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The impact of sleep disruption on mouse physiology, behavior, and welfare

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THE IMPACT OF SLEEP DISRUPTION ON MOUSE PHYSIOLOGY, BEHAVIOR, AND WELFARE

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For Dad

Who taught me how to nerd

For Mike

Who kept me sane

For Addie

Who kept me company

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ABSTRACT

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Laboratory mice are nocturnal, spending most of their daylight hours asleep. But they live in the diurnal world of human investigators and husbandry staff, who primarily work during this rest period. In humans, lack of sleep or sleep that occurs outside the normal circadian sleep period (as in shift work) has adverse effects. These include increased risk of cardiovascular disease, cancer, metabolic disorder, mood disorders, type II diabetes, and obesity. However, it is unknown if mice experience sleep disruption due to these human activities, and, if so, what the adverse effects may be. This is an important question, not only to ensure good welfare for laboratory mice, but also to improve experimental validity. If researchers are inadvertently inducing physiological or cognitive changes in mice through sleep disruption, we may be confounding experimental results in unpredictable fashions. This is particularly relevant to biomedical research, as only eleven percent of drug trials that pass the animal testing stage go on to pass human trials. Part of this discrepancy may be due to sleep disruption-induced changes in mice.

In Chapter 2, we tested two different disruption times, one during the day (their rest period) and one at night (their active period). These changes in disruption time produced no differences in overall amounts of sleep, though there were changes in sleep timing based on sex and type of mouse. These results suggest that disturbance timing does affect sleep, but that response isn't uniform across strains or sexes. However, it is possible that our brief welfare checks may have been too predictable and inconsequential to induce true sleep disruption.

In light of these results, our next experiment (Chapter 3) involved testing more extensive and unpredictable disruptions, as well as using both physiological and behavioral measures, as well as sleep monitoring. In this project, mice were exposed to either a week of predictable disruptions, or a week of those same disruptions, consolidated at the beginning and the end of the day. After 4 days of disruption, we performed a biopsy punch procedure on them to assess wound healing, with mice being assigned to an analgesia or control group. Again, overall sleep did not change for mice in response to disruption. They did, however, display a decrease in activity levels, likely due to the stress of handling and restraint for manual analgesia injection. Additionally, male mice who received analgesia spent more time sleeping than their female counterparts, suggesting that an adequate dose for males may not be sufficient for pain relief in females.

CHAPTER 1. LITERATURE REVIEW

1.1 Abstract

While ethology often measures lying or resting behavior, sleep is a rarely discussed welfare indicator. This review investigates the idea of using sleep as a scientific measure of welfare, in an effort to make use of this prevalence. Sleep functions as a period of restoration, memory consolidation, immune activation, and energy conservation. Sleep timing and duration are controlled by both external and internal factors. As sleep disruption is known to produce adverse outcomes in humans, this is a potentially critical aspect for domesticated animals. Their environment is frequently outside of their control, which may affect their ability to sleep normally. Sleep is challenging to accurately identify externally, but with validated criteria it should be possible. However, while reliable, we feel that sleep is too plastic under various conditions, and too varied within and between species, to be used as a standalone assessment tool. That doesn't mean that it can't be used, though. Like many other welfare measures, such as glucocorticoid levels, when compared to baselines and used in context with other criteria, it has the potential to be a powerful tool in the welfare scientist's toolbox.

Keywords: Animal welfare, sleep assessment, circadian rhythms

1.2 Introduction

Welfare science relies upon the ability to devise and evaluate metrics that permit researchers to objectively assess animal well-being. To get an accurate picture of overall welfare, researchers must evaluate multiple aspects of an animal's physical, mental, and behavioral well-being [1]. This can involve multiple techniques, some of which are labor, time, and/or money intensive. Therefore, to make efficient and effective decisions regarding which welfare assessment tools to use, it's to the advantage of welfare scientists to continue investigating new methods of measurement that may be simpler or measure multiple aspects of welfare with one test. An effective welfare measurement should fulfill the same criteria as an effective diagnostic test; it must be reliable and valid. Furthermore, a measure that can be used across species broadens its usefulness. One potential such measure that fits these criteria is sleep.

All terrestrial mammals (under normal circumstances) sleep for at least part of a 24-hour day, making this behavior a potential welfare measure that is advantageous across species. In addition, sleep is a familiar behavior that is easy to recognize by those who are not formally trained to identify and measure behavior such as animal husbandry personnel, who are likely the ones to be utilizing this behavior as a measure. This review aims to discuss sleep, its features and functions, and to assess its suitability as a welfare measure in captive terrestrial animals.

Marine mammals have dramatically different sleep physiology and patterns than terrestrial mammals. In order to breathe while sleeping, marine mammals display sleep in one hemisphere of the brain at a time[2, 3]. They may go for extended periods of time

without sleeping at all, particularly after parturition[2, 3]. They even appear to display little to no REM sleep[2, 3]. Therefore, we will only review the sleep of terrestrial mammals or marine mammals while they are on land (such as seals and sea lions). For those interested in in further information, Siegel[3] and Siegel[2] both cover marine mammal sleep in general. For a short comparison of sleep across taxa, including non-mammalian species, we suggest Siegel[2]. Campbell and Tobler[4] also cover phylogenetic variation of sleep duration.

1.3 Sleep Form and Function

1.3.1 Defining Sleep

Sleep is visually characterized by three factors: a characteristic body position (such as lying down, or hanging upside down), dramatically reduced physical activity, and an elevated stimulus response threshold[3, 5]. However necessary they may be, these criteria are not sufficient to identify sleep. Visually distinguishing a sleeping animal from an animal that is quietly resting may be effectively impossible; some species sleep standing up and lying down, rendering the postural criteria ambiguous; and an increased stimulus threshold may also happen for other physiological reasons. Therefore, physiological criteria are needed to correctly identify true sleep.

The gold standard of sleep identification uses electroencephalogram (EEG) recordings, which track the electrical activity in the brain. By convention, sleeping waveforms are typically classified by their frequency as alpha, beta, theta, and delta waves (Figure 1). Beta waves occur at the highest frequency, at over 13 Hz. Alpha is the next highest at 8-

13 Hz, followed by theta waves at 3.5-7.5 Hz, and delta waves at less than 3 Hz[6]. There are also morphological classifications of waveforms. K-complexes are large amplitude (tall) delta waves that signal a partial arousal from sleep. Spindles are groups of waves of increasing and then decreasing waves that, taken as a group, resemble a spindle[6]. Researchers use these classifications to score sleep depth, duration, and bout frequency[6].

In animals, sleep is typically classified into two stages, non-REM sleep (NREMS) and REM sleep (REMS). While an awake animal's brain displays low-voltage, fast activity[7], "slow wave sleep" (SWS) or "delta sleep", is marked by slow, rhythmic delta waves – this occurs during NREMS. REMS (rapid eye movement sleep) is named for its characteristic rapid eye movements. REMS EEG waves are asynchronous, meaning that rather than different electrodes presenting a similar pattern and level of activity as they do in NREMS, brain activity is out of sync and very active, much more similar to what is seen in an awake individual. REMS is also marked by lack of muscle tone in most voluntary muscles[5].That has led to REMS also being referred to as "paradoxical sleep", because the level of brain activity doesn't match the level of the rest of the body's activity. While human EEGs allow for discernment of these distinct states via a large number and varied placement of electrodes, animal studies generally use an electromyogram (EMG) electrode to detect muscle movement and electrooculogram (EOG) electrodes to record eye movement; these additional readings can allow for dissection of NREMS and REMS. Mammals experience sleep cycles, which are periods of NREMS followed by REMS; more than one cycle may happen in any given sleep period[8].

1.3.2 Physiological Changes During Sleep

The various stages of sleep are also marked by physiological changes. During NREMS, heart rate, blood pressure, and respiratory rate decrease, eye movement is a slow rolling pattern, and muscle tone is reduced. During REMS, eye movement is frequent, heart rate, blood pressure, and respiratory rate approach near-waking levels, and muscle tone is absent. Not all physiological changes are universal across species, however. For instance, rats experience penile erections during REMS[9], but in armadillos those erections occur during NREMS[10]. In humans, growth hormone release is linked to NREMS[2], but in dogs it occurs during waking hours[11].

Metabolically, sleep also presents drastic changes. Glucocorticoid levels drop during sleep, and begin to rise again an hour or two before waking. Epinephrine and norepinephrine levels also drop over this period. In the meantime, growth hormone (GH), prolactin, melatonin, and leptin levels rise. The increase in these hormones supports immunological effects, including increased production of proinflammatory cytokines, such as IL-1 and tumor necrosis factor (TNF), and T helper cytokines like interferon (IFN) [7, 12-14].

1.3.3 Control of Sleep

A significant amount of research has been aimed at trying to determine what exactly induces sleep[7, 12, 15-34]; the results of that work indicate that there is no one single chemical or neurotransmitter that controls sleep and waking, but rather it is a complex and multi-faceted process. Currently, sleep is thought to be controlled by 2 different processes – Process C, which drives our waking processes and is governed by circadian

rhythms, and Process S, the endogenous drive for sleep that increases as we are awake[18] (for a more in depth review of the control mechanisms of sleep see Brown[7].

1.3.3.1 Process C

Circadian rhythms are patterns that occur with a period of approximately 24 hours. While the sleep/wake cycle is one of the most obvious examples of a circadian pattern, it's far from the only one. Circadian patterns in gene expression have been found in explanted liver, kidney, spleen, thyroid, adrenal, heart, and stomach tissues[35]. Keeping all of these "clocks" synchronized is the job of the area of the brain called the suprachiasmatic nucleus (SCN). The SCN communicates with these peripheral oscillators using a combination of neurotransmitters and hormone secretion to maintain a unified time sense; some of these substances include gamma-aminobutyric acid (GABA), orexin, histamine, serotonin, epinephrine, norepinephrine, acetylcholine, glutamate, and cortisol releasing hormone (CRH). Studies have shown that lesioning the SCN eliminates circadian locomotor patterns, and SCN transplantation into lesioned animals restores them[35]. The SCN itself also oscillates, with a period of a bit more than 24 hours with no external cues; typically it uses information about environmental light/dark status to entrain itself to the 24 hour day. This information comes from specialized photosensitive retinal ganglion cells in the eye that project to the SCN and detect the presence or absence of light, regardless of visual acuity of the organism[36]. This entrainment is why changing time zones requires an adjustment period before we sleep normally again – our SCN is adjusting to a shift in light/dark timing[37].

1.3.3.2 Process S

Separate from our circadian rhythms is our internally driven need for sleep. The longer we are awake, the stronger that drive grows, and the longer and deeper our sleep, the more quickly it dissipates. That drive is referred to as Process S. Process S is governed by the mutual inhibition of sleep- and arousal-enhancing neurological systems. Some of the major actors in Process S include adenosine, nitrous oxide (NO), prostaglandin D2 (PGD2), and cytokines, including IL-1β and tumor necrosis factor (TNF) α [7]. Recent work has also shown that in C57BL/6 mice, not only does the ionic composition of the cerebrospinal fluid (CSF) change during sleep, but by infusing an artificially created CSF with the ionic composition of a sleeping mouse, they could also induce sleep in waking mice[15].

1.3.3.3 C and S Combined

The combination of Processes C and S works to consolidate sleep in humans. Process S "tells" us to sleep, and Process C "tells" us when to do it. This duality means that getting sufficient sleep, but at the "wrong" time can lead to detrimental effects (as seen in shift work [38-41]); so too does getting sleep at the "right" time, but not enough of it [42-44].

1.3.4 Functions of Sleep

For such an ubiquitous behavior, the functions of sleep are not immediately obvious. Entering a stage of consciousness where an organism may be more vulnerable to predation, as well as sacrificing hunting, foraging, or mating time, would seem to induce a dramatic drop in organismal fitness. The fact that it persists, coupled with the

conservation of sleep throughout the terrestrial mammalian taxa, strongly suggests that sleep serves an adaptive function[3, 5, 45].

1.3.4.1 NREMS Function

NREMS may act as a metabolic rate control. Animals with higher metabolic rates produce metabolic byproducts at a similarly higher rate; these byproducts include reactive oxygen species (ROS) which have been implicated in aging. Sleep provides an opportunity for high-metabolism animals to repair the damage done by these ROS, synthesize protective factors, and pre-emptively slow production of ROS [3]. However, others have argued[46] that the studies that produced these results had dramatically different environmental conditions, and once those confounds are controlled for, sleep duration correlates negatively with basal metabolic rate. They argue that instead sleep duration is a mix of trade-offs between foraging time and predation risk, and that species that sleep for shorter durations may compensate for that by sleeping more deeply when they do sleep[46]. For nocturnal animals, sleep may provide a dual function of both energy conservation and predator avoidance. Many predators are diurnal, so sleeping through their active period leads to decreased risk of predation[47]. The early phases of NREMS also seem to be a period of immune system activation; pro-inflammatory cytokines increase and sleep deprived individuals have a decreased response to immunization[12-14, 48-50]. Finally, recent work has shown that sleeping mice (whether naturally sleeping or under anesthesia) experience a 60% increase in the interstitial space of their brains. This leads to a convection current effect with their CSF,

increasing clearance of metabolites including β-amyloid, a major factor in the progression of Alzheimer's[51]. This suggests that sleep may also provide an opportunity for the brain to clear metabolites and refresh the CSF for the demands of the next waking period.

1.3.4.2 REMS Function

REMS is not as obviously adaptive as NREMS, and there has certainly been disagreement in the sleep study field over it[52-56]. Part of the question involves the fact that, during REMS, the brain uses nearly as much energy as when it is in a waking state. This would seem to counteract at least some of the energy-saving potentially accomplished during NREMS. One potential function of REMS is memory consolidation. Studies have found that REMS deprivation in rodents reduces their spatial memory consolidation[57, 58]; however, others have argued that, since rodent results haven't been reproduced in humans, this may not be a universal function[59, 60].

Another potential function of REMS is developmental. Species with altricial (less developed) young tend to have more REMS than those with precocial (more developed) young, a trend which continues into adulthood [3]. Guinea pigs, who are highly precocial, have 1 hour of REMS per day, while the altricial platypus gets 8 hours of REMS per day[3]. Further supporting the developmental hypothesis are studies in monocularly deprived kittens; with one eyelid sewn shut and deprived of REMS, they had a greater reduction in neuronal tissue along the visual pathway than those who were not deprived of REMS[61].

The patterning of sleep stages also suggests a possible function for REMS – preparing the organism for abrupt waking by providing lower stimulus threshold periods during the sleep period[62]. Rats in REMS respond to a startle-inducing stimulus more quickly than those in NREMS [63], which would be an adaptive advantage for a prey species. Even humans have longer REMS periods as the night progresses, until the period length peaks at the end of the sleep phase[3].

1.4 Sleep Dysfunction

In 2004, an estimated 4.52 billion dollars was lost in both direct and indirect costs due to sleep disorders – in Australia alone[64]. This makes sleep disorders an active field of study. In fact, most of our knowledge about the functional consequences of sleep comes from research into sleep dysfunction; we manipulate the organism's sleep, and then investigate the subsequent changes. This review is by no means exhaustive in regards to all the types of sleep disorders, but is instead focused on the dysfunctions that are most relevant to the animal welfare field.

1.4.1 Sleep Deprivation/Restriction

Sleep deprivation refers to the absence of sleep, whether it be total sleep deprivation or REMS deprivation. Partial sleep deprivation, also known as sleep restriction or insomnia, is also frequently studied. Sleep restriction is typically achieved experimentally through the same methods as sleep deprivation, except the deprivation is induced over a shorter period, permitting some sleep (restriction) instead of none (deprivation).

Total sleep deprivation has varying effects, depending upon the species in question. Rats who are completely sleep deprived lose weight, in spite of an increase in appetite; they

develop lesions on their paws and tails; their core body temperature rises and then falls; and will die if deprivation is continued for as short a period as 5 days[65, 66]. Even restricted sleep will kill rats after 4-5 weeks [65]. Sleep deprived rodents also show a decrease in memory acquisition tasks[57]. Male offspring from pregnant rats who are REMS deprived had fewer ejaculations and a longer latency to intromission when exposed to a receptive female than those whose dams were not REMS deprived [67]. Mice perform poorly on novel object place recognition tests if they are REMS deprived after the first object exposure[58]. However, total sleep deprivation in humans shows fewer physiological effects, primarily decreased glucose metabolism in the brain; the main effects in humans are cognitive and psychological, including increased negative affect, decreased performance on cognitive tasks, and decreased memory performance[43]. Why there is a difference between animal and human responses to sleep deprivation isn't clear. However, ecology and psychology may offer some clues.

Recent work has shown that apes sleep less than other primates, and humans sleep the least of all the apes[68]. These researchers hypothesize that the reason behind that is that, as our common ancestors became terrestrial rather than arboreal, sleeping individuals became even more vulnerable to predation. This increased vulnerability imposed selective pressure to decrease the duration of sleep. This paper contends that humans compensated by developing the ability to sleep more "intensely" than other primates. By that, they mean that we oscillate back and forth between NREMS and REMS more frequently, permitting the maximal advantages (cognitive and physiological) conferred

by both states. Perhaps this sleep intensity also provides a protective effect against deprivation.

From a psychological perspective, we know that lack of choice or control over the environment induces stress in animals[69-71]. Humans who are sleep deprived typically have more options for coping with the effects of sleep deprivation than animals do, particularly in experiments where the environment is tightly controlled. That freedom of choice could alleviate some of the stress that may be experienced by an animal who is highly motivated to sleep, but cannot. Additionally, a human under experimental conditions understands why they are being deprived; an animal has no context for its experience of deprivation, which may make the experience more stressful and cause more adverse effects.

1.4.2 Sleep Fragmentation

Sleep fragmentation refers to sleep that occurs in small bouts, rather than longer, consolidated chunks. Total sleep duration may or may not be affected by sleep fragmentation. Sleep fragmentation in humans has been shown to impact healing [72, 73], as well as lead to daytime sleepiness [74]. Mouse studies of sleep fragmentation have shown increases in insulin resistance [75], disorganization of endothelial tissues in their aortas and increased blood pressure[76], and an increase in pro-inflammatory cytokine responses in the hypothalamus, as well as an increase in circulating glucocorticoid levels[77]. This glucocorticoid increase can become a vicious cycle, as increased glucocorticoids lead to reduced sleep[78].

