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Effects of Sleep Fragmentation on the Immune System of Zebra Finches Using Cytokine Gene Expression

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EFFECTS OF SLEEP FRAGMENTATION ON THE IMMUNE SYSTEM OF ZEBRA FINCHES (*TAENIOPYGIA GUTTATA*) USING CYTOKINE GENE EXPRESSION

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EFFECTS OF SLEEP FRAGMENTATION ON THE IMMUNE SYSTEM OF ZEBRA FINCHES (*TAENIOPYGIA GUTTATA*) USING CYTOKINE GENE EXPRESSION

Date Recommended July 19, 2016

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7/28/16

Dean, Graduate Studies and Research Date

I dedicate this thesis to my family, my parents, Raymond and Cassandra Cooper, as well as my sister, Kayla Cooper who have always encouraged me to follow my passion. In addition, I dedicate this work to Dr. Jason Davis and Dr. Sara O'Brien from the Department of Biology at Radford University, as they sparked my interest in research and continue to be a vital resource in furthering my career as a biologist. Lastly, I would like to dedicate this work to my fellow graduate students and friends at WKU. I would like to thank them for the encouragement and input they have given me along this journey.

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Laken N Cooper August 2016 47 Pages

Directed by: Noah Ashley, Cheryl Davis, and Bruce A. Schulte

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Sleep loss is known to trigger an inflammatory response and increase serum corticosterone in both human and murine models. However, very little evidence is available on the potential effects of sleep loss in avian models. This study aims to construct a profile using cytokine gene expression data to determine how birds respond to sleep loss in a controlled environment. I investigated changes in pro-inflammatory (IL-1β and IL-6) and anti-inflammatory (IL-10) cytokine gene expression in the periphery (fat, liver, spleen, and heart) and brain (hypothalamus, hippocampus, and apical hyperpallium) in zebra finches exposed to a novel sleep fragmentation method. Serum corticosterone, body mass, and behavioral profiles also were assessed. Sleep was interrupted periodically for over 12 hours using a sleep fragmentation chamber, which was modified from those typically used in murine studies. This chamber contained a sweeping wire bar that moved the distance of the cage at 2-minute intervals. I predicted that sleep fragmented birds would exhibit elevated pro-inflammatory and reduced anti-inflammatory gene expression relative to those birds that were not sleep fragmented. In addition, I predicted a decrease in body mass and an increase in serum corticosterone levels

because of sleep fragmentation. Contrary to my predictions, sleep fragmentation resulted in lower levels of IL-1β in the apical hyperpallium, but lower levels of IL-10 in the hippocampus. No differences were detected in the adipose tissue of individuals exposed to sleep loss. Sleep fragmentation resulted in an increase in percent body mass lost. Serum corticosterone levels did not differ across groups. These data provide preliminary insight into the inflammatory response that is seen as a result of sleep loss in an avian model. Overall, it appears that as compared to some mammals such as murine rodents, birds are not as susceptible to sleep loss.

INTRODUCTION

Sleep is a natural physiological process utilized by many vertebrate organisms. Despite apparent variability within and between species in sleep duration, the fundamental need for sleep is a conserved phenomenon and consumes a large portion of an individual's life. Sleep can be generally defined as a reversible state of temporary stasis (Siegel 2008). Another accepted component to this definition is the concept of 'sleep rebound'. In other words, following periods of extended wakefulness, individuals will often attempt to recover sleep loss, by either sleeping for longer periods or increasing the frequency of naps throughout the day.

Despite several hypotheses as to why sleep is such an integral part of life, none have been well enough supported to constitute a theory of sleep. Sleep is considered to be an adaptive period used to conserve energy during times of inactivity (Lesku et al. 2012; Lesku & Rattenborg 2014; Siegel 2005). However, shutting down the body during these periods of recovery could potentially increase an individual's risk to predation. In response, there are a variety of adaptations employed by diverse taxa to mitigate this risk. For example, mallard ducks (*Anas platyrhynchos*) show a group effort in balancing the need to sleep with the risk of predation (Rattenborg et al. 1999). Mallards will exhibit different patterns of sleep based upon their position in a group. On the perimeter of a group, individuals participate in unihemispheric slow-wave sleep. Unihemispheric sleep is observationally defined as when an individual sleeps with one eye open, while the other is closed. During this time, electroencephalographic (EEG) patterns show

periods of both fast waves, typically associated with wakefulness and slow-wave patterns indicated of sleep (Bobbo et al. 2008). Mallards in the middle of the flock exhibit “normal” rapid-eye movement (REM) sleep, as indicated by the closure of both eyes (Rattenborg et al. 1999). Unihemispheric sleep is an adaptation unique to most birds and aquatic mammals (which require periodic bouts to the surface to breathe; Oleksenko et al. 1992).

Recently, it has been shown in mammals that sleep may be necessary to allow for recuperation and clearance of metabolic wastes from the brain through cleansing of the cerebral spinal fluid (Xie et al. 2013). Alterations in the amount of time spent sleeping can alter this cleansing period and result in an accumulation of waste products. This build-up of waste products could lead to well-known deleterious effects of sleep loss, such as impaired learning and memory in the short term and increased risk of disease and obesity in the long term (Graves et al. 2003; Markwald et al. 2013; Zielinski et al. 2013). Evidence suggests that sleep quality and duration aid in the maintenance of neurocognitive function and health, which ultimately contribute to the morbidity and mortality of the individual (Simpson & Dinges 2007).

An additional component to the proposed relevance of sleep is the role it occupies in memory consolidation (Brawn & Margoliash 2015; Pack & Abel 2003). The hippocampus plays a large role in the consolidation process by storing recently assimilated information until it can be integrated into long-term memory (Buzsáki 1989; McClelland et al. 1995; Squire & Alvarez 1995). This conversion process takes place during sleep, specifically during slow-wave sleep (Diekelmann

& Born 2010; O'Neill et al. 2010; Walker 2009). According to the synaptic homeostasis hypothesis, sleep is associated with the costs involved in synaptic firing and function. Synaptic firing is an energetically expensive process that cannot be maintained for extended amounts of time. Sleep allows for a decrease in this process providing the body time to recover (Tononi & Cirelli 2003).

A classical concept that may be useful in understanding sleep comes from the inclusion of the category of rapid-body movement (RBM). RBM is classified as unintentional movements, which appear to be highly conserved across all levels of life (Corner 1977). The period of RBM that predominates at the prenatal stage of development seems to develop into the more widely explored process of REM sleep that appears later in life.

It was previously thought that rapid-eye movement and slow-wave sleep (SWS) sleep were unique to birds and mammals (Jones et al. 2008; Lesku et al. 2011). A more recent study shows evidence of REM-like brain waves recorded in the Australian dragon (*Pogona vitticeps*), which now suggest a more distant ancestor shared the ability for REM sleep (Shein-Idelson et al. 2016). The amount of SWS has been thought to mirror sleep intensity and need. This is evident due to the homeostatic increase in SWS following periods of sleep loss.

In vertebrate organisms such as birds and mammals, sleep patterns are typically measured by EEG. However, in lower vertebrates and non-vertebrate species, sleep is primarily measured by the use of behavioral profiles that may be indicative of sleep-like patterns, which are commonly seen in higher vertebrates (Campbell & Tobler 1984). Model organisms in this category have included zebra

fish, round worms, and fruit flies. Briefly, these organisms all exhibit periods of decreased activity, classified by extended periods of motionless states, with an observed increase in arousal time following a given stimulus (Cirelli et al. 2005; Ho & Sehgal 2005; Raizen et al. 2008; Yokogawa et al. 2007; Zhdanova 2011).

Although there are similarities in the components of both avian and mammalian sleep, there are also differences that are unique to each class. In SWS, mammals show the presence of sleep spindles, which are periods of rapid electroencephalographic activity (Fogel & Smith 2011). These periods are absent in birds. In mammals, REM sleep can last up to several minutes, whereas in birds it rarely persists longer than a few seconds (Siegel 2011).

In birds, sleep plays an important role in imprinting (Jackson et al. 2008), song learning (Brawn et al. 2000), and auditory discrimination (Brown et al. 2012; Derégnaucourt et al. 2005; Gobes et al. 2010). Sleep loss can be attributed to migration, mating rituals, and habitat disturbances. Migratory species are often subjected to sleep loss yet do not appear to experience the same effects of sleep-deprived mammals (Rattenborg et al. 2004). For example, the Swainson's thrush (*Catharus ustulatus*), a long distance migratory songbird, has been shown to alter daytime behavior in response to significant loss of sleep at night, which leads to several daytime naps (Fuchs et al. 2006). Although the process of migration has been extensively studied, the physiological and immunological effects of absent or disrupted sleep in birds are largely unexplored.

Within sleep-deprived conditions, there is an observed upregulation in the immune response that is not associated with a pathogenic challenge (Brager et al.

2013). Consequently, an inflammatory response is initiated for an unknown reason. Activation of the immune system in response to pathogens and infection is an energetically expensive process which has been shown to incur many negative fitness-related costs, such as nestling growth (Nilsson et al. 2003; Soler et al. 2003; Tschirren & Richner 2006), parental care (Råberg et al. 2000), breeding success (Bonneaud et al. 2003; Ilmonen et al. 2000; Marzal et al. 2007), and survival (Eraud et al. 2009; Hanssen et al. 2004). Under normal conditions, the immune system changes throughout the day along with the sleep-wake cycle. Disruption of the normal sleep-wake cycle results in altered immune function. Immune cells in the blood exhibit maximum levels in the early evening and reach minimum circulatory levels in the morning. Correspondingly, levels of cytokines, the chemical messengers of the immune system that attract and direct other immune components, follow this cycle and are at maximum levels at night (Simpson & Dinges 2007). In a normal immune response, an acute inflammatory response induces the activation of an immune cascade in response to perceived damage or infection to generate an antigen-specific response.

