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Ahmed Aljohani March 30th, 2021

The effects of circadian rhythm disruptions on monarch butterflies

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2022

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An abstract of
a thesis submitted to the Faculty of Emory College of Arts and Sciences
of Emory University in partial fulfillment
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Bachelor of Science with Honors

Department of Biology

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Abstract

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Organisms have evolved internal biological clocks, known as circadian rhythms, to regulate their physiological functions. Disruptions of circadian rhythms have been associated with the rise of different health complications in multiple vertebrates and mammals, including humans, known as “shift work diseases“. Little is known regarding the developments of such diseases, but one explanation is that the misalignment of circadian clocks directly impacts immune function. Multiple studies have implemented animal models to study this phenomenon in animals. However, most models are heavily focused on a small number of nocturnal species, which may be poor reflections of diurnal situations, thereby highlighting the need for more circadian studies to include diurnal animals. Here we use the diurnal monarch butterflies (*Danaus plexippus*), one of the most acquainted migratory animals, to investigate the possibility of using them as a diurnal animal model for circadian disruptions and immune studies. Monarchs fight off infections from a naturally occurring virulent parasite; however, little is known about the relationship between their circadian rhythm and immunity. We exposed monarchs to multiple circadian disruptions to investigate their immune rhythmicity. Additionally, we observed monarchs’ behavior to determine if monarchs retrain their circadian rhythms after being disrupted. Our results showed a negative effect on the immune response when monarchs larvae were reared in constant darkness; however, we did not observe immune response rhythmicity in monarchs. Behavioral assays showed that monarchs have sleep rebound behavior to retrain their biological clock after a disruption period characterized by constant exposure to light. Further investigations into immune mechanisms using other immune measures such as cell lines or using infected monarch butterflies could clarify how circadian rhythms regulate monarchs’ immune response against parasite infection.

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Acknowledgements

We thank Kandis Adams for teaching the necessary skills used in this experiment, Scott Villa for the recording system training, Joselyne Chavez, and the rest of the de Roode lab for helping with monarch rearing and maintenance, and Erik Edwards for plant maintenance. Moreover, I thank my family, friends, and my advisor Jacobus de Roode for their constant support and encouragement.

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The effects of circadian rhythm disruptions on monarch butterflies

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Introduction

Many organisms, including bacteria, plants, insects, and mammals, have evolved a biological periodicity clock of roughly 24 hours known as circadian rhythm (Dunlap., 1999). It allows organisms to synchronize their physiological and behavioral responses with the Earth's daily rotation. The rhythms exist across various species, from bacteria to plants and insects to mammals, including humans (Johnson et al., 1996; Reppert and Weaver, 2002; Froy et al., 2003, Tataroglu and Emery., 2014; Cohen and Golden., 2015). The importance of the biological clock has been shown in studies that disrupted these rhythms since all levels of biological organization within an organism are subject to circadian regulation, from gene expression to immune function, behavior, and development (Dunlap., 1999; Denlinger et al., 2017; Scheiermann et al., 2018).

Existing research suggests that disruption of the biological clock reduces health and survival while increasing pathogenic infections. Multiple studies on mammals and flies have revealed that disruptions to the circadian rhythm can lead to severe biological consequences. In one study, flies exposed to jet lag survived only 60 days compared with 80 days for controls (Pittendrigh and Minis., 1972). Circadian disruption also impacts host-parasite infections and infectious disease outcomes. In mice, disrupting circadian rhythms enhances herpes and influenza A infections. Primarily, disruption of circadian rhythms causes impairment of the mouse immune response, resulting in increased susceptibility to infections with pathogens (Edgar et al., 2016; Bellet et al., 2013). Similarly, flies

lacking circadian rhythms in locomotor activity show enhanced sensitivity to *Streptococcus pneumoniae* and *Serratia marcescens* infections (Stone et al., 2012).

Humans under circadian disruptions, occurring during chronic shift work or multiple time-zone travel, have shown an increased incidence and severity of several cancers, autoimmune disorders, obesity diabetes, and stroke. The mechanism causing the progress of this so-called “shift work diseases” is unknown. However, one potential explanation is that disrupting the circadian rhythm hinders immune function allowing for such diseases to develop (Stowie et al., 2019). Shiftwork animal models, like mice, have been employed to demonstrate the deleterious effects of circadian disruption on immune rhythmicity. However, most of these models are heavily biased toward a small number of species. Also, most studies have focused on nocturnal animals, which may be poor reflections of diurnal situations. Yan et al. (2020) showed that diurnal animals are not simply reversals of nocturnal animals but have distinctly different rhythms and behaviors, thereby highlighting the need for more circadian studies to include diurnal animals.

Our study used diurnal animals and a natural host-parasite relationship to investigate the effects of constant darkness and frequent schedule changes on the immune system and behavior. We used *Danaus plexippus* (monarch butterfly) and its naturally occurring protozoan parasite, *Ophryocystis elektroscirrha* (OE), to investigate the clock’s role in larvae’s immune response. The monarch butterfly is known for its long-distance fall migration in which it travels up to 4500 km from the eastern United States and Canada to central Mexico (Urquhart., 1976). To make this demanding journey, monarch butterflies use a time-compensated sun compass to maintain southern flight, regulated by their biological clock (Reppert et al., 2016; Shlizerman et al., 2016). When monarch butterflies are infected with (OE), the parasite starts forming spores on the outside of adult

butterflies. The infection passes on when the monarch larvae ingest the parasite spores on the contaminated eggs and plant foliage transmitted from the adult butterfly during the egg-laying process; high parasite doses can decrease larval survival and impede the monarch's ability to migrate (Altizer et al., 2004; Sternberg et al., 2012). Importantly, studies in adult monarchs reveal that disrupting the adult monarch clock leads to blunted clock gene expression, changes in the timing of adult emergence from the pupa, and altered migration flight patterns (Froy et al., 2003). In this study, we investigate the rule of circadian rhythm on monarchs' immune response and their behavior to explore the consequences of the biological clock disruption.

