Short-Term Sleep Loss Alters Cytokine Gene Expression in Brain and Peripheral Tissues and Increases Plasma Corticosterone of Zebra Finch (*Taeniopygia guttata*)*

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ABSTRACT

Lack of sleep incurs physiological costs that include increased inflammation and alterations in the hypothalamic-pituitary-adrenal axis. Specifically, sleep restriction or deprivation leads to increased pro-inflammatory cytokine expression and elevated glucocorticoids in rodent models, but whether birds exact similar costs is unknown. In this study, we examined whether zebra finch (*Taeniopygia guttata*), an avian model species, exhibits physiological costs of sleep loss by using a novel automated sleep fragmentation/deprivation method, wherein a horizontal wire sweeps across a test cage to disrupt sleep every 120 s. We measured 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) of captive finches after 12 h of exposure to a moving or stationary (control) bar during the night or the day. Plasma corticosterone, body mass, and behavioral profiles were also assessed. We predicted that birds undergoing sleep loss would exhibit elevated pro-inflammatory and reduced anti-inflammatory gene expression in brain and peripheral tissues compared with control birds. In addition, we predicted an increase in plasma corticosterone levels after sleep loss. As predicted, sleep loss increased pro-inflammatory gene expression, specifically in adipose tissue (IL-6), spleen (IL-1), and hippocampus (IL-6), but a decrease in anti-inflammatory expression (IL-10) was not detected. However, sleep loss elevated baseline concentrations of plasma corticosterone. Taken together, these results suggest that a diurnal songbird is sensitive to the costs of sleep loss.

Keywords: bird, corticosterone, cytokines, inflammation, interleukin, sleep fragmentation, sleep loss, zebra finch.

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Introduction

Sleep is a fundamental and essential component of vertebrate life cycles, and its curtailment or dysregulation leads to cognitive, metabolic, hormonal, and immunological impairments that are thought to be detrimental to fitness (Faraut et al. 2012). For example, there is accumulating evidence in humans and rodents that inadequate sleep leads to decrements in neurobiological performance, such as motivation, memory, and executive function (Durmer and Dinges 2005; Rolls et al. 2011; Vyazovskiy et al. 2011). In addition, chronic sleep loss is well known to induce a range of metabolic, physiological, and immunological alterations that predispose humans to cardiovascular and metabolic diseases and some cancers (Simpson and Dinges 2007; Mullington et al. 2009; Faraut et al. 2012).

Clinical studies indicate that acute and chronic sleep deprivation/restriction tends to increase pro-inflammatory responses in the brain and peripheral tissues (Frey et al. 2007; Simpson and Dinges 2007; Yehuda et al. 2009; Irwin et al. 2010; Rosa Neto et al. 2010; Motivala 2011; Wisor et al. 2011; Zhu et al. 2012; Zielinski et al. 2012; Hurtado-Alvarado et al. 2013; Carreras et al. 2015; Dumaine and Ashley 2015). Importantly, this response occurs in the absence of infection or injury—the major factors that trigger an inflammatory response (Medzhitov 2008; Ashley et al. 2012). Inflammation is typically activated and coordinated by pro-inflammatory cytokines, namely, interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF-α). Once released, these soluble molecules stimulate a secondary wave of cytokines and recruitment/activation of effector cells such as macrophages in the periphery and microglia in the brain (Medzhitov 2008; Ashley et al. 2012). The primary functions of inflammation during infection or trauma are to rapidly destroy or isolate the underlying source, remove damaged tissue, and then restore homeostasis. Because excessive inflammation is costly and potentially deadly, other signaling molecules, referred to as anti-inflammatory cytokines, such as IL-10 and transforming growth factor-beta (TGF-β), suppress pro-inflammatory cytokines and resolve the inflammatory response (Serhan and Savill 2005). Even though the advantages of inflammation are well understood in response to infection or trauma, the benefits of an inflammatory response due to sleep loss, if any, are unclear.

Besides immunological responses, there are also hormonal changes that occur during forced wakefulness. Activation of the hypothalamic-pituitary-adrenal (HPA) axis, as measured by increased glucocorticoid secretion from the adrenals, is commonly reported in laboratory studies on rodents that examine effects of sleep deprivation or restriction (Meerlo et al. 2002; Andersen et al. 2005; Marquez et al. 2006; Sgoifo et al. 2006; Ashley et al. 2016; but see Ashley et al. 2013*b*). However, this activation is likely due to a combination of factors that are confounded by the method of sleep disruption employed (e.g., flower-pot, multiple platform, human handling; Revel et al. 2009; Colavito et al. 2013) and not necessarily directly tied to effects from sleep loss per se. In addition, chronic activation of glucocorticoids can suppress immune responses (Barnes 1998; Coutinho and Chapman 2011); thus, a firm understanding of immune-endocrine interactions that occur during sleep loss is critical.

Among vertebrates, there is substantial variation in daily sleep duration (Lesku et al. 2006; Siegel 2008, 2009), which suggests that some taxa may cope with sleep deficits more effectively than others. For example, some mammals sleep fewer than 4 h (elephants, giraffes), while others sleep more than 20 h (e.g., bats; Lesku et al. 2006; Siegel 2008, 2009). Few studies have examined physiological, neurocognitive, or immunological costs associated with reduced sleep loss in avian species (e.g., Rattenborg et al. 2004; Jones et al. 2010).

A recurrent theme of ecoimmunology is that activation of the immune system is costly and can potentially create trade-offs with other life-history functions, such as growth and reproduction (Sheldon and Verhulst 1996; Lochmiller and Deerenberg 2000; Ricklefs and Wikelski 2002; Demas 2004; Demas and Nelson 2012; Demas et al. 2012). The underlying assumption of these studies is that an escalating arms race between parasites and their hosts plays an important role in shaping these trade-offs. However, it is unknown whether immune responses from sleep loss (in the absence of infection) are indeed costly and produce similar allocation constraints.

