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Neurobiology of Monarch Butterfly Migration

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Abstract

Studies of the migration of the eastern North American monarch butterfly (*Danaus plexippus*) have revealed mechanisms behind its navigation. The main orientation mechanism uses a time-compensated sun compass during both the migration south and the remigration north. Daylight cues, such as the sun itself and polarized light, are processed through both eyes and integrated through intricate circuitry in the brain's central complex, the presumed site of the sun compass. Monarch circadian clocks have a distinct molecular mechanism, and those that reside in the antennae provide time compensation. Recent evidence shows that migrants can also use a light-dependent inclination magnetic compass for orientation in the absence of directional daylight cues. The monarch genome has been sequenced, and genetic strategies using nuclease-based technologies have been developed to edit specific genes. The monarch butterfly has emerged as a model system to study the neural, molecular, and genetic basis of long-distance animal migration.

INTRODUCTION

The annual migration of the eastern North American monarch butterfly (*Danaus plexippus*) is a visually spectacular phenomenon in terms of both the number of butterflies on the wing in the fall and the mass of butterflies congregated at their overwintering sites in Mexico (3, 66). Not only is the monarch migration a visual splendor, it is also a biological treasure trove (53). In fact, significant progress has been made to establish the migratory monarch butterfly as a model species to study the neurobiological basis of long-distance animal migration (**http://www.reppertlab.org**).

From 2002 to 2010, advances in understanding the monarchs' migration were made by delineating the main compass system used to orient in the fall—a time-compensated sun compass. Elucidating the nature of the circadian clocks used for time compensation, by defining their distinctive molecular mechanism and unconventional location, was central (reviewed in 54).

The current review emphasizes the rapid pace of advances over the last five years. The following were key discoveries: The monarch time-compensated sun compass is bidirectional, because it is used during both the migration south and the remigration north. Anatomical and electrophysiological approaches have been used to define the central complex as the site of the sun compass. A draft sequence of the monarch genome with a full complement of protein-encoding genes has been presented. Reverse genetics using nuclease technology is a tractable method to knock out specific monarch genes. Monarchs can use a light-dependent, inclination magnetic compass as an additional orientation system during migration. The evolutionary history of the migration has been illuminated.

With a genomic blueprint in hand and nuclease-based technologies being honed for editing the monarch genome, we can now define the genetic basis of long-distance migration, at least in the monarch. This review tells the story of how we got here. We underscore the many exciting areas ready for further exploration.

THE YEARLY MIGRATION CYCLE

Most of our knowledge on the monarch migration is derived from studies of the eastern North American population, which is the focus in this review (**Figure 1**). In the fall, environmental factors, including decreasing day length and cooler temperatures, appear to trigger the migration (20). Millions of fall migrants from their northern breeding areas in southeastern Canada and the eastern United States (east of the Rocky Mountains) fly southwards to overwinter in roosts in a small number of coniferous fir groves atop the Transvolcanic Mountains of Michoacán in central Mexico (3, 66) (see sidebar Discovery of the Overwintering Sites in Mexico, below).

DISCOVERY OF THE OVERWINTERING SITES IN MEXICO

The idea that monarch butterflies exhibit active migratory behavior was once controversial (66). However, Fred Urquhart (66) and his colleagues pioneered tagging work with the eastern North American population for decades and revealed the long-distance nature of the monarch migration. This extraordinary effort began in 1937 and ultimately led to the groundbreaking discovery of the monarch overwintering sites in 1975 (66). Moreover, these same tagging techniques were used to determine that monarchs that were tagged at the overwintering sites would in fact remigrate northward during the spring (66). Similar tagging studies are ongoing (e.g., **http://www.monarchwatch.org**) and are important for further delineating the migratory and remigratory routes of the North American populations (**Figure 1**). Urquhart's journey and the discovery of the overwintering sites in Mexico are reenacted in the IMAX movie *Flight of the Butterflies* (61).

