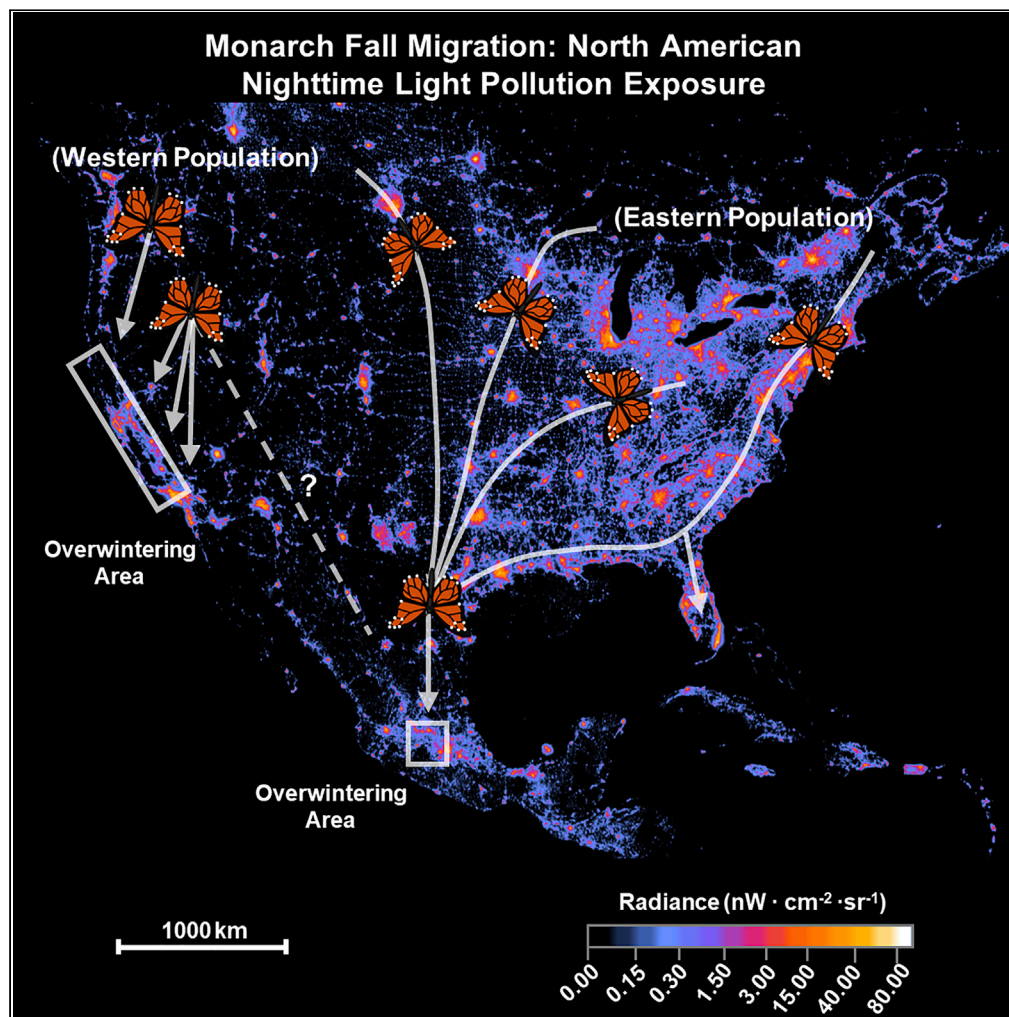


Article

Oriented migratory flight at night: Consequences of nighttime light pollution for monarch butterflies



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Highlights

Nighttime light pollution can disturb diurnal migratory monarch butterflies

Exposure to this pollution induces abnormal activity in normally quiescent monarchs

This pollution acts as sensory noise that perturbs the circadian clock of monarchs

Conservation should consider susceptibility of habitat to nighttime light pollution

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Article

Oriented migratory flight at night: Consequences of nighttime light pollution for monarch butterflies

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SUMMARY

We show that light trespass—a form of nighttime light pollution (NLP)—elicits normal daytime clock-mediated migratory behavior in fall monarch butterflies during their night-cycle. In controlled indoor flight simulator studies isolating the role of NLP on the expression of oriented migratory flight using a time-compensated sun compass, a full-spectrum light source consistent with lights used outdoors at night by the public, triggered proper fall directional flight at night in monarchs. Monarchs remained quiescent when initially placed in the flight simulator in the dark, but flight was immediately triggered when our light source was turned on. This nighttime behavior was identical to that seen in outdoor free-flying fall conspecifics during the day. The light source provided directional cues equivalent to those provided by the sun and could either phase-advance or phase-delay monarchs. Our study highlights the negative consequences of NLP on diurnal animals, especially those that rely on clock-mediated behavior.

INTRODUCTION

Nighttime light pollution (NLP), produced by anthropogenic artificial light sources, is a modern environmental stressor and can range from glare, over-illumination, skyglow, and light trespass (Figure S1, Table S1; Gaston et al., 2012; Kyba and Hölker, 2013). As a form of sensory pollution (Dominoni et al., 2020), NLP can affect individuals by either artificially extending their subjective day, subjecting individuals to aberrant light stimulus events or conditions at night, or prematurely shortening their normal subjective night cycle. A major consequence of these light perturbations at night is that it can induce significant disruptions to the normal circadian rhythms and sleep-wake cycles of the wildlife and humans that are exposed to it (Grubisic et al., 2019), and therefore disrupt daily strategies, behaviors, and physiological processes important for organismal function and survival (Russart and Nelson, 2018).

Animal migration is one example of a taxa-wide natural phenomenon that can be significantly disrupted by NLP, with previous work demonstrating that NLP affects the migratory ability of animals that migrate at night (e.g., nocturnal avian species, Van Doren et al., 2017). NLP produces unnatural light stimuli or landscape light conditions that can interfere with the use of navigational mechanisms that employ nighttime celestial cues (Torres et al., 2020; Voigt et al., 2017). Although a growing body of work supports the detrimental effects of NLP on nocturnal migratory species (St Hilaire et al., 2012), the effects of NLP on species that migrate during the day remain understudied and unknown (Gaston et al., 2013, 2017).

For diurnal species, previous work has shown that light exposure at night can interfere with normal circadian clock function. In controlled experiments, light pulses presented during the night cycle have been shown to either phase-delay or phase-advance individuals across a wide-range of species (Froy et al., 2003; Grone et al., 2011; Glickman et al., 2012; St Hilaire et al., 2012; Kennedy and Hudson, 2016; Tähkämö et al., 2019), resulting in a shift of the phase response curve (PRC) of their circadian clock. This induces a shift in various biological processes such as the timing or performance of behavior. Constant light conditions have also been shown to eliminate proper circadian clock function in animals, thereby disrupting normal behavior altogether (Merlin et al., 2009; Marrus et al., 1996). As such, for diurnal migratory species, the negative effects of NLP might manifest as disrupted circadian clock function, which in turn might disrupt migratory behavior. The negative effects of NLP on the migratory behavior of diurnal animals is possible, as it has been shown that sufficient light perturbations found in urbanized landscapes because of NLP have

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the capability to disrupt nonmigratory clock-mediated behaviors in animals (Dominoni et al., 2013; Gaston et al., 2012). Many diurnal migrants use a sun compass, with the sun producing various visual cues (e.g., azimuthal position, elevation angle, and polarized light patterns) that can guide migratory movement in the proper migratory direction (Guilford and Taylor, 2014; Heinze, 2017). Given the shifting compass position of these solar cues during the day, many individuals use a time-compensated sun compass, as they use timing information from their circadian clock, which is entrained to local photoperiodic conditions, to correct and compensate for these shifts (Merlin et al., 2009; Guilford and Taylor, 2014). Given the key role of this timing information, NLP might target this key vulnerability in the sun compass mechanism, by perturbing or modulating normal circadian clock function such that the performance of oriented migratory movement is affected.

