Trends in Genetics

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REVIEWS

Review

Monarch Butterfly Migration Moving into the Genetic Era

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The genetic architecture and neurogenetics of animal migration remain poorly understood. With a sequenced genome and the establishment of reverse genetic tools, the monarch butterfly has emerged as a promising model to uncover the genetic basis of migratory behavior and associated traits. Here, we synthesize major advances made in the genetics of monarch migration, which includes the discovery of genomic regions associated with migration and molecular mechanisms underpinning its seasonality. We highlight the catalytic role that a rapidly growing number of contemporary genetic and molecular technologies applicable to nonconventional organisms have had in these discoveries, and outline new avenues of investigation to continue moving the field forward.

Highlights

The development of gene editing tools, including CRISPR/Cas9-mediated targeted mutagenesis, for generating loss-of-function mutants in the monarch butterfly has positioned the monarch as a well-suited model organism with which to gain mechanistic insights into the genetic and neurobiological bases of animal migration.

Comparative genomics using whole-genome sequencing, largely driven by the assembly of a draft genome sequence, has revealed genomic regions strongly differentiated between migratory and nonmigratory monarch populations. Over 500 candidate genes were associated with the migratory phenotype and can now be functionally characterized.

Functional genomic studies of the seasonal migration of the Eastern North American population have revealed that circadian clocks mediate the seasonal induction of reproductive arrest exhibited by migrants by affecting the vitamin A pathway in the brain.

Emergence of the Monarch as a Genetic Model System for Animal Migration

Animal migration has evolved as a critical behavioral adaptation for survival in a wide range of taxa and is characterized by a seasonal movement to escape unfavorable conditions. The remarkable navigational abilities used by migratory species to travel long distances and pinpoint their migratory destination with incredible precision have captivated the imagination of generations of scientists and the public alike [1,2]. Although mounting evidence suggests that the morphological, sensory, physiological, and behavioral traits exhibited by migratory species are genetically encoded and turned on at the appropriate time of the year and/or under specific environmental conditions, the genetic and neurobiological bases of migration remains poorly understood [1,3]. While some progress has been made in identifying genes associated with migration in birds and insects [3–6], mechanistic approaches to link genotype to the migratory phenotype are still generally lacking because migratory species are typically not easily maintained in the laboratory and/or amenable to genetic experimentation. One notable exception are the colorful eastern North American monarch butterflies (*Danaus plexippus*), which leave their northeastern American and Canadian summer breeding grounds every autumn and travel up to 3000 miles to reach their overwintering sites in central Mexico [2,7–9]. Catalyzed by the sequencing of a draft genome and the development of **reverse genetics** (see Glossary) tools over the last decade [10–12], the monarch has emerged as a powerful model system to drive the field of animal migration into the realm of genetics [3].

In this review, we synthesize recent discoveries about the genes and pathways involved in dictating several traits underlying the monarch migratory phenotype and outline possible future avenues of genomic and genetic research on monarch migration to obtain mechanistic insights into the mode of action of migratory genes. We also provide an integrated view of current knowledge of the navigational capabilities of monarchs, focusing on time-compensated sun compass orientation, and we highlight how genetic and epigenomic tools can be employed to address current challenges in understanding the intertwined molecular and neurobiological bases of flight orientation.

Migratory Cycle and Neuroethology of the Eastern North American Monarch

Each autumn, coincident with decreasing daylengths (i.e., photoperiods), millions of monarchs in eastern North America and Canada take wing to accomplish one of the longest migrations known

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in any insect (Figure 1, Key Figure). Over the next 2–3 months, these monarchs, which are in a state of reproductive dormancy (i.e., diapause), fly southward from sunrise to sunset, only stopping to nectar for accumulating fat reserves to survive the winter and gathering together to roost for the night, until they reach their overwintering destination at a dozen sites atop volcanic mountains in central Mexico. Year after year, monarchs congregate there in clusters, sometimes on the exact same oyamel trees, and hunker down for the winter [7,8]. By the beginning of spring, when temperatures and photoperiod increase, the same individuals start to become active again, break diapause, mate, and then remigrate northward to the southern USA [2,7,13]. There, milkweed, the monarchs' host plant, is already sprouting in response to the same environmental changes that monarchs experienced, thereby providing the necessary resources for monarch females to lay their fertilized eggs before dying. At least two successive generations of reproductively active spring and nonoriented summer monarchs continue the journey north to repopulate their full northern breeding grounds, presumably by following the northward progression of milkweed emergence across America [14]. The butterflies reaching the breeding areas mate again, and, by late summer, their offspring are reprogrammed into autumn migrants that take flight south as adults, starting the migratory cycle anew [15]. Autumn migrants are always on their maiden voyage; thus, the migratory behavior cannot be socially learned and is instead innate. However, because autumn migrants share the same genetic makeup as their nonmigratory parents, the timing of migratory departure, southward flight orientation, and migratory physiology (e.g., reproductive diapause, fat storage, increased longevity) appear to be triggered by changes in environmental conditions. Similar to the switch in behavior and physiology observed in autumn migrants, the reversal of flight orientation in spring remigrants has been shown to be environmentally induced as well, but, in this case, by prolonged exposure to low temperatures that mimic those experienced at the overwintering sites [16]. Environmental induction of the two-way migration of eastern North American monarchs suggests that **epigenetic** mechanisms triggered by environmental changes regulate migratory behavior in this species [3].

Environmental cues not only play a vital role in triggering seasonal behavioral switches but also provide the compass cues that guide monarchs in their migratory journey. Autumn migrants and spring remigrants use a bidirectional time-compensated sun compass as their primary navigational tool to direct flight orientation [16–20]. Autumn migrants can also use the inclination angle of the Earth's magnetic field for directional information [21], but whether this inclination compass fine-tunes the time-compensated sun compass, serves as a backup mechanism on overcast days (i.e., when the sun is not visible), or underlies a geomagnetic map sense that could help monarchs pinpoint their overwintering area is still unknown [1]. Classical genetic and neurogenetic studies are integral to establishing a comprehensive understanding of the genetic architecture of monarch migration and the molecular and neurobiological bases of the compasses used for monarch navigation. What have we learned from genetic studies so far, and where do we go next to address current challenges and rapidly move the field forward?