The aim of this study was to investigate whether sleep loss in a model bird species, zebra finch (*Taeniopygia guttata*), exacts physiological costs, such as inflammatory responses in the brain and periphery, as well as activation of the HPA axis. Zebra finch are diurnally active but are opportunistic breeders that migrate only short distances for food (Zann 1996). We predicted that 12 h of sleep fragmentation at night would increase the gene expression of pro-inflammatory cytokines (e.g., IL-1, IL-6) in the brain and periphery, reduce the gene expression of an anti-inflammatory cytokine (i.e., IL-10), as well as increase baseline plasma corticosterone titers, which would signify an adrenocortical response to sleep loss. To accomplish this, we devised a novel method of sleep fragmentation/deprivation that involves the use of a programmable, moving bar that periodically sweeps across a test cage and forces zebra finches to hop over the moving bar, thereby awakening them. Importantly, to control for the confound of increased activity from bar movement, another set of birds was also exposed to the moving bar during the day.

Material and Methods

Animals

Adult male ($n = 16$) and female ($n = 16$) zebra finches were housed in a colony room (12L : 12D, lights on at 7 a.m.; 22.0° ± 1°C) at the Western Kentucky University vivarium. This colony was originally acquired from an aviculturist in Washington, and birds were color banded for identification purposes. Birds remained in a communal aviary for 3 mo before the start of the study and were provided with food (bird seed and protein crumble in 1 : 1 ratio) and water ad lib., as well as supplemented with millet sprays, lettuce, 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* (National Research Council 2011) and international ethical standards.

Sleep Fragmentation Cage

To noninvasively reduce sleep in birds, a sleep fragmentation cage was developed based on previous methods in mice that employ the use of a programmable bar that moves across a cage to periodically awake mice at specified intervals (Ramesh et al. 2009; Carreras et al. 2014; Wang et al. 2014; Dumaine and Ashley 2015, 2018). Individual birds were placed in a testing cage (34 cm × 40 cm × 45 cm) that contained a single stationary wooden perch and a 16-gauge wire placed horizontally 1.3 cm above the stationary perch. A flexible wire was used instead of a solid wooden bar because zebra finch would tend to perch on the wooden bar, rather than hop over it. Substitution with a 16-gauge flexible wire was sufficient to prevent finches from perching because it would flex if birds attempted to land on it, making it unstable to balance on the perch. A stationary wooden perch was adjacent to food and water dishes, allowing birds to have ad lib. access to food and water and a place to perch. The wire swept horizontally across the distance of the cage at 2-min intervals for a total of 12 h, and the wire was attached to an automated sleep deprivation base (Lafayette Instruments, Lafayette, IN). This rate of sleep fragmentation is based on previous research on mice to approximate the intermittent arousal rate of humans that exhibit sleep apnea (Ramesh et al. 2009; Kaushal et al. 2012; Wang et al. 2014). One bar sweep lasts ca. 9 s. The cage was placed in a tub of water (3.8 cm deep), which prevented birds from resting on the bottom. Birds were exposed to the moving wire during light or dark phases (see below). To ensure that birds responded in the same way to the sleep loss protocol, a dim blue light that emitted ca. 8 lux was always turned on in the room (both light and dark phases). During light or dark phases, birds typically hopped over the wire or in extremely rare cases (<0.01% of recorded observations) would be pushed down into the water at the bottom of the cage and awaken (see supplemental videos of birds exposed to bar movement at day or night, deposited in the Dryad Digital Repository: https://dx.doi.org/10.5061/dryad.cb5cf20; Cooper et al. 2019). 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 before testing.

Experimental Groups

Birds were exposed to a moving wire during the dark phase (7 p.m. to 7 a.m.), when lights were off and birds were typically resting/sleeping, or during the light phase (7 a.m. to 7 p.m.), when lights were on and birds were generally active. 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 and equally balanced between sexes: (1) light phase and no wire movement (light control; $n = 8$), (2) dark phase and no wire movement (dark control; $n = 8$), (3) light phase with sweeping wire (control to account for increased activity from moving wire; $n = 8$), and (4) dark phase with sweeping wire (sleep fragmentation group; $n = 8$). The same experimental cage was used for all trials.

Behavior

Each 12-h experiment was recorded using an infrared video camera (DNV16HDZ, Bell and Howell). For each hour interval, a random 5-min period was selected for observation. During these 5-min periods, behaviors were documented every 15 s. This produced 20 observations per session, for a total of 240 observations per individual. The four behavioral states quantified during trials were operationally defined: alert, moving, resting, and feeding/drinking (table 1). Behavioral profiles were constructed for each treatment group by using video observations, and the average percentage that each behavior was performed for each hour interval was calculated. 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 percentage of activity. This new variable was used to compare hourly activity levels for each treatment.

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 during <3 min of initial handling. Blood was centrifuged for 30 min at 3,000 *g*, and plasma was removed and stored at −80°C for later analysis. Birds were then deeply anesthetized with isoflurane vapors and killed by rapid decapitation (<1 min after initial brachial sampling). Liver, fat, spleen, and brain tissues were collected and placed into tubes containing RNAlater (AM7020, ThermoFisher Scientific). Liver, fat, and spleen were 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 trials to the nearest 0.01 g, using a digital scale.

Table 1: Ethogram of behavioral states observed and recorded

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

RNA Extraction

Total RNA was extracted from liver, fat, spleen, and brain samples using an RNeasy Mini kit (74106, Qiagen), according to the manufacturer's instructions. The concentration of total RNA was quantified with a Nanodrop 2000 spectrophotometer (ThermoFisher Scientific).