1.4.3 Circadian Mismatch

Circadian mismatch occurs when an individual sleeps at a time that doesn't align with its innate circadian rhythms. A diurnal animal may stay awake at night, or a nocturnal animal might sleep at night. There are concerns about animals in urban landscapes being exposed to dim light at night from street lights, buildings, and cars having their circadian cycles unentrained to the day[79-83]. This mistiming is more thoroughly documented in humans, however, as we have social factors that affect our sleep in ways other animals don't. Individuals who work the night shift, travel across time zones, or stay up late and sleep in later are all situations that animals will rarely encounter.

In humans, mistimed sleep (whether from shiftwork or jet lag) is correlated with increased rates of type II diabetes and metabolic disorder [84, 85], increased risk of breast cancer[41], increased risk of cardiovascular disease[85], and increased prevalence of mood disorders[39]. One potential mechanism of these outcomes is that the desynchronization of the internal oscillators means that, not only is the SCN not synced with peripheral oscillators, but the peripheral oscillators aren't synchronized with each other[86]. For instance, the stomach is prepared for digestion while the liver is beginning glycogen conversion for the sleep phase, causing a drop in blood glucose. Another outcome of this desynchronization may be that cell cycles, which are highly influenced by circadian cycles, become disordered, leading to abnormal cell replication and growth, and cancer[87].

1.5 Evaluating Sleep as a Welfare Indicator

As a fundamental physiological process in land mammals, sleep holds great potential as a welfare indicator. However, whether a measure is useful depends upon both its reliability and validity[88-90]. We will discuss those aspects of sleep as a welfare indicator, addressing multiple techniques as well as their advantages and disadvantages.

1.5.1 Reliability

Reliability revolves around the consistency of results of a measure, whether it be when measured by two different people (inter-rater reliability) or the same person repeatedly (test-retest reliability)[89]. Sleep has the potential to be a very reliable measure. Given a consistent ethogram, EEG scoring method, or computerized algorithms, obtaining the same measures of sleep repeatedly is as plausible as for any other behavior.

1.5.2 Validity

Assessing the validity of a measure asks the question "does this actually measure the variable of interest? [89]" An invalid measure will lack predictive power, whether for outcomes, similar measures, or both. Convergent validity is achieved if the measure of interest gives findings that agree with other validated measures of that indicator and external validity if the findings can be generalized to other situations. Specificity means that the measure accurately reports only the attribute of interest. A measure may have discriminant validity if the measure does not measure unrelated factors, and sensitivity if it reflects changes in the variable in question[90, 91].

1.5.3 Identifying and Measuring Sleep

The gold standard of sleep identification is EEG recording. In animals, the electrodes necessary for this are typically surgically implanted. However, these are expensive, require skill to insert, and necessitate general anesthesia with a subsequent recovery period. Furthermore, at least one study has shown that the gauge of the transmitter cable can affect the behavior of mice that are connected to it[92], making generalization across studies complex. EEG implants will also likely preclude social housing, as multiple animals would likely tangle their transmitter cables; this may alter behavior as well as sleep. Highly social animals, particularly rodents[93-97], production animals[98, 99], and non-human primates[100, 101], display dramatically altered behavior when housed individually. This may well confound any measures obtained under those circumstances. While some efforts have been made to address this complication [102], they still do not allow for group housing, at least for small animals (large animals may potentially be able to carry their recording devices in a backpack or similar device).

One validated alternative to implanted EEG electrodes is adhesive, external electrodes. These have been used to good effect in cattle[103, 104] and owls[105]. These systems do not require anesthesia and permit social housing. While cattle did sometimes rub an electrode off, redundancy allowed data to continue to be collected. The owl study showed an attenuation of signal over time, likely due to feather and skin regrowth at the attachment point. While an improvement over implanted electrodes, these systems are too large to use with small animals like rodents, and cost and convenience may limit their use outside of research settings in larger animals.

Another alternative, though potentially less valid, is abdominal transponders. These contain accelerometers and electrodes and are also used to detect inactivity and/or sleep. Accelerometer data alone is not sufficient to accurately assess sleep status, but with transponders that include electrodes, EEG recording is possible. These too require surgical implantation by a trained individual, with a corresponding recovery time before valid results can be obtained. While each transponder often has its own receiver, allowing animals to be housed socially, these transponders are heavy relative to smaller animals like rodents (1.5-7.8 g, which can approach 10% of their bodyweight) and thus may alter activity patterns. One study has shown that mice with telemetry implants for 5 weeks had heavier spleens than those in control surgical conditions (anesthesia alone, or anesthesia plus sham surgery)[106], suggesting that there may be physiological effects from the presence of the transmitter in the body.

A newer technique for detecting sleep states (though only in mice) has been a combination of infrared beam breaking and video analysis[107], which is up to 90% accurate in determining a mouse's sleep/wake state. A similar technique, using only video data, can also discern NREMS from REMS in mice with similar success[108]. These systems may prove a viable alternative, but are predicated upon having the animal in view at all times, precluding the use of a shelter or a nest. Additionally, these animals must be individually housed for the automated scoring process to work. Both the lack of refuge and individual housing are potential welfare concerns, as well as potentially sleep-altering confounds, and must be evaluated accordingly.

Another non-invasive technique for detecting sleep in mice is a piezoelectric device that detects mouse movements, analyzes their patterns, and uses those patterns to discern whether the mouse is awake or asleep[109, 110]. This method has been validated in mice with EEG/EMG and visual observations. Unlike the video systems, this does allow for mice to be provided with shelters and/or nesting material, but still necessitates individual housing.

EEG, accelerometer, beam breaking and video monitoring, and piezoelectric devices can all provide valid sleep measurements, but they are not without their drawbacks in other arenas. One possible solution to this is a combination of techniques; a physiological measure, such as EEG, combined with behavioral analysis may allow for sufficient correlation between brain activity and visual assessment to allow subsequent studies to use visual assessment alone. However, a researcher pursuing that strategy should keep in mind the caveats that visual assessment correlation is unlikely to be valid between different environments, species, or possibly even strains of the same species.

Another challenge in validating sleep as a measure is choosing what to measure. Total amount of time spent sleeping, sleep bout length, sleep bout number, time spent in different sleep stages, latency to sleep, latency to REMS, circadian patterns of sleep –are all potential measures of sleep, and care must be taken that both within a study, and when generalizing outside of it, the same measurements are being used and compared. This is because different measures can indicate different things – if two animals spend the same proportion of time sleeping, but one has shorter sleep bouts than the other, that suggests sleep fragmentation[43]. An animal with increased latency to REMS may be

suffering from adrenal insufficiency[111], while a decreased REMS latency has been associated in humans with both major depression and PTSD[111, 112]. This does mean that, to properly assess sleep measurements, the evaluator must have some idea of what baseline is for that species (or individual). For captive species in particular, this may be a challenge. Laboratory rodents are nocturnal, but are often disturbed during their normal daytime sleep period by husbandry and research procedures. Cattle need to lie down in order to achieve REMS[113], but spend less time lying down when dry bedding is not available[114]. Producers control light cycles, bedding substrate, and space allocation, all of which may be inadvertently altering sleep from baseline.

Sleep measures are not necessarily convergently valid. Sleep behavior and inactive behavior correspond; this has been shown in multiple studies in the process of validating non-invasive sleep monitoring equipment[103, 107, 108, 115-117]. However a review of inactivity as a measure of welfare by Furiex and Meagher[118] notes, inactivity may have a positive or negative valence for the animal [118]. Meaning that while sleep and inactivity increase, welfare may either be positively or negatively affected, with additional measures needed to assess the directionality of the welfare change. Furthermore, these symptoms in humans are correlated with depression[119]. However, they may also be an indication of recuperation after a stressful experience[111, 112, 120]. To distinguish between those two possible states, a researcher would need additional measures, both in humans and animals. Tests like sucrose preference[121], cognitive bias[122, 123], and glucocorticoid assessment[124] would provide needed context for the changes in sleep behavior.

The external validity of sleep varies. Sleep varies with age[3], species[2-4, 46, 125], and environment (see next section), so generalizations about changes in sleep must take these factors into account. For instance, a researcher may find that a particular intervention leads to an increase in daytime sleeping, a positive welfare finding for nocturnal mice. If that same intervention should lead to the same outcome in a diurnal orangutan [126], that would suggest a negative welfare state. Sleep decreases with age; young animals tend to sleep more than adults, and aging humans and mice sleep less, and in a less circadian fashion, than younger animals[127]. It's not clear what the mechanisms of those changes are, but they may be related to vision loss as animals age; this may decrease the amount of information that the SCN receives from photoreceptors in the retina about the environmental light cycle. Additionally, a decrease in sleep may result from a fear-inducing stimulus[128] or recovery from an illness[129]; context is required to interpret results correctly. For these reasons, sleep data, like glucocorticoid levels, is unlikely to be accurately indicative of welfare when used in isolation. Individual variation could also complicate generalizing sleep findings. Within humans, there are certainly differential responses to sleep loss[43, 44]; it's not unreasonable to suspect that other animals may have substantial inter-specific variation.

Sleep is sensitive to perturbations in animal environment, health, and even mood; when those factors change, sleep often changes with them. Horses housed on straw spend more time in lateral recumbancy (and therefore able to achieve REMS) than those housed on shavings[130], while rats housed on corncob bedding have reduced NREMS

compared to those housed on aspen chips[131]. Gilts with choices of 1, 4, 40, or 400 lux compartments were more often inactive in the 1 or 4 lux areas[132]. Rats exposed to a continuous noise condition had decreased sleep (both REMS and NREMS) after 3 days of exposure[32], and when exposed to an increased noise condition in the morning showed a dramatic drop in REMS[133]. Altitude sickness may cause insomnia[134], a potential concern when transporting animals. Pair-housed beagles in a research environment slept more than those individually housed[135]. All of these findings illustrate how sleep may be impacted by even minor environmental changes.

Health status also plays a large role in sleep behavior, contributing to sleep's sensitivity as a measure. Pain has a negative impact on sleep; rats in a rheumatoid arthritis model slept the same amount as controls, but that sleep was fragmented and spread throughout the day instead of in a circadian fashion[28]. In another study of arthritis model rats, those injected with Freund's adjuvant displayed less overall sleep, and an increased latency to fall asleep; these effects attenuated as time after injection increased [136]. Illness tends to increase sleep; pro-inflammatory cytokines like TNF-α and IL-1 promote sickness behaviors like hypersomnia in both mice and humans[14, 137, 138], and mice who have been knocked out for those receptors sleep less than controls[17]. Sows sleep more as pregnancy progresses[139], with sows sleeping significantly more from week 9 of pregnancy on than they did the first week.

Also contributing to sleep's sensitivity as a welfare measure is stress. Stress has a tremendous impact on sleep. Activation of the HPA axis decreases sleep[78, 112], though not in a linear fashion; adrenalectomy leads to decreased sleep, as do elevated

glucocorticoid levels[140]. Furthermore, steadily increasing levels of stress do not lead to steadily decreasing levels of sleep, and an animal experiencing chronic stress may have a sleep rebound as they begin to acclimate[112]. Stressed rats also have a decreased latency to REMS, as well as an increase in fragmentation of sleep during the day[141].

Measures of sleep have ambiguous discriminant validity. For instance, some studies have shown that dim light at night doesn't affect mouse sleep[142]. However, other studies have shown that dim light at night induces negative affective changes in mice and grass rats[83]. If both of these studies are correct, then the lack of change in sleep in the mice doesn't preclude the development of negative affect.

1.6 Implementing Sleep as a Welfare Indicator

Sleep is extremely plastic. Humans have dramatically different sleeping patterns depending upon their culture; infants and children are encultured into the sleeping habits of their peoples, whether that involves a comparatively fixed rest period or one involving waking and resting as other events prove interesting or disruptive [143]. If sleep weren't comparatively flexible, we would expect humans, regardless of their environment, to sleep in a similar fashion to one another. Food deprived rats decrease their amount of time spent sleeping, but once food is available again, their sleep returns to normal[144]. Some wild rats have even shifted their activity to a diurnal pattern in order to avoid fox predation[145]. This would suggest that, while there is an intrinsic need for and adaptive function of sleep, the amount and timing of it is at least somewhat flexible. This makes sense from an evolutionary standpoint – an animal that couldn't adapt its period of decreased awareness and immobility to current environmental

conditions would have lower fitness than one that was more plastic[8]. It has been noted that there is no universal amount of "enough" sleep for any particular species, either[44]; need for sleep varies based upon individual needs and context. This suggests that we may need to treat sleep according to the Hughes and Duncan[146] model of motivation, with sleep as the behavior that leads to the functional consequences of increased immune function, memory consolidation, CSF renewal, or other factors we aren't aware of yet. Taking that into account, sleep should be treated as any other motivated behavior, with appetitive precursors. If we do that, then perhaps rather than using sleep directly, we can look at its appetitive indicators as clues to whether or not an animal is experiencing a drive to sleep but cannot satisfy it.

Appetitive behaviors of sleep are already documented in the literature. Apes build nests to varying degrees[126, 147, 148]; rodents do as well[106, 149-151], though they do not provide the same exclusive function for sleeping as ape nests. Cattle lie down for REMS[113], as do horses[130]; dogs often circle and cats seek out a high, secure sleeping place[152-154]. A diurnal ape nest-building in the middle of the day might be a clue that something has gone awry for that animal, whether their sleep was disturbed at night and thus their sleep drive remains high, or they are feeling unwell and exhibiting related lethargy or other sickness behavior. Similarly, an ape denied access to nest-building materials, without the ability to express the appetitive precursors to sleep, may display decreased motivation to sleep, until the need for the functional consequences becomes too great. Sleep doesn't appear to have sufficiently robust external validity to use as a standalone indicator of welfare for individual animals. Its variability to changes in welfare, as well as

its plasticity, mean that there is too much potential for false positives and negatives when used in isolation. But that doesn't make it unique among welfare indicators. Mason and Mendl[155] addressed ambiguous measures quite eloquently, particularly in regards to corticosteroid levels, heart rates, weight loss, and prolactin levels. Broom[156] used the excellent example of domestic dog tail wagging as a measure that, alone, is insufficient to identify positive or negative welfare. The presence of stereotypic behavior[157] is another specific measure that, in and of itself, is not diagnostic of the animal's current welfare. Sleep is yet another measure that can be added to the "insufficient in isolation" list. However, used as an indicator of change in the animal's life, it can provide useful information about which individuals may require further attention.

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CHAPTER 2. OUT LIKE A LIGHT? THE EFFECTS ON SLEEP OF BEING A NOCTURNAL MOUSE IN A DIURNAL LAB

2.1 Abstract

Laboratory mice are nocturnal animals living in the diurnal world of investigators and husbandry staff. However, it is unknown if mouse sleep is disrupted by normal human schedules or if this affects their welfare. We hypothesized that the timing of human disruptions would alter mouse sleep patterning. We predicted that mice disturbed during their normal rest period (light period) would either sleep less overall or spend more time sleeping during their typical active period (dark period). We utilized a non-invasive sleep apparatus to continuously monitor sleep, and video recording to monitor behavior, in 48 mice. We used a factorial design to test 2 main treatments: disruption treatment (disturbed with routine husbandry at either 10:00 or 22:00) and nesting material treatment (3, 6, 9, or 12 g). Nesting material was included in the event that a refuge could provide an ameliorating effect. All mice were exposed to each sleep treatment for one week. We tested both sexes of 3 types of mice (CD-1, C57BL/6, and BALB/c). C57BL/6 mice, regardless of sex or disruption timing, slept less overall compared to other mice, and their sleep percentage did not change between treatments. CD-1 female and BALB/c male mice slept more during the day when

disturbed at 10:00; their opposite sex counterparts slept more during the day when disturbed at 22:00. Sleep analysis over 24 hours showed multiple differences, particularly in the periods immediately after lights on and including either disturbance time. Nesting material increased sleep bout length in CD-1 mice with 12 g compared to those with 3 g. Disruptions did not influence frequency of stereotypic or nesting behavior, though mice disturbed at night spent more time inactive during lights on than those disturbed during the day. These results suggest that disturbance timing does affect sleep, but varies between mouse type and sex, and our brief disruptions may have been too predictable and inconsequential to induce true sleep disruption.

Keywords: animal welfare, mouse sleep, circadian rhythms, sleep disruption

2.2 Introduction

Sleep is a critical physiological state for terrestrial mammals [1]. Consequences of sleep disruption or deprivation can include metabolic dysfunction, altered physiology, impaired cognition , and even death[2-4]. Shift work in humans (and its subsequent nighttime exposure to light and disruption of circadian rhythms) has been associated with increased cardiovascular disease[5]; increased risk of breast cancer[6]; increased prevalence of depression and anxiety[7]; increased prevalence of gastrointestinal disorders [8]; and impaired glucose metabolism [9]. Laboratory mice are nocturnal, therefore our manipulation of them during daylight hours, and subsequent forcing of them to be active during their natural inactivity period, suggests an analogy to shift work. Furthermore, there is the potential for similar negative consequences in mice. By the Fraser et al.[10] definition, these conditions lead to decreased welfare by negatively

impacting physical and mental wellbeing. While this alone is a sufficient reason to be concerned with sleep patterning in laboratory mice, sleep disruption may also be altering their utility as model organisms. Therefore, adequate and appropriate sleep for laboratory mice is a crucial component for both applicable and ethical research.

Provision of a refuge or shelter has been shown to decrease exhibition of stress behaviors in cats [11] and a decrease in anxiety behaviors during open field tests in mice [12, 13]; it seems plausible that a refuge (in the form of a fully enclosed nest) may ameliorate some of these potential negative outcomes in laboratory mice. The amount of nesting material provided is integral to its effectiveness [14]; knowing this, we chose to test varying amounts of nesting material, in an effort to determine how much material may be required to offset resting period disruptions for mice.