Population Genetics for Migratory Gene Discovery

Initiated by the release of the monarch's draft genome sequence almost a decade ago [10,22], efforts to identify the genes and pathways underlying monarch migratory traits have capitalized on the existence of monarch populations around the world with different migratory phenotypes [6]. Aside from the iconic eastern North American monarch population, two other populations undergo a seasonal migration, albeit of shorter distances: one in North America west of the Rocky mountains, which migrates to the California coast, and another in Australia, whose migration direction is seasonally reversed compared with that of North American populations [23]. Several others, which appear to be nonmigratory and have formed through three independent dispersal events from ancestrally migratory monarchs, can be found in Central America,

Glossary

ATAC-seq: molecular technique relying on a hyperactive Tn5 transposase to assess genome-wide chromatin accessibility.
Bisulfite sequencing: bisulfite treatment of DNA followed by sequencing to determine the pattern of DNA methylation.
CRISPR/Cas9: genome editing tool using a designed RNA molecule to guide a DNA endonuclease enzyme to a specific sequence of DNA.
CUT&RUN: molecular technique relying on the endonuclease activity of micrococcal nuclease and specific antibodies to profile the epigenome and/or identify binding sites of transcription factors or proteins of interest.
Enhancers: short regions of DNA on which proteins can bind to increase the probability of transcription of a target gene.
Epigenetic: heritable and/or environmentally induced external modifications to DNA that turn genes 'on' or 'off' without altering the DNA sequence.
Haplotypes: combinations of specific alleles in an organism that are inherited together from a single parent.
Homology-directed repair: cellular mechanism to repair double-stranded DNA breaks through homologous recombination.
Population genomics: study of the genome-wide genetic composition of biological populations and the changes in genetic composition that result from the operation of selection.
Quantitative genetics: study of genetic control of quantitative traits that vary continuously across segregating generations.
Reverse genetics: molecular method used to test the function of a gene by genetically engineering changes in its sequence to disrupt its function.
RNA-seq: sequencing technique used to identify the presence and quantity of RNA in a given biological sample.
Transposon-based transgenesis: technique allowing genes to be transferred to a host organism's chromosome.

Key Figure

Annual Migratory Cycle of North American Monarch Butterflies

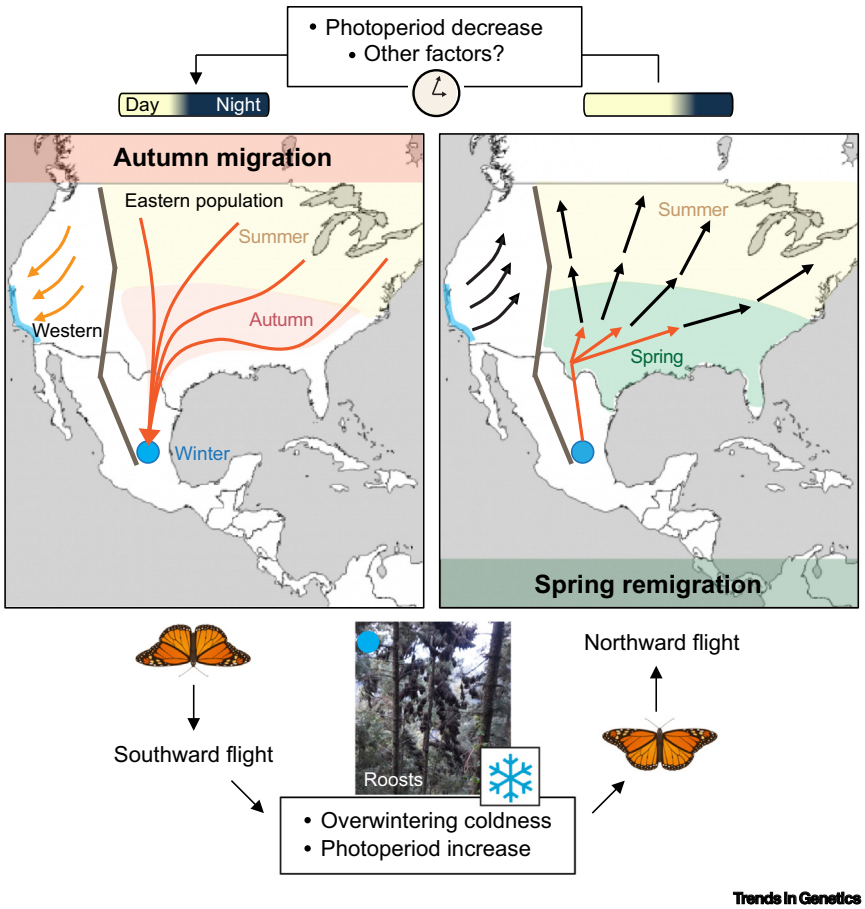


Figure 1. North American monarchs start migrating southward in the autumn, coincident with decreasing daylength sensed by an endogenous timer functioning with circadian clock genes. Monarchs east of the Rocky Mountains (gray line) navigate over long distances (red arrows) to their overwintering sites in Mexico (blue circle). In the spring, when temperatures and photoperiod increase, the same individuals become reproductive, mate, and reverse their flight orientation northward. The switch in compass orientation in seasonal migratory forms has been shown to result from prolonged exposure to coldness that mimic those experienced at the overwintering sites, underscoring the critical importance to increase conservation efforts of the overwintering sites that are threatened by logging. On their way back to the USA, fertilized remigrant females lay their eggs on milkweed plants before dying (red arrows). Subsequent generations of spring and summer butterflies progress northward following the latitudinal emergence of their host plants to repopulate the northern summer breeding grounds (black arrows). Autumn migration and spring and summer breeding ranges are denoted by colored areas. Monarchs west of the Rocky Mountains also migrate southward in the autumn, overwinter along the California coast (blue line), and remigrate northward in the spring, but the migration distances are much shorter than those traveled by eastern North American monarchs. Modified from [3]. Photo credit: Aldrin Lugena.

