

# Demystifying Monarch Butterfly Migration

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Every fall, millions of North American monarch butterflies undergo a stunning long-distance migration to reach their overwintering grounds in Mexico. Migration allows the butterflies to escape freezing temperatures and dying host plants, and reduces infections with a virulent parasite. We discuss the multigenerational migration journey and its evolutionary history, and highlight the navigational mechanisms of migratory monarchs. Monarchs use a bidirectional time-compensated sun compass for orientation, which is based on a time-compensating circadian clock that resides in the antennae, and which has a distinctive molecular mechanism. Migrants can also use a light-dependent inclination magnetic compass for orientation under overcast conditions. Additional environmental features, e.g., atmospheric conditions, geologic barriers, and social interactions, likely augment navigation. The publication of the monarch genome and the development of gene-editing strategies have enabled the dissection of the genetic and neurobiological basis of the migration. The monarch butterfly has emerged as an excellent system to study the ecological, neural, and genetic basis of long-distance animal migration.

## Introduction

Universally famous, the fall migration and overwintering roosts of the eastern North American monarch butterfly (*Danaus plexipus*) are eye-popping. So, it is hard to believe that the location of the monarch's overwintering grounds was a mystery until January of 1975. That was when one overwintering site was discovered in the mountains of central Mexico by Kenneth and Catalina Brugger. They were part of the team led by Fred Urquhart, who had been searching for the monarch's overwintering sites for 38 years, including 24 years of tagging studies with citizen science involvement. Urquhart used the August 1976 issue of *National Geographic Magazine* [1] as a visual platform to announce the discovery of the overwintering area to the outside world — of course, the locals were all too familiar with this spectacle. And what a spectacle it is, with tens to hundreds of millions of colorful butterflies collectively roosting and completely covering entire trees and the forest floor beneath in coniferous oyamel fir groves. Over the next decade, Lincoln Brower, William Calvert and colleagues mapped the precise locations of the major overwintering sites of the eastern population atop a few mountain ranges in the center of the Transverse Neovolcanic Belt of Michoacán Mexico [2].

In this review, we translate the visually spectacular migration of the monarch (Figure 1A) into equally compelling biological terms. We cover important ecological factors that affect the migration, including host plant interactions and the monarch's struggle with an obligate, protozoan parasite. Then there are the mechanistic studies of the neural and genetic basis of the migration, which focus on the remarkable navigational capabilities of these long-distance travelers. The neural mechanisms underlying the navigational aspects of the migration have been reviewed recently [3]. We revisit these navigational issues and, using an inclusive, integrated approach, highlight our current understanding of the ecological, neural, and genetic basis of the migration focusing on studies of the eastern North American

population. We emphasize the major advances and pose the next challenges to address. We believe that understanding the monarch migration will provide general biological principles that may apply broadly to other long-distance migratory species.

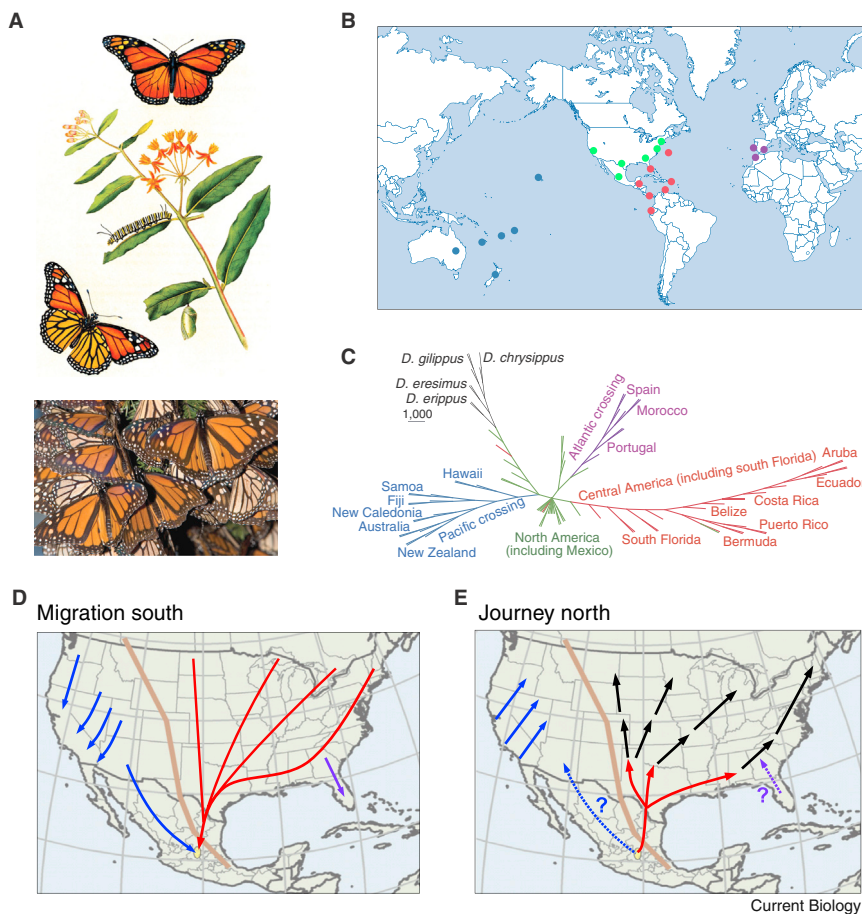
## Evolutionary History

Remarkably, the eastern monarch migration is ancient, dating back a million years [4]. Although monarchs are best known for their long-distance migratory population in eastern North America, they also form a smaller population west of the Rocky Mountains, a major portion of which migrates to the Pacific Coast of California (Box 1) [5]. In addition, monarchs have formed apparent non-migratory populations around the world, in locations in South Florida and Central America, southwestern Europe and North Africa and many Pacific Islands [6,7] (Figure 1B). The publication of an expressed sequence tag resource [8] and the monarch genome [9] provided the essential means to study monarch population genetics and their evolutionary history, using a variety of genetic markers including microsatellites [10–12] and genome-wide single nucleotide polymorphism (SNP) variations [4]. Indeed, the whole genome sequencing of 80 monarchs from around the world suggested that monarchs originated in North America and were ancestrally migratory, with subsequent Central American, Pacific Ocean and Atlantic Ocean dispersals to form apparent non-migratory resident populations [4] (Figure 1C); this evolutionary history was confirmed independently by microsatellite analyses [10]. 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 northerly range.