Reverse Transcription

A reverse transcriptase kit (4368814, ThermoFisher Scientific) was used to synthesize cDNA from total RNA. The total RNA concentration of the tissue was diluted to the following concentrations (in ng/μL): hypothalamus, 10.8; hippocampus, 3.2; apical hyperpallium, 12.7; liver, 4.7; spleen, 19.9; and fat, 3.1. 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 Polymerase Chain Reaction

Amplification was performed on an ABI 7300 real-time polymerase chain reaction (PCR) system using Taqman Universal PCR Master Mix (4369016, Applied Biosystems). 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 ThermoFisher Scientific for IL-1β, IL-6, IL-10, and peptidyl proline isomerase A (PPIA; housekeeping gene), using annotated sequences within the zebra finch genome that spanned two exons. Primer/probes passed all quality control tests outlined by ThermoFisher Scientific. 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 IL-1β, IL-6, and IL-10 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 (PPIA) levels (Zinzow-Kramer et al. 2014).

ELISA

Plasma corticosterone concentration was determined using an ELISA kit (ADI-900-097, Enzo Life Sciences) with 96% recov-

ery 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. Plasma 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 by using a four-parameter logistic curve fit. Intraassay variation was 5.1%.

Statistical Analyses

All data are expressed as mean ± standard error. For variables reported below, three-way ANOVAs were initially used to test for differences among groups, with main effects of light/dark, moving/stationary bar, and sex (male/female) evaluated. However, there were no significant differences or interactions with sex, so this factor was not considered in subsequent analyses. Therefore, two-way ANOVAs with light/dark phase and moving/stationary bar as main factors were used to compare percent body mass change, plasma corticosterone concentration, and relative cytokine expression in peripheral and brain tissues of zebra finch. If significant differences were found, then Bonferroni post hoc tests were used to determine which groups were significantly different from one another. A repeated-measures ANOVA was used to compare differences in activity levels among the four treatment groups. Time was the repeated measure. A two-way ANOVA was used to test for effects of time of day, bar movement, and their interaction upon total percent activity. If significant, then Bonferroni post hoc tests were used to determine which groups were significantly different from one another. Logarithmic transformation was necessary for some variables (plasma corticosterone, IL-6 in spleen, IL-10 in apical hyperpallium and hippocampus) that initially failed to exhibit normality and/or homogeneity of variances.

Results

Body Mass

Body mass varied significantly between light- and dark-exposed birds (two-way ANOVA, light/dark, $F_{1, 28} = 256.60, P < 0.0001$; fig. 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 ($P < 0.05$, fig. 1). The sweeping bar also produced a slight loss in body mass irrespective of light/dark phase ($F_{1, 28} = 6.40, P = 0.018$). There was no significant interaction between light/dark phase and moving/stationary bar ($F_{1, 28} = 0.35, P = 0.90$).

Behavior

Behavioral ethograms of finches (table 1; fig. 2*a*–2*d*) provide an indication of the different behaviors that occur during 12 h of light versus dark and exposure to a moving or stationary bar.

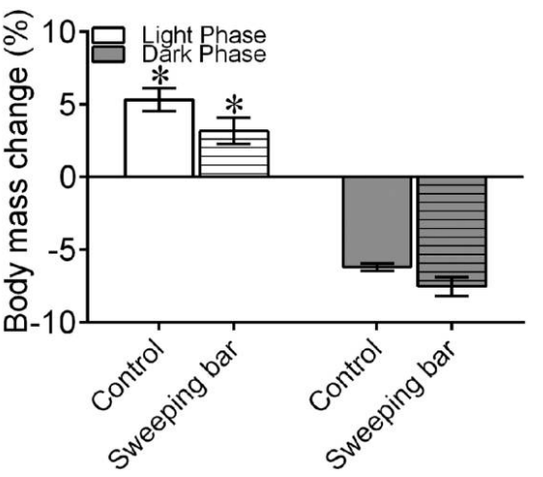

Figure 1. Percentage change in body mass (mean ± SE, $n = 8$ per group) of zebra finches at the end of day (hour 12) or the end of night (hour 0) with or without exposure to a moving bar. An asterisk indicates a significant effect of time of day as determined by two-way ANOVA followed by a Bonferroni post hoc test. $P < 0.05$ is considered statistically significant.

Behaviors exhibited during the day (fig. 2*a*) included locomotory, feeding, and alert behaviors (12-h % mean ± SE; light phase, stationary bar: locomotion, 23.46% ± 1.81%; feeding, 21.71% ± 2.384%; alert, 52.58% ± 1.35%; resting, 2.25% ± 1.49%; light phase, moving bar: locomotion, 28.46% ± 2.00%; feeding, 25.75% ± 2.43%; alert, 45.79% ± 1.84%; and resting, 0%; fig. 2*a*, 2*b*), whereas during the night, birds were mostly resting (12-h % means; dark phase, stationary bar: locomotion, 0%; feeding, 0.37% ± 0.10%; alert, 10.33% ± 2.57%; resting, 89.53% ± 2.63%; dark phase, moving bar: locomotion, 6.58% ± 0.40%; feeding, 0.52% ± 0.24%; alert, 56.25% ± 3.32%; resting, 36.3% ± 3.63%; fig. 2*c*, 2*d*). Introduction of a sweeping bar during the day had little effect on light-associated behaviors (fig. 2*b*), whereas during the night, birds subjected to the moving bar increased locomotory activity (from 0% to 6.5%), reduced resting behavior (from 90% to 36%), and increased alert behavior (from 10% to 56%; fig. 2*d*). There was no significant interaction across treatment and time in hourly percent activity (repeated-measures ANOVA, $F_{3, 29} = 0.52, P = 0.99$), but a significant difference existed in treatment (repeated-measures ANOVA, $F_{3, 29} = 63.407, P < 0.0001$; fig. 2*a*–2*d*). Since there was no significant effect of time, each hour value was binned into an average percent for each individual for the entire 12-h period (fig. 2*e*). Time of day (two-way ANOVA, $F_{1, 28} = 439.80, P < 0.0001$) and bar movement (two-way ANOVA, $F_{1, 28} = 256.6, P < 0.0001$) significantly affected total percent activity but not their interaction (two-way ANOVA, $F_{1, 28} = 0.01, P = 0.91$). Post hoc tests indicated that among birds exposed to darkness, total % activity was increased in birds subjected to a moving bar (7.08% ± 0.56%) versus a stationary bar (0.13% ± 0.10%; Bonferroni post hoc tests, $P < 0.05$; fig. 5*e*). In addition, a moving bar caused a slight increase in total activity levels in light-exposed birds (stationary bar, 48.46% ± 2.72%, vs. moving bar,