The acute phase response (APR) is a highly conserved component of the innate immune system and is activated during the early stages of infection in vertebrates. The APR response can be dangerous for the host if not appropriately down regulated (Sorci & Faivre 2009). Under normal conditions the response typically subsides within 24-48 hours (Baumann & Gauldie 1994). Detection of pathogen or danger associated molecular patterns (PAMPs or DAMPs) occurs by means of binding of ligands to the leucine rich repeat domains of toll-like receptor

proteins (TLRs). These can be expressed on macrophages or other immune cells. Lipopolysaccharide (LPS) has been extensively used to stimulate an immune response due to its ability to trigger the APR through an interaction with TLR-4 (Kadowaki et al. 2001; Medzhitov et al. 1997). The acute phase response is typically activated by pro-inflammatory cytokines. These include but are not limited to, interleukin-1 (IL-1), interleukin-6 (IL-6) and tumor necrosis factor alpha (TNFα), which regulate the recruitment of immune cells, and trigger inflammatory responses (Ashley & Wingfield 2007). In addition, the acute phase response involves the secretion of acute-phase proteins (APP) from the liver. APPs are a class of proteins whose plasma concentrations increase or decrease in response to inflammation. These acute phase proteins function in a variety of defense-related activities such as limiting the dispersal of infectious agents, repair of tissue damage, killing of microbes, and restoration of the healthy state (Bayne & Gerwick 2001). Because many pro-inflammatory cytokines are present following sleep alterations, it is generally accepted that insufficient sleep results in an acute inflammatory response. However, the mechanism underlying the induction of an inflammatory response has yet to be elucidated. In either condition, the increase in pro-inflammatory cytokines (ex: IL-1β, IL-6, TNF-α) leads to a persisting, non-specific inflammatory response. If the inflammatory condition persists, then tissue damage will eventually result from the cytotoxic products of the inflammatory state (Murphy 2012).

Not only does insufficient sleep lead to an increase in the number of immune cells and an upregulation in cytokine gene expression, but it also stimulates

changes in other hormone levels. For instance, insufficient sleep alters satiety hormone levels, affecting food intake (Leproult & Cauter 2010). Interestingly, ghrelin (a hunger signaling hormone) increases in rats, stimulating food intake, yet increased ghrelin in sleep deprived chicken reduces food intake (Kaiya et al. 2013).

Sleep loss is known to be a stressful event and therefore can initiate the hypothalamus-pituitary-adrenal (HPA) axis. Upon activation, corticotropin-releasing hormone (CRH) is released from the hypothalamus, which stimulates the production of adrenocorticotropic hormone (ACTH) from the anterior pituitary gland. This endocrine cascade leads to secretion of cortisol in humans and corticosterone in birds from the adrenal cortices. This process can be halted by a series of negative feedback loops that regulate the anterior pituitary and hypothalamus. Corticosterone (CORT) is the major glucocorticoid in birds and is used as an indicator of stress levels in birds (Holmes & Phillips 1976). CORT is associated with behavioral and physiological changes in energy demands (Harvey et al. 1984) and is usually released rapidly into the bloodstream from adrenal tissues in response to a variety of perturbations (Wingfield et al. 1983; Wingfield et al. 1994).

In order to investigate if birds incur any costs associated with sleep loss, a novel sleep fragmentation chamber was constructed to simulate sleep loss by essentially waking individuals throughout the night. This technique minimizes human interference, which could lead to compounding variables to account for during analysis. The specific aim of this research was to determine how the immune system was affected by experimental sleep fragmentation in birds.

After 12 hours of subjecting birds to fragmented sleep, I hypothesized that there would be a significant shift towards the pro-inflammatory cytokine profile compared with non-sleep-fragmented controls. Because of this, I further hypothesized that the relative expression of IL-1β and IL-6, the pro-inflammatory cytokines of interest, would increase in all tissues (liver, fat, spleen, and brain) following sleep fragmentation. The relative expression of IL-10, an anti-inflammatory cytokine, was expected to decrease in all tissues after sleep loss. Additionally, because sleep loss is a stressful condition, I hypothesized that there would be an increase in serum corticosterone levels following sleep fragmentation relative to non-sleep fragmented (control) birds.

MATERIALS AND METHODS

Animals

Adult zebra finches (n = 40) were housed in a colony room (12 h of light (L):12 h of dark (D), lights on at 0700; 22.0 °C +/- 1°C) at the Western Kentucky University vivarium. After a two-week acclimation period, birds were color-banded for identification purposes. Birds remained in a communal aviary before starting the study and were provided with food (bird seed and protein crumble in 1:1 ratio) and water *ad libitum*, as well as supplemented with millet sprays and cuttlebone. This study was conducted under the approval of the Institutional Animal Care and Use Committee (Animal Welfare Assurance # A3558-01) at Western Kentucky

University, and procedures followed the National Institutes of Health's "Guide for the Use and Care of Laboratory Animals" and international ethical standards.

Sleep Fragmentation Cage

Individuals were placed into a testing cage (34 cm x 40 cm x 45 cm) that was modified to disrupt sleep for a 12 h period. The sleep fragmentation cage contained a single stationary perch and a 16-gauge wire placed approximately 1.3 cm above the stationary perch. The stationary perch is adjacent to food and water dishes, allowing birds to have access to food and water *ad libitum.* The wire swept horizontally across the distance of the cage at 2 min intervals, and it was attached to an automated sleep deprivation base (Lafayette Instruments). In addition, the cage was placed in a tub of water (3.8 cm deep) which prevented birds from resting on the bottom. Birds had to hop briefly over the wire or they would be pushed down into the water at the bottom of the cage and awaken. The control group was exposed to the same cage, but modified so that the wire remained stationary. Control and experimental groups were acclimated to the cage 24 h prior to testing.

Sleep Fragmentation

Birds were exposed to sleep fragmentation during the dark phase (7 pm to 7 am) when lights were off and birds were typically resting/sleeping, and during the light phase (7 am to 7 pm) when lights were on. The latter treatment was used to control for potential activity level differences between light and dark periods (see Results). Four different treatment groups were used; two control groups (L-Con: light control

and D-Con: dark control; the wire never moves) and two experimental (L-Move: light with moving wire and D-Move: dark with moving wire). The same experimental cage was used for all trials.

Tissue Collection

Immediately following the end of the 12 h period, birds were taken from cages and blood was collected in capillary tubes from the brachial vein and then placed on ice. This sample was obtained < 3 min of initial handling. Blood was centrifuged for 30 min at 3000 x g, and plasma was removed and stored at -80°C for later analysis. Birds were deeply anesthetized with isoflurane vapors and killed by rapid decapitation. Liver, fat, spleen, heart, and brain tissues were collected and placed into tubes containing RNAlater (Thermo Fisher Scientific, AM7020). Liver, fat, spleen, and heart tissue was stored at -80°C whereas the brain was furthered processed. The hypothalamus, hippocampus, and apical hyperpallium were dissected from each brain and stored in RNAlater at -80°C. Body mass was also recorded before and after experimentation to the nearest 0.01g using a digital scale.

RNA Extraction

Total RNA was extracted from liver, fat, spleen, and brain samples using an RNeasy Mini kit (Qiagen, #74106) according to the manufacturer's instructions. Total RNA was extracted from the heart using an RNeasy Fibrous Tissue Mini kit

(Qiagen, #74704). The concentration of total RNA was quantified with a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific).

Reverse Transcription

A Reverse Transcriptase Kit (Thermo Fisher Scientific, #4368814) was used to synthesize cDNA from total RNA. The total RNA concentration of the tissue was diluted to the following concentrations: hypothalamus: 1.8ng/µL; hippocampus: 3.2ng/µL; apical hyperpallium: 12.7ng/µL; liver: 4.7ng/µL; spleen: 19.9 ng/µL; fat: 3.1ng/µL and heart: 3.6ng/µL. The reaction was carried out according to the manufacturer's instructions. The amplification conditions for the thermocycler were 25°C for 10 min, 37°C for 120 min and 85°C for 5 min.

Real-time PCR

Amplification was performed on an ABI 7300 Real-Time PCR system using Taqman Universal PCR Master Mix (Applied Biosystems, #4369016). Amplification conditions involved universal two-step real-time PCR cycling: 50°C for 2 min, 95°C for 10 min followed by 40 cycles of 95°C for 15 s, and 60°C for 1 min. Primer/probes were custom designed through Thermo Fisher Scientific for PPIA, IL-1 β, IL-6, and IL-10, with assay identification numbers of AIRSBD, AIVI5WP, AT97QH, and AIS09J9, respectively. Cytokine probes (IL-1β, IL-6, and IL-10) were labeled with the fluorescent reporter dye FAM at the 5' end and PPIA was labeled with the fluorescent reporter dye VIC. The relative expression of IL1β, IL6, and IL10 was quantified by comparison to a standard curve generated using

serial dilutions of cDNA (1: 1; 1: 10; 1: 100; 1: 1,000; 1: 10,000) and normalization to the endogenous control (peptidyl proline isomerase A: PPIA) levels.

ELISA

Serum corticosterone concentration was determined using an ELISA kit (#ADI-900-097, Enzo Life Sciences) with 96% recovery for corticosterone (0.3 ng/mL sensitivity). The reagents and standards were prepared according to the manufacturer's instructions. Cross reactivities for the kit were < 28.6% deoxycorticosterone, < 1% aldosterone, and < 2% progesterone. Serum samples were diluted 1:40. The reaction was carried out in duplicate according to the kit instructions and the average absorbance of the plate was determined using a plate reader and subtracting the absorbance at 450 nm from the absorbance at 570 nm per the manufacturer's instructions (BioTek Synergy H1 Hybrid Reader). Corticosterone concentrations were extrapolated from a standard curve using a four-parameter logistic curve fit and multiplied by the dilution factor (1:40).

Behavior

Each 12-hour experiment was recorded using an infrared video camera (Bell and Howell, DNV16HDZ). For each hour interval, a random 5-minute period was selected for observation. During these 5-minute periods, behaviors were documented every 15 secsonds. This produced 20 observations per session, for a total of 240 observations per individual. I operationally defined four behavioral states for use in this experiment: alert, moving, resting and feeding/drinking.

Table 1. Definitions of behavioral states observed and recorded.

Alert:	Frequent head movements and an absence of locomotor activity.
Moving:	Locomotor activity.
Resting:	Absence of head/wing movement locomotor activity.
Feeding/drinking:	Visits to either food or water dishes.

Statistical Analyses

All data are expressed as mean ± standard error (SE). Two-way ANOVAs were used to detect differences among groups, with main effects of light/dark and sleep-fragmentation/no sleep-fragmentation. Two-way ANOVAs were used to compare the mean relative cytokine expression between the control and experimental trials for adipose tissue, heart, brain and spleen. If significant differences were found, then a Fisher's PLSD post-hoc test was used to determine which groups were significantly different from one another. A repeated measures ANOVA was used to compare differences in activity levels between treatment groups. Time was the repeated measure. If significant, then Fisher's PLSD post-hoc tests were used to determine which groups were significantly different from each other.