## The Contemporary Migration Cycle

Unique among insects and resembling that of birds, the monarch migration is one of a kind. Why? —because of the distances traveled (up to ~4,500 km) [13], combined with its anticipated,





**Figure 1. Natural and evolutionary history of the monarch butterfly.**

(A) Upper: Life cycle or one complete generation in the monarch butterfly. Complete metamorphosis from egg to larva (5 instars) to pupa (chrysalis) to adult. The male butterfly (upper right) has visible black spots on his hind wings that are missing in females (lower left, underwing view). The larva is feeding on milkweed. Photograph of engraving from James Edward Smith, *Natural History of the Rarer Lepidopterous Insects of Georgia*; from the *Observations of John Abbot, 1797*. Lower: A cluster of overwintering migrants coving a tree trunk in Michoacán, Mexico. Photo credit: Thomas Marent, Minden Pictures (Getty Images). (B) World-wide distribution of monarch butterflies. Sample sites: Blue dots, Pacific Islands; red dots, Central America; green dots, North America; purple dots, Southwest Europe and North Africa. Not demarcated are additional monarch sites in Guam, Palau, and Taiwan. Modified from Pierce *et al.* [10]. (C) Neighbor-joining consensus tree of all *D. plexippus* individuals, based on 1,000 bootstrap replicates of genome-wide SNP data of butterflies from the sampling sites shown in (B). Four different *Danaus* species served as outgroup. Reproduced from Zhan *et al.* [4]. (D) Migration south. Eastern North American monarch butterflies east of the Rocky Mountains (brown line) migrate long distances during the fall from their northern ranges (red arrows) to their overwintering sites in central Mexico (yellow oval). Some Eastern migrants do not end up at the overwintering sites in central Mexico, but fly off-course and end up in Florida [101], where they presumably join the year-long resident monarch populations in Florida (purple arrow). Western 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 USA, Western migrants have been shown to also reach the overwintering sites in

Mexico [25,102]. Reproduced from Reppert *et al.* [3]. (E) Journey north. Eastern migrants remain at the overwintering sites in Mexico until spring, when these butterflies return northwards to reproduce, with females laying fertilized eggs on newly emerged milkweed in the southern United States (red arrows). The offspring of these butterflies will continue the journey northwards to further re-populate the northern sections of the monarch habitat range (black arrows). It is speculated that monarchs from populations in Florida may also travel northwards during the spring, presumably to also take advantage of newly emerged milkweed. Reproduced from Reppert *et al.* [3].

repetitive nature. Each year, southerly bound migrants arrive at their overwintering sanctuary around November 1, coinciding with the celebrations of Day of the Dead, and northerly based re-migrants begin to leave Mexico around March 15 (Figure 1D). Although the distance traveled by monarchs is reminiscent of that of many migratory birds, the monarch migration differs because it is mostly multigenerational (Figure 1E). Thus, while individual birds complete one or more complete round-trip migrations during their lifetime, it takes 3–5 generations for most monarchs to complete the full journey [14,15]. Some butterflies, however, apparently make a complete round trip in one generation [15]. It is important to note that one generation of monarchs comprises one complete life cycle, from egg to larva (5 instars) to pupa (chrysalis) to adult butterfly (Figure 1A, upper). The spring and summer generations are continuously brooded, while the butterflies of the fall (migratory) generation have arrested reproductive development (diapause), as detailed below.

Monarch caterpillars are specialist feeders of milkweed host plants (Box 2), which die back seasonally in North America. Consequently, in the fall, dying milkweed, along with approaching

freezing temperatures, limit continuation of the monarch life cycle beyond the emergence of migrant butterflies in their northern range. The migratory generation is actually initiated in late summer-developing caterpillars and/or pupae that are responding to shortening day length, decreasing temperature, and senescing host plants so that the newly emerged migrants can anticipate winter by entering reproductive diapause and beginning the migration south [16]. This reproductive diapause is characterized by arrested egg and accessory gland development and increased lipid reserves, and results from decreasing titers of juvenile hormone (JH) [9,17,18]. Suppression of JH synthesis also prevents aging in migratory monarchs, which enables them to survive the 6–8 months they need to migrate south, overwinter and remigrate north in the spring [19]. The draft sequence of the monarch genome has provided identification of all the genes and their protein products proposed to be involved in JH biosynthesis and degradation [9]. Preliminary transcriptional profiling studies suggest a sexually dimorphic pattern of JH biosynthesis in migrants, and follow up studies are needed [9].

**Box 1. West versus East.**

While monarchs are best known for the long-distance migration of tens to hundreds of millions of butterflies from eastern North America to Mexico, up to several hundred thousand monarchs inhabit breeding grounds west of the Rocky Mountains and migrate shorter distances to Monterey Pine and Eucalyptus evergreen groves along the Pacific Coast in California [2,103,104] (Figure 1D). Eastern monarchs may fly over 4,000 km to reach the Mexican overwintering sites [13], while many western monarchs reach the California Coast by flying less than 500 km, although some may fly over 1,600 km [105]. Whether eastern and western monarchs form genetically distinct populations remains unresolved. Tagging programs have indicated that there is at least some exchange between eastern and western monarchs, with monarchs tagged in Arizona being recovered in both California and Mexico [102]. In addition, population genetic analyses using neutral microsatellite markers did not detect significant genetic differentiation between eastern and western monarchs [11]. As with eastern migratory monarchs, the population size of western migratory monarchs has declined over recent decades, from several millions in the 1980s to hundreds of thousands in recent years [104].

After several weeks of southward flight, migrants arrive for a four-month stay at their mountain top overwintering roosts, where temperatures hover just above lethal freezing. Those low temperatures, buffered by roosting behavior, are needed to maintain the arrested reproductive state, prevent high activity and premature depletion of lipid reserves, and ultimately reverse sun compass direction (see below). With the warming temperatures of spring, overwintering butterflies become reproductively active, mate and leave the overwintering sites to remigrate northwards [20,21]. The remigrant females oviposit primarily on milkweed in the southern USA [22]. Being multigenerational (see above), it takes at least another two generations of reproductively active spring and summer butterflies to recolonize the full northern breeding range (Figure 1E). These monarchs are likely not migrating north, but instead are following the progressive, northerly emergence of milkweed to repopulate their northern breeding range, although this issue requires further study [14]. The migration cycle is then complete and repeats itself again in the fall. Impressively, the annual migration concentrates up to hundreds of millions of butterflies from a 4.5 million km<sup>2</sup> breeding range into less than a dozen overwintering sites that together measure roughly 1 km<sup>2</sup> [23]. While overwintering colonies occupy distinct forest patches, these colonies are fully mixed with respect to natal origin [14,24]. Migration is not only essential for the survival of monarchs in their temperate North American climate, but also provides additional benefits by reducing the prevalence of virulent infections by a protozoan parasite (Box 3).

**Navigating to Mexico**

Migratory monarchs possess remarkable navigational abilities, emanating from a brain no larger than the head of a pin. Migratory animals are often classified as either true navigators or compass navigators [25]. True navigators, including white-crowned sparrows, loggerhead sea turtles and spiny lobsters [26–28], know both the travel direction and their geographic location in relation to their goal, while compass navigators use compasses to orient in the direction of their goal [25]. Compasses indeed are used for orientation by the millions of monarchs, migrating long distances to their overwintering grounds in central Mexico. Such compasses take advantage of predictable environmental cues that can be used as guide posts for directionality over the course of the day and throughout the duration of the migration. In fact, for proper orientation, monarchs possess two compass

systems — a dominant one that exploits skylight cues and a clock, while another uses a geomagnetic cue. But, are monarchs also true navigators with an actual ‘map’ sense? A map sense could help explain how monarchs know where and when to stop at their overwintering sites in Mexico. How migrants find their overwintering grounds year after year remains a glaring unknown of the migration.