Monarch butterflies (*Danaus plexippus*) are a famous example of long-distance migratory animals that use a time-compensated sun compass as a primary mechanism to guide their migratory flight during the day (Perez et al., 1997; Reppert et al., 2004; Froy et al., 2003; Reppert and de Roode, 2018; Taylor et al., 2019). A multigenerational migratory species, perhaps the most spectacular and well-known aspect of monarch migration is the iconic fall migration of monarchs in Eastern North America. Here, millions of fall monarchs living east of the Rocky Mountains leave their summer breeding grounds in Southern Canada and the Northern United States, often traveling up to 4,000 km during a maiden voyage to migrate southwards to a handful of overwintering sites in Central Mexico (Guerra, 2020). A key component of the time-compensated sun compass used by fall monarchs is the antennal clock mechanism that provides the relevant timing information for the sun compass, with previous work showing that direct exposure to light cues entrains and mediates proper antennal clock function (Merlin et al., 2009; Guerra et al., 2012).

In this study, we conducted indoor controlled laboratory flight simulator studies in which we were able to simulate and isolate the effects of light trespass perturbation on diurnal migratory animals at night. Using fall monarchs as a model, we tested the hypothesis that light trespass conditions are relevant environmental sensory perturbations that can adversely affect diurnal migratory animals. As a form of NLP, light trespass consists of light being cast where it is unwanted or not needed during the night, when and where it would otherwise be dark (Table S1; Gaston et al., 2012) and emanate from human infrastructure such as buildings to streetlamps resulting from nighttime anthropogenic activities (Gaston et al., 2013, 2017). This form of sensory pollution is ubiquitous across the landscape, particularly in urbanized areas but is also present in suburban and rural habitats. Therefore, light trespass is a relevant form of NLP that can be experienced by fall monarchs while resting at night during migration, such as when roosting in a tree that is immediately adjacent to a streetlamp or when in a pollinator garden in close proximity to the lights of homes, businesses, and street traffic.

We first examined whether a full-spectrum light source, consistent with ubiquitous and common anthropogenic artificial light sources used at night, could elicit normal oriented migratory flight (southwest directionality using a time-compensated sun compass; Guerra, 2020) in fall monarchs during the day. As the sun is the brightest sky parameter that monarchs can use for directionality during the day, we predicted that our artificial full-spectrum light source, presented as the sole and brightest light source during daytime testing, would elicit normal southwards flight orientation in fall monarchs. We simulated two daytime conditions by testing monarchs with two opposing azimuthal positions with corresponding sun elevations in the morning (ZT3, Figure 1A) and the late afternoon (ZT9, Figure 1C) using our light source. Given the apparent shifting of the sun's position in the sky during the day in the Northern Hemisphere, we predicted that monarchs tested during the morning and presented with a morning azimuthal position would keep the light source to their left when orienting southwards (Figure 1A). In contrast, monarchs tested during the afternoon and provided an afternoon azimuthal position would keep the light source to their right to maintain southwards directionality (Figure 1C). As the sun can provide other cues that affect migratory flight directionality in monarchs besides azimuthal position, we also tested monarchs during the day in which we manipulated the angle of our artificial light source (elevation). The angle of elevation of the sun at noon appears to control the expression of oriented migratory flight during the fall migration, with oriented flight no longer observed when the sun's elevation is outside a specific sun angle window at solar noon (SN), i.e., between 46° and 57° (Sun Angle at Solar Noon, SASN, hypothesis; Taylor et al., 2019; Figure 1B). Finally, we tested if fall monarchs use the light source as a directional cue in a time-compensated manner as they do with the sun in daytime experiments using monarchs with a delayed light:dark (LD) cycle in our flight simulator trials.

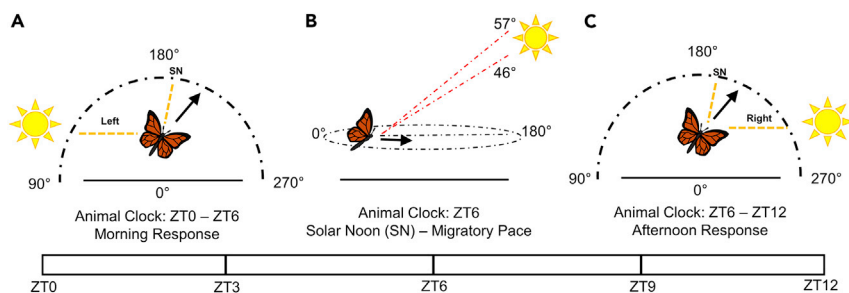


Figure 1. Circadian clock of the monarch butterfly and their flight orientation response in the field to the sun (time-compensated sun compass) during diurnal migration

Flight orientation response of monarch butterflies in their (A) subjective morning at ZT3 where individuals will keep the sun to their left as they fly their migratory southwest direction, (B) maintenance of orientation during the solar noon where the sun angle is between the 46–57° window, and (C) orientation response of butterflies in the afternoon at ZT9 where individuals keep the sun to their right as they fly southwest.

We then tested fall monarchs at night to examine how light trespass might affect their behavior by testing them with our light source at three different ecologically relevant time points during their night cycle (ZT15, ZT18, and ZT21). These light trespass stimuli represent realistic NLP perturbations that fall monarchs can experience during migration, such as the light from buildings that can artificially extend the subjective day (e.g., ZT15 light trespass stimulus) or light from streetlamps that can come on later at night which can simulate the earlier onset of the next morning (e.g., ZT18 and ZT21 light trespass stimuli). These three conditions were tested to see if light trespass might elicit normal oriented flight at night, but also to specifically test the effects of light trespass on the circadian clock of monarchs. For this suite of conditions, we predicted that light at ZT15 would phase delay monarchs, whereas light trespass at ZT18 and ZT21 would phase advance them. These phase-shifts would be reflected in the way monarchs orient their flight in response to light trespass. If fall monarchs were to behaviorally respond in this manner, this pattern of results would be consistent with what occurs with other species in response to light perturbation at night (Levine et al., 1994; Suri et al., 1998) and demonstrate the negative effects of NLP on diurnal migratory animals.