South America, the Caribbean, Europe, North Africa, and throughout the Pacific islands [6,24,25] (Figure 2A). The apparent loss of migration in these populations has provided a unique opportunity to study the genetics of monarch migration through **population genomics** approaches. Resequencing of 80 individuals from migratory and nonmigratory populations identified about 5 Mb (~2%) of the genome encompassing 536 genes as targets of divergent natural selection,

significantly associated with shifts in migratory behavior [6]. Among these 5 Mb, an outlier region of 21 kb showed multiple signatures of strong divergent selection (Figure 2B) and an enrichment of shared alleles in populations that originated from dispersal events, suggestive of a divergence in **haplotypes** between nonmigratory populations and the migratory eastern North American population [6]. Three candidate genes that could be involved in the migratory phenotype are located within this 21-kb region and encode an F-box protein (FBXO45), whose homologue in mice is a component of an E3 ubiquitin ligase complex that is selectively expressed in the nervous system and regulates neurotransmission [26]; a transmembrane protein of unknown function; and the α1-subunit of collagen type IV, which is essential for muscle morphogenesis and function in *Drosophila* [27] (Figure 2C). On the basis of differential expression in flight muscles of migratory and nonmigratory monarchs that are correlated with flight metabolic rates, collagen type IV α1 has been proposed to regulate flight efficiency during long-distance migration [6]. Differential expression studies using **RNA-seq** between migratory populations, including North American and Australian populations, and nonmigratory populations could illuminate which of the 536 candidate genes should be prioritized for *in vivo* functional characterization. However, because the migratory phenotype encompasses a suite of adapted traits that include morphology (e.g., wing size and shape), development, sensory processing (e.g., circadian clocks, skylight cues, and magnetic sensing), physiology (e.g., metabolism, regulation of reproduction), and behavior (e.g., orientation and navigation, flight endurance), these differential expression studies should be performed in a variety of tissues and across developmental stages.

As previously proposed, classical **quantitative genetics** using populations varying in their migratory phenotypes are another unexplored, yet potent, way to identify candidate genes underlying different aforementioned traits associated with migration in monarchs [1,28]. Performing crosses between migratory and nonmigratory monarchs, or between migratory monarchs varying in their migration distances (e.g., eastern and western North American populations), quantifying phenotypic traits related to migration in parental and F2 generations, and mapping quantitative trait loci across the genome should not only extend the list of candidate migratory genes but also correlate them with specific traits. Most of the population genetics studies have so far focused on signatures of selection in coding DNA [5,6]. Expanding detection of selection signals in noncoding genomic regions will be equally important because evolutionary changes in **enhancers**, which regulate gene expression in higher eukaryotes, could be associated with the variation in migratory phenotypes [29–31]. Ultimately, candidate migratory genes and genomic regions should be functionally characterized *in vivo*. This should be facilitated by the development of contemporary genome editing tools in the monarch, including **CRISPR/Cas9**, for the generation of loss-of-function mutants by gene knockout or the introduction of precise mutations via **homology-directed repair** [11,12,32], and by the availability of a semiartificial diet for raising monarchs and maintaining large colonies of mutants in laboratory conditions[i] as an alternative to greenhouse-grown plants when those cannot be generated in sufficient quantity [12]. In addition, being able to induce migratory behavior from laboratory-raised monarchs will be necessary to test the effect of specific mutations. Although we do not yet know which cues are necessary for triggering migratory behavior in laboratory conditions, this could be accomplished by raising mutant monarchs along with their wild-type siblings in greenhouses under natural conditions in late summer. Finally, the rich and well-documented biology of the eastern North American seasonal migratory monarch also offers unique opportunities to dissect the genetic basis of monarch migration [1,2,33]. As discussed later, studies focused on this seasonal migratory population have already provided glimpses of the molecular basis of some migratory traits and are likely to be central in building a comprehensive picture of the biological basis of monarch migration and its genetic and neurogenetic underpinnings.