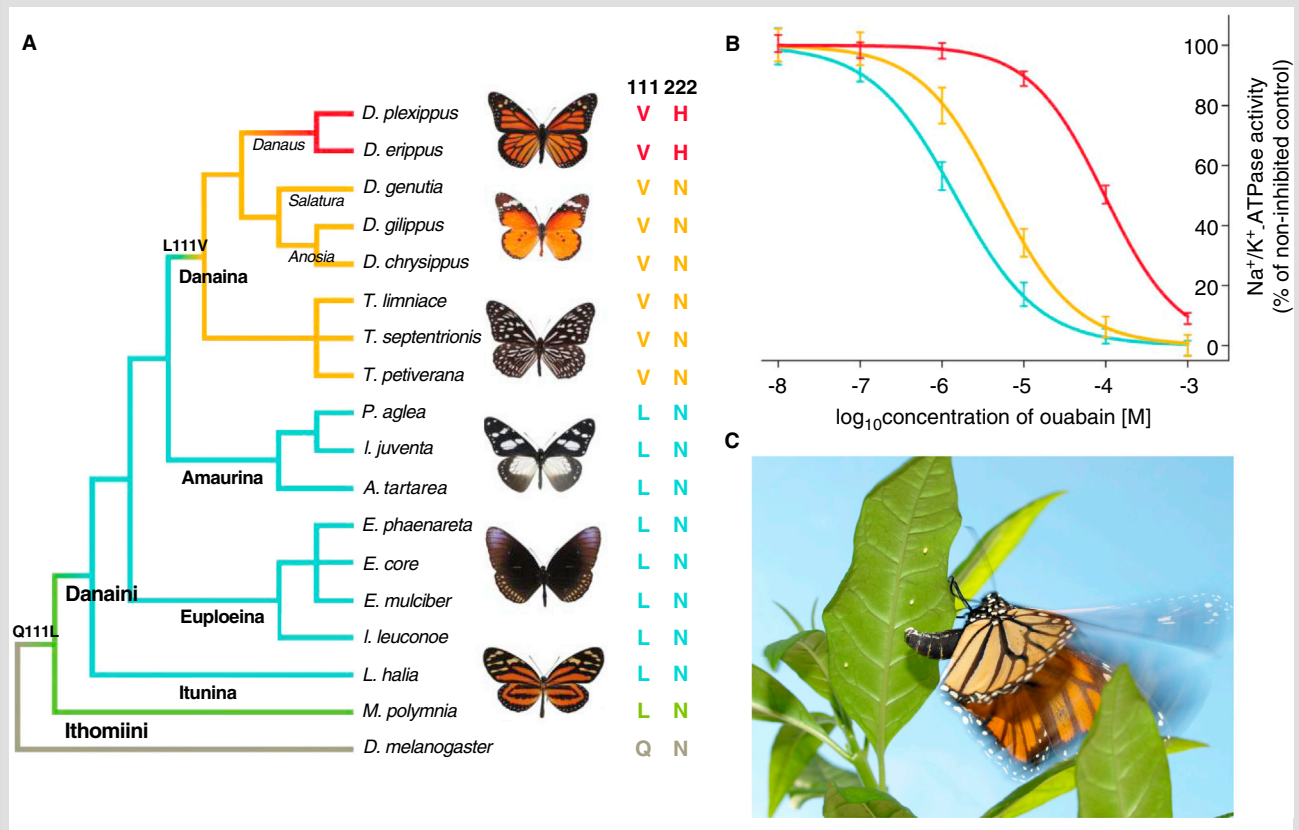
One interpretation of tagging studies carried out over the last ~50 years suggests that fall migrants may indeed use a map sense, as monarchs from across their breeding grounds have predictably different southerly orientation directions [29]. In general, those from the Northeastern US orient southwest, whereas those from the Midwest/West are more likely to orient due south or even southeast (Figure 1D). However, one study has reported that migrants longitudinally displaced west by 2,500 km across Canada do not adjust their orientation to compensate for the displacement [30], stimulating some debate concerning the experimental design of this investigation [29,31]. Clearly, more extensive displacement studies are required.

**Using a Time-Compensated Sun Compass**

Monarchs use a time-compensated sun compass as the major compass system for directionality during both the southward fall migration and the northward spring remigration [21]. Because of the daily rotation of the earth about its axis, it appears as though the azimuthal (horizontal) position of the sun moves across the horizon from East to West. So, for an animal to use the sun’s position for a fixed, daily heading, the circadian clock has evolved as a timing mechanism necessary for adjusting or compensating for the sun’s movement. Hence, the sun compass is time compensated. The azimuthal position of the sun is the dominant skylight cue used for directionality [3]. Yet, scattered sunlight generates the skylight polarization pattern, which on partly cloudy days, when the sun is obscured, but while blue sky is still visible, may provide a backup directional cue [32,33]. A time-compensated sun compass was first proposed by Karl von Frisch in foraging honey bees [34] and by Gustav Kramer in migratory birds [35]. The genetically determined, unrelenting directional orientation, day in and day out, has made the migratory monarch an ideal system for studying the mechanistic details of this compass system.

Behavioral experiments of sun compass orientation have been performed using both disappearance bearings of released migrants [21,36,37] and the orientation of tethered

## Box 2. Monarchs and milkweed.



While adult monarchs utilize a wide variety of flowers as nectar sources, monarch caterpillars are specialist feeders of milkweed plants, mostly in the genus *Asclepias*. Milkweeds have evolved a diverse array of defenses against herbivores, including the white latex from which 'milk'-weeds derive their name [106]. In addition, many milkweeds have tough leaves or carry large numbers of hairy outgrowths, and most milkweed species contain considerable concentrations of cardiac glycosides, a class of chemicals also known as cardenolides [107]. Because of their toxic nature, milkweeds are generally avoided by generalist herbivores, but a small number have evolved the ability to avoid, tolerate and resist the defenses of milkweed [108]. Indeed, milkweed specialists ranging from monarchs to aphids hijack the milkweeds' defenses to protect themselves against natural enemies [109]. Monarchs have become a textbook example of warning coloration and plant-derived toxicity to predators [110]. Monarch larvae sequester cardenolides from their milkweed diet [22], and broadcast their toxicity through the black, white and yellow stripes in larvae, and the bright orange marked with black and white accents in adults [111]. Classical studies have demonstrated that avian predators quickly learn to associate the bright colors with bitter taste and emesis, leading to prey avoidance [110,112].