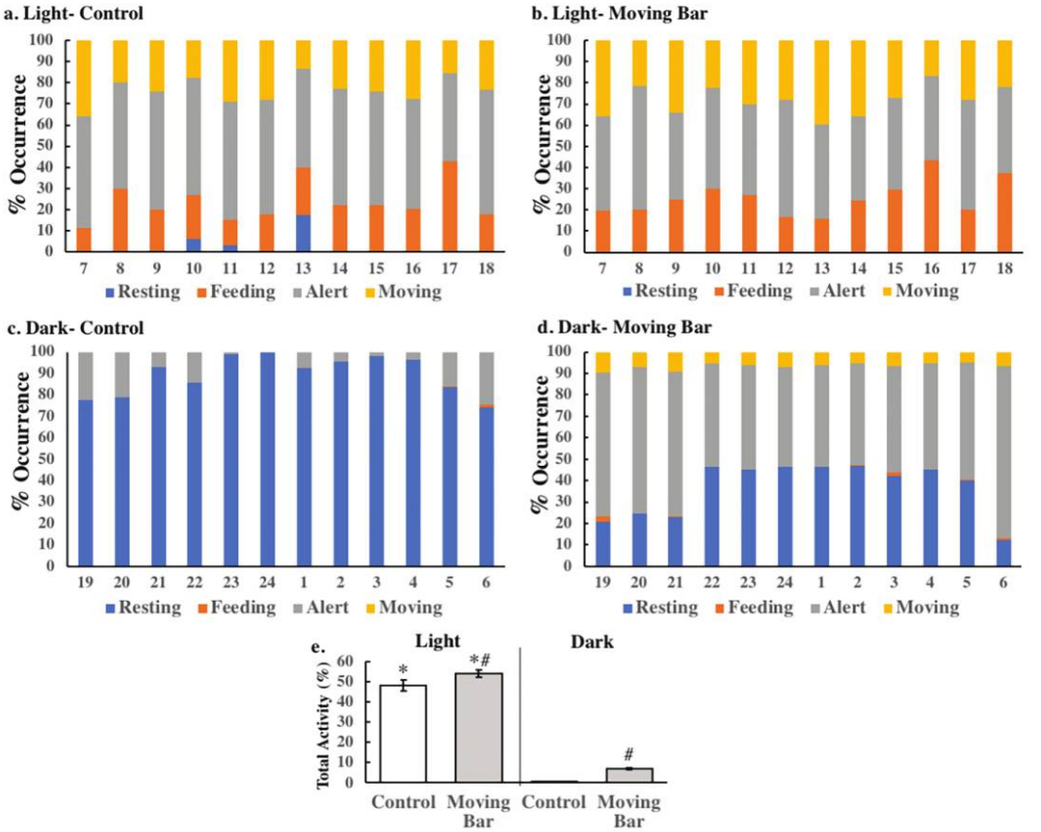

Figure 2. *a–d*, Behavioral profiles of zebra finch ($n = 8$ per group) for each treatment group over a 12-h period. Bars are stacked columns that reflect the percentage of behaviors elicited per hour. The *X*-axis denotes time of day (*a*, *b*) or time of night (*c*, *d*). *e*, Total activity is estimated as the total proportion of active behaviors (moving, feeding) for the entire 12-h period (mean ± SE, $n = 8$ per group). Asterisk and pound sign indicate a significant effect of time of day and moving bar, respectively, as determined by two-way ANOVA followed by a Bonferroni post hoc test. $P < 0.05$ is considered statistically significant.

54.21% ± 1.84%; Bonferroni post hoc test, $P < 0.05$; fig. 2*e*). Last, total activity of birds exposed to a stationary or moving bar during the day was significantly greater than total activity when exposed to a stationary or moving bar during the night (Bonferroni post hoc tests, $P < 0.05$; fig. 2*e*).

Peripheral Gene Expression

In adipose tissue, the sweeping bar did not significantly alter IL-1β or IL-10 mRNA expression (two-way ANOVAs, $F_{1,28} < 0.69$, $P > 0.41$; fig. 3*a*, 3*c*), but there was a significant elevation of IL-6 expression (two-way ANOVA, $F_{1,28} = 5.84$, $P = 0.02$; fig. 3*b*). Among finches exposed to the dark phase, adipose IL-6 expression was increased for birds subjected to a moving bar relative to a stationary bar (fig. 3*b*; Bonferroni post hoc test, $P < 0.05$). There was also a significant interaction between the two main factors for adipose IL-1 mRNA expression (two-way ANOVA, $F_{1,28} = 9.66$, $P = 0.004$), but no post hoc differences were detected.

There was a significant effect of time of day on IL-6 expression in the heart (two-way ANOVA, $F_{1,28} = 9.128$, $P = 0.005$). More specifically, control birds during the light phase exhibited increased levels in heart IL-6 expression relative to controls during the dark phase (Bonferroni post hoc test, $P < 0.05$; fig. 3*e*). IL-1 and IL-10 expression in the heart was not affected by main effects or their interactions (two-way ANOVA, $F_{1,28} < 3.15$, $P > 0.09$).

In liver, there were significant interactions between bar movement and light/dark phase for the three genes measured (two-way ANOVA, all $F_{1,28} > 5.70$, all $P < 0.024$). Post hoc tests indicated that hepatic IL-1 gene expression was elevated in control birds exposed to the light phase versus the dark (Bonferroni post hoc test, $P < 0.05$; fig. 3*g*). During the dark phase, IL-6 and IL-10 gene expression was elevated in birds exposed to a stationary bar compared to a moving bar or to birds exposed to a stationary bar during the light phase (Bonferroni post hoc tests, all $P < 0.05$; fig. 3*h*, 3*i*).