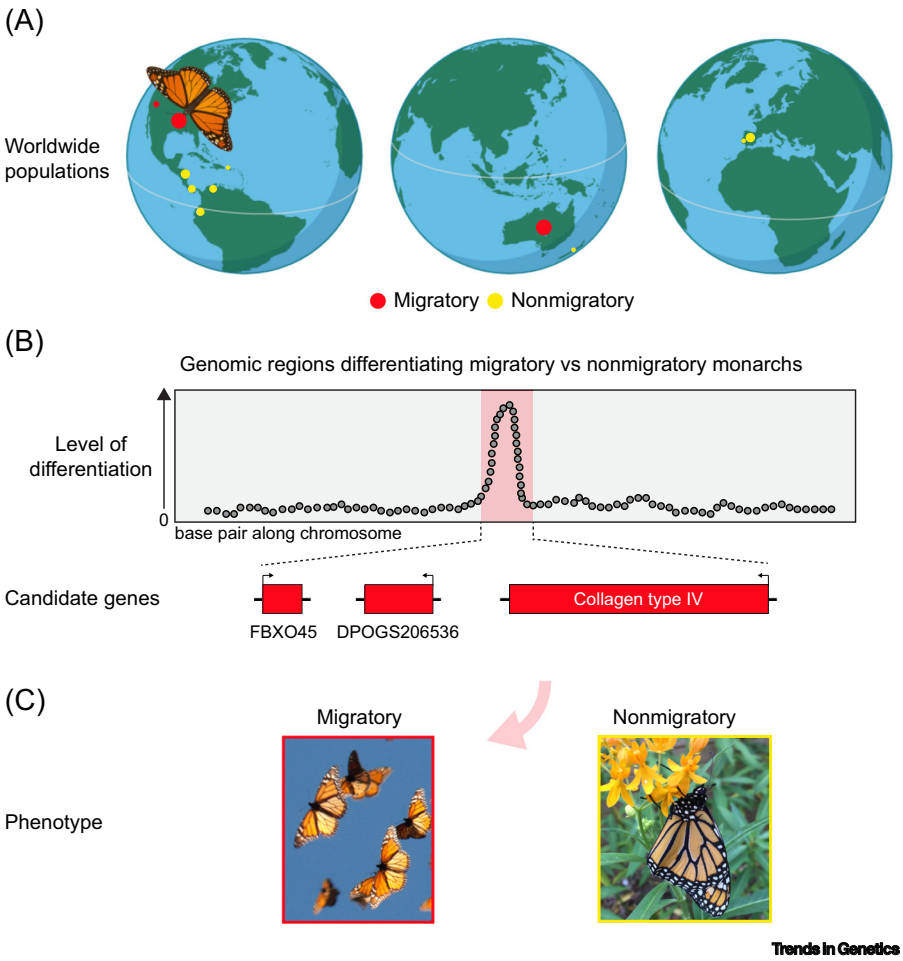


Figure 2. Genetic Dissection of Monarch Migration Using Population Genomics. (A) Monarch populations that differ in their migratory phenotypes are distributed around the globe. In North America, two migratory populations separated by the Rocky Mountains undergo seasonal migrations: the eastern population, best known for its spectacular long-distance migration, overwinters in central Mexico; and the western population, which migrates over much shorter distances, overwinters on the California coast. A third migratory population is present in Australia. However, monarchs also exist throughout Central America, South America, the Caribbean, Europe, North Africa, and throughout the Pacific islands, where they appear to have formed nonmigratory populations through three dispersal events from the ancestral eastern North American migratory population. Whether they lack the ability to migrate or simply do not express this behavior in their local environments remains an open question. (B) The variation in migratory phenotypes across populations has been leveraged for comparative population genomics studies. Regions of the genome strongly differentiating North American monarchs and monarchs from nonmigratory populations were identified by resequencing the genome of these individuals and applying quantitative measures of sequence differentiation. The most highly differentiated region contained three genes encoding the F-box protein FBXO45, an uncharacterized transmembrane protein (DPOGS206536), and the α1-subunit of collagen type IV. Modified from [6]. (C) Because of their strong association with a shift in migratory behavior, these genes (together with those found in other differentiated genomic regions) may underpin the genetic basis of monarch migration. Photo courtesy of Monarch Watch (left image) and Guijun Wan (right image).

Circadian Clocks: Seasoned to Perfection

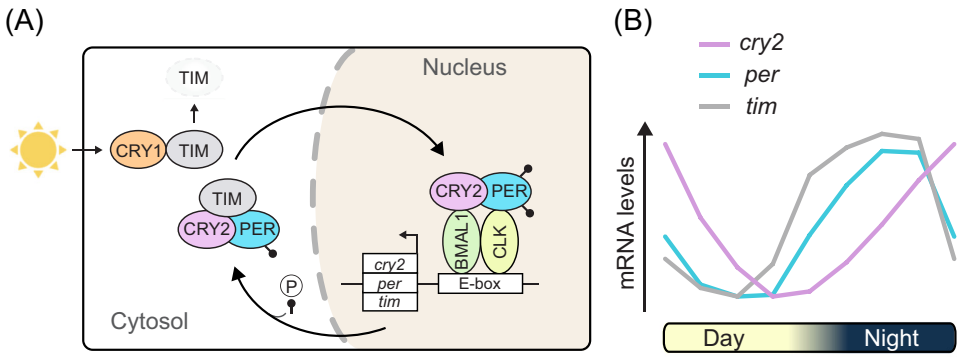
Timing is integral to the seasonal migration of eastern North American monarchs [3] because the onset of migratory behavior and departure from breeding grounds is tightly linked to the change in season (Figure 1), a response akin to that of many migratory birds [34]. To follow timing schedules, animals keep track of the time of day and seasonal variations in

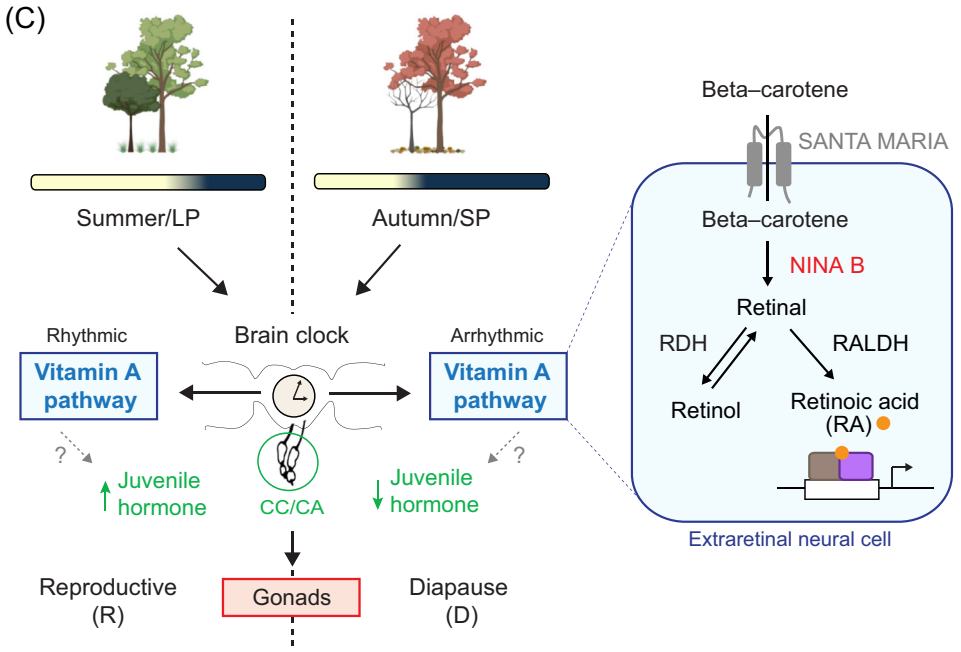
daylength using endogenous timers such as circadian clocks [35,36]. Due to the key role of circadian clocks in monarch sun compass navigation and potentially in seasonal timing [1,2], the molecular mechanisms of monarch clock function have been defined using a complement of *in vitro* and *in vivo* approaches, including the use of the monarch DpN1 cell line [37], *Drosophila* transgenesis [38], and nuclease-mediated targeted mutagenesis in monarchs [11,12,39]. Similar to that found in *Drosophila* and mammals [36,40], the monarch clock relies on a negative transcriptional/translational feedback loop in which circadian activators drive the cell autonomous 24-h rhythmic transcription of circadian repressors that then shut down their own transcription (Figure 3A). What distinguishes the monarch clock from the clocks of *Drosophila* or mammals is the existence of two functionally distinct CRYPTOCHROMES: a light-sensitive *Drosophila*-like CRYPTOCHROME (named CRY1 and absent in mammals) that functions as a blue-light circadian photoreceptor and a light-insensitive mammalian-like CRY (named CRY2 and absent in *Drosophila*) that serves as a potent circadian repressor [12,38,41].

