CIRCADIAN DISRUPTION, DIET, AND EXERCISE

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by

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Introduction

Circadian rhythms are endogenously generated biological cycles with periods that are close to 24 hours in length. These rhythms can be expressed at behavioral, physiological, or molecular levels. Circadian rhythms are synchronized with the 24-hour day by zeitgebers (German for time-giver), such as the rising and setting of the sun. Endogenous rhythms are robust, and persist in the absence of an environmental time cue. The expression of a rhythm in such constant conditions is called a free-running rhythm. Circadian rhythms have endogenous periods (\(\tau\)) that are unique to each individual and species, and are usually close to but not exactly 24 hours (Aschoff, 1967).

Biological rhythms in physiology and behavior are driven by a master circadian pacemaker located in the suprachiasmatic nucleus (SCN) of the hypothalamus. Lesioning the SCN eliminates behavioral, physiological (Moore & Eichler, 1972; Stephan and Zucker, 1972), and molecular rhythms (Sakamoto et al., 1998) in animals that are deprived of external time cues. The SCN is located in the anterior hypothalamus, close to the third ventricle and superior to the optic chiasm (Cassone et al, 1988).

Various types of stimuli act as circadian zeitgebers, including scheduled feeding (Stokkan et al., 2001; Damiola et al., 2000), temperature (Francis & Coleman, 1997; Buhr et al., 2010), scheduled exercise (Edgar & Dement, 1991), or introduction of a novel
running wheel to an animal’s cage (Mrosovsky et al., 1989). However, light serves as the primary zeitgeber that entrains circadian rhythms. The SCN receives photic information from the daily environmental light-dark cycle. When light enters the retina, it activates photoreceptive ganglion cells (Berson et al., 2002), which transmit signals to the SCN via the retinohypothalamic tract (Rusak & Boulos, 1981). Activation of this pathway leads to increases in the expression of the period genes Per1 and Per2 (Albrecht et al., 1997; Miyake et al. 2000), which results in adjustments to the phase of the SCN necessary to keep it in sync with the environment. In turn, the SCN uses neuronal and humoral signals to signal other central and peripheral clocks, synchronizing them in a coordinated fashion.

Core clock mechanism

The discovery of the core clock gene mechanism has allowed for in-depth analyses of circadian rhythms. The core clock mechanism is regulated by transcriptional feedback loops of clock genes and their protein products, which have a positive (excitatory) or negative (inhibitory) function (Albrecht, 2002). Clock and Bmal1 are two essential positive elements in regulating circadian rhythmicity in mammals. Clock and Bmal1 dimerize to form a transcription factor that binds to the enhancer box (E-box) located in the promoter region of Period genes (per1 & per2) and Cryptochrome genes (cry1 & cry2). Per and Cry genes both serve as negative elements of the clock mechanism. The protein products of Per and Cry accumulate in the cytoplasm and form
heterodimers, which translocate back to the nucleus and interact with the Clock/Bmal1 heterodimer. This interaction prevents further activation of Per and Cry transcription (Albrecht & Eichele, 2003; Reppert & Weaver, 2001). In this thesis, I examine whether changes in an animal’s housing conditions can alter the parameters of this feedback loop, asking whether or not these conditions can alter the fundamental function of circadian clocks.

![Figure 1: The circadian clock is regulated by transcriptional feedback loops of the clock genes and their protein products.](image)

**Oscillation of clock genes in SCN and peripheral clocks**

Clock genes are robustly expressed in a rhythmic fashion both in SCN and peripheral tissues (Buijs et al., 2001). In the SCN this oscillation has been observed both in vivo (Reppert & Weaver, 2001), and in vitro (Yamaguchi et al., 2003). Since the SCN synchronizes peripheral clocks, clock gene expression in the SCN tend to peak earlier than in peripheral tissues (Balsalobre, 2002). In this thesis, I will measure clock gene
expression in both peripheral tissues and the SCN to examine if it is altered by different housing conditions. To use as a reference for analyzing changes in clock gene expression, I summarized the average zeitgeber time (ZT) of each gene’s peak transcription level in Table 1.

**Table 1: Mouse clock genes and their average ZT of peak mRNA expression.**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Average ZT at peak or transcription level</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SCN</td>
<td>Peripheral Tissue</td>
</tr>
<tr>
<td>Bmal1</td>
<td>18</td>
<td>22-02 (heart, lung, &amp; liver)</td>
</tr>
<tr>
<td></td>
<td>Constative</td>
<td>21-03 (heart, lung, &amp; liver)</td>
</tr>
<tr>
<td>Per1</td>
<td>4-8</td>
<td>12 (heart, lung, &amp; liver)</td>
</tr>
<tr>
<td>Per2</td>
<td>9-12</td>
<td>12 (heart, lung) 12-16 (liver)</td>
</tr>
</tbody>
</table>

**Analyzing entrainment**

Animals entrain to new LD cycles by adjusting the internal clock on a daily basis to match the new light cycle. A phase advance is a shift of the clock to an earlier time, similar to traveling eastbound (e.g., New York to France). A phase delay on the other hand is the shift of the clock to a later time, similar to traveling westbound (e.g., France to New York). Many studies have distinguished differences between rodent responses to these two phase shifts (Reddy et al., 2002; Antle et al., 2009; Kott et al., 2012). During a 6 hour phase shift, mice fully re-entrain to a phase advance in 7 days and only 3 days to
a phase delay. Behavioral adjustments to phase shifts are also accompanied by corresponding readjustments of Cry and Per genes (Reddy et al., 2002). In addition, resynchronization of the SCN core and shell after a phase shift is achieved in 10-13 days following a 6 h advance, but only 7 days after a 10 h delay (Nagano et al., 2003). Signaling cascades downstream to the activation of NMDA receptors also differ between phase delays and phase advances (Weber et al., 1995; Ding et al., 1998).

Entrainment of circadian rhythms can be measured in vivo through a variety of behavioral and physiological variables, including locomotor activity, body temperature, and drinking rhythms. However, behavior is not always directly representative of the phase of the clock in the SCN due to masking effects. Masking occurs when an environmental variable has a direct influence on a circadian behavior, effectively concealing the expression of the underlying endogenously generated rhythm. Examples of masking factors include: light, background noise (Landstrom et al., 1988), temperature (Mavjee & Horne, 1994), lighting (Leproult et al., 1997), motivational behavior (Hayashi et al., 1998), or even food (Paz & Berry, 1997) (van Dongen & Dinges, 2000). In addition to measuring the effects of chronic jet lag on body weight, I examine the animal’s entrainment to the shifting light-dark cycle. Since light suppresses activity in mice, the onset of wheel running as driven by the circadian clock may be masked. Therefore, entrainment should also be measure at activity offset, which is an unmasked indicator of behavioral entrainment (Reddy et al., 2002).
Home cage influence on peripheral and SCN clock gene expression

Since circadian laboratories across the world study different aspects of circadian rhythms, they also vary in methodology when measuring entrainment. As a result, laboratories may be equipped differently depending on the nature of their study. Cages may come in different sizes and running wheels may or may not be used. Mice in small cages have decreased locomotor activity compared to large caged animals (Poom et al., 1997). Wheel running influences energy expenditure (Levin, 1991), entrainment (Mrosovsky et al. 1989), and motivational behavior of animals (Belke, 1996). The purpose of my study is to examine if having a different size cage or access to a running wheel will have an effect on clock gene expression. The influence of running wheel or cage size on clock gene expression has not previously been documented. This was a factor that is commonly overlooked when comparing results across circadian studies. If results between differently housed studies are not parallel with each other, then housing of the animals in circadian experiments should be considered when measuring clock gene expression.