RESULTS

Sleep fragmentation increases percent body mass loss.

Body mass varied significantly between light versus dark-exposed birds (two-way ANOVA, light/dark, $F_{3, 36} = 55.057$, $p < 0.0001$; Figure 1). Post-hoc tests revealed

that birds lost body mass when exposed to 12 h of darkness compared with birds subjected to 12 h of light, which gained body mass ($p < 0.05$, Figure 1). Furthermore, among birds exposed to dark, sleep fragmentation (D-Move) induced a greater loss in body mass compared with controls (D-Con). Among birds studied during the light period, body mass change was not affected by movement of the bar ($p = 0.3451$).

Sleep fragmentation induces no change in gene expression in adipose tissue.

Sleep fragmentation resulted in no significant change in IL-1β expression in adipose tissue in fat (two-way ANOVA, log-transformed, $F_{7, 32} = 1.303$, $p = 0.2622$; Figure 2A) or liver (two-way ANOVA, log-transformed, $F_{7, 32} = 0.0001$, $p = 0.9786$; Figure 2B) among groups. A significant interaction was detected in the spleen (two-way ANOVA, log-transformed, light/dark * SF/no SF, $F_{7, 21} = 6.077$, $p = 0.0224$; Figure 2C). Post-hoc tests revealed a difference between the control (L-Con) and experimental (L-Move) groups during the light trials (Fisher's PLSD; $p = 0.0133$, Figure 2C). Similarly, no difference was detected in IL-6 expression in adipose tissue (two-way ANOVA, log-transformed, $F_{7, 31} = 0.021$, $p = 0.8847$; Figure 2D). Although there appears to be an increase in the D-Move group, no significant findings were present and the graphical representation is skewed because of the large degree in variation between males and females, which was also not significant. A significant interaction was detected in the liver (two-way ANOVA, log-transformed, light/dark, $F_{7, 24} = 4.562$, $p = 0.0431$; Figure 2E). Post-hoc tests

revealed a difference between D-Con and L-Con groups (Fisher's PLSD; p = 0.0455) and between D-Con and L-Move groups (Fisher's PLSD; p = 0.0195). A significant interaction was also detected in IL-6 expression in the spleen (two-way ANOVA, log-transformed, light/dark * SF/No SF, $F_{7, 18} = 4.679$, p = 0.0442; Figure 2F). Post-hoc tests revealed differences between the following groups; D-Con and L-Move (Fisher's PLSD; p = 0.0275), D-Move and L-Move (Fisher's PLSD; p = 0.0273), and L-Con and L-Move (Fisher's PLSD; p = 0.0211). No significant differences were detected in IL-10 expression in adipose tissue (two-way ANOVA, log-transformed, $F_{7, 26}= 0.074$, p = 0.7875; Figure 2G) or liver (two-way ANOVA, log-transformed, $F_{7, 32}= 3.616$, p = 0.0663; Figure 2H). A three-way interaction effect was detected in the spleen (three-way ANOVA; log-transformed; $F_{7, 22}= 5.668$; p = 0.0.0264, Figure 2I). Post-hoc tests revealed that this difference was attributed to a sex difference in the L-Move group. Males in this group showed higher IL-10 expression compared to females (Fisher's PLSD; p = 0.0313).

Sleep fragmentation results in tissue specific changes in the brain.

Sleep fragmentation resulted in decreased expression of IL-1β in the apical hyperpallium (two-way ANOVA, log-transformed, light/dark * SF/No SF, $F_{7, 32}= 7.825$, p = 0.0087; Figure 3C). Post-hoc tests revealed that this difference was between D-Move and D-Con groups (Fisher's PLSD; p = 0.0147) and also between D-Con and L-Con groups (Fisher's PLSD; p = 0.0395). There was no significant difference in the hippocampus (two-way ANOVA, log-transformed, $F_{7, 32}= 0.069$, p = 0.7939; Figure 3A) or the hypothalamus (two-way ANOVA, log-transformed, $F_{7,}$

$_{26}$= 0.074, p = 0.7875; Figure 3B) in IL-1β expression. No changes were detected in IL-6 expression in any of the areas of the brain measured: hippocampus (two-way ANOVA, log-transformed, $F_{7, 28}$= 1.677, p = 0.2059; Figure 3D), hypothalamus (two-way ANOVA, log-transformed, $F_{7, 25}$= 1.192, p = 0.2854; Figure 3E), and apical hyperpallium (two-way ANOVA, log-transformed, $F_{7, 32}$= 0.955, p = 0.3358; Figure 3F). A three-way interaction was detected in IL-10 expression in the apical hyperpallium (three-way ANOVA, log-transformed, light/dark x SF/No SF x sex, $F_{7, 24}$= 6.058; p = 0.0214, Figure 3I). However, post-hoc tests revealed no significant differences between any two groups. In the hippocampus, several significant differences were detected between multiple groups (two-way ANOVA, log-transformed, light/dark x sex, $F_{7, 25}$= 5.892, p = 0.0227; Figure 3G). Post-hoc tests revealed that the differences were between D-Move and D-Con (Fisher's PLSD; p = 0.0346), D-Move and L-Con (Fisher's PLSD; p = 0.0005), and L-Con and L-Move groups (Fisher's PLSD; p = 0.0014). Similarly, an overall ANOVA revealed a significant interaction within the hypothalamus (three-way ANOVA, log-transformed, light/dark x SF/No SF x sex, $F_{7, 30}$= 6.882, p = 0.0136; Figure 3H). Post-hoc tests determined that the differences were between D-Move and L-Con (Fisher's PLSD; p = 0.0318) and L-Con and L-Move groups (Fisher's PLSD; p = 0.0037).

Sleep fragmentation results in no significant change in expression in cardiac tissue.

Following sleep fragmentation, no significant differences were found in the expression of IL-1β (two-way ANOVA, log-transformed, $F_{7, 30}$= 0.998, p = 0.3256; Figure 4A), IL-6 (two-way ANOVA, log-transformed, $F_{7, 31}$= 0.999, p = 0.3256; Figure 4B) or IL-10 (two-way ANOVA, log-transformed, $F_{7, 31}$= 1.736, p = 0.1973; Figure 4C) in heart tissue. A summary of findings of cytokine gene expression in various tissues are shown in Table 2.

Sleep fragmentation results in no difference in serum corticosterone levels.

Sleep fragmentation did not alter plasma corticosterone concentration (two-way ANOVA, log-transformed, $F_{7, 31}$= 1.850, p = 0.1836; Figure 5).

Sleep fragmentation and behavior

Behavioral profiles were constructed for each treatment group using video observations (Figure 6.) Each graph (A-D) represents the average percentage that each behavior was performed for each hour interval (Figure 7). Total percent activity was then calculated for each group. To calculate this variable, the behaviors assigned as active (moving and feeding/drinking) were added together to create a total percent activity. This new variable was used to compare activity levels for each treatment for each hour interval. A repeated measures ANOVA was used to determine whether differences were present across the 12 h period. The repeated measures ANOVA revealed no interaction across treatment and time

(repeated measures ANOVA, category for activity * category, $F_{3, 33}$= 0.515, p = 0.9890; Figure 7), but did show a significant difference between each treatment category (repeated measures ANOVA, $F_{3, 36}$= 63.407, p < 0.0001; Figure 7). Given that no difference was found across time, each hour value was combined into an average percent for each individual, creating a value for average total activity for the 12 h period (Figure 8). A one-way ANOVA was used to test for differences among each treatment group (one-way ANOVA, $F_{3, 36}$= 63.621, p < 0.0001). Post-hoc tests revealed that differences were present between D-Move and D-Con (Fisher's PLSD; p = 0.0471), D-Move and L-Con (Fisher's PLSD; p < 0.0001), D-Move and L-Move (Fisher's PLSD; p < 0.0001), and D-Con and L-Con (Fisher's PLSD; p < 0.0001), D-Move and L-Move (Fisher's PLSD; p < 0.0001) groups. Average total activity did not differ between L-Move and L-Con groups (Fisher's PLSD; p = 0.0590).

Table 2. The effects of 12-hour sleep fragmentation on cytokine gene expression.

Tissue	**Effect**
Fat	-
Liver	-
Spleen	-
Heart	Trend: Decrease in IL-1β
Hypothalamus	-
Hippocampus	Decrease in IL-10; Trend: Increase in IL-6
Apical Hyperpallium	Decrease in IL-1β; Trend: Increase in IL-10

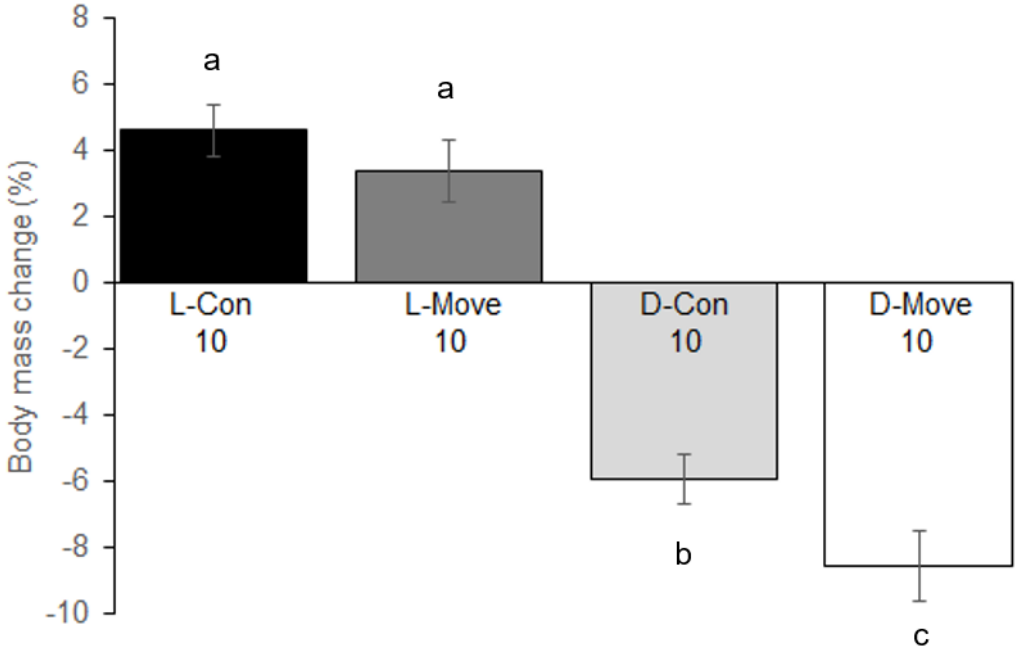

Figure 1. Sleep fragmentation induces change in body mass. Values are shown as mean body mass change (%) ± SE for each group, $p < 0.05$. Shared symbols indicate no significant difference between groups. The numbers included on the bar of each column indicate the sample size of each group.

