum of two weeks is needed for all constituents of the blood to return to normal. If less than the maximum amount of blood is withdrawn the animal will replace blood constituents at the rate of 1000 µl/1000g/day (or 1 ml/kg/day). During the experiments, we will be first conducting the submandibular blood draw on one day which will be used for the cholesterol testing and ELISA tests (to allow for only one draw and less stress). Then, glucose tolerance can be done on a different day of the same week (2-3 days later depending upon the size of the mouse, 25 µl blood replenishment per day for a 25 gram mouse) so that the mice will experience only one blood draw type per day.

A diabetic test strip takes approximately 5 µl of blood, which allows for multiple collections during a single day, but the investigators will limit it to 4 per day. If the full collection using diabetic test strips occurs, then the investigators will wait at least two days to repeat. Blood flow is stopped by applying finger pressure on a sterile gauze pad placed at the blood sampling site for approximately 10-20 seconds before the mouse is returned to its home cage. It is essential to be able to recognize the clinical signs of shock and anemia and to be able to take appropriate action.

Signs of shock include a fast and irregular pulse, pale dry mucous membranes, cold skin and extremities, restlessness, hyperventilation, and a sub-normal body temperature. This usually occurs if more than 10% of body blood was removed in one day (200 µl or more), which will not occur during this study. If more than 10% of the total blood volume has been removed unintentionally, a routine replacement with the same volume of warm (30-39 C) normal buffered saline (0.9% w/v) would constitute good animal care. Signs of anemia include pale mucous membranes of the conjunctiva or inside the mouth, pale tongue, gums, ears or footpads (non-pigmented animals), intolerance of exercise and, at the more extreme level, an increased respiratory rate when at rest. If the animal cannot be rescued, it will be euthanized as described below.

Procedure and Test Design (if applicable)

This protocol will involve blood collections of very small volumes (20 µl or less per day). Due to advances in blood biochemistry analysis, it is possible to analyze, for example glucose, insulin and drug levels, from small (5-20 ul) blood samples. The small sample volume allows serial samples to be collected from the same animal (rather than composite bleeds from several animals) and the use of satellite animals to be reduced or avoided, thereby reducing the total number of animals required for toxicokinetic and pharmacokinetic studies. The small sample volume also provides a refinement, because warming prior to sampling can be avoided.

The mouse is removed from home cage and placed on top of a work station. The mouse is gently restrained by either a tube rodent holder where the tail can be easily accessed or by the base of the tail. The mouse may be transferred to a wire cage top to reduce its movement. Inanimate restrainers can be used, although manual restraint reduces distress more effectively. As the investigators get more comfortable conducting small tail bleeds, the need for the restrainer will be reduced over time. Blood flow is stopped by applying finger pressure on a soft tissue placed at the blood sampling site for approximately 10-20 seconds before the mouse is returned to its home cage. All sterile supplies, including needles, test strips, and capillary tubes will be used only once per mouse, and disposed of properly in a biohazard container.

Please refer to the following video guide:
http://www.nc3rs.org.uk/bloodsamplingmicrosite/page.asp?id=1335

The following blood collection procedures will be conducted on all mice:

1. Blood Collection for Glucose Monitoring: The tip of the tail is pricked with a sterile 23-25 gauge needle or lancet. A 5 ul, sample obtained by capillary action into a test strip for reading. The average of two readings (10 µl total) will be used to reduce error. The initial needle prick should be as near to the tip of the tail as possible to avoid damage to the tail on repeated sampling. To obtain a 5 ul blood sample there is no need to pre warm the mouse. This test can be conducted on mice of any age (5-weeks and beyond).

2. Intraperitoneal Glucose Tolerance Test: This test is designed to determine how sensitive an individual is to spikes in their blood sugar after a fast, and it is common to perform this test on a human to test for diabetes (although usually conducted orally in humans). Mice will be fasted for 12 hours prior to the test. After the 12-hour fast, the mice will experience a baseline blood glucose reading as described above, which will correspond with time 0. The mice will then be injected intraperitoneally (IP) (10 ml/kg for each mouse) with 2 mg/g of glucose in PBS, using a 1 ml syringe. The mouse can be contained in a restraint device with holes for IP needle injections and to prevent mouse movement, which may cause injury to the mouse if it moves around too much. As the investigators gain experience in this method, they can avoid restraining the mouse by letting the mouse grab onto a wire cage top with its front paws, grab the skin cuff on the back of the neck with the forefinger and thumb, use their ring finger to hold the legs down, and their pinky to hold the tail. Blood glucose samples will be taken again 15 min and 60 min later, for a total of three 5 µl collections per mouse. The mice will be allowed to recover for at least 1 week before the next glucose tolerance test. This protocol will be conducted in mice aged over 8 weeks of age only; the reason being that mice younger than 8-weeks of age show no difference in a glucose tolerance test.