Wheel running as a measure of behavior

Running wheels have been widely used as an easy and non-complex way to measure general locomotor activity of rodents (Sherwin, 1998). Given voluntary access to a running wheel, mice will move the equivalent of several kilometers per day. Wheel running accelerates re-entrainment of an animal to a new LD cycle (Mrosovsky et al.
1987). Free access to running wheels by blinded rats also shows shortening of free-running period (Yamada et al., 1988). The presence of a wheel also serves as a novel factor altering entrainment of an animal (Mrosovsky et al., 1989). Running wheels have effects on the animal’s energy balance, general activity, and activation of rewarding system of laboratory animals (Novak et al., 2012). At times, running wheels have been used as a form of exercise. Obese rodents have reduced body weight gain and adiposity when given voluntary access to a running wheel (Haskell-Luevano et al., 2009; Patterson and Levin, 2008). The increased energy expenditure due to wheel running also results in increase in food intake, thus regulating body weight (Levin, 1991; Zachwieja, 1997).

Running wheel access provides extended opportunities for locomotor activity, further improving welfare of laboratory animals (Sherwin, 1996). Furthermore, mice find wheel-running to be naturally rewarding. It serves as a motivated behavior that activates neuronal reward systems (Belke, 1996, Rhodes et al., 2003), and decreases anxiety-like effects (Binder et al., 2004; Duman et al., 2008). This thesis investigates whether the documented influences of home cage running wheels ultimately have an effect on the core clock mechanism.

*Circadian disruption and metabolic impairment*

While studying how home cage variety influences clock gene output, I became interested in the negative physiological effects of a disrupted circadian clock. Two familiar models of clock disruption are jet lag and shiftwork. Jet lag is a temporary
condition in which the sleep/wake cycle of a person is disrupted due to rapid long
distance travel across time zones. As a result, the person’s endogenous clockwork is
desynchronized from the environmental light cycle. Symptoms of jet lag include daytime
fatigue, insomnia, malaise, and gastrointestinal disturbance. (Sack, 2009). Similar to jet
lag, the circadian rhythms of shift workers are also greatly disrupted. The chronic and
repeated shifting of the clock of a shift worker results in the aggravation of variety of
symptoms. These include increased susceptibility to cardiovascular disease and
metabolic disorders (Ha & Park, 2005; Esquirol, 2009). A population based study showed
a correlation between obesity and shift workers for both men and women. Shift workers
tend to have higher triglycerides and lower concentration of HDL cholesterol compared
to non-shift workers (Karlsson et al., 2001), and night shift nurses have a higher
prevalence for weight gain (Niedhammer et al., 1996). Furthermore, in 2007 the World
Health Organization classified shift work as a probable carcinogen to humans (IARC,
2010).

Chronic Jet Lag

For this thesis, I wanted to investigate the disrupting effects of jet lag on
metabolism. To simulate jet lag, I used a forced desynchronization protocol, sometimes
referred to as chronic jet lag. This procedure simulates the sleep/wake cycle of an
individual who is constantly traveling across time zones and constantly adjusting their
endogenous clockwork to compensate for the demands of the shift. Experiments using
chronic jet lag in animal models has showed suppression of the immune system (Castanon-Cervantes et al., 2010), increased tumor development (Smalaand et al., 1996; Wu et al., 2012), decreased life expectancy (Davidson et al., 2006), and increased incidence of metabolic disorders (Preuss et al., 2008; Scheer et al., 2009; Gonnissen et al., 2012).

Jet lag and metabolism

Feeding and metabolism are both directly linked to circadian rhythms (Damiola et al., 2000; Buijs et al., 2006; Kaneko et al., 2009; Arble et al., 2009; Froy, 2010). Both gene expression and secretion of the hormones leptin and ghrelin cycle in a rhythmic fashion (Kalra et al. 2003). Clock disruption by jet lag causes impairment in proper metabolic function. Short-term circadian misalignment in humans increases meal-induced glucose and insulin levels and decreases leptin levels and the duration of sleep (Scheer et al., 2009). Increased glucose and insulin are signs of reduced insulin sensitivity. Decreased leptin increases appetite thereby increasing food intake. Decrease in duration of sleep is associated with obesity and diabetes (Knutson et al., 2006; Kohatsu et al., 2006). Chronically maintaining these conditions may result in aggravated metabolic impairment. Many previous experiments using different clock disruption paradigms also found damaging effects on metabolism. Clock mutant mice develop metabolic syndrome and become highly susceptible to obesity (Turek et al., 2005). Altering the timing of feeding can induce weight gain (Arble et al. 2009). Exposure to
artificial periods that deviate from 24 hours disrupts an animal’s natural rhythm. A 20-hour LD cycle results in defective metabolic function and instability of entrainment (Karatsoreos et al., 2011). Also, subjecting the animals to a LD 3:3 schedule promotes glucose intolerance and other diabetic markers regardless of the change in body mass and food consumption (Oishi & Itoh, 2013).

When an animal is exposed to a new LD cycle, endogenous clocks take time to adjust their period and phase to align with the new LD schedule. Simulating chronic jet lag causes repeated adjustments every time the LD cycle is shifted. Entrainment to a phase shifted LD cycle is accompanied by desynchronization of distinct SCN sub regions, with the ventral SCN adjusting to the new LD cycle first, followed by the dorsal SCN a few days later (Nakamura et al., 2004). This desynchronization results in conflicting output signals from the different regions of the SCN. There is also an initial misalignment of the SCN and peripheral clock oscillators during a phase shift. The peripheral clocks take longer to fully entrain to a new LD cycle in comparison to the SCN. This makes sense, given that peripheral clocks take their guidance from the SCN. There are also different rates of entrainment depending on the specific peripheral tissue (Yamazaki et al., 2000). Although this misalignment may be transient, subjecting an animal to repeated phase shifts over a period of time results in exacerbated symptoms as compared to a single jet lag event (Davidson et al., 2009).

During a phase shift, the dark phase is shortened depending on the direction of the shift; with the first half of the original dark phase exposed to light when the clock is
delayed, or the 2nd half exposed to light when the clock is advanced. In the first few days of the shift, the animal is exposed to some “light at night” (LAN), which eventually disappears as soon as the endogenous clock becomes synchronized with the new LD cycle. While the LAN phenomenon might seem transient and insignificant, increased exposure to LAN during repeated shifts may aggravate underlying effects. Animal models that have been exposed to LAN showed depressive like responses (Fonken & Nelson, 2013), increased body mass (Fonken et al., 2010), and increased susceptibility to cancer (Barsch et al., 2009). Therefore repeated exposure to LAN every time the clock is shifted may contribute to the exacerbated effects of chronic jet lag.

*Effects of High-Fat Diet Consumption*

The negative effects of a high fat diet (HFD) in health are widely recognized. Obesity, insulin resistance, diabetes, and heart disease are only a few of the many conditions that result from a HFD. In this thesis, the potential negative effects of chronic jet lag are combined with those of HFD consumption. This combination is hypothesized to result in metabolic impairment and exacerbated weight gain compared to non-shifted light and normal diet (ND) fed mice. Various studies have documented the effects of HFD on circadian rhythms. Mice maintained on a HFD have a lengthened period beginning as early as one week into the diet treatment. A higher percentage of total locomotor activity was also observed during the light phase of HFD fed mice. HFD mice tend to consume more food during the light phase and have decreased food intake
during the subjective night phase (Kohsaka et al., 2007). It is possible that the alteration of feeding patterns caused by HFD contributes to increased weight gain. HFD fed mice also show disruptions in the rhythmicity of feeding hormones insulin, glucose, and leptin (Kohsaka et al., 2007). HFD mice entrain slower to a jet-lag shift compared to ND fed mice. When subjected a 6h advanced LD cycle, the HFD fed mice took one full day more to re-entrain to the new LD compared to ND fed mice (Mendoza et al., 2008). High consumption of HFD may also causes changes in the expression of core clock genes. However, the degree of clock gene alteration caused by HFD is still controversial. Core clock gene expression in the mediobasal hypothalamus, liver, and epididymal fat of male was dramatically altered after six weeks of HFD treatment in male C57BL/6 mice (Kohsaka et al., 2007); also after 11 months in HFD, clock genes were altered in the liver and kidneys of male C57BL/6 mice (Hsieh et al., 2010). In contrast, an eight week HFD treatment of female mice showed only minimal core clock gene alteration in the adipose tissue and liver (Yanagihara et al., 2006).