RESULTS

Artificial light is sufficient for normal daytime migratory behavior

We first tested fall migrant monarchs during the day in controlled indoor flight simulator trials testing them for the use of a time-compensated sun compass, using a full-spectrum artificial light source to replace the sun as their source of directional cues. Monarchs tested in the morning with a morning azimuthal light position (ZT3, sun at 10:00; azimuth 110°, elevation 29°, Figure S2A) significantly oriented southwest as a group (231°, Rayleigh's test, $r = 0.725$, $p = 0.0012$, $n = 13$; Figure 2A). Monarchs tested in the afternoon with an afternoon azimuthal light position (ZT9, sun at 17:00; azimuth 245°; elevation 31°, Figure S2B) also significantly oriented southwest as a group (217.0°, Rayleigh's test, $r = 0.747$, $p < 0.001$, $n = 13$; Figure 2B). The southwards orientation of both morning and afternoon tested monarchs were similar (ZT3 v. ZT9; Mardia-Watson-Wheeler [MWW]: $W = 1.02$, $p = 0.600$; Figure S3A) and were consistent with how we predicted monarchs would orient their flight behavior relative to the azimuthal position of a directional light source during the day (Figure 1B and 1C). In addition, our results were equivalent to previously published data for fall migrant monarchs that fly southwest when examined outdoors under clear, sunny skies (Figure S4), illustrating the functional significance of artificial light on monarch flight directionality.

Artificial light features influence migratory flight directionality

To determine the importance of light source elevation on flight behavior, we tested fall monarchs at two representative dates to test the SASN hypothesis, which corresponded to SN sun elevation conditions in Southwest Ohio where monarchs used in our trials were caught (see Supplemental information). When tested with our light source with an SASN elevation corresponding to 15-September (ZT6 SN, azimuth 190°; elevation 53°; Figure S3A), the time of peak monarch migration in Southwest Ohio, monarchs were significantly oriented southwest as a group (228.5°, Rayleigh's test, $r = 0.612$, $p < 0.01$, $n = 12$; Figure 2C). This mean orientation was consistent with the mean southwest orientations observed for our 15-September positions for morning azimuth (ZT3 v. Sept-15 ZT6 SN: [MWW] $W = 2.42$, $p = 0.299$) and afternoon azimuth

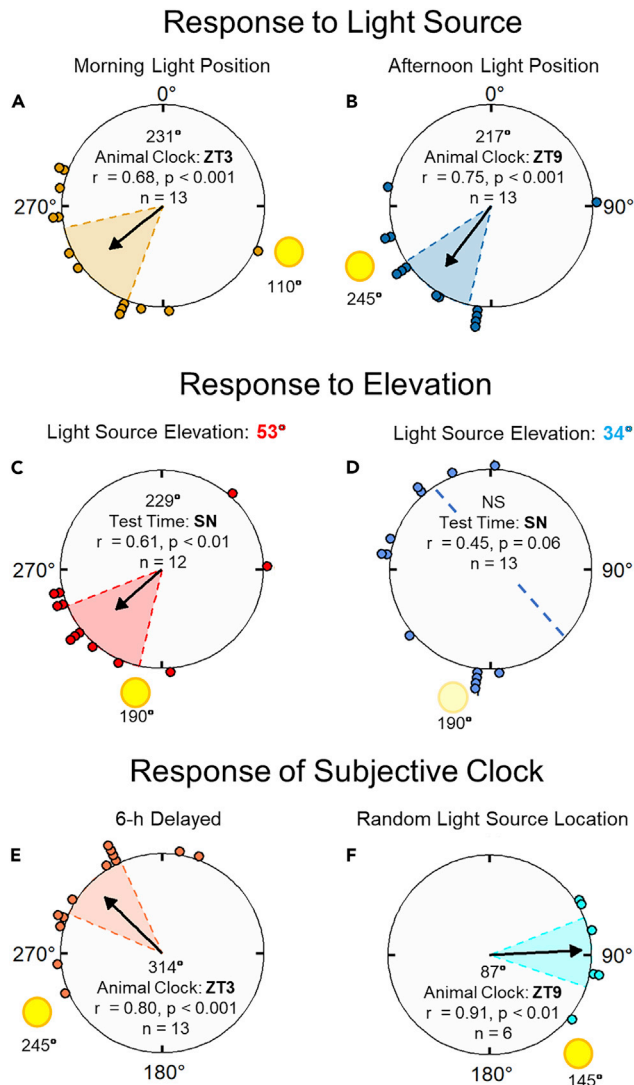


Figure 2. Circle diagrams representing the mean flight orientation response in indoor flight simulator trials with our artificial light source

Light source location is represented by a yellow circle, each arrow indicates the mean vector ($\pm 95\%$ confidence interval) of individual vectors (dots) of tested butterflies. Circle diagram without an arrow consists of butterflies flying nondirectionally as a group. The statistical significance of each mean vector was tested using Rayleigh's test (r is the length of the mean vector, with p value and sample size, n , for each mean vector).

(A) Monarch tested with the light source at the morning azimuthal position of 110° .

(B) Monarchs tested with an afternoon azimuthal light position at 245° .

(C) Monarch butterflies tested with the 15-September sun angle of 53° .

(D) Monarch butterflies tested with the 5-November sun angle of 34° .

(E) Animals housed with a 6-h delayed light:dark cycle tested during their subjective morning during the actual afternoon with an afternoon sun position.

(F) Butterflies tested with the light source at a random location (145°) tested in their subjective afternoon. See also

[Figure S4](#).

(ZT9 v. Sept-15 ZT6 SN: [MWW] $W = 0.831$, $p = 0.660$). In contrast, when monarchs were tested with a SN sun elevation from 5-November in Southwest Ohio (ZT6 SN, azimuth 190° ; elevation 34° ; [Figure S3B](#)), a time during the fall migratory period in which fall migrants would already be arriving at their overwintering sites in Central Mexico, monarchs no longer displayed mean southwest directionality (Nondirectional, Rayleigh's test, $r = 0.46$, $p = 0.0613$, $n = 13$; [Figure 2D](#)). The 34° elevation of our light source represented a migratory scenario where the SASN is outside the hypothesized 46° – 57° threshold window that elicits

oriented migratory flight (Figure 1B). Taken together, not only do our results support the sun providing a variety of cues that affect migratory flight directionality in fall monarchs but that artificial light can also provide the same salient cues that mediate migratory flight directionality as the sun.

Migratory monarchs time-compensate artificial light cues

To show that fall monarchs can use a time-compensated sun compass when tested indoors using our artificial light source, we tested butterflies that had a 6-h delayed LD cycle in flight simulator trials in the afternoon (light source placement: azimuth 245°; elevation 31°) during their subjective morning (ZT3). If monarchs housed under a 6-h delayed LD cycle are time-averaging the position of the sun for southwards-oriented flight, then monarchs should display mean flight orientation that is shifted 90° clockwise. Our 6-h delayed LD monarchs had a mean orientation (314°, Rayleigh's test, $r = 0.801$, $p < 0.0001$, $n = 13$; Figure 2E) that was shifted with the appropriate magnitude and direction (shifted 97° from the ZT9 group with a mean orientation of 217°; Figure 2B); this was consistent with the expected mean orientation response of monarchs migrating during the morning in relation to the sun (Figure 1A) and with what we observed in monarchs tested during the morning with a morning azimuthal light position (Figure 2A). We also tested monarchs during their subjective afternoon (ZT9) with the light source positioned at a randomly chosen location (azimuth 145°) to examine how monarchs would orient relative to the light source. The monarchs were significantly oriented as a group (87.2°, Rayleigh's test, $r = 0.907$, $p < 0.01$, $n = 6$; Figure 2F) as expected if interpreting the light source's location as an afternoon azimuthal position (Figure 1C). Therefore, our results demonstrate that monarchs respond to our artificial light source in a time-compensated manner, as they do when using the sun's position as a cue for maintaining proper southwards fall migratory orientation.