In the first experiment, we explored the consequences of circadian disruption on butterflies to explore if they express a sleep rebound behavior. A previous study showed that the monarch caterpillar's clock is more challenging to phase shift than the adult stage, and therefore it might be harder to observe behavioral changes in caterpillars (Adams et al., 2021). Therefore, we used adult monarchs and subjected them to frequent schedule changes for eight days. We put monarchs in netted cages separated by sex to avoid unwanted mating. At first, monarchs were subjected to 48 hours of light-dark controls (LD) for adjustment (0700h to 2100 h), followed by 48 hours of disruptive constant light (LL), then 48 hours again under light-dark controls (LD) to observe any sleep rebound behavior. Lastly, the monarchs were exposed to 48 hours under constant darkness. Their movements were recorded by a bird-view camera placed above each cage for the experiment duration.

For the second experiment studying the effects of circadian disruption on the immune function of monarchs, we focused on the larval stage because larvae are the only monarch life stage that can be infected by*O. elektroscirrha* (Adams et al., 2021), meaning that the larval stage serves as the starting point for the parasite life cycle in which the immune system will be fighting off the infection. Newly

hatched monarch larvae were divided into three groups: stab-control group under light-dark condition (LD), treatment group under light-dark condition (LD), treatment group under constant darkness condition (DD). Monarch larvae were reared in incubators until they reached the fifth instar, the last larval stage of their pre-pupal development. The treatment groups were injected at four different time points within 24 hours with LPS (lipopolysaccharide), a bacterial endotoxin that triggers an immune response and is used in many immune studies (Stowie et al., 2019). Their blood was analyzed for an immune response using LB agar plates with *E.coli* bacteria to measure the zone of inhibition that did not contain bacterial growth. The established importance of the biological clock driving monarch biology and the occurrence of a natural debilitating parasite make monarchs ideal for studying the role of the biological clock in regulating the immune response and host-parasite interactions.

Methods:

Animal Husbandry

We carried out two experiments to determine the effect of circadian rhythm disruption on monarchs' behavior and monarchs' immune response. Monarch larvae were the outbred offspring of monarchs originally collected from St. Marks, Florida. Eggs were laid on *Asclepias curassavica,* which is a tropical plant that serves as the monarch's larval host plant. These plants were grown in the Emory University greenhouse.

Circadian disruption effects on monarch behavior (April 2021). Wild-caught butterflies collected from St. Marks, Florida, mated in 0·6 m3 mesh cages. Mating pairs were transported to a separate cage, and after dissociation, the male was removed. *A.curassavica* was provided for female oviposition. Experimental larvae were picked randomly from different lineages and reared on *A.curassavica* in

clear plastic tubes (5-inch diameter, 22.5-inch height) until pupation. After pupation, pupae were glued to the lids of solo 16oz cups with hot glue. Adult monarchs were transferred to separate glassine envelopes a few hours after emergence and held in a DigiTherm® incubator at 12°C. We then checked the abdomens of each monarch for signs of *Ophryocystis elektroscirrha* (OE) spores. We housed uninfected monarchs (n=32) in medium-size (91.5 cm × 30.5 cm2) mesh pop-up cages. Each cage contained four monarchs of the same sex to avoid unwanted mating that might disrupt the results with equal numbers from each sex (females n= 16, males n=16). The cages were placed in temperature- and humidity-controlled walk-in climate chambers at 25°C, with TL835 fluorescent white light tubes (with roughly 2000 lumens experienced by individual monarchs). The goal of the first experiment was to observe the changes in monarch behavior under different circadian conditions (figure 1). Therefore, recording cameras were placed to give each cage a bird-view of the monarchs. Recording remained continuous until the end of the experiment. We chose adult monarchs because they are more likely to express behavioral changes related to circadian rhythm disruptions than caterpillars (Niepoth et al., 2017)

At the beginning of the experiment, monarchs were given 48 hours under light-dark (LD) control conditions (0700h to 2100 h), followed by 48 hours under constant light (LL). After that, monarchs were subjected again to 48 hours of light-dark (LD) control conditions (0700h to 2100 h) to observe any sleep-rebound behavior. Lastly, monarchs were put under constant darkness for 48 hours. Monarchs were fed with 10% honey water that we replenished daily. At the end of the experiment, the footage was collected for analysis, and monarchs were placed in glassine envelopes in the DigiTherm® incubator. To measure monarchs' behavioral changes, we observed each cage's footage and calculated the number of seconds when there is a movement in the cage. The movements were

divided into day movement (0700h to 2100 h) and night movement (2100h to 0700 h) to resemble the respective normal circadian conditions.