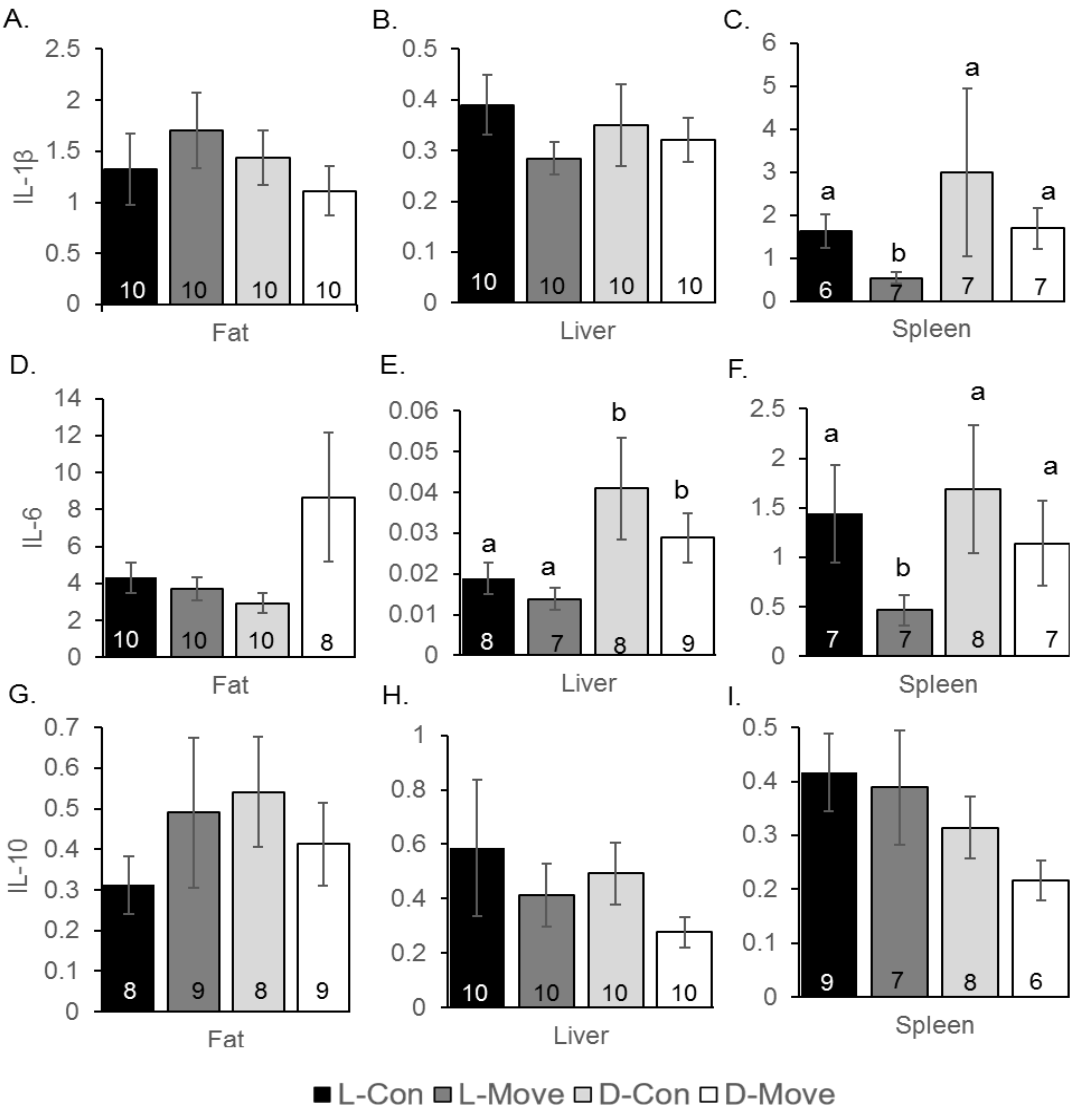

Figure 2 (A-I). Cytokine gene expression data in adipose, liver, and spleen tissue. Data are shown as mean ± SE for each group. Gene expression was undetectable by RT-PCR in some tissue samples, resulting in a decreased sample size in these groups. Shared or no symbols indicate no significant difference between groups. The numbers at the base of the column indicate the sample size of the group.

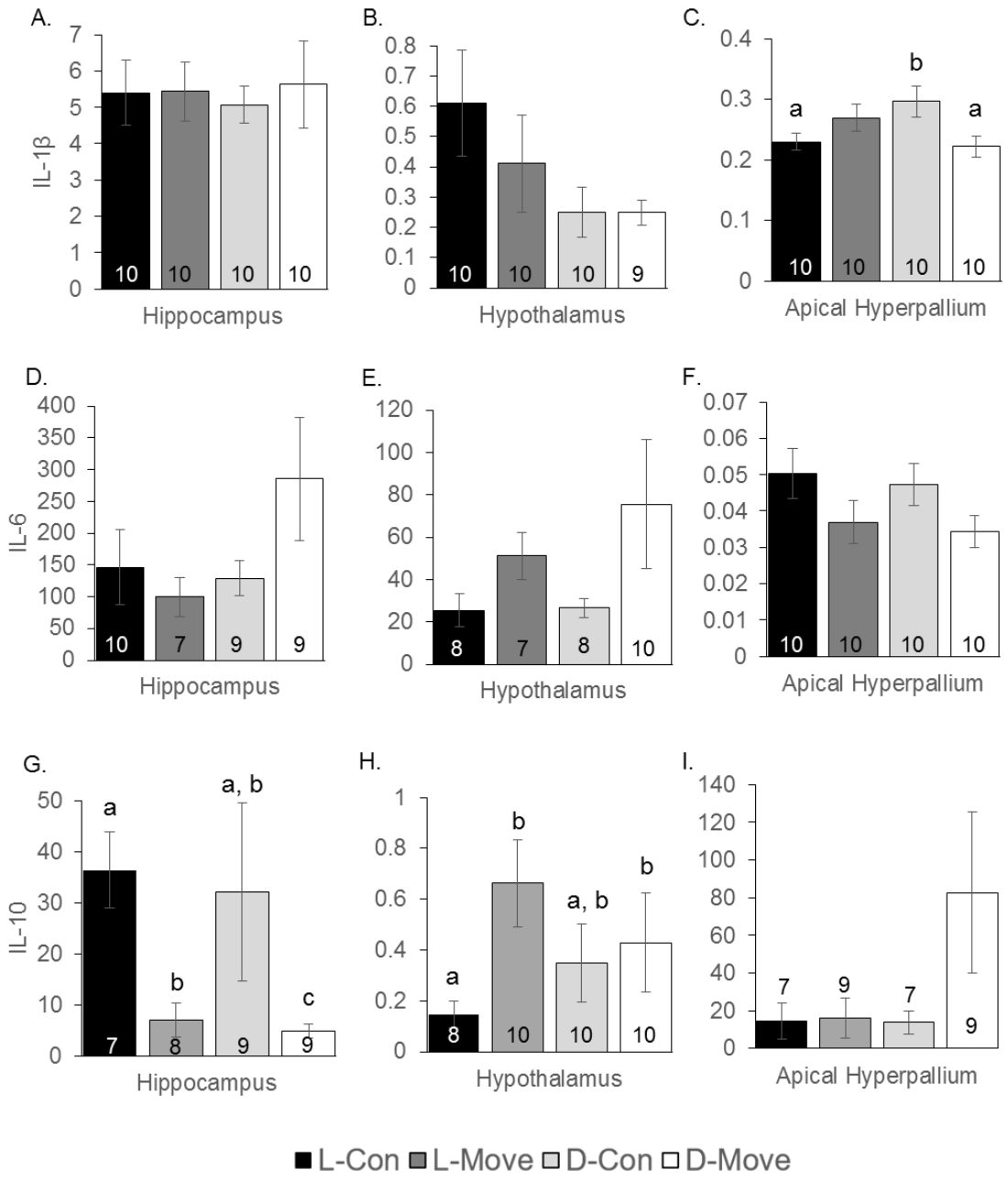

Figure 3 (A-I). Cytokine gene expression data in the brain. Data are shown as mean ± SE for each group. Gene expression was undetectable by RT-PCR in some tissue samples, resulting in a decreased sample size in these groups. Shared or no symbols indicate no significant difference between groups. Sample size of the group at the base of the column.

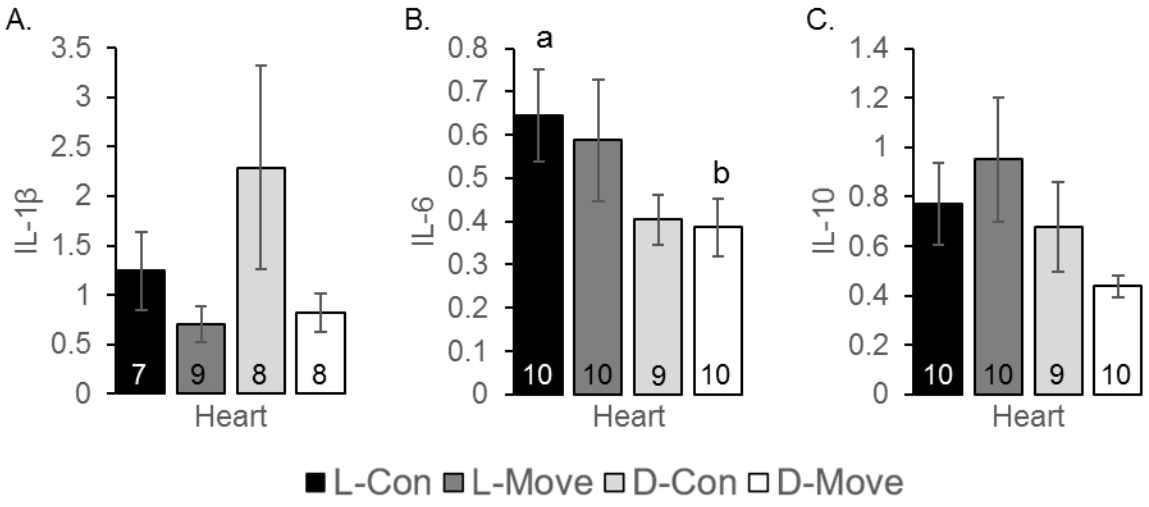

Figure 4 (A-C). Cytokine gene expression data in cardiac tissue. Data shown as mean ± SE for each group. Gene expression was undetectable by RT-PCR in some tissue samples, resulting in a decreased sample size among these groups. Shared or no symbols indicate no significant difference between groups. The numbers at the base of the column indicate the sample size of the group.

