The Effect of a Disrupted Light-Dark Schedule on Fear in Mice

by

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A Thesis

Presented to the Faculty of Mount Holyoke College

in partial fulfillment of the requirements

for the degree of Bachelor of Arts

with Honors

Program in Neuroscience and Behavior

Mount Holyoke College

South Hadley, Masachusetts

ACKNOWLEDGMENTS

I thank my thesis advisor, Will Millard, for continually pushing me to be more and do more. Your comments encouraged me to be thorough and to pay attention to the smallest detail. Over this year, I have grown tremendously as a scientist, and also as an individual. I also thank the members of my thesis committee, Karen Hollis and Jeff Knight, for their encouragement and thoughtful comments throughout the year. I also thank Deborah Piotrowski, the curator of the vivarium, for her patience and grace in accommodating the requirements of my project.

I thank my lab partners, Irem Durman, Jackie Jackter, and Mahdiya Ahmed for their help and dedication to this project. To my classmates, Amee Phan, Skylar Robinson and Rosa Frias, I say thank you. Your companionship was absolutely instrumental to my success.

Hearty thanks to my friends and family, whose love and encouragement have seen me through this process.

I thank Mr. and Mrs. Harap for their genourous financial support towards my project.

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ABSTRACT

The objective of the present study was to assess the effect of a disrupted light-dark (LD) schedule on fear and body weight in mice. Female and male Swiss Webster mice ($N = 71$) were exposed to either a normal 12 : 12 hr LD schedule or a disrupted 12 : 12 hr LD schedule for 35 days, after which fear was assessed in two behavioral assays. To assess to the effect of LD disruption on body weight, body weights were measured every 5 days during the course of the LD disruption. Possible differences between females and males were also explored. For all but one variable in the behavioral tests, results showed that LD disruption no effect on fear. However, increased defecation frequency in animals exposed to the disrupted schedule shows a fear-increasing effect of LD disruption. Increased time in the periphery as well as increased defecation in males in the open field show that males were more fearful than females. However, more time in the light section of the light-dark box and longer latency to enter the dark section of the box show that males were less fearful than females in the light-dark box. Females made more frequent entries to the light section, indicative of reduced fear as well as higher locomotor activity in females than males in the light-dark box, a finding which is consistent with the literature. LD disruption also did not affect body weight. Because of contrasting results from variables in the behavioral tests, the effect of the disrupted LD schedule on fear in the present study is unclear.

INTRODUCTION

From algae to mammals, many organisms have physiological processes that occur in roughly 24 hr cycles and are thus said to have circadian rhythms (Arble, Ramsey, Bass, & Turek, 2010; Sharma, 2003). These circadian rhythms are maintained internally by groups of cells, which act as time keepers, coordinating physiological processes within the organism and synchronizing the processes with environmental cues such as the light-dark (LD) cycle and time of food presentation (Arble et al., 2010). Such environmental cues provide organisms with information about the light and dark periods and are thus called Zeitgebers, a German word meaning *time-givers* (Aschoff, 1960; Lakin-Thomas & Brody, 2004). By synchronizing their biological processes with environmental factors, organisms can better adapt to changes in the environment and thus increase reproductive success and survival (Arble et al., 2010; Kalsbeek, van der Spek, Lei, Endert, Buijs, & Fliers, 2012). For instance, by synchronizing internal processes with the LD cycle, some kinds of cyanobacteria are able to confine nitrogen fixation to the dark phase of the LD cycle, as oxygen produced during photosynthesis in the light phase would inhibit nitrogen fixation during the day (Mitsui et al., 1986). Similarly, for diurnal chipmunks, synchronization of reduced locomotor activity with the dark cycle reduces the risk of detection by nocturnal predators like weasels (DeCoursey, Walker, & Smith, 2000).

The present study examines the effect of a disrupted LD schedule on fear and metabolism in mice. Thus, the following sections of the introduction review relevant literature on circadian rhythm disruption induced either genetically or by disrupting LD schedules. The first section addresses circadian rhythm regulation in mammals and examines how behaviors such as shift work and trans-meridian travel common in humans result in circadian rhythm disruption by desynchronizing the LD and sleep-wake cycles. The second section reviews literature that shows that altering LD conditions results in disrupted circadian rhythms in animals, establishing light as the main Zeitgeber in animals. The third section reviews literature on the effects of circadian rhythm disruption on fear, a question addressed in the present study. This section also reviews methodology used in assessing fear in animals. The fourth section reviews existing literature on the effects of altered LD conditions on metabolism, a question also addressed in the present study. A possible explanation for the effects of altered light condition on fear and metabolism could be that altered LD conditions cause sleep loss in animals (Castanon-Cervantes et al., 2010). If this is the case, then it is the sleep loss caused by altered LD conditions, and not the altered conditions themselves, that causes the observed effects. Therefore, the next section reviews literature that examines whether altered LD conditions result in sleep loss in animals.

The sixth section reviews literature on sex differences in the effect of altered LD conditions on fear. The section also reviews sex differences in results

from different behavioral assays. In the last section, the hypotheses assessed by the present study are given and the methodology used, briefly described.

1. Circadian Regulation in Mammals

In mammals, the suprachiasmatic nuclei (SCN) form the region in the brain that coordinates circadian rhythms (Buijs & Kalsbeek, 2001; Laposky, Bass, Kohsaka & Turek, 2008). Lesion studies showed that damage to the SCN resulted in the abolition of nocturnal drinking, locomotor activity patterns, and corticosterone rhythms, thus implicating the SCN as the regulatory center of the circadian system (Moore & Eichler, 1972; Silver & Schwartz, 2005; Stephan & Zucker, 1972). The SCN, located in the anterior hypothalamus, above the optic chiasm, receives information about the LD cycle through the retinohypothalamic pathway (Laposky et al., 2008). Formed by non-rod, non-cone photoreceptors known as retinal ganglion cells, the retinohypothalamic pathway is made up of two major branches—the medial and lateral branches—that proceed from the retina and innervate four regions of the hypothalamus, including the SCN (Canteras, Ribeiro-Barbosa, Goto, Cipolla-Neto & Swanson, 2011; Pierson, Thompson, Hankins, & Foster, 2005; Provencio, Cooper, & Foster, 1998). A study that used an anterograde tracer, *Phaseolus vulgaris* leucoagglutinin, to label SCN projections initially confirmed the transmission pathway between the SCN and different hypothalamic regions (Buijs et al., 2006; Watts, Swanson, & Sanchez-Watts, 1987). When the SCN receives light information from the retina,

it transmits a circadian signal to endocrine neurons and other regions of the hypothalamus, which in turn, communicate with other parts of the brain, peripheral clocks and hence, the rest of the organism (Buijs & Kalsbeek, 2001). The SCN thus regulates various circadian cycles in the organism such as those that control locomotor activity, cortisol and melatonin secretion, sleeping, and waking (Buijs & Kalsbeek, 2001; Pittendrigh, 1960).

The regulation of the sleep-wake cycle is especially peculiar in humans because of the presence of artificial lighting and behaviors like shift work and rapid trans-meridian travel, unique to humans. Unlike other animals, humans alter light conditions—extending or contracting the duration of the light or dark period—at will (Honma, Honma, & Wada, 1987; Wever, Polasek, & Wildgruber, 1983). Also, because of work and social activities, humans are sometimes active at night when diurnal animals are naturally less active. Trans-meridian travelers often experience a desynchronization between sleep-wake cycles and the external LD cycle and shift workers are faced with having to adjust irregular work hours and readjust to regular schedules when they are off work for short periods (Harrison & Gorman, 2012). Because shift workers often adjust their eating habits to their work schedules, metabolic diseases like diabetes as well as some gastrointestinal disorders have been correlated to the circadian rhythm dysfunction experienced during shift work (Arbel et al., 2010; Choobineh, Soltanzadeh, Tabatabaee, Jahangiri, & Khavaji, 2012; Knutsson & Bogglid, 2010;

Preuss et al., 2008). Cardiovascular diseases and higher incidences of colon and breast cancer have also been associated with circadian rhythm dysfunction in shift workers and frequent trans-meridian travelers (Haus & Smolensky, 2006; Preuss et al., 2008). Although circadian rhythm dysfunction has been studied using human subjects, for practical reasons, animal models of circadian rhythm dysfunction have also been developed to enhance understanding of circadian regulation (Arble et al., 2010; Kudo, Loh, Truong, Wu, & Colwell, 2011). Thus, research on circadian regulation has been carried out using animals such as rats, mice, chipmunks and the fruit fly, *Drosophila melanogaster* (Arble et al., 2010; DeCoursey et al., 2000; Kudo, Loh, Truong, Wu, & Colwell, 2011; Sivaperumal, Subramanian, Yadav, & Sharma, 2011).

The reviewed literature identifies the SCN as the primary brain region for circadian rhythm regulation and introduces the roles of environmental cues such as the LD cycle in the entrainment of the SCN. Shift work and trans-meridian travel desynchronize people's activity patterns with the external LD cycle and thus disrupt circadian rhythms. Animal models of different types of circadian dysfunction have been developed to aid deeper understanding of circadian regulation.

2. Does LD Schedule Alter Circadian Rhythms?

Constant light causes the circadian period—the time to complete one circadian cycle—to shorten in diurnal animals and lengthen in nocturnal animals,

a phenomenon referred to as Aschoff's rule (Emery, Stanewsky, Hall, & Rosbash, 2000; Konopka, Pittendrigh, & Orr, 1989; Pittendrigh, 1960). Research with *Drosophila* also shows that light signals that occur before subjective dawn, the period that coincides with the dark-light transition in a normal LD cycle, results in a phase advance in the eclosion rhythm of the organism. Light signals occurring after subjective dawn result in a phase delay while light signals that coincide with subjective dawn, result in no resets in the organism's eclosion rhythm (Pittendrigh, 1960). Another study using male chaffinches, which are diurnal birds, also showed that under conditions of constant illumination, the period of the circadian clock was longer when a lower light intensity was used than when a higher light intensity was used. With the lower intensity, the activity period, that is, the period in which the bird was moving around in its cage, was shorter, and the rest period longer, than with the higher intensity (Aschoff, 1960).

A study in male F344 rats also shows the effects of shifting LD schedules on circadian rhythms (Tsai, Tsai, Hwang, Huang and Tzeng, 2005). Rats in the experimental group were exposed to biweekly 12 hr shifts of the LD schedule for 13 weeks while rats in the control group were exposed to a constant 12 hr LD schedule. Results showed that the temperature rhythm of rats in the experimental group exhibited a phase delay in the first 4 - 5 weeks and subsequently, a mixture of phase delays and phase advances. The circadian heart rate and activity rhythms

also responded in similar ways to the LD shifts, suggesting that LD shifting resulted in continual circadian phase shifts in the rats.

In another study, the effects of an abrupt LD shift on the SCN were assessed in male Wister rats (Nagano et al., 2003). Animals were first entrained to a 12 hr LD schedule for two weeks and then either exposed to a 10 hr phase delay, by shifting the lights-on time forward by 10 hr, or a 6 hr phase advance, by shifting the lights-on time backward by 6 hr. To determine the effect of the shifted LD schedule on circadian rhythms, researchers monitored the expression of the circadian protein, *rPer1*, which cycles continuously in the SCN, with high expression of the protein occurring during the day and low expression during the night. Results showed that the abrupt LD shift desynchronized the rhythms of the two subdivisions of the SCN—the ventrolateral SCN and the dorsomedial SCN. The ventrolateral SCN, which receives direct retinal innervation, responded rapidly to the light shift, while the dorsomedial SCN, which is not directly innervated from the retina, responded slowly to the light shift. During the desynchrony, the rats exhibited increased night time rest, as well as shortened activity patterns and elongated rest periods. To determine the time it takes the subdivisions to resynchronize, researchers monitored the expression profile of *rPer1* mRNA. Circadian rhythms in the two subdivisions resynchronized after 7 days, for the rats exposed to the 10 hr phase delay and after 13 days, for the rats that were exposed to the 6 hr phase advance. These results show that changes in

LD conditions lead to desynchrony within the subdivisions of the main biological pacemaker, the SCN.

Research shows that this desynchrony also occurs in peripheral organs in the body (Davidson, Castanon-Cervantes, Leise, Molyneux, and Harrington, 2009). In one study, after entrainment to a 12 : 12 hr LD cycle, mice were exposed to a 6 hr phase advance, after which rhythms in the SCN and body organs such as the thymus, spleen, lung and esophagus were assessed. Rhythms in the different organs showed varying responses to the light shift, and remained desynchronized until 8 days after the phase advance. Neurons in the ventral and dorsal regions of the SCN exhibited a 2 hr phase difference and regained synchrony with each other 8 days after the phase advance. This study again shows the desynchronizing effects of a single LD shift on the circadian system.

In another study that models shift work more closely, internal desynchronization was assessed in rats forced to work during their sleep phase (Salgado-Delgado, Angeles-Castellanos, Buijs, and Escobar, 2008). For the 5-week duration of the experiment, male Wistar rats were forced to exercise during their sleep phase by putting them in rotating drums for 8 hr of the light cycle for five consecutive days of the week, to model the human experience of night-work. Control rats were either left undisturbed in their home cages or forced to work during the dark phase of the LD cycle. The LD cycle was kept constant throughout the course of the experiment. Animals forced to work during the sleep

phase showed progressively less nocturnal activity over the course of the experiment and shifted most of their food intake to the light phase of the LD cycle. These rats also exhibited glucose arrhythmicity as well as reversed rhythmicity for triacylglycerols. Animals that were forced to work in the dark phase or left undisturbed did not show such changes in their rhythms. However, for the experimental and control groups, circadian rhythms of nocturnal corticosterone were similar and the temporal patterns of clock proteins, *per1* and *per2*, remained synchronized with the LD cycle. Similar to results described above, these results show that internal desynchronization can occur when activity patterns are not properly synchronized with the LD cycle.

The studies reviewed above show that altering light conditions, whether once or several times, alters circadian rhythms in the SCN as well as in peripheral organs.

3. Do Disrupted Circadian Rhythms Affect Fear?

Several mood disorders like seasonal affective disorder, bipolar disorder and major depressive disorder have circadian rhythm disruption as a common symptom (McClung, 2007; Turek, 2007). Also, many of the treatments used to treat mood disorders involve shifting rhythms in the SCN, suggesting that the circadian system is involved in mood regulation (McClung, 2007). Thus, literature on the effects of circadian rhythm disruption on emotion, specifically fear, is reviewed here. In these studies, researchers induced circadian rhythm

disruption in rodents either genetically or by altering light conditions. Here, fear is defined as rodents' aversion to brightly lit, open spaces, similar to the human aversion to threatening situations such as to open or public spaces as in agoraphobia (American Psychiatric Association, 1994).

Circadian rhythm disruption has been shown to affect fear in rodents. To study fear in non-verbal animals, behavioral paradigms like the open field, the light-dark box, and the elevated plus maze have been developed (Lister, 1990). The open field consists of an open space, surrounded by walls to prevent the subject from escaping, which may be further divided into smaller regions. Though initially developed to study fear in rats, the open field is now used in studies on animals like rabbits, calves, honeybees and fruit flies (Hall, 1934; Prut & Belzung, 2003; Soibam et al., 2012; Walsh & Cummins, 1976). Defecation, locomotor activity and time spent in the center of the open field are recorded and used to assess the animal's fear. When placed in a novel environment, an animal will tend to freeze and defecate more. The animal will also spend more time in the periphery of the space than in the center, a behavior known as thigmotaxis. Thus, an animal that moves around in the open field, spends less time in the periphery, and defecates less exhibits less anxious behavior (Lister, 1990; Prut & Belzung, 2003). An animal that is less anxious would also be more active in the open field (Lister, 1990).

Discrepancies in results from the open field test and other measures of fear like the elevated plus maze however raise the question of validity, specifically, construct validity, that is, whether the open field is actually measuring what it is thought to measure (Carola, D'Olimpio, Brunamonti, Mangia & Renzi, 2002). For example, one study showed that while a neuroactive drug, chlordiazepoxide increased the frequency of open arm visits in the elevated plus maze, a behavior indicative of reduced fear, the same dose of the drug did not affect behavior in the open field (Lalonde and Strazielle, 2010). However, another study showed correlations between results from the elevated plus maze and the open field (Carola, D'Olimpio, Brunamonti, Mangia, and Renzi, 2002). Because of these inconsistencies, some studies used more than one behavioral paradigm to assess fear. For example, one study used a battery of tests including the open field, elevated plus maze and light-dark box to assess the effect of chlordiazepoxide on fear in mice (Hussin, Fraser, Ramos, & Brown, 2012). Thus, in the present study, in addition to assessment in the open field, fear in mice was assessed in the light-dark box.

The light-dark box, another behavioral paradigm used to measure fear in non-verbal animals, consists of a box divided into two compartments, one with high illumination and the other with low illumination (Bourin, & Hascoet, 2003; Crawley & Goodwin, 1980; Lister, 1990). Different studies have varied the proportions of the lit and darkened compartments, with some using equal sizes for

both sections and others using larger lit compartments (Belzung, Misslin, Vogel, Dodd, & Chapouthier, 1987; Crawley & Goodwin, 1980). The test measures behavior that is both a factor of the animal's aversion to brightly lit spaces and its motivation to explore a novel environment (Bourin & Hascoet, 2003; Lister, 1990). Measures assessed from the test include time spent in each of the compartments, number of transitions between compartments and latency to enter one or the other compartment. An animal that spends more time in the lit compartment than in the dark compartment is regarded as exhibiting less anxious behavior (Bourin & Hascoet, 2003; Lister, 1990).

Circadian mutants, which express certain circadian malfunctions, have been vital in research exploring the relationship between circadian disruption and fear. For example, in one study, a strain of mice called After hours, *Afh,* with a mutation which resulted in a lengthened circadian rhythm of 27 hr, was tested in several behavioral paradigms including the open field (Keers et al., 2012). They found that *Afh* mice made more transitions to the inner zone of the open field and also spent more time in the inner zone than wild type mice. Results from the light-dark box also suggested that circadian disruption reduces fear in mice. Animals spent more time in the lit compartment of the light-dark box and entered the lit compartment more frequently than wild type mice.

Another study examined the effect of circadian disruption on fear in the mutant mice, *PK2(-/-)*, which lack the gene for prokineticin 2, an important

signaling molecule in circadian system (Li, Hu, & Zhou, 2009) . *PK2(-/-)* mice displayed less fear, making more entries into the open arms of the elevated plus maze and also spending more time in the open arms than wild type mice. Mice also spent more time in the lit part of the light-dark box and had shorter latencies to enter the lit part than wild type mice. Similarly, in another study, mice that lacked a gene important in the circadian system, *Clock,* exhibited behaviors associated with reduced fear—they spent more time in the center of the open field and entered the open arms of the elevated plus maze more frequently than wild type mice (McClung, 2011; Roybal et al., 2007).