Goals

Laboratories use a wide range of housing conditions when conducting experiments in circadian rhythms. Animals are housed in different size cages that may or may not have a running wheel for measuring locomotor activity. Running wheels have an effect on energy balance, general activity, and an animal’s reward systems (Novak et al., 2012). Furthermore, entrainment of the animal is accelerated with access to a
running wheel (Mrosovsky et al., 1989). Varying housing conditions may have an influence on the core clock gene mechanism, thus altering the fundamental function of circadian clocks. Both the SCN and peripheral clock gene expression may be affected by the extra activity induced by the presence of a running wheel. In this study I will examine whether the clock gene expression is altered in several peripheral tissues (liver, lungs, and heart) and the SCN by cage size or the access to a running wheel. Since free-running periods are affected by the presence of a running wheel, wheel running may have a greater influence on clock gene expression in the SCN compared to peripheral clocks. I predict that access to a running wheel alters clock gene expression in the SCN, but not in the heart, liver, or lung. The goal of this study is to examine if housing conditions should be considered as a source of variability in clock gene results across different laboratories.

Jet lag disrupts circadian rhythms and promotes damaging effects in physiology including impaired metabolic function. Simulating chronic jet lag on laboratory animals suppresses the immune system (Castanon-Cervantes et al., 2010), tumor development (Smalaand et al., 1996; Wu et al., 2012), increases mortality rate (Davidson et al., 2006), and causes metabolic disorders (Preuss et al., 2008). Furthermore, consumption of a HFD can alter an animal’s patterns of feeding, locomotor activity, period, and entrainment (Kohsaka et al., 2007; Mendoza et al., 2008). In this thesis, I hypothesize that weight gain as a result of a HFD will be exacerbated during simulated jet lag, as
compared to normal fed control mice. Running wheels will be used to examine
difference in general locomotor activity between HFD and ND fed mice.

The running wheels used for measuring activity impede the increase of weight
gain, due to its effects in energy balance and body weight regulation (Levin, 1991;
Zachwieja, 1997). Therefore, examining the damaging effects of chronic jet lag on HFD
fed mice should be exacerbated with the absence of a running wheel. I hypothesize that
weight gain as a result of HFD will be further exacerbated during simulated jet lag in the
absence of running wheels. Excessive weight gain promotes insulin resistance and
increases the susceptibility for diabetes. Therefore I will also examine alteration in
glucose tolerance to further support the interaction between chronic jet lag and diet.
Overall, the goal of these studies is to address the role of chronic jet lag in metabolic
impairment as indicated by body weight increases, altered body composition
distribution, and impaired glucose tolerance.
Methods

Experiment #1

Animals

Seventy five mice (C57BL/6J strain) were bred in the Kent State University animal facility from stock purchased from Jackson Laboratories. They were group-housed in a 12:12 light-dark (LD) cycle with food and water available \textit{ad libitum}. Animals included 36 females and 39 males, aged 10 to 17 weeks.

Experimental design

For the duration of the experiment, the animals were single-housed in three difference caging conditions: small cages (11.5”L x 7.5”W x 5”H), large cages (19”L x 10.5”W x 6.125”H), and large cages (19”L x 10.5”W x 6.125”H) with an access to a wheel. All cages were made of a high temperature polycarbonate plastic. There were 3 males and 3 females used for each group, except for the ZT 0 time point, which had 3 additional males added. They were kept in a 12h light:12h dark light cycle for 5 weeks. The animals were given a week to acclimate to their new surroundings, and then another 4 weeks in their corresponding cage conditions. At the end of the 5th week, all animals were sacrificed at ZT 0 (light onset), ZT 6 (middle of light phase), ZT 12 (onset of dark), and ZT 18 (middle of dark phase) via cervical dislocation. The brains, as well as
samples or liver, lung, and heart tissue, were collected, flash frozen, and stored at –80°C.

Figure 2: Experimental design used for Experiment #1. Mice housed in each conditions (LW, LC, & SC) were sacrificed at 4 different timepoints (ZT0, ZT6, ZT12, & ZT18). The liver, lung, heart, and SCN in brains were harvested from each animal. 4 different clock genes (Per1, Per2, Clock, and Bmal1) were measure for mRNA expression for the time of sacrifice.
Liver, lung, and heart gene expression

Approximately 20 mg of liver tissue and 25 mg of lung and heart tissue from each mouse were used to measure the expression of clock genes. Tissue was homogenized using a sonicator and RNA was isolated using Qiagen Rneasy RNA purification kits, with the inclusion of a DNAse treatment step. The samples were tested for purity using a 1 µl aliquot in a Nanodrop Spectrophotometer ND-100 (Thermo Scientific). Samples having 260/280 ratio higher than 2.0 and a 260/230 ratio between 2.0-2.2 were used. Purified RNA samples were reverse transcribed into cDNA in a 20 µl reaction using High Capacity cDNA Reverse Transcriptase Kits (Applied Biosystems). Lastly, quantitative real-time PCR was used to assess clock gene expression at each time point (Per1, Per2, Clock, and Bmal1) using primer/probe sets from Applied Biosystems. A total of 6 µl of aliquoted cDNA was used for a 60 µl reaction for each gene, which was then distributed in 20 ul triplicate wells. GAPDH was used as a control gene for qPCR analysis.

SCN gene expression

Laser capture microscopy was used to capture the SCN region in order to isolate SCN mRNA for gene expression. Frozen mouse brains were first sliced in a cryostat at 12 µm thick. Sections were mounted on positively charged glass slides and kept frozen either in dry ice or in a -80°C freezer for no longer than 14 days if not used immediately. Sections were counterstained with hematoxylin to aid in visualization of the SCN.
Sections were submerged in 75% ethanol for 30 seconds, and then rinsed with deionized water. The sections were submerged in the hemotoxylin stain for 90 seconds, then rinsed in water twice to remove excess hemotoxylin. After staining the sections were put through a dehydration series to remove excess water. The sections were submerged in 75% ethanol for 30 seconds, 95% ethanol for 30 seconds, and then in 100% ethanol for 1 minute. Sections were submerged in xylenes for at least 5 minutes but no longer than 2 hours before laser capture.

Slides were placed in the laser capture microscope (Arcturus Autopix, MDS Analytical Technologies) and the xylenes allowed to evaporate. Six to nine consecutive SCN regions per brain were identified then captured using Capsure HS LCM Caps (Molecular Devices, MDS Analytical Technologies). Images before and after capture, were taken to confirm SCN region capture. Captured tissue was lysed and purified using an RNA purification kit (Picopure, MDS Analytical Technologies) with the inclusion of a Dnase treatment step. The samples were tested for purity using a 1µl aliquot in a Nanodrop Spectrophotometer ND-100 (Thermo Scientific). Samples having 260/280 ratio higher than 2.0 and a 260/230 ratio between 2.0-2.2 were used. Purified RNA samples were reversed transcribed into cDNA in a 20 µl reaction using High Capacity cDNA Reverse Transcriptase kits (Applied Biosystem). Lastly quantitative real-time PCR was used to assess clock gene expression at each time point (Per1, Per2, Clock, and Bmal1) using primer/probe sets from Taqman. A total of 6 µl of aliquoted cDNA was
used for 60 µl reaction for each gene, which was then distributed in 20µl triplicate wells. GAPDH was used as a control gene for qPCR analysis.