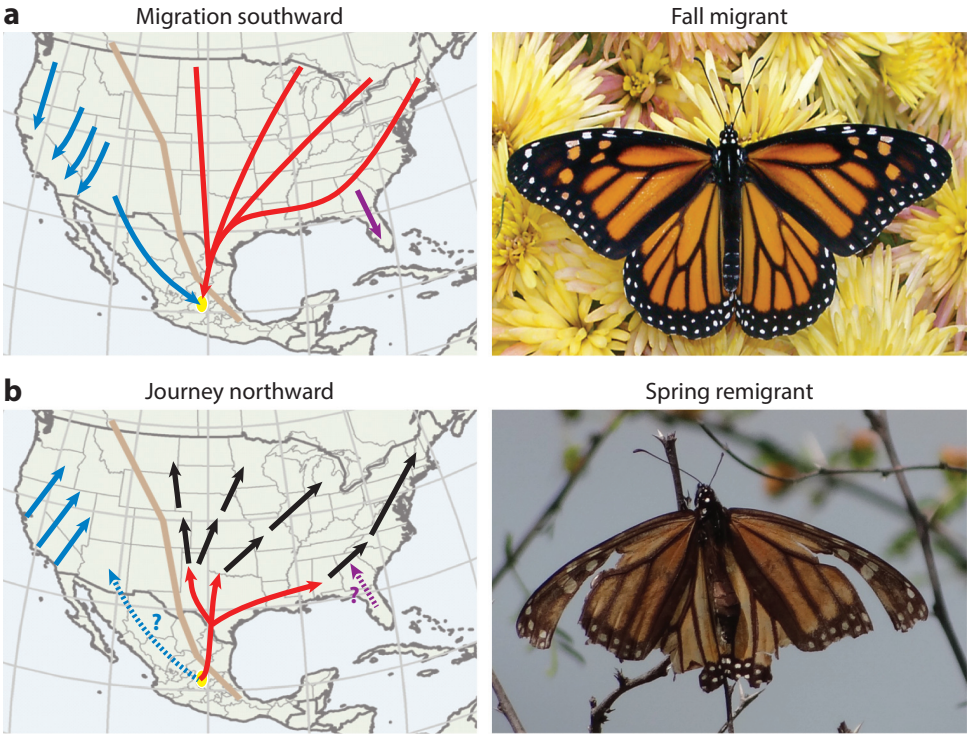


Figure 1

Annual migration cycle of North American monarch butterflies. (*a*) Migration southward. (*Left*) North American monarch butterflies east of the Rocky Mountains (*brown line*) migrate long distances during the fall from northern ranges (*red arrows*) to their overwintering sites in central Mexico (*yellow oval*) (3, 66). Some eastern migrants do not reach the overwintering sites in central Mexico but fly off course and end up in Florida (35), where they presumably join year-long resident monarch populations (*purple arrow*). North American monarch butterflies west of the Rocky Mountains also migrate during the fall and overwinter in protected roosts along the Pacific Coast in California (*blue arrows*). As indicated by tagging and recovery efforts in the southwestern United States, western migrants also reach the overwintering sites in Mexico (**http://www.swmonarchs.org**) (9). (*Right*) A typical fall migrant butterfly. (Photo courtesy of David R. Weaver and used with permission.) (*b*) Journey northward. (*Left*) Eastern migrants remain at the overwintering sites in Mexico until spring, when these butterflies return northward to reproduce, with females laying fertilized eggs on newly emerged milkweed (genus *Asclepias*) in the southern United States (*red arrows*). The offspring of these butterflies will continue the journey northward to further repopulate the northern sections of the monarch habitat range (*black arrows*). It is speculated that monarchs from Florida may also travel northward during the spring, presumably to take advantage of newly emerged milkweed (**http://www.monarchwatch.org**) (*purple dashed arrow*). Western migrants also leave their overwintering sites along the Pacific Coast during the spring and return north to repopulate their home range (*blue arrows*). It is unknown whether migrants overwintering in Mexico return north by a westerly route, which would take them west of the Rocky Mountains (*blue dashed arrow*). (*Right*) A spring remigrant monarch. Note the rather tattered and worn appearance of this older and well-traveled monarch relative to the fall migrant above. (Photo courtesy of Harlen and Altus Aschen and used with permission.)

When spring arrives, these butterflies become reproductively active, mate, and leave the overwintering sites to remigrate northward, with the females ovipositing on milkweed (genus *Asclepias*), the primary larval food source, in the southern United States. Because of the distances traveled and the anticipated, repetitive, cyclic nature of the journeys, the monarch migration is birdlike (33, 65). However, it differs from that of birds in that it is multigenerational; it takes at least another two generations of spring and summer butterflies that are likely following the progressive, northerly emergence of milkweed to repopulate their entire range and complete the migration cycle (**Figure 1**). The migratory cycle then begins anew in the fall.

NAVIGATIONAL MECHANISMS

Maps and Compasses

Similar to other long-distance migratory species, monarchs have been proposed to use a map for navigation (54). A map would allow migrants to sense their location relative to their destination and know where to end their southward fall migration (coniferous fir groves in central Mexico). It is unclear, however, whether migrant monarchs possess a true or complete map sense (5, 44, 66; for more on the use of maps by migratory species, see 8). A recent study suggests that monarchs do not have a map sense: Longitudinally displaced migrants did not adjust their oriented flight to compensate for this displacement when tested in flight simulator trials (44). Yet, the map sense issue for monarchs has not been clearly resolved and needs further study (45, 47).

Migrants may rely on various compass senses, alone or together with a map sense, for navigation during migration. A considerable body of work demonstrates that migrant monarchs possess two compass senses: a time-compensated sun compass (13, 23, 24, 42, 44, 46, 51, 63, 75) and an inclination-based magnetic compass (22) (**Figure 2**). These compasses allow migrant monarchs