Splenic IL-1 expression was affected by a significant interaction of time of day and bar movement (two-way ANOVA, $F_{1,28} = 12.06$, $P = 0.0018$). Birds exposed to a sweeping bar at night exhibited higher IL-1 expression compared with birds subjected to a sweeping bar during the day and dark phase,

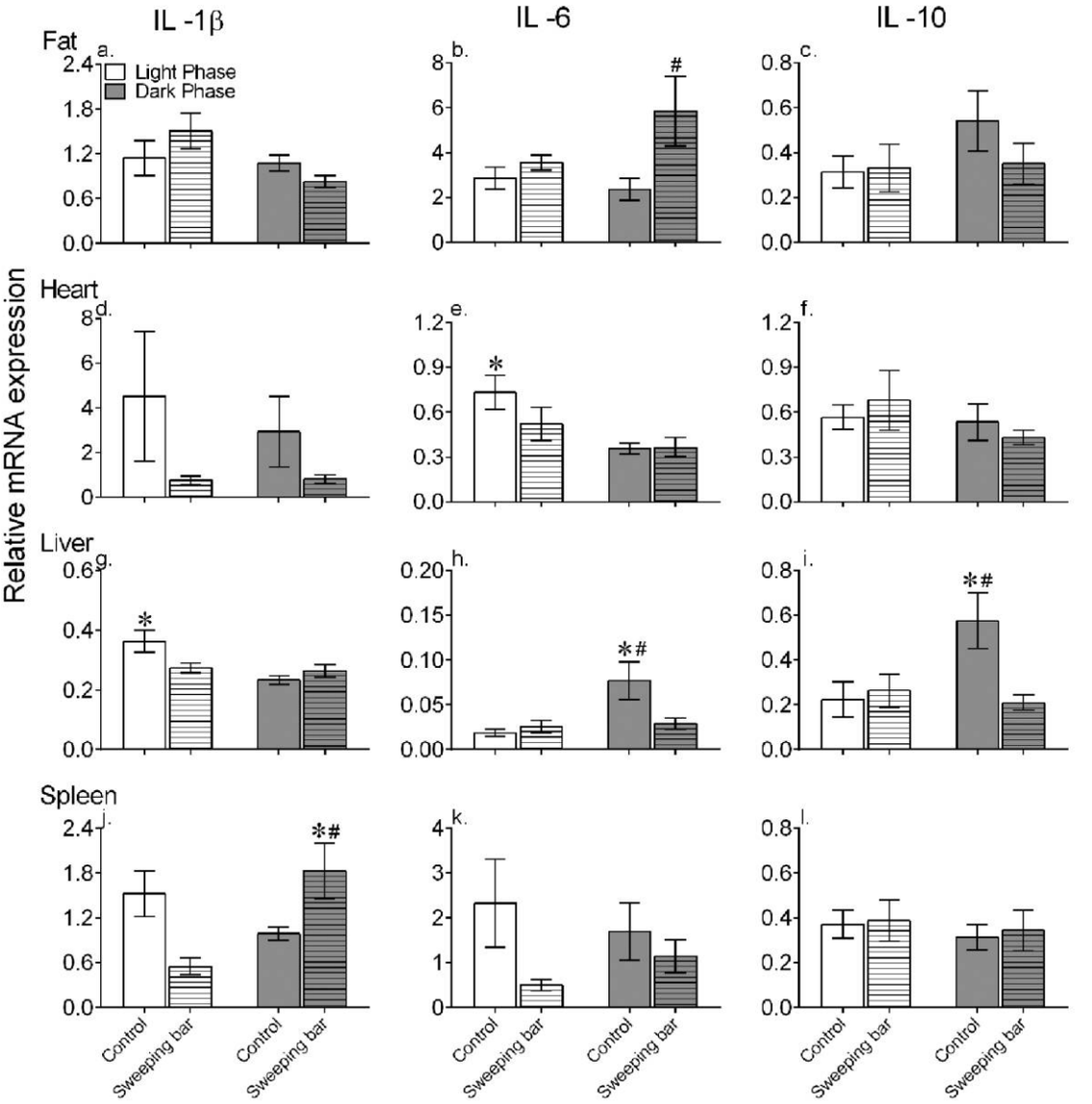

Figure 3. Peripheral IL-1β, IL-6, and IL-10 mRNA expression levels (mean $\pm$ SE, $n = 8$ per group) in fat (*a–c*), heart (*d–f*), liver (*g–i*), and spleen (*j–l*) of zebra finches at the end of day (hour 12) or the end of night (hour 0) with or without an exposure to a moving bar. Asterisk and pound sign indicate a significant effect of time of day and moving bar, respectively, as determined by two-way ANOVA followed by a Bonferroni post hoc test. $P < 0.05$ is considered statistically significant.

control (no bar movement) birds (Bonferroni post hoc test, all $P < 0.05$; fig. 3*j*). There were no significant main effects or interactions of IL-6 or IL-10 expression in the spleen.