**Chronic jet lag experiment (Experiment #2 & #3)**

*Animals*

One hundred male mice (C57BL/6J strain) were purchased from the Jackson Laboratory (Bar Harbor, Maine, USA). Animals were group-housed in the Kent State University animal facility in a 12h-light/12h-dark cycle and fed *ad libitum*. At the beginning of the experiment the animals used were 50-52 days of age. All animals were allowed to acclimate for 7 days prior to the actual experiment. During this time, all animals were fed normal rodent chow *ad libitum*. Experimental protocols for this study were approved by The Kent State Institutional Animal Care and Use Committee.

*Light Treatments*

The mice were randomly assigned to one of three light treatment groups: a stable LD control group, a phase advanced group, or a phase delayed group. For the control LD group, the animals kept in a 12:12 LD cycle for the entire duration of the experiment. The phase advanced group was subjected to 6 hour advances of the LD cycle every 7 days. This was accomplished by shortening the dark phase by 6 hours on the day of the shift. After the 6 hour dark phase, the lights turned on and marked the light phase of the new LD cycle. The phase delayed group was subjected to 6 hour
delays of the LD light cycle every 7 days. This was accomplished by 6 hour lengthening of the light phase on the day of the shift. After the 18 hour light phase, the lights turned off and marked the dark phase of the new LD cycle (See Figures 3 & 4).

**Body Composition Determination**

Initial and final body composition parameters such as lean mass and fat mass were measured for this experiment. Body composition was measured using magnetic resonance spectroscopy body composition analysis (Echo MRI-700, Houston, TX). The animals were placed gently in one end of a cylindrical holder. The holders were inserted in the EchoMRI machine and scanned for 3.5 minutes. After the scan, whole body fat and lean mass data were calculated and saved.

**Glucose Tolerance Test**

The animals were fasted for 6 hours prior to testing. This length of fasting time allows for the most robust differences in plasma glucose when comparing the glucose tolerance of mice in normal chow versus HFD (Andrikopoulous S. et al., 2008). Food was taken away at ZT 20. The animals were allowed to drink *ad libitum* during the fast. Each animal was weighed prior to testing to measure the appropriate dosage of glucose to be administered via intraperitoneal injection. Glucose dosage was determined by glucose-body mass ratio of 2g/kg, and was administered at the 0-min time point. Tail prick blood
samples were collected at -10 min, 15 min, 30 min, 60 min, and 120 min. An Alphatrak glucometer and Alphatrak glucose test strips were used to measure blood glucose.

Experiment #2

A total of 60 mice (20 per light cycle) were used. The animals were individually housed in 19”L x 10.5”W x 6. 125”H cages with free access to a running wheel. The running wheels monitored general locomotor activity using ClockLab software. 10 animals in each light treatment group received normal rodent chow, and the other 10 received a HFD (20 kcal% from protein, 35 kcal% from carbohydrate, and 45 kcal% from fat). Diet treatment began at week 1, right after the acclimation period. Both diets were given ad libitum through the whole duration of the experiment. At week 2, the LD cycle is shifted as directed in each light treatment protocol. The phase advanced and phased delayed groups were shifted every week for 7 weeks, for a total of 6 LD shifts. Body masses were measured at the beginning of the experiment (prior to the diet treatment), and every week right before the light was shifted. Body composition measurements were taken before the experiment (prior to the diet treatment) and after the 7th week.
Figure 3: Diagram depicts the chronic phase advance protocol. Dark shaded regions are dark phases and clear regions are light phases. The clock is shifted every 7 days in the advanced direction. Yellow regions represent the shortened dark phase every time the clock is advanced by 6 hours.
**Chronic Phase Delay**

**Figure 4:** Diagram depicts the chronic phase delay protocol used for this study. Dark shaded regions are dark phases and clear regions are light phases. The clock is shifted every 7 days in the delayed direction. Yellow regions represent the lengthened light phase every time the clock is delayed by 6 hours.
Experiment #3

A total of 40 mice (20 per group) were subjected to 2 different light treatment groups: control LD and phase delayed. The animals were individually housed in small shoebox size cages (11.5”L x 7.5”W x 5”H) with no access to running wheels. 10 animals in each light treatment group received normal rodent chow, and the other 10 received a HFD. Diet treatment was administered at week 1, right after the acclimation period. Both diets were given ad libitum through the whole duration of the experiment. At week 2, the LD cycle was shifted for the phase delayed group. The phase delayed group was shifted every week for 7 weeks, for a total of 6 LD shifts. Body masses were measured at the beginning of the experiment (prior to the diet treatment), and every week right before the light was shifted. Body composition measurements were taken before the experiment (prior to the diet treatment) and after the 7th week. The light was shifted 2 more times for another 2 weeks. After the 9th week, the glucose tolerance test (GTT) was performed on all groups. GTT was performed at least 5 days after the last shift to ensure complete entrainment after the most recent shift. The animals were fasted for 6 hours prior to GTT.

Data Analysis

Gene expression data were analyzed using a two-way ANOVA (with time point and housing conditions as factors). Tukey-Kramer Multiple Comparison Tests were used to determine differences between experimental housing groups at the same time
points. Weight gain was analyzed using a three-way ANOVA (with diet, light treatment, and week as factors). Dunnett’s Two-Sided Multiple-Comparison Test was used to determine differences between light groups in each diet treatments over the 7 week experiment.

For body composition, data were analyzed using a two-way ANOVA (with diet and lights treatments as factors). Dunnett’s Two-Sided Multiple Comparison Test was used to determine differences between light treatment groups in each diet treatment. When comparing wheel vs. no wheel animals, the wheel factor was added to each comparison. A three-way ANOVA was used to analyze body composition between wheel versus no wheel animals (with lights, diet, and wheel as factors). Again Dunnett’s Two-Sided Multiple Comparison Test was used to determine differences between light groups and diet groups with or without a wheel. Body weight between wheel and no wheel animals were analyzed using four-way repeated measures ANOVA (with diet, lights, wheel, & week as factors). Mean and standard error of mean were shown and P-values less than 0.05 were considered statistically significant. NCSS statistical software was used to perform all ANOVA and multiple comparison analyses.

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>SC</td>
<td>Small Cage</td>
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<tr>
<td>LC</td>
<td>Large Cage</td>
</tr>
<tr>
<td>LW</td>
<td>Large Cage with Wheel</td>
</tr>
<tr>
<td>HFD</td>
<td>High Fat Diet</td>
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<tr>
<td>ND</td>
<td>Normal Diet</td>
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<tr>
<td>PA</td>
<td>Phase advance</td>
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<td>PD</td>
<td>Phase Delay</td>
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<td>LD</td>
<td>Light-Dark</td>
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<td>GTT</td>
<td>Glucose Tolerance Test</td>
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<tr>
<td>AUC</td>
<td>Area under a curve</td>
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**Table 2:** Abbreviations used in this thesis and their corresponding definitions.
RESULTS