Light trespass can either phase-delay or phase-advance monarchs

After establishing that fall monarchs can fly in the appropriate migratory direction by employing their time-compensated sun compass in response to our artificial light source during the day, we then investigated the effects of light trespass on monarchs by testing them using our light source at night. In night trials, we tested a total of 78 fall monarchs for our different light trespass perturbation treatments at ZT15, ZT18, and ZT 21 (light source position: morning azimuth, azimuth 110°, elevation 29°). These butterflies remained quiescent when initially introduced in the flight simulator in the dark and 96% (75 out of 78 monarchs tested at night) of individuals flew immediately when the artificial light source was turned on. Although tested at night, these monarchs all flew in an equivalent manner as the monarchs that we tested during the day. Of these monarchs tested at night, 63% of monarchs (47 out of the 75 monarchs that immediately flew) reliably met our trial criteria of 1-min burn-in continuous flight, followed by at least 5 min of continuous flight for trial analysis, which was comparable to the behavior of monarchs tested during the day. We observed that 93% of these monarchs (42 out of the 47 fliers that met our continuous flight trial criteria) flew directionally in our light trespass trials.

Monarchs tested at ZT15 were significantly oriented as a group (15°, Rayleigh's test, $r = 0.73$, $p < 0.01$, $n = 10$; Figure 3A), keeping the position of the light source to their right, flying relative to the light source as if tested during the afternoon like butterflies in our daytime trials (Figures 1C and 2B). This result is consistent with our prediction that exposure to light trespass at ZT15 would phase-delay monarchs, as the flight behavior of butterflies at this point during the night was still consistent with the flight behavior of monarchs in the afternoon. At ZT15, the effect of NLP is consistent with prolonging the perceived day of monarchs. ZT15 corresponds with the highest estimated summed neuronal firing rate that is similar to ZT9 during the day.

At ZT18, monarchs were significantly oriented as a group (233°, Rayleigh's test, $r = 0.64$, $p < 0.01$, $n = 12$; top circle diagram, Figure 3B), but this time kept the position of the light source to their left, flying relative to the light source as if tested like butterflies tested in the morning with a morning azimuthal position (Figures 1A and 2A). For trials at ZT18, we additionally tested monarchs but where the position of the light source was moved to an afternoon azimuthal position (245°; bottom circle diagram, Figure 3B). Despite the different azimuthal position, monarchs were also significantly oriented and flew with a mean direction of 296° ($r = 0.90$, $p < 0.001$, $n = 10$; Figure 3B) keeping the light source to their left, consistent with flight in response to a 6-h delay (Figure 2E). At ZT21, monarchs were also significantly oriented as a group (232°, Rayleigh's test, $r = 0.67$, $p < 0.01$, $n = 10$; Figure 3C), and similarly kept the light source to their left like monarchs tested at ZT18. These results from both ZT18 and ZT21 are consistent with our prediction that light trespass

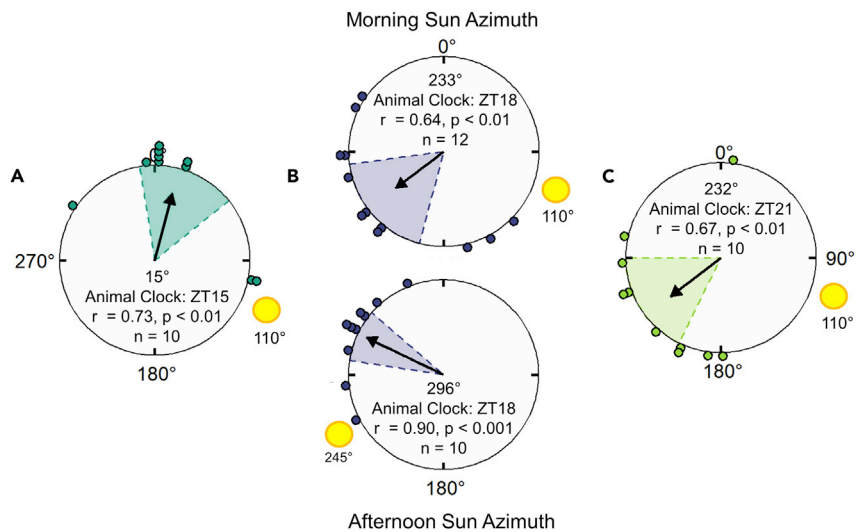


Figure 3. Migratory flight orientation response at night in response to a light trespass event

(A) Butterflies tested at their ZT15.

(B) Butterflies tested at their ZT18 in the morning (top circle diagram) or afternoon (bottom circle diagram) sun azimuthal position.

(C) Butterflies tested at their ZT21. Circle diagrams show the mean flight vector of tested butterflies in each treatment condition as in Figure 2.

exposure would phase-advance monarchs. Here, despite being tested at both the middle and latter parts of the night cycle, monarchs flew as if being tested in the morning. NLP exposure thus functions to advance monarchs to perceive an earlier start of the next daytime period.

DISCUSSION

With our controlled, indoor nighttime flight simulator trials, we were able to specifically test the effect of light trespass on fall monarchs, an environmental variable that is difficult to isolate under natural conditions, as day and night outdoor conditions present monarchs with significantly different sensory contexts along multiple sensory modalities. We demonstrate that exposure to light trespass can negatively impact migratory monarchs during their fall migration, as it can immediately elicit normal diurnal migratory flight behavior at night, the time of day when monarchs are typically quiescent and are at rest whether in the wild or in laboratory conditions. The ability of light trespass to elicit normal migratory flight behavior in monarchs was observed throughout their night cycle and only occurred in the presence of light. Here, light trespass at the beginning of the night (ZT15) induced afternoon-like flight orientation (phase-delay), but light trespass at the middle (ZT18) and end (ZT21) of the night produced premature morning-like flight orientation (phase-advance). The ability of light trespass to induce normal diurnal behavior at night in our monarchs is consistent with the ability of light stimuli presented at night to phase-delay or phase-advance the circadian clock (Froy et al., 2003; Grone et al., 2011; Glickman et al., 2012; Kennedy and Hudson, 2016; St Hilaire et al., 2012; Helfrich-Förster, 2018; Tähkämö et al., 2019), an endogenous mechanism that controls a multitude of downstream biological processes in all organisms.