Previous research performed on sleep in laboratory animals has involved implantation of electrodes and/or transmitters in order to perform EEG and EMG testing [15-24]. Anesthesia alone has been shown to disrupt mouse thermoregulatory processes for a week after implantation of a transmitter [25]. Bioenergetic homeostasis may take longer to re-establish; mice in one study never achieved the same weight as their non-implanted counterparts [25], and mice in another study didn't show any effect of treatment until four weeks post-operatively [26]. Another complication of transmitter implantation is the weight of the transmitter itself. Commercially available transmitters can weigh as much as 3.4 g; for a 20 g mouse, this is a body weight increase of ~ 15%. Abruptly increasing bodyweight in this fashion creates an unknown energetic demand on mice, further complicating the interpretation of experimental data. While time is

typically given in experiments to allow animals to return to thermal and energetic homeostasis, it's difficult to assess how much time is "enough", as well as what effects monitoring equipment itself may have upon behavior.

Video monitoring provides a non-invasive method of observing behavior, and has also been used for rodent sleep assessment [27-29]. Although non-invasive, this method requires the animal to be visible to the camera at all times. The subjectivity of identifying a sleeping animal exclusively through behavioral monitoring by necessity entails the difficulty of discerning an animal at quiet rest from one that is sleeping. Additionally, the need to be able to see subjects would preclude the use of any sort of nesting material or opaque structure as enrichment, as well as bedding substrate that could be used to build a nest.

These complications from surgical implantation or video monitoring meant that we were interested to try a different, non-invasive form of sleep monitoring apparatus, where mice are able to move freely and have nesting material without interfering with data collection.

We hypothesized that the timing of disturbances for routine husbandry would alter the sleep patterning of mice, and that increasing provision of nesting material would provide increasing protection from this disturbance. We predicted that mice disturbed during the day (their natural inactive period) would have disrupted sleep patterns compared to those disturbed at night (their natural active period).

2.3 Materials and Methods

2.3.1 Ethical Statement

This study was approved by the Purdue Animal Care and Use Committee, and conformed to all guidelines put forward by both the committee and the Guide for the Care and Use of Laboratory Animals [30]. At the start of study, animals were free of a list of common mouse infectious agents; further details may be found at http://www.criver.com/files/pdfs/rms/hmsummary.aspx. All mice were monitored daily by trained members of the research team for food and water consumption and overall health status, with no adverse conditions or health outcomes noted.

2.3.2 Animals and Housing

This experiment utilized 2 different strains and 1 stock of mice, BALB/cAnNCrl (BALB/c), C57BL/6NCrl (C57BL/6), and Crl:CD-1(ICR) (CD-1) of both sexes, obtained at 6 weeks of age (Charles River, Kingston, NY). We also tested 4 different amounts of nesting material: 3, 6, 9, and 12 g (Enviro Dri, Shepherd Specialty Papers, Watertown, TN). Each treatment combination of variables (type of mouse, sex, and nesting material amount) had two replicates for a total of 48 mice (Table 2.1). This factorial design enabled us to maximize statistical power while minimizing use of animals.

Mice were housed in one of two non-invasive sleep monitoring apparatuses (Figure 2.1, Signal Solutions, Lexington KY). Each apparatus individually houses 4 mice, allowing 8 mice to be tested simultaneously. We chose to use this non-invasive sleep assessment apparatus that allowed us to determine sleep disruptions and changes without surgical implantation or increased burden on the mice (Flores et al., 2007). The apparatus uses a

piezoelectric mat underneath the cage to detect vibrational movement of the mouse. Customized software (MouseRec Data Toolbox, Signal Solutions, Lexington KY) uses an algorithm to process the signal and discern sleeping respiratory patterns from waking respiratory patterns; this algorithm has been validated using EEG, EMG, and visual evaluation [31]. Visual barriers were in place between cages, but audible and olfactory contact was still possible. Each cage included a built in food hopper and water bottle opening. Because the apparatus detects movement through pressure rather than video signal, it also allows mice in a sleep study to have vision-obstructing nesting material. Provision of nesting material improves mouse thermoregulatory abilities [26, 32] and provides valuable behavioral enrichment [12, 33-37]. Additionally, because this apparatus scores sleep via an algorithm, there is no opportunity for observer bias, as there is with video coding. This provides an opportunity for unaltered, continuous sleep assessment over several weeks. However, this apparatus does require mice to be housed singly, rather than socially. It is also sensitive to vibrations from other sources, so care must be taken with placing the apparatus away from wind currents and not on surfaces with equipment that generates vibrations, such as computers or fans. Mice were also continuously video recorded while in the apparatus using a DVR system (USA Vision Systems, Inc, Irvine CA) and 8 IR CCTV cameras (ClearVision CV-BC700VIRA), one for each cage.

Each cage was bedded with 32 g of laboratory grade aspen shavings (Envigo, Indianapolis IN) with the experimental amount of nesting material added. Mice were provided with an 18 % protein laboratory diet (Harlan 2018, Indianapolis IN) and reverse

osmosis filtered water ad libitum. Lights were kept on a 12:12 light/dark cycle, with lights on at 07:00 and off and 19:00 hours. The room was maintained at 68-74° F, and 28-65 % humidity. A homemade red (750 nm) LED light box (http://www.diyphotography.net/build-a-pro-quality-light-source-with-this-awesome-diy-led-light-panel-tutorial/) on an automatic timer provided illumination from 22:00 to 23:00 hours during both the acclimation and testing period to allow the experimenter to see in the room without introducing a white lighting source.

2.3.3 Procedures

Mice were weighed upon arrival, and then placed into their randomly assigned cages within the sleep apparatus and their assigned nesting treatment. Assignment to cages and nesting treatment amounts was done utilizing a random integer list generator (www.random.org). After 4 days of acclimation, continuous sleep and behavioral data recording began. During acclimation, mice were exposed to the opposite condition of their first assigned disturbance treatment; this ensured that the first day of recording involved a change of disruption time treatment for all groups.

Disturbance treatments consisted of checking food, water, and animal health and conducting husbandry tasks between either 10:00 - 11:00, the day disturbance condition, or 22:00 - 23:00 hours, the night disturbance condition. Those windows corresponded to either 3 hours after lights on and 3 hours after lights off. We chose this timing because 3 hours after lights on, mice have completed their peak nest building activity and are settling in for their sleep period[38]. Three hours after lights off, they are well into their active period and likely to be awake[38]. The first treatment phase that

mice were exposed to was randomized to compensate for order effects and balanced across the six groups. After 7 days, the disturbance treatment was switched to the opposite timed disturbance, so that each mouse experienced both disturbance times. On days 1 and 8, investigators weighed mice, performed a wellness exam, and provided clean bedding and new nesting material. At the conclusion of treatments, BALB/c and C57BL/6 mice were euthanized using inhaled carbon dioxide to effect. CD-1 mice were retained for subsequent testing in a different study.

2.3.4 Data Collection

Behavioral data were collected from a total pool of 14 days (per animal) of 24-hour video recordings. Focal animal scan sampling at 20 minute intervals was used to create a behavior time budget consisting of general activity, nest building activity, and maintenance behaviors, as well as animal location in or out of nest (Table 2.2). One-zero sampling was used to document stereotypic behavior over a two-minute interval prior to the time budget scan sample every 20 minutes. Behavioral data collection was limited to days 1, 3, and 6 of each disruption treatment. Video coders achieved >90% inter-rater agreement prior to video analysis, and were blinded to which sleep disruption the mice were experiencing while they coded. Nests were also scored from video at 2 time points (1:00 and 13:00), and scored according to previously developed criteria[39, 40]. Briefly, the nest is scored by assessing how close to a complete dome the nest is. Nesting material that has not been interacted with at all is a 0, material that has been interacted with but not built with is a 1, a flat nest is a 2, a cup-shaped nest whose sides are less than half of

a sphere is a 3, an incomplete dome nest, whose walls are more than half a sphere is a 4, and a complete dome is 5.

Sleep data were collected continuously over the full two weeks for each group of mice.

2.3.5 Statistical Analysis

2.3.5.1 Sleep Data

Sleep data were analyzed using JMP statistical software (JMP®, Version 10). We used a General Linear Model (GLM) with full factorial combinations of: type of mouse, sex, nesting material amount, lights on/off, day of treatment, 2 hour epoch, and disturbance treatment was conducted for mean total bout length, mean daytime bout length, mean nighttime bout length, mean total sleep percentage, mean daytime sleep percentage, and mean nighttime sleep percentage. Because we only collected behavioral data on days 1, 3, and 6 of each disturbance treatment, we also limited our sleep analysis to those days. Type of mouse, sex, and amount of nesting material were nested within cage (the experimental unit) and cage was treated as a random factor. Apparatus and cage location were included as blocking factors, and removed from analysis if they were non-significant. The assumptions of GLM (normality of error, homogeneity of variance, and linearity) were confirmed post-hoc[41]. Sleep bout length was angularly transformed in order to meet these assumptions. Significant ($P<0.05$) effects were then analyzed using post-hoc Tukey tests or Bonferroni-corrected test slices, as needed. All values are given as least squares means and standard error.

2.3.5.2 Behavioral Data

The number of observations for each category (general activity, maintenance, nesting behavior, inactive, unknown) were divided by the total number of observations for each mouse on a daily basis. Unknown observations were excluded from the behavioral analysis to preserve independence. Similarly, number of observations made in the nest were divided by total observations to calculate a proportion of time spent in nest. Stereotypic behavior observations (present or absent) were analyzed using a Generalized Linear Model (GLIM) as a binomial logistic regression with logit link function and Firth-adjusted bias.

Behavioral data were analyzed using JMP statistical software (JMP®, Version 10). A full factorial GLM analysis of the following factors: type, sex, nesting material amount, and disturbance timing was conducted on proportion of time spent in different behavioral categories, nest scores, and proportion of observations of the mouse in nest. Type of mouse, sex, and amount of nesting material were again nested within cage. Cage (and therefore individual mouse) was treated as a random factor. Apparatus and cage location were included as blocking factors. Nest score was included as a covariate in the analysis of location in or out of the nest, to determine if better built nests altered nest usage. Assumptions of GLM (normality of error, homogeneity of variance, and linearity) were confirmed post-hoc[41]. Significant effects ($P<0.05$) were then analyzed using post-hoc Tukey tests and Bonferroni corrected test slices and pairwise comparisons. All values are given as least squares means and standard error.

2.4 Results

2.4.1 Overall Sleep

The only significant main effect on the overall percentage of time spent sleeping over the full week of treatment was type. C57BL/6 mice spent less time sleeping than BALB/c or CD-1 mice (GLM, $F_{(2, 23)} = 15.87$, $P < 0.0001$). Overall bout length had a significant four-way interaction between type, sex, disturbance time, and amount of nesting material (GLM, $F_{(6,24)} = 4.12$, $P = 0.0055$). Test slices ($F_{(15,35.13)} = 3.33$, $P = 0.0016$) indicated those differences arose within CD-1 mice only. Bonferroni-corrected pairwise comparisons within CD-1 mice showed male mice with 12 g of nesting material had longer bout lengths when they were disturbed at night than those with 3 g.

2.4.2 Nighttime Sleep

When we analyzed the percentage of time spent sleeping at night, we found a significant interaction of sex, type, and disturbance time (GLM, $F_{(2, 24)} = 90.8$, $P < 0.0001$). Post-hoc Tukey tests showed that all males of all three types slept differently depending upon when they were disturbed; C57BL/6 and CD-1 males slept more at night when they were disturbed at night, while BALB/c males slept more at night when they were disturbed during the day. Female BALB/c and CD-1 mice also differed with disturbance time, with BALB/c mice sleeping more at night when they were disturbed at night, while CD-1 mice slept more at night when disturbed during the day. There was no difference in sleep for C57BL/6 females. Additionally, there were differences in nighttime sleep within the types. BALB/c females disturbed during the day slept less at night than their male counterparts, and CD-1 females disturbed during the day slept more at night than

the males. When they were disturbed at night, female BALB/c mice slept more at night than males. C57BL/6 and CD-1 female mice slept less than their male counterparts did at night when they were disturbed at night. We also found a significant four way interaction between sex, type, disturbance time and nesting material (GLM, $F_{(6,24)}$=2.66, P=0.04); however, Bonferroni-corrected pairwise t-tests showed that this significance arose solely from type differences, rather than nesting material or disturbance time differences.

Nighttime bout length also had a four way interaction between sex, type, disturbance time, and nesting material amount (GLM, $F_{(6,24)}$=5.17, P=0.0015). Bonferroni-corrected pairwise comparisons showed that this difference was, as in overall bout length, a difference between male CD-1 mice with 3 and 12 g of nesting material who were disturbed at night.

2.4.3 Daytime Sleep

Percentage of time spent sleeping during the day also had a significant interaction between type of mouse, sex, and disturbance time (GLM, $F_{(2,24)}$=60.86, P<0.0001, Figure 2.2). Post-hoc Tukey tests found that female C57BL/6, female CD-1, and male BALB/c mice all slept more during the day when disturbed at night, while male C57BL/6, male CD-1, and female BALB/c mice slept more during the day when disturbed during the day.

Daytime bout length also showed a significant three way interaction between sex, type of mouse, and disturbance time (GLM, $F_{(2,24)}$=22.68, P<0.0001, Figure 2.3). Among the male mice, BALB/c had longer bout lengths when disturbed at night than

when disturbed during the day; there was no difference in bout length for male C57BL/6 and CD-1 mice. For female mice, there was no difference within the types of mice between daytime and nighttime disruption. Within types, male BALB/c mice had longer daytime bout lengths than females when they were disturbed at night, and CD-1 males had longer daytime bout lengths than females when disturbed during the day. There were no differences between male and female C57BL/6 mice.

2.4.4 Sleep Over 24 Hours

Analyzing the percentage of time spent sleeping over 2 hour epochs throughout the entire day showed two significant interactions – type of mouse by 2 hour epoch (GLM, $F_{(22,811)}$=4.69, P<0.0001, Figure 2.4), and sex by 2 hour epoch by disturbance time (GLM, $F_{(11,811)}$=4.07, P<0.0001, Figure 2.5). For type of mouse by epoch, we used Bonferroni-corrected test slices to determine which epochs had significant differences. Those test slices showed that epochs 7-8 ($F_{(2,715.7)}$=5.65, P=0.0037), 9-10 ($F_{(2, 715.7)}$=17.44, P<0.0001), 11-12 ($F_{(2,715.7)}$=15.17, P<0.0001), 13-14 ($F_{(2,715.7)}$=5.92, P=0.0028), and 23-0 ($F_{(2,715.7)}$=7.65, P=0.0005) were all significant. From there, we performed Bonferroni corrected pairwise t-tests to determine from where that significance arose We took a similar approach to the sex by epoch by disturbance time interaction. In this case, the test slices indicated that the only difference was in the 19-20 epoch. Bonferroni corrected pairwise t-tests showed that, in that epoch, male mice disturbed at night slept more than when they were disturbed during the day, and that male mice disturbed during the day slept less than female mice disturbed during the day. We also found an interaction between day of treatment and 2 hour epoch (GLM, $F_{(66, 7454)}$=5.63, P<0.0001,

Figure 2.6). Test slices narrowed the differences down to the 7-8 ($F_{(6,7454)}$=3.30, P=0.003), 9-10 ($F_{(6,7454)}$=5.26, P<0.0001), 11-12 ($F_{(6,7454)}$=6.03, P<0.0001), 17-18 ($F_{(6,7454)}$=3.60, P=0.001), 19-20 ($F_{(6,7454)}$=24.20, P<0.0001), and 21-22 ($F_{(6,7454)}$=13.06, P<0.0001) epochs, and Bonferroni-corrected pairwise t-tests allowed us to tell that in the 7-8 epoch, mice slept more on day 1 than day 6; in the 9-10 epoch, mice slept more on days 1 and 3 than 6; in the 11-12 epoch, mice slept more on day 3 than on days 1 or 6; in the 17-18 epoch, mice slept more on day 6 than day 1; in the 19-20 epoch, mice slept more on day 6 than on day 3, and more on day 3 than on day 1; and finally, in the 21-22 epoch, mice slept more on day 6 than on either 1 or 3.

We also analyzed sleep bout length over two-hour epochs. We found a significant effect of type of mouse by 2 hour epoch (GLM, $F_{(22, 811)}$=8.85, P<0.0001, Figure 2.7), as well as type of mouse by sex by nesting material amount (GLM, $F_{(6,20)}$=3.73, P=0.012). For the latter, the differences all arose from C57BL/6 mice having shorter bout lengths than the other two types of mice. For the former, test slices and Bonferroni-corrected pairwise comparisons showed differences in the 1-2 epoch ($F_{(2,443.8)}$=11.13, P<0.0001), with CD-1 mice having longer bouts then; the 5-6 epoch ($F_{(2,443.8)}$=5.84, P=0.0031), with C57BL/6 mice having shorter bouts than BALB/c mice; the 7-8 epoch ($F_{(2,443.8)}$=14.93, P<0.0001), with C57BL/6 mice having shorter bouts than BALB/c mice; the 9-10 ($F_{(2,443.8)}$=32.10, P<0.0001), 11-12 ($F_{(2,443.8)}$=33.05, P<0.0001), 13-14 ($F_{(2,443.8)}$=31.39, P<0.0001), 15-16 ($F_{(2,443.8)}$=42.20, P<0.0001), and 17-18 ($F_{(2,443.8)}$=25.89, P<0.0001) epochs, with C57BL/6 mice having shorter bouts than BALB/c and CD-1 mice; the 19-20 epoch ($F_{(2,443.8)}$=10.64, P<0.0001), with C57BL/6 mice having

shorter bouts than CD-1 mice; and the 23-0 epoch ($F_{(2,443.8)}$=14.55, P<0.0001), with CD-1 mice having shorter bouts than the other strains.