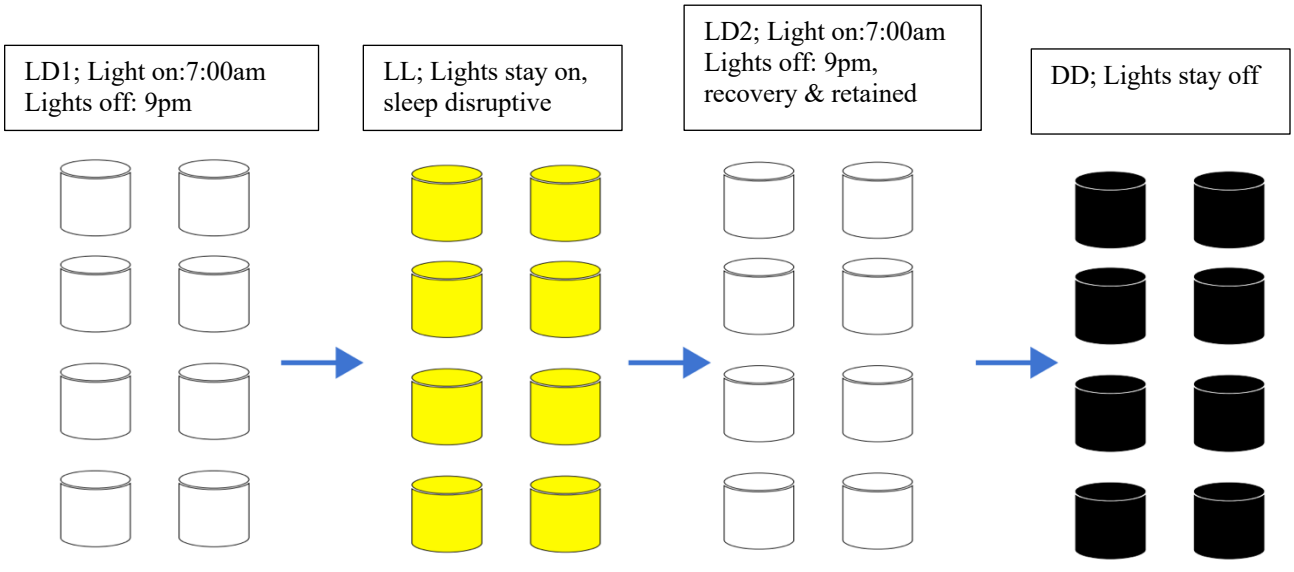

Figure 1 Experimental Method for the behavioral analysis

Effect of circadian disruptions on immunity experiment (October 2021). Newly hatched caterpillars from three different lineages were divided into three groups: stab-control group (n=24, light-dark (LD) condition (Z0= 0700h to Z14= 2100 h)), treatment control (n=48, LD condition), treatment disturbance group (n=48, constant darkness (DD) condition). Each group was placed in its own separate light- and humidity -controlled incubator with cold-cathode compact florescent light bulbs that produce white light (with roughly 400 lumens experienced by individual monarchs) (Tritech Research, Inc). Two caterpillars were housed together in clear 16-ounce solo cups with the temperature maintained at 27°C. A digital thermometer was placed inside each incubator to monitor the internal temperature. No significant temperature changes in lights-on or lights-off were recorded. Temperature only changed by +-1°C. Caterpillars were fed with *A.curassavica* leaves from the plants grown in the Emory University greenhouse.

Immune stimulation and humoral zone of inhibition

After the caterpillars reached fourth/fifth instars, the 24 hours sample collection started. Twelve caterpillars were collected from the two treatment groups at four different times during the day (Z4=11am, Z10=5pm, Z16=11pm, Z22=5am). Six caterpillars were injected with 1µl of LPS (lipopolysaccharide), a bacterial endotoxin that triggers an immune response, and treatment controls were injected with 1 µl water. Four hours post-injection, one µl of hemolymph was taken from each caterpillar, including the stab-control group, and loaded onto LB agar plates containing *E.coli*. The plates were incubated at 37°C for 48 hours to allow bacterial growth and then moved to 4°C. The area that did not have any bacterial growth around the loaded sample was measured in mm2 using the software ImageJ after taking a picture using a 3.5X-180X dissecting scope.

Statistics

Most statistical tests were conducted with JMP software (JMP 15, SAS Institute Inc., Cary, NC). Complete factorial analysis of variance (ANOVA) or Tukey's range test were used to assess monarch behavior between day and night and the zone of inhibition to compare the immune response across the different collection times (4 levels: Zt= 4, 10, 16, 22), LPS treatments (2 levels: control and treated) and their two-way interaction as the explanatory variables. Group differences were considered significant at $p \leq 0.05$.

Results

Circadian disruption effect on monarch behavior

Monarch movement. The collected behavioral data consisted of the number of seconds of movement in the cage overall and day movement (0700h to 2100 h), and night movement (2100h to 0700 h). In the light-dark controls, and constant light (LD1, LD2, LL), we observed a significant drop in night

movement in comparison to day movements; however, in constant darkness (DD), no significant difference was found between day and night (Figure 2). During the respective nighttime in constant light (LL), monarchs expressed higher movement than the night movement in the controls. This could be a result of increased physiological stress due to the imposed sleep-deprived condition. No significant difference was found between male and female movements across the different circadian disruptions (Figure 3).