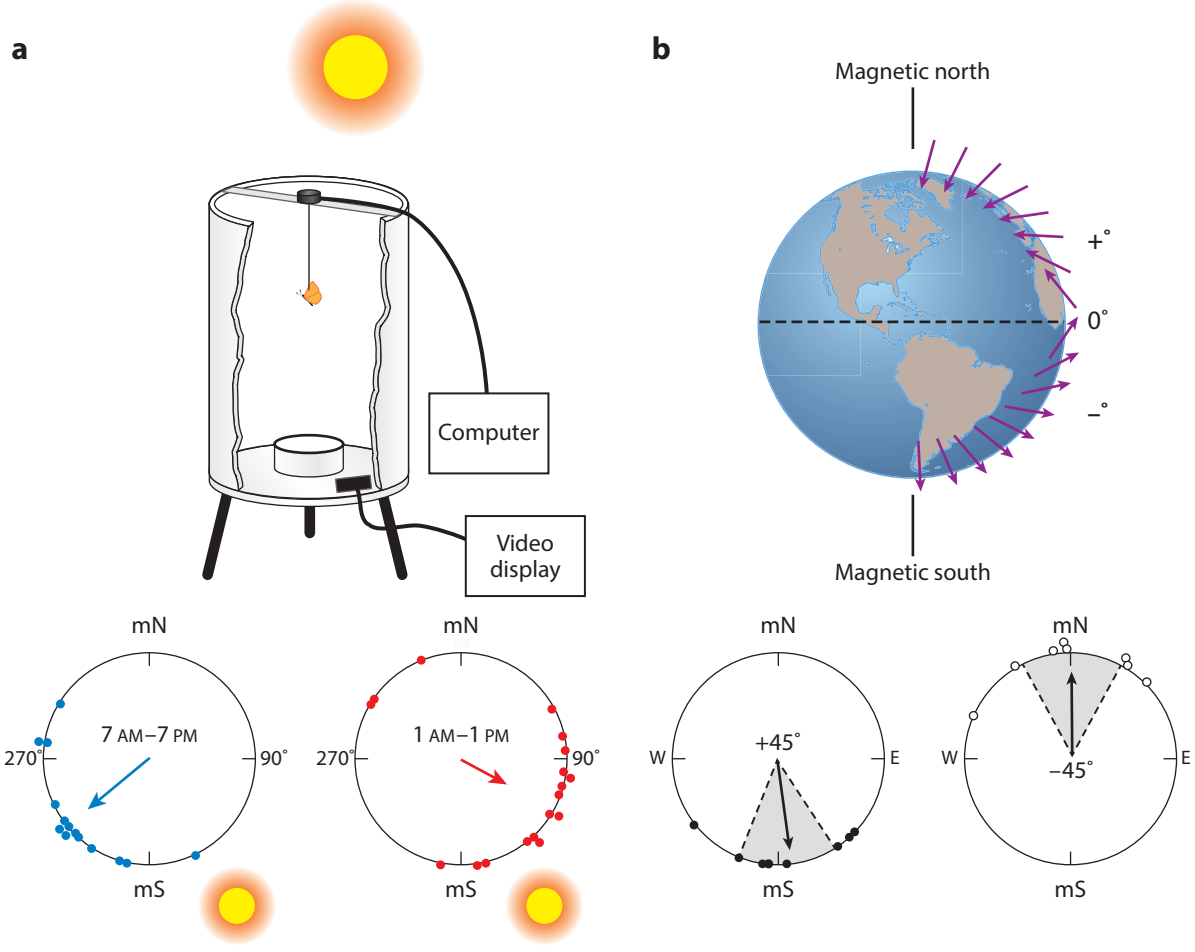
Figure 2

Sun compass and magnetic compass orientation in migrant monarchs. (*a*) The time-compensated sun compass. (*Top*) Cutaway view of a flight simulator. An individual monarch is tethered in an opaque plastic barrel, which excludes buildings and trees from view during testing. The butterfly can fly and rotate freely but is unable to move vertically or horizontally. A video camera records the monarch's flight behavior, and a directional recording device captures flight orientation data, which are stored via computer. (*Bottom*) Migrant monarchs use a time-compensated sun compass for orientation. Monarchs housed under normal light/dark conditions in the laboratory, with sunup simulated at 7:00 AM and sunset simulated at 7:00 PM, flew in the migration-appropriate southwesterly direction (*blue dots*). These monarchs were tested at approximately 10:00 AM and appropriately perceived the time as 10:00 AM, given the azimuthal position of the sun. Monarchs housed in the laboratory with a light/dark cycle shifted by 6 h (sunup simulated at 1:00 AM and sunset simulated at 1:00 PM) shifted their flight orientation counterclockwise (*red dots*) when tested at approximately 10:00 AM. This counterclockwise shift from southwest to southeast is consistent with these monarchs perceiving the time to be 4:00 PM, as indicated by the sun's position, and demonstrates the time-compensated aspect of the sun compass. Modified from Reference 54. (*b*) The inclination magnetic compass. (*Top*) Migrant monarch butterflies use the inclination angle of the Earth's geomagnetic field to approximate their latitudinal position on Earth during migration. Inclination angles range from 0° at the equator (*dashed line*) to 90° at the magnetic poles. Inclination angles (*purple arrows*) in the Northern Hemisphere are designated positive, with the magnetic North Pole (mN) being +90°, whereas inclination angles in the Southern Hemisphere are designated negative, with the magnetic South Pole (mS) being −90°. (*Bottom*) The orientation behavior of monarchs in indoor flight simulator trials under artificial magnetic field conditions is consistent with an inclination-based magnetic compass. Monarchs orient toward the equator and away from mN when tested under positive inclination angle conditions, a bearing that will bring them toward the overwintering sites in Mexico (*black dots*). When the artificially generated inclination angle was reversed 180°, providing monarchs with altered inclination angle directional information, monarchs reversed their orientation and flew northward (*open dots*). This reversal of flight orientation in response to the reversal of the inclination angle in monarchs is clear evidence for the existence of an inclination compass, as demonstrated in other migratory animals that use a magnetic compass for orientation (69). Modified from Reference 22. For circlegrams in panels *a* and *b*, a single dot represents the orientation of an individual monarch that flew continuously for at least 5 min; length of arrows indicates degree of mean group orientation. In panel *b*, shaded areas represent a 95% confidence interval.

to use reliable environmental cues, such as the sun's position in the sky and the inclination angle of the Earth's magnetic field, to fly in the appropriate migratory direction. Migrant monarchs may use these multiple modalities for directionality, such that the compasses complement each other. One compass sense might fine-tune the other, or one compass system might be a backup for a dominant compass sense (25).

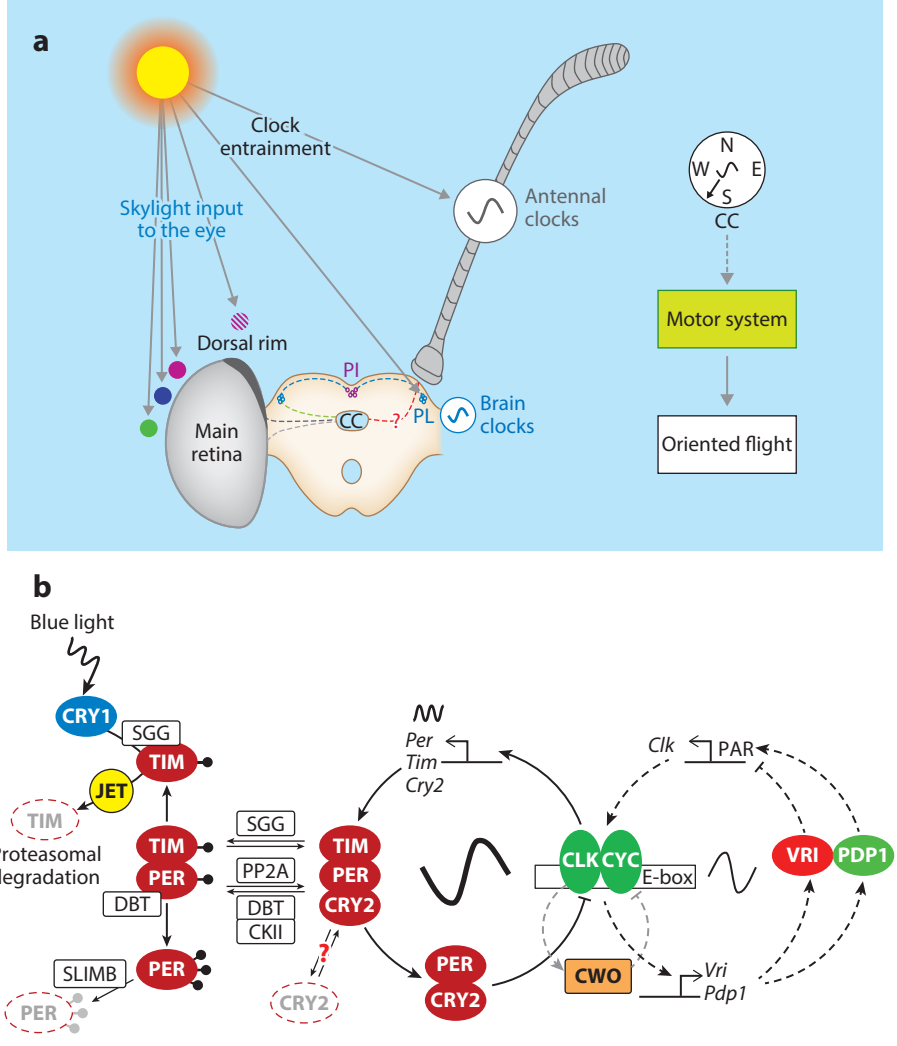
A Time-Compensated Sun Compass

Monarch butterflies are diurnal and predominantly use daylight cues for orientation during migration using a time-compensated sun compass. Specifically, the sun's azimuthal (horizontal) position in the sky is the dominant source of directional information during both the southward fall migration and the northward spring remigration (13, 24, 46, 55, 63). When the sun is obscured but some blue sky is visible, skylight polarization patterns resulting from scattered sunlight may be a cue providing directional information (28). Because the sun's position in the sky constantly shifts throughout the day, migrant monarchs use a circadian clock mechanism to adjust their flight relative to shifting skylight cues, such that their sun compass is time compensated (13, 23, 24, 42, 44, 46, 51, 63, 75) (**Figure 2*a***). The monarch's use of a time-compensated sun compass for