In the monarch, CLOCK (CLK) and BMAL1 transcription factors heterodimerize and activate the rhythmic transcription of *cry2*, *period* (*per*), and *timeless* (*tim*) genes (Figure 3A,B). Upon translation, CRY2, PER, and TIM form cytosolic complexes that translocate back into the nucleus, where CRY2 inhibits, 24 h later, CLK:BMAL1-mediated transcriptional activation [38]. The clock is reset daily when the blue-light circadian photoreceptor CRY1 mediates TIM degradation upon light exposure, leading to the subsequent degradation of PER and CRY2 and allowing a cycle of transcription to start anew [38] (Figure 3A,B). Importantly, the discovery of a mammalian-like CRY in monarchs has now been extended to all insects, with the exception of fly species belonging to the brachyceran lineage [39,42]. The *in vivo* characterization of monarch CRY2 through nuclease-mediated reverse genetics [12,39] has also revealed functional similarities with mammalian CRYs in their mode of repressive action on CLK:BMAL1 [43–45], suggesting the interesting possibility that the monarch could be used as a complementary model to the mouse in order to understand circadian repression relevant to mammals, including humans. Additional putative players of the clock, such as genes and their products involved in post-translational modifications and degradation of core clock components, homologous to those in *Drosophila* and the mouse [36,40], have also been identified in the monarch genome [10]. Functional characterization of these genes should continue to further the understanding of the monarch clockwork and may reveal additional surprises.

Seasonality and the Molecular Basis of Reproductive Diapause

Reproductive quiescence (also called 'diapause' in insects) is a hallmark of the migratory phenotype [46,47]. Like migratory flight, diapause is a seasonal response exhibited by many species in anticipation of unfavorable seasonal conditions [46]. Consistent with the classical view that photoperiod is a major environmental signal used by animals living at temperate latitudes to predict the onset of an unfavorable season and regulate the diapause response in insects, eastern North American migratory monarchs enter into overwintering diapause in the autumn, coincidentally to decreasing photoperiod [35]. Although entry into full diapause likely depends on a combination of decreased daylength, decreased temperature, and senescing milkweeds [15], female monarchs raised under short photoperiod develop significantly less mature oocytes than when raised under long photoperiod [48]. This diapause-like response has been harnessed to show that circadian clocks and/or clock genes in the monarch brain are necessary for photoperiodic measurement [48]. Inactivation of the clock in the monarch butterfly using loss-of-function mutants for the circadian activators CLK and BMAL1 and the circadian repressor CRY2 abolishes photoperiodic responses in reproductive





	Wild type	*Cry2*$^{-/-}$	*Clk*$^{-/-}$	*Bmal1*$^{-/-}$	*Nina B1*$^{-/-}$
LP	R	D	R	R	R
SP	D	D	R	R	R

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Figure 3. Circadian Clocks and the Induction of Seasonal Reproductive Diapause. (A) The core molecular mechanism of the monarch circadian clock relies on a feedback loop in which the CLOCK (CLK) and BMAL1 heterodimer drives the rhythmic transcription of the *cryptochrome 2* (*cry2*), *period* (*per*), and *timeless* (*tim*) genes. CRY2, PER, and TIM form complexes in the cytosol. Upon PER phosphorylation, PER and CRY2 are translocated into the nucleus and repress CLK:BMAL1-mediated transcription. The blue-light circadian photoreceptor CRYPTOCHROME 1 (CRY1) resets the clock by mediating TIM degradation upon light exposure. (B) Expression profiles of *cry2*, *per*, and *tim* mRNA levels over a 24-h day. (C) The circadian clock or clock genes in the brain are involved in the induction of reproductive diapause exhibited by autumn migrants. The brain clock helps monarchs distinguish long photoperiods (LP) in the summer from short photoperiods (SP) in the fall. The brain clock affects photoperiodic responsiveness by regulating, in a photoperiod-dependent fashion, the expression of genes involved in the vitamin A pathway. β-Carotene is transported into extraretinal neural cells of the adult brain via SANTA MARIA and

(Figure legend continued at the bottom of the next page.)