Brain Gene Expression

There was a significant interaction between time of day and bar movement for IL-1 expression in the apical hyperpallium (two-way ANOVA, $F_{1,28} = 7.14$, $P = 0.012$; fig. 4*a*). Among birds exposed to dark, IL-1 expression was elevated in birds subjected to a stationary bar versus moving bar (Bonferroni post hoc test, $P < 0.05$; fig. 4*a*). For IL-6 expression, there were significant effects of bar movement (log-transformed, two-way ANOVA, $F_{1,28} = 14.42$, $P < 0.007$) and time of day (log-transformed, two-way ANOVA, $F_{1,28} = 4.37$, $P = 0.046$) but no significant interaction (log-transformed, two-way ANOVA, $F_{1,28} = 1.41$, $P = 0.25$; fig. 4*b*). More specifically, IL-6 expression was elevated in dark-exposed birds with no bar movement compared with birds subjected to a moving bar in the dark (log-transformed, Bonferroni post hoc test, $P < 0.05$; fig. 4*b*). There were no significant main effects or interactions

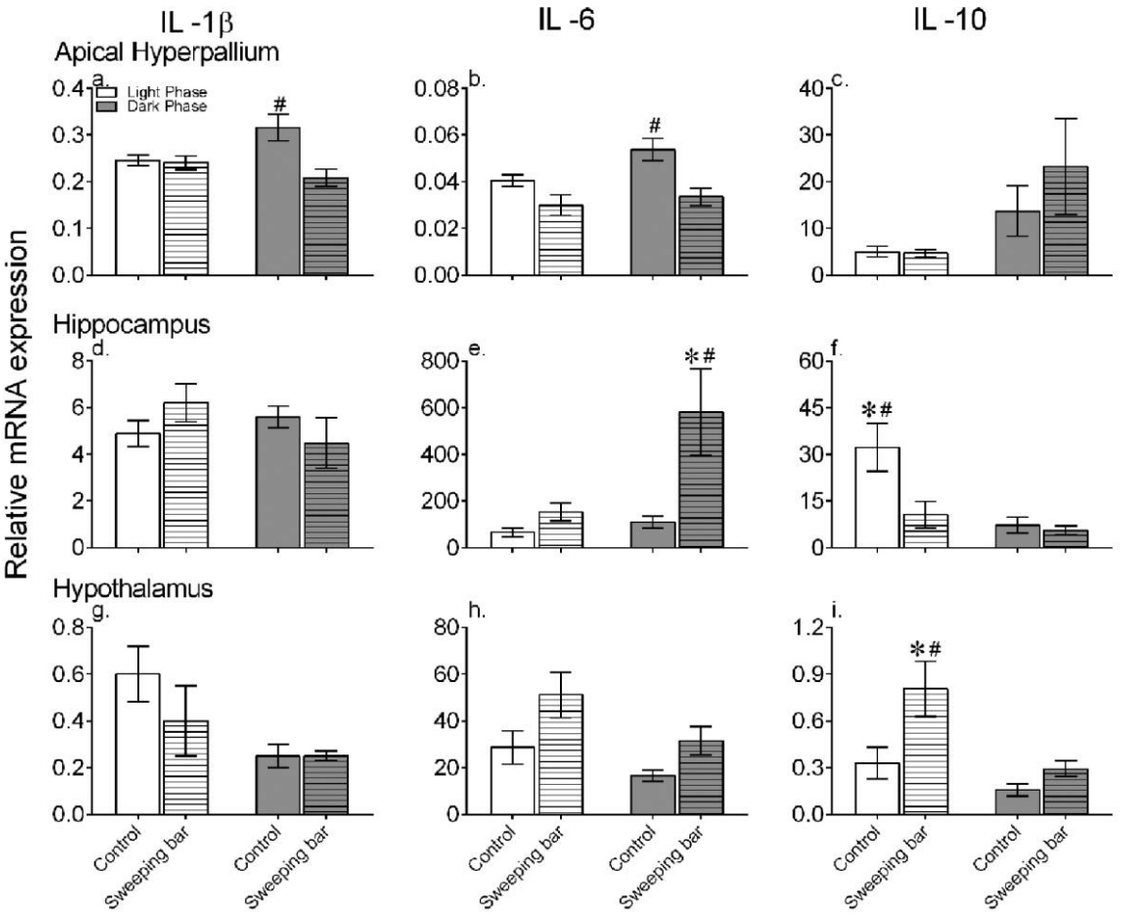

Figure 4. Brain IL-1β, IL-6, and IL-10 mRNA expression levels (mean ± SE, $n = 8$ per group) of apical hyperpallium (*a–c*), hippocampus (*d–f*), and hypothalamus (*g–i*) in brains of zebra finches sampled at the end of day (hour 12) or the end of night (hour 0) with or without an exposure to a moving bar. Asterisk and pound sign indicate a significant effect of time of day and moving bar, respectively, as determined by two-way ANOVA followed by a Bonferroni post hoc test. $P < 0.05$ is considered statistically significant.

for IL-10 mRNA expression in the apical hyperpallium (two-way ANOVA, all $F_{1,28} < 2.02$, all $P > 0.16$; fig. 4*c*).

In hippocampus, there were no significant effects or interactions in IL-1 expression (two-way ANOVA, all $F_{1,28} < 2.4$, all $P > 0.13$; fig. 4*d*). However, there was a significant interaction between time of day and bar movement in hippocampal IL-6 expression (two-way ANOVA, $F_{1,28} = 4.94$, $P = 0.035$; fig. 4*e*). Specifically, birds in the dark phase and exposed to a moving bar exhibited elevated hippocampal IL-6 expression relative to dark-phase birds subjected to a stationary bar and light-phase birds exposed to a moving bar (Bonferroni post hoc tests, all $P < 0.05$; fig. 4*e*). For IL-10 expression in the hippocampus, there were significant effects from time of day (log-transformed, two-way ANOVA, $F_{1,28} = 6.80$, $P = 0.015$) and from bar movement (log-transformed, two-way ANOVA, $F_{1,28} = 5.81$, $P = 0.023$) but no interaction effect (log-transformed, two-way ANOVA, $F_{1,28} = 3.15$, $P = 0.88$; fig. 4*f*). Finches subjected to a stationary bar during the light phase exhibited higher IL-10 expression compared with birds exposed to a moving bar during the day or a stationary bar at night (Bonferroni post hoc tests, all $P < 0.05$).