Experiment #1

In this experiment, expression of *Per1, Per2, Clock, and Bmal1* in several peripheral tissues (liver, lungs, and heart) and the SCN were measured in mice housed in large cage with wheels (LW), large cage (LC), and small cage (SC) at 4 different time points (ZT 6, ZT 12, ZT 18, ZT 0). *Per1, Per1, Clock, and Bmal1* all exhibited significant variation across the day consistent with 24 h rhythmicity in the peripheral tissues and the SCN. There were no significant differences in *Per1* and *Per2* expression across housing conditions in all peripheral tissues and the SCN (Figure 5, 7, 9, &11). There were significant differences in *Clock* and *Bmal1* expression, most of which occurred at either ZT 12 or ZT 18. In the liver and heart, there were no significant differences in *Clock* expression (Figure 6a & 10a). However, in the lungs, *Clock* expression was significantly increased in SC mice compared to LW and LC at ZT 18 (p < 0.000001) (Figure 8a). In the SCN, *Clock* gene expression was significantly decreased in SC mice compared to LW at ZT 18 (p=0. 014) (Figure 12a). There were no significant differences in *Bmal1* expression across housing conditions in the heart (p=0.29) (Figure 10b). In the liver, *Bmal1* expression was significantly decreased in SC mice compared to LW and LC at ZT 0 (p = 0.00063) (Figure 6b). In the lungs, *Bmal1* gene expression was significantly decreased in LW mice compared to LC and SC at ZT 18 (p < 0.000001); and significantly decreased in
SC mice compared to LW and LC at ZT 0 (p < 0.000001) (Figure 8b). In the SCN, Bmal1
gene expression was significantly decreased in SC mice compared to LW and LC at ZT 18
(p = 0.00011) (Figure 12b). Taken together, the differences in clock gene expression
between the housing conditions seemed to be due to the small cage being different in
size and not so much due to the presence of a running wheel. Most significant
differences were also observed at ZT 18 (mid active phase) and ZT 0 (end of active
phase). Overall, changes in clock gene expression from different housing conditions
were detected in the liver, lungs, and SCN, but not in the heart (Table 3).
Both Per1 (a) and Per2 (b) in the liver of LW, LC, and SC mice exhibited significant differences in expression depending on time of day (p < 0.05). There was no significant interaction between housing conditions and time of day on Per1 and Per2 expression (Per 1, p = 0.76; Per2, p = 0.61 respectively). Error bars represent standard error of mean.
Figure 6: Both Clock (a) and Bmal1 (b) in the liver of LW, LC, and SC mice exhibited significant differences in expression depending on time of day (p < 0.05). There was no significant interaction between housing conditions and time of day on Clock expression (Clock, p = 0.30). However during ZT 0, Bmal1 expression in SC was significantly lower compared to both LW and LC (p < 0.001). * Significant difference, p < 0.05. Error bars represent standard error of mean.
Figure 7: Both Per1 (a) and Per2 (b) in the lungs of LW, LC, and SC mice exhibited significant differences in expression depending on time of day (p< 0.05). There was no significant interaction between housing conditions and time of day on Per1 and Per2 expression (Per 1, p = 0.49; Per2, p = 0.77). Error bars represent standard error of mean.
Figure 8: Both Clock (a) and Bmal1 (b) in the lungs of LW, LC, and SC mice exhibited significant differences in expression depending on time of day (p < 0.05). At ZT 18, Clock expression was significantly higher in SC mice compared to both LW and LC (Clock, p = 0.00053). Bmal gene expression was significantly decreased in LW mice compared to both LC & SC at ZT 18 (p < 0.000001), and significantly lower in SC animals than both LW & LC at ZT 0 (p < 0.000001). *Significant difference, p < 0.05. Error bars represent standard error of mean.
Figure 9: Both *Per1* (a) and *Per2* (b) in the heart of LW, LC, and SC mice exhibited significant differences in expression depending on time of day (p< 0.05). There was no significant interaction between housing conditions and time of day on *Per1* and *Per2* expression (*Per 1, p = 0.28; Per2, p = 0.11*). Error bars represent standard error of mean.
Figure 10: Both *Clock* (a) and *Bmal1* (b) in the heart of LW, LC, and SC mice exhibited significant differences in expression depending on time of day (*p* < 0.05). There was no significant interaction between housing conditions and time of day on *Clock* and *Bmal1* expression (*Clock*, *p* = 0.91; *Bmal1*, *p* = 0.29). Error bars represent standard error of mean.
Figure 11: Both Per1 (a) and Per2 (b) in the SCN of LW, LC, and SC mice exhibited significant differences in expression depending on time of day (p< 0.05). There was no significant interaction between housing conditions and time of day on Per1 and Per2 expression (Per 1, p = 0.96; Per2, p = 0.067). Error bars represent standard error of mean.
Figure 12: Both Clock (a) and Bmal1 (b) in the SCN of LW, LC, and SC mice exhibited significant differences in expression depending on time of day (p < 0.05). At ZT 18, Clock gene expression was significantly decreased in SC compared to LW (Clock, p = 0.0014). Bmal1 gene expression was also significantly decreased in SC mice compared to both LW & LC at ZT 18 (Bmal1, p = 0.000098). * Significant difference, p < 0.05. Error bars represent standard error of mean.
Table 3: Summary of all significant interaction between housing conditions and time point in the liver, lungs, heart, and SCN.
Experiment # 2

Body Weight

In this experiment, changes in body weight were evaluated across two diet treatments (high fat diet (HFD) and normal diet (ND)) and three lighting conditions (simulated chronic jet lag - phase advanced (PA), simulated chronic jet lag - phase delayed (PD), and a constant LD control). 10 mice were used for each diet-light treatment. All groups gained weight in the course of the 7 week experiment regardless of diet or light treatment (p < 0.000001) (Figure 13 & 14). HFD fed mice gained significantly more weight compared to ND fed mice in PA, PD, and LD groups (p < 0.000001). There was no significant difference in weight gain between light treatments groups in ND fed animals (Figure 13). However, HFD-PD had an increased weight gain compared to both HFD-PA and HFD-LD (p=0.0026). Weight gain between HFD-PA and HFD-LD was not significantly different (Figure 14).

Body composition

Change in body composition at the end of the experiment was also evaluated (Figure 15). As expected, HFD mice all showed significantly greater fat mass compared to ND fed mice (p < 0.000001). However, there was no significant in fat mass between light treatment groups in ND or HFD (p = 0.36) (Figure 15b). PA and PD both had significantly increased lean mass compared to LD (p = 0.032) (Figure 15a).
Activity

Wheel-running activity of each diet-light treatment group was also evaluated (Figures 16, 17, & 18). The average amount of activity was analyzed for the ND-LD (Figure 19a) compared to HFD-LD (Figure 19b) mice at days 1-7, days 21-27, and days 37-43 of the diet treatment (Figure 20). Average activity significantly decreased from the days 1-7 to days 37-43 of the diet treatment for both ND-LD and HFD-LD. Activity at days 37-43 significantly decreased from days 1-7 for ND-LD, but remained comparable to days 1-7 for HFD-LD (Figure 19). Furthermore, upon observation of the actograms, no qualitative differences in resetting to delaying or advancing light dark cycle were detected across feeding conditions.
Figure 13: Normal Diet fed mice all significantly gained weight over the 7 week experiment ($p < 0.000001$). There was no significant interaction between light treatments and week throughout the course of the experiment for ND mice. Error bars represent standard error of mean.
Figure 14: HFD fed mice all significantly gain weight over the 7 week experiment (p < 0.000001). There was no significant difference in weight gain between HFD fed PA and LD group. However there was a significant increase in weight gain in HFD-PD compared to both HFD-PA and HFD-LD (p = 0.0026) in weeks 3 (a week after the 2nd PD), 5, & 6. * Significant difference, p < 0.05. Error bars represent standard error of mean.
Figure 15: HFD mice gained significantly greater fat mass (b) compared to ND (p < 0.000001). However, ND had gained significantly more lean mass (a) compared to HFD (p = 0.025). For mice in HFD, both PA and PD had gained more lean mass compared to LD (p = 0.032). However, there was no light treatment did not show to have any influence in fat mass (p = 0.36). Significant difference: *, p < 0.05; ***, p < 0.0001. Error bars represent standard error of mean.
Figure 16: Actograms of ND fed mice (a) and HFD fed mice (b) on constant LD for 7 weeks. Shaded areas indicate the hours of lights off, and white areas indicate hours of lights on. Black bars represent activity.
Figure 17: Actograms of ND fed mice (a) and HFD fed mice (b) on a chronic phase advance treatment for 7 weeks. Shaded areas indicate the hours of lights off, and white areas indicate hours of lights on. Black bars represent activity. No qualitative differences in resetting to delaying or advancing light dark cycle were detected across feeding conditions.
Figure 18: Actograms of ND fed mice (a) and HFD fed mice (b) on a chronic phase delay treatment for 7 weeks. Shaded areas indicate the hours of lights off, and white areas indicate hours of lights on. Black bars represent activity. No qualitative differences in resetting to delaying or advancing light dark cycle were detected across feeding conditions.
Figure 19: Average amount of activity for ND-LD (a) and HFD-LD (b) animals at days 1-7, days 21-27, and days 37-43 of diet treatment. Activity significantly decreased from days 1-7 to days 37-43 for both ND and HFD. Days 21-27 activity is significantly decreased compared to days 1-7, but is comparable to days 37-43 in ND-LD. However in HFD-LD mice, average activity on days 21-27 remained consistent with days 1-7 and is significantly higher than day 37-43. Error bars represent standard error of mean.
Figure 20: There is no significant difference in average activity between ND-LD & HFD-LD mice during days 1-7 (a), days 21-27 (b), & days 37-43 (c) of diet treatment. Error bars represent standard error of mean.
Experiment #3