output (Figure 3C), similar to that observed in a few other insect species [49–53]. How circadian clocks, known to rhythmically regulate many biological pathways, could regulate the photoperiodic responses had remained elusive until recently. RNA-seq studies aimed at identifying rhythmic gene expression in brains of summer monarchs, autumn migrants, and monarchs raised in long and short photoperiods identified the vitamin A pathway as being differentially regulated in a photoperiod-dependent manner [48]. The key role of this clock-controlled pathway in mediating the photoperiodic induction of diapause was further genetically validated with a CRISPR/Cas9-mediated loss-of-function mutant of the gene *nina B1*, encoding the rate-limiting enzyme that converts β-carotene into retinal, which lost the ability to enter diapause under short photoperiod (Figure 3C) [48]. As in *Drosophila* [54], the entry into diapause in monarchs results from a juvenile hormone (JH) deficiency in the corpora cardiaca–corpora allata complex and a likely downregulation of insulin-like peptides in the brain [10,55,56]. The link between the clock-controlled vitamin A pathway and JH regulation is still unknown. A first step to determine how vitamin A affects the diapause response will be to define the role of retinal in the brain, which could function in two ways: either as the chromophore of an opsin-based deep brain photoreceptor for photoperiodic measurement or to produce retinoic acid that could regulate a seasonal transcriptional program and/or the seasonal plasticity of a neuronal network in the brain as in mammals [57,58]. Support for the regulation of the photoperiodic control of seasonal reproduction by extraretinal photoreceptors is not without precedent, as shown in avian species such as ducks and Japanese quails [59]. Knocking out opsin-encoding genes in the monarch could help sort out which of these two roles the vitamin A pathway plays in the photoperiodic control of diapause response in insects.

Neurogenetics of Flight Orientation: Focus on the Bidirectional Time-Compensated Sun Compass

Migratory flights require a suite of coordinated traits that include elongated wing morphology, proper flight muscle physiology, and the ability to use biological compasses and maps to navigate toward their overwintering destination [6,9,60]. While the existence of a true map sense in monarchs is still under debate [1,61–63], the compasses exploited by migratory monarchs to maintain directionality during their long-distance migration are defined and rely on the use of either sun/skylight information [9,20,64,65] or the Earth's geomagnetic field [21]. Behavioral studies of the orientation of migratory monarchs, either tethered in a flight simulator or released for disappearance bearings measurements, have established that monarchs use a time-compensated sun compass as the major compass system for both southward autumn and northward spring orientations [16,17,19] (Figure 4A). Directional cues from the daylight sky, which provide information about the position of the sun, are sensed by two anatomically distinct areas of the eyes (the dorsal rim for polarized light and the main retina for the sun's azimuthal position) and integrated in the central complex (CX), a midline structure of the insect brain [64,66–69] (Figure 4B). These directional cues are not fixed over the course of the day, however, because the sun's azimuthal position changes from sunrise to sunset. Fixed flight direction is maintained through time compensation of the sun's movement by circadian clocks, with those located in the antennae playing a major role in this process [16–19,70] (Figure 4A). Grounded in neurophysiology and neuroanatomy, progress has been made in identifying individual neurons within the CX

converted to retinal by the rate-limiting enzyme NINA B. Retinal can either be interconverted into retinol by a retinol dehydrogenase (RDH) or converted into retinoic acid (RA) by a retinaldehyde dehydrogenase (RALDH). RA binds to retinoid receptors to regulate transcription of target genes. Functional disruption of the clock and of the vitamin pathway disrupts photoperiod responsiveness. The connection between vitamin A and juvenile hormone deficiency, characteristic of diapausing monarchs, remains unknown. Modified from [48]. CC/CA, corpora cardiaca/corpora allata.