Cardenolides exert their toxic effects in most animals by interfering with the  $\text{Na}^+/\text{K}^+$ -ATPase sodium pumps, but monarchs and other milkweed specialists have mutations that reduce cardenolide binding [8,9]. Evolution of cardenolide insensitivity evolved in a step-wise manner during the macroevolution of milkweed butterflies, with monarchs having at least two non-conservative mutations that strongly reduce their sensitivity to cardenolides. (A) A cladogram of the Danaini tribe of butterflies, with branches color-coded with respect to  $\text{Na}^+/\text{K}^+$ -ATPase (reproduced from Petschenka *et al.* [113]). Amino acid substitutions at positions 111 and 122 are indicated to the right of the cladogram, showing the stepwise evolution of this ion channel. (B) The sensitivity to the commercially available cardenolide ouabain of the three types of  $\text{Na}^+/\text{K}^+$ -ATPase found in Danaini, with colors corresponding to the colors in the cladogram in (A) (reproduced from Petschenka *et al.* [113]). The high level of resistance to cardenolides allows monarchs not only to feed on milkweeds, but also to sequester cardenolides for their own defenses [113]. Although the sequestration of cardenolides by monarchs has been mostly studied in the context of predation, recent studies suggest that these toxins also provide protection against infection with a virulent protozoan parasite (Box 3). Monarchs reared on milkweed species with higher concentrations of heart-arresting cardenolides experience lower infection rate, less parasite growth and fewer disease symptoms than those reared on milkweeds with lower concentrations [114,115]. Furthermore, when given a dual choice between

(Continued on next page)



## Box 2. Continued

milkweeds that vary in their anti-parasitic effects, infected female monarchs prefer to lay their eggs on the anti-parasitic species (C; photo by J.C. de Roode), thereby reducing infection and disease symptoms in their offspring [116]. These studies have also shown that despite their high level of insensitivity, monarchs are not fully resistant to cardenolides, with highly toxic cardenolides reducing adult monarch lifespan [117].

monarch migrants in a flight simulator [18,21,30,38–42] (Figure 2). Shifting of the timing of the 24-hour light–dark cycle consistently results in predictable shifts in orientation behavior (Figure 2A). The results of such clock-shift experiments show the existence of a time-compensated sun compass in monarchs. Summer monarch butterflies, by contrast, do not show oriented flight [18].

We begin to dissect the mechanisms behind the time-compensated sun compass starting with the sensing of skylight cues for directionality (Figure 3). The main retina of the compound eye of the monarch senses the sun's azimuthal position. The dorsal rim area of the eye is anatomically and molecularly specialized to sense the angle of partially plane polarized ultraviolet light (the electric *E* vector) [32,33,43]. However, whether migrants can respond behaviorally and orient to skylight polarization is unclear [32,42]. Sensory signals from the eye-sensed directional cues are transmitted via complex circuitry to the brain's central complex, a mid-line structure believed to be the central component of the monarch's sun compass where skylight cues are integrated (Figure 3A,C).

The insect central complex is a broadly conserved region consisting of a highly ordered neuroarchitecture [44–46]. Intracellular recordings from monarch central complex input neurons show that single neurons integrate both azimuthal position and the *E*-vector angle [47]. Moreover, these dual responses are integrated to create a consistent representation of skylight cues and their changing relationship to each other in the central complex throughout the day (Figure 3D). These data strongly support the notion that this brain region is indeed the core structure for the monarch sun compass [47].

A standardized, average-shape representation of the neuropils in the central complex of the monarch brain has been developed [48]. This standardized version provides useful reference volumes of these neuropils and a common reference for the mapping and characterization of neuron structure in the central complex. Registration of these neuron morphologies on the standardized neuropils has identified the major input and output pathways of the monarch central complex, along with its intrinsic neurons [48]. The general anatomical features of the monarch central complex are consistent with those found in the central complex of other insect species, in which this structure is believed to act as a sensory-motor integration center. Examples include the central complex association with sun compass navigation in the desert locust, with place learning and visual landmark orientation in *Drosophila*, with polarized moonlight foraging in nocturnal dung beetles, and with antennae-based obstacle navigation in cockroaches [49]. To understand how spatial and temporal information are integrated to guide oriented flights in fall migrants and spring remigrants, translating this wiring map into functional circuits is needed by the continued use of electrophysiological as well as imaging and molecular approaches, which are now achievable [3].

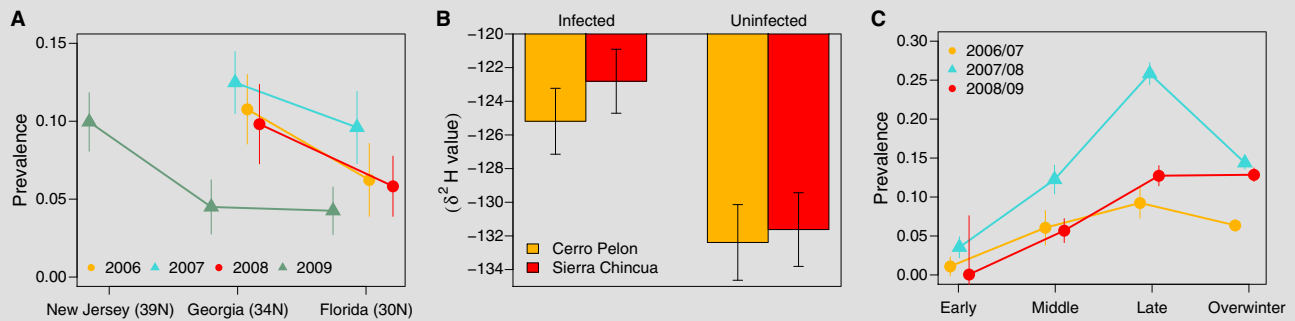
Surprisingly, the chronometer for the sun compass system in the monarch resides in circadian clocks in the antennae, not those in brain [39,40]. Monarchs without antennae or which have had both antennae painted black (to block light input) do not exhibit the proper southerly orientation [40] (Figure 2B). A single, functional antenna, however, is sufficient for proper time compensation and oriented flight, but conflicting timing between the two antennae disrupts orientation [39]. Thus, each antenna appears to provide independent timing information to a brain integration site that ultimately interacts with the output side of the sun compass circuit [39].

As previously stated, migrant monarchs use their time-compensated sun compass during BOTH the fall migration southward and the spring remigration northward (Figure 2C). This northward recalibration of time-compensated sun compass directionality has been shown to rely on the exposure of migrant monarchs to coldness for three weeks, temperature conditions similar to those found at the overwintering sites of monarchs during their overwintering period [21]. Aged fall monarchs not receiving the cold treatment, but who were instead housed in the laboratory under fall-like conditions during the overwintering period and examined outdoors the following spring, continued to orient southwards, while their remigrant counterparts returning from the overwintering sites in Mexico were on their northward journey [21]. Collectively, the results show that without coldness exposure at the overwintering sites the migration cycle would end.

In addition to the recalibration of the bidirectional sun compass via coldness, spring remigrant monarchs also use antennal clocks for time compensation [21]. Unknown are aspects of the time-compensated sun compass circuit affected by coldness to recalibrate directionality. As previously suggested [3], transcriptional profiling 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) [50] and the anatomical location of the relevant temperature sensor (maybe in the antennae).

A fascinating parallel occurs between cold recalibration of the sun compass direction and the cold-required vernalization of milkweed. The subsequent return northwards of spring remigrants by increasing photoperiod likely co-evolved with the timing and onset of newly emerged milkweed in the Southern US in spring [50]. This allows remigrant females to oviposit on newly emerged milkweed at the appropriate time and place. Knowledge of the 'cold trigger' in the remigration of the monarch butterfly and its dependence on the proper seasonal development of milkweed plants during the spring underscores how vulnerable the migration may be to climate change [50].