orientation during migration has been shown using both vanishing bearings of released butterflies (24, 51) and orientation behavior of tethered monarchs in a flight simulator (13, 23, 24, 42, 44, 46, 51, 63, 75), as originally developed by Mouritsen & Frost (46). Summer nonmigratory butterflies, on the other hand, do not show oriented flight (75).

Although the circuitry involved in the processing and integration of directional and timing information that produces directed migratory flight remains to be determined, our understanding of the anatomical layout of the neuronal network of the time-compensated sun compass of migrant monarchs has increased (**Figure 3*a***). The compound eyes sense skylight cues: The main retina detects the azimuthal position of the sun, and anatomically and molecularly specialized UV-expressing photoreceptors in the dorsal rim area detect the angle of plane polarized UV light [the electric (E)-vector] (37, 55, 62). Information extracted from these directional cues is transmitted through complex circuitry to the brain's central complex, a midline structure that is the proposed site of the sun compass where skylight cue information is processed and integrated with other relevaxnt directional information for flight (10, 27–29).



The insect central complex is a highly conserved region consisting of a group of neuropils that possesses a highly stereotyped neuroarchitecture (27). Intracellular recordings from monarch central complex input neurons have shown that single neurons integrate both the azimuthal position of the sun and the E-vector angle (28). Furthermore, these dual responses are integrated to create a consistent representation of skylight cues in the central complex throughout the day. These data strongly support the notion that this brain region is indeed the neural substrate for the monarch sun compass (28).

Heinze et al. (27) developed a standardized, average-shape representation of the neuropils in the central complex of the monarch brain. This standardized version has provided useful models of the reference volumes of these neuropils (27) and is a common reference for the mapping and characterization of neuron morphologies in the central complex. Registration of these neuron morphologies on the standardized neuropils has led to the identification of both the major input and output pathways of the central complex and the neurons in this neural circuitry that are intrinsic to the central complex (27). The central complex in the monarch has wiring features consistent with those of the central complex of other insect species in which it is believed to mediate similar behaviors involving visual cues and three-dimensional spatial awareness—e.g., spatial memory, visual learning, integration of multimodal stimuli, and motor control (10, 27, 43). Translating this wiring map into functional circuits by means of electrophysiological, imaging, and molecular approaches—all of which are now feasible—is imperative to understand how spatial information is integrated with temporal information to guide oriented flights in fall migrants and spring remigrants. In fact, we are now modeling this integration in our laboratory (E. Shizerman, J. Phillips-Portillo, D.G. Forger & S.M. Reppert, unpublished data).

A startling discovery of the time-compensated sun compass system was the finding that skylight directional cues sensed by the eyes and relayed to the central complex region are time compensated

←

Figure 3

The time-compensated sun compass system of migrant monarchs. (*a*) Model outlining the components used for sun compass navigation by migrant monarchs. The compass mechanism involves the sensing of skylight cues by the monarch eye that include the color gradient of the sky (*violet*, *blue*, and *green circles*) or the position of the sun and the polarization pattern of UV light (*striped violet circle*), and the brain integration of skylight-cue-stimulated neural response in the central complex (CC; *gray dashed lines*). Time compensation for the sun compass is provided by circadian clocks that are found in both antennae. The integrated time-compensated sun compass information is transmitted to the motor system to induce oriented flight. The brain circadian clocks are found in the pars lateralis (PL) and communicate with the pars intercerebralis (PI). The PL may also communicate with the central complex. Modified from Reference 71. (*b*) Schematic of the proposed clockwork mechanism in the monarch butterfly, including the core transcriptional/translational feedback loop (*thick arrows*) and the modulatory feedback loop (*dashed arrows*), both incorporating monarch orthologs of all described *Drosophila* clock genes (71). CLOCK (CLK) and CYCLE (CYC) heterodimers drive the transcription of *period* (*per*), *timeless* (*tim*), and *type-2 cryptochrome* (*cry2*), which upon translation form complexes and 24 h later cycle back into the nucleus, where CRY2 inhibits CLK:CYC-mediated transcription. Light entrainment is mediated by type-1 cryptochrome (CRY1), which promotes TIM degradation. Casein kinase II (CKII), doubletime (DBT), and the protein phosphatase 2A (PP2A) are involved in the posttranslational modifications of PER and TIM, and supernumerary limbs (SLIMB) and jetlag (JET) signal their degradation. The gene(s) involved in CRY2 degradation are unknown (*red question mark*). The modulatory feedback loop regulates the expression of CLK through VRILLE (VRI) and PDP1. Monarch *vri* has five consensus CACGTG E-box elements in its promoter, and *pdp1* has five in its first intron, through which CLK and CYC could drive their transcription; each transcription factor also contains poly(ADP-ribose) (PAR) DNA-binding domains that could modulate CLK transcription by binding to PAR-like binding sites present in the monarch *clk* promoter. Clockworkorange (CWO) modulates the amplitude of the clock. Modified from Reference 71.

via circadian clocks located in the antennae, and not in the brain (23, 42) (**Figure 3*a***). Monarchs without antennae or with both antennae painted black (to block light input) do not exhibit the proper southerly orientation in flight trials (42). A single functional antenna, however, is sufficient for proper time compensation and properly oriented flight, but conflicting timing between the two antennae disrupts proper orientation (23). These findings suggest that each antenna provides independent timing information to the brain that ultimately interfaces with the output side of the sun compass circuit (23).