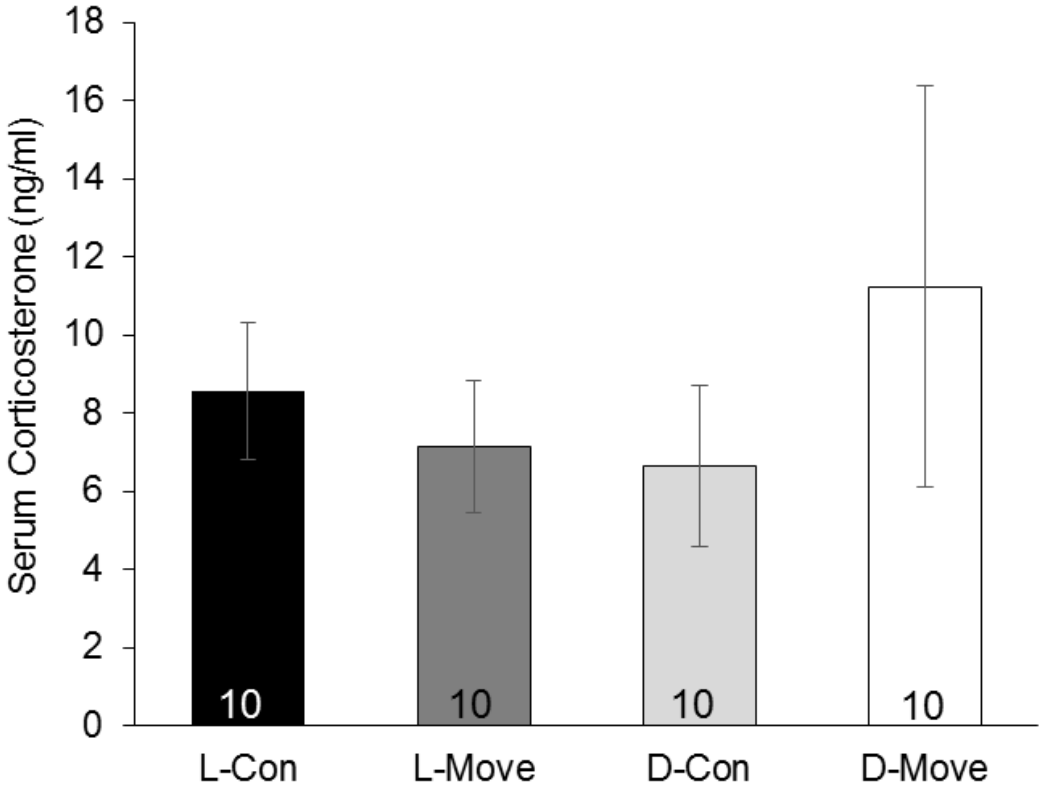

Figure 5. Serum corticosterone concentrations show no significant alterations following sleep fragmentation. Data are shown as mean (ng/ mL) ± SE for each group. Numbers at the base of the column indicate sample size.

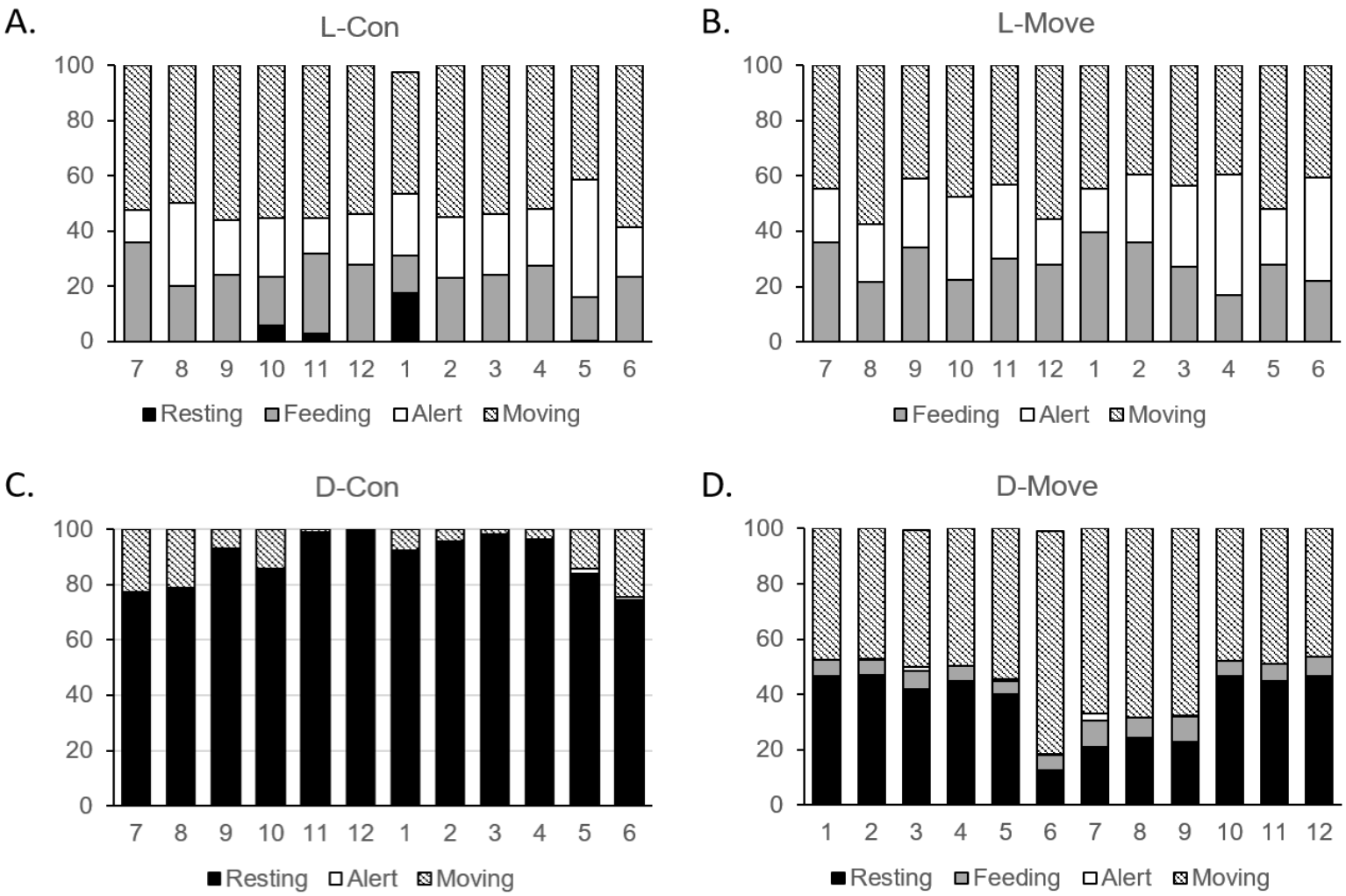

Figure 6. Behavioral profiles for each treatment group across 12 h period. Data are shown as mean percentages. Behaviors are indicated in figure legends.

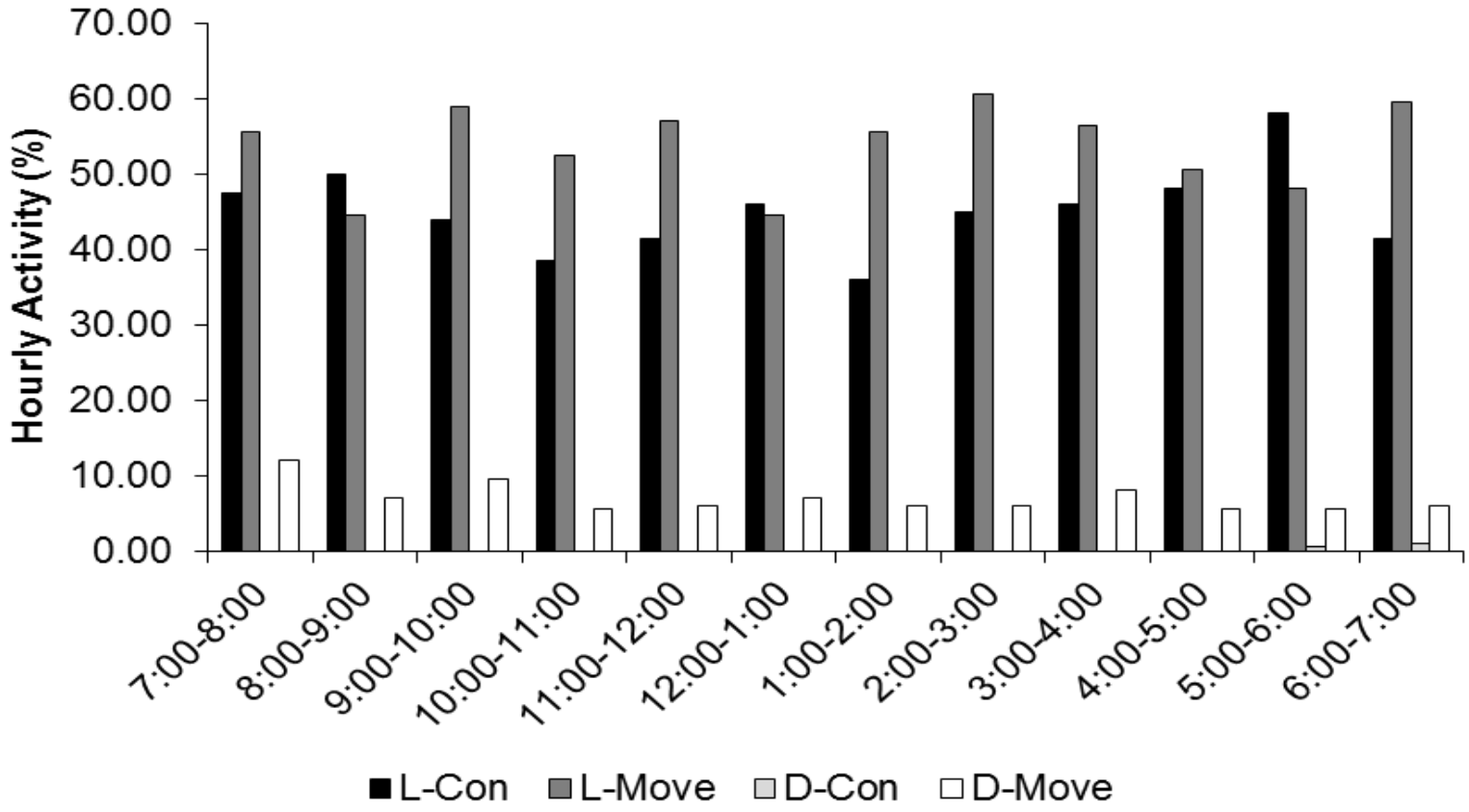

Figure 7. Total percent activity for each hour of behavioral observations. Data are shown as mean (%) for each group. Activity is defined as the total proportion of active behaviors (moving, feeding/drinking) for each hour. This represents an average of behaviors for the individuals in each group.