Body weight

In this experiment I examined if weight gain as a result of HFD will be exacerbated by subjecting the animals to a chronic phase delay protocol in the absence of a running wheel. The results showed a significant increase in weight gain in the PD group compared to LD on weeks 2 and 3 in both HFD (Figure 21) and ND (Figure 22) fed animals. Body weight gain on weeks 4, 5, and 6 were not significantly different between PD and LD groups (Figure 21 & 22). The end point weight gain was not significantly different between light groups, showing that the increase in body weight caused by the initial PD shift was temporary.

Body composition

Change in body composition at the end of the experiment was also evaluated (Figure 23). As expected, the HFD mice in both PD and LD had significantly higher levels of fat mass compared to ND fed mice ($p < 0.000001$). ND mice significantly gained more lean mass compared to HFD ($p = 0.00078$). Lean mass was significantly increased in PD compared to LD animals, irrespective of diet ($p = 0.016$) (Figure 23a). However, my results showed no significant difference in fat mass between light treatments ($p = 0.47$) (Figure 23b).
Glucose tolerance test

After 10 weeks of diet treatment, I also performed an intraperitoneal glucose tolerance test following a 6h fast to examine any impairment in plasma glucose clearance. There was a rise in plasma glucose levels > 400 mg/dl, with the peak occurring at 15 min for both ND-LD and ND-PD. For the HFD fed animals there was a rise in plasma glucose levels > 515 mg/dl, with a sustained peak at 15 min and 30 min for both HFD-LD and HFD-PD mice. There was a significant increase in plasma glucose level in HFD compared with the ND fed animals for both PD and LD groups (Figure 24a). Clearance of plasma glucose in ND fed animals was significantly faster compared to HFD fed animals for both light treatment (Figure 24a).

Using the area under the curve (AUC), I was able to easily summarize the average glucose tolerance of each group. Both HFD-LD and HFD-PD had significantly higher area under the curve compared to both ND-LD and ND-PD (p < 0.000001) (Figure 24b). This suggests that the high plasma glucose in HFD groups maybe a result of an impairment in the glucose clearance mechanism. Also, HFD-PD had a slightly greater AUC compared to HFD-LD (p=0.067). This mirrors the slight increase in body weight for by HFD PD compared to HFD-LD (Figure 21).
Wheel vs no wheel

Comparing body weight and body composition of animals that have access to wheel versus no wheel access, showed that wheel running impedes weight gain in HFD mice ($p < 0.00001$) (Figure 26). Regardless of access to running wheels, HFD mice significantly gained more weight compared to ND ($p < 0.000001$). HFD mice significantly gained fat mass ($p < 0.000001$). In contrast, ND had a significantly higher lean mass compared to HFD ($p = 0.016$). Both wheel and no wheel experiments exhibited an significant increase in lean mass in PD mice compared to ND (wheel, $p = 0.032$; no wheel, $p = 0.00078$). Fat mass was not significantly different between light treatment groups (wheel, $p = 0.36$; no wheel, $p = 0.47$).
Figure 21: No wheel-HFD fed mice significantly gained weight in the course of the 7 week experiment (p < 0.000001). No wheel HFD-PD mice significantly gained more weight at week 2 compared to no wheel HFD-LD (p = 0.0020). * Significant difference, p < 0.05. Error bars represent standard error of mean.
Figure 22: No wheel-ND fed animals significantly gained weight in the course of the 7 week experiment (p < 0.000001 for both PD & LD). No wheel ND-PD mice significantly gained more weight at weeks 2 and 3 compared to no wheel ND-LD (week 2, p = 0.0020; week 3, p = 0.00037). * Significant difference, p < 0.05. Error bars represent standard error of mean.
Figure 23: Lean mass (a) and fat mass (b) composition of mice without wheels. HFD fed animals gained significantly higher fat mass compared to ND (p < 0.000001). ND gained significantly more lean mass compared to HFD (p = 0.00078). PD mice gained more lean mass than LD mice (p = 0.016). However, light treatments did not show any influence in change in fat mass (p = 0.47). Significant difference: *, p < 0.05; **, p < 0.001; ***, p < 0.000. Error bars represent standard error of mean.
Figure 24: Plasma levels during GTT (a) and AUC (b) for glucose tolerance comparison between groups. There was a significant increase in plasma glucose level in HFD compared to the ND fed animals for both PD and LD groups. Clearance of plasma glucose was significantly faster in ND compared to HFD mice. HFD mice had significantly higher AUC compared to ND-LD and ND-PD (p < 0.000001). * Significant difference, p < 0.05. Error bars represent standard error.
Figure 25: HFD significantly gained more weight compared to ND (p < 0.000001). Access to running wheels significantly impeded weight gain in HFD mice (p = 0.00041). Significant difference: **, p < 0.001; ***, p < 0.00001. Error bars represent standard error of mean.
Figure 26: Lean mass (a) and fat mass (b) difference between wheel and no wheel animals. HFD significantly gained more fat mass ($p < 0.000001$), but had significantly lower lean mass ($p = 0.016$) compared to ND. Animals with access to wheels had significantly lower lean mass and fat mass compared to animals with no wheels (lean mass, $p = 0.0093$; fat mass, $p < 0.000005$). Significant difference: $^*$, $p < 0.05$; $^{**}$, $p < 0.01$, $^{***}$, $p < 0.0001$). Error bars represent standard error of mean.
**Discussion**

*Experiment #1*

Experiments in circadian rhythms have been conducted using a wide range of housing conditions. Animals are housed in different size cages that may or may not be equipped with a running wheel. If housing conditions alter basic clock function, then that could have important implications for comparisons of research findings across different laboratories, potentially altering the interpretation of some of those findings. The influence of running wheel or cage size on clock gene expression has not previously been documented. In Experiment 1, I examined whether cage size or access to a running wheel influenced clock gene expression in three peripheral tissues and the SCN. I predicted that access to a running wheel would alter clock gene expression in the SCN, but not in the heart, liver, or lungs.

All four clock genes that were measured exhibited significant variation across the 24-hour day in the SCN and all peripheral tissues of mice housed in large cages with wheel (LW), large cages (LC), and small cages (SC). The peak expression of these genes mirrored what had previously been reported in previous studies (Table 1). Most significant differences between the housing conditions were observed around the peak of the active phase (ZT 18). This observation suggests that the amount of activity in all groups during the active phase may influence clock gene expression.
Interactions between housing conditions and time point were observed in the liver (Figure 6b), lungs (Figure 8), and SCN (Figure 12), but not in the heart (Figure 9 & 10). My results also showed that housing conditions mainly influenced the expression of *Clock* and *Bmal1*, without affecting *Per1* and *Per2*. Furthermore, the significant differences between housing conditions appear to be more due to the size of the cage rather than wheel access.