Time of day significantly affected hypothalamic IL-1 expression (two-way ANOVA, $F_{1,28} = 6.28$, $P = 0.018$), with an elevation occurring during the light phase (fig. 4*g*). However, bar movement or its interaction with time of day did not significantly affect IL-1 expression in the hypothalamus (two-way ANOVA, all $F_{1,28} < 1.01$, all $P > 0.32$; fig. 4*g*). For hypothalamic IL-6 and IL-10 expression, there were significant effects of time of day and bar movement (two-way ANOVA, all $F_{1,28} > 5.29$, all $P < 0.30$) but no significant interactions (two-way ANOVA, all $F_{1,28} < 2.56$, all $P > 0.11$; fig. 4*h*, 4*i*). Although no post hoc differences were detected for hypothalamic IL-6 expression (Bonferroni post hoc tests, all $P > 0.05$; fig. 4*h*), finches exposed to the light phase and a sweeping bar exhibited increased IL-10 expression compared with birds subjected to a moving bar at night or to a stationary bar during the day (Bon-

Table 2: Summary of the effects of time of day (light/dark phase), bar movement (or no movement [control]), and their interaction on cytokine gene expression in peripheral and brain tissues of zebra finch

Tissue	Effect	Factor(s)
Periphery:		
Fat	Increase: IL-1	Interaction*
	Increase from sleep loss: IL-6	Moving bar
Heart	Increase, light: IL-6	Time of day
Liver	Decrease from moving bar, light: IL-1	Interaction
	Decrease from sleep loss: IL-6, IL-10	Interaction
Spleen	Increase from sleep loss: IL-1	Interaction
Brain:		
Apical hyperpallium	Decrease from sleep loss: IL-1	Interaction
	Decrease from sleep loss: IL-6	Moving bar, time of day
Hippocampus	Increase from sleep loss: IL-6	Interaction
	Decrease from moving bar, light: IL-10	Moving bar, time of day
Hypothalamus	Increase, light: IL-1	Time of day*
	Increase: IL-6	Moving bar, time of day*
	Decrease from moving bar, light: IL-10	Moving bar, time of day

*No significant effect after post hoc testing.

ferroni post hoc tests, all $P < 0.05$; fig. 4*i*). See table 2 for a summary of gene expression results and a supplemental table (deposited in the Dryad Digital Repository: https://dx.doi.org/10.5061/dryad.cb5cf20; Cooper et al. 2019) for summary of statistics.

Plasma Corticosterone

Concentrations of plasma corticosterone were affected by a significant interaction between time of day and bar movement (log-transformed, two-way ANOVA, $F_{1,28} = 9.66$, $P = 0.004$; fig. 5). Post hoc tests indicated that birds exposed to the sweeping bar at night exhibited elevated corticosterone levels compared with birds subjected to a stationary bar at night (Bonferroni post hoc test, $P < 0.05$; fig. 5).

Discussion

The findings of our study indicate that acute sleep loss leads to inflammatory responses in select areas of the brain and periphery, as well as activation of the HPA axis. Therefore, our hypothesis that sleep reduction in birds can exact physiological and immunological responses, as previously shown in rodents, is supported. More specifically, the results are also consistent with previous studies investigating the effect of acute sleep fragmentation in mice by using the sweeping bar method (Dumaine and Ashley 2015), albeit there are some notable differences between mice and birds (see below).

Our use of the novel "sweeping bar" method to induce sleep loss in birds was effective from a behavioral standpoint. Birds at night subjected to a sweeping bar (every 120 s for 12 h total) exhibited increased locomotion and feeding behaviors and concomitantly rested less than birds exposed to a stationary bar. During the day, a moving bar increased overall activity levels slightly but did not alter resting behaviors relative to a stationary bar. In addition, changes in body mass were affected by light or dark phase but not by bar movement. Together, these data indicate that birds are being awakened at night (but not the day) and that the increased activity that is occurring as a result of the moving bar is not affecting body mass. It was unexpected that birds would exhibit such large variation in body mass after exposure to 12 h of light versus darkness. However, given the small size of these birds, high metabolic rate, and lack of feeding during the night, a 5%–7% decline in body mass is within the range of variation observed in zebra finch after nightly fasting (Metcalfe and Ure 1995; Dall and Witter 1998). Although behavior

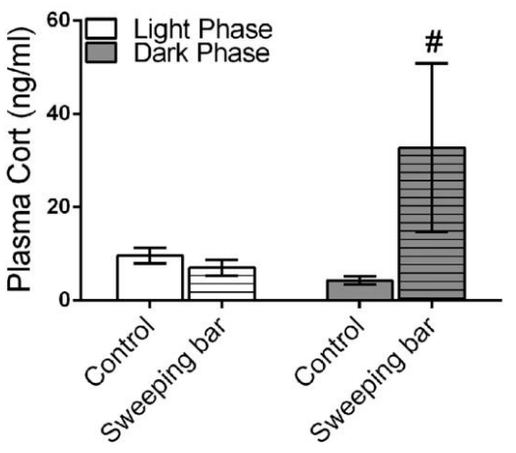

Figure 5. Plasma corticosterone levels in zebra finches (ng/mL; mean ± SE; $n = 8$ per group) sampled at the end of day (hour 12) or the end of night (hour 0) with or without exposure to a moving bar. Two-way ANOVA, followed by a Bonferroni post hoc test, tested the effects of time of day, moving bar, and their interaction. Pound sign indicates a significant effect of moving bar. $P < 0.05$ is considered statistically significant.

can provide proxies for sleep/wake states, this procedure will need to be refined by assessing electroencephalographic and electromyographic biopotentials in subjects to accurately score vigilance states (rapid-eye movement [REM], non-REM, and wakefulness; Newman et al. 2008). Thus, without collecting additional data, it is unknown whether birds in our study were experiencing fragmented sleep or complete sleep deprivation.