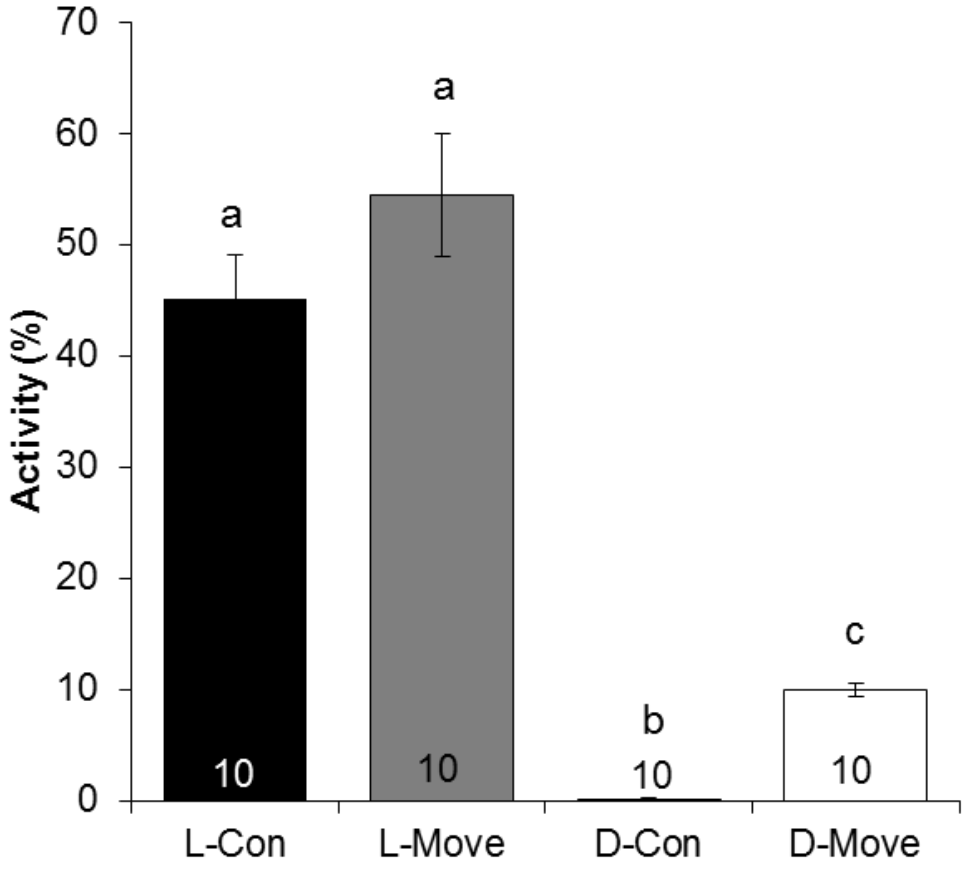

Figure 8. Percent activity by group. Shared symbols indicate no significant difference between groups. The numbers at the base of the column indicate the sample size of the group. Activity is defined as the total proportion of active behaviors (moving, feeding/drinking). This represents the total average of behaviors for each treatment group.

DISCUSSION

This study provides evidence for both a decrease in pro-inflammatory cytokines and an increase/decrease in anti-inflammatory cytokines from experimental sleep fragmentation in brain, but not peripheral tissue. Differences between brain and peripheral cytokine activation following sleep loss have also been reported in studies using a murine model (e.g., Dumaine & Ashley 2015). Results from the present study show that IL-1β gene expression was downregulated in the apical hyperpallium in individuals that were exposed to a 12-hour period of sleep fragmentation compared to other groups. There was a decrease in the anti-inflammatory cytokine (IL-10), in the hippocampus and a trend for an increase in the anti-inflammatory cytokine in the apical hyperpallium.

The hippocampus is involved with memory (Turner 1969) and spatial recognition (Squire & Zola-Morgan 1991) whereas the apical hyperpallium is typically associated with intelligence (Striedter 2006) and song regulation (Lovell & Mello 2011). Although not confirmed, it appears that sleep loss may affect immune activation in these areas of the brain differently and that potentially these areas of the brain are more sensitive to sleep loss than in the periphery. It would be interesting to examine whether the behavioral output regulated by these brain regions, such as memory and song regulation, were differentially affected by sleep loss.

My results regarding cytokine gene expression were only partially aligned with the proposed predictions. However, other studies have shown that sleep deprivation indeed does not lead to an increase in cytokine gene expression in the

Siberian hamster (*Phodopus sungorus*) (Ashley et al. 2013). In experimental trials regarding the skin disease psoriasis, it was found that mice (Balb/C strain) exposed to sleep deprivation showed an increase in pro-inflammatory cytokines and a decrease in the anti-inflammatory cytokine, IL-10 (Hirotsu et al. 2012). These conflicting results may be attributed to the methods employed, but also could be specific responses that are unique to each species.

Time spent sleeping has been shown to be negatively associated with body mass based off a comparative study of vertebrate representatives from various classes (Savage & West 2006). An additional comparative study that represents a wider range of sampling from the animal kingdom reported a positive association with body mass (Capellini et al. 2008). Chronic sleep fragmentation has been shown to increase orexigenic behavior, ultimately resulting in weight gain in mice (Carreras et al. 2014; Wang et al. 2014). There is also evidence that the response to sleep loss and the amount of sleep gained is a sex-dependent variable that can vary based on time of year and resource availability (Beck et al. 2003; Campero et al. 2008; Harmon et al. 2011).

However, in the zebra finch it was found that those individuals subjected to a 12-hour period of sleep fragmentation lost a significantly higher percentage of body mass compared to those kept in the dark without interrupted sleep. There are certainly examples that show birds lose body mass in response to stressful situations (Dickens et al. 2009). As to weight loss in birds at night that were not experiencing sleep fragmentation, it has previously been proposed that sleeping birds need to maintain energetic demands and to meet this demand they must

utilize fat storages (Peters 1983). Due to the lack of differences in weight change during the light groups, it would be a reasonable assumption that weight loss in the present study can be attributed to sleep fragmentation, and less likely that it is due to the stress of human interaction. The total activity levels of each group provide good evidence that the novel sleep fragmentation method developed for this experiment was successful in disrupting sleep in an avian model.

The current study revealed no difference in corticosterone profiles because of sleep fragmentation. Not all studies which have examined the effects of sleep loss have shown an elevation in levels of corticosterone. For example, in Siberian hamsters (*Phodopus sungorus*), Ashley et al. (2013) reported that 24 hours of sleep deprivation did not affect serum corticosterone levels compared with non-sleep-deprived controls. In contrast, Dumaine and Ashley (2015) reported increased corticosterone levels in C57/BL6j mice exposed to acute sleep fragmentation. The present study only evaluated effects from acute sleep fragmentation (12 hours). Thus, a longer bout of sleep loss could potentially activate the HPA axis, but this requires further exploration.

Although the body of evidence supporting the importance of sleep continues to develop, the scientific community is still unable to provide concise explanations regarding the specific functions of sleep. Previous studies have indicated the importance of sleep as a process that can clear buildup of waste products in the brain through the glymphatic system (Xie et al. 2013). Sleep is proposed to be an adaptive period of inactivity, which is unique in its ability to reduce activity and body/brain metabolism, while still permitting a high level of responsiveness relative

to other dormancy states, such as hibernation and torpor (Siegel 2009). Sleep loss induces a wide array of both physiological and behavioral responses that are often disadvantageous for the overall health of the individual (Nair et al. 2011; Simpson & Dinges 2007; Zielinski et al. 2013).

In mammals, it is well-known that sleep deprivation results in an acute inflammatory response, characterized by an increase in pro-inflammatory cytokines, such as IL-1β, TNF-α, IL-6, and an increase in serum glucocorticoids (Brager et al. 2013; Carreras et al. 2014; Hirotsu et al. 2012; Mullington et al. 2010; Simpson & Dinges 2007).

Overall, it appears that birds may have an internal mechanism that allows them to cope with short-term fluctuations in acute sleep loss. Such a mechanism seems necessary for birds to temporarily forego sleep during migration (Lyamin et al. 2005). This raises the question as to whether non-migratory birds have similar adaptations to cope with sleep loss. In a study using a disk-over-water (DOW) method for sleep deprivation, Newman et al. (2009) showed that pigeons appear to be more resistant to sleep deprivation compared to rats. Pigeons lacked physical signs that are generally associated with sleep deprivation, such as metabolic and thermoregulatory changes. The present study only subjected individuals to a temporary period of sleep fragmentation, which other than changes in body mass, may have not been long enough to elicit a stronger response. In conclusion, the present study provides preliminary insight into the avian response to short-term sleep fragmentation.

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APPENDIX

Appendix 1. Two-way ANOVA results table representing the effect of sleep fragmentation on body mass change.