Our results demonstrate that NLP can negatively affect the migratory biology of diurnal migratory animals, a phenomenon that has not received much attention in the study of this ubiquitous, contemporary anthropogenic environmental stressor. Diurnal migrants, such as the monarch, who rely on clock-mediated mechanisms for proper migratory behavior, e.g., a time-compensated sun compass, are therefore vulnerable to NLP that they might experience during migration. NLP is relevant during the fall migration, as fall monarchs can experience repeated, nightly exposure to NLP from anthropogenic artificial light on their journey (Figure 4A). For example, Eastern fall monarchs, those butterflies living east of the Rocky Mountains, can experience significant and frequent NLP sensory perturbations like light trespass (e.g., from streetlamps; Figure 4A, left) within their migratory corridors along the Eastern seaboard, the Great Lakes Region, and the Midwestern central flyway in North America (Figure 4A, right). Moreover, these monarchs will converge in Texas and then get funneled into Mexico, two other regions

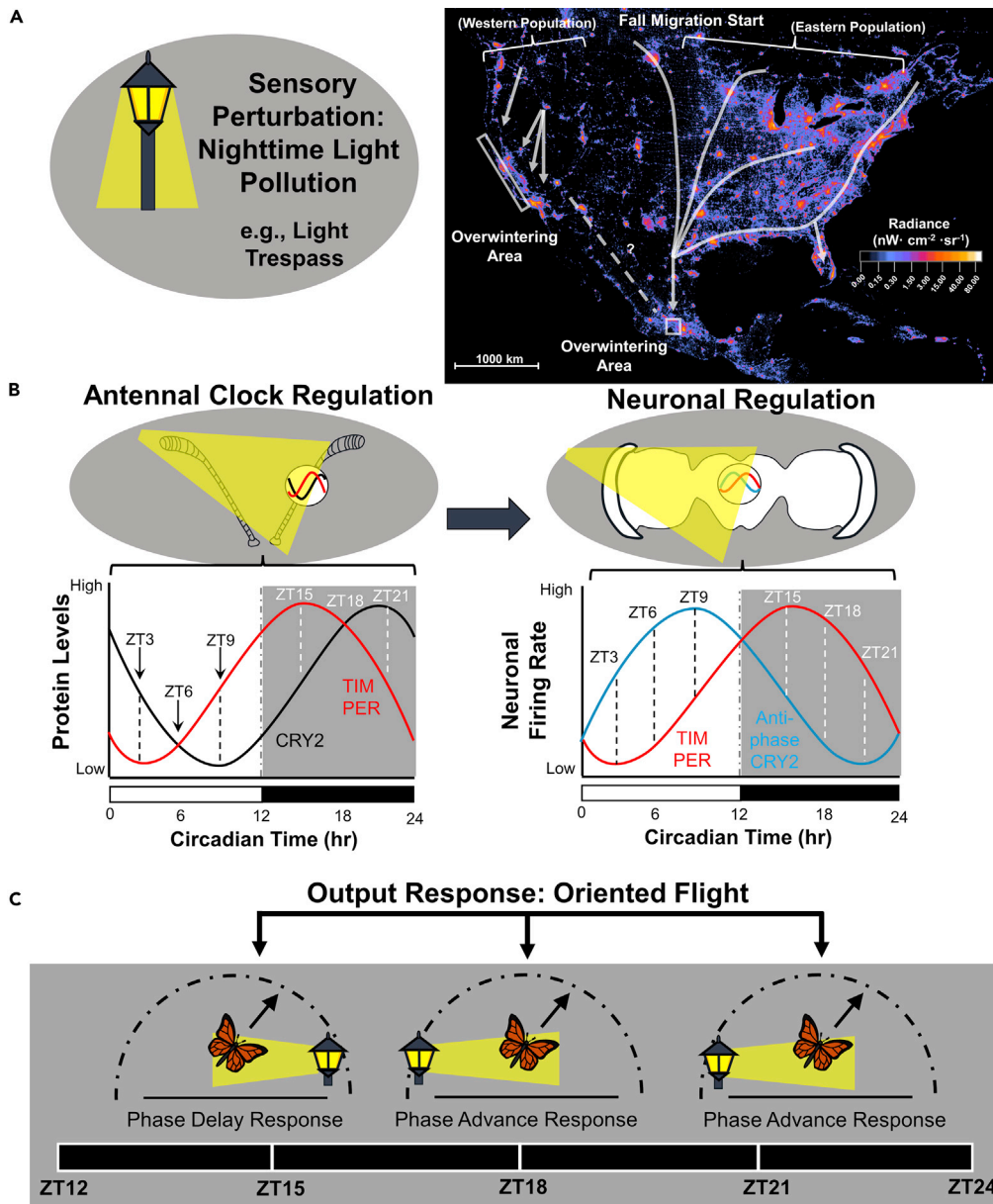


Figure 4. Sensory integration of nighttime light pollution related to antennal clock function and neuronal regulation in the monarch butterfly, and the flight orientation response of monarchs during the night phase (A) An example of the nighttime sensory perturbation (light trespass, left) that is ubiquitous throughout the Eastern and Western monarch fall migration routes (white transparent lines) across North America (right; light pollution data as radiance, $nW \cdot cm^{-2} \cdot sr^{-1}$). The extent of nighttime light pollution that monarchs can be exposed to is widespread in North America, for both Eastern and Western populations of monarchs, and is present in rural, suburban, and urban areas. (B) Interaction between the molecular-mediated clock and neuronal regulation of monarchs showing the relationship between diurnal and nocturnal regulation. (C) Oriented flight at night based on the regulation of the molecular-mediated clock and neuronal regulation in response to the location of a sensory perturbation (i.e., light trespass) at ZT15, ZT18, and ZT21.

monarchs fly through that can expose them to NLP during their migration to the overwintering sites in Central Mexico (Figure 4A, right). Although our study examines the effects of NLP on the migratory flight behavior of Eastern monarchs (e.g., we tested monarchs captured within the Greater Cincinnati Area). Western monarchs living west of the Rocky Mountains, in particular monarchs migrating in California,

also likely experience frequent NLP perturbation such as light trespass during fall migration (Figure 4A right).

When might fall monarchs be susceptible to NLP perturbations such as light trespass? Under natural conditions, fall monarchs are most likely exposed to NLP when they form their nightly roosts during their southward migration (Figure S5). Although a systematic analysis of observed roost sites at night remains to be done that fully characterizes the potential for exposure of monarchs to NLP (e.g., identifying if a source of NLP, such as a street lamp or well-lit building, is in close proximity to a tree in which monarchs are roosting in), our analysis of observed fall monarch roost sites (Sheehan and Weber-Grullon, 2021) show that nightly roost sites do occur in areas with NLP, which can vary in radiance level (Figure S5A). For example, many roost sites observed between 2012 and 2020 were found in locations with high levels of NLP, especially those roosts found in urban areas (see Figure S5B and Supplemental information). Although it is unknown if the observed NLP in these locations affected the monarchs roosting at these sites (see the following paragraphs for potential effects of NLP exposure on monarchs), our analysis does demonstrate that monarchs can be exposed to NLP even at high levels under real-world conditions (Figure S5B). As many monarchs will roost in urban areas (Davis et al., 2012), this supports the potential for fall monarchs being exposed to significant NLP radiance levels during actual migration.