Although progress has been made defining the eye-sensing, sun compass and circadian clock components, still missing, as

**Box 3. Monarch migration and infectious disease.**

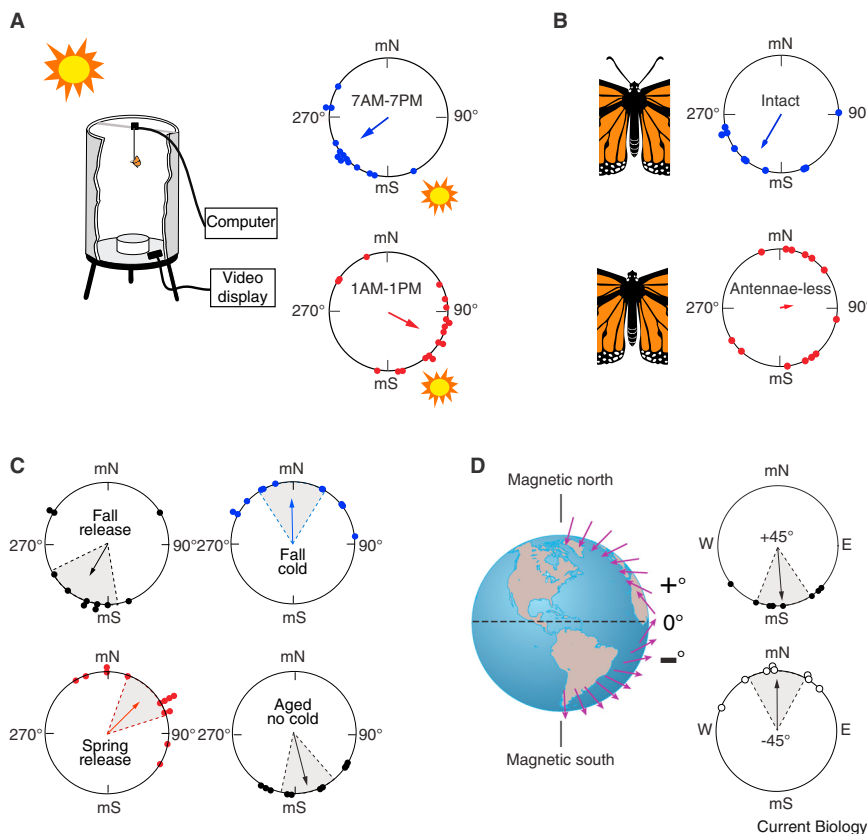
Monarchs are commonly infected with the apicomplexan parasite *Ophryocystis elektroscirrha* [118]. This protozoan parasite forms dormant spores on the abdomen of monarch butterflies [119]. When infected females oviposit on milkweed host plants, they passively transfer spores onto their eggs and the surrounding milkweed foliage. Parasite spores are ingested after hatching and parasites subsequently undergo asexual and sexual reproduction to produce a new generation of dormant spores on the outside of the monarch abdomen [119]. Because *O. elektroscirrha* replicates to high numbers (infection with a single spore can lead to the formation of millions of spores on the abdomen [120]), it is highly detrimental to monarchs. Infected monarchs suffer reduced survival as adults, and reduced mating ability [121,122]. Most pertinent to this review, parasite infection reduces adult lifespan and flight ability [122,123], which can have grave consequences for monarch migration. In fact, infected monarchs are less likely to complete their journey to the overwintering sites in Mexico. In particular, during the migratory season, parasite prevalence decreases with decreasing latitude (A) [124]. Moreover, stable isotope analyses were done for monarchs obtained at the overwintering sites at Sierra Chincua and Cerro Pelon in Mexico. In these analyses, more negative  $\delta^2\text{H}$  values of monarch wings indicate that monarchs had bred at higher latitude, thus showing that infected monarchs at the Mexican overwintering sites originate predominantly from more southerly breeding grounds (B) [125].

The process by which migration removes infected individuals is known as ‘migratory culling’ [126]. While parasite infection reduces the migratory ability of individual monarchs, migratory culling is beneficial for the migratory population, reducing the overall rate of parasitism. Animal migration can also reduce parasite prevalence by allowing animal hosts to escape parasite-infested habitats, a process called ‘migratory escape’ [126]. Studies have shown that this process also occurs in monarchs: parasite prevalence in North America increases over the breeding season (C) [124], partly due to a buildup of parasite spores on milkweed plants and ongoing parasite transmission in breeding populations [127]. Because milkweeds die in the fall, and monarchs migrate south, they effectively escape their parasite-infested habitats, and temporarily break the parasite transmission cycle [118]. Global variation in parasite prevalence is consistent with migratory culling and escape, with migratory monarch populations generally harboring lower prevalence than non-migratory populations [118,128].

noted, is a biologically tangible clock-to-compass circuit. Thus, a working mathematical model of the time-compensated sun compass has been proposed [51]. For this model, a receptive field of the compound eye that encodes solar azimuth was first constructed. Then, a theoretical neural circuit that integrates azimuthal and circadian signals to correct flight direction was derived. The computer-generated model demonstrated an integration mechanism, which produces robust trajectories toward the southwest regardless of time of day and included a modification for remigration. Comparison of model simulations with flight paths in the flight simulator shows analogous behaviors. Hopefully, this model will translate into relevant biological terms; that is, help define the actual connecting circuits. In the meantime, this model could be put to the test by constructing a monarch drone, using the model parameters, to determine whether the drone can use the sun’s position outdoors to fly directionally in a time-compensated manner.

### A Distinctive Circadian Clock Mechanism

Because of the necessity of the circadian clock for the monarch sun compass system, the monarch clockwork mechanism needed definition. Indeed, studies of the monarch clockwork have been informative, as they have revealed a molecular clock mechanism that was unique among animals at the time of its discovery. Its distinctive character originates from the clockwork’s utilization of two different CRYPTOCHROME (CRY) flavoproteins; monarchs have both a type 1 *Drosophila*-like CRY (designated CRY1) that functions as a circadian photoreceptor and a type 2 vertebrate-like CRY (designated CRY2 and NOT found in *Drosophila*) that functions as the major transcriptional repressor of the clock feedback loop [52,53]. Similar to *Drosophila* and mammals [54,55], the core clock mechanism in both the brain and antennae of monarchs relies on a negative transcriptional feedback loop (Figure 3E), which drives cell autonomous rhythms in the mRNA and protein levels of a set of core clock components (Figure 3F). For the negative feedback loop of the monarch