	DF	Sum of Squares	Mean Square	F-Value	P-Value	Lambda	Power
Light/Dark	1	1263.926	1263.926	158.961	<0.0001	158.961	1.000
SF/No SF	1	37.043	37.043	4.659	0.0385	4.659	0.544
Sex	1	6.794	6.794	0.855	0.3622	0.855	0.140
Light/Dark * SF/No SF	1	5.206	5.206	0.655	0.4244	0.655	0.119
Light/Dark * Sex	1	6.214	6.214	0.782	0.3833	0.782	0.132
SF/No SF * Sex	1	10.703	10.703	1.346	0.2545	1.346	0.192
Light/Dark * SF/No SF * Sex	1	6.539	6.539	0.822	0.3713	0.822	0.136
Residual	32	254.438	7.951				

Interleukin-1

Appendix 2. Two-way ANOVA results table representing the effect of sleep fragmentation on IL-1β expression in the fat.

	DF	Sum of Squares	Mean Square	F-Value	P-Value	Lambda	Power
Light/Dark	1	0.005	0.005	0.066	0.7988	0.066	0.057
SF/No SF	1	0.004	0.004	0.047	0.8299	0.047	0.055
Sex	1	0.281	0.281	3.757	0.0615	3.757	0.455
Light/Dark * SF/No SF	1	0.185	0.185	2.465	0.1262	2.465	0.316
Light/Dark * Sex	1	0.030	0.030	0.396	0.5336	0.396	0.092
SF/No SF * Sex	1	0.007	0.007	0.087	0.7694	0.087	0.059
Light/Dark * SF/No SF * Sex	1	0.097	0.097	1.303	0.2622	1.303	0.188
Residual	32	2.395	0.075				

Appendix 3. Two-way ANOVA results table representing the effect of sleep fragmentation on IL-1β expression in the liver.

	DF	Sum of Squares	Mean Square	F-Value	P-Value	Lambda	Power
Light/Dark	1	0.003	0.003	0.076	0.7842	0.076	0.058
SF/No SF	1	0.034	0.034	0.833	0.3683	0.833	0.137
Sex	1	0.013	0.013	0.309	0.5823	0.309	0.082
Light/Dark * SF/No SF	1	0.038	0.038	0.921	0.3443	0.921	0.147
Light/Dark * Sex	1	1.514E-4	1.514E-4	0.004	0.9521	0.004	0.050
SF/No SF * Sex	1	0.009	0.009	0.214	0.6468	0.214	0.073
Light/Dark * SF/No SF * Sex	1	3.014E-5	3.014E-5	0.001	0.9786	0.001	0.050
Residual	32	1.319	0.041				

Appendix 4. Two-way ANOVA results table representing the effect of sleep fragmentation on IL-1β expression in the spleen.

	DF	Sum of Squares	Mean Square	F-Value	P-Value	Lambda	Power
Light/Dark	1	3.040	3.040	3.023	0.0968	3.023	0.367
SF/No SF	1	6.842	6.842	6.803	0.0164	6.803	0.705
Sex	1	3.157	3.157	3.139	0.0910	3.139	0.379
Light/Dark * SF/No SF	1	6.112	6.112	6.077	0.0224	6.077	0.652
Light/Dark * Sex	1	4.126	4.126	4.102	0.0557	4.102	0.478
SF/No SF * Sex	1	1.987	1.987	1.976	0.1745	1.976	0.255
Light/Dark * SF/No SF * Sex	1	1.431	1.431	1.423	0.2462	1.423	0.196
Residual	21	21.122	1.006				

Appendix 5. Two-way ANOVA results table representing the effect of sleep fragmentation on IL-1β expression in the hippocampus.

	DF	Sum of Squares	Mean Square	F-Value	P-Value	Lambda	Power
Light/Dark	1	0.002	0.002	0.045	0.8338	0.045	0.055
SF/No SF	1	0.001	0.001	0.017	0.8972	0.017	0.052
Sex	1	4.270E-4	4.270E-4	0.010	0.9205	0.010	0.051
Light/Dark * SF/No SF	1	3.985E-4	3.985E-4	0.009	0.9232	0.009	0.051
Light/Dark * Sex	1	0.112	0.112	2.649	0.1134	2.649	0.336
SF/No SF * Sex	1	0.218	0.218	5.177	0.0297	5.177	0.592
Light/Dark * SF/No SF * Sex	1	0.003	0.003	0.069	0.7939	0.069	0.057
Residual	32	1.351	0.042				

Appendix 6. Two-way ANOVA results table representing the effect of sleep fragmentation on IL-1β expression in the hypothalamus.

	DF	Sum of Squares	Mean Square	F-Value	P-Value	Lambda	Power
Light/Dark	1	0.371	0.371	2.876	0.0999	2.876	0.360
SF/No SF	1	0.027	0.027	0.213	0.6478	0.213	0.072
Sex	1	0.155	0.155	1.201	0.2816	1.201	0.176
Light/Dark * SF/No SF	1	0.171	0.171	1.325	0.2585	1.325	0.190
Light/Dark * Sex	1	0.123	0.123	0.956	0.3357	0.956	0.150
SF/No SF * Sex	1	0.090	0.090	0.696	0.4106	0.696	0.123
Light/Dark * SF/No SF * Sex	1	0.009	0.009	0.069	0.7943	0.069	0.057
Residual	31	3.996	0.129				

Appendix 7. Two-way ANOVA results table representing the effect of sleep fragmentation on IL-1β expression in the apical hyperpallium.

	DF	Sum of Squares	Mean Square	F-Value	P-Value	Lambda	Power
Light/Dark	1	0.001	0.001	0.082	0.7767	0.082	0.059
SF/No SF	1	0.010	0.010	0.813	0.3740	0.813	0.135
Sex	1	0.001	0.001	0.088	0.7685	0.088	0.059
Light/Dark * SF/No SF	1	0.092	0.092	7.825	0.0087	7.825	0.784
Light/Dark * Sex	1	0.011	0.011	0.925	0.3433	0.925	0.147
SF/No SF * Sex	1	0.007	0.007	0.570	0.4558	0.570	0.110
Light/Dark * SF/No SF * Sex	1	0.046	0.046	3.897	0.0570	3.897	0.469
Residual	32	0.377	0.012				

Appendix 8. Two-way ANOVA results table representing the effect of sleep fragmentation on IL-1β expression in the heart.

	DF	Sum of Squares	Mean Square	F-Value	P-Value	Lambda	Power
Light/Dark	1	0.002	0.002	0.015	0.9038	0.015	0.052
SF/ No SF	1	0.220	0.220	1.631	0.2110	1.631	0.223
Sex	1	0.020	0.020	0.149	0.7024	0.149	0.066
Light/Dark * SF/ No SF	1	0.378	0.378	2.798	0.1044	2.798	0.352
Light/Dark * Sex	1	0.164	0.164	1.214	0.2790	1.214	0.178
SF/ No SF * Sex	1	0.006	0.006	0.046	0.8325	0.046	0.055
Light/Dark * SF/ No SF * Sex	1	0.003	0.003	0.021	0.8847	0.021	0.052
Residual	31	4.186	0.135				

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Appendix 9. Two-way ANOVA results table representing the effect of sleep fragmentation on IL-6 expression in the fat.

	DF	Sum of Squares	Mean Square	F-Value	P-Value	Lambda	Power
Light/Dark	1	2.653	2.653	0.778	0.3849	0.778	0.131
SF/No SF	1	1.316	1.316	0.386	0.5392	0.386	0.090
Sex	1	3.195	3.195	0.937	0.3409	0.937	0.148
Light/Dark * SF/No SF	1	1.937E-5	1.937E-5	5.678E-6	0.9981	5.678E-6	0.050
Light/Dark * Sex	1	0.104	0.104	0.031	0.8622	0.031	0.053
SF/No SF * Sex	1	0.699	0.699	0.205	0.6541	0.205	0.072
Light/Dark * SF/No SF * Sex	1	3.407	3.407	0.999	0.3256	0.999	0.154
Residual	30	102.338	3.411				

Appendix 10. Two-way ANOVA results table representing the effect of sleep fragmentation on IL-6 expression in the liver.

	DF	Sum of Squares	Mean Square	F-Value	P-Value	Lambda	Power
Light/Dark	1	0.572	0.572	4.562	0.0431	4.562	0.526
SF/ No SF	1	0.059	0.059	0.473	0.4982	0.473	0.099
Sex	1	0.003	0.003	0.026	0.8744	0.026	0.053
Light/Dark * SF/ No SF	1	0.001	0.001	0.005	0.9436	0.005	0.051
Light/Dark * Sex	1	0.006	0.006	0.052	0.8222	0.052	0.055
SF/ No SF * Sex	1	0.007	0.007	0.056	0.8149	0.056	0.056
Light/Dark * SF/ No SF * Sex	1	0.139	0.139	1.107	0.3032	1.107	0.164
Residual	24	3.011	0.125				

Appendix 11. Two-way ANOVA results table representing the effect of sleep fragmentation on IL-6 expression in the spleen.

	DF	Sum of Squares	Mean Square	F-Value	P-Value	Lambda	Power
Light/Dark	1	5.490	5.490	3.776	0.0678	3.776	0.440
SF/ No SF	1	7.075	7.075	4.866	0.0406	4.866	0.543
Sex	1	3.479	3.479	2.393	0.1393	2.393	0.297
Light/Dark * SF/ No SF	1	6.803	6.803	4.679	0.0442	4.679	0.526
Light/Dark * Sex	1	2.779	2.779	1.911	0.1837	1.911	0.246
SF/ No SF * Sex	1	3.608	3.608	2.482	0.1326	2.482	0.306
Light/Dark * SF/ No SF * Sex	1	3.232	3.232	2.223	0.1533	2.223	0.279
Residual	18	26.171	1.454				

Appendix 12. Two-way ANOVA results table representing the effect of sleep fragmentation on IL-6 expression in the hippocampus.

	DF	Sum of Squares	Mean Square	F-Value	P-Value	Lambda	Power
Light/Dark	1	0.284	0.284	0.432	0.5162	0.432	0.095
SF/ No SF	1	0.005	0.005	0.008	0.9309	0.008	0.051
Sex	1	0.320	0.320	0.488	0.4904	0.488	0.101
Light/Dark * SF/ No SF	1	0.016	0.016	0.024	0.8768	0.024	0.053
Light/Dark * Sex	1	0.006	0.006	0.009	0.9244	0.009	0.051
SF/ No SF * Sex	1	0.001	0.001	0.002	0.9683	0.002	0.050
Light/Dark * SF/ No SF * Sex	1	1.100	1.100	1.677	0.2059	1.677	0.227
Residual	28	18.369	0.656				

Appendix 13. Two-way ANOVA results table representing the effect of sleep fragmentation on IL-6 expression in the hypothalamus.