Proper housing conditions for laboratory animals are outlined in the *Guide for the Care and Use of Laboratory Animals*. This includes adequate activity space, ready access to food and water, and appropriate vertical height (Bayne, 1996). Both large and small cages used for this experiment met these criteria. Few previous experiments have examined the influence of cage size on behavior of laboratory animals. Furthermore, there is no literature to date on cage size effects on clock gene expression. Mice in SC have decreased locomotor activity compared to LC animals (Poom et al., 1997), which is consistent with similar studies that used different model animals such as monkeys (Crockett et al., 1995) and dogs (Bebak & Beck, 1993) that were also kept in isolated cages. Rats also showed reduced responses to stressors if they are housed in SC (Sharp et al., 2003). With this supporting evidence, it is possible that the decrease in activity and stress response as a result of small cage housing can be a contributing factor to changes in levels of clock gene expression. For this experiment, the role of the running wheels is to provide extra exercise, which amplifies the amount of activity even further.
Interestingly, the extra exercise, increased energy expenditure, and motivational effects of wheel running (Novak et al., 2012) did not significantly influence clock gene expression in the liver, heart, and SCN. The only effect of the running wheels was shown by the suppression of *Bmal1* expression in the lungs of LW mice at ZT 18 compared to both LC and SC (Figure 7a). Since the peak of *Bmal1* expression for LW was still at ZT 0—typical for *Bmal1* rhythmicity in the lungs (Table 1), it is a possibility that the different housing induced the peaks to shift because the peaks for LC and SC are not clearly shown, unlike for LW. But verification of this statement would require further measurements of more than just 4 time intervals across the 24 hours.

Housing conditions mainly influenced the expression of *Clock* and *Bmal1*, without affecting *Per1* and *Per2*. Significant differences between housing conditions were mostly exhibited as reduced *Clock* or *Bmal1* expression in SC compared to LW and LC. Both *Clock* and *Bmal1* are essential elements of the core clock mechanism. *Bmal1* knockout mice are arrhythmic both in the behavioral and molecular levels (Bunger et al., 2000); while *Clock* knockout mice experience a shortened period length (DeBruyne et al., 2006). *Bmal1* expression in the SCN peaks at night and is reduced upon light stimulus (Tamaru et al., 2000). The night time is also the most active phase of mice, indicating that increased *Bmal1* levels coincide with high behavioral activity levels. Furthermore, *Bmal1* knockout mice have decreased activity levels both in a LD cycle and in constant darkness (Bunger et al., 2000). Therefore, it is a possibility that the decreased activity that may have resulted from limited cage size, lead to a feedback on the expression of
Bmal1 itself. Clock mutated mice do not show a similar response of activity outputs (Vitaterna et al., 1994). The significant differences in Clock expression between housing conditions may simply be due to Clock’s close interaction with Bmal1 in a functional clock mechanism. Clock and Bmal1 act as transcription factors that bind to the E-box of various genes not exclusive to Per and Cry genes. Therefore, the influence of housing conditions on Clock and Bmal1 may be due to the heterodimer’s role as a regulator for many physiological functions including locomotor activity.

My results showed that housing conditions had no influence in Per1 or Per2 expression. This is a notable finding in that most studies that examine the phase of peripheral clock gene expression do so using the period genes as endpoint measurements. Some important experimental effects may be missed by not measuring other clock components such as Clock and Bmal1. Per1 or Per2 knockout mice do not show changes in the amount of locomotor activity (Zheng et al., 2001; Zheng et al., 1999). Per1 expression normally peaks between ZT 4-8 in the SCN and ZT 12 in the peripheral tissues, while Per2 expression peaks between ZT 9-12 in the SCN and around ZT 12 in peripheral tissues. Since Per1 and Per2 peak during the animal’s non-active phase, any effect that is relative to the amount of locomotor activity may be minimal at this time point. Also unlike Clock and Bmal1, which serve as transcription factors for many non-clock genes, Per1 and Per2 mainly serve as an inhibitor of Clock-Bmal1 dimerization giving Per genes a main role as a period regulator. The lack of direct
interaction between *Per* genes and other non-clock genes, maybe the reason why the *Per1* and *Per2* expression were not altered by the housing conditions.

Significant differences in clock gene expression between housing conditions were observed in the liver, lungs, and SCN, but not in the heart. The oscillation of clock genes in the SCN has an advanced phase compared to peripheral clocks due to its synchronizing role (Balsalobre, 2002). However, clocks in the liver, lungs, and heart also respond to other physiological functions such as feeding and metabolism (Rudic et al., 2005). The relation between locomotor activity and feeding (Stokkan et al., 2001) may contribute to changes in peripheral clock parameters. If housing conditions alter the amount of activity during the active phase, changes in peripheral clock function should be observed. Surprisingly, my results showed no significant difference in clock gene expression in the heart between housing conditions (Figure 9 & 10). This is consistent with another study that found that hypertrophy of the heart, which is the organ’s response to increased workload, had very little effect on clock genes. (Young et al., 2001). This is interesting because the process of hypertrophy itself involves increased size of the cardiac muscles, which requires changes in both gene expression and protein synthesis. Taken together, all supporting evidence suggest that that clock gene expression in the heart is more resistant to alteration compared to the liver and lungs. The resistance of the heart to clock gene alteration may be a result of the critical role of the heart to continuously function properly.
Because gene expression was only measured at 4 different time points, I used differences in relative expression at each particular ZT as a determining factor of significance. Another way to analyze gene expression data is to look at changes in the phase of peak expression. However, to do so would entail measurement of clock gene expression across a greater range of time points. Doing so would capture a more precise rhythm that shows actual peak time, changes in phase, and change in expression levels.

This experiment examined how different housing conditions, in terms of running wheel access and cage size, alter clock gene expression. But, this study did not identify the actual reason or mechanism for alteration of clock genes. Further experimentation is needed with addition of more controlled groups. One major limitation of our study is the lack of a true control group. A proper control group would be a LC with a locked running wheel. Doing so would examine if any interaction was mainly caused by the presence of the wheel alone. Another control group would be a SC with access to a running wheel and SC with a locked wheel; however, due to equipment and other resource limitations these experimental groups could not be added.

To better strengthen my conclusions it would also be useful to measure and compare general locomotor activity in each housing conditions. Because some groups would require no wheel access, a different method of measuring activity would be needed. Furthermore, examination of the clock genes in the skeletal muscle would also be of interest due to its direct relation to exercise.
Experiment #2

Jet lag disrupts circadian rhythms and has negative effects on physiological function. Simulating chronic jet lag suppresses the immune system (Castanon-Cervantes et al., 2010), increases the likelihood of tumor development (Smalaand et al., 1996; Wu et al., 2012), hastens mortality (Davidson et al., 2006), and causes metabolic disorders (Preuss et al., 2008). Disruption of circadian rhythms has been linked to impairment in metabolic function. Furthermore, consumption of HFD alters patterns of feeding and activity, period, and entrainment (Kohsaka et al., 2007; Mendoza et al., 2008). In this thesis, I hypothesized that weight gain as a result of a HFD will be exacerbated during simulated jet lag, compared to ND fed control mice. Running wheels were also used to examine the amount of activity between diet treatment groups.

Interestingly the HFD-PD group had significantly increased weight gain compared to both HFD-PA and HFD-LD group. No significant difference in weight gain was observed between the HFD-PA and HFD-LD (Figure 14). There were no significant differences in weight gain between all three light treatment groups of ND fed animals (Figure 13). HFD mice showed significantly greater fat mass compared to ND mice. There was no significant difference in fat mass between light treatment groups (Figure 15b). However, both chronic jet lag groups (PA and PD) exhibited significantly increased lean mass compared to LD control (Figure 15a). There was also no significant difference in average locomotor activity between HFD-LD and ND-LD (Figure 20). However, activity of both diet groups significantly decreased after the first week of treatment (Figure 19).
The increased weight gain in the HFD-PD group supports previous findings of Tsai et al. that repeated phase shifts in male F344 rats speed up body weight gain compared to rats kept in stable LD (Tsai et al., 2005). In contrast, female CD2F1 mice that were subjected to weekly 12 hour LD shifts exhibited decreased body weight (Nelson & Halberg, 1986). The discrepancy in results could be due to the different sex or species of animals, or difference in protocol used. The increase in weight gain may be due to alteration in metabolic function. My results are consistent with reports that chronic jet lag negatively affects normal metabolic function (Scheer et al., 2009; Gonnissen et al., 2012). My findings also correlate with existing epidemiological studies on the damaging effects of shiftwork on metabolism (Karlsson et al., 2001; Niedhammer et al., 1996). The underlying mechanisms of how chronic jet lag causes such impairments are still not well understood.