Studies that exposed animals to changing LD schedules also suggest a fear-reducing effect of circadian disruption. In a study assessing the effects of circadian disruption on the brain, metabolism and behavior of mice, male C57BL/6 mice were either exposed to a 10 : 10 hr LD cycle or 12 : 12 hr LD cycle for 9 weeks (Karatsoreos, Bhagat, Bloss, Morrison, & McEwen, 2011). To assess behavior, mice were tested in the open field and light-dark box after the light treatment. Results showed that the amount of time spent in the center of the open field or in the light section of the light-dark box did not differ for animals exposed to either LD cycle. However, animals exposed to the 10 : 10 hr LD cycle entered the center of the open field more rapidly than animals that were exposed to the 12 : 12 hr LD cycle. Similarly, animals exposed to the shorter LD cycle showed a decreased latency to enter the light section of the light-dark box than

animals exposed to the 12 : 12 hr LD cycle. Decreased latency to enter the center of the open field and the light section of the light-dark box therefore show a fear-reducing effect of circadian disruption.

The above studies support a fear reducing effect of circadian dysfunction in rodents. However, a majority of studies on circadian rhythm disruption use genetically altered mice. Very few studies use normal mice to assess the effect of altered LD schedules on fear. Thus the present study assessed the effects of a disrupted LD schedule on fear in genetically unaltered mice using the open field and light-dark box.

4. Circadian Rhythm Disruption And Body Weight

The study in which experimental animals were exposed to a 10 : 10 hr LD schedule also showed that LD disruption leads to increased body weight (Karatsoreos, Bhagat, Bloss, Morrison, & McEwen, 2011). Mice exposed to the shortened LD schedule showed an increase in body weight that only became noticeable four to five weeks after light treatment had begun. The increase in body weight became significant in the sixth week, and continued rapidly through the tenth week. The experimental group also showed an increase in plasma leptin and insulin, hormones that are important in fat and glucose regulation, suggesting that disrupted LD schedules might affect metabolism.

The effects of circadian disruption in *Clock* mice with a mutation on an important circadian gene, *Clock,* which results in longer circadian periods of

about 27 to 28 hr were also examined (Turek et al., 2005). (A period is the time taken to complete one cycle) When given a high-fat diet, *Clock* mice gained more weight than wild type mice and displayed high blood levels of cholesterol, glucose, leptin and triglycerides as well as low insulin levels, which are indicators of metabolic dysfunction. Turek and colleagues suggests that altered feeding rhythms, that is, increased food intake in the light phase, might be responsible for the observed weight gain. Similarly, in their study with F344 rats, Tsai, Tsai, Hwang, Huang and Tzeng (2005) found that rats exposed to repeated LD shifts, had greater body weights even up to Day 10 of recovery than rats that kept under a constant LD schedule.

In contrast, some studies show that frequent LD shifts do not affect body weight in healthy animals. However, when animals are already exposed to health challenges such as disease, repeated LD shifts appear to exacerbate the challenges. For example, in one study, mice were entrained to a 12 : 12 hr LD cycle for two weeks, and then kept either under a 12 : 12 hr LD cycle that shifted every five days or a fixed 12 : 12 hr LD cycle for three months. Results showed that LD shifts do not affect body weight in healthy mice. However, when the mice were given Dextran sodium sulfate (DSS) to induce colitis, an inflammatory bowel disease, mice in the shifted cycle group weighed significantly less than those in the fixed cycle group (Preuss et al., 2008). Another study found that LD

shifts did not affect body weight in CD1F2 mice, suggesting that LD shifts do not have an effect on healthy animals (Nelson & Halberg, 1986).

Similarly, a study by Davidson et al. (2006) also found that circadian disruption affected aged animals more severely than young animals. Young (8 - 12 weeks) and old mice (27 - 31 weeks) were exposed to either a 6 hr phase advance or 6 hr phase delay for 8 weeks. LD phase shifts affected old mice more than young mice, with older mice displaying a significantly higher mortality rate than younger mice. In another study, young mice (3 – 6 months) and old mice (22 – 28 months) were exposed to a 6 hr phase advance in the LD cycle (Sellix et al., 2012). Results showed that the change in the SCN as a result of the LD shift was larger in old mice than in young mice. Also, re-entrainment after the LD shift took a longer time in old mice than in young mice. As with colitis in the study by Preuss et al. (2008), old age may have exacerbated the effects of disrupted LD schedules (Davidson et al., 2006).

Studies on rodents show inconsistent results in the effect of altered LD cycles on body weight, with some studies showing an effect and others showing no effect. Thus, the present study assessed the effect of circadian disruption, as a result of repeated LD shifts, on body weight in mice.

5. Does Circadian Rhythm Disruption Cause Sleep Loss?

Research shows that the negative effects of circadian disruption are not as a result of sleep loss, that is, circadian disruption does not lead to a reduction in

the total amount of sleep for the animal. In a study, *Per2*^{Luc} knock-in mice (mice genetically engineered to express a protein that traces the protein *mPer2* along its circadian pathway) were exposed to one 6 hr phase advance or weekly 6 hr phase advances for 4 weeks (Castanon-Cervantes et al., 2010). Core body temperature, locomotor activity and sleep patterns of the mice were collected via surgically implanted telemeters. Histological procedures were also conducted at different stages in the experiment. While central and peripheral circadian rhythms were altered by the light treatment, total duration of sleep during the experiment was similar to baseline values of the individual mice. Interestingly, on two non-consecutive days during the fourth shift phase, rapid eye movement (REM) sleep increased. From the data obtained, Castanon-Cervantes et al. hypothesized that rather than circadian disruption resulting in sleep loss, the phase of their sleep-wake cycle might be shifting to accommodate the light shift, as did the animals' core body temperatures.

Similarly, another experiment confirmed that circadian arrhythmicity in rats exposed to constant light conditions did not affect total amounts of REM sleep, non-rapid eye movement (NREM) sleep and wake episodes (Mueller, Mear, & Mistlberger, 2011). Furthermore, they found that REM sleep deprivation, achieved by placing the rats on a platform surrounded by water, reduced cell proliferation in the hippocampus. However, circadian disruption as a result of exposure to constant light conditions did not affect hippocampal cell proliferation,

suggesting that the mechanism by which REM sleep deprivation affects hippocampal cell proliferation does not involve circadian arrhythmicity. Loh et al. (2010) also found that while a 6 hr phase advance in the LD cycle shifted rest patterns in C57BL/6 mice, the advance did not result in sleep deprivation. Similar to findings of Castanon-Cervantes et al. (2010), a 6 hr phase delay in the LD cycle rather led to an increase in the amount of sleep.

These studies show that the effects of circadian rhythm disruption caused by shifting LD schedules is not a result of sleep loss.

6. Do Females And Males Respond Differently?

Sex Differences in Response to Disrupted LD Schedules and Circadian Rhythm Disruption. Although the literature suggests that differences exist between sexes in response to circadian rhythm disruption, most of these differences are observed in human studies (Karlsson, Knutsson, & Lindahl, 2001). Not many studies have explored sex differences in response to LD disruption (Nelson & Halberg, 1986). However, in a study evaluating drug-induced circadian disruption, Ahowesso et al. (2011) administered irinotecan, a topoisomerase I inhibitor used in cancer treatments, to mice at sex-specific dosing times. Circadian disruption was assessed using rest-activity patterns, body temperature, body corticosterone and liver mRNA expressions of certain clock genes. The results showed a minimal circadian rhythm disruption in both sexes when irinotecan was administered at a previously determined optimal dose time.

However, when irinotecan was administered at a previously determined worst dose time, severe circadian rhythm disruption was observed in female mice while there was no difference between optimal and worst dose time responses for male mice. As majority of the studies on circadian rhythm disruption and LD shifting have used male mice this study explored the effects of repeated LD shifts, and thus circadian disruption, on fear levels of male and female mice.

Sex Differences in Behavioral Assays. Studies have shown that different sexes behave differently in tests of fear like the elevated plus maze and the light-dark box. In a study assessing the anxiogenic or fear-increasing effects of predator stress in mice, male and female mice were randomly assigned to three groups and for 10 min, were exposed to a cat, a room with a cat in it or were not exposed to a cat. Results showed that male and female mice responded anxiously towards different stimuli. Female mice appeared to be more anxious when they were exposed to a room with a cat in it than when they were exposed to the other conditions while male mice appeared to be more anxious when exposed to the cat than when exposed to the other stimuli (Adamec, Head, Blundell, Burton, & Berton, 2006). This finding suggests possible differences in the way fear is modulated in male and female mice, causing certain stimuli to be more anxiogenic for one sex than the other.

Another study evaluated baseline behavioral responses of rats in several behavioral paradigms including the open field test (Simpson, Ryan, Curley,

Mulcaire, and Kelly, 2012). The results showed that in the open field test, females moved a greater distance and at a greater velocity than males. However, males and females did not differ in time spent in or frequency of transitions into the center of the open field. The same result was observed with the elevated plus maze, with the females being more active than the males but no significant difference between the two groups was observed in connection with number of entries into the open arms of the maze.

Sex differences might also be mediated by the estrus cycle in females. A study assessing the influence of the estrus cycle on behavior in two strains of mice found that behavior of females of one strain was different depending on phase of the estrus cycle. However, behavior in the other strain was unaffected by the estrus cycle (Meziane, Ouagazzal, Aubert, Wietrzych, & Krezel, 2007). Another study also showed differences in exploratory behavior of individually housed female and male mice (Palanza, Gioiosa, & Parmigiani, 2001). Although males showed increased exploratory behavior when given the choice to stay in their cage or explore an open field, females tended to stay in their home cages. In addition, this decreased exploratory behavior in females was influenced by the phase of females' estrus cycle.

The above studies examined sex differences in the effect of circadian rhythm disruption and in different behavioral tests. Although the literature shows sex differences in some variables used to measure fear in different behavioral

tests, the available literature is inconclusive as to whether sex differences exist in response to altered LD schedules. Thus, the present study assessed whether females and males respond differently to altered LD schedules.

7. The Present Study

The objectives of the present study were to assess the effects of a disrupted LD schedule on fear and body weight, in young adult male and female mice. Animals were exposed to a 12 hr phase disruption every 5 days for 5 weeks. To assess the effect of the disrupted LD schedule on body weight, body weight measurements were taken once every 5 days, on the fifth day of the phase shift. At the end of the light treatment, to assess the effects of the disrupted light conditions on fear, mice were tested once in the open field for 10 minutes, divided into two consecutive 5-minute periods.

As described earlier, to measure fear, percent time in the periphery of the open field, frequency to enter the center, latency to enter the center and defecation frequency were obtained. To measure locomotor activity, total distance moved during the trial was also obtained. As an additional measure of fear, the same mice were tested for five minutes in the light-dark box two days later. The variables obtained to measure fear were percent time spent in the light section of the light-dark box, frequency to enter the light section, latency to enter the dark section and defecation frequency. Possible differences between females and males were also explored, making the design of the experiment a full factorial design.

The effect of the LD disruption on body weight over the course of the study was also assessed. These results, as with results from the fear tests, were evaluated to assess sex differences. Results from genetically altered mice show a fear-reducing effect on mice. However, evidence from unaltered mice is not sufficient to make a prediction on the effect of the LD disruption on fear. However, it is expected that female mice would be more active than male mice in the open field and light-dark box but no significant difference in fear between the males and females.

METHOD

Subjects

Eight-week old male and female ND4 Swiss Webster mice were obtained from Harlan Laboratories, Indianapolis, IN. Mice were housed separately in polypropylene cages (11-1/2 [L] x 5 [W] x 7-1/2 [H] in., Lab Products Inc., Seaford, Delaware) with stainless steel wire bar covers and corncob bedding (Harlan Laboratories, Indianapolis, IN). Room temperature was controlled (70 ± 5 °F) as well as humidity (37 ± 5%). Tekland Global Rodent diet (Harlan Laboratories, Indianapolis, IN) and water were available at all times. Cage litter was changed every 10 days.

Depending on the light treatment condition, mice were exposed to either 12-hr LD disruptions every 5 days or a control schedule for the course of the experiment. Male and female mice were randomly assigned to one of the two conditions. The two treatment groups were housed in the same room prior to the start of the light treatment but were transferred to two different but similar rooms once the light treatment began. After the light treatment, animals were tested in the open field and light-dark box. The animals were naïve prior to testing in the open field.

The procedure was replicated three times resulting in total $N = 71$. The control group contained 33 animals and the experimental group, 38 animals. There were 35 females and 36 males.

Apparatus

Open Field. The open field was used to assess fear in mice. The apparatus consists of an open box (40 [L] x 40 [W] x 20 [H] cm) made of clear acrylic placed on a gray background (see Figure 1). Two identical 60 W incandescent lamps above the open field provided illumination during trials. The amount of light reflected by the floor of the open field was 1.58 candelas per square meter (Minolta Luminance Meter, Model LS-100). A video camera, placed 107cm above the field, recorded the movements and behaviors of the mice during trials.

An automated video tracking system, EthoVision®, received the signal from the camera in an adjoining room (Noldus, 2001). For the purpose of tracking, the open field was divided into two areas—the periphery and center. The boundary between the areas was not visible to the mice in the open field (see Figure 1).

Following each trial, the walls and floor of the open field were wiped with an ammoniated solution.

Light-Dark Box. The light-dark box was used as another procedure to measure fear. The light-dark box is identical to the open field previously described. However, to create distinct light and dark regions, half of the box was

covered with black cardboard, with a piece of cardboard fixed over the top to create a roof. The other half of the box, the light section, was left uncovered (see Figure 2).

Illumination from light reflected by the floor of the light-dark box was 9.91 candelas per square meter in the light section and 0.09 candelas per square meter in the dark section. The video tracking system described previously was used with the light-dark box.

Procedure

Animals were first acclimated to light conditions in the housing facility and then exposed to the LD treatment. After the light treatment, animals were tested in the open field and light-dark box.

LD Schedule. Female and male mice were acclimated to a 12 : 12 hr LD cycle for 10 days, during which lights were on from 0700 hr EST to 1900 hr EST. The animals then were randomly assigned to the control LD schedule or the disrupted LD schedule. On Day 11, the animals were transferred to one of two similar rooms, where they remained for the 35 days of the light treatment and the two non-consecutive days of testing. The length of treatment was chosen because previous research found changes in body temperature rhythms and rhythms in the suprachiasmatic nucleus and peripheral organs after less than 4 weeks of light-dark disruption (Castanon-Cervantes et al., 2010; Davidson et al., 2010).

Figure 1. Open field (40 [L] x 40 [W] x 20 [H] cm) showing the center and the periphery. The yellow and blue lines were not visible to the subject in the open field.

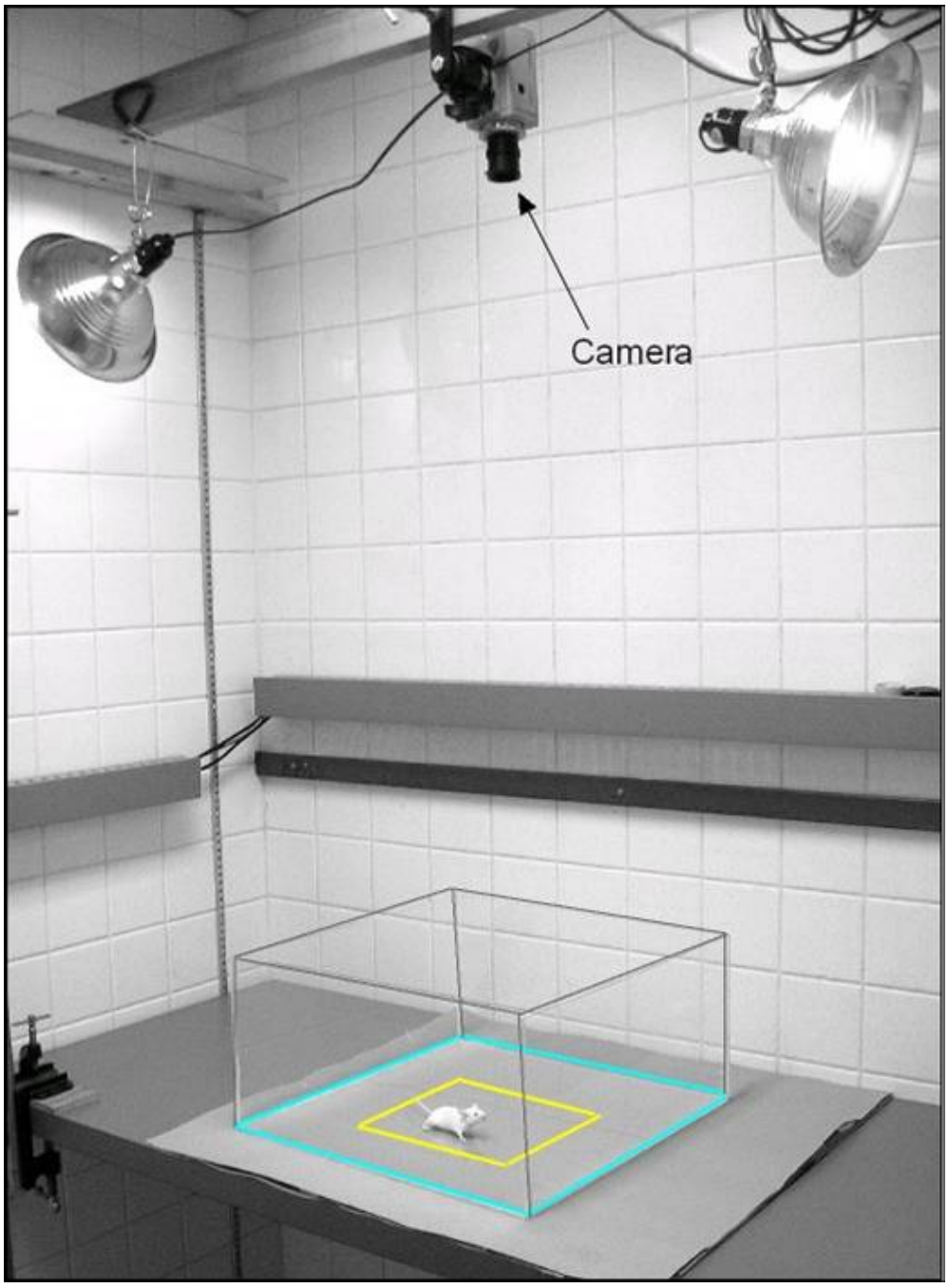

Camera

Figure 2. Light-dark box (40 [L] x 40 [W] x 20 [H] cm) used to assess fear in mice. Half of the box was covered with black cardboard to create distinct light and dark sections.