For diurnal migrants, the sun is the brightest light source during the day and can provide the necessary information (e.g., orientation cues) and stimuli (e.g., brightness levels that affect flight propensity and motivation) for properly oriented migratory flight. The artificial light source used in our trials to produce light trespass stimuli is consistent with artificial lights commonly used outdoors at night and is orders of magnitude less bright than other commonly found artificial lighting in the environment (Table S1). Therefore, the ability of our artificial light source to elicit normal migratory flight behavior in monarchs (sun compass driven flight behavior both during the day and at night) highlights the particular broad risks and general dangers of NLP to migratory animals, even those that migrate during the day. Furthermore, our far-reaching results suggest that at night, sufficiently bright artificial light sources with the appropriate wavelengths (Figure S2) produce identical cues (e.g., light elevation cues that mediate oriented flight; Figure S4) and stimuli (e.g., lighting conditions at night which sufficiently motivate animals to fly as if during the day) normally provided by the sun. Therefore, artificial light at night, even as a single isolated source, can effectively act as the sun during the normal night cycle of organisms, with animals like monarchs unable to distinguish between the sun and artificial light.

Under natural conditions, it is rather unlikely that fall monarchs actively fly and migrate at night despite being exposed to NLP. To our knowledge, there are no documented reports of monarchs flying at night during the fall migration, and temperatures at night during the fall are often insufficiently high to support sustained monarch flight. Although nighttime temperatures are typically cooler at night than during the day, for many migratory species, nighttime temperatures during the migratory period are still within the range of temperatures that permit migratory movement (Masters et al., 1988). Moreover, specific landscape effects (e.g., urban heat island effect) and climate change are producing higher temperatures at night (Oke et al., 1991), conditions which can increase the effectiveness of NLP in perturbing migratory phenomena, now and in the future. For example, other diurnal butterflies that can undergo long-distance migrations similar to the monarch have been recorded to fly at night (e.g., flight in response to electric lights; Ryrholm and Källander, 1986), particularly at night during conditions that can facilitate migration (e.g., favorable trade winds), suggesting that some parts of their migratory journey might be done at night (e.g., red admiral and painted lady butterflies migrating between Africa and Europe – Ryrholm and Källander, 1986; Stefanescu et al., 2007; Talavera and Vila, 2017). NLP, particularly in urban areas, might therefore affect the migratory biology of these diurnal butterfly species, warranting further research examining the effects of NLP on other diurnal migratory lepidopteran species.

How might NLP specifically affect diurnal migratory animals such as the monarch? Ultimately, the disruption to the normal night cycle of diurnal migrants induced by NLP can cause many negative effects, which can lead to decreased migratory success. NLP can act as an environmental perturbation of the circadian clock, inducing a misinterpretation of sun-compass cues and actual time of day during daily diurnal migration because of a shift of the rhythmicity of the internal circadian clock (Merlin et al., 2009; Guerra et al., 2012). NLP can also cause increased daily energy expenditure, premature arousal at night when quiescent, or flight (actual or preparation) to occur during suboptimal conditions. Finally, frequent and prolonged perturbation of the circadian clock because of environmental stressors such as NLP can lead to many adverse health effects in animals (Dominoni et al., 2016).

Most importantly, our results are consistent with NLP, specifically targeting the internal circadian clocks that provide the relevant timing information for the sun compass orientation mechanism. The antennal clocks are what provide fall monarchs with the proper timing information in the sun compass mechanism (Merlin et al., 2009; Guerra et al., 2012). Unlike in fruit flies, where an early light pulse can cause a phase shift based on just TIM and PER activity (Levine et al., 1994; Suri et al., 1998; Emery et al., 2000; Tang et al., 2010), the monarch antennal clocks (peripheral clocks) use a negative transcriptional feedback loop where the absence of light drives the transcription of *cryptochrome 2* (*cry2*), *timeless* (*tim*), and *period* (*per*), and in the presence of light, the blue-light circadian photoreceptor cryptochrome 1 (CRY1) degrades TIM, enabling CRY2 and PER to enter the nucleus of the cell and repress transcription (Zhu et al., 2008; Merlin et al., 2009, 2020), forming a robust 24-h circadian oscillator that cyclically modulates levels of CRY2, TIM, and PER proteins. It is important to note that oscillation of *cry2* versus *tim* and *per* differ in their peak mRNA expression, which occurs at different time points within a 12L:12D light cycle (Merlin et al., 2009; Shlizerman et al., 2016). It is predicted that the neuronal signal produced from antennal clock function is directly related to the summed phase concentration of TIM and PER, and the antiphase of CRY2, which are staggered by 6-h (Shlizerman et al., 2016). Thus, the behavioral responses of monarchs that we observed at ZT3 and ZT21, ZT6 and ZT18, and ZT9 and ZT15 are clearly linked with the summation of protein levels and align with the mathematical model (Shlizerman et al., 2016) with light trespass stimuli acting as the relevant light cue (Figure 4B). This temporal information is then incorporated in the monarch central complex (Heinze and Reppert, 2011) with visual solar information sensed by the eyes (Sauman et al., 2005) to produce and maintain proper southwest flight orientation during the fall migration (Shlizerman et al., 2016). Although performed under laboratory conditions, our behavioral results support that if monarchs are roosting in the presence of NLP, e.g., near a streetlight, even if monarchs do not actually fly away, NLP exposure can physiologically perturb and shift their internal circadian clock.

Our results further suggest that NLP might act as an environmental stressor that interferes with proper migration in monarchs at multiple life history stages. Interestingly, as remigrant monarchs also use the same time-compensated sun compass mechanism to orient northwards during the spring remigration (Guerra and Reppert, 2013), NLP can negatively affect multiple legs of the annual monarch migratory cycle. NLP might also adversely affect the development of relevant aspects of the monarch migratory syndrome, namely the hallmark trait of oriented migratory flight, which relies on a properly functioning circadian clock (Guerra, 2020). As decreasing photoperiod is one important contributing factor to the development of the migratory syndrome (Goehring and Oberhauser, 2002), NLP such as light trespass experienced by developing monarchs throughout the landscape, might significantly interfere with the sensing of this clock-relevant environmental signal and interfere with the induction of the migratory phenotype.

Recent work has demonstrated that indoor, captive-reared monarchs have reduced southwards flight orientation when tested outdoors during the fall (Tenger-Trolander and Kronforst, 2020; Oberhauser, 2019), whereas captive-reared monarchs might regain proper southwards flight orientation once released outdoors (Wilcox et al., 2021). These results suggest that rearing conditions for captive-reared monarchs are relevant for the expression of proper migratory flight orientation. Specifically, monarchs reared in captivity, both indoors and outdoors, might be sensitive to the specific lighting conditions, either natural or artificial light that they experience. This suggests that captive reared monarchs from efforts such as participatory programs related to conservation and monarchs reared for commercial purposes should adhere to best practices related to light exposure. Best practices for captive rearing would include the use of ecologically relevant photoperiod lengths and eliminating exposure to light perturbations during the subjective night phase. These measures are critical for monarchs that are reared and released outdoors during the fall. This will aid in the proper acquisition of the migratory syndrome and the correct entrainment of habitat specific circadian rhythms such that accurate sun-compass orientation can be achieved upon release.