2.4.5 Behavior

Type of mouse and sex significantly affected the behavior budget (GLM, $F_{(6, 1993)}$=6.50, P<0.0001; Figure 2.8). CD-1 male mice were inactive more and performed maintenance behaviors less than females. Female C57BL/6 mice spent more time in general activity than males; and BALB/c mice spent more time in general activity than CD-1. A significant sex by nesting material by behavior interaction (GLM, $F_{(9,1993)}$=1.95, P=0.041), indicated that male mice with 12 g of material spent more time inactive than males with other amounts, or females with any amount. The interaction of ype of mouse by nesting material by behavior (GLM, $F_{(18, 1993)}$ = 4.10, P < 0.0001) showed that C57BL/6 mice with 12 g of nesting material spent more time inactive than those with 9 or 3 g, but not 6. It also showed that C57BL/6 mice with 3 g of nesting material spent more time in maintenance behavior than those with 12 g, BALB/c mice with 6 g spent more time in maintenance behaviors than those with 12 g, CD-1 mice with 12 g spent more time in maintenance behaviors than BALB/c or C57BL/6 with 12 g, CD-1 mice with 3 and 12 g of material spent less time in general activity than their BALB/c counterparts. For type of mouse by lighting status by behavior (GLM, $F_{(6, 1993)}$ = 23.65, P < 0.0001, Figure 2.9), all types of mice spent more time inactive during lights on than lights off, and BALB/c mice spent less time inactive during lights off than the other types of mice. CD-1 mice in the dark phase spent more time in maintenance than the other two types after lights off, and all three kinds spent more time in maintenance during lights off than

lights on. All mice spent more time in general activity during lights off, and BALB/c mice spent more time in general activity during lights off than the other kinds of mice. Nesting material by lighting status by behavior (GLM, $F_{(9, 1993)} = 2.49$, $P = 0.0078$) showed similar results, with all mice inactive more often during lights on than lights off, and mice with 12g of nesting material inactive more than those with lesser amounts. Mice also engaged in maintenance behaviors and general activity more often during lights off than lights on. Evaluating disruption time by lighting status by behavior (GLM, $F_{(3, 1993)} = 7.42$, $P < 0.0001$), we found that mice disturbed at night spent more time inactive during lights on than those disturbed during the day. Finally, lighting status by behavior by day of treatment (GLM, $F_{(6, 1993)} = 2.11$, $P = 0.0049$) was significant, with mice spending more time inactive during lights on days 3 and 6 than they did on day 1, and more time inactive after lights off on day 6 than on 1 and 3. Mice engaged in more maintenance and general activity during lights off than lights on regardless of day of treatment.

When analyzing the stereotypy observation data, we found one significant factor, lighting status ($X^2_{(1)}=5.52$, $P=0.019$), with mice being more likely to be observed stereotyping during lights off than lights on.

In or out of nest observation data also yielded multiple significant interactions. Sex by type of mouse by lighting status (GLM, $F_{(2, 418)}=5.94$, $P=0.0029$) showed an effect, but post-hoc Tukey tests indicated that the differences here were all due to lighting status, with mice being observed in their nests more with the lights on than off. Sex by nesting material by lighting status (GLM, $F_{(3, 418)}=3.10$, $P=0.027$) and type of mouse by nesting material by lighting status (GLM, $F_{(6, 418)}=4.18$, $P=0.0004$) had the same post-hoc

results. Sex by disruption time by lighting status (GLM, $F_{(1, 418.1)}$=5.55, P=0.019) revealed that male mice were observed in their nests more often during lights on when they were disturbed at night, rather than during the day. Finally, disruption time by lighting status by day of treatment (GLM, $F_{(2, 418.4)}$=4.96, P=0.0074) showed that, once again, mice were more often observed in their nests during lights on than lights off, but mice disturbed during the day were observed in their nests during lights on less often on day 1 than day 3 or 6 of treatment. The covariate of nest score was also significant (GLM, $F_{(1, 435.7)}$=25.07, P<0.0001), with proportion of observations in the nest increasing with nest score.

Nest scores had one significant two way interaction between nesting material amount and day of disruption ($F_{(6,419)}$=2.85, P=0.0099). Post-hoc Tukey test showed that all the differences arose on day 1 of treatment, with mice with 12 and 9g of nesting material having higher scores than those with 3g. There were also several 3-way interactions. Strain by lighting status by day of treatment ($F_{(4,419)}$=3.06, P=0.016) was significant, with post-hoc Tukey showing that all those differences arose on day 1, with mice having lower nest scores during lights off on day 1 than any other times. We also found an interaction of disruption time by lighting status by day of treatment ($F_{(2,419)}$=7.74, P=0.0005, Figure 2.10), again with day 1 having lower scores than day 3 or 6. Sex by lighting status by day of treatment ($F_{(2,419)}$=4.33, P=0.015, Figure 2.11) showed that on day 1, mice of both sexes had lower nest scores when the lights were off rather than on, and female mice scored lower than males with lights off on that day. Additionally, overall nest scores were lower on day 1 than day 3.

2.5 Discussion

This project took on the challenge of a lack of baseline knowledge about "normal" sleep for laboratory mice. Presumably undisturbed lab mice would, eventually, settle into a circadian rhythm that would reflect their natural tendencies. What's not known is whether those natural tendencies are actually observed in the laboratory. It seems likely that they have diverged at least some from their wild-type cousins, either from inadvertent selection for mice who tolerate daytime disruptions or through mutations, like the loss of melatonin in C57BL/6 mice [42].

Our primary hypothesis, that mice who were disturbed during their normal rest period during the day would sleep less than those disturbed after dark, was not supported by our data. In some ways, this is a positive welfare finding, as it means that the timing of current husbandry practices may not be as disruptive as we feared. However, the shifts in sleep patterning between disruption treatments do suggest that mice are affected even by very short and mild human activities; even so, their responses to these disruptions are not uniform across type of mouse or sex. It's not clear if this means that some types of mice are more sensitive than others, though that seems likely, particularly in the case of BALB/c mice who are often used as anxiety models [43]. That does not explain, however, the sex differences in BALB/c and CD-1 mouse responses to disturbances.

Percentage of time spent sleeping over 24 hours roughly paralleled bout length (Figures 4 and 7). This is another positive welfare finding, in the sense that, as percentage of time spent sleeping increased, so did bout length. This implies sleep

consolidation instead of fragmentation. Fragmentation leads to adverse effects in humans and mice [2, 18, 44-46], so the lack of fragmentation in these mice is positive.

We also did not find that increasing amounts of nesting material decreased the effects of disruption on sleep. There are several potential explanations for this. First, it's possible that our disruptions weren't strong enough for a retreat space to have any benefit. It's also possible that there was some protective effect, but it was less than the disruptive effect of our actions. Or it may be that, by providing nesting material, we blunted the effects of disruption without eradicating them. We believe the most likely positive effects from nesting substrate would have been found with Enviro-Dir, as Enviro-Dri provides an opportunity for naturalistic nesting behavior as well as improved quality nests [39]. It may, however, be worthwhile to compare our results to those of mice who have already been tested with the same apparatus but given other types of material and see if those results are comparable.

Considering our behavioral data, there are several findings of note. Firstly, maintenance behaviors were observed more often in CD-1 mice than in the other strains. In this study, maintenance behaviors included grooming, drinking, and eating. This last is particularly relevant, as three CD-1 mice developed food-grinding behavior over the course of the study. In mice, food-grinding occurs when a mouse chews but does not consume its pelleted diet, resulting in increased food use without concomitant weight gain, as well as a large amount of crumbs, or "orts", remaining on the cage floor [47, 48]. In our study, CD-1 mice who did not food grind used an average of 98.3g over 2 weeks; food-grinding mice however averaged 177g over the same period of time. These

mice likely spent more time at the food hopper as they performed this behavior, inflating maintenance behavior for CD-1 mice.

Inactive behavior of male mice with 12g of nesting material was increased compared to males with lesser amounts. This may be a reflection of mice with larger amounts of material feeling more secure and therefore resting more soundly, but this is not reflected in the sleep data. Another possibility is that a mouse with 12g of material was able to build a nest with a large enough central cavity that they could perform some subtle movement without moving the exterior of the nest, thus causing us to code them as "Inactive" when they were not. The provision of nesting material makes this an unavoidable conundrum.

Stereotypic behavior was rare among these mice, with mice stereotyping more when lights were off, corresponding with their typical active period. This relative lack of stereotypic behavior may be related to the relatively young (6 weeks) age of these animals. ICR(CD-1) mice at 40 days old were spending less than 10% of their time engaged in stereotypic behavior[49]. Additionally, CD-1 mice (who may be driven to perform wire-gnawing behavior in particular[49, 50]) may have been stymied in this case because the lid of the apparatus is a metal plate with circular holes cut out for ventilation, rather than the typical wire bars.

We were also interested to see the differences in behavior based upon day of treatment. Day 1 involved the mouse being weighed and having its cage changed, while days 3 and 6 consisted solely of a visual welfare check and food and water supplementation as needed. That mice spent less time inactive during lights on day 1

makes sense for the week where disruptions happened during daylight hours. But one would expect a concurrent decrease in inactive time during lights off during the other week of treatment; this doesn't appear to have been the case. It appears that these mice found the cage change and weighing disruptive enough that, even hours after cage change, though they were already likely awake and active when it occurred, they had difficulty settling down into their nest. Sleep data separated by day and 2 hour epoch at least partially supports this (Figure 6), as there are clearly visualized differences between days. Interestingly, as time since cage change increases, sleep shifts later into the day, and further into the period immediately after lights out. Day 1 also shows a clear drop in sleep in the 9-10 epoch, when cage change would occur for the daytime disruptions, but doesn't show any irregularities in the 21-22 epoch, when cage change occurred during nighttime disruptions. So, even if the mice find both disruptions times equally stressful, it would appear that only the daytime one affected their sleep patterning. However, we must note that cage changes and disruption time changes occurred on the same day – day 1. It is possible that the combination of cage change and change in disruption time produced an effect where only one or the other would not. But this was the case for the transition into both daytime and nighttime disruptions, so either way, the daytime disruption was different for some reason than the nighttime was.

Finally, an increase in the amount of nesting material provided did not increase the amount of nesting behavior observed, nor did it change the number of observations of the mouse in the nest. However, observations in the nest did co-vary with nest score.

This suggests that supplying a larger quantity of nesting material does not necessarily lead to more performance of behavioral enrichment, nor does it mean a mouse will spend more time in its nest. However, once the nest is built, a higher-scoring nest does seem to correlate with the mouse spending more time in the nest. It's not clear if this is causative – a higher scoring nest alleviates cold stress better [26, 32], so perhaps the mouse then chooses to spend more time in it. Or perhaps a mouse that spends more time on building also spends more time in its nest; however, our data don't support this conclusion. The only significant effect of nesting material treatment on nest scores was on the first day mice received the material. Mice with 12 or 9g had higher nest scores on day 1 than those with 6 or 3g; but those differences disappear by day 3. This may be why we saw little effect on sleep due to amount of nesting material; the mice were able to make adequate nests even with the smaller amounts.

This study illustrates the complexity of sleep and welfare assessment in laboratory mice. Our hypotheses about sleep deprivation were not supported, but there was clearly a response to disturbance timing in these mice. The fact that mild disruptions (daily welfare check) can elicit a change suggests that mice are more sensitive to our actions than we may give them credit for, and even minor disturbances, especially if frequent, should be considered, particularly in sleep and behavioral research.

2.6 References

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Table 2.1: Factorial combinations of treatments. Each amount of nesting material was used with 2 males and 2 females.

Mouse Type	CD-1		C57BL/6		BALB/c	
Nesting Material	3, 6, 9, 12g		3, 6, 9, 12g		3, 6, 9, 12g	
Sex	Male	Female	Male	Female	Male	Female
Replicates	2	2	2	2	2	2

Table 2.2: Ethogram for behavior coding. Includes both scan sampling and 1/0 sampling behaviors. Maintenance and nesting categories had subsets of behaviors, but subsets were binned into categories for analysis.

Behavior Category	Description	Measurement
General Activity	Animal is actively engaged in behavior that is not maintenance or nesting behavior. Includes stereotypy.	Scan sampling, 20 minute intervals
Maintenance	Animal is engaged in eating, drinking, or grooming	Scan sampling, 20 minute intervals
Nesting	Animal is engaged in manipulating or processing nesting material	Scan sampling, 20 minute intervals
Inactive	Animal is not moving, except for respiration	Scan sampling, 20 minute intervals
Unknown	Animal is engaged in activity, but the specific behavior cannot be determined	Scan sampling, 20 minute intervals
Stereotypy	Animal is engaged in apparently functionless, repetitive (at least 3 repetitions) behavior	1/0 sampling, 2 minute periods at 20 minute intervals
In Nest	Fifty percent or more of animal's body is in contact with nest	1/0 sampling, 20 minute intervals

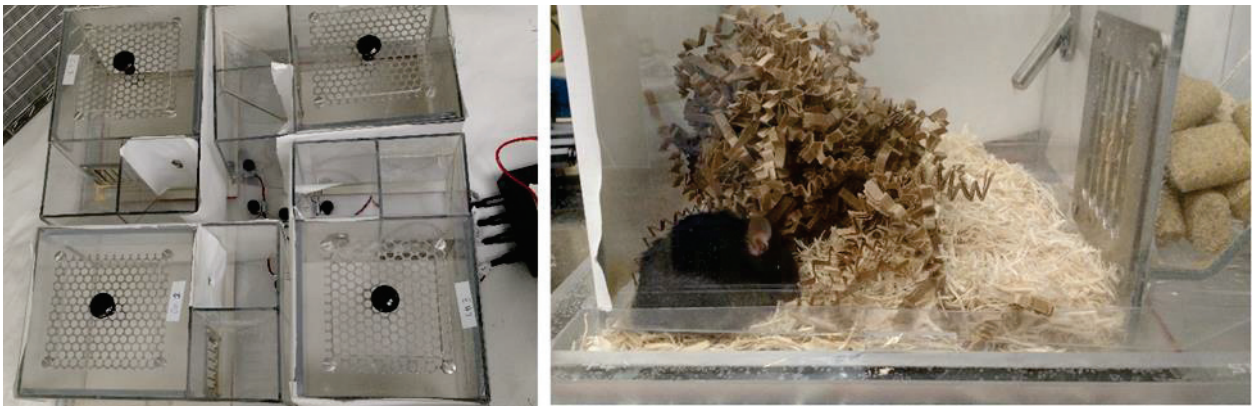

Figure 2.1: Sleep apparatus, top view (L) and C57BL/6 mouse in apparatus with nesting material (R)

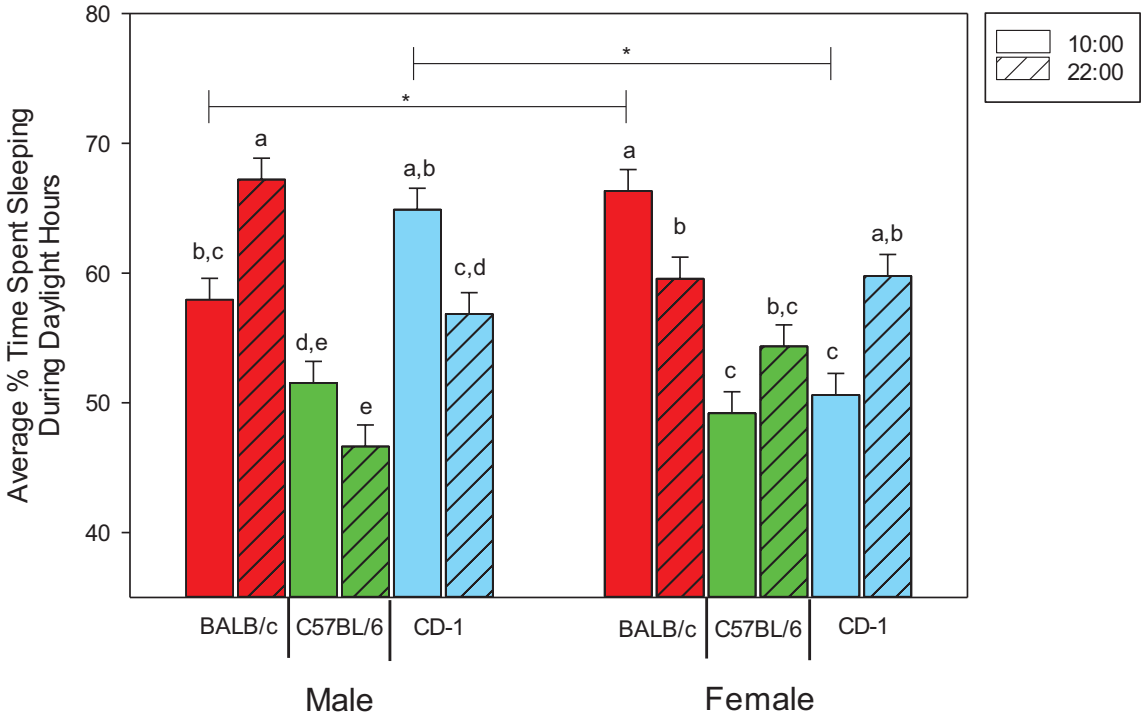

Figure 2.2: Average percentage of time spent sleeping during daylight hours. Solid bars represent daytime disturbances (10:00) and hashed bars represent nighttime disturbances (22:00). Differing letters indicate within sex differences, bars with asterisks indicate between sex differences (Tukey, $P<0.05$). Data represented are LSM and SE.

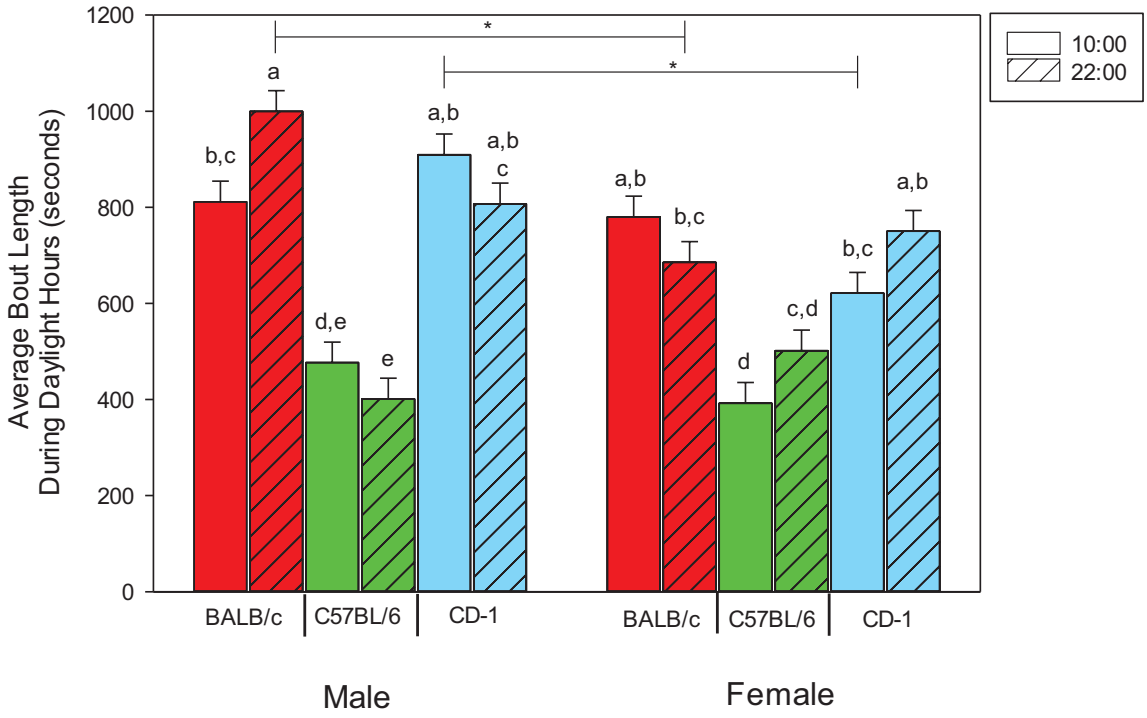

Figure 2.3: Average sleep bout length during daylight hours. Solid bars represent daytime disturbances; hashed bars represent nighttime disturbances. Differing letters indicate within group differences, asterisks indicate between group differences (Tukey, $P<0.05$). Data represented are LSM and SE.