Mice exposed to the undisrupted LD schedule continued on the schedule to which they had been acclimated (see Figure 3). Cages were placed on aluminum racks with two or three shelves. At the level of the top shelf, illumination from light reflected by the floor of the room was 85.13, 70.19, and 73.97 candelas per square meter for Replications 1, 2 and 3 respectively. At the level of the bottom shelf, illumination was 46.54, 42.61 and 39.85 candelas per square meter for Replications 1, 2 and 3 respectively. The rack used in the third replication had three shelves and illumination at the level of the middle shelf was 48.12 candelas per square meter.

For mice exposed to the disrupted LD schedule, light phases shifted by 12 hr every 5 days. Lights were on either from 0700 hr EST to 1900 hr EST or from 1900 hr EST to 0700 hr EST (see Figure 4). At the level of the top shelf, illumination from light reflected from the floor of the room was 83.29 and 74.04 candelas per square meter for Replications 1 and 3 respectively. At the bottom shelf, illumination was 51.29 and 45.51 candelas per square meter for Replications 1 and 3 respectively. Illumination levels for the room with the disrupted LD schedule in Replication 2 are not available.

Because not all the available rooms were the same size, the isolation rooms used in Replication 1 were slightly bigger than rooms used in Replications 2 and 3, which had similar rooms.

Interobserver Agreement. Four recorders were trained to adhere to the experimental protocol by running 6 four-minute trials in the open field and light-dark box. To assess interobserver agreement, recorders were tested in pairs, such that each recorder was tested with the other three recorders twice. Fecal boli were counted at the end of trials. There was a 100% agreement among observers in the number of fecal boli counted.

Measure of Body Weight. Body weight measurements in grams were obtained the day before a phase change. Mice were weighed once every five days during the light phase.

Measures of Fear. At the end of the light treatment, fear was assessed in the open field and light-dark box (Bourin, & Hascoet, 2003; Crawley & Goodwin, 1980; Lister, 1990; Prut & Belzung, 2003).

***Open field*.** For a trial the animal was placed in the bottom right-hand corner of the open field. Each trial was 10 min long. The following measures were obtained: frequency of entries into the center and periphery of the open field, latency of entry to the center as well as percent time spent in each zone. Total distance traveled in each trial was also obtained as a measure of locomotor activity. For the purpose of analysis, each trial was divided into two 5-min periods.

After each trial, number of fecal boli was counted and the animal was returned to its cage.

Figure 3. Undisrupted LD schedule. White bars represent the light phase and black bars represent the dark phase of the LD schedule.

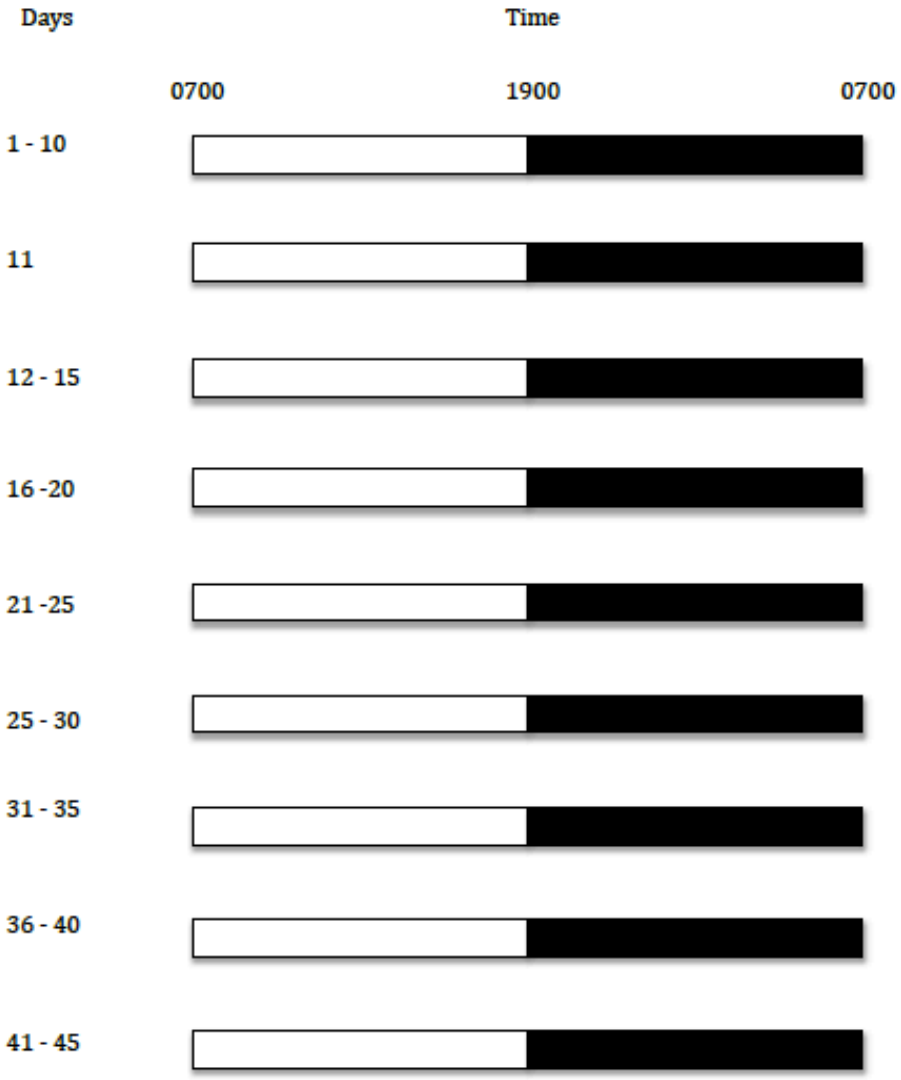
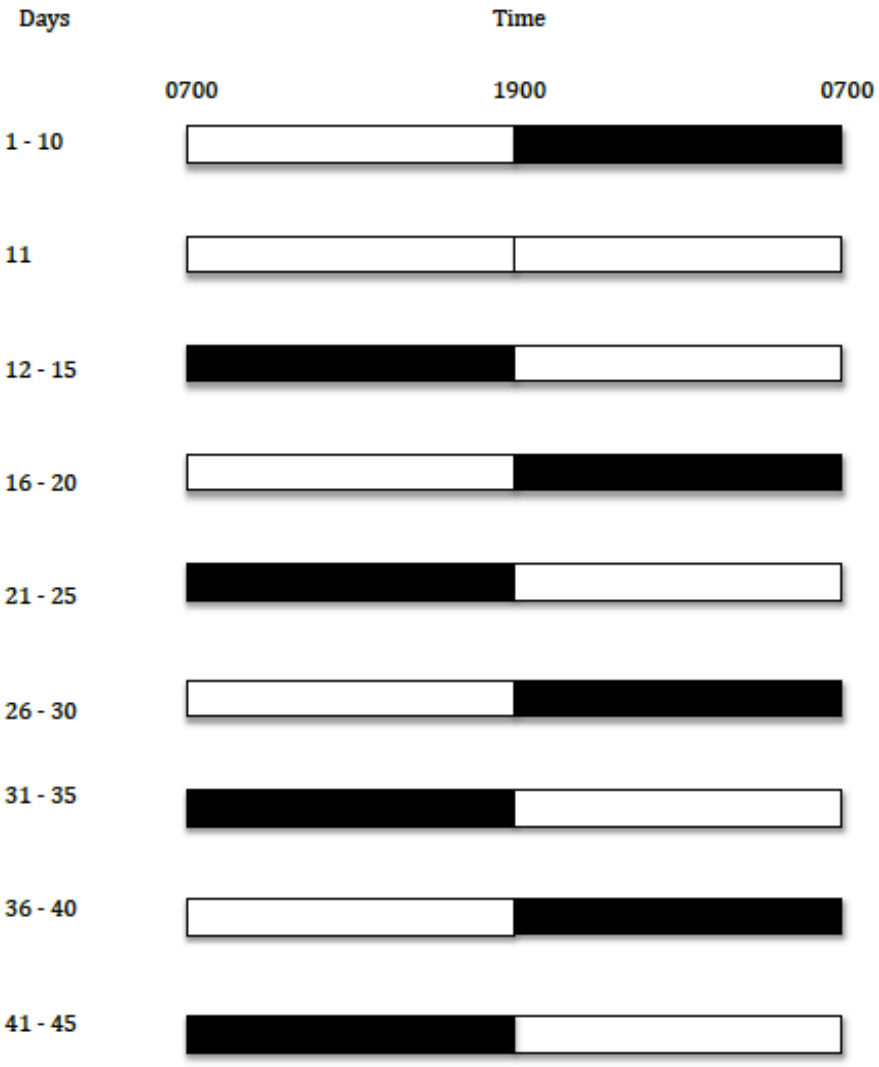

Days
Time
0700
1900
0700
1 - 10
11
12 - 15
16 -20
21 -25
25 - 30
31 - 35
36 - 40
41 - 45

Figure 4. Disrupted LD schedule. White bars represent the light phase and black bars represent the dark phase of the LD schedule.


Days
Time
0700
1900
0700
1 - 10
11
12 - 15
16 - 20
21 - 25
26 - 30
31 - 35
36 - 40
41 - 45

Light-dark box. Two days after testing in the open field, mice were tested in the light-dark box in 5-min trials. For a trial the animal was placed in the bottom right-hand corner of the box, in the light section of the box. The following measures were obtained: percent of time spent in the light section, latency of entry to the dark section, and frequency of transitions into the light section of the box.

Because animals were introduced into the light-dark box in the light section, freezing (the initial immobility of the animal), which would normally indicate fearfulness, would increase animals' recorded duration in the light section, and thus indicate reduced fearfulness. To avoid wrongly regarding freezing behavior in the light section as decreased fearfulness, a second percent time variable was derived from the obtained measures by subtracting the latency to enter the dark section from the total trial time and using that value as the total trial time in calculating the percent of time spent in light section. After each trial, number of fecal boli was recorded and the animal returned to its cage.

Data Analysis

Measures of Fear.

Open field. Percent of time spent in the periphery and frequency of entry into the center, the design was a 2 (LD condition: control vs. disrupted) x 2 (Sex: female vs. male) x 2 (Period: first five-minute period and second five-minute period) x 3 (Replication: 1, 2, 3) mixed design. Data from the first and second periods were also summed to give a total score for the trial.

For latency to enter the center and defecation frequency, the design was a 2 (LD condition: control vs. disrupted) x 2 (Sex: female vs. male) x 3 (Replication: 1, 2, 3) mixed design. LD Condition, Sex, and Replication were between-subjects factors while Period was a within-subjects factor.

Light-dark box. Percent time in periphery (with and without latency), frequency of entry to light section and latency of entry to dark section, the design was a 2 (LD condition: disrupted vs. undisrupted) x 2 (Sex: male vs. female) x 3 (Replication: 1, 2, 3) mixed design. LD Condition, Sex, and Replication were between-subjects factors. The Tukey test was used wherever a post-hoc test was needed.

Correlation analysis. As earlier addressed, in addition to the open field, the light-dark box was used as a second measure of fear. To evaluate the correlation of open field and light-dark box variables, a correlation analysis was performed.

Measure of Body Weight. For body weights obtained, the design was 2 (LD condition: disrupted vs. undisrupted) x 2 (Sex: male vs. female) x 7 (Time period: 1 - 7). LD condition and Sex were between-subjects factors while Time period was a within-subjects factor. A Newman-Keuls analysis was used as a within-subjects post-hoc test.

Weight gain for each time period was also derived by subtracting the body weight measured in the previous time period from the newly measured body weight.

All data were analyzed using IBM SPSS Statistics (Version 19). An alpha level of 0.05 was used for all statistical tests.

RESULTS

The objectives of the present research were to assess the effects of a disrupted LD schedule on fear in mice. The effect of the disrupted LD schedule on body weight was also examined as well as possible differences between male and female mice. Fear was measured using the open field test and the light-dark box. Based on the literature, it was hypothesized that animals exposed to the disrupted LD schedule would be less fearful than mice exposed to the undisrupted LD schedule. In the results below, an effect of replication is only mentioned when statistically significant.

Effect of Light-Dark Schedule on Fear

Open Field. Fear in the open field was assessed using percent time spent in periphery, latency to enter the center, number of transitions to center, and defecation frequency. To assess locomotor activity, total distance moved was also. According to the literature, less time spent in the periphery, more frequent transitions into the center, and less fecal boli indicate reduced fear (Lister, 1990, Prut & Belzung, 2003). The results for each variable are reported below.

Percent time in periphery. Animals exposed to the disrupted LD schedule did not spend significantly more time slightly more in the periphery than animals exposed to the control LD schedule, F (1, 59) = 1.54, $p > .05$, indicating that the LD schedule did not affect fear (see Figure 5). Males spent significantly more

time in the periphery than females, F (1, 59) = 3.99, p = .05, indicating that males were more fearful than females (see Figure 6). The LD condition x Sex interaction was not significant, F (1, 59) = 2.87, p > .05 (see Figure 7).

Percent time mice spent in the periphery did not differ significantly between the first 5-min period (M = 94.65, SEM = 0.42) and the second (M = 94.00, SEM = 0.44). The Period x LD condition, F (1, 59) = 1.39, p > .05 and Period x Sex, F (1, 59) = 0.10, p > .05 and Period x LD condition x Sex, F (1, 59) = 0.01, p > .05, interactions were also not significant.

Animals in the first replication (M = 95.63, SEM = 0.58) spent significantly more time in the periphery than animals in the third replication (M = 92.97, SEM = 0.90), resulting in a significant replication effect, F (2, 59) = 4.33, p < .05. The Period x LD condition x Replication interaction was also significant, F (2, 59) = 3.91, p < .05.

Latency to enter the center. Animals exposed to the disrupted LD schedule did not differ significantly from those exposed to the control schedule in latency to enter the center, F (1, 59) = 2.19, p > .05 (see Figure 8). Also, females and males did not differ significantly in latency to enter the center, F (1, 59) = 0.10, p > .05 (see Figure 9). The LD condition x Sex interaction was also not significant, F (1, 59) = 0.47, p > .05 (see Figure 10).

Frequency to enter the center. Animals exposed to the disrupted LD schedule did not make significantly more transitions to the center of the open field

than animals exposed to the undisrupted LD schedule, F (1, 59) = 0.79, $p > .05$, indicating that disruption of LD schedule did not have an effect on fear in mice (see Figure 11). There was also no significant difference between sexes in frequency of transitions to the center, F (1, 59) = 3.98, $p > .05$ (see Figure 12). The LD condition x Sex interaction was also not significant, F (1, 59) = 1.99, $p > .05$ (see Figure 13).

Animals made significantly more transitions into the center in the second 5-min period than in the first 5-min period, F (1, 59) = 11.76, $p < .05$, indicating reduced fear over a 10-min period (see Figure 14). However, the Period x LD condition, F (1, 59) = 0.63, $p > .05$, Period x Sex, F (1, 59) = 0.33, $p > .05$, and Period x Sex x LD condition, F (1, 59) = 0.07, $p > .05$, interactions were not significant.

Animals in the third replication (M = 24.48, SEM = 4.30) made significantly more transitions into the center than animals in the first replication (M = 12.77, SEM = 1.39), resulting in a significant effect of replication, F (2, 59) = 5.29, $p < .05$.

Figure 5. Percent time in periphery (mean % ± *SEM*) as a function of LD condition.

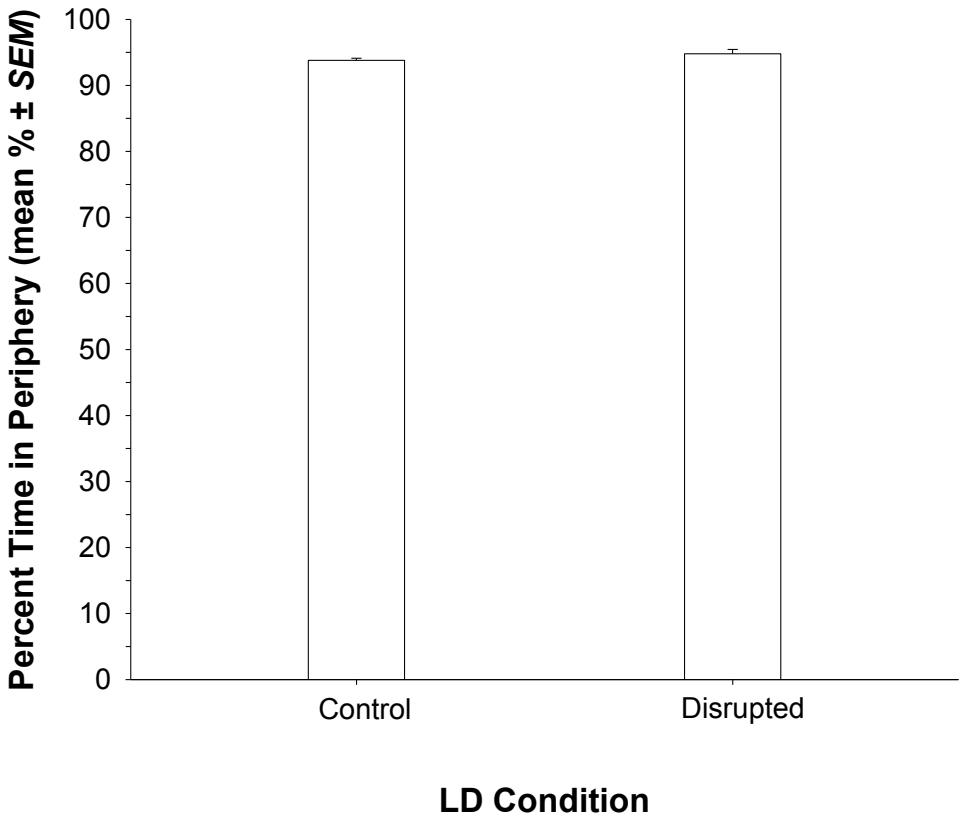

Percent Time in Periphery (mean % ± SEM)
100
90
80
70
60
50
40
30
20
10
0
Control
Disrupted
LD Condition

Figure 6. Percent time in periphery (mean % ± *SEM*) as a function of Sex.