Migratory species have been experiencing substantial declines across taxa as modern anthropogenic stressors such as NLP, exacerbated by urbanization, have negatively impacted migratory pathways and corridors (Bauer et al., 2019). Conservation efforts in areas that provide habitat refuge or stopover sites for migratory and resident species (e.g., roosting sites) should consider the location, timing, positioning, and type of lights (i.e., wavelength and lumens, Table S1; Figure S1) used to illuminate specific areas at night. In addition, conservation

strategies focused on habitat creation should take into consideration the current location of lights and avoid building habitats such as pollinator gardens in areas that are susceptible to NLP events.

Our study reveals that diurnal migrants are primarily at risk because of NLP interfering with their internal circadian clock, a negative effect of NLP that is not readily seen by observers, especially if free-living individuals cannot locate sufficient nocturnal refuge that protects them from encroachment and exposure to NLP. For ectotherms, maintaining lower core temperatures at night allows individuals to reduce energy consumption during migration. A premature arousal and flight response (e.g., behavioral and physiological responses at the level of the preparation for flight) at night because of exposure to NLP can deplete energy stores but also increase the chances of mortality depending on the location in space of the NLP source. In our study, butterflies only flew when the artificial light was turned on, where individuals began flying immediately in the presence of our artificial light, not when they were initially placed in the flight simulator in the dark. This highlights the specific role of artificial light at night at perturbing normal behavior, with exposure to NLP capable of affecting animals acutely and immediately. Although the emphasis in our study is on the time-compensated sun-compass of a migratory species, as NLP can adversely affect other clock-mediated behaviors (Gaston et al., 2012), NLP might be affecting the biological processes of other diurnal species that also use time of day information to facilitate important movement behavior, such as species that disperse (fish, Mouritsen et al., 2013; sea turtles, Krenz et al., 2018) or forage (bees, Dyer and Dickinson, 1994; ants, Wehner and Müller, 1993) during the day.

Limitations of the study

Although a charismatic and much-appreciated animal, as they are diurnal, there is a significant lack of information on fall migratory monarchs at night, particularly of any observations of monarchs flying at night and while in nightly roosts, e.g., monarchs in roosts that can be exposed to NLP. Nevertheless, we show in our controlled studies the negative effects of NLP on fall migratory monarchs, especially in regards to how NLP can directly target their circadian clocks and alter their nighttime behavior. Observational studies and experimental work conducted under natural conditions, which directly examine the effects of the spectrum of environmental NLP conditions on monarchs, would be extremely beneficial. Such work would allow us to better characterize and functionally verify how NLP acts as a form of sensory pollution that can affect monarch migratory performance and success.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.isci.2022.104310>.

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AUTHOR CONTRIBUTIONS

Conceptualization, A.F.P., S.M.S., and P.A.G.; Methodology, A.F.P., S.M.S., and P.A.G.; Formal Analysis, A.F.P. and S.M.S.; Investigation, A.F.P. and S.M.S.; Resources, A.F.P., S.M.S., and P.A.G.; Data Curation, A.F.P. and S.M.S.; Writing – Original Draft, A.F.P. and S.M.S.; Writing – Review & Editing, A.F.P., S.M.S., and P.A.G.; Visualization, A.F.P. and S.M.S.; Supervision, A.F.P. and P.A.G.

DECLARATION OF INTERESTS

The authors declare no competing interests.

INCLUSION AND DIVERSITY

One or more of the authors of this paper self-identifies as an underrepresented ethnic minority in science. We worked to ensure sex balance in the selection of non-human subjects.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Deposited data		
Summary of Orientation Data	Supplemental information of this paper	
Software and algorithms		
R Project for Statistical Computing (version 3.6.0)	R Core Team	RRID:SCR_001905

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources should be directed to and will be fulfilled by the lead contact, Dr. Patrick A. Guerra (patrick.guerra@uc.edu).

Materials availability

This study did not generate new materials or unique reagents.

Data and code availability

- All of the raw data collected for this study are publicly available as [Supplemental information](#) as of the date of publication ([Data S1](#)).
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the [lead contact](#) upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Animal models

Wild-caught fall adult monarch butterflies (*Danaus plexippus*) were captured at the University of Cincinnati Center for Field Studies (N39.28, W84.74) between 1-September and 26-October during the 2019 and 2020 fall monarch migratory periods. Adult butterflies were caught as they migrated through the area during the fall. For all experiments, butterflies were kept in glassine envelopes housed in environmental chambers (Percival model I36LL; Perry IA, USA) under fall-like conditions consisting of a constant 12L:12D photoperiod with day temperatures set to 21°C and night temperatures set to 12°C. Monarchs (mixed sex) were fed a 25% honey solution, a minimum of twice a week and checked daily. Previous studies have found no difference in the group or individual directionality between the sexes ([Zhu et al., 2009](#)), so the subsequent analyses used combined male-female flight orientation responses.

METHOD DETAILS

Indoor flight simulator trials

Our flight simulator design was based on that used in [Mouritsen and Frost \(2002\)](#) and [Froy et al. \(2003\)](#) and used a new, non-invasive tethering technique ([Parlin et al., 2021](#)). Trials were conducted in a laboratory dark room with the flight simulator set in the middle of the room ([Figures 2A–2E and 3](#)) with the exception of one set of trials that were conducted inside a concrete planetarium (Burnet Woods Wolff Planetarium, Cincinnati, OH, USA; [Figure 2F](#)). Geomagnetic north was controlled by zeroing the flight simulator to geomagnetic north at the animal at the beginning of each trial. We simulated the sun by using an artificial light source consisting of a full spectrum halogen bulb (120V 250W R7S, 4,000 lumens; Feit Electric Company, Inc., Pico Rivera, CA, USA) shop light (250W 4-in-1 Work Light, LG Sourcing, Inc., N. Wilkesboro, NC, USA), with UVA/UVB ([Guerra et al., 2014](#)), that was attached to a tripod ([Figure S1](#)). Using information from a broad online product search of relevant light trespass sources (<https://www.ledlightexpert.com/>) and information provided by the National Optical Astronomy Observatory (NOAO; <https://www.noao.edu/education/qtkit.php>), we found that our artificial light source was consistent with common

anthropogenic light trespass sources (Table S1). We also verified that our simulated light trespass event during trials, in particular its intensity, was consistent with multiple common light trespass scenarios (Figure S1). We obtained measurements (Figure S1A) using a light meter (VWR International, Traceable® Dual-display Light Meter, 21800 - 014) and curated data (Figure S1B) using the Acuity Brands Visual Floodlight Tool (<https://www.visual-3d.com/tools/vertical/default.aspx>). We initially selected three LED lights from a single manufacturer (top and middle: PSLED P (1&7) 44 30K PSLEDUBV; bottom: PMLED P5 44 30K PMLED UBV; all: Acuity Brands Holophane), with similar lighting patterns, and varying lumen ratings. Using the Floodlight Tool, the illuminance value was then calculated for a point directly horizontal to the center of the light at the four distances we tested (Figure S1B), to exemplify how the intensity of our simulated trespass event corresponds and is consistent with different light trespass events.