The pathways that relay this vital timing information from the circadian clocks of the antennae to the central complex, as well as the neural substrates that allow for the integration of this timing information with directional daytime skylight cues, remain unknown. Relevant timing information from the separate antennae may be relayed neuronally via direct axonal projections from the antennal clocks to the central complex, through a more intricate multisynaptic circuitry, or independently from the nervous system via yet-to-be identified diffusible molecular components that are generated and/or regulated in a clock-dependent manner (23). Further studies are warranted to distinguish between these possibilities.

A Unique Circadian Clock Mechanism

The necessity of the circadian clock for time compensating the sun compass has driven the efforts to elucidate the monarch molecular clock mechanism. This work has identified a clock mechanism that is unique among animals (**Figure 3*b***) in that it exploits two different CRYPTOCHROME proteins (CRY). Monarchs have both a type 1 *Drosophila*-like CRY (CRY1) that functions as a circadian photoreceptor and a type 2 vertebrate-like CRY (CRY2; not found in *Drosophila*) that functions as the major transcriptional repressor of the clockwork feedback loop (76, 77). Similar to mechanisms in *Drosophila* and mammals (1, 49), the core mechanism in both the brain and antennae of monarchs relies on a negative transcriptional feedback loop that drives self-sustaining rhythms in the mRNA and protein levels of a set of core clock components. In the proposed monarch model (**Figure 3*b***), the transcription factors CLOCK (CLK) and CYCLE (CYC) form heterodimers that drive the transcription of the *period* (*per*), *timeless* (*tim*), and *cry2* genes. Translated PER, TIM, and CRY2 form complexes in the cytoplasm and cycle back into the nucleus, where CRY2 inhibits CLK:CYC-mediated transcription (76), similar to what CRY does in mammals (49).

The discovery of type 2 vertebrate-like CRY proteins in insects, derived from the initial finding of CRY2 in monarch butterflies (77), has altered our view of how circadian clocks of nondrosophilid insects work and has redefined our understanding of the evolution of animal CRYs (70). Two to four cells in the dorsolateral region of the central brain [the pars lateralis (PL)] contain the major circadian clocks in the butterfly brain (**Figure 3*b***); these are the brain cells in which the transcriptional repressor CRY2 cycles into the nucleus to regulate the molecular clock feedback loop (76).

Much of the molecular clock mechanism was initially derived from a combination of in vitro and in vivo approaches, including use of the monarch DpN1 cell line (48), which contains a light-driven diurnal clock, and use of *Drosophila* transgenesis, to strengthen the differential clockwork functions of the monarch CRY proteins. Genetic studies using nuclease technology to knock out CRY2 have confirmed that the flavoprotein is in fact an essential clock gene and the major transcriptional repressor of the monarch clockwork (41; see sidebar The Monarch-Derived DpN1 Cell Line, below).

Based on studies in *Drosophila* and laboratory mice (*Mus musculus*), the clockwork mechanism also involves a second, modulatory feedback loop (1, 49). The draft genome of the monarch has identified the putative clock components involved in this second loop, VRILLE and PDP1, based

THE MONARCH-DERIVED DpN1 CELL LINE

DpN1 is a monarch cell line (48), originally derived from embryos, that has proven indispensable in exploring the monarch butterfly's circadian clock mechanism. In DpN1 cells under a light/dark cycle, clock gene mRNAs and proteins oscillate in a manner that parallels their behavior in the monarch brain. This close correlation makes the cell line a useful system in which to study the molecular and biochemical details of the monarch clock transcriptional feedback loops in light/dark (focusing on the role of CRY2), as well as its intracellular light input pathway (focusing on the role of CRY1). No other light-sensitive insect cell line has been described so far. Moreover, DpN1 can be used to assess signaling pathways and novel protein interactions and to test genomic strategies. It has also been indispensable for characterizing the specificity of monarch-directed antibodies against clock proteins. For example, antigen specificity on Western blots can be verified by showing that band intensity of the endogenous protein is reduced by specific RNAi knockdown in DpN1 cells (76).

largely on homologies with *Drosophila* components (**Figure 3*b***). Studies to elucidate their function in DpN1 cells are now needed. Moreover, the genes for many enzymes that are likely involved in the posttranslational modification and degradation of the core negative feedback components by providing essential delays in the 24-h kinetic of the feedback loop have also been identified in the monarch genome; their precise functions need to be clarified. Results from these studies should nicely complement what is already known in the fly and mouse clocks, with the possibility of unexpected surprises (e.g., discovery of monarch CRY2 and its function).

The monarch butterfly may use CRY proteins as output molecules that define clock circuits in the brain (58, 76). Because the circadian clocks necessary for time compensation of sun compass orientation do not reside in the PL (42) but are located in the antennae, much of the brain's clock-CRY circuitry is likely involved in aspects of migration (e.g., reproductive diapause and longevity; see below) that are regulated separately from navigation (36, 50, 75) and in the circadian regulation of additional activities, including the daily timing of adult eclosion, the sleep-wake cycle, and metabolic rhythms. The brain clock circuit might also be involved in sensing the decreasing photoperiod in the fall, seemingly important for triggering the migration south, as previously mentioned (20).

The monarch genome also includes genes that encode orthologs of both the prepropeptide and the receptor for pigment dispersing factor (PDF), a key output system in the *Drosophila* brain essential for clock circuitry and driving locomotor activity rhythms (30, 60). Mapping monarch PDF peptide and receptor expression and defining their roles in butterfly clock circuitry, which are now feasible, may reveal a critical clock output system in monarchs.

A Bidirectional Time-Compensated Sun Compass

Remarkably, migrant monarchs use their time-compensated sun compass during both the fall migration southward and the spring remigration northward. This recalibration of time-compensated sun compass directionality relies on exposure of migrant monarchs to cold temperatures consistent with those found in the high-altitude coniferous forests in Mexico during their overwintering period there (24). Although the changing photoperiod during overwintering is not necessary for compass recalibration, it is likely important for timing the monarch's actual departure north (reviewed in 25). Age-matched fall monarchs that did not receive the overwintering cold temperature treatment, but were instead housed in the laboratory under fall-like conditions during the overwintering period and tested the following spring, continued to orient southward, whereas their

wild remigrant counterparts returning from the overwintering sites were on their northward journey (24). Thus, the migration cycle would be broken without cold exposure at the overwintering sites.