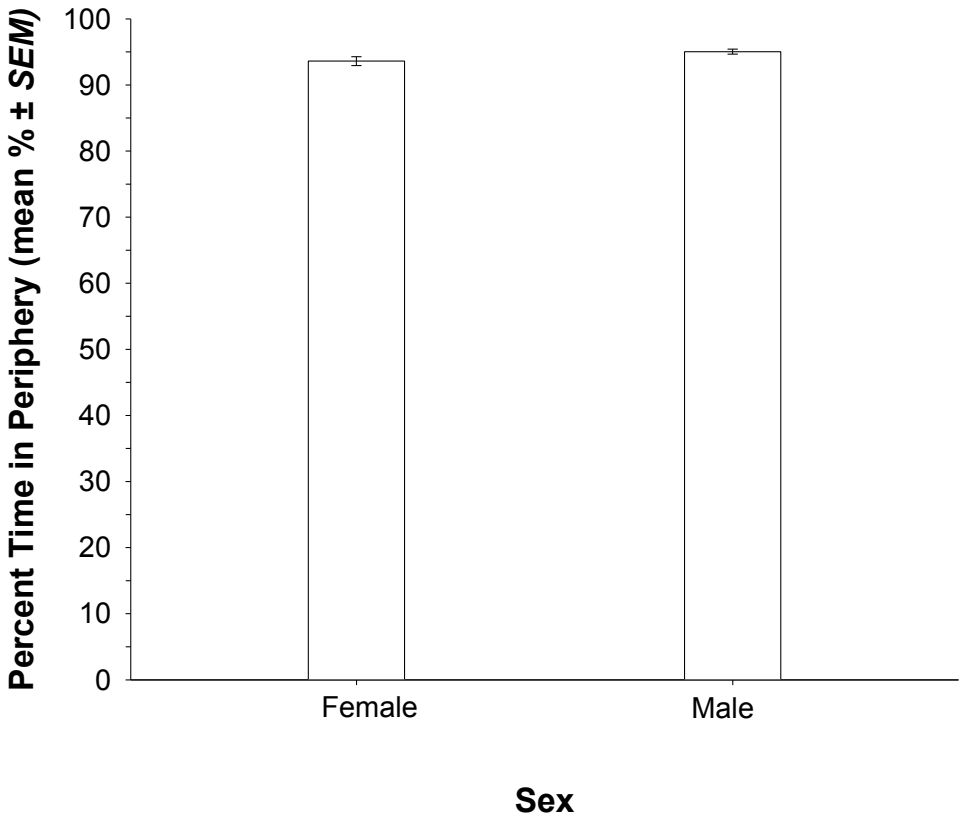

Percent Time in Periphery (mean % ± SEM)
100
90
80
70
60
50
40
30
20
10
0
Female
Male
Sex

Figure 7. Percent time in periphery (mean % ± *SEM)* for the LD condition x Sex interaction.

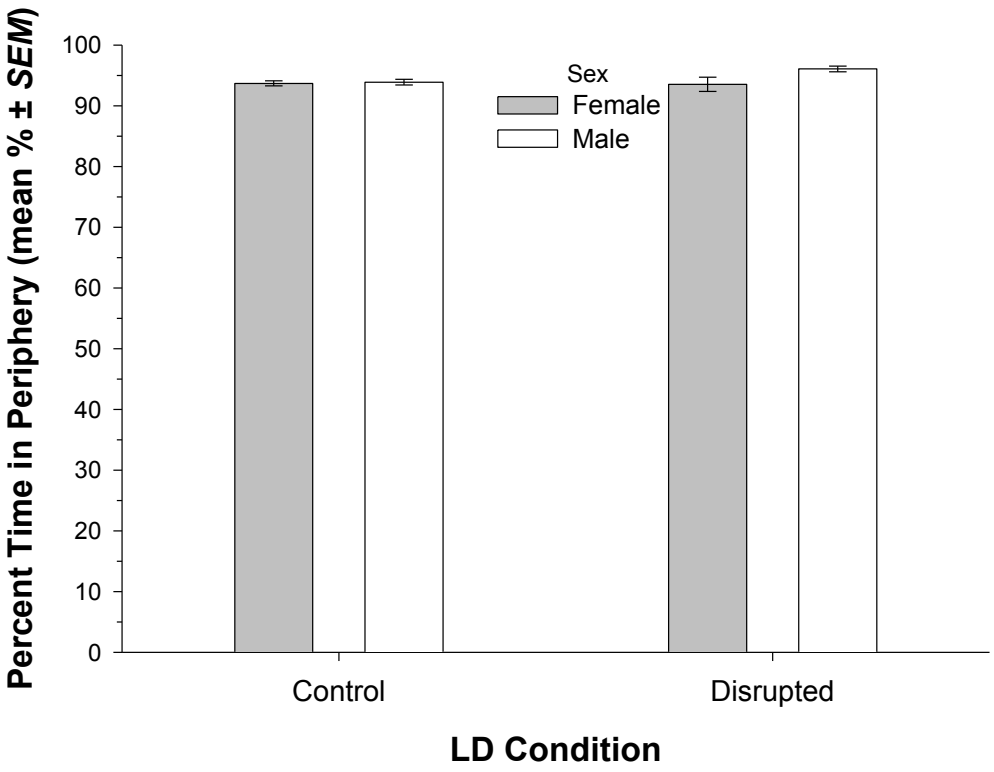

Percent Time in Periphery (mean % ± SEM)
100
90
80
70
60
50
40
30
20
10
0
Sex
Female
Male
Control
Disrupted
LD Condition

Figure 8. Latency to enter the center (mean sec ± *SEM)* as a function of LD condition.

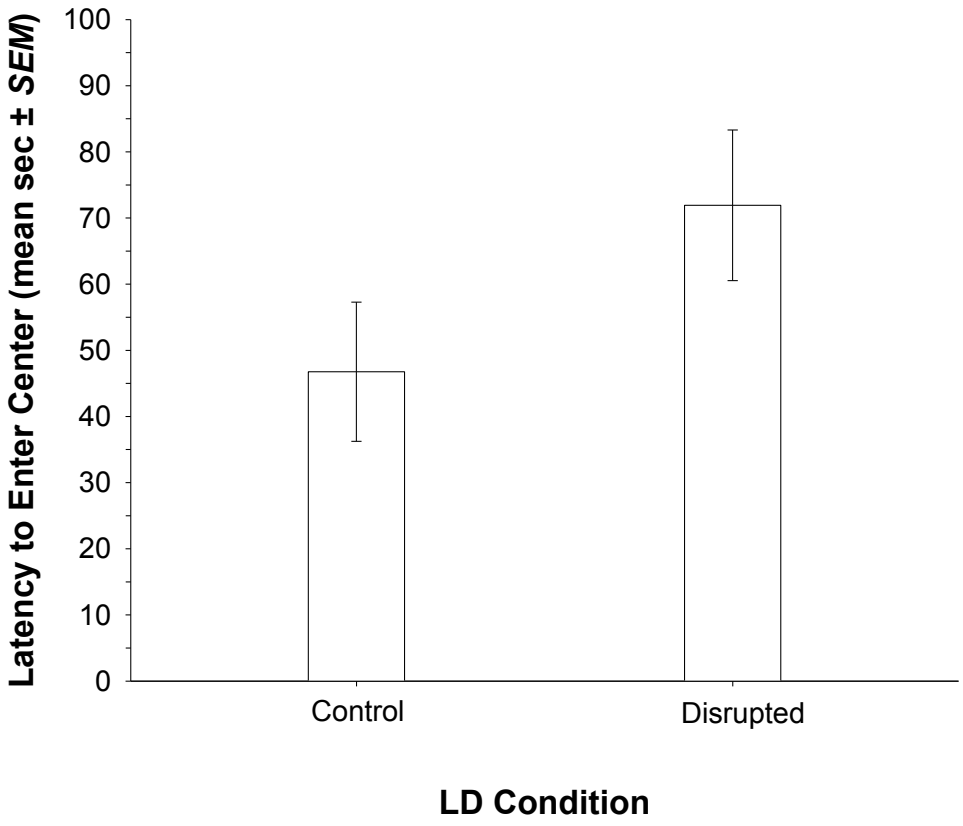

Latency to Enter Center (mean sec ± SEM)
100
90
80
70
60
50
40
30
20
10
0
Control
Disrupted
LD Condition

Figure 9. Latency to enter the center (mean sec ± *SEM*) as a function of Sex.

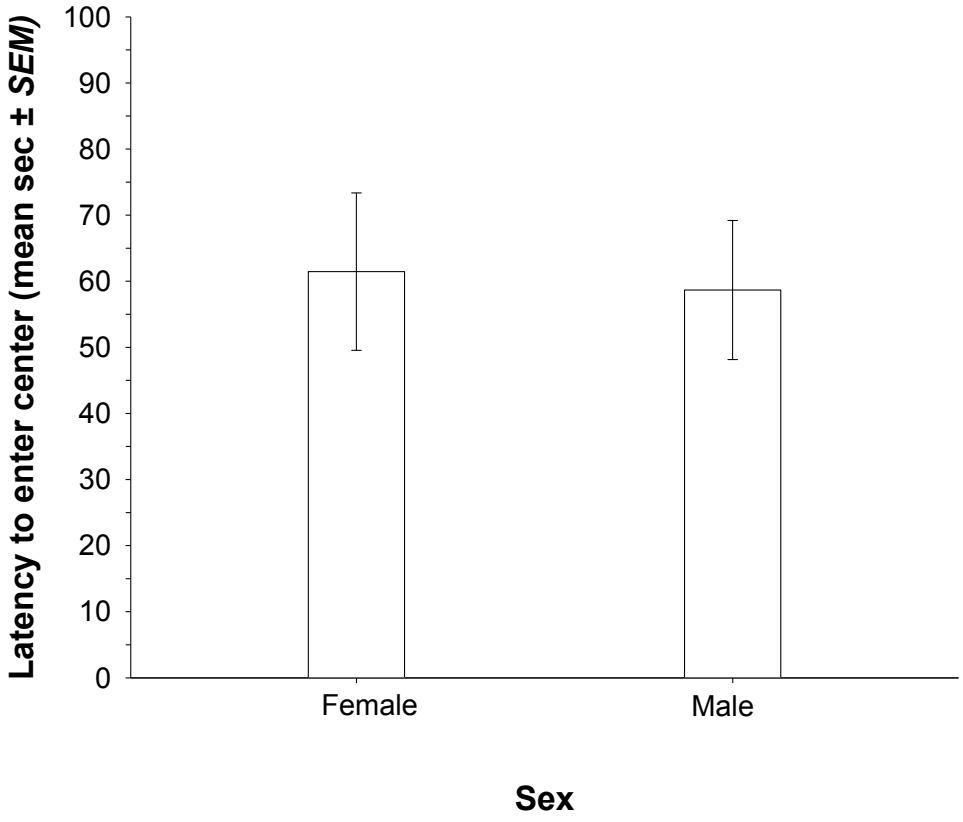

Latency to enter center (mean sec ± SEM)
100
90
80
70
60
50
40
30
20
10
0
Female
Male
Sex

Figure 10. Latency to enter the center (mean sec ± *SEM*) for the LD condition x Sex interaction.

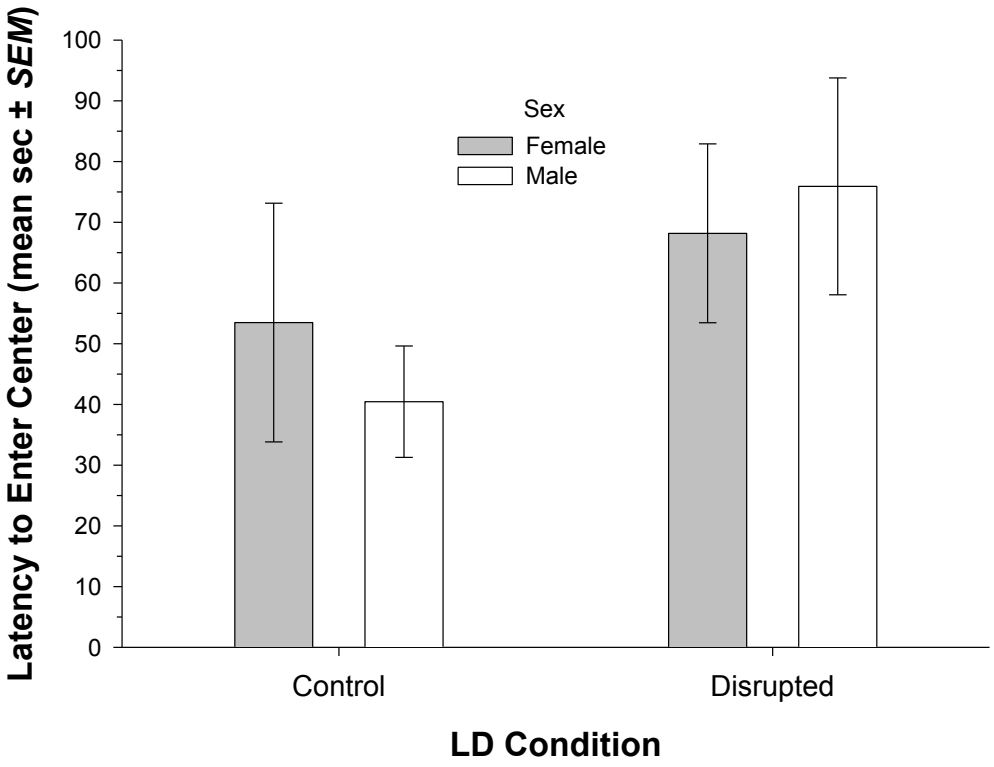

Latency to Enter Center (mean sec ± SEM)
100
90
80
70
60
50
40
30
20
10
0
Sex
Female
Male
Control
Disrupted
LD Condition

Figure 11. Frequency to enter the center (mean ± *SEM*) as a function of LD condition.

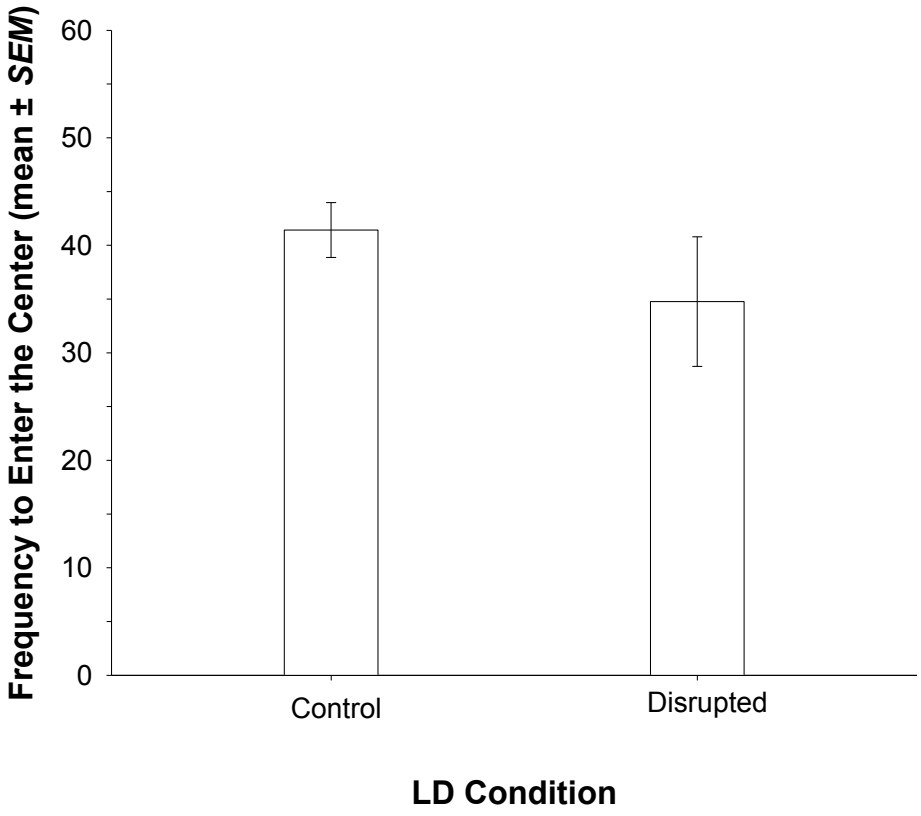

Frequency to Enter the Center (mean ± SEM)
60
50
40
30
20
10
0
Control
Disrupted
LD Condition

Figure 12. Frequency to enter the center (mean ± *SEM*) as a function of Sex.

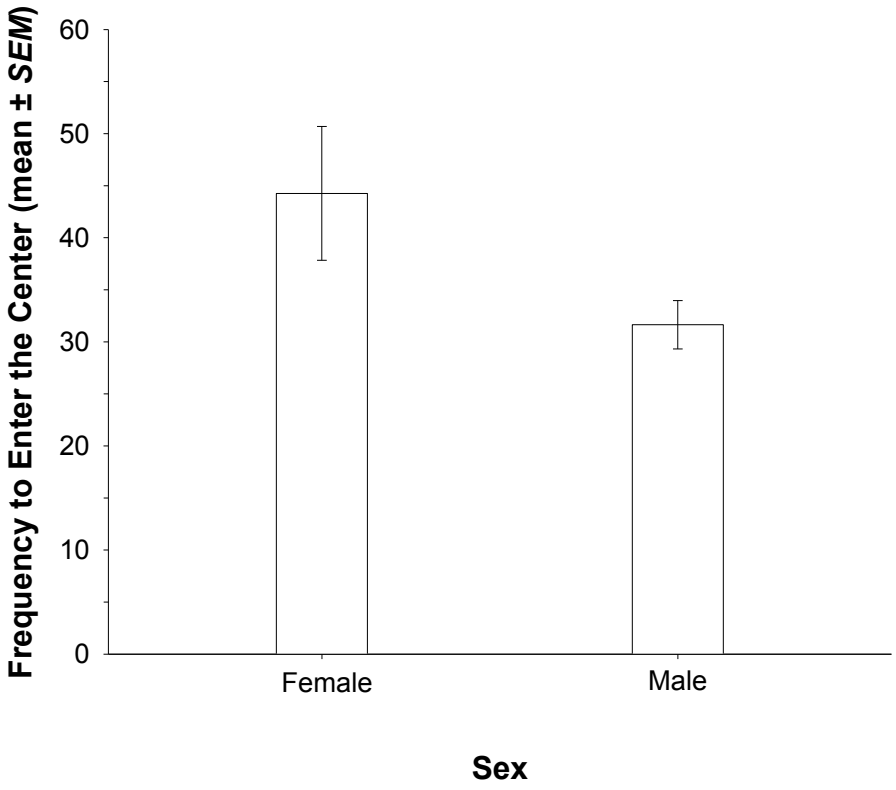

Frequency to Enter the Center (mean ± SEM)
60
50
40
30
20
10
0
Female
Male
Sex

Figure 13. Frequency to enter the center (mean ± *SEM*) for the interaction LD condition x Sex.