Acute sleep loss (i.e., bar movement at night for 12 h) increased pro-inflammatory gene expression in adipose tissue (IL-6), spleen (IL-1), and hippocampus (IL-6). Upregulation of these cytokines is characteristic of a typical acute inflammatory response observed in mammals in response to sleep deprivation (Irwin et al. 1996, 2006; Simpson and Dinges 2007; Mullington et al. 2009) or fragmentation (Carreras et al. 2014; Dumaine and Ashley 2015, 2018). For example, among mice exposed to the same rate of sleep fragmentation (30/h) and a similar sweeping bar method for 24 h, a significant upregulation of IL-1 gene expression in adipose tissue and hypothalamus was detected (Dumaine and Ashley 2015). Other methods of sleep disturbance (e.g., use of a rocking cage) have also increased IL-6 secretion in the murine hippocampus but not the cortex (Zhu et al. 2012). The upregulation of pro-inflammatory cytokines in adipose tissue is a common response from sleep fragmentation studies that is likely due to the invasion of classical pro-inflammatory M1 macrophages into adipose tissue (Lumeng and Saltiel 2011; Zhang et al. 2014). Accumulating evidence also suggests that sleep loss can lead to alterations in cytokine signaling that can predispose individuals toward neuroinflammation (Wisor et al. 2011; Zhu et al. 2012; Zielinski et al. 2014). It is well known that hippocampal neuroinflammation can lead to neurological deficits that can manifest into pathological conditions (Barrientos et al. 2015; Kim and Won 2017; Valero et al. 2017). These changes have been linked to the activation of microglia and subsequent gliosis (Wadhwa et al. 2017). Like mammals, the hippocampus in birds plays a key role in spatial memory, and hippocampal size can vary over the annual cycle in food-storing birds (Smulders et al. 1995; Sherry and Hoshooley 2010). Future research is warranted to investigate whether neuroinflammation from sleep loss could affect hippocampal performance in birds by assessing spatial memory after sleep loss.

In several peripheral and brain tissues, there were significant changes in cytokine gene expression that were not directly related to sleep fragmentation (i.e., a moving bar at night). For example, there was significant upregulation of both pro-inflammatory (IL-1, IL-6) and anti-inflammatory (IL-10) expression in control birds during the day compared with the dark, which suggests an underlying diurnal or possibly circadian rhythmicity in their expression. Accumulating evidence suggest that the immune system and cytokine activity exhibit diurnal and circadian variation (Keller et al. 2009; Nakao 2014; Labrecque and Cermakian 2015; Man et al. 2016; Nilsonne et al. 2016; Segal et al. 2018). The liver, heart, and hippocampus of finches showed a diurnal elevation in IL-1, IL-6, and IL-10 expression, respectively. Conversely, in the dark, IL-6 and IL-10 expression was elevated in the liver of finches, as well as an upregulation of IL-1 and IL-6 in the apical hyperpallium, a homologue of the mammalian cortex (Dugas-Ford et al. 2012). This increased expression was suppressed in birds that were exposed to sleep fragmentation. These data indicate that sleep fragmentation inhibited the elevation of the cytokines in these tissues. The reasons for this are unclear but could involve a suppressive role for glucocorticoids, which were elevated in birds exposed to the moving bar (see below).

Anti-inflammatory cytokines are also elevated during the course of an inflammatory response to regulate overexcessive pro-inflammatory responses (Medzhitov 2008; Ashley et al. 2012). A previous study has documented increased expression of anti-inflammatory cytokines (e.g., TGFβ) in the hypothalamus and hippocampus of mice exposed to a similar sweeping bar method of sleep fragmentation (Dumaine and Ashley 2015). It has been hypothesized that, given the sensitivity of the brain to inflammatory insults, anti-inflammatory cytokines are upregulated to mitigate inflammation that may occur as a result of sleep loss (Dumaine and Ashley 2015). However, this protective effect was not observed in the brains of zebra finch. Besides time-of-day effects in IL-10 expression (as discussed above), there seemed to be an upregulation of IL-10 expression in the hypothalamus as a result of exposure to the moving bar during the day (but not night). This is the only result that we can attribute to a possible confound of increased activity from the moving bar.

To date, most studies that have documented increased glucocorticosteroids from experimental sleep deprivation or restriction have been unable to adequately separate the effects of sleep loss from the method used to induce sleep (Meerlo et al. 2002; Andersen et al. 2005; Dumaine and Ashley 2015, 2018; Ashley et al. 2016). For example, the act of handling may keep an animal awake, but it is also a perceived stressor by the animal. In our study, we show for the first time that preventing sleep in birds (through use of a moving bar) increases the HPA axis, because a similar treatment in active birds does not elicit the same response. This suggests a direct effect of sleep loss on stimulating the HPA response. Given this result, it is possible that increased corticosterone could regulate the inflammatory response to sleep loss, but this possibility will require further study.

The fact that zebra finch display inflammatory and adrenocortical response to sleep loss does not necessarily rule out that other species of birds may be more resilient to these physiological costs. Zebra finch are diurnal, opportunistic breeders that only travel short distances for food (Zann 1996). This species does not experience migration at night that is characteristic of many migratory songbirds nor does it exhibit "reproductive sleeplessness" on its breeding grounds as do arctic-breeding birds (Lesku et al. 2012; Ashley et al. 2013*a*). For example, Gambel's white-crowned sparrows (*Zonotrichia leucophyrs gambelii*) spend 60% less time sleeping when migrating without experiencing declines in cognitive function (Rattenborg et al. 2004). In addition, arctic-breeding birds, such as Lapland longspurs (*Calarius lapponicus*) and pectoral sandpipers (*Calidris melanotos*), reduce sleep to meet the intense competition and demands of a highly truncated breeding season in the Arctic (Lesku et al. 2012; Ashley et al. 2013*a*). Therefore, future directions should assess whether birds that are adapted to shorter sleep durations (e.g., migratory and polar-breeding birds) are less sensitive to the effects of sleep

loss than nonmigratory birds such as zebra finch. Last, more studies need to be conducted that elucidate the dynamics of immune-endocrine-sleep interactions in birds and their role in shaping energy budgets and life-history decisions.

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