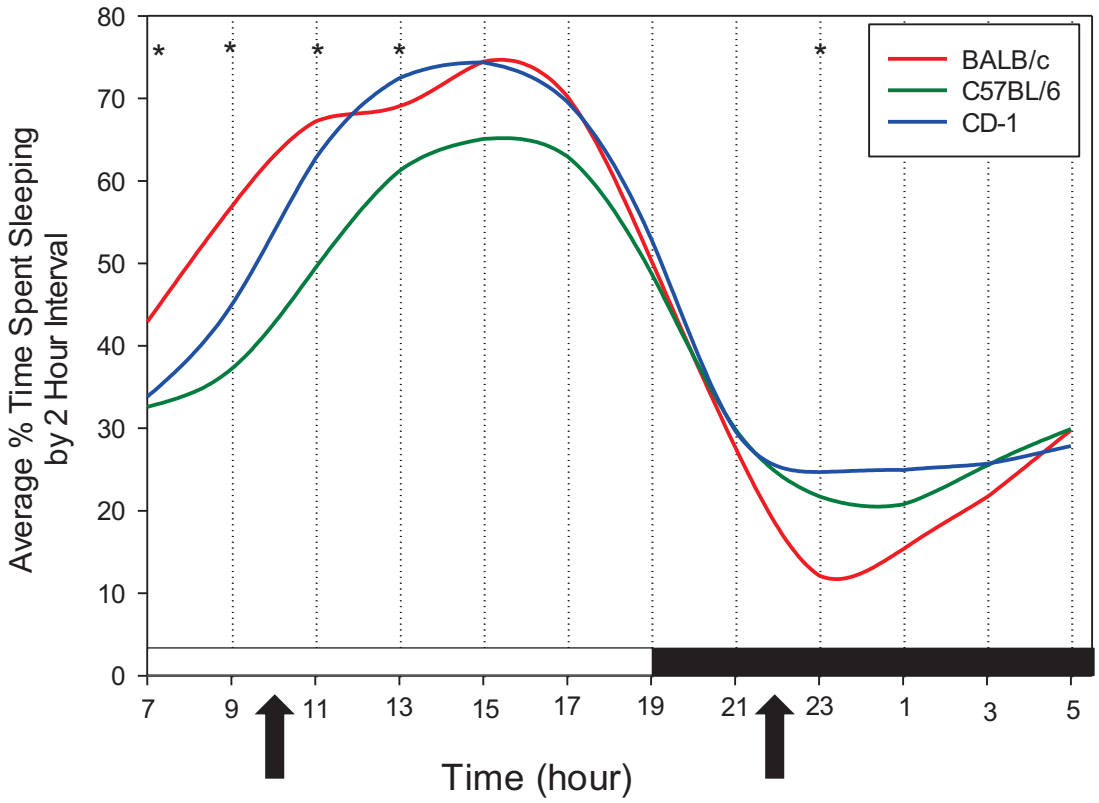

Figure 2.4: Average percentage of time spent sleeping per 2 hour interval. Open bar on the x-axis indicates lights on and the closed bar indicates lights off. Arrows indicate disruption times. Asterisks indicate sleep differences between types of mice in that epoch (Bonferroni corrected test slices, $P<0.004$). Data presented are LSM.

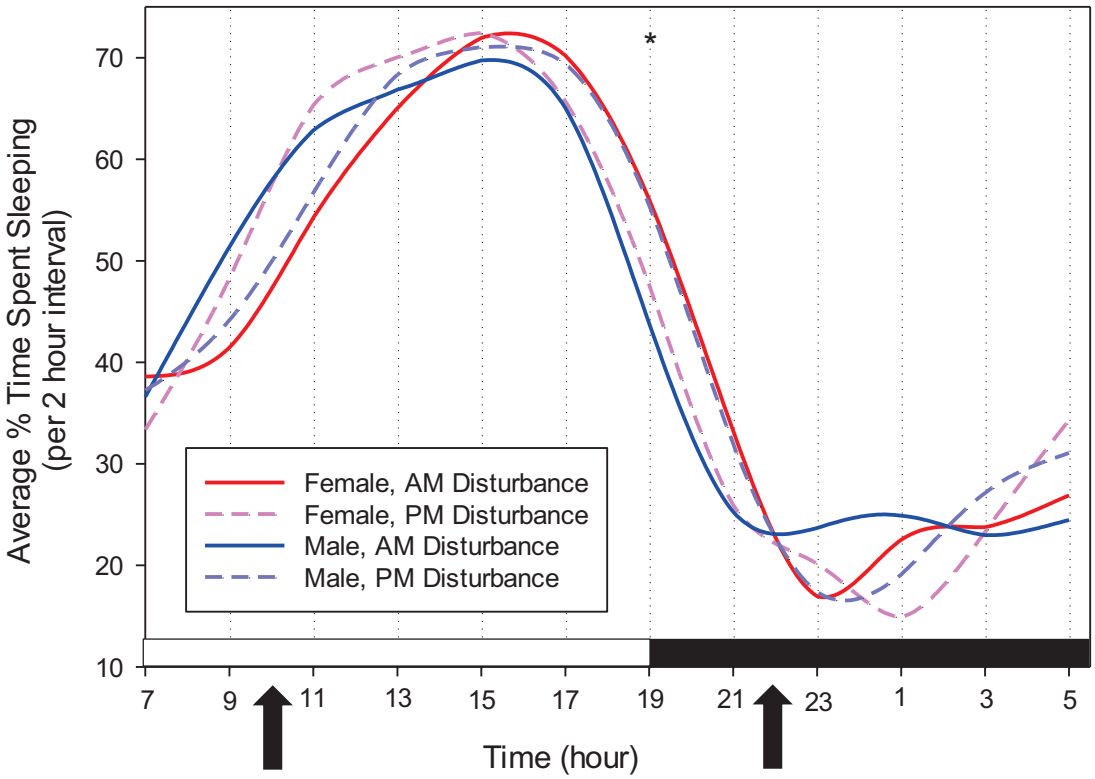

Figure 2.5: Average percentage of time spent sleeping per 2 hour interval. Open bar at the bottom indicates lights on, closed bar indicates lights off. Arrows indicate disturbance times. Asterisks indicate differences in bout length during that interval (Bonferroni corrected test slices, $P<0.004$). Solid lines indicate daytime disturbance, hashed lines indicate nighttime disturbance. Data presented are LSM.

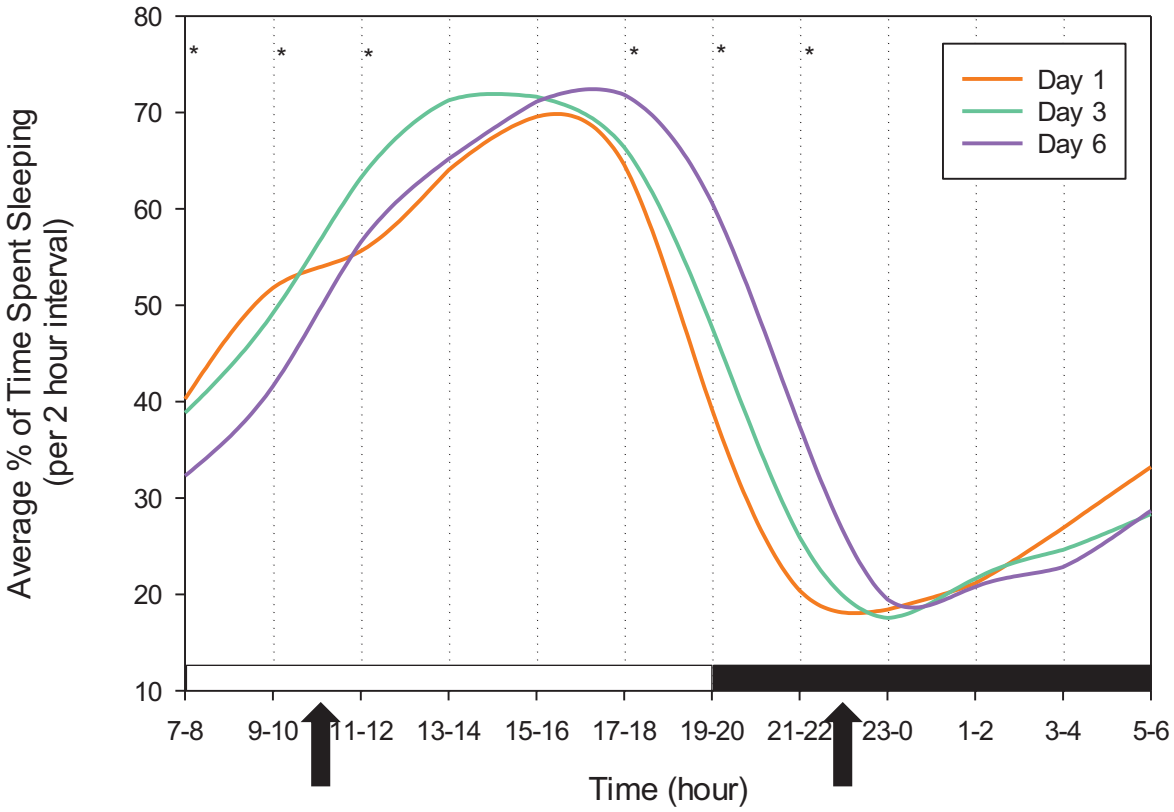

Figure 2.6: Percentage of time spend sleeping per 2 hour interval by day of treatment. Open bar at the bottom indicates lights on, closed bar indicates lights off. Arrows indicate disturbance times. Asterisks indicate differences in sleep in that epoch (Bonferroni corrected test slices, P<0.004). Data presented are LSM.

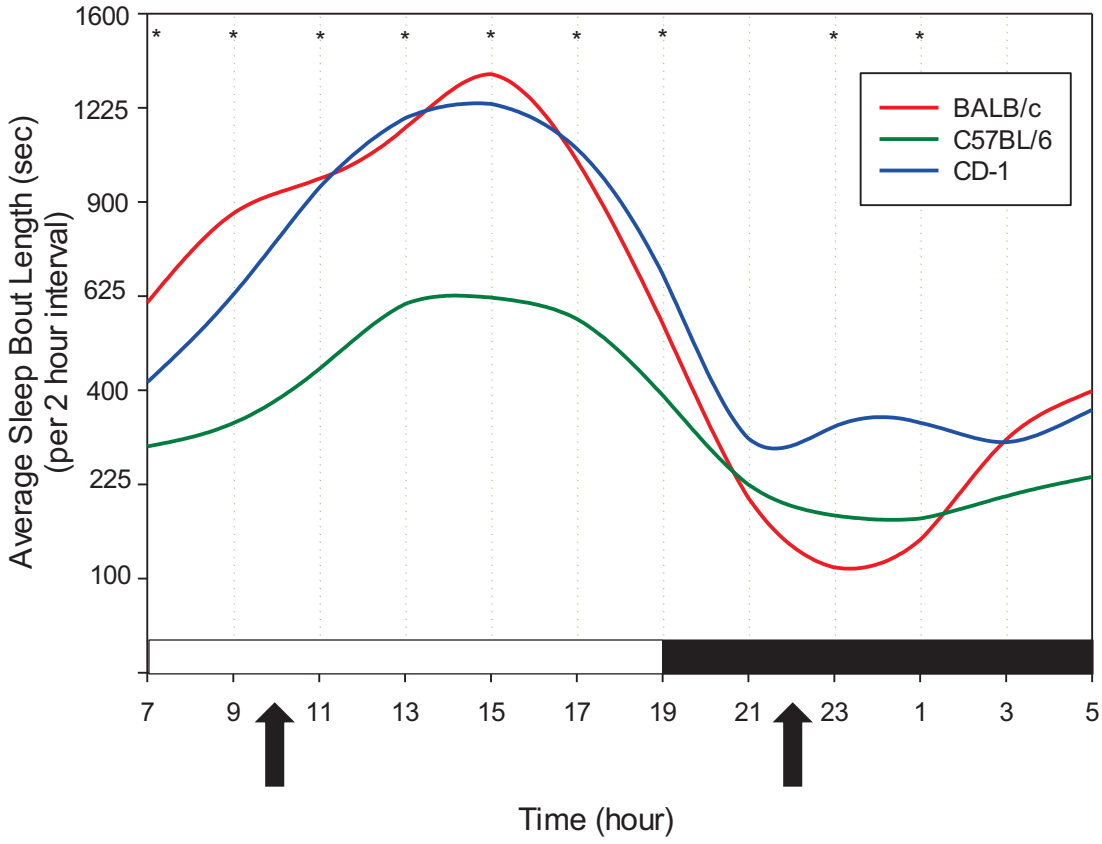

Figure 2.7: Average bout length by 2 hour interval. Open bar at the bottom indicates lights on, closed bar indicates lights off. Arrows indicate disturbance times. Asterisks indicate differences in bout length during that epoch (Bonferroni corrected test slices, $P<0.004$). Data were angular transformed, y-axis is backtransformed for clarity. Data presented are LSM.

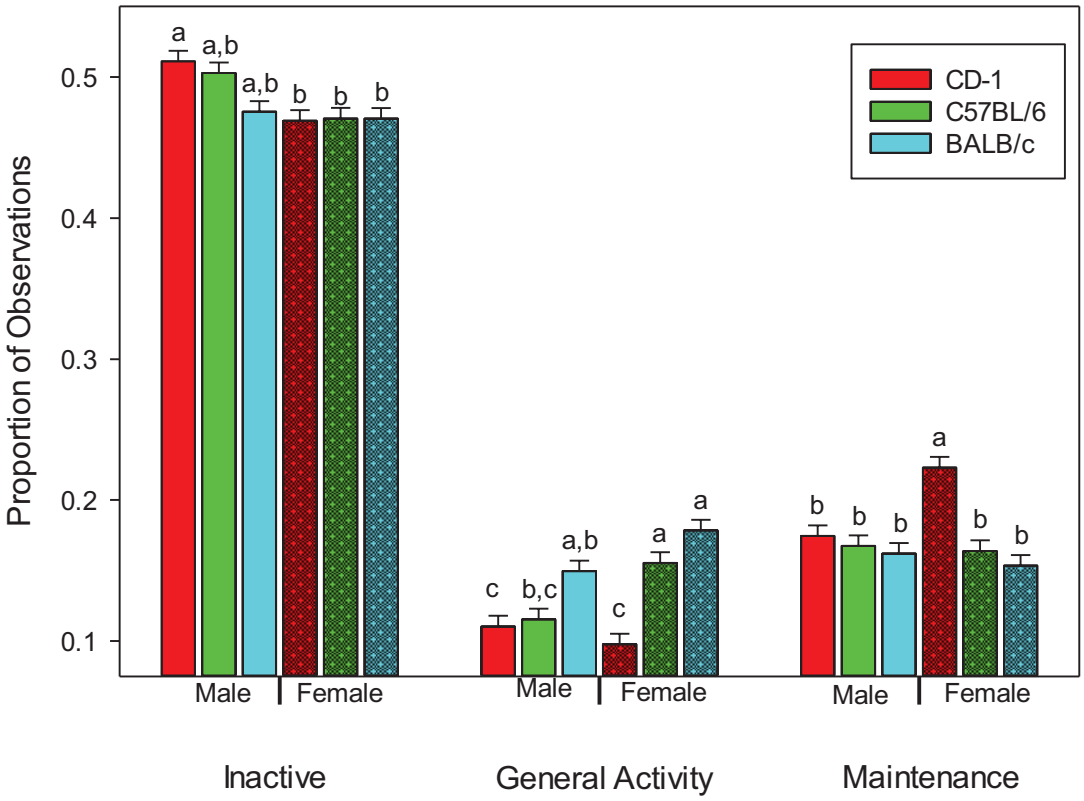

Figure 2.8: Proportion of observations of behavior categories. Solid bars indicate males, shaded bars indicate females. Different letters indicate significant (P<0.05, Tukey) differences within groups. Data presented are LSM and SE.