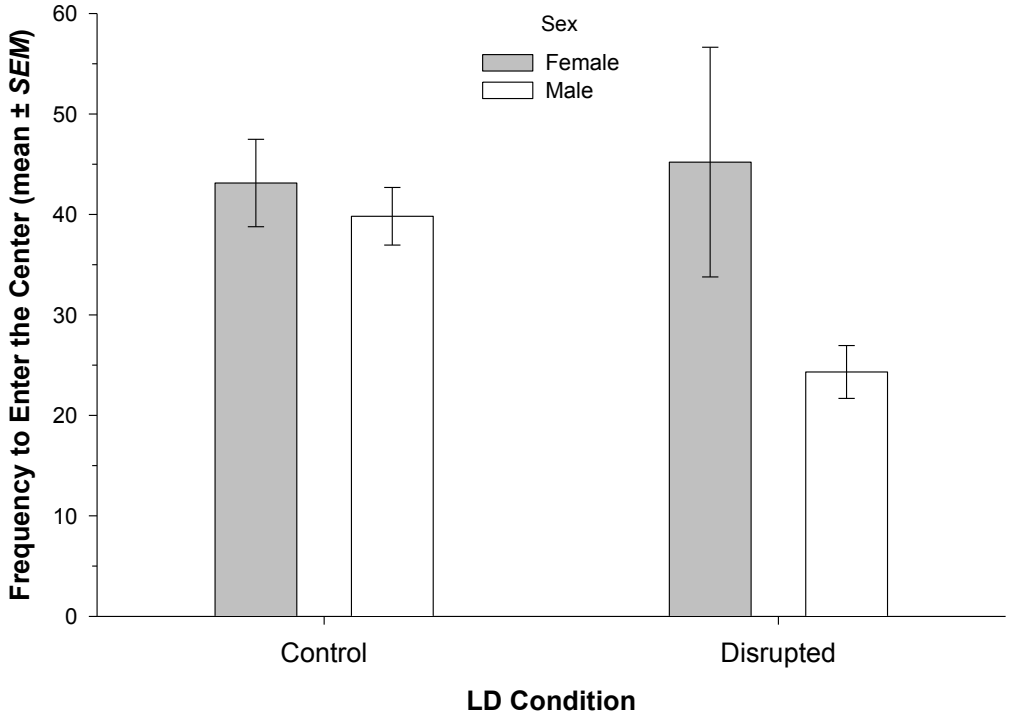
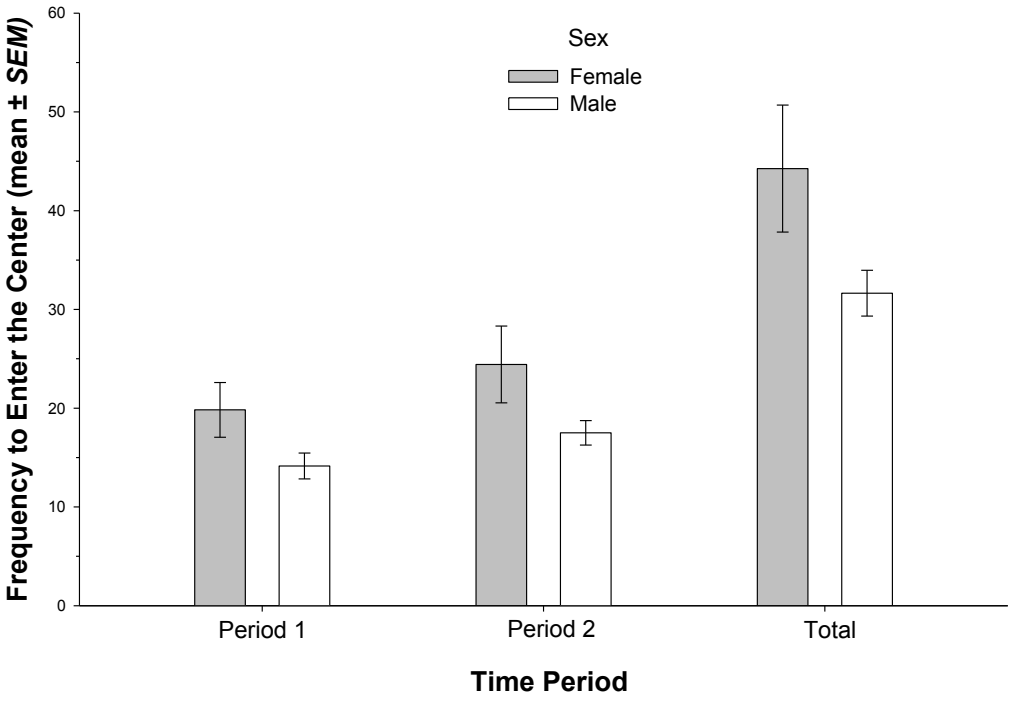

Sex
Female
Male
Frequency to Enter the Center (mean ± SEM)
60
50
40
30
20
10
0
Control
Disrupted
LD Condition

Figure 14. Frequency to enter the center (mean ± *SEM*) as a function of time period. The time period "Total" is a sum of the first and second periods.


Sex
Female
Male
Frequency to Enter the Center (mean ± SEM)
60
50
40
30
20
10
0
Period 1
Period 2
Total
Time Period

***Defecation frequency*.** Animals exposed to the disrupted LD schedule produced significantly more fecal boli than animals exposed to the undisrupted schedule, F (1, 59) = 14.15, $p < .05$, indicative of increased fear in the former group (see Figure 15). Males (M = 3.28, SEM = 0.52) also produced significantly more fecal boli than females (M = 1.29, SEM = 0.36), F (1, 59) = 11.92, $p < .05$, indicating increased fear in males. The LD condition x Sex interaction was not significant, F (1, 59) = 1.33, $p > .05$ (see Figure 16).

Animals in the second replication (M = 3.30, SEM = 0.67) produced significantly more fecal boli than animals in the first replication (M = 1.42, SEM = 0.45), resulting in a significant effect of replication, F (2, 59) = 3.73, $p < .05$.

***Total distance moved*.** There was no significant difference in total distance moved between animals exposed to the disrupted LD schedule and animals exposed to the undisrupted LD schedule, F (1, 59) = 0.04, $p > .05$, indicating that LD condition did not affect fear in mice (see Figure 17). There was also no significant difference between females and males in total distance moved, F (1, 59) = 3.64, $p > .05$ (see Figure 18). The interaction, LD condition x Sex, F (1, 59) = 0.33, $p > .05$, was also not significant.

Figure 15. Defecation frequency (mean ± *SEM*) as a function of LD condition.

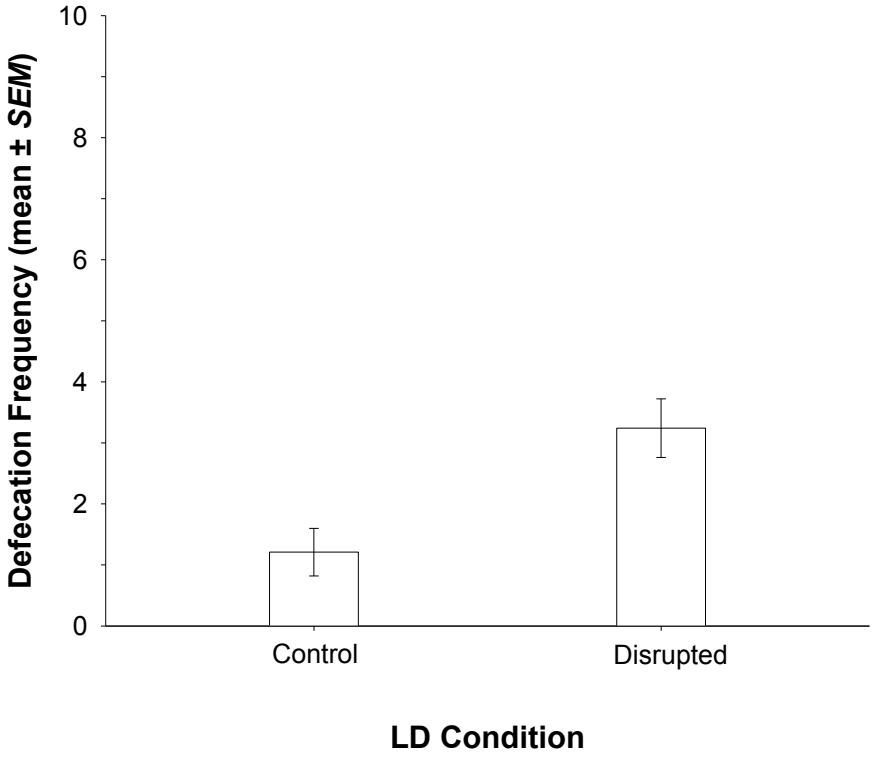

Defecation Frequency (mean ± SEM)
10
8
6
4
2
0
Control
Disrupted
LD Condition

Figure 16. Defecation frequency (mean ± *SEM)* for the LD condition x Sex interaction.

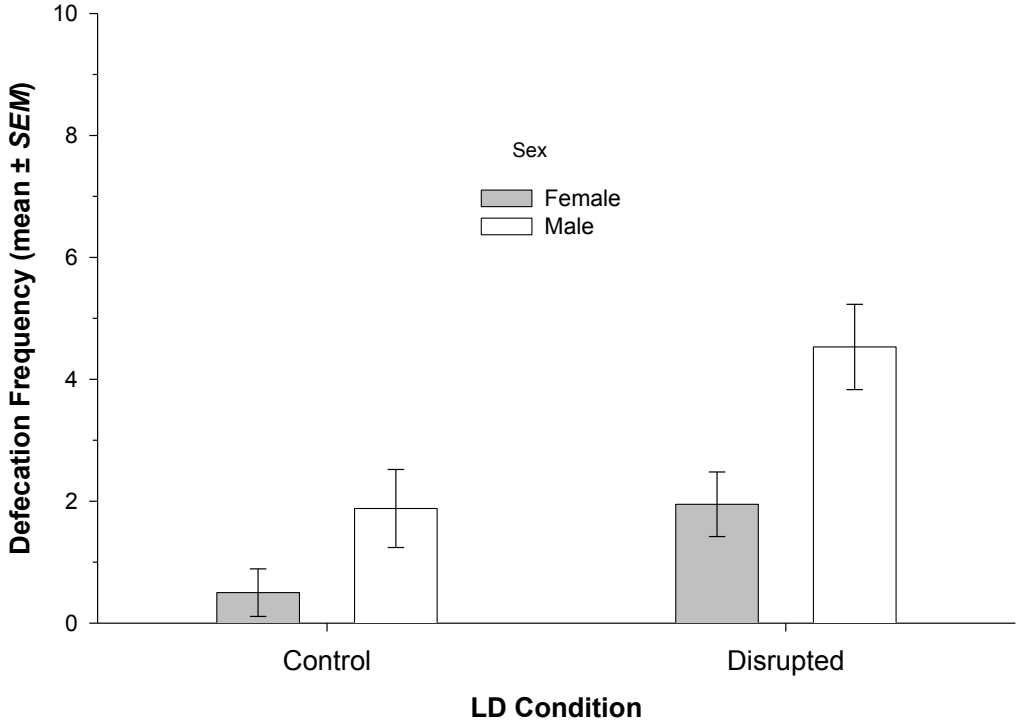

10
8
6
4
2
0
Defecation Frequency (mean ± SEM)
Sex
Female
Male
Control
Disrupted
LD Condition

Figure 17. Total distance moved (mean cm ± *SEM*) as a function of LD condition.

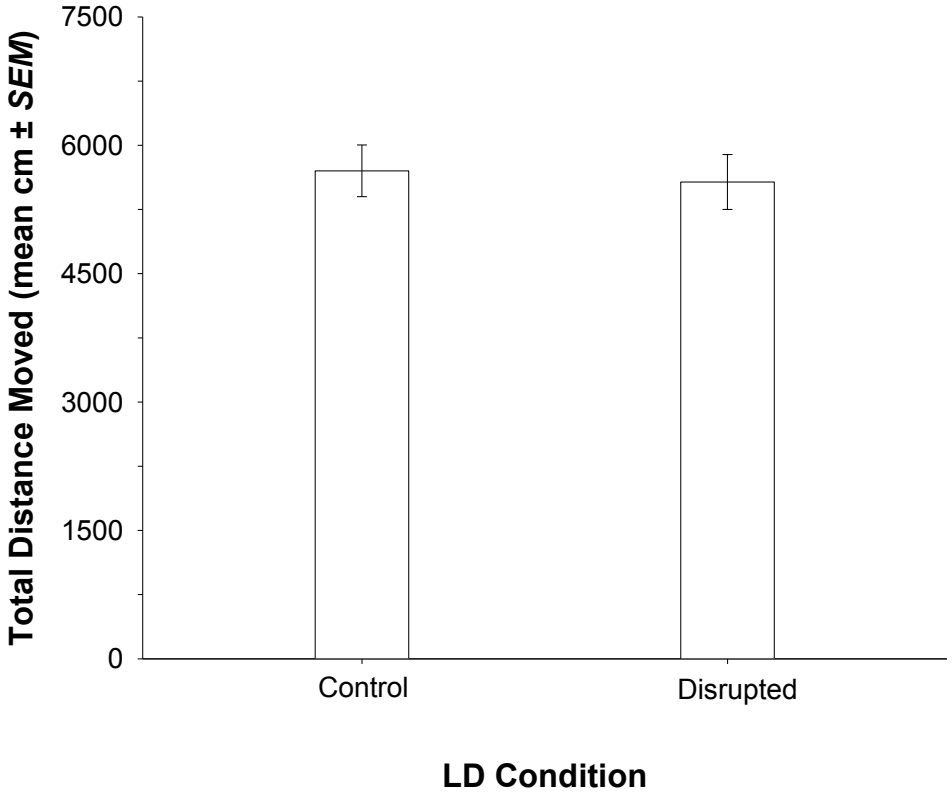
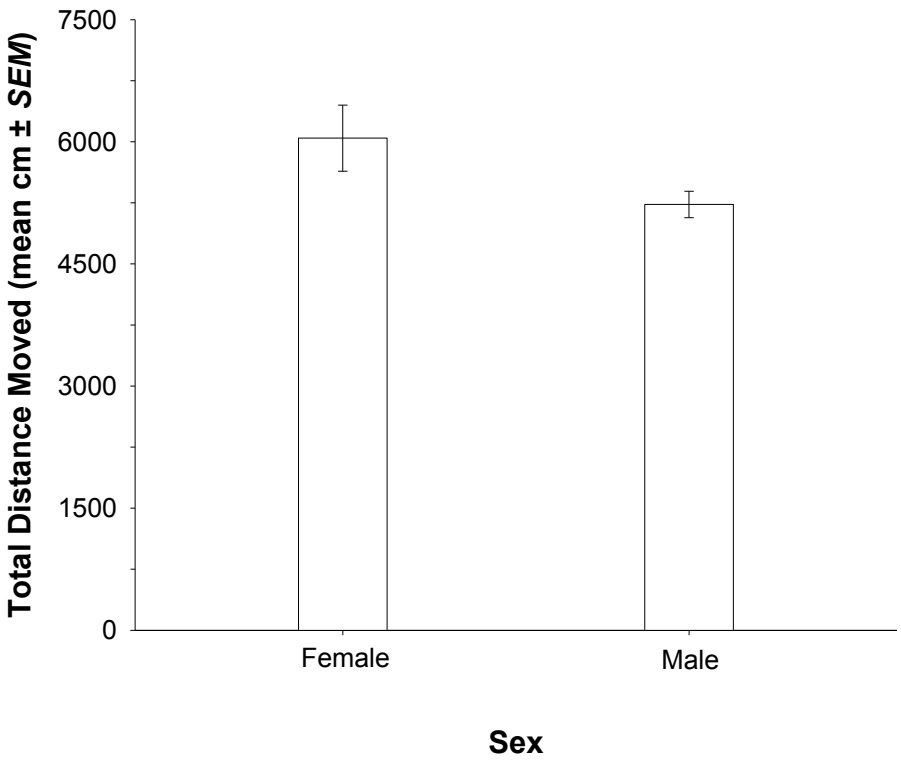

7500
6000
4500
3000
1500
0
Total Distance Moved (mean cm ± SEM)
Control
Disrupted
LD Condition

Figure 18. Total distance moved (mean cm ± *SEM*) as a function of Sex.


Total Distance Moved (mean cm ± SEM)
7500
6000
4500
3000
1500
0
Female
Male
Sex

Animals moved significantly more during the second 5-min period than during the first, F (1, 59) = 6.71, $p < .05$ (see Figure 19). However, the Period x LD condition, F (1, 59) = 0.03, $p > .05$, Period x Sex, F (1, 59) = 0.57, $p > .05$, and Period x LD condition x Sex, F (1, 59) > 0.00, $p > .05$, interactions were not significant.

For all but one variable in the open field—defecation frequency—the analysis of variance showed no significant difference between the two LD condition groups, indicating that disruption of LD cycle did not affect fear in mice. However, animals exposed to the disrupted LD schedule produced significantly more boli than animals exposed to the disrupted LD schedule, indicating a fear-increasing effect of the LD schedule disruption. Increased time in periphery and greater number of fecal boli in males indicate that males were more fearful than females. Latency of entry to the center, frequency of entry to center and total distance moved showed no difference between sexes.

Light-Dark Box. Fear was assessed using percent time spent in the light section of the box, frequency of entry to the light section, latency to enter the dark section and defecation frequency. Data from some animals were not included in some of the analyses because of a program malfunction. For two of the trials, the program failed to detect the mouse before it went to the dark section. For such trials, rather than record that the animal was in the dark section, the program recorded missing samples for the animal. Results for each variable follow.

Percent time in light section (with latency). Animals exposed to the disrupted LD schedule did not spend significantly more time in the light section than animals exposed to the undisrupted schedule, F (1, 59) = 0.07, $p > .05$, indicating that disruption of the LD schedule did not affect fear in mice (see Figure 20).

Males spent significantly more time than females in the light section, F (1, 59) = 8.04, $p < .05$, indicating that males were less fearful than females (see Figure 21). The LD condition x Sex interaction, F (1, 59) = 1.40, $p > .05$, was not significant.

Animals in the third replication (M = 39.39, SEM = 2.01) spent significantly less time in the light section than animals in the first replication (M = 47.45, SEM = 2.69), resulting in a significant effect of Replication, F (2, 59) = 4.53, $p < .05$. The LD condition x Replication interaction was also significant, F (2, 59) = 3.23, $p < .05$.

Percent time in light section (without latency). Datum from one animal was excluded from the analysis because of program malfunction (N = 70).

Animals exposed to the disrupted LD schedule did not spend significantly more time in the light section than animals exposed to the undisrupted schedule, F (1, 58) = 0.14, $p > .05$, indicating that disruption of the LD schedule did not affect fear in mice (see Figure 22).

Figure 19. Distance moved (mean cm ± *SEM*) as a function of time period. Time period, "Total" is derived from the sum of the first and second periods.