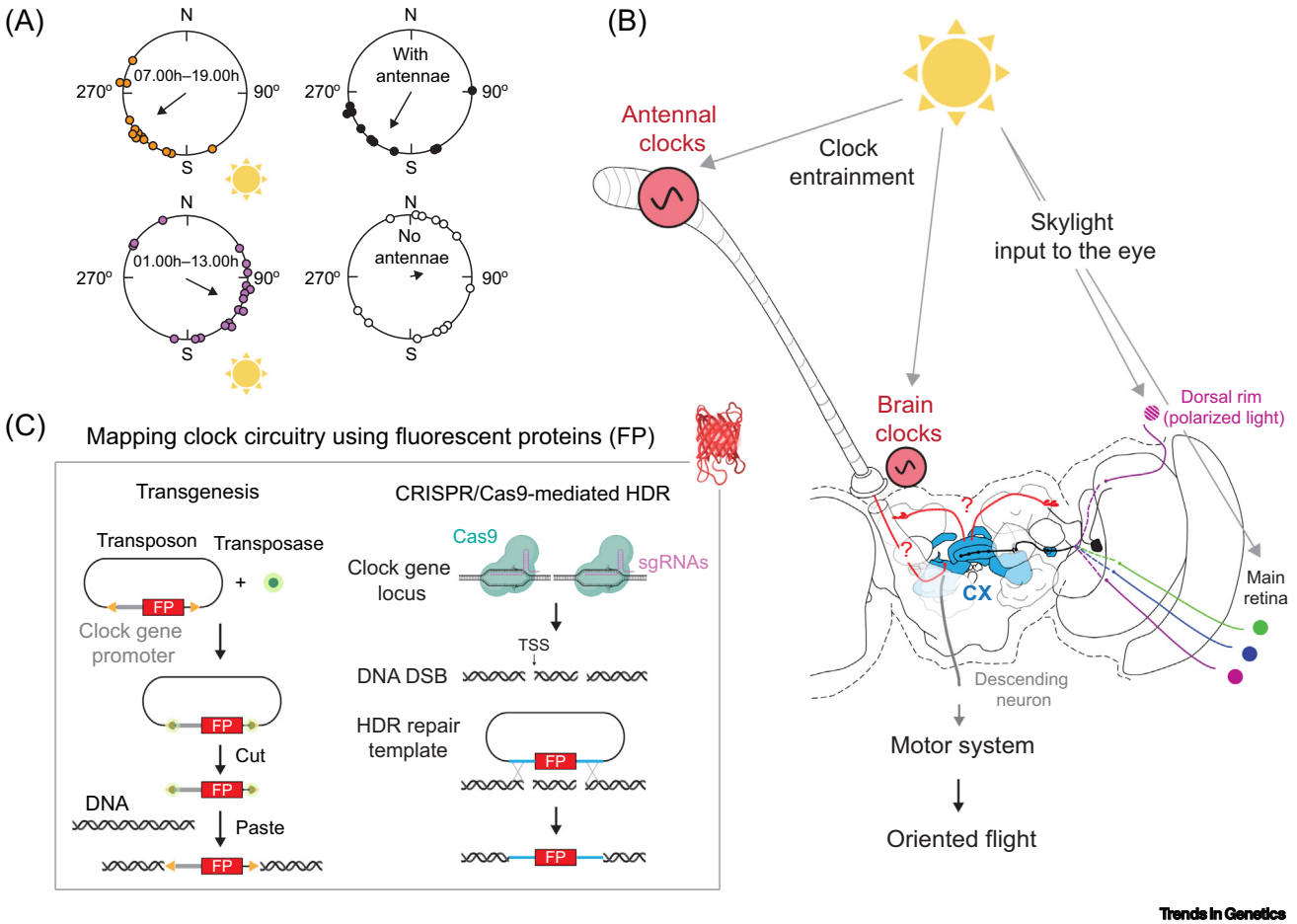

Figure 4. Integration of Timing and Sun Compass Information for Flight Orientation. (A) Migrant monarchs housed in autumn light/dark (LD) cycles with lights on at 07.00h and lights off at 19:00h and flown in a flight simulator in the morning orient in the proper southwesterly migratory direction (upper left). When housed in clock-shifted LD cycles advanced by 6 h, monarchs interpret this morning sun as an afternoon sun and shift their orientation counterclockwise, demonstrating time compensation of sun compass orientation (lower left). Modified from [2]. By contrast to autumn migrants with intact antennae, antenna-less migrants are disoriented as a group, showing that the antennae contain the timer for sun compass orientation (upper and lower right). Modified from [18]. Colored dots indicate the orientation of individuals; arrow indicates mean orientation of the group. (B) Skylight cues are sensed by the eyes (UV polarized light by the dorsal rim and colors of the light or the sun itself by the main retina) and integrated into the central complex (CX; blue). Circadian clocks in the antennae provide the major timing information for sun compass orientation behavior, but brain clocks could have a minor contribution. The neural pathways connecting circadian clocks to the CX remain to be determined (red lines with question marks). Ultimately, the integrated signal is transmitted via descending neurons (gray line) to motor circuits to generate oriented flight behavior. Modified, with permission, from [88]. (C) Genetic tools for genomic integration, including transposon-based transgenesis and CRISPR/Cas9-mediated homology-directed repair (HDR), could be employed to mark clock neurons with fluorescent proteins and map the clock circuitry. Abbreviations: DSB, double-stranded break; sgRNA, single-guide RNA; TSS, transcription start site.

that integrate both azimuthal position and light polarization angle [66,67], but where exactly in the brain time compensation of sun compass information occurs (i.e., in input neurons of the CX, in the CX itself, or in the output descending neurons to the motor system) remains a mystery. The continued development of genetic tools in the monarch to mark clock neurons with membrane-tagged fluorescent proteins could help illuminate the neural circuit connecting clocks to the sun compass (Figure 4C). The clock neuronal circuitry could be mapped by integrating fluorescent proteins under the control of clock gene promoters into the monarch genome, either randomly using piggyBac **transposon-based transgenesis** [71,72] or in a targeted fashion at the endogenous clock loci using