My results suggest that repeated PD caused an increase in weight gain in mice. Weight gain of HFD-PA on the other hand did not show any significant difference from HFD-LD. The difference in the results may be rooted to the different mechanism involved between an adjustment to a PA compared to a PD. Downstream to the activation of NMDA receptors, a PD involve release of calcium through ryanodine receptors (Ding et al., 1998); while a PA involve the cGMP-dependent protein kinase signaling pathway (Weber et al., 1995). Differences in the molecular mechanism between these two phase shifts may explain the variation in responses to a PD versus a PA adjustment. A study in humans showed that appetite and total body expenditure did
not differ between PA and PD groups. However, the PD group displayed increased plasma glucose concentration and decreased leptin concentration; while the PA group exhibited high insulin levels compared to PD and LD (Gonnissen et al., 2012). High glucose and low leptin in PD, and high insulin levels in PA, both suggest impairment in glucose uptake.

In contrast to my results, other studies suggest that PA has more disruptive effects compared to PD. Aged C57BL/6 mice that were subjected to chronic jet lag displayed hastened mortality in PA compared to the PD group (Davidson et al., 2006). Also mice are able to re-entrain to a PD faster than a PA (Reddy et al., 2002), which could be an indicator that a PD requires an easier adjustment for mice compared to a PA. Determining which direction of phase shift contributes to more to changes in metabolic function needs further investigation. Other documented side-effects of chronic jet lag such as disruption of feeding hormones (Gonnissen et al., 2012), sleep deprivation, desynchronization between central and peripheral clocks (Nakamura et al., 2004; Yamazaki et al., 2000), “light at night” effects (Fonken et al., 2013), or suppression of the immune system (Castanon-Cervantes et al., 2010), could also act as contributing factors to the increased weight gain. Also, it should be noted that mice have a tau that is less than 24 hours. Therefore, it is possible that a PA is less disruptive because mice naturally advance in free-running conditions. A weekly PD may require more difficult adjustments compared to weekly PA, thereby causing a greater disruptive effects. Further investigation is needed to examine if increased weight gain is reflective of the
natural free-running direction of the animal. To better explain the link between chronic jet lag and weight gain, an in-depth examination of other metabolic criteria including feeding hormones, food intake, and measure of activity is needed.

As expected, fat mass in HFD fed animals was significantly higher compared to ND fed mice in all three light treatment groups (Figure 15b). Interestingly, both chronic jet lagged groups exhibited increased lean mass compared to LD (Figure 15a). Gonnissen et al. found that total energy expenditure did not differ between PA and PD animals (Gonnissen et al., 2012). In my experiment, the animals were given running wheels that provided increased opportunity for energy expenditure. My results also showed no significant difference in the amount of wheel-running activity between HFD and ND fed animals (Figure 20).

Experiment #3

Results from Experiment #2 showed that chronic PD exacerbates weight gain in HFD animals. Wheel running increases energy expenditure, thereby regulating food intake and body weight regulation (Levin, 1991). In this study, I examined if HFD-PD would exhibit an even greater increase in weight gain if the running wheels were not present. I hypothesized that without access to running wheels, weight gain as a result of chronic PD will be further exacerbated compared to normal LD control mice. Since weight gain and obesity are positively correlated with diabetes, I also examined if PD animals without wheel access will have impaired glucose tolerance. Since chronic PA did
not show any significant effect on weight gain compared to the LD group in, this experiment only examined the negative effects of a chronic PD.

Similar to Experiment #2, PD groups displayed significant increase in weight gain compared to LD groups (Figure 21 & 22). However, the increase was minimal and only occurred at weeks 2 & 3, which followed the 1\textsuperscript{st} and 2\textsuperscript{nd} shift respectively. These findings suggest that one event of PD is enough to promote weight gain. Tsai et al. (2005) found that male F344 rats had increased weight gain when subjected to twice-weekly LD shifts: a phase advance then a phase delay (Tsai et al., 2005). It is possible that they exhibited weight gain because the animals were exposed to two consecutive events of each phase shift. My chronically jet lagged animals may have found the repeated shifts easier, especially if repeated in the same direction. Responses to chronic jet lay may be adaptive. This hypothesis is consistent with another study which found shorter duration of the readjustment to phase shifts following the initial shift (Yan, 2011).

The GTT results showed that HFD-PD animals had a slightly impaired glucose tolerance compared to HFD-LD (Figure 24). Though not significant, this slight difference mirrors the small increase in weight gain suggesting that the effect of PD may be minimal. HFD animals also had a slower rate of glucose clearance compared to ND animals, displaying the destructive effects of HFD consumption. My results are consistent with a study by Surwit et al., where HFD fed C57BL/6J mice showed impaired glucose tolerance and insulin resistance as type II diabetes markers (Surwit et al., 1988).
It is surprising that that the PD animals without wheels had only a minimal increase in weight gain, compared to PD animals with wheel access that had a more significant increase in weight gain. This suggests that interaction between chronic phase delays and wheel access is complex. HFD resulted in increased weight gain regardless if a wheel was present or not. However, the presence of the wheel resulted in attenuation of weight gain, with greater effects in HFD compared to ND fed mice (Figure 25). This is mostly likely due to the extra exercise opportunity given by free access to a running wheel. To further study the relation between chronic phase delay and wheel access, it would be worthwhile to measure the autonomic outflow to muscle when running wheels are used between PD and LD groups. In contrast, a study by Jung & Luthin found that wheel access did not attenuate weight gain in HFD fed mice. In their study, the daily caloric intake was significantly higher in HFD mice, but there was no difference in food consumption between wheel or no wheel mice (Jung & Luthin, 2010).

To sum up all my results, the increased weight gain by chronic PD was not dependent on either wheel access or diet.

For both Experiment 2 and 3, fat mass was greatly increased by HFD but was not influence by light treatment (Figure 23b). Similar to Experiment 2, no wheel PD mice gained more lean mass compared to LD (Figure 23a). Lean mass is consistently increased in chronic jet lagged mice compared to LD control, regardless of phase shift direction. This is very interesting, since increased weight gain seemed to be more direction dependent. Lean mass was also significantly increased in ND group compared to HFD
groups (Figure 23a). HFD not only increased fat mass, but it may also decrease lean mass. Animals with access to wheels had significantly gained less lean mass and fat mass compared to animals with no wheels (Figure 26). This displays the exercise effect of wheel running and is supported by a previous study on Syrian hamsters, where wheel running resulted in decrease in body fat mass (Coutinho et al., 2006). These results suggest that the presence of a wheel, as a form of exercise, alters body composition.

Conclusions

When evaluating clock gene expression, housing conditions need to be taken in consideration in experimental set ups to eliminate variability in results. The size of the cage may contribute to the alteration of clock genes in liver, lung, and SCN, especially during the active phase. Cage size mostly affected Clock and Bmal1 expression, without affecting Per1 and Per2, thus suggesting that measurements of Clock and Bmal1 expression may be more suitable when examining phase of clock gene.

Weight gain is highly increased by HFD. This weight gain is exacerbated when animals are subjected to chronic phase delay. However, increase of weight gain as a result of phase delay is minimal and is not dependent on wheel access or diet. Wheel running serves as an exercise component and attenuates increased weight gain. Disruption of the clock by simulated chronic jet lag consistently resulted in increase in lean mass regardless of the direction of the shift. Increased weight gain by HFD is accompanied by an increase in fat mass and impairment in glucose tolerance, thus
showing promotion of pre-diabetic symptoms. This study supports the well-documented negative effects of circadian disruption by simulating chronic jet lag.
References