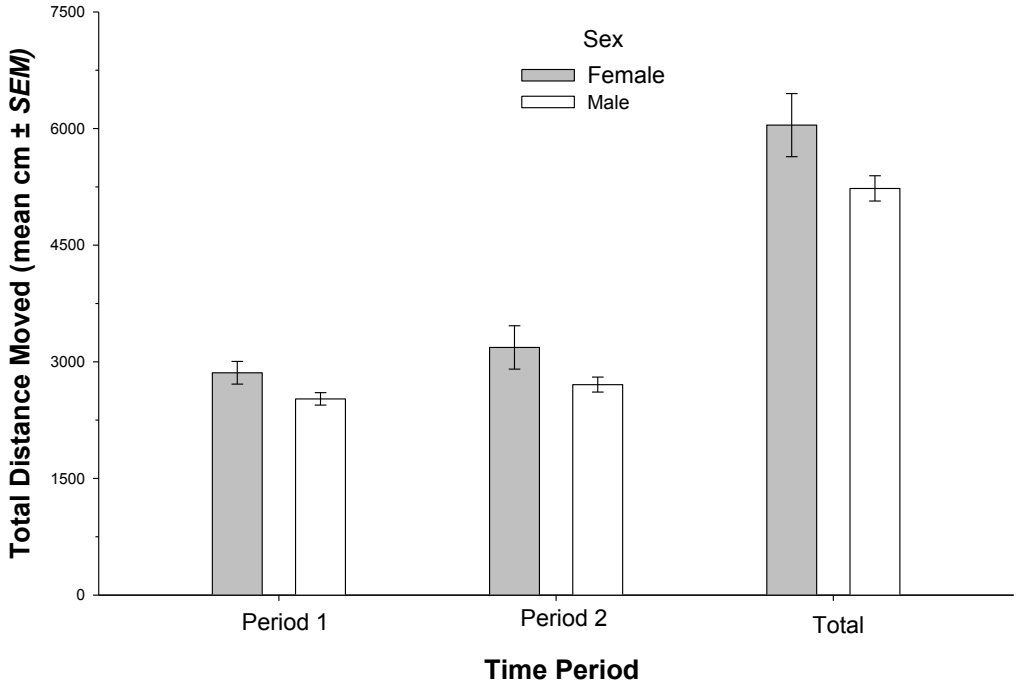

Sex
Female
Male
Total Distance Moved (mean cm ± SEM)
7500
6000
4500
3000
1500
0
Period 1
Period 2
Total
Time Period

Figure 20. Percent time in the light section (with latency) (mean % ± *SEM*) as a function of LD condition.

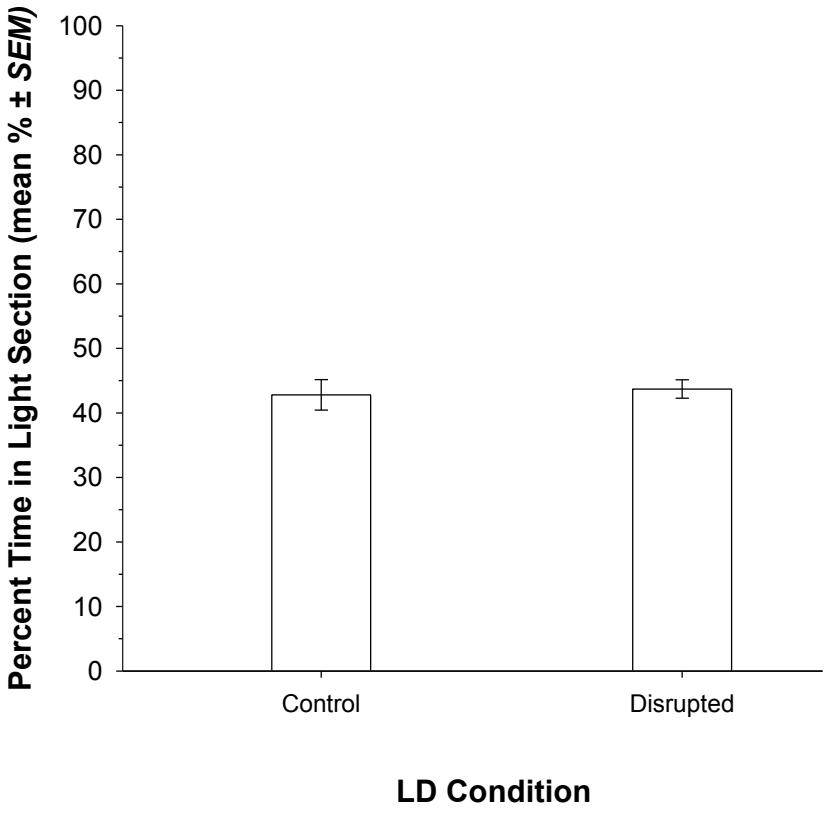

Percent Time in Light Section (mean % ± SEM)
100
90
80
70
60
50
40
30
20
10
0
Control
Disrupted
LD Condition

Figure 21. Percent time in the light section (with latency) (mean % ± *SEM*) as a function of Sex.

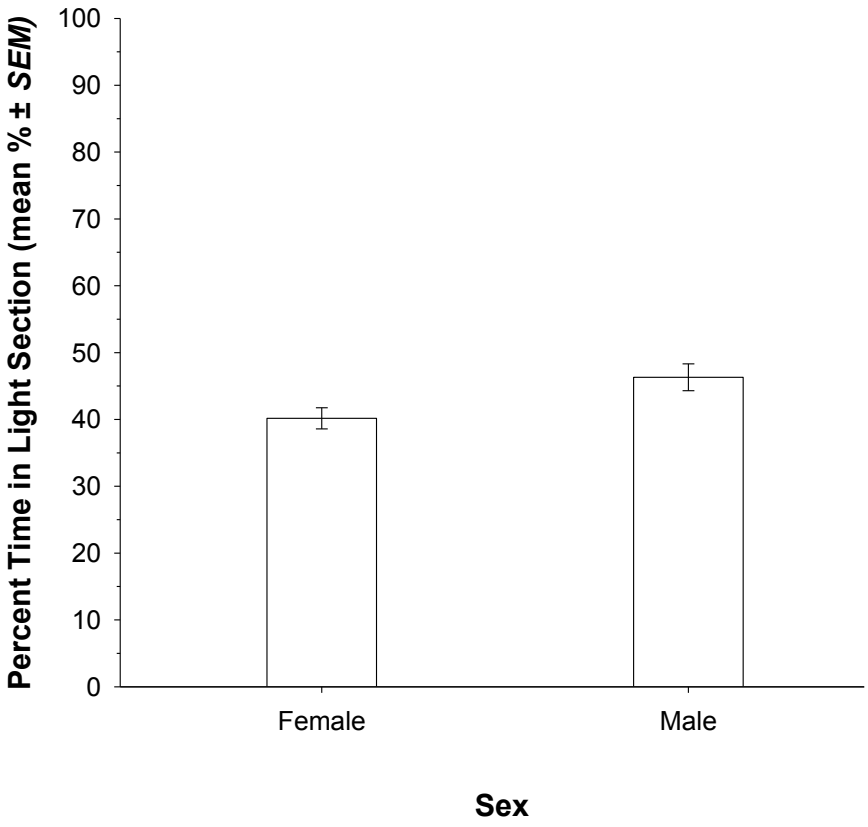
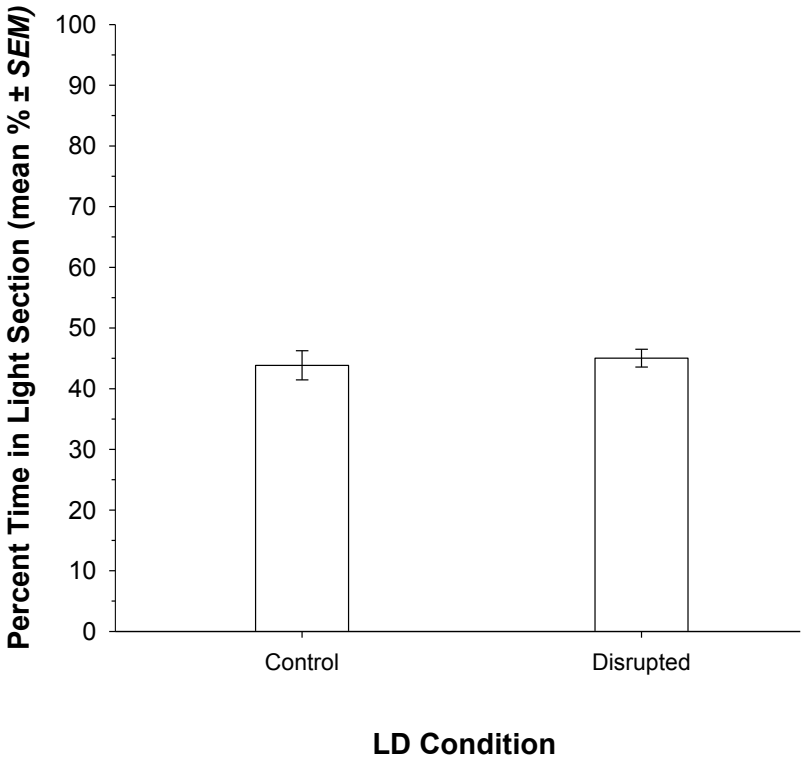

Percent Time in Light Section (mean % ± SEM)
100
90
80
70
60
50
40
30
20
10
0
Female
Male
Sex

Figure 22. Percent time spent in the light section (without latency) (mean % ± *SEM*) as a function of LD condition.


Percent Time in Light Section (mean % ± SEM)
100
90
80
70
60
50
40
30
20
10
0
Control
Disrupted
LD Condition

Males spent significantly more time than females in the light section, F (1, 58) = 10.49, $p < .05$, indicating that males were less fearful than females. The LD condition x Sex interaction was not significant, F (1, 58) = 1.23, $p > .05$.

A Tukey post-hoc test showed that animals in the third replication spent significantly less time in the light section than animals in the first replication, resulting in a significant effect of replication, F (2, 58) = 5.26, $p < .05$. The LD condition x Replication interaction was not significant, F (2, 58) = 2.92, $p > .05$.

***Frequency to enter light section*.** Datum from one animal was excluded from the analysis of variance for this variable because of a program malfunction (N = 70).

There was no significant difference in frequency of transitions to the light section between animals that were exposed to the disrupted LD schedule and those that were exposed to the undisrupted LD schedule, F (1, 58) = 0.20, $p > .05$ (see Figure 23). However, females made significantly more transitions into the light section of the box than males, F (1, 58) = 4.59, $p < .05$, indicating less fear in females than males (see Figure 24). The LD condition x Sex interaction was not significant, F (1, 58) = 0.02, $p > .05$.

Figure 23. Frequency to enter the light section (mean ± *SEM*) as a function of LD condition.

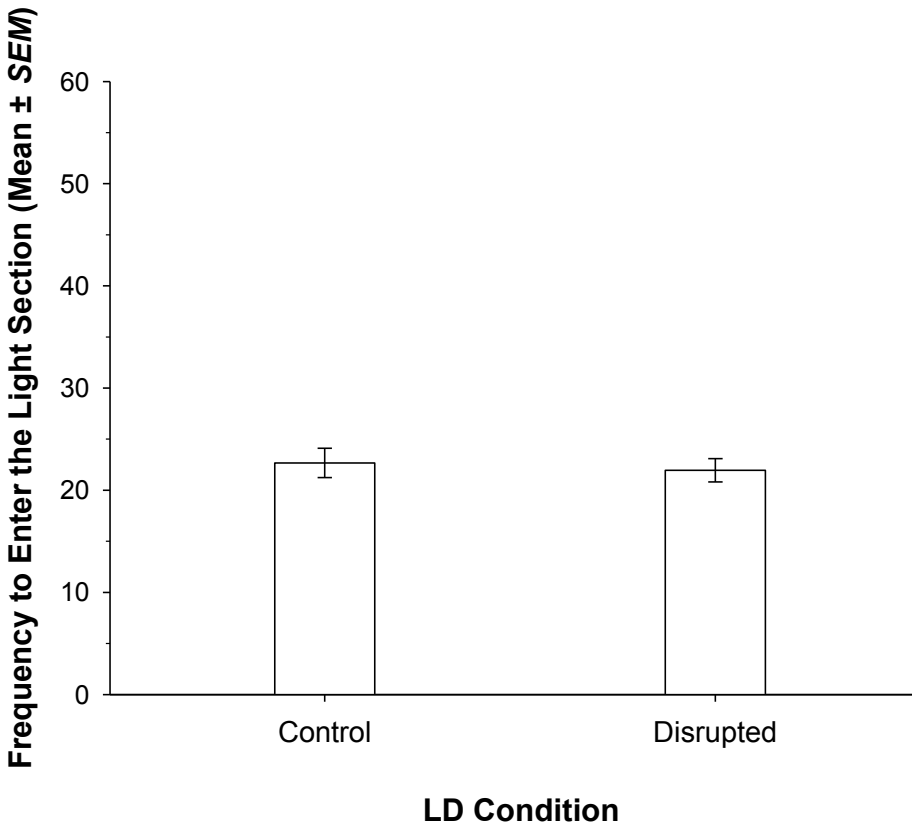
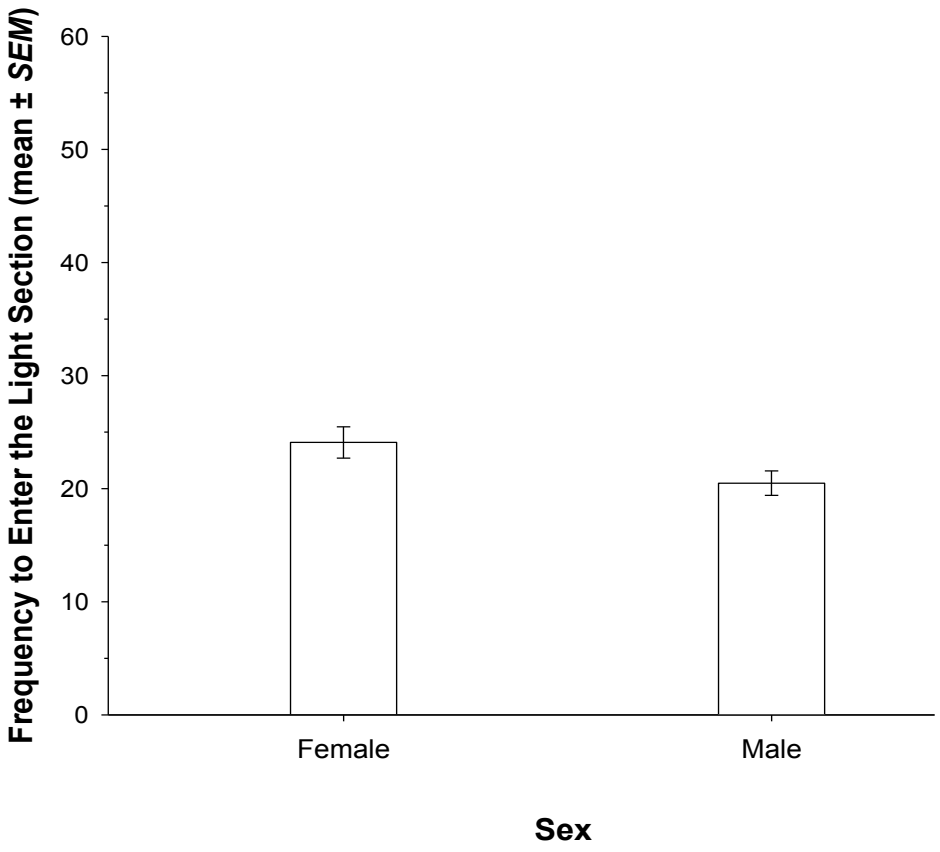

Frequency to Enter the Light Section (Mean ± SEM)
60
50
40
30
20
10
0
Control
Disrupted
LD Condition

Figure 24. Frequency to enter the light section (mean ± *SEM*) as a function of Sex.


Frequency to Enter the Light Section (mean ± SEM)
60
50
40
30
20
10
0
Female
Male
Sex

***Latency to enter dark section*.** Data from two animals were excluded from the analysis because of a program malfunction ($N = 69$).

There was no significant difference in latency to enter the dark section between animals exposed to either the disrupted or undisrupted schedule, F (1, 57) = 0.38, $p > .05$, indicating that LD schedule disruption did not affect fear in the mice (see Figure 25). Males had significantly longer latencies to enter the dark section than females, indicating reduced fear in males and resulting in a significant sex effect, F (1, 57) = 13.60, $p < .05$ (see Figure 26). The LD condition x Sex interaction was not significant, F (1, 57) = 0.13, $p > .05$.

The LD condition x Sex x Replication interaction was significant, F (2, 57) = 3.771, $p < .05$.

***Defecation frequency*.** Animals exposed to the disrupted LD schedule produced significantly more fecal boli than animals that were exposed to the undisrupted LD schedule, F (1, 59) = 8.37, $p < .05$, indicating a fear-increasing effect of LD schedule disruption (see Figure 27). There was no significant difference in number of fecal boli between males ($M = 1.56$, $SEM = 0.38$) and females ($M = 0.80$, $SEM = 0.23$), F (1, 59) = 3.50, $p > .05$. The LD condition x Sex interaction was not significant, F (1, 59) = 0.50, $p > .05$.

Figure 25. Latency to enter dark section (mean sec ± *SEM*) as a function of LD condition.

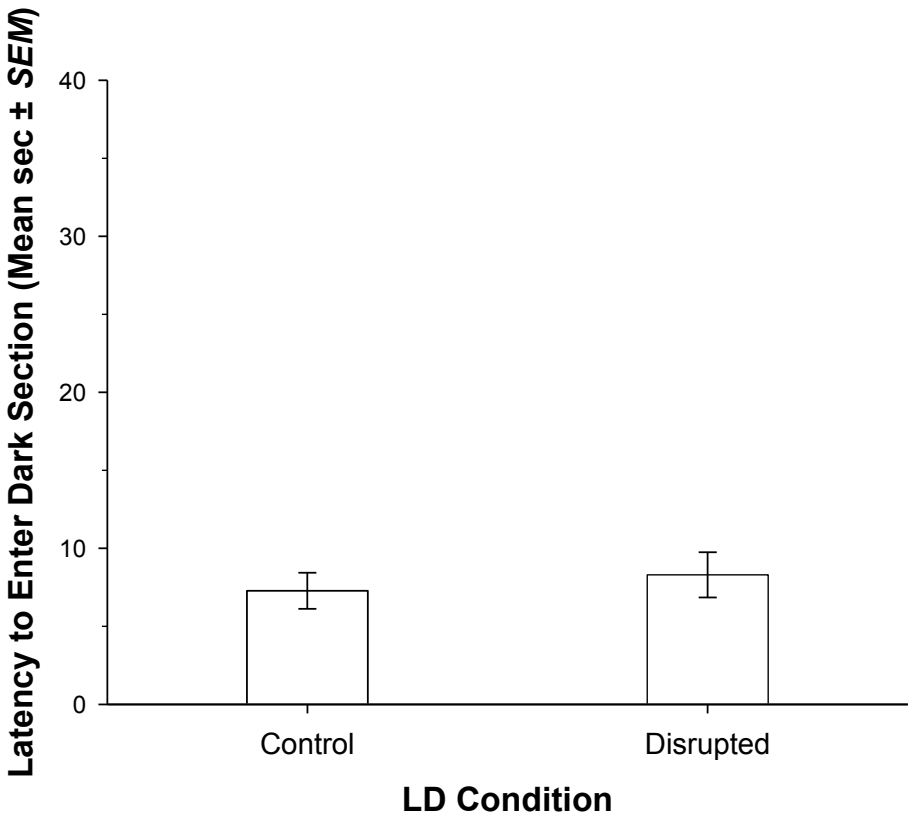

Latency to Enter Dark Section (Mean sec ± SEM)
40
30
20
10
0
Control
Disrupted
LD Condition

Figure 26. Latency to enter dark section (mean sec ± *SEMs*) as a function of Sex.