RNA-seq: a technology that uses high-throughput DNA sequencing to identify and quantify mRNAs (the transcriptome) from a whole genome

The time-compensated sun compass used by remigrant monarchs utilizes the same substrates for proper compass usage as during the fall (24). However, it remains to be determined what aspects of the time-compensated sun compass circuit are affected by cold to recalibrate directionality. Furthermore, transcriptional profiling by RNA-seq between southbound and northbound migrants may help dissect the molecular mechanism by which low temperature causes the switch in flight direction (e.g., using temperature-sensitive transient receptor potential channels) (25) and locate the relevant temperature sensor (perhaps in the antennae). The cold trigger in the remigration of the monarch butterfly and its dependence on the proper seasonal development of milkweed plants (*Asclepias* spp.)—the previously indicated primary food source of monarch larvae (25)—during the spring underscores how vulnerable the migration may be to global warming and overall global climate change.

A Magnetic Compass

Migrant monarchs use an Earth-strength, light-dependent, inclination-based, magnetic compass (**Figure 2*b***). This was shown using flight simulator trials during which diffuse nondirectional light was provided, while monarchs were exposed to artificially generated magnetic fields (polarity, inclination, intensity) (22). The results demonstrated that fall migrants can use the inclination angle of the Earth's magnetic field to obtain directional information, which they use to guide their latitudinal movement during the fall migration. A key finding in this regard is that a functional magnetic sense requires that monarchs be exposed to UV-A/blue light (wavelengths between 380 and 420 nm), supporting the idea that the magnetic sense is a light-based photochemical reaction in monarch butterflies, likely involving the CRY proteins (22). Either monarch CRY1 or monarch CRY2 transgenes can restore light-dependent magnetosensitivity in CRY-deficient *Drosophila* using the same UV-A/blue wavelength spectrum (16, 17). The roles of monarch CRY proteins in magnetoreception, at least in transgenic *Drosophila*, are independent of circadian clock function (17). Intriguingly, the monarch antennae have been identified as the structures that contain the relevant light-dependent magnetosensors for the magnetic compass to operate properly (22). Knowledge of the primary location of the relevant light-sensitive component of the magnetosensor in monarchs allows for evaluation of both the molecular and genetic mechanisms of magnetoreception and the involvement of CRY at a level not yet possible in any other migratory species.

Flight simulator experiments involving clock-shifted butterflies showed that migrants fly in the predicted adjusted orientation despite being exposed to the Earth's unmanipulated magnetic field (13, 23, 24, 42, 46, 63, 75). Thus, the magnetic compass is likely a backup mechanism to the primary time-compensated sun compass, which may dominate only when directional daytime skylight cues are unavailable, e.g., during overcast conditions (22, 59). Additionally, the magnetic compass may be an important calibrator of the time-compensated sun compass in monarchs, fine-tuning time-compensated sun compass usage (25).

The magnetic sense of monarchs may also underlie an overall geomagnetic map sense. Monarchs may use this geomagnetic map sense to assist in locating the handful of coniferous fir grove overwintering sites in Mexico. It has also been postulated that there may be olfactory cues, originating from the unique forest habitat and/or from the mass of decaying dead butterflies at the site, that guide the monarchs to this forest (44, 54).

Other Navigational Mechanisms

Monarchs may use other navigational mechanisms during migration besides their time-compensated sun compass and magnetic compass. For example, given that migrant monarchs use thermals to facilitate long-distance passive flight, they likely have a strategy to detect winds with the correct speed and direction to assist their migratory flight (5, 18, 19, 66). Johnston's organs in the antennae of several insect species are important mechanosensors used to detect wind (7, 14, 40, 56).

Monarchs may also use navigation or orientation information acquired in social interactions while migrating, such as during flight or within overnight roosts. Such migration-relevant information might be transferred as olfactory cues in the form of volatile conspecific pheromones. Antennae may play an important role in the detection of these conspecific cues, given that they are important olfactory sensors in lepidopterans (6) and several olfactory receptor genes of the pheromone clade have been identified in the monarch genome (71).

Monarchs may use landmarks during migration. For example, the funneling of migrant monarchs through Texas during the late stages of the fall migration may be due to their use of bodies of water or mountain ranges for navigation. Similarly, migrants may use cues derived from geophysical landmarks—e.g., the light reflected off a mountain top or a body of water at specific times of day (26).

JUVENILE HORMONE AND MIGRATION

Juvenile hormone (JH) regulation is crucial in migratory butterflies to orchestrate the multiple physiological processes required for successful migration, including reproductive arrest, an increase in longevity, and increasing fat stores used for flight. These are likely induced in the migratory monarch by downregulation of the insulin-signaling pathway and the demonstrated JH deficiency (31, 32), as documented in *Drosophila* (12). In response to environmental factors (e.g., temperature and photoperiod), insulin signaling could be reduced through a decrease in the production and/or secretion of insulin-like peptides and/or a decrease in the expression of associated receptors, which would reduce JH biosynthesis in the corpora cardiaca–corpora allata complex, leading to both reproductive shutdown and reduced aging (54). Indeed, transcriptional studies suggest that levels of insulin-like peptide-1 are decreased in migrant monarchs, consistent with its involvement in regulating JH biosynthesis (71).

Curiously, JH deficiency is not necessary for persistent time-compensated sun compass orientation in migrants (50, 75); migrants treated with a JH analog (methoprene) that induces full reproductive function still orient southward over the course of the day. Although it is possible that JH deficiency is involved in the induction of directed flight for sun compass orientation, persistent JH deficiency is not required for the maintenance of time-compensated sun compass orientation, supporting the notion that sun compass orientation is mediated by a separate mechanism (and clock system—antennae versus brain).

With the draft sequence of the monarch genome, virtually all the genes proposed to be involved in JH biosynthesis (2) have been identified and annotated (71). Unexpectedly, transcriptional profiles of these enzymes in summer (nonmigratory) and migratory monarchs reveal a sexually dimorphic pattern of JH biosynthesis (71). Enzyme levels are globally downregulated in males, whereas females appear to maintain low JH levels by increasing turnover or binding to JH-binding proteins. This finding in monarchs suggests that sexual dimorphism of the molecular pathway of JH regulation may be used by other insects and is an area that deserves further study.

GENETIC BASIS OF THE MIGRATION

In the fall, migratory monarch butterflies are on their maiden voyage, suggesting a genetically driven epigenetic basis of migration (54). Demonstrating that all generations of the migratory population, including spring and summer, have the genetic capacity of expressing the migratory syndrome if exposed to the appropriate environmental stimuli during development or early adulthood would validate this hypothesis. Epigenetic events leading to the migratory state could involve DNA methylation, histone and chromatin modifications, microRNA (miRNA) function on gene regulation, and/or RNA editing. Epigenetic mechanisms could lead to differences in gene expression levels between migratory and summer butterflies that could in turn reveal the underlying genetic basis of the migration.