3. Blood Collection for ELISA and Cholesterol Tests: Unfortunately, the procedure previously described (Proposal 2014-03) was ineffective in acquiring sufficient blood necessary for the ELISA test. A new protocol using the Medipoint Golden Rod Lancets will be used. These devices allow for the safe removal of up to 400 µl of blood from the submandibular vein of a mouse. For this protocol, the Cholesterol and ELISA testing blood collection will occur at most once every four weeks in order to give the mice ample recovery time. Preparation with a sterile gauze may be conducted prior to using the Medipoint Golden Rod Lancet on the mouse, if necessary.

Using the Submandibular site for bleeding, this lancet eliminates the problems experienced when using the scalpel. Instead, the lancet comes with specific point lengths of 5 mm (mice aged 2-6 months) and 5.5 mm (older than 6 months). The different point lengths are used for different size mice. The design of the lancet allows the investigator to stick the mouse with enough pressure to insure a good

blood draw. As in human blood drawing lancets, the design makes it so the puncture is only as deep as the point of the lancet. Additionally, this technique is safer for the mouse than retro-orbital bleeding, and easier than Saphenous vein collection, as this method requires shaving and is cumbersome. Lastly, it is the method that produces the least increase in corticosterone and blood glucose levels, making this method the most appropriate for use in determining the effects of treatments and stimuli on diabetic symptoms.

http://www.medipoint.com/html/mouse_phlebotomy.html

http://www.medipoint.com/html/for_use_on_mice.html

With the aforementioned blood collection techniques, there can be some adverse side effects, but they are extremely rare in occurrence. There is a less than 1% chance of infection or hemmorage if these procedures are followed correctly and conducted sterilely (new needles, sterile gloves, clean hands and arms, etc.). In addition, there is minor discomfort experienced during the tail prick, which is analogous to having a lance pierce the finger tip of a diabetic checking their blood sugar. There can also be stress during the handling or containment for the injections or blood collections but they can be minimal if the procedure is done efficiently and quickly.

The following treatment procedures will not be conducted on all mice, randomized into these specific groups for each of the experiments. Each mouse will be tested only using one of the Chronobiological Stressors, combined with an alcohol and/or food protocol (i.e., a mouse will not be exposed to both constant light and shift-lag, but it can be exposed to constant light and a high-fat diet and a bottle of ethanol):

1. Chronobiological Stressors: 1a) Shift-Lag or Simulated Jet Lag Protocol: This protocol consists of a 6-hour phase advance in the Light-Dark (LD) cycle repeated every three weeks. For example, the normal LD cycle is from 0600 to 1800 h; after three weeks, the new LD cycle will be from 0000 to 1200, while three weeks after that, the new LD cycle will be 1800 to 0600 hours. This shift in the LD cycle is equivalent to jet-lag experienced from a trip from Boston, MA (USA) to Munich, Germany, every three weeks. Circadian stressors such as jet-lag and shift work are known to alter food cravings (increased intake of carbohydrates and fats), and alcohol drinking (producing increases or decreases, based upon initial preference). This protocol will aim to uncover whether circadian stressors affect plasma glucose and insulin levels, produce increases in body weight or food consumption, and affect glucose tolerance in diabetic and non-diabetic mice. The aforementioned blood tests will be conducted on these mice to determine the effects of the simulated jet-lag.

1b) Constant Conditions: The mice will be placed into constant darkness (in order to observe their free-running rhythm) or constant light (a circadian stressor). Constant darkness (DD) is not a stressor, and a common way to observe the behavioral circadian clock. DD will determine if different diets or alcohol have an effect on the

behavioral circadian rhythm. Other mice will be exposed to constant light (LL). LL is a stressor; exposing the mice to LL will determine how light all day can affect food preference, alcohol drinking, and diabetes symptoms.

1c) Differing Photoperiods: Most of the world does not live in an area with a 12:12 LD cycle. Most organisms are exposed to different day-lengths. In this protocol, mice will be exposed to either a long day (18:6 LD) or a short day (6:18 LD) in order to test the effects of photoperiodism on diabetic symptoms, alcohol, and food-preference (compared to control 12:12 LD). In addition, in order to control for the effects of light on the mice (is it the timing of the light or the amount of light), a skeleton photoperiod will be used. A skeleton photoperiod is a LD cycle with one-hour light pulses that indicate lights on and lights off (dawn and dusk). For example, a 12:12 skeleton photoperiod will have a one-hour light pulse at 6 am, and one at 6 pm, while the rest of the time is in the dark. A 18:6 skeleton photoperiod will have a light pulse at 6 am, and one at midnight, while a 6:18 skeleton photoperiod will have one at 6 am and noon.