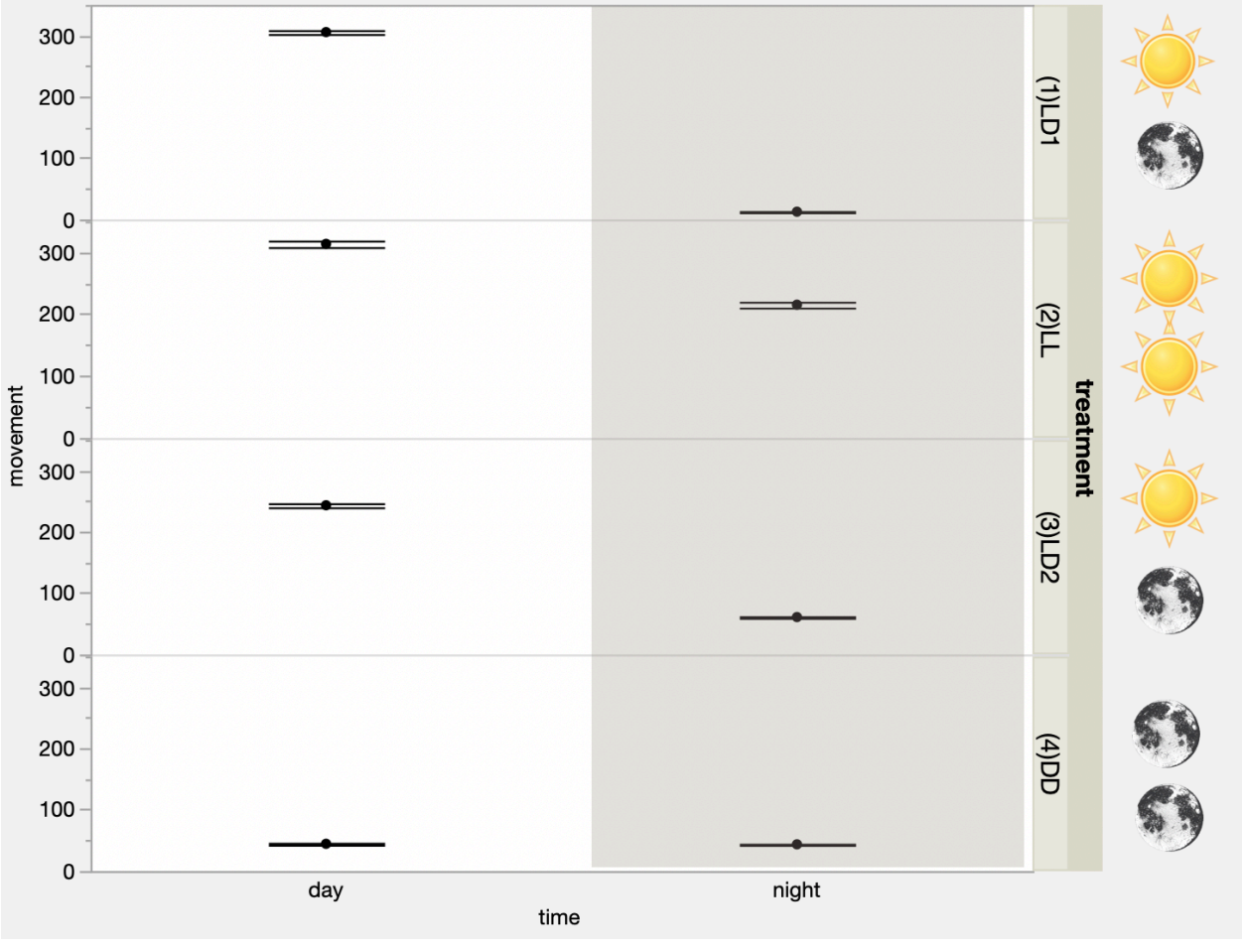

Figure 2. The effects of multiple schedule changes on monarch movements in day vs night (n=32). LD1 and LD2 represent the light dark-controls, LL constant light, and DD constant darkness. White is respective day time and shaded is respective nighttime. Each value is mean +/- SEM. Full factorial ANOVA: day and night movement in LD1, LL, LD2 $p < 0.0001$, DD condition $p = 0.4871$. Abbreviations: ANOVA = analysis of variance; SEM = standard error of the mean.

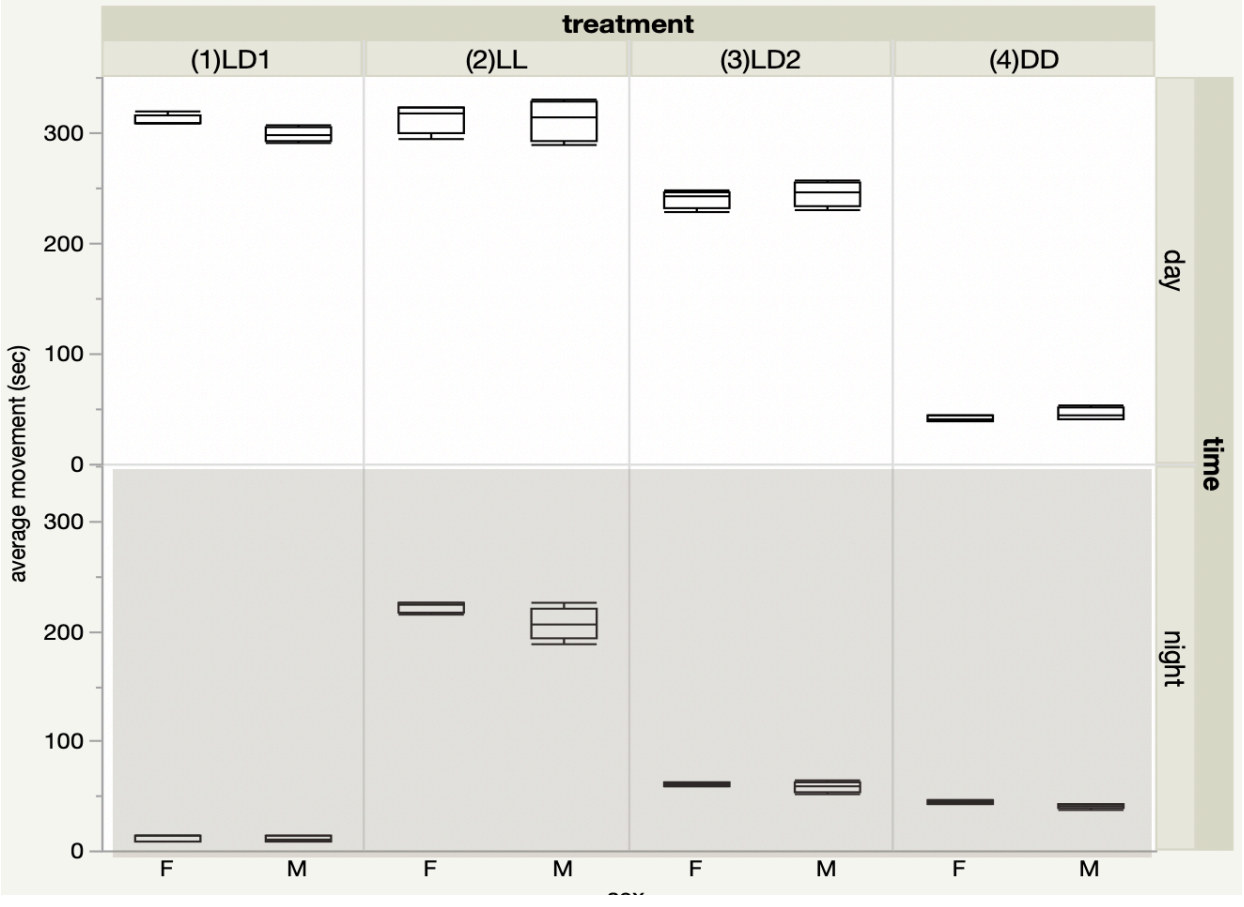

Figure 3. The effects of multiple schedule changes on the movement of male monarchs (M, n=16) and female monarchs (F, n=16). LD1 and LD2 represent light-dark controls, LL constant light, and DD constant darkness. White is respective day time and shaded is respective nighttime. Each value is mean + SEM. Full factorial ANOVA: day and night movement in LD1, LL, LD2, DD $p < 0.0001$. Abbreviations: ANOVA = analysis of variance; SEM = standard error of the mean.