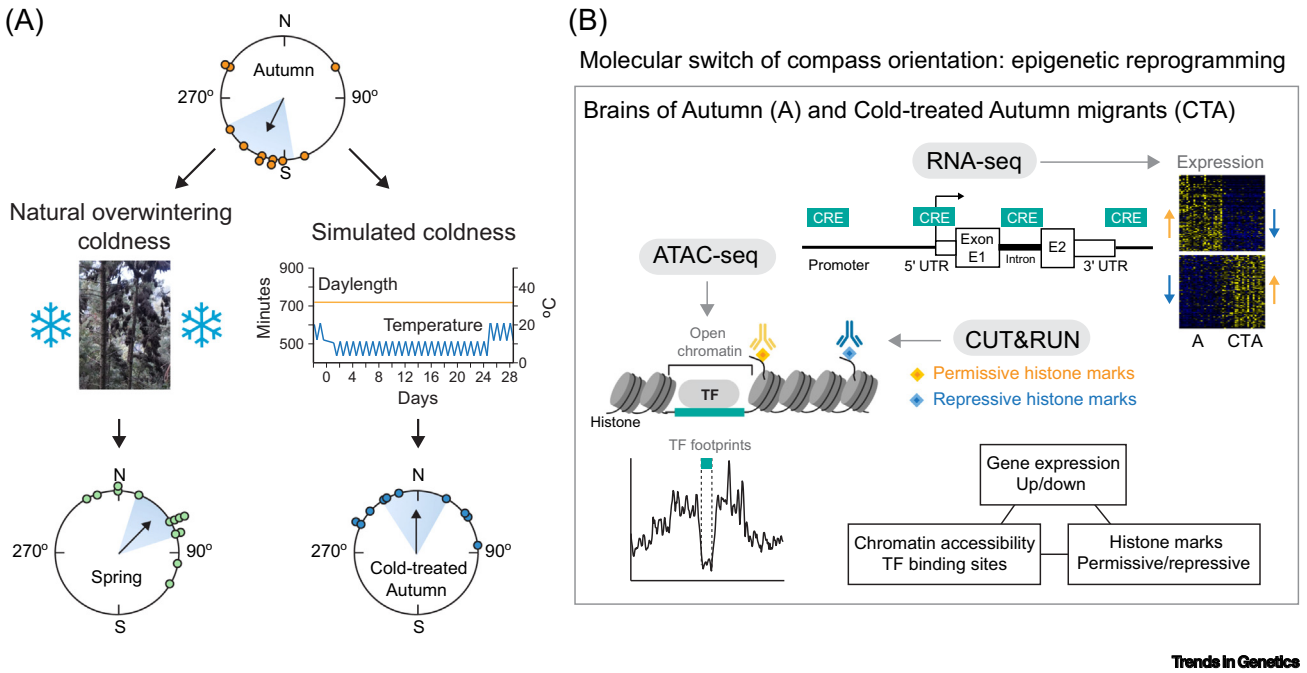

Figure 5. Coldness-Induced Reprogramming of Seasonal Flight Orientation to Study the Molecular Basis of Sun Compass Orientation. (A) Migrants orient southwesterly in the autumn and reverse their flight northeasterly in the spring after prolonged exposure to overwintering coldness conditions (left). Autumn migrants subjected to simulated overwintering-like coldness for 24 days in constant photoperiod also reverse their flight orientation northward (right). Modified, with permission, from [16]. (B) The switch in flight orientation upon exposure to environmental coldness suggests an epigenetic reprogramming of flight orientation. The genes, *cis*-regulatory elements (CREs) and putative transcription factors (TFs) that control their expression, which may be involved in this molecular switch, could be identified through integrated approaches combining RNA-seq, ATAC-seq, and CUT&RUN in brains of autumn migrants and cold-treated autumn migrants. RNA-seq quantifies differential gene expression between conditions. ATAC-seq detects open chromatin regions and TF footprints for the identification of putative TFs. CUT&RUN profiles the epigenome through the use of antibodies against conserved histone marks enriched in permissive or repressive chromatin regions. Abbreviations: UTR, untranslated region.

CRISPR/Cas9-mediated homology-directed repair [73] (Figure 4C). The resulting identification of the brain regions to which antennal and brain clock neurons project could then guide electrophysiological recordings in wild-type monarchs and already available clock-deficient mutants [11,12,39] to precisely define the neurons in which clock–compass integration occurs.

The bidirectionality of the time-compensated sun compass orientation could also be exploited to decipher how sun compass orientation is regulated at the molecular level. The environmental condition that switches flight orientation from southward in autumn migrants to northward in spring remigrants has been identified as a sustained exposure to overwintering-like coldness [16] (Figure 5A). The molecular mechanism by which low temperature causes the switch in flight direction could rely on temperature-dependent splicing, RNA editing patterns, or regulation of gene expression via either noncoding RNAs or epigenetic mechanisms [3,74]. Transient exposure of animals to environmental factors has been shown to induce and maintain behavioral states by changing the neuronal epigenetic landscape that transcriptionally regulates genome-wide gene expression [75]. In addition, post-transcriptional events such as splicing in *Drosophila* and RNA editing in octopuses have been shown to be involved in temperature adaptation [76,77]. Performing RNA-seq studies in the brains of autumn migrants, autumn migrants reprogrammed into spring remigrants by cold treatment, and wild-caught spring remigrants could be used to

detect differentially expressed genes and noncoding RNAs in addition to cold-dependent RNA splicing or editing events (Figure 5B). Epigenetic regulation of gene expression in neurons in the brain has also been shown in other behavioral contexts to result in long-term and robust behavioral changes [78–82]. Epigenetic changes driving the seasonal switch in monarch flight orientation could occur either through the activation of specific transcription factors that can reprogram gene regulatory networks or through alteration of chromatin structure via DNA methylation or histone post-translational modifications (Figure 5B). Cutting-edge technologies such as **bisulfite sequencing** [83], **ATAC-seq** [84,85], and **CUT&RUN** [86,87] could be applied to the monarch to profile the epigenome in the brains of each seasonal form and correlate differential gene expression to its mechanism of regulation (i.e., through epigenetics or noncoding RNAs). The integration of such approaches holds great promise to reveal the underlying genetic basis of flight orientation. In this review, we have described the current understanding of the genetic basis of monarch migration and highlighted how genetic and epigenomic approaches could be deployed to provide new insights into the poorly understood molecular and neurobiological bases of flight orientation.

Concluding Remarks

The recent 'genomic revolution' and rise in cutting-edge genetic and molecular technologies applicable to nonconventional model systems have started to unlock the potential of the monarch butterfly as a key organism to move the genetics of migration forward. Progress has already been made in identifying genomic regions associated with the migratory phenotype, clarifying the role of the circadian clock in seasonal responses, and providing new insights into the molecular basis of seasonal reproductive diapause. The application of contemporary genetic tools such as CRISPR/Cas9 has also positioned the monarch as a benchmark migratory species for the functional characterization of candidate genes and neural circuits. Despite these substantial advances, several fundamental and fascinating questions in the field of migration genetics remain unanswered (see Outstanding Questions). The time is ripe to address them, as an ever-growing number of cutting-edge molecular, genetic, and genomic tools can now be combined with neurobiology and behavioral studies in the monarch.

Outstanding Questions

What cues trigger migratory behavior and at which developmental stages are the cues sensed? Are circadian clocks involved in triggering migration and/or timing migratory departure?

Which genes and/or *cis*-regulatory elements control migratory flight orientation, its seasonal switch, and migratory distances?

How does the vitamin A pathway in the monarch brain mediate seasonal responses? Does it function to generate a deep brain photoperiodic photoreceptor, or does it control seasonal transcriptional programs and/or neural plasticity?

How is time and sun compass information integrated into the nervous system to allow monarchs to maintain flight orientation over the course of the day?

Did nonmigratory populations present across the globe lose the ability to migrate, or do they simply not express this behavior under their local environments?

What are the molecular bases of magnetoreception in monarch butterflies?

How do monarchs pinpoint their overwintering grounds without ever having been there and with such precision that they often congregate on the same trees as their great-grandparents?

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Resources

[i]https://monarchwatch.org/

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