2. Alcohol Drinking Experiment: This protocol will be similar to ones described in previous IACUC proposals, including IACUC case number 2013-07. Mice will be given access to 10%-ethanol in either a free-choice condition (2 bottles, 1 bottle of 10%-ethanol, 1 bottle water, positions switched weekly) or in forced conditions (1 bottle of 10%-ethanol only). Initially, mice will be exposed to free-choice ethanol in order to uncover ethanol preference in TH mice, and to determine if lower amounts of ethanol can affect blood glucose and insulin levels. No study has determined the alcohol preference of TH mice. Previous experiments from other institutions (Seggio et al., 2009, Bragger et al., 2010) have used forced ethanol conditions without causing decreased health in mice for these circadian protocols. Seggio et al., 2009 determined that "free-choice" ethanol consumption does not produce differences in the circadian rhythm because the mice do not drink pharmacologically relevant levels of ethanol, while the forced mice did. The B6 mouse is the most ethanol tolerant inbred strain, able to drink forced ethanol without side effects for over 15 weeks. This protocol will aim to uncover the effects of alcohol consumption on blood glucose and insulin levels, and glucose tolerance in diabetic and non-diabetic mice.

3. Food Preference and Obesity Testing: In this protocol, mice will be given access to a variety of Test Diet (St. Louis, MO), which have all of the nutritional needs of mice (vitamins, minerals, etc), but vary in their carbohydrate, protein, and fat composition. The diets the mice will be exposed to the following diets: Standard Chow (5001), 45% kcal Fat (58V8), 60% kcal Fat (58Y1), Western Diet, Control, and High Cholesterol (5TJN/5TJS/5TJT), South Beach Diet (5TVX), American Diet (5TLN), and Atkins Diet (5TJP). The food can be given ad libitum, in a choice-preference test, or during a restricted access study, where the animal will be given only 4-hours maximum access to the food per day. In this protocol, it is known that mice will eat in excess to their normal daily allotment, so starvation will not be a concern. These different foods will be given in order to test the effects of diet on

circadian disruption, alcohol drinking, and genetic background regarding diabetic and immune symptoms and processes.

Biological Data Collected (if applicable)

Biological data collected will include:

1. Plasma insulin levels at different times of day, via ELISA test. Insulin is a hormone necessary for glucose uptake into cells. Type II Diabetics show increased insulin and insulin resistance, making it difficult to absorb glucose properly.

2. Plasma leptin levels at different times of day, via ELISA test. Leptin is a hormone necessary for metabolism. Leptin acts as an appetite suppressant and studies show that decreases in leptin can lead to obesity.

3. Plasma glucose levels at different times of day, via glucose monitor. Commonly known as blood sugar, these levels are known to spike after eating carbohydrate laden meals and are altered in diabetics.

4. Plasma C-peptide levels at different times of day, via ELISA test. C-peptide is a peptide necessary for proper insulin function and structure. C-peptide is reduced in type I diabetics, but elevated in type II diabetics.

5. Plasma IgG levels at different times of day, via ELISA test. These are antibodies secreted by B-cells and play a role in specific immune functions.

6. Plasma IgM levels at different times of day, via ELISA test. These are antibodies secreted by B-cells and play a role in specific immune functions.

7. Plasma IgE levels at different times of day, via ELISA test. This is an antibody used to fight off parasitic worms.

8. Time (in days) for re-entrainment to a shifted LD cycle. The ability to resynchronize to a shifted LD cycle is necessary for individuals who change time zones and/or who do night shift-work.

9. Activity levels via running wheel or home cage activity. Activity will be determined by counting the number of wheel turns per day in running wheel cages, or by number of IR beam crosses in the non-running wheel cages.

10. Amount of Drinking solutions and food consumed.

11. Body mass.

12. Plasma triglyceride, HDL, and total cholesterol, via the CardioChek home analyzer.

Animal Fate at Experiment Conclusion

At the conclusion of the experiment, the research animals will be singly euthanized in a separate room, using CO2 hypoxia treatment from a gas cylinder, as prescribed in the 2013 AVMA Guidelines to Euthanasia (pages 24-26). The CO2 will remain flowing for at least two minutes after all movement has stopped. The animal will remain in the chamber after euthanizing is complete for at least three-minutes and monitored for zero movement to ensure death. The animal will then be decapitated immediately afterwards to completely ensure death. The deceased animal will be placed into an appropriate plastic bag and stored in a freezer until transport to an incinerator can be arranged. The euthanasia would be conducted by an appropriately qualified CITI trained researcher.

Signatures

I certify to the best of my knowledge the information presented herein is an accurate reflection of the proposed research project. I will comply with the Bridgewater State University IACUC protocols.

_____ _____ _____

PI name printed PI signature Date

By signing below, I certify that I am aware of this application for animal research under IACUC protocols.

_____ _____ _____

Department Chair *(print name)* Department Chair *(signature)* Date