**Figure 2. Orientation behavior in migrants.**

(A) The time-compensated sun compass. (Left) Cutaway view of the flight simulator, designed after [41]. Individual monarchs are tethered to monitor their flight orientation in a plastic barrel, which excludes buildings and trees from view during testing. A butterfly can fly and rotate freely, but is unable to move vertically or horizontally. During flight trials, the monarch's flight behavior is monitored via video recording, and a directional recording device captures the flight orientation data of the monarch, which is collected via computer. (Right) Migrant monarchs use a time-compensated sun compass for orientation. (Upper) Monarchs housed under normal light/dark conditions in the lab, with a simulated sunup at 7:00 AM and a simulated sunset at 7:00 PM, flew in the migration-appropriate southwesterly direction (blue dots). These monarchs were tested at ~10 AM, and appropriately perceived the time as 10 AM given the azimuthal position of the sun in the sky. (Lower) Monarchs housed with a 6 h clock-shifted light/dark cycle in the lab, with a simulated sunup at 1:00 AM and a simulated sunset at 1:00 PM, shifted their flight orientation counterclockwise (red dots) when also tested at ~10 AM. This counterclockwise shift from southwest to southeast is consistent with these monarchs perceiving it to be 4:00 PM as indicated by the sun's position, and demonstrates the time-compensated aspect of the sun compass. Modified from Reppert *et al.* [3]. (B) Antennae contain the chronometer for time compensating the sun compass. Orientation data from flight simulator trials of monarchs with antennae (upper; blue dots) or without antenna (lower; red dots). Modified from Merlin *et al.* [40]. (C) Coldness triggers the remigration north. (Left) (upper) Disappearance bearings of monarch released outdoors in

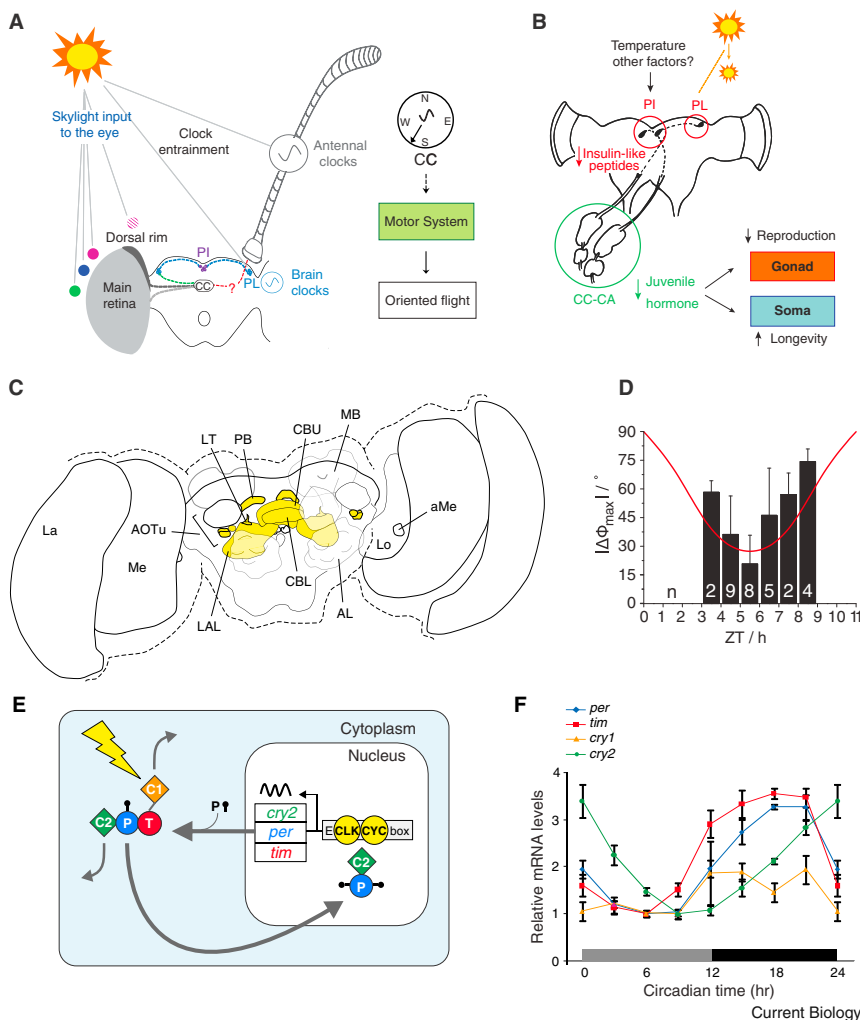
Worcester, Massachusetts in the fall (black dots) or (lower) from Austin, Texas in the spring (red dots). (Right) Orientation data from flight simulator trials of (upper) fall migrants exposed to 23 days of cold (blue dots) or (lower) aged migrants maintained in the laboratory under fall conditions throughout the overwintering period and then flown outdoors in the spring (black dots). Modified from Guerra and Reppert [21]. (D) The inclination magnetic compass. (Left) 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 in the Northern hemisphere possess a positive designation, with the North magnetic pole being +90°, while inclination angles in the Southern hemisphere possess a negative designation, with the South magnetic pole being -90°. (Right) The orientation behavior of monarchs in indoor flight simulator trials under artificial magnetic field conditions is consistent with an inclination-based magnetic compass. (Upper) Monarchs orient equatorward away from the North magnetic pole when tested under positive inclination angle conditions, a bearing that will bring them towards the overwintering sites in Mexico (black dots). (Lower) 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. Modified from Guerra *et al.* [65]. For circlegrams in (A), (B), (C, right) and (D), 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; mN is magnetic North. For orientation data in (C) and (D) shaded areas are 95% confidence intervals.

clockwork, heterodimers of the transcription factors CLOCK (CLK) and CYCLE (CYC) drive transcription of the *period* (*per*), *timeless* (*tim*), and *cry2* genes. Once translated, PER, TIM, and CRY2 proteins form cytoplasmic complexes that cycle back into the nucleus 24 hours later, where CRY2 inhibits CLK:CYC-mediated transcription [52], like CRY's action in mammals [55]. The discovery of type 2 vertebrate-like CRY proteins in non-drosophilid insects, originating from the CRY2 work in monarch butterflies [53], has transformed our view of how circadian clocks in these other insects work and has enlightened our understanding of the evolution of animal CRYs [56].

The monarch molecular clock mechanism was initially derived from both *in vitro* and *in vivo* approaches, including the use of the monarch DpN1 cell line [57], which contains a light-driven diurnal clock, and the use of *Drosophila* transgenesis, to bolster the differential clockwork functions of the monarch CRY proteins [52].

Most recently, genetic studies using nuclease technologies to knock out CRY2 have confirmed that the flavoprotein is an essential clock gene and the major transcriptional repressor of the monarch clockwork [58,59], while additional nuclease studies have revealed the molecular details of CRY2's repressive action [60]. Moreover, the monarch genome has provided the genes and protein products likely involved in the post-translational modification and degradation of core components of the negative feedback loop, the components of a secondary, modulatory feedback loop similar to that found in *Drosophila* and the mouse [54,55], and a potential key output signaling pathway of the molecular clock — pigment dispersing factor and its receptor — whose potential importance in monarchs is based on studies of the *Drosophila* clock [3,61].