All sun azimuth measurements were based off the geomagnetic north measured at the animal's location in the flight simulator. We measured the sun angle by placing a protractor at the top of the encoder maintaining that angle to the middle of the light source. This set the virtual horizon at the top of the flight simulator. We also confirmed that the monarch could see the light source by making sure the light source was above the horizon of the flight simulator using Pythagorean's theorem. Azimuth (horizontal angle) and altitude (vertical angle) values were obtained by using the R package 'suncalc' (Agafonkin and Thieurmel, 2017), which provided the necessary parameters based on geographic coordinates (Cincinnati, OH, USA), date (Sept-5: local peak monarch migration; Nov-5: monarch arrival at overwintering site), and time of day (10:00, 13:00, 17:00).

The 6-hour light:dark (LD) delayed experiments were done by testing the animals at their subjective ZT3 and then testing them in the flight simulator with the ZT9 sun position (azimuth 245°; elevation 31°). For tests that occurred during the dark cycle, monarchs were handled in the dark using only a red-light in order to see when placing individuals in the flight simulator (<30 seconds handling time from incubator to simulator) and then had the artificial light immediately turned on. After the 10-minute trial, the light source was immediately turned off, the animal removed, and then placed back in the incubator (<30 seconds handling time from simulator to incubator). Total exposure time to the light source and time outside of the incubator was less than 12 minutes. For animals tested at their ZT15, ZT18, and ZT2, individuals were tested within ± 30 -minutes of the exact time point of their night cycle. Throughout all the trials, the ambient temperature at the butterfly's location in the flight simulator was $22.34 \pm 0.93^\circ\text{C}$. Throughout the entire experimental procedure, we had a 39.0% success rate (112 out of 287 – all daytime and nighttime trials combined) in monarchs that flew under indoor conditions across all the experiments combined, similar to other studies that report trial success (Guerra and Reppert, 2013; Parlin et al., 2021).

Artificial light source spectral properties

We measured the spectral properties of our artificial light source using a spectrometer (Ocean Optics Inc., Dunedin, FL, USA) and an optic fiber (QP230-1-XSR, 235 microns; Ocean Optics Inc.) with cosine corrector (CC-3-UV-S; Ocean Optics Inc.). For each of the treatment positions (i.e., morning azimuthal, afternoon azimuthal, 53° solar noon azimuthal, and 34° solar noon azimuthal position on 5-November; Figures S2 and S3) we took five measurements, all at the animal's location in the flight simulator with the probe facing the light source as the device recorded the data. For 53° solar noon and 34° 5-November solar noon azimuthal positions, our measurements were based on the sun angle at solar noon experiments in which azimuthal position was 190°. We used the 'pavo' R-package (Maia et al., 2013) to convert the values from radiance to photon flux, and then averaged the measurements across all wavelengths to generate the appropriate curves (Figures S2 and S3).

QUANTIFICATION AND STATISTICAL ANALYSIS

Data analysis

To be counted as a trial, a monarch had to fly continuously in the flight simulator for at least 5 minutes. From each trial, we analyzed a 5-minute segment of continuous flight. For each individual, we calculated the mean vector and Z-Score. If a Z-score is below 500, this indicates that the virtual path taken by the butterfly was non-directional, whereas a Z-score greater than 500 indicates a directional response (Zhu et al., 2009; Parlin et al., 2021). Butterflies tested with the morning azimuthal position had a mean Z-score (\pm SEM) of 1897.6 ± 230.2 (Figure 2A), and monarchs tested with the afternoon azimuthal position had a mean Z-Score of 1407.12 ± 200.88 (Figure 2B). Butterflies tested with the Sept-15 SASN had a Z-score of 1556 ± 257.3 and the butterflies tested with the Nov-5 SASN had a mean Z-score of 1675.9 ± 188.9 (Figures 2C and 2D).

6-hour LD delayed butterflies had a mean Z-Score of 1424.5 ± 244.7 (Figure 2E), and butterflies tested with the afternoon azimuthal position had a mean Z-Score of 1920.1 ± 195.3 (Figure 2F). For monarchs tested at night, butterflies tested with a morning azimuthal position at ZT15 had a Z-score of 1233.11 ± 509.42 , at ZT18 had a Z-score of 1431.43 ± 195.86 , and at ZT21 had a Z-score of 1194.66 ± 388.37 (Figure 3). For the afternoon azimuthal position for ZT18, the mean Z-score of the butterflies was 1649.81 ± 231.26 (Figure 3). In each treatment condition, we calculated the mean and the 95% group vector confidence interval. We used a Rayleigh's test to test the significance of the mean vector ($\alpha = 0.05$). For all trials, we used Mardia-Watson-Wheeler (MWW) test to determine whether the group vectors flew in the same directions and applied a Bonferroni correction for pairwise comparisons. All statistical analyses were conducted utilizing the R 'circular' package (Agostinelli and Lund, 2017) and performed in program 'R' v3.6 (R Core Team, 2019).

Indoor and outdoor flight directionality

To ensure that our results were representative of monarchs using natural solar cues, we compared outdoor disappearing bearing (Figure S4A; Guerra and Reppert, 2013) and outdoor flight simulator (Figure S4B; Guerra et al., 2012) data from previous studies with our data from monarchs tested indoors with our artificial light source at the afternoon azimuthal position (Figure S4C). The two previously published data sets of monarchs tested outdoors did not differ in the mean vector between the disappearing bearing and flight simulator for monarchs tested (Figure S4; [MWW] $W = 2.52$, $df = 2$, $p = 0.28$). When we compared our indoor data with outdoor disappearing bearing data, we found no difference in the mean afternoon vector (Figure S4; [MWW] $W = 1.58$, $df = 2$, $p = 0.46$). When we compared our indoor flight simulator data with the outdoor flight simulator data, we also found no difference in the mean afternoon vector (Figure S4; [MWW] $W = 0.14$, $df = 2$, $p = 0.93$). In all cases, the mean southwest group vector of the tested fall monarchs did not differ between the outdoor disappearance, outdoor flight simulator, and indoor flight simulator trials (Figure S4).

Monarch roosts and nighttime light pollution

During fall migration, monarchs can roost in areas that are not completely dark at night, thus exposing them to light trespass stimuli that can negatively affect them, e.g., perturb their internal circadian clock. We assessed the occurrence of monarch roosts nearby potential nighttime light pollution sources from 2012 to 2020 using the VIIRS Day-and-Night Band (DNB) (Cao et al., 2013, 2014; Uprety et al., 2013). Monarch roost data were collected from publicly available data (Sheehan and Weber-Grullon, 2021; www.journeynorth.com). Data were thinned down to only observations with 10 or more individual monarchs, and from 2012 to 2020 as these were the years where VIIRS monthly cloud-free DNB composites were available. We parsed out the monarch roosts by year (2012–2020) and month (August, September, October, and November) to then subsequently extract the radiance ($\text{nW} \cdot \text{cm}^{-2} \cdot \text{sr}^{-1}$) value at each location based on the corresponding VIIRS DNB composite. We show example plots of monarch roost locations near locations of low to high nighttime light pollution (Figure S5A) and the corresponding counts of monarch roosts in varying ranges of radiance values (Figure S5B).