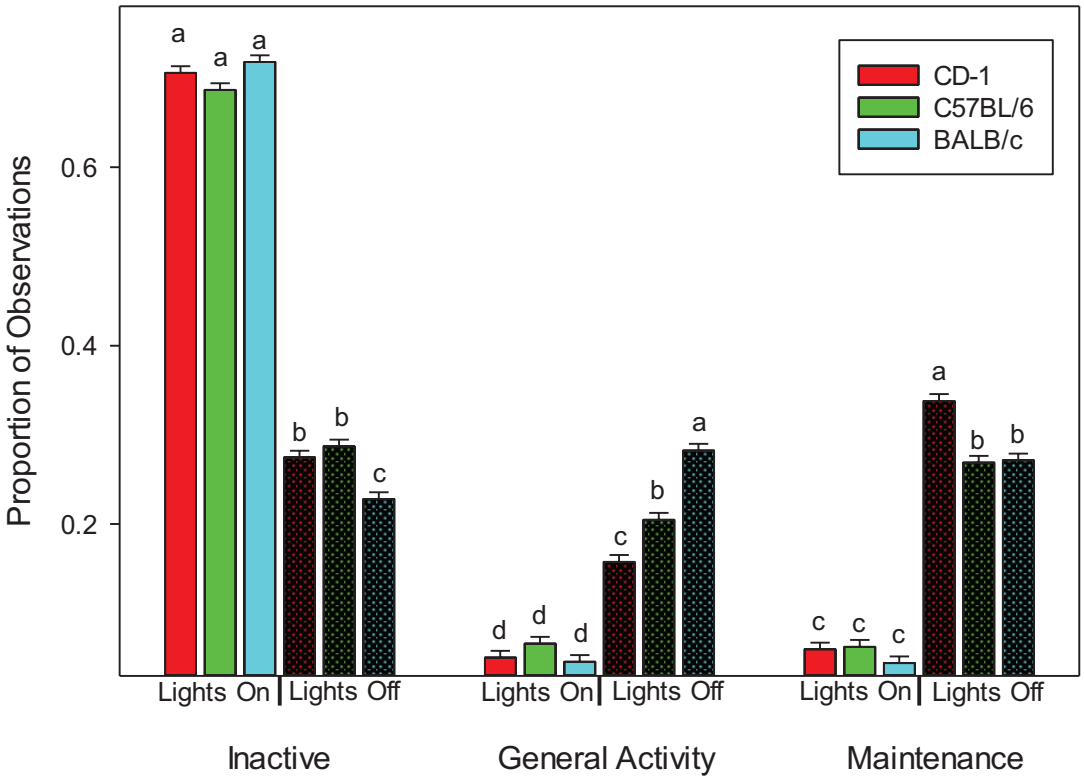

Figure 2.9: Proportion of time observed in behavior categories. Solid bars indicate lights on, shaded bars indicate lights off. Different letters indicate significant ($P<0.05$, Tukey) differences within groups. Data presented are LSM and SE.

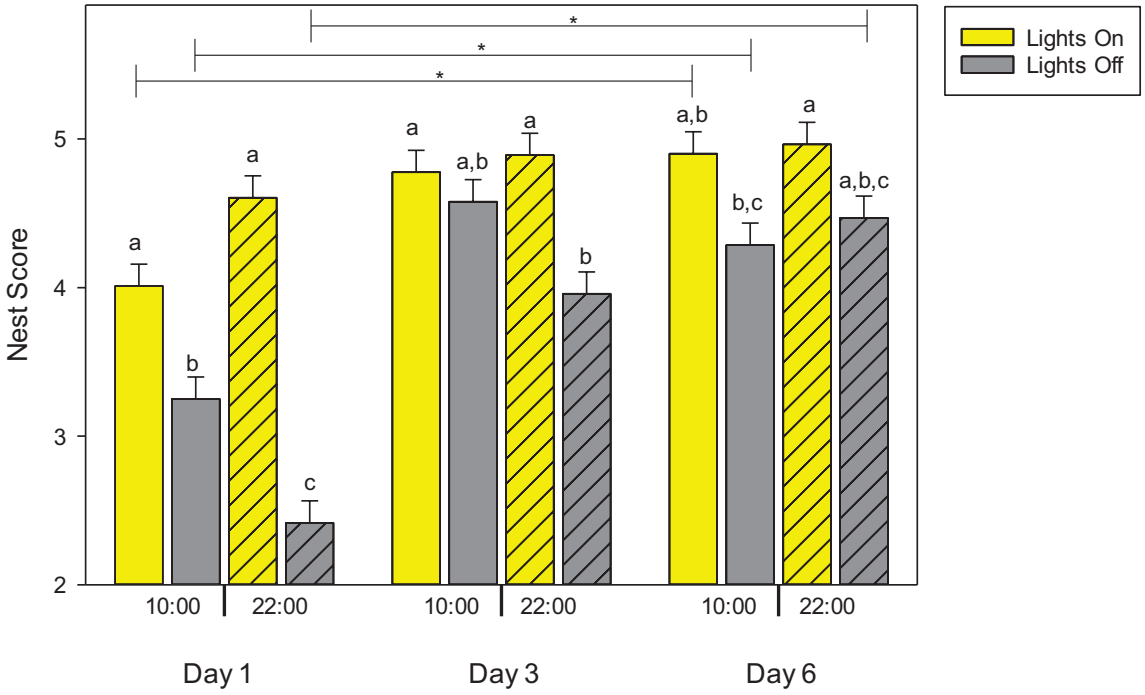

Figure 2.10: Nest scores affected by lights, disruption time, and day of treatment. Solid bars represent daytime disturbances (10:00) and hashed bars represent nighttime disturbances (22:00). Differing letters indicate within day differences, bars with asterisks indicate between day differences (Tukey, $P<0.05$). Data represented are LSM and SE.

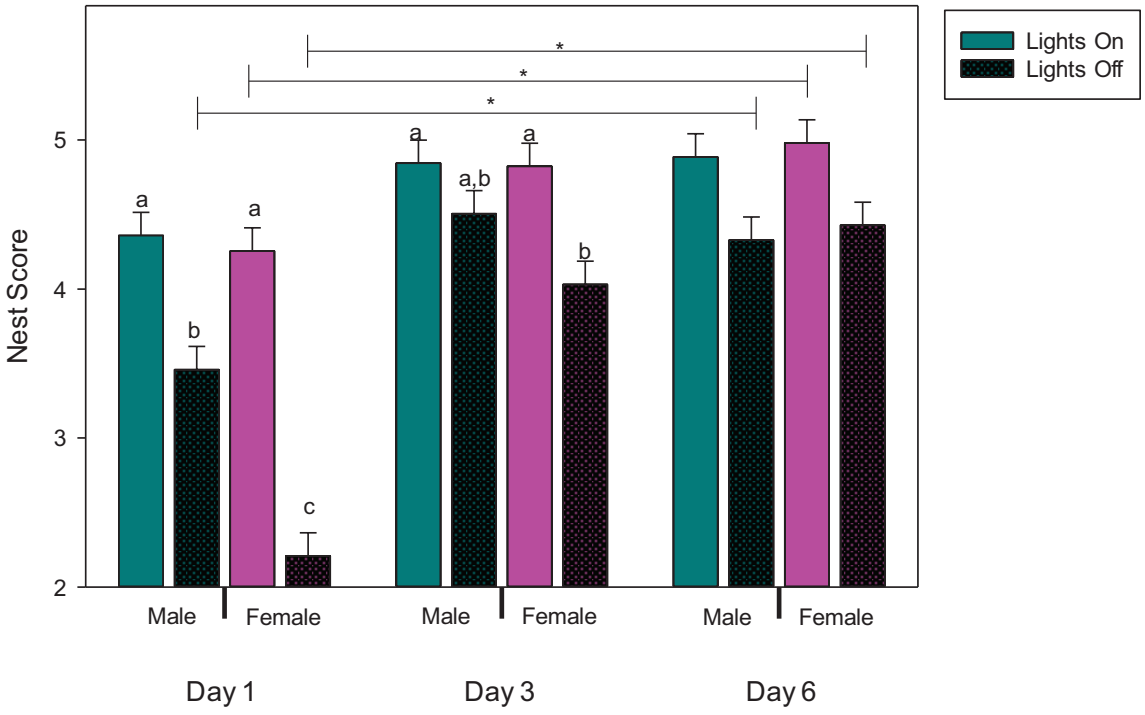

Figure 2.11: Nest scores affected by sex, lights, and day. Solid bars represent lights on and filled bars represent lights out. Differing letters indicate within day differences, bars with asterisks indicate between day differences (Tukey, $P<0.05$). Data represented are LSM and SE.

CHAPTER 3. SLEEPING THROUGH ANYTHING: THE EFFECTS OF UNPREDICTABLE DISRUPTIONS ON MOUSE SLEEP, HEALING, AND AFFECT

3.1 Abstract

Many aspects of the laboratory environment are not tailored to rodent needs, behaviorally or physiologically. Mice are nocturnal, but live in a diurnal environment to accommodate human activity. However, it's unknown how disruptions from the human-mouse circadian mismatch affect mouse physiology and welfare. There is a real potential that unpredictable human activities during the day may disrupt mouse sleep, inducing physiological changes like slowed wound healing, as well as decreasing affect. We tested 32 C57BL/6 mice of both sexes in a non-invasive sleep apparatus to see if this was the case. Mice were exposed to 7 days of either predictable or unpredictable disruptions, with a biopsy punch procedure on day 4 of the week to allow us to assess wound healing. We also tested administration of a non-steroidal analgesic against a control group, to see if there was an interaction between pain and sleep disruption. On day 7, mice were euthanized and we collected both the wound tissues as well as the adrenal glands. We found that the predictability of disruption had no effect on mouse sleep, wound healing, or adrenal cortex:medulla ratio in this experiment. It's possible that the disruption period didn't last long enough to induce chronic stress responses in these

mice. Analgesia did have an effect however, with male mice who received analgesia sleeping more than their female counterparts; this may be related to sex differences in pain perception. Overall, these were positive welfare findings, since the mice didn't show any signs of chronic stress with either disruption treatment.

3.2 Introduction

Many aspects of the laboratory environment are not tailored to rodent needs, behaviorally or physiologically. Mice experience cold stress at normal laboratory temperatures, depleting their energetic resources for reproduction[1-3]. Routinely provided corncob bedding is aversive and decreases sleep[4, 5], and typical handling[6, 7] induces stress. Furthermore, laboratory mice are nocturnal, but live in a diurnal environment to accommodate human workers. However, it's unknown how disruptions from the human-mouse circadian mismatch affect mouse physiology and welfare. Sleep fragmentation (the interruption of sleep either through waking or transitioning to a lighter sleep stage) induces physiologic, metabolic, and (if experienced during gestation) epigenetic effects, including slowed wound healing[8-12]. Other stressors are also known to slow wound healing, including restraint stress [13, 14] and pain [15-20]. However, there is a major gap in the literature regarding the impact of routine human activity on mouse sleep.

Sleep fragmentation in mice has typically been studied as a model for humans with frequent arousals from sleep (every 1-2 minutes), similar to what is experienced by individuals with sleep apnea or periodic limb movements [21]. Mechanized disruptions are generally used to study sleep fragmentation in mice [8, 9, 22], and therefore disruptions are

potentially predictable. This detail is important because it is not an accurate representation of the environment mice in a vivarium are exposed to. Mice probably experience some level of predictability, since human work often happens on a regular schedule, but certainly not to the degree of being awakened precisely every 2 minutes over 12 hours. Additionally, these mechanically awakened mouse studies did not include the presence of humans, which is dramatically different from what a typical laboratory mouse experiences. This may be an important aspect of investigations since Chesler et al. [7] found that 42 % of data variability in a thermal nociception study was attributed to environmental factors, primarily due to different people running parts of the experiment.

We know that unpredictability is stressful for animals; rats who receive unpredictable shocks develop ulcers[23] and anhedonia[24], and rats given a choice of shock will choose a predictable shock over an unpredictable shock[25, 26]. Typical vivariums involve multiple unpredictable disruptions. Animals from several projects may be housed in the same room, meaning researcher activities may not be coordinated. Running water, cleaning equipment, and even who caretakers are can vary on a daily basis. Not only are mice experiencing unpredictability of disruption, but these disruptions are also occurring during the light phase of the day, when mice would ordinarily be sleeping. This combination may be sufficient to induce sleep fragmentation and stress that we aren't accounting for in welfare assessment or experimental design.

One method of assessing the physiological effects of stress is through measuring wound healing; increased stress leads to slow or imperfect healing[27-32]. However,

another factor in wound healing is pain. Pain slows the healing process in humans [18-20] and alters general behavior. After experiencing a painful procedure, mice burrow less and build less complex nests[33-36], and are slower to incorporate new nesting material into an existing nest[37]. Additionally, pain interferes with sleep [38, 39], and sleep deprivation can induce hyperalgesia in rats[40]. This suggests that a vicious cycle may exist between these factors and requires that the interaction between pain, sleep, and healing be considered in this area of research.

The effects of sleep disruption are not solely physiological. Work in both humans [41-44] and rodents [45] has shown cognitive changes after sleep deprivation and disruption, and sleep dysfunction is also associated with mood disorders in humans [46-48]. These findings indicate that an investigation of the potential welfare implications of sleep disruption should also include assessment of changes in mental well-being.

Our hypotheses were that unpredictable disruptions are more disruptive to mouse sleep than predictable disruptions. We also hypothesized that pain following from lack of post-operative analgesia would negatively affect nesting behavior and sleep patterning. We predicted that mice who experienced frequent, unpredictable disruptions during their normal rest period would sleep less and/or have more fragmented sleep during the day and have higher indicators of stress than those whose disruptions occurred at predictable times. We also predicted that mice who received analgesia (rather than a saline ? injection) would sleep more during their normal rest period and have lower indicators of stress.

3.3 Materials and Methods

3.3.1 Ethical Statement

This study was approved by the Purdue Animal Care and Use Committee, and conformed to all guidelines put forward by both the committee and the Guide for the Care and Use of Laboratory Animals [49]. At the start of study, animals were free of a list of common mouse infectious agents; further details may be found at http://www.criver.com/files/pdfs/rms/hmsummary.aspx. All mice were monitored daily by trained members of the research team for food and water consumption and overall health status, with no adverse conditions or health outcomes noted.

3.3.2 Experimental Design, Animals, and Housing

Two main treatments, in a factorial design, were assessed in C57BL/6NCrl mice of both sexes (6 weeks of age; Charles River, Kingston, NY); 1) sleep disruption (unpredictable or predictable) and 2) analgesia administration (analgesia or saline). Each factorial combination had four replicates for a total of 32 mice (Table 3.1). Mice were tested from May to June of 2016.

Mice were housed in one of the two sleep monitoring apparatuses (Figure 3.1). Each apparatus houses 4 mice, allowing us to test 8 mice simultaneously. The apparatus uses a piezoelectric mat underneath the cage to detect vibrational movement of the mouse and therefore mice must be housed singly. Customized software (MouseRec Data Toolbox, Signal Solutions, Lexington KY) uses an algorithm to process the signal and discern sleeping respiratory patterns from waking respiratory patterns; this algorithm has been validated using EEG, EMG, and visual evaluation [50]. A different algorithm also

permits quantification of activity level, where higher numbers indicate greater intensity of activity. Visual barriers were in place between cages, but audible and olfactory contact was still possible. Each cage included a built in food hopper and water bottle opening. Each cage was bedded with 32g of laboratory grade aspen shavings (Harlan, Indianapolis IN) and 8g of nesting material (Enviro Dri, Shepherd Specialty Papers, Watertown, TN). Mice were provided with an 18% protein laboratory diet (Harlan 2018, Indianapolis IN) and reverse osmosis filtered water *ad libitum*. Lights were kept on a 12:12 light/dark cycle, with lights on at 05:00 and off and 17:00 hours. The room was maintained at 72± 2° F, and 36-64% humidity. Upon arrival (Day -4 – see Figure 3.2), mice were randomly assigned to an analgesia treatment using a random number generator (www.random.org). Mice were weighed and placed in their cage within the sleep apparatus, no longer than 1 hour before the lights were turned off.

3.3.3 Procedures

3.3.3.1 Disruption Treatments

Sleep disruptions began immediately after arrival. Because all testing was conducted in a single room, all 8 mice in a test group were exposed to the same disruption treatment (unpredictable or predictable) simultaneously. Both treatments consisted of daily exposure to 4 of a possible 8 disruptions – presence of a stranger, a recorded conversation playing in the room, a radio playing pop music, cage changing noises, presence of a t-shirt that was worn by a man, running water, a running cage changing station with ventilation hood, and floor disinfection (Table 3.2). These

disruptions were chosen based upon activities that occur in a typical vivarium and factors that are known to alter mouse behavior (such as the presence of a male investigator, or a shirt worn by one [51]). The order and duration of the disruptions were initially scheduled in a random fashion, but the schedule itself was consistent across disruption treatments. For instance, all mice experienced the same disruptions on the same day during the experiment, for the same durations. The only difference was whether they were spread throughout the day (unpredictable) or consolidated at the beginning and end of the day (predictable). No disruption was repeated in the same day, and there were a total of 4 disruptions per day. Potential durations of disruption were 15, 30, 45, or 60 minutes; floor disinfection and running water only lasted 15 minutes due to practical and environmental considerations. In the unpredictable disruption group, the interval between disruptions was also randomized, with intervals between them of either 45, 60, 90, or 120 minutes. For the predictable disruption group, disruptions occurred between 2.5-3.5 hours after the lights were turned on (7:30 – 8:30) or within an hour of turning the lights off (16:00-17:00), with two disruptions scheduled for the morning period, and two for the evening period. The exception to this schedule was on the morning of the punch biopsy procedure; no disruptions were scheduled.

3.3.3.2 Punch Biopsy Procedure

After 4 days of acclimation and disruptions (Day 0), all mice were anesthetized with isoflurane in an induction chamber and maintained on isoflurane administered via

nose cone. We clipped and sterilized the cervical area of each mouse, placed them in lateral recumbency, and pulled the dorsal skin away from the animal, as if scruffing them. We then utilized a 3mm biopsy punch (Sklar Surgical Instruments, West Chester, PA) to push through both layers of skin, creating 2 symmetrical 3 mm full-thickness wounds. The wounds were not sutured, stapled, or glued. Surgical order was randomized to account for order effects. During this procedure, we used a chemical hand-warmer (HotHands, Kobayashi Americas, Dalton GA) to provide thermal support to the mice. Mice then received an analgesic injection of carprofen at 10mg/kg or an equivalent volume of saline subcutaneously, depending upon the animal's assigned analgesia treatment; these injections were located on the caudal part of the mice, to avoid manipulating the biopsy area post-operatively or any possible leakage of medication from the biopsy sites. All mice, regardless of analgesia group, received 0.05 mL of 2% lidocaine gel topically applied to each wound for short-term local analgesia. Mice were then moved to heated recovery cages until they were ambulating normally. Once mice recovered, they were returned to their home cage. Two post-operative anesthesia recovery checks were performed two hours apart. Sleep disruptions resumed as scheduled.