The monarch butterfly may also use the CRY proteins as output clock circuit molecules in the brain [52,62]. Because the



**Figure 3. Monarch circadian clock functions, neural organization of compass, and the molecular clock.**

(A) Model of the time-compensated sun compass. The compass mechanism involves the monarch eye sensing of skylight cues, including color gradient or the sun itself (violet, blue, and green circles) and the polarization pattern of ultraviolet (UV) light (violet circle crosshatched), and the brain integration of skylight cue-stimulated neural response in the central complex (CC; gray dashed lines). In addition, time compensation is provided by circadian clocks located in the antennae. The integrated time-compensated sun compass information is relayed to the motor system to induce oriented flight. The brain circadian clocks are located in the pars lateralis (PL) and communicate with the pars intercerebralis (PI). The PL may also communicate with the central complex. Reproduced from Zhan *et al.* [9]. (B) Proposed endocrine regulation of reproductive arrest and longevity in migratory monarch butterflies. Decreasing day-length (decrease in sun size) is sensed by circadian clocks in the pars lateralis (PL). This information is relayed to the pars intercerebralis (PI) in which the production and/or secretion of insulin-like peptides is decreased, resulting in a decrease of juvenile hormone (JH) biosynthesis in the corpora cardiaca-corpora allata (CC-CA) complex. JH deficiency affects target tissues, leading to reproductive quiescence and increased longevity. Reproduced from Zhan *et al.* [9]. (C) Location of the sun compass in monarch brain. A frontal view is present with compass components colored in yellow. PB, protocerebral bridge; CBU, upper division of the central body; MB, mushroom body; La, lamina; Me, medulla; aMe, accessory medulla; Lo, lobula; LoX, lobula complex; AOTu, anterior optic tubercle; LAL, lateral accessory lobe; LT, lateral triangle; pPC, posterior protocerebrum; AL, antennal lobe. Reproduced from Merlin *et al.* [45]. (D) E-vector tuning and azimuth tuning and adjustment of E-vector tuning over the course of the day as measured by intracellular recordings from compass input neurons. The angle between E-vector tuning and mean azimuthal tuning is

defined as a  $|\Delta\phi_{\max}|$ -value. The red line represents the prediction of the angular difference between the mean perceived E-vector for the region of sky viewed by the monarch dorsal rim area and the solar azimuth over the course of the day. The values were calculated for the latitude and season from which migratory monarchs had been captured. ZT, Zeitgeber time of the 11-hr light:13-hr dark lighting cycle in which the monarchs were maintained; 0 represents lights on. Bars, mean  $\pm$  SD. Reproduced from Merlin *et al.* [45]. (E) The main gear of the clock mechanism in brain and antennae is an autoregulatory transcription feedback loop in which CLK and CYC heterodimers drive the transcription of the *period* (*per*), *timeless* (*tim*), and *cryptochrome 2* (*cry2*) genes through E box enhancer elements. TIM (T), PER (P), and CRY2 (C2) form complexes in the cytoplasm, and CRY2 is shuttled into the nucleus where it shuts down CLK:CYC-mediated transcription. PER is progressively phosphorylated and likely helps translocate CRY2 into nucleus. The *cryptochrome 1* (*cry1*) gene encodes CRY1 (C1), a circadian photoreceptor, which, upon light exposure (lightning bolt), causes TIM degradation to gain access to the central clock mechanism. The two small curved arrows represent potential output functions of CRY1 and CRY2. Modified from Reppert *et al.* [76]. (F) Clock gene mRNA profiles in antennae. Values are relative to the minimal level for each gene and are the mean  $\pm$  SEM of four antennae. Points at circadian time (CT)0 are replotted at CT24. Horizontal bars: gray, subjective day; black, subjective night. Reproduced from Merlin *et al.* [40]. Similar clock gene patterns occur in brain.

circadian clocks necessary for time-compensation of sun compass orientation do not involve brain clocks, specifically those located in the pars lateralis (PL), but are located in the antennae [40], much of the brain clock-CRY circuitry is likely involved in JH-mediated aspects of migration (e.g., reproductive diapause and longevity; Figure 3B) that are regulated separately from navigation [3]. In fact, treatment of JH-deficient migrants with a JH analog activates reproductive function, but does not alter time-compensated sun compass orientation, which persists unabated [18,63]. The PL brain clock-CRY circuitry may also be involved in additional circadian activities, including the daily timing of adult eclosion, the sleep-wake cycle, and metabolic rhythms. The PL brain clock circuit might also be involved in

sensing the decreasing photoperiod in the fall, apparently important for triggering the migration south, as previously mentioned [16]. Because the initiation of migration-specific physiology appears to occur in caterpillars and pupae rather than adult butterflies, it will be important to study the brain clock-CRY circuitry in these life stages as well. In fact, one recent study of monarch caterpillars has revealed cyclical feeding behavior and the cycling of clock gene expression [64].

### An Inclination-Based Magnetic Compass

A clock-independent compass system is also exploited by migratory monarchs — a light-dependent, inclination-based, magnetic compass that can be used at Earth-strength intensity



(Figure 2D). This compass system was shown using flight simulator trials indoors during which the monarch's magnetic field parameters (polarity, inclination, and intensity) were artificially generated, and in which no directional light cues were accessible [65]. The results showed that fall migrants can use the inclination angle of the Earth's magnetic field for directional information to guide their latitudinal movement south during the fall migration. The functional magnetic sense requires UV-A/blue light (wavelengths between 380 and 420 nm), not considered in prior studies of a magnetic sense in monarchs [41,42], supporting the idea that the magnetic sense is a light-based photochemical reaction process, likely involving the CRY proteins [65]. Previous studies have shown that either monarch CRY1 or CRY2 transgenes can restore light-dependent magnetosensitivity in CRY-deficient *Drosophila* using the same UV-A/blue wavelength spectrum [66,67]. Furthermore, the monarch antennae appear to contain the relevant light-dependent magnetosensors for proper operation of the magnetic compass [65]. Knowing the principal location of the light-sensing component of the magnetosensor allows for evaluation of both the molecular and genetic mechanisms of magnetoreception and the involvement of CRY in the migratory monarch [3].

Previous flight simulator experiments, involving clock-shifted butterflies, show that migrants fly in the predicted, adjusted orientation despite being exposed to the Earth's unmanipulated magnetic field [18,21,38–42]. Thus, the magnetic compass is likely a backup mechanism subservient to the dominant time-compensated sun compass. Accordingly, the inclination compass may only be apparent when directional daylight sky cues are unavailable, e.g., under overcast conditions, during which observational studies have shown that free-flying monarch migrants continue to fly directionally [68]. Also, the magnetic compass may help calibrate the time-compensated sun compass by fine-tuning time-compensated sun compass usage [50].

An interesting possibility to consider is that the magnetic sense of monarchs may assume dominance over the sun compass during the last leg of the migration (~500 km) and underlie an overall geomagnetic map sense. Such a geomagnetic map sense could be key for locating the small number of overwintering groves in Mexico.

### Additional Navigational Processes

Prevailing wind direction (e.g., out of the northeast in the fall) and changes in atmospheric pressure are important factors that augment monarch migration [69–71]. In fact, migrants passively ride thermals to aid long-distance travel. And strategies have evolved to detect wind direction and compensate for drift due to prevailing wind speed and direction [70,72]. The antennae may play a major role in sensing wind and barometric changes, because insect antennae can sense sound, wind and gravity, in addition to their well-known chemosensory function in olfaction [73–75].