We further investigated the effect of circadian disruptions on monarch behaviors by comparing their different activities on individual days and nights (Figure 4). In the first schedule change of light-dark controls (LD1), we found a clear difference in monarch movement between day and night overall. Moreover, we found a significant reduction in monarch behavior on the second day compared to the first day. This can be attributed to the monarchs being introduced to a new environment and moving outside their envelopes on the first day, and their becoming accustomed on the second day. During the constant light disruption (LL), monarchs showed a similar pattern to light-dark controls (LD1) during the day. However, they showed a higher movement during the respective nighttime relative to when they were under light-dark controls (LD1). Additionally, their night movement was

significantly lower than during the day, indicating monarchs sensed that time should be regular nighttime, but the light might have disrupted them.

Monarchs showed a significant drop in their activity during the first day of the following schedule change: light-dark controls (LD2). Monarchs moved less than the observed day's movement during the first light-dark controls (LD1), implying induced stress caused by constant lights on monarchs. The second day of LD2 showed a similar pattern to LD1, suggesting their circadian rhythm was retrained. When put under constant darkness (DD), monarchs almost stopped moving. No significant difference was found between the individual timepoints except between the first day and the second night.

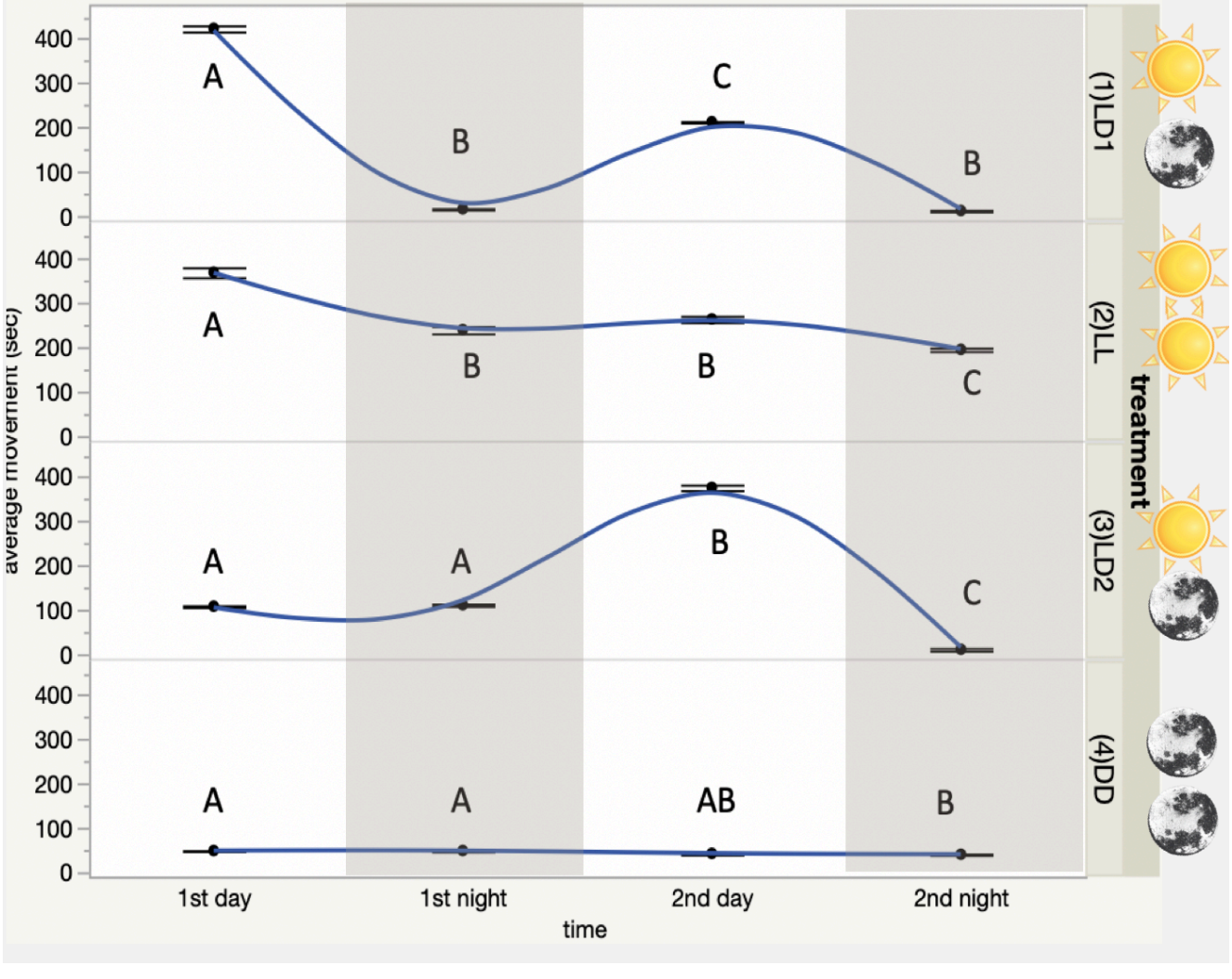

Figure 4. Monarch movement changes on individual days and nights under circadian disruptions (n=32). LD1 and LD2 represent the light-dark controls, LL constant light, and DD constant darkness. White is respective day time and shaded is respective nighttime. Each value is mean + SEM. Full factorial ANOVA: different letters resemble a significance group ($p < 0.05$) within each treatment (LD1, LL, LD2, DD). Abbreviations: ANOVA = analysis of variance; SEM = standard error of the mean.