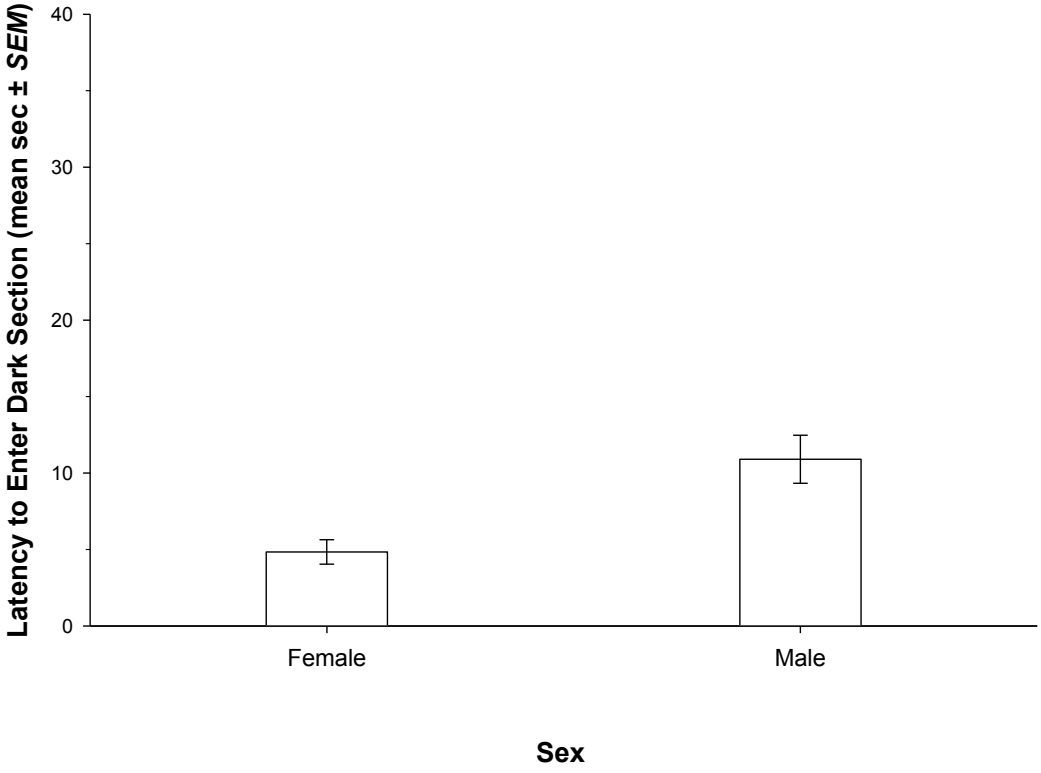
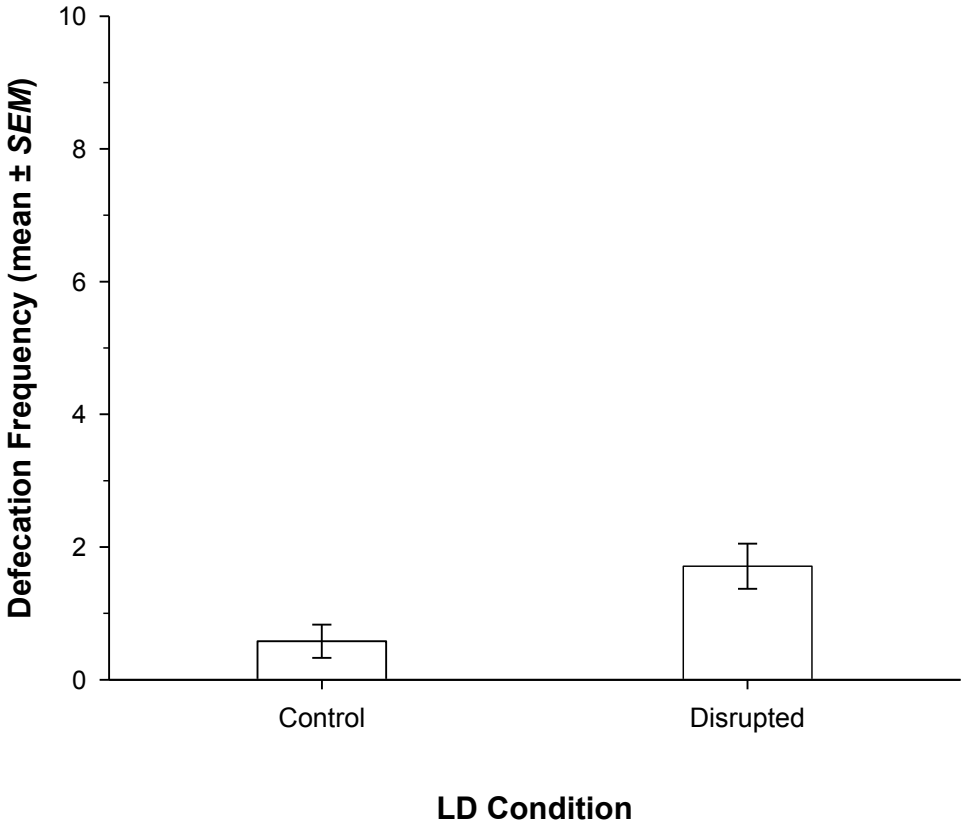

Latency to Enter Dark Section (mean sec ± SEM)
40
30
20
10
0
Female
Male
Sex

Figure 27. Defecation frequency (mean ± *SEM*) as a function of LD condition.


10
8
6
4
2
0
Defecation Frequency (mean ± SEM)
Control
Disrupted
LD Condition

Animals in the second replication (M = 1.91, SEM = 0.47) produced significantly more fecal boli than animals in the second replication (M = 0.67, SEM = 0.27), resulting in a significant effect of replication, F (2, 59) = 3.16, p = .05.

Only defecation frequency showed any effect of LD condition, with animals exposed to the disrupted LD schedule producing significantly more fecal boli than animals exposed to the undisrupted schedule, a behavior indicative of a fear-increasing effect of LD schedule disruption. The other variables showed no effect of LD condition, indicating that LD schedule disruption had no effect on fear. Males showed significantly longer latencies to enter the dark section and spent significantly more time in the light section than females, indicating that males were less fearful than females. However, females made more transitions into the light section than males, indicative of reduced fearfulness as well as locomotor activity in females.

Correlation Analysis. As previously discussed, because of discrepancies in results from the open field used in different laboratories, the light-dark box was used as a second measure of fear. Thus, to evaluate the correlation between the two assays, a correlation analysis was performed. Also, to evaluate relationships among variables in each assay, correlation analyses of variables in each assay were performed.

Open field variables. Percent time spent in the periphery had a significant positive correlation with latency to enter the center but a significant negative correlation with frequency to enter the center of the open field (see Table 1). Frequency to enter the center had a significant positive correlation with distance moved in the open field (see Table 1).

Light-dark box variables. Although percent time in the light section was positively correlated with frequency to enter the light section, this correlation was not significant (see Table 2).

Percent time in the light section was also positively correlated with latency to enter the dark section and defecation frequency, but the correlation in each case was not significant (see Table 2).

Open field and light-dark box variables. Percent time in periphery of the open field had a significant positive correlation with percent time in light section of the light-dark box in the first 5-min period but not the second 5-min period (see Table 3). Frequency to enter the center of the open field had a significant positive correlation with frequency to enter the light section of the light-dark box. Defecation frequency in both assays was positively correlated. Distance moved in the open field had a positive correlation with frequency to enter the light in the light-dark box (see Table 3).

Effect of LD Schedule on Body Weight

Mice were weighed once every 5 days and measured 8 times over the course of the experiment. The first measurement served as the baseline body weight of the mice. There was no significant difference in absolute body weight between mice exposed to the disrupted LD schedule and mice exposed to the undisrupted schedule, F (1, 59) = 0.41, $p > .05$ (see Figure 28). The LD condition x Sex interaction was also not significant, F (1, 59) = 0.00, $p > .05$.

Time period, F (6, 354) = 39.16, $p < .05$ and the Time period x LD condition interaction, F (6, 354) = 5.56, $p < .05$ were significant. However a Newman-Keuls analysis confirmed that within time periods, the control and experimental groups did not differ significantly. The Time period x Sex interaction was also significant, F (6, 354) = 5.92, $p < .05$ as was the time period x Replication interaction, F (12, 354) = 2.94, $p < .05$.

LD condition did not affect weight gain, F (1, 59) = 0.17, $p > .05$ (see Figure 29). However, females gained significantly more weight than males from the beginning to the end of the light treatment, F (1, 59) = 29.21, $p < .05$ (see Figure 29). The LD condition x Sex interaction was not significant, F (1, 59) = 1.55, $p > .05$.

Table 1. Correlation matrix of open field variables. An asterisk (*) indicates a significant relationship.

Variables	1	2	3	4	5	6	7	8	9	10	11
(1) Percent Time in Periphery (Period 1)	1										
(2) Percent Time in Periphery (Period 2)	.57*	1									
(3) Percent Time in Periphery (Total)	.88*	.88*	1								
(4) Frequency to Enter Center (Period 1)	-.91*	-.67*	-.88*	1							
(5) Frequency to Enter Center (Period 2)	-.73*	-.81*	-.87*	.83*	1						
(6) Frequency to Enter Center (Total)	-.84*	-.78*	-.92*	-.78*	.97*	1					
(7) Latency to Enter Center	.55*	.09	.36*	-.52*	-.24	-.37*	1				
(8) Fecal Boli	.10	.07	.10	-.11	-.10	-.11	.10	1			
(9) Distance Moved (Period 1)	-.67*	-.48*	-.65*	.80*	.72*	.79*	-.47*	-.14	1		
(10) Distance Moved (Period 2)	-.52*	-.41*	-.53*	.62*	.74*	.72*	-.22	-.12	.79*	1	
(11) Distance Moved (Total)	-.61*	-.46*	-.61*	.72*	.77*	.79*	-.33*	-.14	.91*	.97*	1

Table 2. Correlation matrix of light-dark box variables. An asterisk (*) indicates a significant relationship.

Variables	1	2	3	4
(1) Percent Time in Light Section	1			
(2) Frequency to Enter Light Section	-.08	1		
(3) Latency to Enter Dark Section	.01	-.25*	1	
(4) Fecal Boli	.21	-.11	-.12	1

Table 3. Correlation matrix of open field and light-dark box variables. An asterisk (*) indicates a significant relationship.

Variables		Light-Dark Box			
		Percent Time in Light Section	Frequency to Enter Light Section	Latency to Enter Dark Section	Fecal Boli
Open Field	Percent Time in Periphery (Period 1)	.25*	-.38*	.14	.31*
	Percent Time in Periphery (Period 2)	.15	-.41*	.08	.14
	Percent Time in Periphery (Total)	.23	-.44*	.13	.25*
	Frequency to Enter Center (Period 1)	-.18	.40*	-.14	-.28*
	Frequency to Enter Center (Period 2)	-.26*	.51*	-.09	-.21
	Frequency to Enter Center (Total)	-.24*	.49*	-.12	-.25*
	Latency to Enter Center	-.02	-.22	.17	.20
	Fecal Boli	.14	-.20	.03	.67*
	Distance Moved (Period 1)	-.09	.52*	-.24*	-.26*
	Distance Moved (Period 2)	-.11	.52*	-.06	-.19
	Distance Moved (Total)	-.11	.55*	-.13	-.23

Figure 28. Absolute body weight (mean g ± *SEM*) for Time period x LD condition. Each time period is 5 days long.

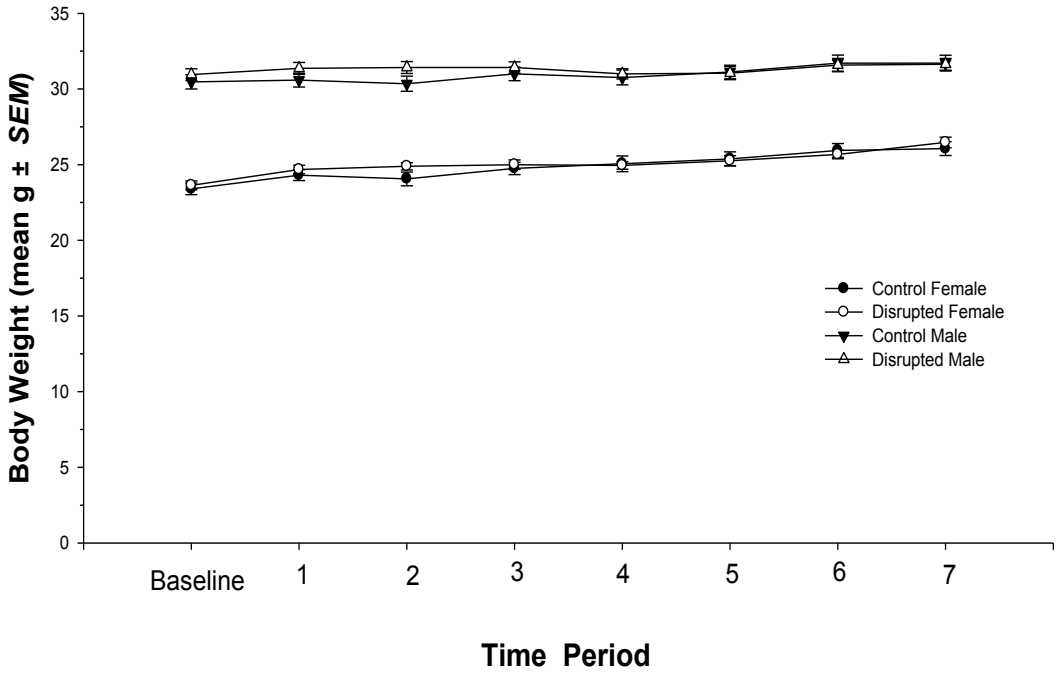
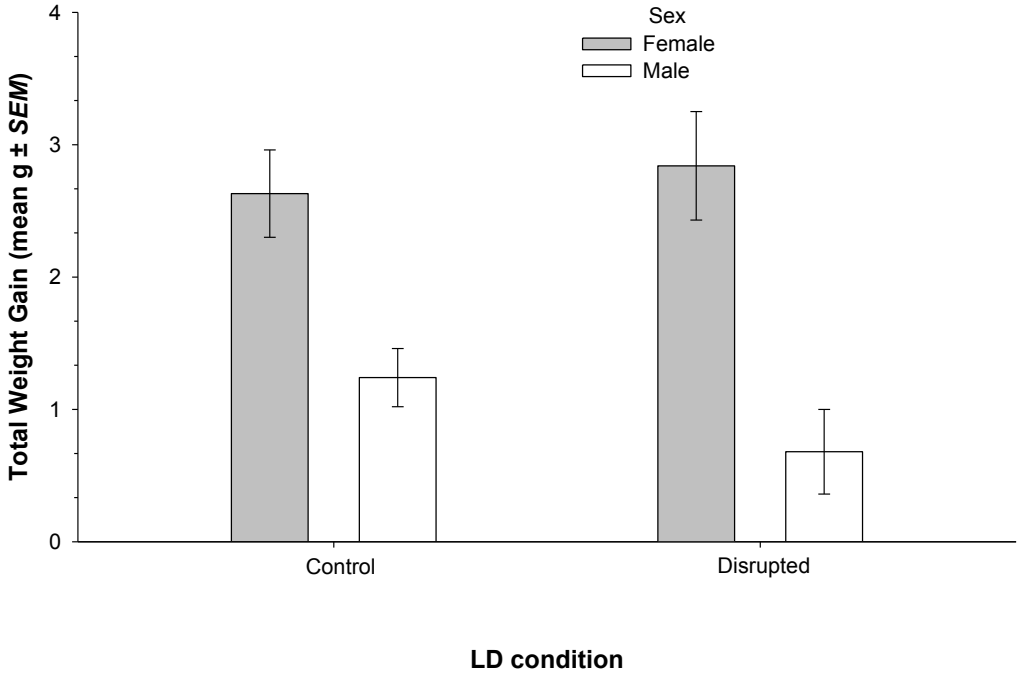

Body Weight (mean g ± SEM)
35
30
25
20
15
10
5
0
Baseline
1
2
3
4
5
6
7
Time Period
Control Female
Disrupted Female
Control Male
Disrupted Male

Figure 29. Weight gain (mean g ± *SEM*) from start to end of light treatment for LD condition x Sex. Each time period is 5 days long.


Sex
Female
Male
Total Weight Gain (mean g ± SEM)
4
3
2
1
0
Control
Disrupted
LD condition

Time period, F (6, 354) = 6.19, $p < .05$, and the Time period x LD condition interaction were significant, F (6, 354) = 3.10, $p < .05$. Again, a Newman-Keuls analysis confirmed that the control and experimental groups did not differ significantly in weight gain, within each time period. The control group gained the most weight during the third time period (between Days 10 and 15) and lost weight during the second time period (between Days 5 and 10) (see Figure 30).

Animals exposed to the disrupted LD schedule gained the most weight in the first time period and lost weight in the fourth time period (see Figure 30). The Time period x Sex interaction, F (6, 354) = 2.21, $p < .05$, was also significant but after a Newman-Keuls analysis, no difference was found between the sexes within each time period. Females gained the most weight within the first 5 days of treatment and gained the least weight in the second time period (between Days 5 and 10) (See Figure 31). Males gained the most weight during the sixth time period (between Days 25 and 30) and lost weight during the second time period (see Figure 31). The Time period x Sex x Replication interaction, F (12, 354) = 2.61, $p < .05$, was also significant.

Therefore LD condition did not affect body weight. Female mice gained more weight over the course of the experiment than male mice.

Figure 30. Weight gain (mean g ± *SEM*) for Time period x LD condition. A time period is 5 days long.