3.3.3.3 Analgesia Treatment

Mice assigned to the analgesia treatment group received 10 mg/kg carprofen subcutaneously on Day 0 (after wounding), Day 1, and Day 2. Mice in the analgesia control group received an equal volume of saline subcutaneously on the same days as

the analgesia mice. On Day 1 and 2, a dorsal access mouse restrainer (Braintree Scientific, Braintree MA) was used to hold the mice while we administered a subcutaneous injection in the caudal region, avoiding manipulation of the biopsy area and any risk of medication leaking from the surgical site.

3.3.3.4 Behavioral Testing

Sleep and activity data were collected continuously via the sleep apparatus. We began data collection once the final mouse was housed on Day -4 (prior to 17:00) and ended it by 9:00 the morning of Day 3, prior to euthanasia.

Mice were TINT tested[37] to assess pain and general welfare. In brief, in the TINT we provide a small amount of new nesting material to mice 2-3 hours after lights on and give them 10 minutes to integrate this new material into the existing nest. A positive TINT score means the material has been incorporated, and suggests positive welfare. A negative TINT score suggests that the mice in that cage are experiencing more negative welfare, and personnel should investigate further. For this project, an investigator would enter the room, cut a Nestlet (Ancare, Bellmore, NY) into 4 equal squares, deliver one of those pieces to each cage, and leave the room for 10 minutes. Upon returning to the room, we assessed whether or not the Nestlet had been integrated into the nest. In this case, 'integrated' means had been transported to the main body of the nest. TINT testing occurred daily at 8:00. This time corresponds with peak nest-building behavior [36]. The scores on Days -3 to -1 were considered 'practice', as mice have been shown to

shorten their latency to incorporate material with repeated exposures [37, 52], so data presented from day -1 are used as their baseline TINT.

Sucrose preference testing was used to assess anhedonia[53]. We did this by providing mice with 5g of sugary cereal (Froot Loops, Kelloggs, Battle Creek MI) between 16:00 and 17:00 (prior to lights out), and then weighing the remainder between 7:30 and 8:00 the next morning. This allowed us to calculate the amount of cereal consumed each night; a decrease in consumption is indicative of anhedonia. We conducted these tests on Day -1, Day 1, and Day 2.

3.3.3.5 Tissue Collection

On Day 3, mice were euthanized via carbon dioxide anoxia. Immediately after euthanasia, mice were weighed and the punch biopsy area was excised, as well as surrounding tissues. Adrenal glands were also collected, in order to assess HPA axis activation[54, 55].

All tissues were fixed in 10% neutral buffered formalin and embedded in paraffin. Sections 5 µm thick were stained with hematoxylin and eosin according to standard methods. Microscopic examination was performed by a board-certified veterinary pathologist and the interpretation was based on standard histopathological morphology. The pathologist was blinded to the treatment groups. Wound width and re-epithelialization were quantified for all mice. Wound width was defined as the distance between wound margins in which the original epidermis was intact. Re-epithelialization was calculated as amount of newly formed epidermis as a percentage

of the wound margin. Newly formed epidermis was defined as less than 3 cell layers thick of squamous epithelium devoid of stratum corneum.

Both adrenal glands were sectioned en toto and representative sections were cut 50 micrometers deep. One adrenal gland per mouse was used to calculate an average cortex to medulla length ratio. Three cortical lengths and 3 cross-sectional medulla lengths were averaged and a ratio was calculated for each mouse.

3.3.4 Statistical Analysis

All data, with the exception of TINT success/failure, were analyzed using up to 3rd degree factorial General Linear Model (GLM) in JMP (version 11, SAS Institute Inc) of the following factors: sex, disruption treatment, analgesia treatment, experiment day and (for sleep and activity data) lights on or off. To calculate food consumption, regular diet and sucrose cereal consumption were combined to calculate the total intake, where applicable. Individual mouse was the experimental unit and was used as a random factor, with sex, disruption treatment, and analgesia treatment nested within it. Cage location and sleep apparatus were used as blocking factors. Bodyweight was included as a covariate with food consumption, sucrose consumption, and adrenal cortex:medulla ratio. We used square root transformation for sleep bout length and activity level data, and log transformation for adrenal cortex:medulla ratio, in order to meet the assumptions of GLM. The assumptions of GLM (normality of error, homogeneity of variance, and linearity) were confirmed post-hoc[56]. Significant effects were then analyzed using post-hoc Tukey tests. All values are given as least squares means and standard error.

TINT success/failure was analyzed using up to 3rd degree factorial Generalized Linear Model (GLIM) for binomial logistic regression, with Firth-adjusted bias, for the following factors: sex, disruption treatment, analgesia treatment, and day of experiment. Cage was used as a fixed factor, with sex, disruption treatment, and analgesia treatment nested within it. Cage location and apparatus number were used as blocking factors. Non-significant 3rd degree interactions were removed from the model, which produced a lower AICc number, denoting an improved model fit[57]. Pairwise planned contrasts were subsequently conducted on levels of significant factors to assess where differences arose, and were Bonferroni corrected for multiple comparisons.

3.4 Results

3.4.1 Sleep Measures

We found multiple effects on proportion of time spent sleeping. Sex by analgesia treatment (GLM, $F_{(1, 21)}$=6.38, P=0.0196) showed that males who received analgesia slept more than females with analgesia. No other differences between control animals or within the sexes were observed. Sex by lights on/off was also significant (GLM, $F_{(1, 184)}$=5.34, P=0.0219), with males sleeping more during lights off than females. Lights on/off by day of experiment (GLM, $F_{(3, 184)}$=26.99, P<0.0001; Figure 3.3) showed that animals slept less during lights on on Day 1 than baseline, or Days 2 or 3. Additionally, animals slept less during lights off at baseline than they did on days 2 and 3. And mice slept more during lights on than lights off for Days 1, 2, and 3. Finally, I found a 3 way interaction between disturbance treatment, analgesia treatment, and lights on/off

(GLM, $F_{(1, 184)}$=14.32, P=0.0002). However, this effect was solely due to lights on/off, with mice sleeping more when lights were on.

Mean sleep bout length had multiple significant interactions. Lights on/off by day of experiment (GLM, $F_{(3, 184)}$=18.42, P < 0.0001; Figure 3.4) showed that, during lights on, mice had the shortest bout lengths on Day 1; during lights out, their bout lengths were shortest on Day 2. There was also a significant interaction between sex, analgesia treatment, and lights on/off (GLM, $F_{(1, 184)}$=4.48, P=0.0356). However, post-hoc Tukey analysis showed no differences between groups. Sex by analgesia treatment (GLM, $F_{(1,78.49)}$=5.59, P=.0205) showed that female mice who received analgesia had shorter sleep bouts than those in the control group; there was no difference in the male mice, or within treatments.

3.4.2 Activity Levels

Mean activity level analysis showed several significant factors. Lights on/off by day of experiment (GLM, $F_{(3, 184)}$=8.41, P<0.0001; Figure 3.5) showed a decrease in activity during lights off for Days 1 and 3. During lights on, mice were more active on Day 1 than on Days 2 or 3. Additionally, sex by analgesia treatment by day of experiment (GLM, $F_{(3,184)}$=3.64, P=0.0139; Figure 3.6) demonstrated that female mice in the analgesia control group were less active on Day 1 than they were at baseline, and males in the analgesia treatment group were less active on Day 3 than at baseline. Finally, disruption treatment by analgesia treatment by lights on/off (GLM, $F_{(1, 184)}$=5.85, P=0.0166) showed only one difference – that mice in the unpredictable disruption plus

analgesia control group were more active during lights off than lights on; there were no other differences between lights on/off or treatment groups.

3.4.3 Sucrose Consumption

Sex (GLM, $F_{(1,24)}$=5.49, P=0.0277) was a significant main effect on sucrose consumption, with females (2.3 g, ± 0.07) consuming more than males (2.06 g, ± 0.07). Day of experiment was also significant (GLM, $F_{(2,50)}$=10.78, P=0.0001), with mice consuming more sucrose on Days 1 (2.33 g, ± 0.07)and 2 (2.24 g, ± 0.07) than at baseline (1.98 g, ± 0.07). Bodyweight was included as a covariate, but was not significant.

3.4.4 TINT Scoring

TINT success analysis had multiple significant effects. Mice at baseline were more likely to pass their TINT than on Days 1, 2, or 3 (GLIM, $X^2_{(3)}$=25.17, P<0.0001). Assessing sex by disruption treatment (GLIM, $X^2_{(1)}$=6.82, P=0.0090), Bonferroni-corrected contrasts were not significant. Sex by analgesia treatment (GLIM, $X^2_{(1)}$=11.98, P=0.0005) showed that males given analgesia were more likely to succeed than controls. Additionally, females in the control group were more likely to successfully integrate nesting material than their male counterparts. Finally, disruption treatment by analgesia treatment (GLIM, $X^2_{(1)}$=7.84, P=.0051) was significant, but Bonferroni-corrected contrasts were not significant.

3.4.5 Food Consumption

Total food consumption had two significant main effects, sex (GLM, $F_{(1, 20.91)}$=4.99, P=0.0366) and day of experiment (GLM, $F_{(3, 77.35)}$=40.90, P<0.0001). Female mice (3.84 g, ± 0.10) consumed more than males (3.46 g, ± 0.10); mice at baseline (4.22

g, ± 0.10) and Day 2 (4.04 g, ± 0.10) consumed the most, followed by those on Day 3 (3.55 g ± 0.10) and then Day 1 (2.78 ± 0.10). Bodyweight was a significant covariate (GLM, $F_{(1, 21.76)}$=8.86, P=0.007); as bodyweight increased, so did food consumption.

3.4.6 Bodyweight

Bodyweight was affected by 3 main effects. Sex (GLM, $F_{(1, 27.71)}$=53.26, P<0.0001) showed that males (20.36 g ± 0.26) were heavier than females. Day of experiment (GLM, $F_{(3,75)}$=32.21, P<0.0001) indicated that mice weighed more at baseline (19.0 g ± 0.18) than Day 1 (18.74 g ± 0.18), and less than on Days 2 (19.30 ± 0.18) and 3 (19.46 ± 0.18). Finally, disruption treatment (GLM, $F_{(1, 27.71)}$=7.81, P=0.0093) was significant, with mice in the unpredictable disruption group (20.36 g ± 0.26) weighing more than those in the predictable group (18.49 g ± 0.26).

3.4.7 Histopathology

There were no significant factors in either percent re-epithelialization or adrenal cortex:medulla ratio.

3.5 Discussion

Few of our hypotheses were supported by our results. Proportion of time spent sleeping and sleep bout length were unaffected by disruption treatments, which was where we had expected to see the strongest results. This may be an example of anthropomorphism on our part. For instance, when we would expect the opposite, Pajor et al[58] found that yelling at dairy cattle was more aversive than a control condition, while being struck on the rump was not. It would seem that we similarly

misjudged how disruptive these mice would find the presence of humans making noise in the room with them.

Interestingly, analgesia turned out to be significant, even for a minor procedure like a biopsy punch. Punches are used for wound healing studies[13, 14, 28, 29, 59-62], but also for identification[49]. However, there is no consensus on analgesia protocols for mice who have had this procedure[13, 63, 64]. This seems to be a concern, since male mice who received analgesia spent more time sleeping than their female counterparts. Additionally, females who got analgesia had shorter sleep bout lengths than controls. This may be related to sex differences in pain perception. Females, in both humans and rodents, have been reported to perceive pain more intensely than males [65-68]. So while male mice might have experienced sufficient pain relief from the carprofen dosage, the females may not. Although other literature suggests that the dosage of carprofen we used may not completely eliminate pain following a laparatomy[69], we felt that a punch biopsy would be a less painful procedure; this was namely because, while it does break the skin, it does not pierce a muscle layer as in a laparotomy. Higher doses were considered before the start of the experiment but concerns of gastrointestinal upset and renal toxicity ultimately resulted in our choice of a more moderate dose[69]. After consulting with our attending veterinarian, we decided to provide a higher dose than what is recommended by ACLAM[70] (5mg/kg) but less than a dose that might cause the toxic effects of an overdose. Opioid analgesics, such as buprenorphine, were also considered but due to its associated behavioral changes [71-73], it was likely to have interfered with our behavioral measures. In the future, either higher doses of analgesia

or perhaps a combination of non-steroidal and opioid medication could achieve effective relief, particularly for female mice, without altering behavior.

Histopathology measures were unaffected by any of our treatments. The sleep disruptions, and subsequent stress that these treatments were meant to induce, may not have been sufficient enough to induce adrenal morphology changes, and were more acute than chronic. In studies where adrenal changes have been noted, durations of stressors have been at least 2 weeks[74-77], and when a stressor only lasted for one week, changes were not observed[78]. As far as the wound re-epithelialization, the location of the wound may have affected our results. We hoped to avoid having the mice exacerbate their wounds and delay healing by placing the punch closer to the neck than the flanks. However, wound healing is dependent upon multiple factors, and particularly in rodents, wounds contract quickly due to their panniculus carnosus[29]. This is a layer of muscle that permits their skin to contract for healing. A wound splint process may have been helpful to prolong the healing process and more accurately assess re-epithelialization (more like in humans), [29]. However, it's also possible that we didn't induce sufficient stress in the mice, and therefore wound healing was not impaired.

Sucrose consumption results were also unexpected. We predicted that mice would have higher baseline consumption than any post-operative time point, regardless of treatments. Instead, we found exactly the opposite. Perhaps these mice needed repeated exposures to overcome any food neophobia[79], needing time to learn that the cereal was highly palatable. Alternatively, this sucrose consumption pattern may be a reflection of how long mice actually need to acclimate to a new environment after

transport. Baseline sucrose testing began for our mice approximately 3 days after arrival, with disruptions already occurring. These mice may not have been disturbed enough to change their sleeping patterns, but still were stressed in some fashion and therefore consumed less sucrose. A potential caveat to these theories, however, is that glucose is required for the physiological stress response. Wilcox, et al. found that socially stressed calves consumed more molasses than control calves[80], presumably in order to fuel that adrenal response. Perhaps that mechanism was at play in these mice; rather than not expressing anhedonia, they were expressing a stress response. However, this increased glucose consumption secondary to chronic stress has not been demonstrated in mice, and our other measures seem to agree that this was not a particularly stressful experiment for our mice. It may be worthwhile to explore this potential interaction between stress and sucrose preference in mice further, however.

Similarly, our results for TINT success rate were lower than expected. The validation work on TINT demonstrated that mice at baseline were successful the overwhelming majority of the time[37, 52]; this was not the case for our mice. However, the mice in the referenced work had been present at the study facility for much longer than ours had (personal communication from the author) and were almost certainly more acclimated to their environment.

One thing that was not surprising was the decrease in activity levels on days 2 and 3. While perhaps counterintuitive, because presumably the mice were healing and should have been feeling better, those days corresponded with the first restraint and injections the mice received. Mice responded negatively to these events, urinating,

defecating, and vocalizing. This was the only time vocalizations were observed during the project. This suggests that the mice found the restraint extremely aversive, and their subsequent activity levels may be a reflection of that. We know that mice react differently to different types of handling[81], and that nest scores can be reduced after being handled by a novel individual[6]; this drop in activity may be a manifestation of their apparent aversion to unconditioned handling.

While our results didn't support our hypotheses, they do raise some interesting questions regarding acclimation periods, sex differences in pain response, and just how disruptive human activity actually is to mice. This project would suggest that direct interaction and restraint with the mice is more stressful than mere investigator presence or noise. However, this was only conducted with one strain of mice, over a relatively short time period. It is possible that mice in longer term projects may experience those events differently. At this time, we can't make many recommendations, other than considering longer acclimation periods prior to commencing research, and investigating the longer term effects of carprofen use at higher doses for effective analgesia, particularly for female mice.

3.6 References

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Table 3.1: Factorial design with number of replicates per combination

Disruptions	Predictable				Unpredictable			
Sex	Male		Female		Male		Female	
Analgesia	Y	N	Y	N	Y	N	Y	N
Replicates	4	4	4	4	4	4	4	4

Table 3.2: Disruption descriptions, durations, and number of occurrences. When disruptions occurred more than once, different durations were possible; if that was the case, all duration times are listed. All mice experienced all disruptions.