	DF	Sum of Squares	Mean Square	F-Value	P-Value	Lambda	Power
Light/Dark	1	0.032	0.032	0.154	0.6984	0.154	0.066
SF/ No SF	1	0.642	0.642	3.072	0.0919	3.072	0.377
Sex	1	0.013	0.013	0.061	0.8073	0.061	0.056
Light/Dark * SF/ No SF	1	0.073	0.073	0.347	0.5612	0.347	0.086
Light/Dark * Sex	1	0.014	0.014	0.068	0.7960	0.068	0.057
SF/ No SF * Sex	1	0.019	0.019	0.089	0.7681	0.089	0.059
Light/Dark * SF/ No SF * Sex	1	0.249	0.249	1.192	0.2854	1.192	0.174
Residual	25	5.228	0.209				

Appendix 14. Two-way ANOVA results table representing the effect of sleep fragmentation on IL-6 expression in the apical hyperpallium.

	DF	Sum of Squares	Mean Square	F-Value	P-Value	Lambda	Power
Light/Dark	1	0.270	0.270	4.319	0.0461	4.319	0.511
SF/ No SF	1	0.052	0.052	0.839	0.3667	0.839	0.138
Sex	1	0.171	0.171	2.742	0.1078	2.742	0.346
Light/Dark * SF/ No SF	1	0.001	0.001	0.009	0.9257	0.009	0.051
Light/Dark * Sex	1	0.009	0.009	0.146	0.7052	0.146	0.065
SF/ No SF * Sex	1	0.067	0.067	1.079	0.3070	1.079	0.163
Light/Dark * SF/ No SF * Sex	1	0.062	0.062	0.998	0.3256	0.998	0.155
Residual	31	1.937	0.062				

Appendix 15. Two-way ANOVA results table representing the effect of sleep fragmentation on IL-6 expression in the heart.

	DF	Sum of Squares	Mean Square	F-Value	P-Value	Lambda	Power
Light/Dark	1	0.005	0.005	0.142	0.7090	0.142	0.065
SF/ No SF	1	0.219	0.219	6.305	0.0173	6.305	0.684
Sex	1	0.131	0.131	3.762	0.0613	3.762	0.455
Light/Dark * SF/ No SF	1	0.002	0.002	0.050	0.8251	0.050	0.055
Light/Dark * Sex	1	0.125	0.125	3.580	0.0676	3.580	0.436
SF/ No SF * Sex	1	0.075	0.075	2.159	0.1515	2.159	0.282
Light/Dark * SF/ No SF * Sex	1	0.033	0.033	0.955	0.3358	0.955	0.150
Residual	32	1.113	0.035				

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Appendix 16. Two-way ANOVA results table representing the effect of sleep fragmentation on IL-10 expression in the fat.

	DF	Sum of Squares	Mean Square	F-Value	P-Value	Lambda	Power
Light/Dark	1	0.384	0.384	4.449	0.0447	4.449	0.518
SF/No SF	1	8.456E-6	8.456E-6	9.798E-5	0.9922	9.798E-5	0.050
Sex	1	1.874	1.874	21.715	<0.0001	21.715	0.998
Light/Dark * SF/No SF	1	0.146	0.146	1.696	0.2042	1.696	0.228
Light/Dark * Sex	1	0.248	0.248	2.876	0.1019	2.876	0.357
SF/No SF * Sex	1	0.030	0.030	0.352	0.5583	0.352	0.086
Light/Dark * SF/No SF * Sex	1	0.006	0.006	0.074	0.7875	0.074	0.058
Residual	26	2.244	0.086				

Appendix 17. Two-way ANOVA results table representing the effect of sleep fragmentation on IL-10 expression in the spleen.

	DF	Sum of Squares	Mean Square	F-Value	P-Value	Lambda	Power
Light/Dark	1	0.011	0.011	0.076	0.7843	0.076	0.058
SF/No SF	1	0.128	0.128	0.892	0.3520	0.892	0.144
Sex	1	0.623	0.623	4.357	0.0449	4.357	0.515
Light/Dark * SF/No SF	1	0.127	0.127	0.891	0.3523	0.891	0.143
Light/Dark * Sex	1	0.438	0.438	3.064	0.0896	3.064	0.381
SF/No SF * Sex	1	0.104	0.104	0.726	0.4006	0.726	0.126
Light/Dark * SF/No SF * Sex	1	0.517	0.517	3.616	0.0663	3.616	0.440
Residual	32	4.578	0.143				

Appendix 18. Two-way ANOVA results table representing the effect of sleep fragmentation on IL-10 expression in the liver.

	DF	Sum of Squares	Mean Square	F-Value	P-Value	Lambda	Power
Light/Dark	1	0.021	0.021	0.272	0.6071	0.272	0.078
SF/No SF	1	0.232	0.232	3.005	0.0970	3.005	0.367
Sex	1	0.467	0.467	6.048	0.0223	6.048	0.652
Light/Dark * SF/No SF	1	0.035	0.035	0.452	0.5084	0.452	0.096
Light/Dark * Sex	1	0.062	0.062	0.804	0.3795	0.804	0.132
SF/No SF * Sex	1	0.059	0.059	0.765	0.3914	0.765	0.128
Light/Dark * SF/No SF * Sex	1	0.438	0.438	5.668	0.0264	5.668	0.621
Residual	22	1.699	0.077				

Appendix 19. Two-way ANOVA results table representing the effect of sleep fragmentation on IL-10 expression in the hippocampus.

	DF	Sum of Squares	Mean Square	F-Value	P-Value	Lambda	Power
Light/Dark	1	0.581	0.581	2.720	0.1116	2.720	0.339
SF/No SF	1	3.704	3.704	17.329	0.0003	17.329	0.988
Sex	1	0.068	0.068	0.319	0.5774	0.319	0.083
Light/Dark * SF/No SF	1	0.410	0.410	1.918	0.1783	1.918	0.252
Light/Dark * Sex	1	1.260	1.260	5.892	0.0227	5.892	0.644
SF/No SF * Sex	1	0.052	0.052	0.241	0.6278	0.241	0.075
Light/Dark * SF/No SF * Sex	1	0.003	0.003	0.015	0.9023	0.015	0.052
Residual	25	5.344	0.214				

Appendix 20. Two-way ANOVA results table representing the effect of sleep fragmentation on IL-10 expression in the hypothalamus.

	DF	Sum of Squares	Mean Square	F-Value	P-Value	Lambda	Power
Light/Dark	1	.141	.141	.532	.4716	.532	.105
SF/No SF	1	1.923	1.923	7.261	.0114	7.261	.749
Sex	1	.012	.012	.045	.8327	.045	.055
Light/Dark * SF/No SF	1	1.130	1.130	4.268	.0476	4.268	.505
Light/Dark * Sex	1	.055	.055	.209	.6506	.209	.072
SF/No SF * Sex	1	.103	.103	.388	.5379	.388	.091
Light/Dark * SF/No SF * Sex	1	1.823	1.823	6.882	.0136	6.882	.724
Residual	30	7.944	.265				

Appendix 21. Two-way ANOVA results table representing the effect of sleep fragmentation on IL-10 expression in the apical hyperpallium.

	DF	Sum of Squares	Mean Square	F-Value	P-Value	Lambda	Power
Light/Dark	1	1.290	1.290	3.085	0.0918	3.085	0.377
SF/No SF	1	0.508	0.508	1.214	0.2815	1.214	0.176
Sex	1	0.397	0.397	0.949	0.3396	0.949	0.148
Light/Dark * SF/No SF	1	0.021	0.021	0.050	0.8246	0.050	0.055
Light/Dark * Sex	1	1.083	1.083	2.591	0.1206	2.591	0.324
SF/No SF * Sex	1	0.254	0.254	0.608	0.4430	0.608	0.112
Light/Dark * SF/No SF * Sex	1	2.533	2.533	6.058	0.0214	6.058	0.656
Residual	24	10.036	0.418				

Appendix 22. Two-way ANOVA results table representing the effect of sleep fragmentation on IL-10 expression in the heart.

	DF	Sum of Squares	Mean Square	F-Value	P-Value	Lambda	Power
Light/Dark	1	0.248	0.248	3.094	0.0884	3.094	0.384
SF/No SF	1	0.010	0.010	0.127	0.7243	0.127	0.063
Sex	1	0.160	0.160	2.001	0.1672	2.001	0.264
Light/Dark * SF/No SF	1	0.024	0.024	0.302	0.5865	0.302	0.082
Light/Dark * Sex	1	0.001	0.001	0.013	0.9106	0.013	0.051
SF/No SF * Sex	1	0.236	0.236	2.942	0.0963	2.942	0.367
Light/Dark * SF/No SF * Sex	1	0.139	0.139	1.736	0.1973	1.736	0.235
Residual	31	2.482	0.080				

Corticosterone

Appendix 23. Two-way ANOVA results table representing the effect of sleep fragmentation on serum corticosterone levels.

	DF	Sum of Squares	Mean Square	F-Value	P-Value	Lambda	Power
Light/Dark	1	920.496	920.496	1.570	0.2196	1.570	0.216
SF/No SF	1	265.849	265.849	0.453	0.5058	0.453	0.097
Sex	1	1097.964	1097.964	1.872	0.1811	1.872	0.250
Light/Dark * SF/No SF	1	723.923	723.923	1.234	0.2751	1.234	0.180
Light/Dark * Sex	1	786.676	786.676	1.341	0.2556	1.341	0.192
SF/No SF * Sex	1	770.409	770.409	1.314	0.2605	1.314	0.189
Light/Dark * SF/No SF * Sex	1	1085.135	1085.135	1.850	0.1836	1.850	0.247
Residual	31	18179.863	586.447				

Behavior

Appendix 24. Repeated Measures ANOVA table.

	DF	Sum of Squares	Mean Square	F-Value	P-Value	Lambda	Power
Category	3	264703.542	88234.514	63.407	<0.0001	190.220	1.000
Subject(Group)	36	50096.250	1391.562				
Category for Activity	11	1320.625	120.057	0.315	0.9826	3.464	0.175
Category for Activity * Category	33	6473.958	196.181	0.515	0.9890	16.979	0.527
Category for Activity * Subject(Group)	396	150988.750	381.285				