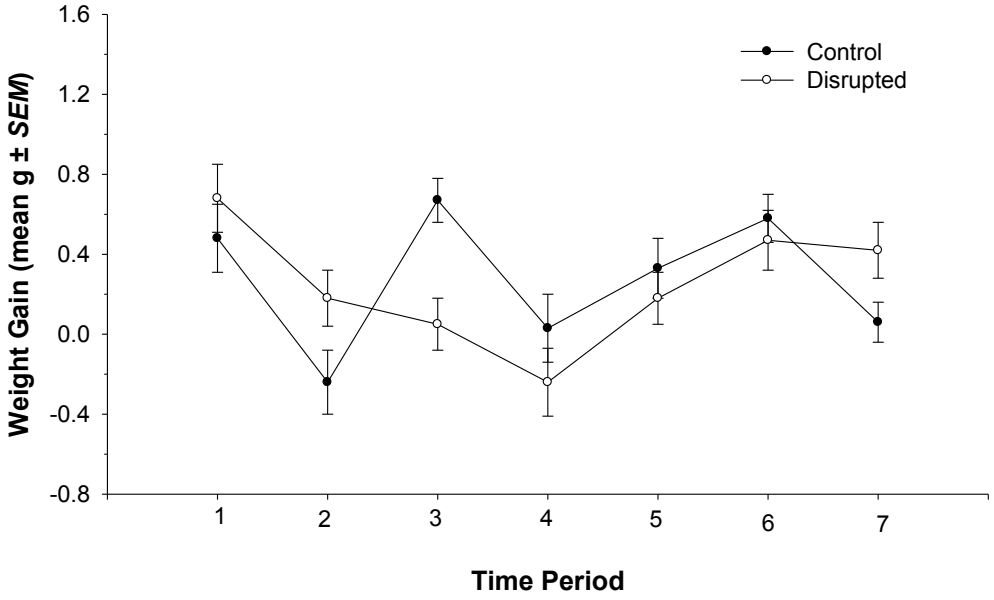
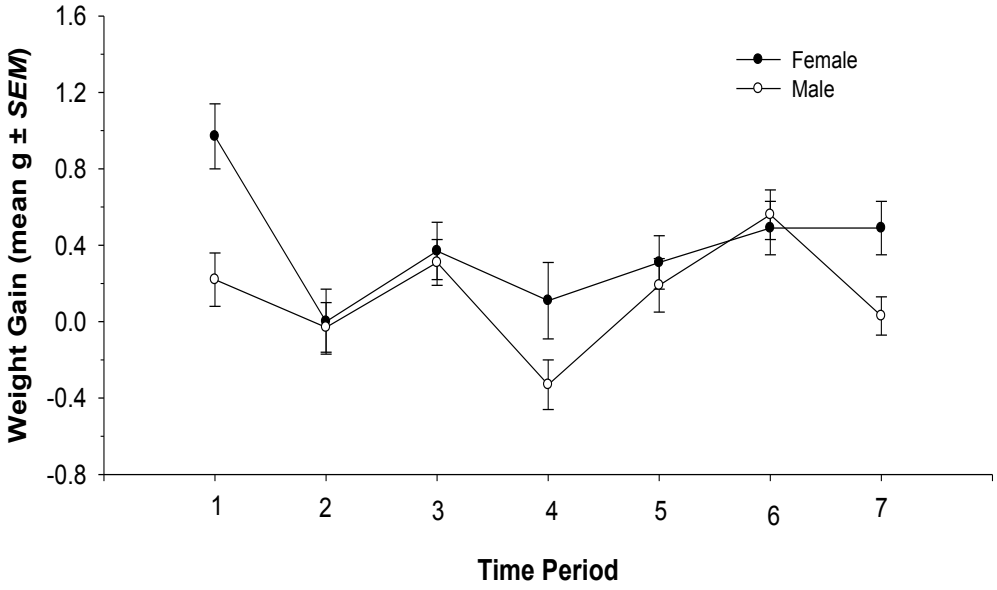

1.6
1.2
0.8
0.4
0.0
-0.4
-0.8
Weight Gain (mean g ± SEM)
Control
Disrupted
1
2
3
4
5
6
7
Time Period

Figure 31. Weight gain (mean g ± *SEM*) for Time period x Sex. A time period is 5 days long.


1.6
1.2
0.8
0.4
0.0
-0.4
-0.8
Weight Gain (mean g ± SEM)
Female
Male
1
2
3
4
5
6
7
Time Period

DISCUSSION

The objectives of the present study were to examine the effect of a disrupted LD schedule on fear and metabolism in mice and also to examine possible differences between females and males.

Measurement of Fear

There was no effect of LD schedule in both behavioral assays with one exception, defecation frequency. In the open field, there was no difference between experimental and control groups in percent time spent in the center and in the number of entries into the center. Similarly, in the light-dark box, there was no difference between experimental and control groups in percent time spent in the light section or in the number of entries into the light section of the box. These results show that disruption of LD schedule does not affect fear in mice, which is contrary to results by Karatsoreos et al. (2011) and Li et al. (2009), both of which support a fear-reducing effect. However, results from the present study are consistent with results from a study on the previously mentioned circadian mutant, Clock, that showed that circadian disruption had no effect on fear behaviors in the open field and the elevated plus maze (Easton, Arbuzova, and Turek, 2003). The study also found that circadian rhythm disruption increased exploratory activity in mice, a finding that the current results do not support. LD condition did not significantly affect locomotor activity in the open field nor did it

affect frequency of entries into the center of the open field or the light section of the light-dark box. However, because the mice used in the Easton et al. (2003) study were circadian mutants, results from the study might not be directly comparable to results in the present study that used wild type mice.

Animals also traveled significantly more in the second 5-min period of the trial than in the first 5-min period. The increased locomotor activity, might be because the freezing behavior initially evoked by the novelty of the open field, was replaced by an exploratory behavior as the trial continued. Research shows that wild mice tend to freeze when placed in a novel environment (Smith, 1978). The length of this freezing behavior varied among subjects in the present study, with some mice freezing for varying amounts of time and others not freezing at all.

For both the open field and light-dark box, only defecation frequency was significantly affected by LD condition, with higher numbers of fecal boli produced by mice in the disrupted LD condition suggesting a fear-increasing effect of LD schedule disruption. This result is not consistent with past research that shows a fear-reducing effect (Karatsoreos et al., 2011; Li et al., 2009). However, defecation frequency might also be influenced by time of food intake and thus might not be a reliable measure of fear (Lister, 1990; Turek et al., 2005). In the study by Turek et al. (2005), the circadian Clock mutants had a shifted food intake period, and therefore consumed more food during the light period of the

LD cycle than during the dark period, when control animals consumed the most food. Though Turek et al. (2005) suggest this shifted food intake as a possible explanation for the observed body weight gain, in the present study, shifted time of food intake might also have affected defecation frequency in the open field. If the same shifted food intake occurred in the present study, then the experimental animals, which were tested during the dark phase of the LD cycle, might have eaten during the preceding light phase and consequently produced more fecal boli at the time of testing than control animals. However, because time of food intake was not monitored in this study, it is unknown whether food intake time was shifted in the mice exposed to the disrupted LD schedule.

Although the results showed that LD condition did not significantly affect latency to enter the dark section of the light-dark box or the center of the open field, the latency variable may also not be a very reliable measure of fear. In the open field, if animals took a long time to enter the center zone because they had just been awoken, such behavior would increase recorded time in the periphery and be wrongly regarded as indicating increased fearfulness. Similarly, in the light-dark box, animals may not have entered the dark section of the box because of sleepiness or because of initial freezing. (In one of the trials, the animal spent the first half of the 5-min trial in the same corner of the open field it was placed in.) In the case of sleepiness, increased time in light section would be wrongly regarded as decreased fearfulness. In the second case, where an animal could have

taken longer to enter the dark section because of freezing, such a behavior, which would normally indicate fear, will be wrongly regarded as an indication of reduced fear. However, this possible source of error does not affect the interpretation of the present results, as even after excluding the latency period from the total trial time, LD condition did not significantly affect the time spent in the light section.

Correlation analysis. A positive correlation was observed between percent time in the periphery of the open field and percent time in the light section of the light-dark box. One would expect that if both variables were measures of fear, they would be negatively correlated, that is, as percent time in the periphery increased, percent time in the light section would decrease. The positive correlation between the two variables therefore suggests that the two variables are not equivalent measures of fear. This result could also be as a result of a learning effect on the mice. Mice were more fearful in the novel open field but less so when placed in the light-dark box two days later.

Frequency to enter the center was negatively correlated with percent time in the periphery, which meant that as both variables were in agreement as measures of fear in the open field. As percent time in the periphery increased, frequency to enter the center also decreased, and as such both behaviors were indicative of increased fear. Frequency to enter the center was also positively correlated with frequency to enter the light section of the light-dark box, which

means that the latter variables might also be a good measure of fear in the dark section.

Defecation frequency in both the open field and light dark box were positively correlated but were not significantly correlated with percent time in the periphery or in the light section of the light-dark box. This result indicates that defecation frequency might not be a reliable measure of fear in either of the assays.

Measure of Body Weight

LD condition did not affect body weight of mice in the present study. This finding is consistent with previously reviewed studies that show that altering LD conditions does not affect body weights in healthy mice (Nelson & Halberg, 1986; Preuss et al., 2008). Another study also showed that mice exposed to a disrupted LD schedule only began to gain significantly more weight than control mice six weeks into the light treatment (Karatsoreos et al., 2009). This suggests that a more prolonged period of exposure to the light treatment might be necessary to affect body weight.

Also, females gained more weight over the course of the experiment than males. This finding is contrary to a previous study by Hong, Stubbins, Smith, Harvey, and Nunez (2009) in which males gain more weight than intact and ovariectomized females, when exposed to either a 30% calorie-restricted, low fat or high fat diet.

Sex Differences in Behavioral Assays

Results from the open field showed that males spent significantly more time in periphery and produced more fecal boli than females, indicating that males were more fearful than females. However, results from the light-dark box showed that males spent more time than females in the light section of the box and took longer to enter the dark section, indicating the opposite that males were less fearful than females. Although these results may point to the possibility that females and males may act differently in various behavioral tests, males might have been more sensitive to the carry-over effect from the open field test two days earlier.

As previously mentioned, sex differences observed must be interpreted with care as factors such as hormone levels, might be a confound. Open field behavior has been found to be affected by the estrus cycle in females, with the increased fear observed in the estrus and proestrus periods (Kazuya et al., 2009; Rosskothen et al., 2008). Since the estrus cycles of the female mice were not monitored, variation among female subjects in the open field and light-dark box might be because the mice were at different stages of the estrus cycle. Results from open field testing are also not consistent with results from a study by An et al. (2011) that investigated sex differences in fear behavior in two inbred strains of mice. Results showed no differences between the sexes in percent time spent in the center of the open field and frequency of transitions between zones.

Results from the light-dark box that showed that females made more entries into the light section than males could also be taken as an indication of increased locomotor activity in females, a finding that supports the initial hypothesis and past research (Simpson et al., 2012).

Replication Effect

For some of the variables in the open field and light-dark box, animals in Replications 1 and 3 differed significantly from each other. While the same protocol was repeated each time, it is possible that differences among experimenters in handling the animals might have made testing conditions different for one group than the other. Another possibility is that slightly higher light intensities in the isolation rooms used in Replication 1 might have altered the experience of animals in that replication. However, one would expect that because illumination conditions were similar in Replications 2 and 3, statistical analysis would also have obtained a significant difference between Replications 1 and 2, which was not the case.

The fact that the replications occurred at different times of the year, however small the time difference, might also contribute to the significant replication effect observed. With more available experimenters and access to greater number of computers and Ethovision® software packages, this problem might be solved, though different setups might also result in different results.

Future Studies

Previous research suggests that disruption of LD schedules leads to physiological disruptions such as desynchronization of circadian rhythms and increases in metabolic markers like leptin and insulin (Davidson et al., 2009; Karatsoreos et al., 2011). The present study only assessed the effect of disrupted LD schedules on behavior. Though results show that LD disruption for 35 days does not affect fear in mice, in a future study, I would like to examine whether the same light treatment alters metabolic markers and other physiological markers in the animals.

In the study by Karatsoreos et al. (2011), researchers found that weight gain in the animals exposed to the disrupted LD schedule became noticeable between the fourth and fifth week of treatment and only became significant after the sixth week. It is possible that 35 days was too short a period to see an effect. Therefore in future studies, I would explore the effect of a longer period of LD disruption on fear in the mice.

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APPENDIX

Open Field Protocol Thesis Research Spring 2013
Contacts:
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Pre-Trial:

1. Retrieve lab coat from 128. Proceed to 215.
2. Put on the computer and open Ethovision. Open correct experiment: Click 'File' and in the file names at the bottom of the window click '2012f-try2.ews,'
3. Make sure the experiment 'Openfield_Kehinde 2013' is bolded. This means it's active. If it is not, right click 'Openfiled_Kehinde2013' and select 'Set as active.'
4. In 215 B, turn on power and camera. Ensure light dial is pointed at the middle 'T.'
5. Clean the open field, making sure to clean the sidewalls as well as the floor. Ensure that the open field is aligned with the marks on the table. Place anything on the table on the floor. Only the open field should be on the table.
6. Turn off florescent lights in the corridor of 215 and in the main vivarium corridor (The light switch is by the red door farther away from 214)
7. Get red light torch from the back of 214's door. Wash hands in 214 and proceed to 218. Leave lights off in 218 corridor. Plug and turn on red lamp on the counter in 218.
8. Locate a cart in 218. You will transport the animal on this cart.
9. Go to the appropriate isolation room in 218 and get the first animal. Place cage on cart and wheel to 215.

Trial:

1. "Computer person" should prepare to collect data with Ethovision while the "Animal person" is in 218.
    a. Under workspace tab, go to view "Arena Profile."
    b. Under experiment menu, select "Acquire data", under Tracking menu, go to Update Detection Variables; make sure that the values are: Low Limit (91) and High Limit (255); click OK.

    c. Click Play (the sideways triangle symbol).
    d. Enter Experimenter ID and Mouse ID (assigned alphabet) in the pop-up window; do NOT click OK yet; leave pop-up window as is.
2. The "Animal person" should bring the first cage in from 218 to 215 and place cage beside open field.
3. Click OK on Experimenter ID pop-up window. The "Introduce Mouse" window appears.
4. Place mouse in bottom right hand corner of open field. Click OK in "Introduce Mouse" window. Leave cage on the floor and exit 215 B quietly, shutting the door until it is only slightly open.
5. Count number of center/periphery transitions during trial using counter and record on data sheet after **each period.** (You should have **2 values** of center/periphery transition for every trial.
6. Take note of number of samples not found (it's in the second box, not the top box) at the end of **each period**. Record on data sheet. (Again, you should have **2 values** for the number of samples not found per trial.)

After trial:

1. Remove mouse from open field. Count and record number of fecal boli. Clean open field with ammoniated solution, making sure to clean floor and sidewalls. Check that open field is aligned with the marks on the table.
2. Return mouse to appropriate isolation room in 218.
3. Bring new mouse into 215 and repeat trial procedure.

After all trials:

1. Make sure that all cages are returned to the correct isolation room in 218 and counters are wiped down, and that all doors are securely closed.
2. In Ethovision, under View Data:
    a. Under "Experiment", select "Visualize"
    b. Under "Data", click "Select tracks" and choose appropriate tracks
    c. Under "Experiment " select "Analyze Data" and click Yes to save
    d. Under "Analysis" select "Calculate"
3. Save data
4. In 215B, ensure lights and camera are off, open field is clean, and all lights in 215 are off.

Light-Dark Box Protocol Thesis Research Spring 2013

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Pre-Trial:

1. Retrieve lab coat from 128. Proceed to 215. Ensure you have data sheet and counter with you.
2. Put on the computer and open Ethovision. Open correct experiment: Click 'File' and in the file names at the bottom of the window click '2012f-try2.ews,'
3. Make sure the experiment 'Lightdarkbox_Kehinde 2013' is bolded. This means it's active. If it is not, right click 'Lightdarkbox_Kehinde2013' and select 'Set as active.'
4. In 215 B, turn on power and camera. Ensure light is turned all the way up.
5. Clean the light-dark box, making sure to clean the sidewalls as well as the floor. Ensure that the box is aligned with the marks on the table. Place anything on the table on the floor. Only the light-dark box should be on the table.
6. Turn off florescent lights in the corridor of 215 and in the main vivarium corridor. (The light switch is by the red door farther away from 214) Make sure the motion sensors aren't active. Walk towards the sensor to make sure.
7. Get red light torch from the back of 214's door. Wash hands in 214 and proceed to 218. **Leave the light in 218 off**. Plug and turn on red lamp on counter in 218.
8. Locate cart in 218. You will transport the animal on this cart.
9. Go to the appropriate isolation room in 218 and get the first animal. Place cage on cart and wheel to 215.

Trial:

1. "Computer person" should prepare to collect data with Ethovision while the "Animal person" is in 218.
    a. Under workspace tab, go to view "Arena Profile."
    b. Under experiment menu, select "Acquire data."
    c. Click Play (the sideways triangle symbol). Ethovision will ask you to remove animal. Make sure nothing is in the box and that the box is aligned with the arena definition on the screen. Click OK.

    d. Enter Experimenter ID and Mouse ID (assigned alphabet in uppercase) in the pop-up window; do NOT click OK yet; leave pop-up window as is.
2. The "Animal person" should bring the first cage in from 218 to 215 and place cage beside light-dark box.
3. Click OK on Experimenter ID pop-up window. The "Introduce Mouse" window appears.
4. Place mouse in bottom right hand corner of open field. "Computer person," click OK in "Introduce Mouse" window. Leave cage on the floor and exit 215 B quietly, shutting the door until it is only slightly open.
5. Count number of transitions into the lit section of the light-dark box during trial using counter and record on data sheet. Take note of the first time the mouse goes into the dark. Record it on data sheet.
6. Animal person" should monitor "number of samples not found" value during trial. Take note of number of samples not found (it's in "Object information" box) at the end of each trial. Record on data sheet.

After trial:

1. Remove mouse from box. Count and record number of fecal boli. Clean open field with ammoniated solution, making sure to clean floor and sidewalls. Check that box is aligned with the marks on the table.
2. Return mouse to appropriate isolation room in 218.
3. Bring new mouse into 215 and repeat trial procedure.

After all trials:

1. Make sure that all cages are returned to the correct isolation room in 218 and counters are wiped down, and that all doors are securely closed.
2. In Ethovision, under View Data:
    a. Under "Experiment", select "Visualize"
    b. Under "Data", click "Select tracks" and choose appropriate tracks
    c. Under "Experiment " select "Analyze Data" and click Yes to save
    d. Under "Analysis" select "Calculate"
3. Save data

In 215B, ensure lights and camera are off, light-dark box is clean, and all lights in 215 are off.