Effect of circadian disruptions on immunity

Monarch's general immune response LD vs DD. Figure 5 represents the different components of the Humoral Zone of Inhibition assay used to measure the monarch's immune response. The *E.coli* bacteria are labeled with A, the Zone of Inhibition (ZOI) with B, and the loaded sample with A. The size of the collected zone of inhibition area reflects the magnitude of the immune response. As shown in Figure 6, the light-dark condition (LD) control showed a weaker immune response when compared to controls (con) injected with water versus the group injected by LPS. This is anticipated as water should not produce a significant immune response compared to the LPS treated group. The caterpillars exposed to LPS under light-dark conditions had a larger zone of inhibition than the group under constant darkness, suggesting that the circadian disruption weakened the immune system. Furthermore, the group that was not stabbed did not produce any zone of inhibition. This treatment was needed to eliminate the probability that stabbing itself could induce an immune response.

Monarch circadian immune response. We failed to observe a rhythmic immune response across the different injection times (figure 7). In light-dark condition (LD), the caterpillars injected with LPS showed a higher immune response in comparison to controls (con) with a significant increase at Zt=10 (5 pm, $p<0.05$). In constant darkness (DD), the control and LPS groups showed a similar immune response, suggesting that constant darkness weakened the caterpillars' immune response. An exception happened at Zt=22 (5 am, LD), with the LPS treatment showing a lower immune response than the controls.

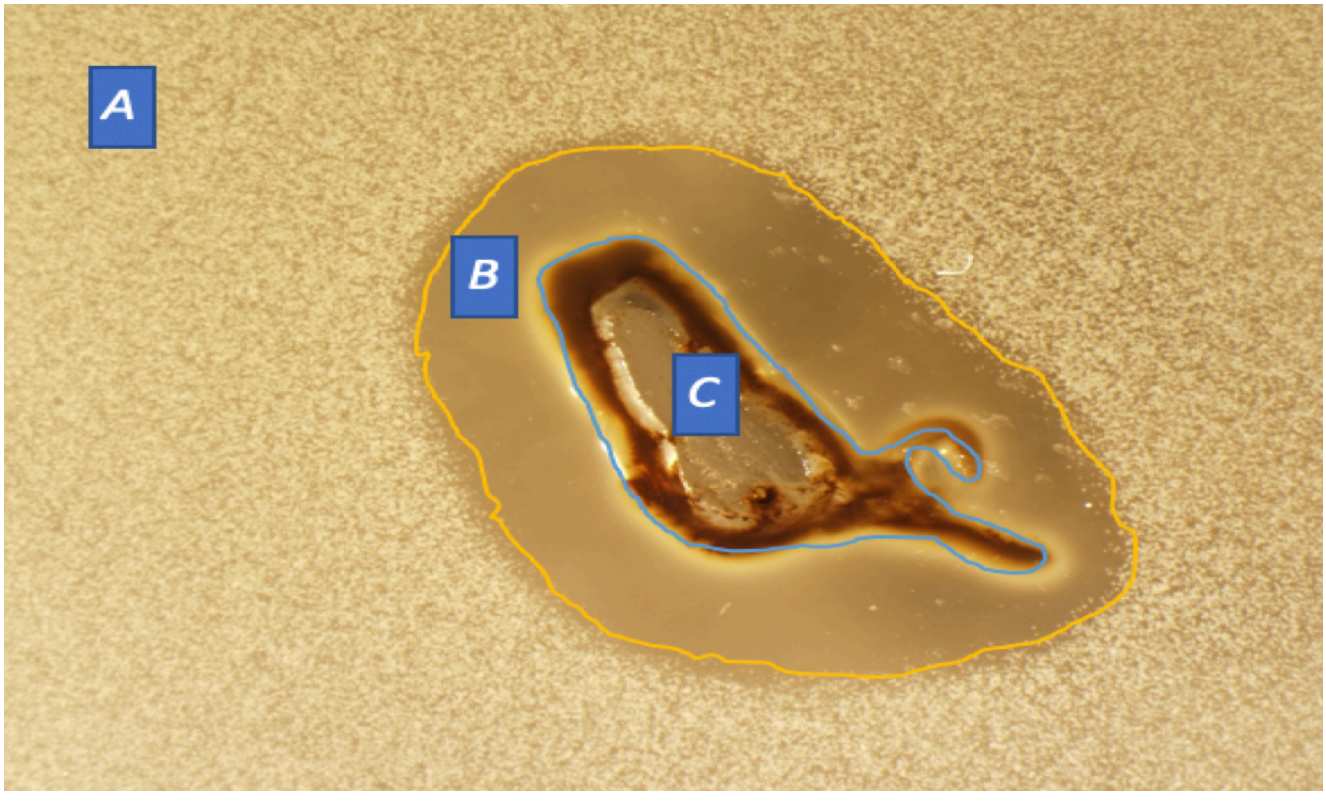

Figure 5. Sample of ZOI taken from one of LD samples.