Approaching Texas, millions of butterflies are coalesced by two barriers — the Gulf of Mexico on their left and the Rocky Mountains/Sierra Madre Orientals on their right, first described by Calvert [69] (Figure 1D). From that point onward, in addition to the potential use of a geomagnetic map sense mentioned above, a 'destination beacon' has been postulated [76] that

guides the butterflies to their overwintering grounds. Some laboratories have focused on olfactory cues as the signal given off at the overwintering sites by the oyamel trees that reside in the unique coniferous forest in which the butterflies roost [30,76,77]. The precise nature of the speculated olfactory signal still needs to be determined, however, if it actually exists. Examining the orientation behavior of fall migrants displaced 100 km south of the overwintering grounds may be informative.

Social interactions among the gregarious fall migrants may also be an important consideration for successful migration, as also previously proposed [3]. Migrants form overnight roosts during the journey south and are surrounded by thousands of their mates as they fly from Texas southward [2]. Again, the antennae may loom large for sensing olfactory cues in the form of volatile, conspecific pheromones. Several olfactory receptor genes of the pheromone clade have been identified in the monarch genome [9] and need to be deorphanized to help understand function. Could social interactions give rise to a leader-follower hierarchy of the migration?

### Migrating with Genomic and Genetic Tools

In the fall, migratory monarch butterflies are on their original journey, suggesting that there is a genetically driven basis to the migration [76]. Events triggered in monarchs leading to the migratory state could involve DNA methylation, histone and chromatin modifications, microRNAs (miRNAs), and/or RNA editing. Study of differences in gene expression levels between migratory and summer butterflies could help determine the underlying genetic basis of the migration.

The draft sequence of the 273 Mb genome of the monarch butterfly predicted over 16,000 protein-coding genes [9]. MonarchBase (<http://monarchbase.umassmed.edu>) provides an updated version of the genome assembly upon which all related data integration (e.g., annotations and microRNAs) is now based [78]. The utility of the genome for aiding the understanding of various aspects of monarch biology has been discussed throughout this review. One potentially important feature of the genomic analysis predicted that monarchs are unlikely to use methylation to regulate the migratory state [9]. miRNAs in summer and migratory monarchs were also characterized, and over 100 were identified and half of those exhibited >1.5-fold differences in mean expression levels between summer and migratory monarchs [9]. Three miRNAs are notable, because, based on homology and studies in other animals, they are linked to muscle activity, cold tolerance, and fat metabolism. These miRNAs still need further evaluation.

As previously stated, nuclease technology, namely zinc finger nucleases [59], transcriptional activator-like effector nuclease, and CRISPR-associated RNA-guided nuclease Cas9 (CRISPR/Cas9) [58], has genetically defined CRY2 as the main transcriptional repressor in the monarch clockwork. The CRISPR/Cas9 system leads to efficient genome editing in the monarch [58] and will help elucidate the molecular and cellular mechanisms of the long-distance migration. Additionally, generating a nuclease-mediated homologous recombination strategy with CRISPR/Cas9 to knock-in reporter tags into genomic loci (e.g., clock gene ones) will hasten neuronal circuit mapping [3].

### Loss of Migration to Study Migration

As mentioned before, monarchs are ancestrally migratory and formed apparent non-migratory populations around the world through three independent dispersal events. These proposed independent losses of migration provide a unique opportunity for population genetic and genomic studies to reveal additional genes related to migration. So far, the population genomic SNP study based on 80 monarch genomes already identified genomic regions strongly differentiated between migratory and apparent non-migratory populations [4]. This included 2.1% of the genome encompassing over 500 genes, including those involved in morphogenesis and neurogenesis. A single 21-kb genomic segment showed clear signatures of divergent selection. The gene showing the most marked divergence encodes collagen type IV  $\alpha$ -1, which is important for muscle function. Further gene expression and metabolic studies suggested greater flight efficiency as one of the features of the migration. RNA-seq studies of transcriptional differences among migratory and apparent non-migratory populations could help sort out which of the 500 candidates should be subjected to a more rigorous assessment of function.

Animal migration is best described as a syndrome that includes traits related to morphology, development, growth, energy metabolism, locomotion, navigation and regulation of reproduction [79]. Quantitative genetics approaches show that migration has a strong genetic component across many different species [80]. In fact, recent studies have shown that genes involved in muscle development, energy metabolism and circadian rhythm tend to show genetic divergence or differential expression patterns between migratory and non-migratory individuals (e.g. [81–84]). Thus, in addition to continued work on the neurobiological aspects of navigation, further work in monarchs is required to reveal the biological basis of other migration traits. For instance, several studies have revealed within- and between-population variation in wing size, with monarchs that fly greater distances possessing larger wings than apparent non-migratory monarchs [13,85,86]. Further traits that require study are induction of reproductive diapause, and flight speed, endurance and efficiency. One fruitful approach to study such traits is to combine crosses between monarchs from migratory and non-migratory populations with Quantitative Trait Locus analysis. Such approaches could lead to a novel suite of candidate migration genes, which can subsequently be tested through reverse genetics.

### Monarch Conservation

The study of monarch migration is not only relevant to our improved understanding of the mechanisms by which animals migrate but is also relevant to the conservation of this iconic species. The population size of migratory monarchs in eastern North America has dwindled from roughly 300–900 million in the 1990s to 33–200 million in recent years [23]. This decline is most likely a result of illegal logging at the Mexican overwintering sites, climate change, agriculture-induced loss of milkweed host plants in North America, and reduced availability of nectar sources along the fall migration flyways [87–89]. Ongoing are conservation efforts to mitigate these decline-related threats (e.g., establishing monarch waystation habitats) [87,90,91]. Because of these threats, some have coined

the monarch migration an endangered phenomenon [92], while others have challenged this claim [93]. Regardless, a group of organizations and scientists have petitioned the US Fish and Wildlife Service to protect monarchs under the Endangered Species Act [94].

Following recent advances in merging evolutionary biology with conservation biology [95–98], a crucial aspect of monarch biology is to determine the adaptive capacity of the butterflies. This includes asking how much adaptive genetic variation monarch populations harbor. While studies have shown that monarchs likely lost their migration phenotypes during their worldwide dispersals, it remains unknown whether this migration loss is reversible; that is, do apparent non-migratory populations contain genetic variants that would be able to regain migration if released in North America? This is an important question to ask, because a focal, catastrophic climatic event in North America may have consequences not only for the migration, but also for the species itself, which fortunately has a global distribution. A recent study showed that monarchs in Australia retain their ability to enter reproductive diapause when exposed to shortening day length [99]. Furthermore, monarchs from Guam displayed similar clock gene expression profiles in antennae to those found in Western North American monarchs [99]. Nonetheless, whether these patterns would allow these apparent non-migrant monarchs to migrate when reared and released in North America remains an open question.

### Biology on the Wing

The genetic and neurobiological architecture of most migratory species remains poorly understood [79,80,100]. Monarchs, in contrast, have emerged as a model organism to gain mechanistic insights into the migration, due to their suitability for experimentation (e.g., use of a semi-artificial diet to rear monarch larvae continuously in the laboratory) and the rapid development of contemporary tools, including gene editing. While great progress has been made on many fronts over the past decade, what we are witnessing is only the end of the beginning. Remarkably, the monarch migration is slowly but surely revealing its secrets, and an exciting, robust era of monarch research is upon us.

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