Initial studies of differences in gene expression were performed using a brain-expressed sequence tag library for microarray construction and subsequent transcriptional analysis (74, 75). However, the resultant analysis was of limited value because of incomplete genome coverage and a focus on brain expression. What was needed was a more complete genomic blueprint.

The Monarch Genome

In 2011, the draft sequence of the 273-Mb genome of the monarch butterfly was presented along with a set of over 16,000 protein-coding genes (71). The biological interpretation of the genome focused on the monarch's migration biology. The utility of the genome for aiding the understanding of various aspects of monarch biology has been discussed throughout this review.

One potentially important aspect of the genomic analysis predicted that monarchs have a low level of DNA methylation and are thus unlikely to use methylation as an epigenetic strategy (71). Sequencing and computation methods were used to characterize miRNAs in summer and migratory monarchs. Over 100 miRNAs were identified, and half of those exhibited >1.5-fold differences in mean expression levels between summer and migratory monarchs (71). Of those with known function, 3 miRNAs stood out; based on homology and studies in other animals, they appeared to be associated with muscle activity, cold tolerance, and fat metabolism. All of these may be biologically relevant to the migration and will require further evaluation.

To make the extensive data associated with the genome accessible to the general biological and lepidopteran communities, MonarchBase (**http://monarchbase.umassmed.edu**) was established (72). MonarchBase provides an updated version of the genome assembly upon which all related data integration (e.g., annotations and miRNAs) is now based.

Population Genetics

The draft genome was expected to provide a solid background for population genetics studies between migratory and nonmigratory populations (71). In fact, results of population genetics studies have been surprising (52, 73). Because monarchs (*D. plexippus*) occur worldwide, it was possible to perform whole-genome sequencing on 80 monarchs from diverse populations from across the globe, including both migratory and nonmigratory ones, and to analyze genome-wide single nucleotide polymorphism (SNP) variation to reveal the evolutionary history of monarchs and their migration (73). The monarch butterfly appears to have originated in North America and had a migratory ancestor, with the migration dating back at least one million years. Approximately 20,000 years ago, after the last glacial maximum, the North American population expanded and the migration extended further northward from Mexico to ultimately fill out its current northern range.

Genomic regions strongly associated with migration have been identified (73). These make up 2.1% of the genome and comprise more than 500 genes, including those involved in morphogenesis and neurogenesis. A single 21-kb genomic segment stood out as showing signatures of divergent selection. The gene showing the most marked divergence encodes collagen type IV α-1, which is important for muscle function. Further gene expression and metabolic studies suggested greater flight efficiency among migratory monarchs as one of the hallmarks of the migration. It is still unclear whether other genes at the loci under selection could be involved in navigation. An RNA-seq study of transcriptional differences between migrants, remigrants, and nonmigrants should help sort out which of the 500 candidate genes should be subjected to a more rigorous functional assessment.

Genetic Manipulations

Given their wide success in model and nonmodel organisms, engineered nucleases have emerged as powerful tools for dissection of gene function in vivo. Zinc-finger nucleases (ZFNs), transcription activator–like effector nucleases (TALENs), and RNA-guided nucleases (RGENs) derived from the clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein 9 (Cas9) system enable site-specific genetic modifications by inducing DNA double-stranded breaks (DSBs), which allow for precise genome manipulation when repaired by the cellular machinery. DSBs stimulate the introduction of small random insertions/deletions through nonhomologous end joining repair or the introduction of donor constructs through homology-directed repair (**Figure 4**) (reviewed in 15, 34).

A big challenge in applying these technologies to nonmodel insects, however, is to develop reliable strategies for nuclease delivery and mutation screening. Such approaches have been successfully developed in only a few insect species (38, 39, 41, 57, 64, 67, 68). As previously mentioned, microinjection of mRNAs encoding ZFNs into fertilized eggs at the "one nucleus" stage effectively induced targeted mutagenesis at the *cry2* locus in the monarch and genetically defined CRY2 as the main transcriptional repressor in the monarch clockwork (**Figure 4**) (41).

Despite this important progress, TALENs and RGENs are attractive alternatives to ZFNs. RGENs, for which only the gRNA needs to be customized to target the genes of interest, will be prime candidates because they allow for unprecedented efficiency of targeted mutagenesis in another lepidopteran species, *Bombyx mori* (67). Continuing to expand the genetic tools available for easier and more efficient genome editing in the monarch will aid a systematic dissection of the molecular and cellular mechanisms underlying the monarch's long-distance migration. Ultimately, generating a nuclease-mediated homologous recombination strategy to knock in fluorescent tags at genomic loci (e.g., clock gene loci) will accelerate the circuit mapping of the navigational neuronal network used by migratory monarchs.

CONSERVATION

Over the last two decades, the overall number of monarch butterflies in North America, as estimated from the number of migratory eastern North American fall migrants at overwintering sites in Mexico, has been on a steady decline (4), with a record low observed in 2013. This has raised concern that the extraordinary phenomenon of monarch migration is in danger in North America, in particular the long-distance migration of the eastern North American population. In addition, although the idea that monarchs themselves are in danger of extinction is unfounded, it has nonetheless prompted significant and well-publicized efforts to designate monarch butterflies as a protected species under the US Endangered Species Act. It is important to note, however, that