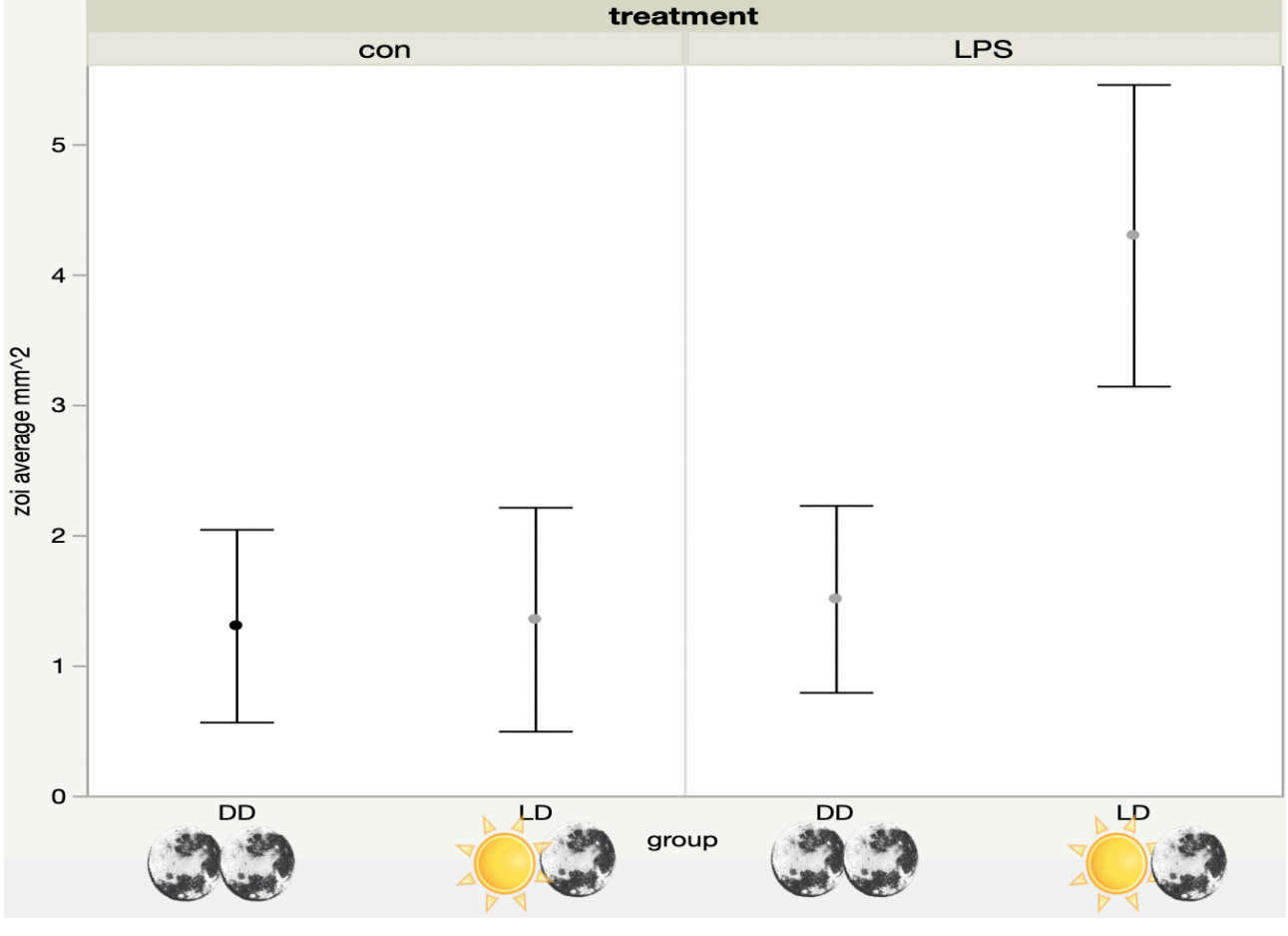

Figure 6. Zone of inhibition (ZOI) assay in DD vs LD. Effects of circadian disruptions on the zone of inhibition measured using imageJ 1.52k. Caterpillars in the treatment group (LPS), treated with LPS, and control group (con), treated with water, were reared in two conditions: light-dark controls (LD, n=32) and constant darkness (DD, n=32). Each value is mean + SEM. LPS had a significantly higher ZOI in LD ($p < 0.05$). Abbreviations: ANOVA = analysis of variance; SEM = standard error of the mean.