Disruption	Description	Duration	Number of Occurrences
Cage change	Investigator removes mice from cage, supplies fresh bedding and nesting material, replaces mouse	30 min	1
Cage change noise	Investigator rattles cages containing corncob bedding and lids	45 min, 60 min	3
Conversation	Smartphone used to play back each of two specific stand up comedy tracks (65-72 dB at cage level)	45 min	2
Exhaust fan	Exhaust fan of cage changing station turned on (62 dB at cage level)	30 min, 60 min	3
Floor cleaning	Investigator uses power washer to distribute cleaning solution, scrubs floors with scrub brush, rinses with bucketed water, then squeegees floor dry	15 min	1
Male t-shirt	Investigator places t-shirt that was worn the night before in the room near the cages	30 min, 60 min	3
Music	Antenna radio tuned to local rock music station	15 min, 30 min, 45 min, 60 min	4
Running water	Water left running in stainless steel sink (58-62 dB at cage level)	15 min	3
Stranger	Unfamiliar person sits or stands quietly in room without interacting with mice	15 min, 45 min	2
Unfamiliar smell	Investigator sits quietly in room while wearing strongly scented lotion	30 min	1

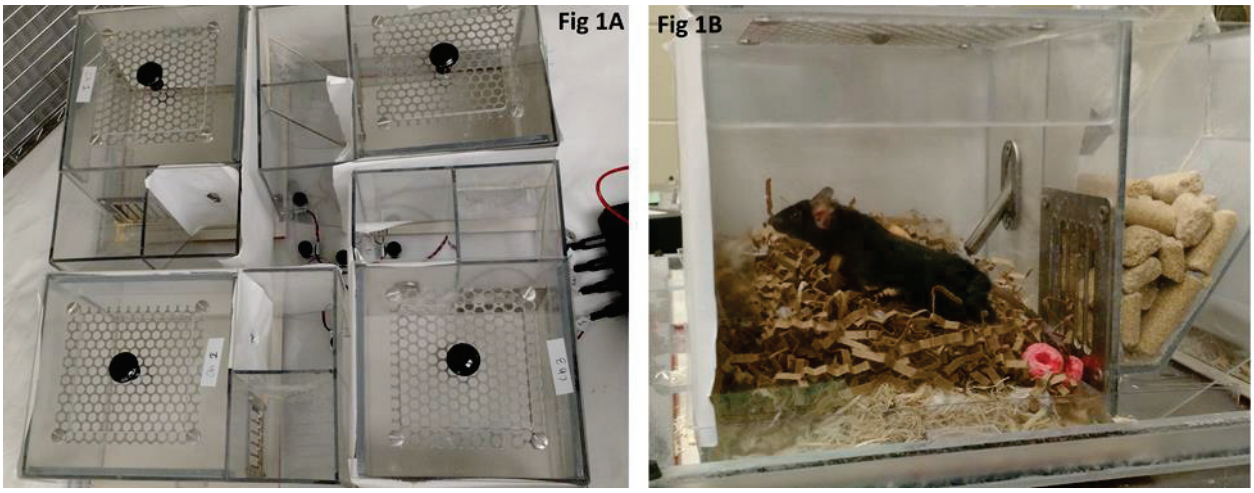

Figure 3.1: Sleep apparatus viewed from above (1A) and a close up side view of an individual mouse cage (1B). Sugary cereal used for the sucrose preference test can be seen in 1B

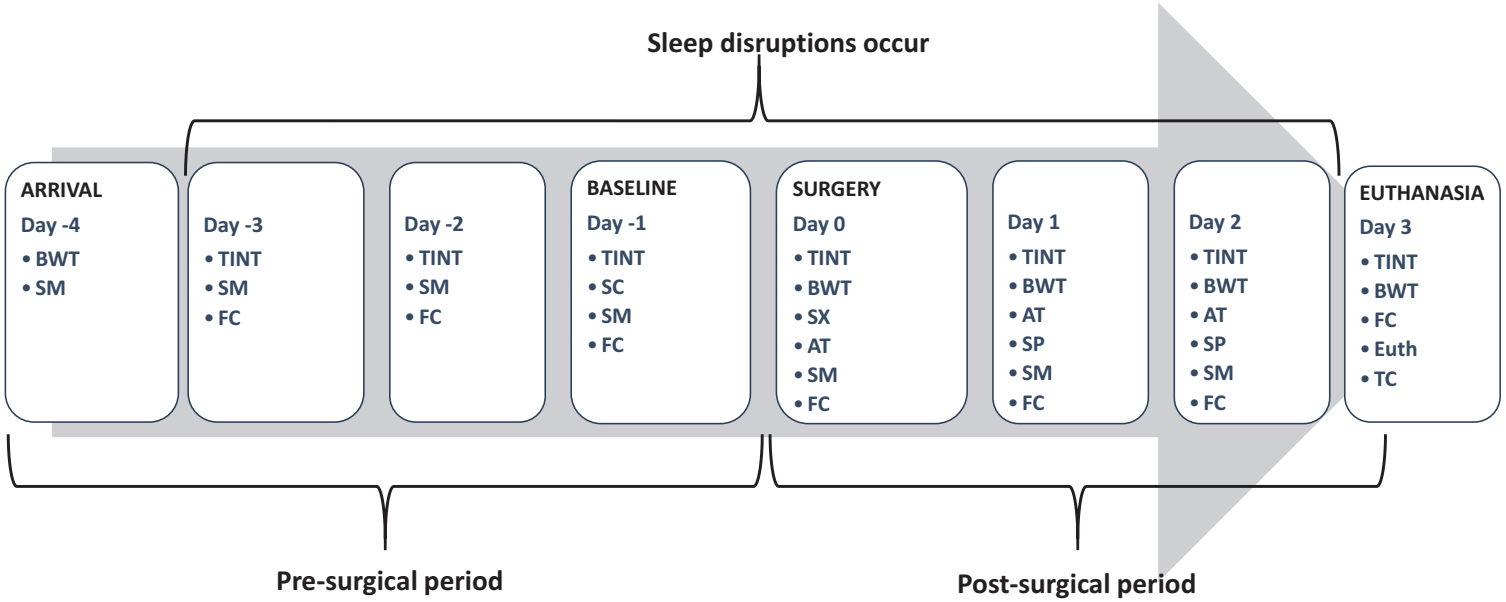

Figure 3.2: Experimental Timeline. Lists all measurements made on each day of experiment. Day -1 is considered baseline. Mice arrive on day -4. Abbreviations: **BWT –** bodyweight; **SM –** sleep monitoring; **TINT –** Time to Integrate Nesting Material Test; **FC –** food consumption; **SC –** sucrose consumption; **SX –** surgery; **AT –** analgesia treatment;

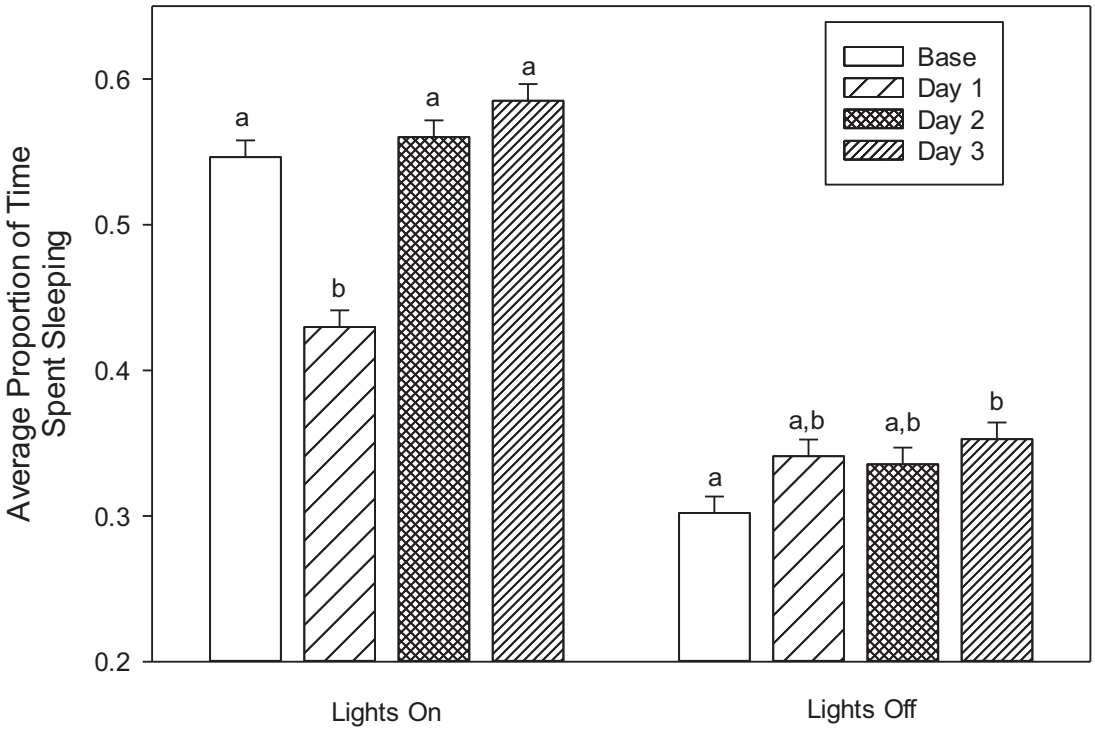

Figure 3.3: Average proportion of time spent sleeping by lights on/off and day of experiment. Different letters indicate significant (Tukey, P<0.05) differences within categories. Data presented are LSM and SE.

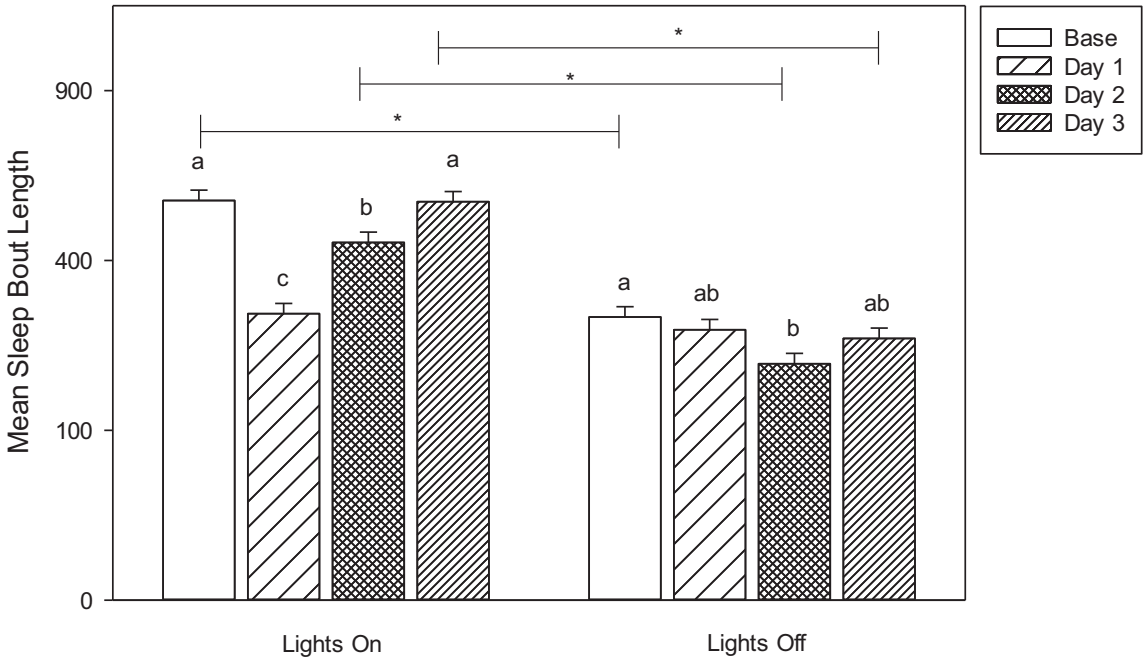

Figure 3.4: Average sleep bout length by lights on/off and day of experiment. Different letters indicate significant (Tukey, P<0.05) differences within categories, bars indicate differences between categories. Data were angularly transformed for analysis, Y-axis is backtransformed for clarity. Data presented are LSM and SE.

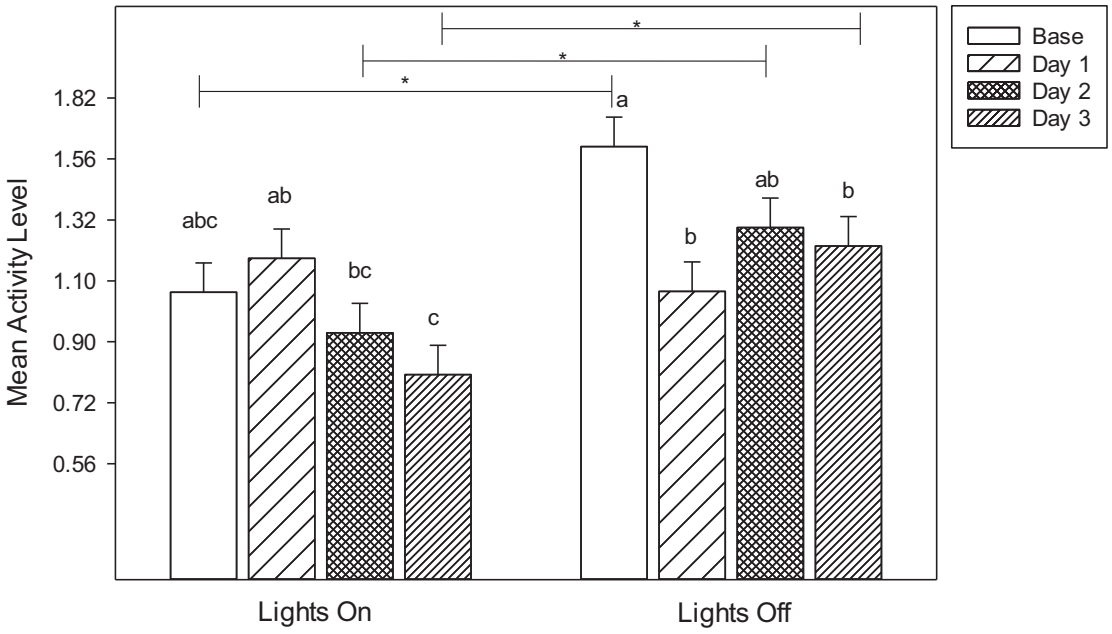

Figure 3.5: Mean activity level by lights on/off and day of experiment. Different letters indicate significant differences within categories, bars with asterisks indicate differences between categories (Tukey, $P<0.05$). Data were square root transformed for analysis; y-axis is backtransformed. Activity level is a linear measurement from 0 to 3; higher values indicate higher levels of activity. Data presented are LSM and SE.

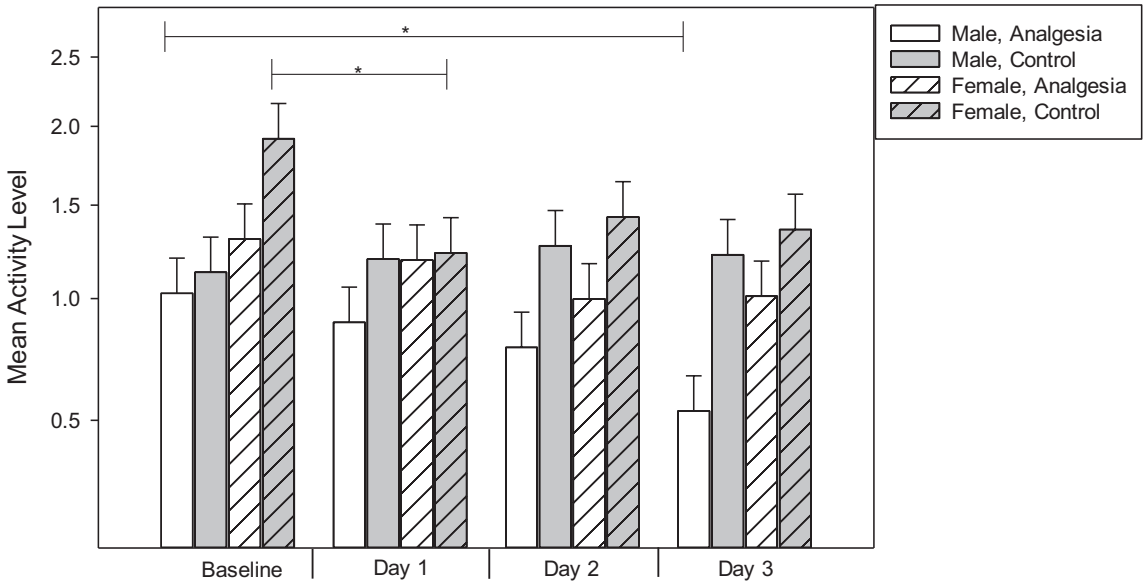

Figure 3.6: Activity level by day of experiment, sex, and analgesia treatment. Bars with asterisks indicate differences between categories (Tukey <0.05). Data were square root transformed for analysis; y-axis is backtransformed. Activity level is a linear measurement from 0 to 3; higher values indicate higher levels of activity. Data presented are LSM and SE.

CHAPTER 4. CONCLUSIONS

Sleep is an important consideration in animal welfare. The effects of not getting enough sleep, or having sleep displaced into abnormal time periods, are well documented in humans[1-5]; we have few reasons to think some of those may not apply to animals as well.

But sleep is also an important consideration for the validity of animal-based research. If we are altering animal physiology by changing their sleep patterns, we may be confounding our research in ways that we aren't accounting for in experimental design or analysis. This concern was what led to our studies of the effects of human activities on mouse sleep.

For these mice, the findings were positive. When they were disturbed during their rest period, they still slept the same amount as when they were disturbed during their active period. There were no differences in the incidence of stereotypy during the daytime and nighttime disruptions, and even mice with small amounts of nesting material got the same overall amount of sleep as mice with larger amounts. However, there was a clear difference in sleep patterning on the first day of treatment, compared to days 3 and 6. While this may reflect the change in disruption time, that seems unlikely, since the differences only show up after the morning disruption. This does

suggest that the mice found cage change and weighing more disruptive during the day than at night.

Our study on unpredictable disruptions also turned up positive results for the mice. Unpredictable disruptions didn't affect sleep, activity, or affect any more than predictable ones did. In fact, the biggest stressor for those mice appears to have been the handling and restraint involved with analgesia administration. We also saw a disparate response to analgesia between male and female mice, suggesting that the pain relief that we chose wasn't equally effective across the sexes.

These projects raise some interesting new questions to explore. What if we moved the restraint and handling to the dark period? Cage change seemed to be less disruptive after dark, perhaps handling would be as well.

Also, the safety of higher doses of non-steroidal anti-inflammatory drugs should be explored. As opioids are frequently inappropriate for behavioral studies, and female rodents experience pain more intensely than males do[6-10], it's important to know if the higher doses described by Matsumiya et al[11] are safe for prolonged use.

Finally, we would urge more work on acclimation periods and stress in laboratory rodents. The mice in our second experiment didn't TINT successfully at the rate that previous work has shown, for either solo or group housed animals[12], even with practice sessions prior to baseline assessment. It's possible that the mice hadn't yet fully acclimated to their new environment, even though we waited the standard 72 hours before baseline testing.

While we didn't find dramatic changes in sleep duration or patterns in our projects, this was a very small subset of mouse populations and potential disruptions. Disruptions that include handling the mice, such as injection, gavage, and blood collection, should be included in future work. It seems likely that those will be perceived as more stressful, and therefore have more impact on sleep. The effects on sleep of unpredictable high-stress experiences should also be explored, as predictability and control have tremendous influence over welfare[13-18]. It's possible that, even with aversive experiences like gavage or blood collection, predictability of the stressor could ameliorate any subsequent sleep disruption.

From an alternative perspective, animal welfare scientists have been addressing the replication crisis that biomedical sciences are experiencing; one possible cause of this is that we have restricted variability too much in our models[19]. Perhaps we can help address that lack of variability by artificially inducing mild changes in sleep patterning, or by adjusting laboratory light cycles and routines to mimic outdoor conditions. Perhaps giving mice the cues to adopt seasonal patterns of sleep and other behavior, as humans have the ability to do, would make transitioning drug trials from animals to humans more successful.

The work described in this thesis is only the tip of the iceberg of mouse sleep and welfare research, but it addressed some of the very basic questions, and suggests future directions to explore. We can hope that this research will provide the foundation for further work on animal welfare, as well as information for current researchers about the welfare of their animals.

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