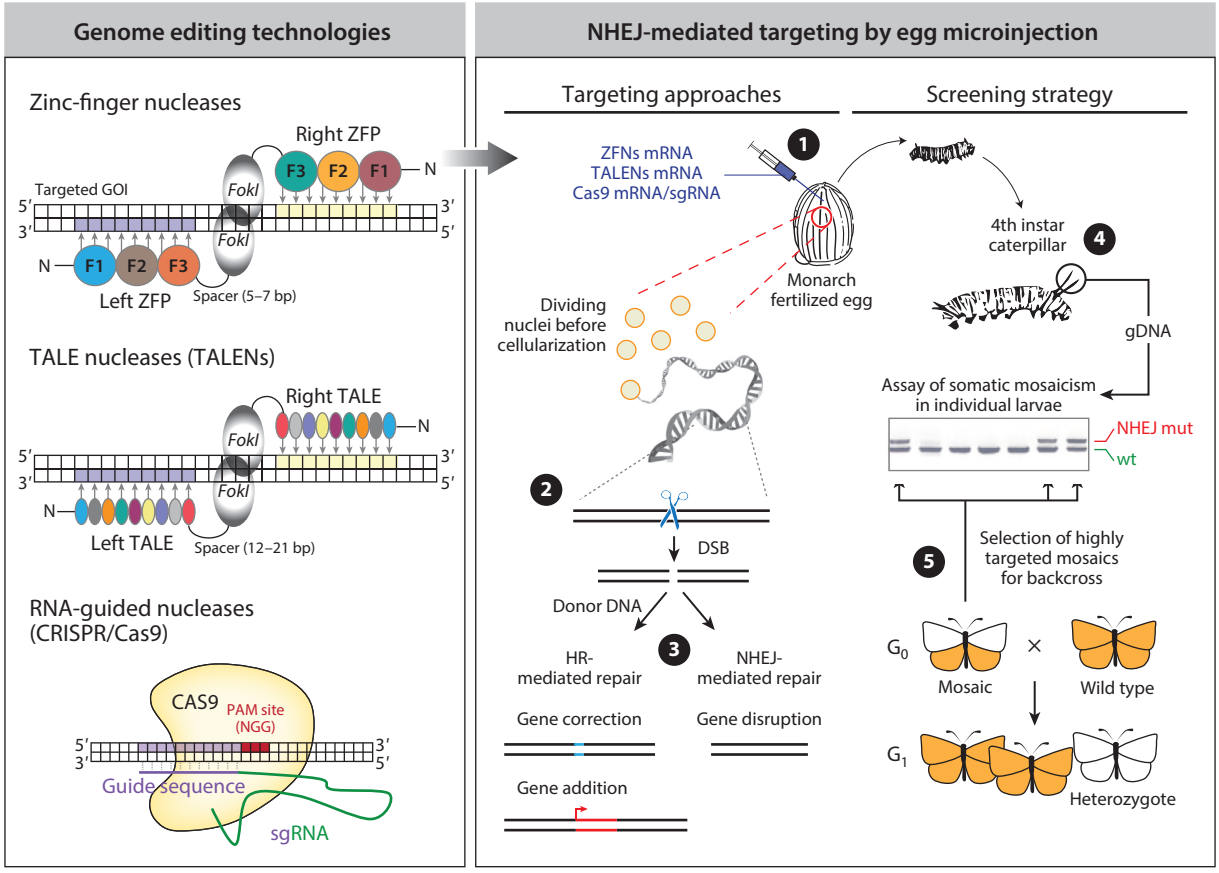


Figure 4

Targeted genome editing in monarch butterflies. (*Left*) Available genome editing technologies to manipulate genes in vivo in nonmodel insects. (*Top*) Zinc-finger nuclease (ZFN) pair bound to its target sequence. Each ZFN is composed of an array of three to four zinc-finger proteins (ZFPs), each recognizing a 3-bp DNA sequence, linked to a *FokI* nuclease domain that cleaves double-stranded DNA upon dimerization in the spacer. (*Middle*) Transcription activator–like effector nuclease (TALEN) pair bound to its target sequence. Each TALE array is composed of 33–35 amino acid repeats, each recognizing a single base pair in the target sequence, linked to a *FokI* nuclease domain that cleaves double-stranded DNA upon dimerization in the spacer. (*Bottom*) RNA-guided nuclease, composed of a CRISPR (clustered regularly interspaced short palindromic repeat)-associated protein 9 (Cas9) and a single-chain guide RNA (sgRNA). The guide sequence in the sgRNA is complementary to a 20-bp target sequence that harbors a protospacer adjacent motif (PAM; 5′-NGG-3′) site. (*Right*) Targeting and screening strategy for establishing nonhomologous end joining (NHEJ)-mediated knockout lines. ❶ Newly fertilized eggs are injected during the first nuclei division to promote targeting of germ cell precursors with ZFN-encoding mRNAs, TALEN-encoding mRNAs, or Cas9-encoding mRNAs along with a sgRNA. ❷ Upon binding on the targeted genomic sequence, ZFNs, TALENs, and the RNA-guided Cas9 induce DNA double-stranded breaks (DSBs) that stimulate cellular DNA repair mechanisms, including error-prone NHEJ-mediated and homologous recombination (HR)-mediated repair. ❸ NHEJ-mediated repair of DSBs can introduce insertions/deletions that lead to frameshifts and subsequent gene disruptions. Gene correction and/or addition can also be achieved through HR-mediated repair by coinjecting the nucleases along with an exogenous DNA donor template. Larvae surviving the injection are reared to the fourth instar ❹, a stage at which noninvasive genotyping is performed to screen for highly targeted somatic mosaics using PCR-based assays on genomic DNA (gDNA). ❺ To reduce labor-intensive breeding efforts, the larvae presenting the highest degree of NHEJ-mediated mutagenic targeting (NHEJ mut) in somatic cells are selected, reared to adulthood (G_0), and backcrossed to wild-type monarchs (wt). The G_1 progeny are screened for targeted mutations, and mutated alleles are sequenced. Modified from Reference 41.

the monarch species is not necessarily threatened; rather, their migratory biology is in jeopardy (21).

Research into the neurobiology of the orientation capacities of migrant monarchs has yielded important information that can serve to inform efforts to preserve the long-distance migration. For example, climate change and human-induced sensory pollution appear to be important factors that may detrimentally affect monarch butterflies, given that such environmental factors may impede the orientation capabilities of migrating monarchs. Because cold is the key trigger for the recalibration of the time-compensated sun compass used during remigration, climate change may hinder this recalibration process. Therefore, this important finding exposes a key vulnerability of the monarch's migratory cycle to temperature change associated with climate change. Warmer temperatures at the overwintering sites could prevent or alter the northward return of migrants during the spring; warmer temperatures in the southern United States during winter may obviate migration to Mexico and induce monarchs to form resident, nonmigratory populations all year round, as seen in Florida (35). Moreover, the coldness requirement for sun compass recalibration, coldness that is unique to and provided by the microenvironments found at the overwintering sites, underscores the necessity of preserving these habitats for monarchs.

Just as human-induced electromagnetic noise appears to hamper the orientation of other species that possess a magnetic compass sense (11), such sensory interference may negatively affect the magnetic sense of monarchs during migration. This information may further conservation efforts with respect to continued human urban planning and technology use on large spatial scales. Future research will bolster efforts to preserve this remarkable phenomenon in the iconic monarch butterfly.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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