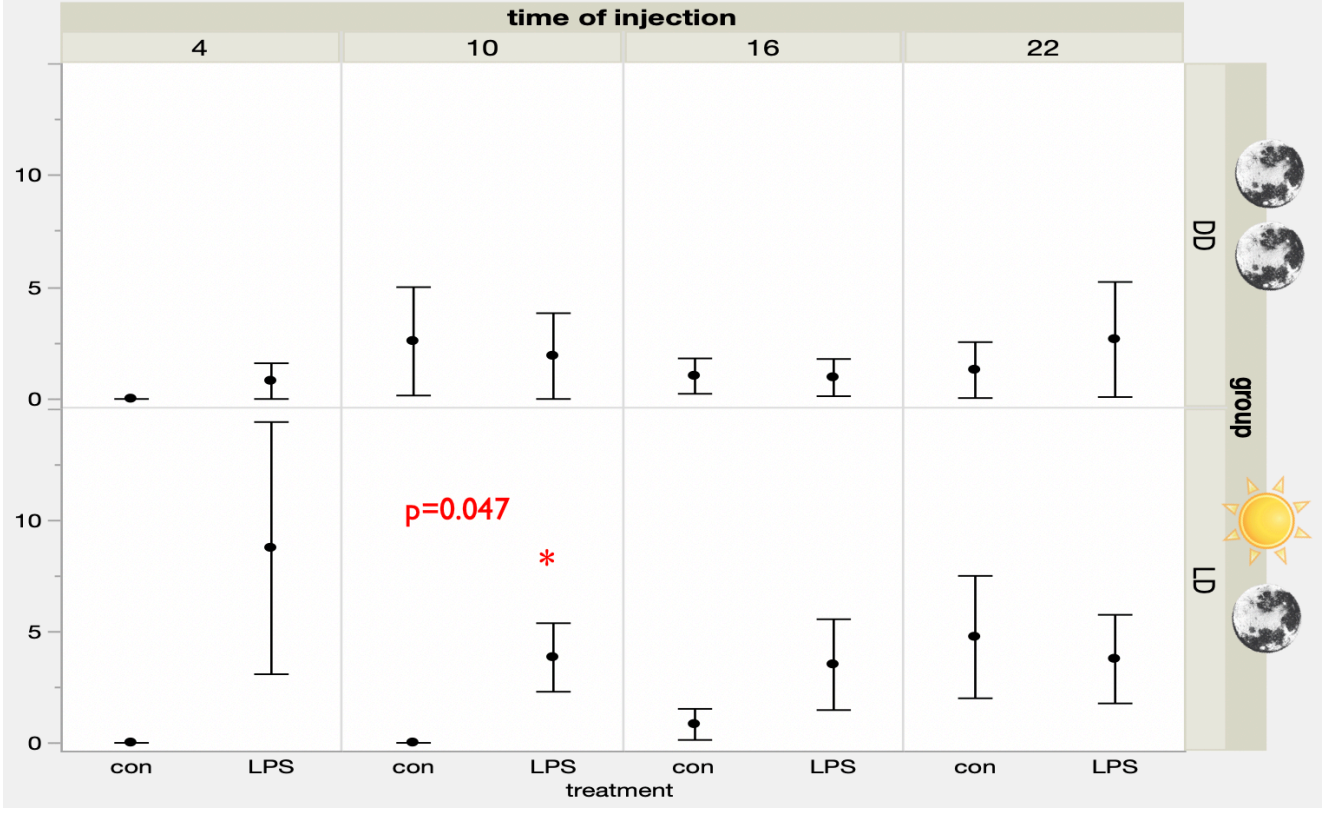

Figure 7. Zone of inhibition (ZOI) assay in LPS vs control across different injection times. Effects of circadian disruptions on the zone of inhibition measured using imageJ 1.52k. Caterpillars in the treatment group (LPS), treated with LPS, and control group (con), treated with water, were reared in two conditions: light-dark controls (LD) and constant darkness (DD). Different caterpillars were injected at four timepoints (Zt=4, 10, 16, 22). Samples were collected four hours post-injection and plotted. Each value is mean + SEM. The LPS group had a significantly higher ZOI in LD at Zt=10 ($p < 0.05$). Abbreviations: ANOVA = analysis of variance; SEM = standard error of the mean; Zt0= 7am.

Discussion

Circadian disruption has shown different health effects in mammals. Mice exposed to constant light in early development stages experienced disrupted development of mouse immune tolerance and decreased survival (Mizutani et al., 2017; Castanon-Cervantes et al., 2010). Similarly, the circadian rhythm plays a vital role in monarch migration and development. Disruptions of the rhythm lead to

changes in the timing of adult emergence from the pupa and altered migration flight patterns (Froy et al., 2003). While studies have shown that circadian disruption affects monarchs and blunts their clock genes, little is known about the rhythmicity of their immune system. In this study, we examined the effect of circadian disruption on monarch immune responses and their behavior.

The larval stage in monarchs' serves as the starting point of *Ophryocystis elektroscirrha* (OE) infection; the monarch can only be infected with OE during its larval stage (Adams et al., 2021). Our work failed to observe rhythmicity in caterpillar immune response. However, we observed a decrease in immune function in caterpillars under circadian disruption (constant darkness, DD), suggesting a possible immune response regulation by the circadian rhythm.

A time-compensated sun compass regulates the demanding annual migration by monarchs. It allows them to keep a southern flight during their long-distance fall migration to central Mexico from the eastern United States and Canada. One possible hypothesis is that light plays a critical role in regulating monarch physiological functions from development to adult emergence and migration. The light could play an essential role in regulating monarch immunity as one study found constant light to increase infected monarch larval and pupal development time without increasing parasite growth. Incontrast, increased parasite growth was observed under constant darkness (Adams et al., 2021). Our study observed a weaker immune response in the absence of light, signifying that it might play a crucial role in immunity.

In a similar pattern, monarchs expressed a significantly lower movement under constant darkness than the normal light-dark controls. Previous research has shown that the diurnal honeybee stops moving during the night due to an obscure vision caused by a low level of light that hinders their

ability to detect objects and dangers (Liporoni et al., 2020). This might be the case for monarchs as well. Additionally, monarchs might prefer to stay put and sleep to avoid dangerous nocturnal predators. When adult monarchs were under constant light (LL), their movement decreased during the respective night time, indicating their recognition that it should be time for sleep, based on their circadian rhythm. Nevertheless, they did not stop moving ultimately, unlike the (LD1) nighttime, denoting that light disrupts their behavior.

A downside to these conclusions is that little is known about monarch sleep. We have managed to find they only stop moving during the night. However, adult monarchs expressed a sleep rebound behavior after the constant light conditions (LL) induced continuous stress. These diurnal insects exhibited a lower movement time during the day for the second standard light-dark (LD2) condition, possibly to retrain their circadian rhythm since they showed a significantly higher movement 24 hours after being in the control's conditions.

Light pollution is a phenomenon that is increasing right now in the world, and more studies should be conducted to confirm if light pollution is disturbing the monarch's wild population by affecting their sleep patterns and disrupting their migration and development. Additionally, it is not clear yet whether monarchs' immune response is regulated by circadian rhythm, and few conclusions can be drawn about light's effects on monarch immunity as ZOI is just one immune measure; further analysis should include other immune measures. The study of circadian disruption effects on immunity is complicated by the inability to control for immunologically relevant behavioral and environmental factors (Stowie et al., 2019). Cell lines provide an excellent approach to this problem, allowing for direct accurate measurements of the immune response as well as the genetic components of the clock and subsequent effect on cellular function in a precise, cell-type-specific

fashion. Future steps will focus on using a constant light (LL) experimental group with the same experimental design to determine the effects of light on monarch immunity using OE-infected monarch larvae. Working with infected monarchs in the future will help detect the effects of light on monarch health and how it affects the immune system. Furthermore, the definition of sleep for monarchs needs to be identified and explored to confirm their sleep rebound ability.

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