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Report

Coldness Triggers Northward Flight in Remigrant Monarch Butterflies

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Summary

Each fall, eastern North American monarch butterflies (*Danaus plexippus*) migrate from their northern range to their overwintering grounds in central Mexico [1–3]. Fall migrants are in reproductive diapause, and they use a time-compensated sun compass to navigate during the long journey south [4–6]. Eye-sensed directional cues from the daylight sky (e.g., the horizontal or azimuthal position of the sun) are integrated in the sun compass in the midbrain central complex region [7, 8]. Sun compass output is time compensated by circadian clocks in the antennae so that fall migrants can maintain a fixed flight direction south [9, 10]. In the spring, the same migrants remigrate northward to the southern United States to initiate the northern leg of the migration cycle. Here we show that spring remigrants also use an antenna-dependent time-compensated sun compass to direct their northward flight. Remarkably, fall migrants prematurely exposed to overwintering-like coldness reverse their flight orientation to the north. The temperature microenvironment at the overwintering site is essential for successful completion of the migration cycle, because without cold exposure, aged migrants continue to orient south. Our discovery that coldness triggers the northward flight direction in spring remigrants solves one of the longstanding mysteries of the monarch migration.

Results and Discussion

We began our investigations by asking whether monarch butterflies overwintering in Mexico remigrate northward in the spring utilizing a time-compensated sun compass (Figure 1A). We performed outdoor release trials near Austin, Texas in April 2011 to track the flight direction (disappearance bearing) of wild-caught remigrants (see Figure S1 available online) housed indoors in either spring-like or phase-shifted lighting cycles. High ambient temperatures precluded the use of a flight simulator for the study of flight orientation of remigrants in Texas. Nonetheless, we showed that tracking disappearance bearing was as accurate a determinant of directionality as measuring orientation behavior in a flight simulator (see Supplemental Experimental Procedures).

For direct comparison with remigrants, we tracked disappearance bearings from fall migrants captured in Minnesota and tested in Massachusetts in September 2011. These migrants were housed in the laboratory under fall-like light-dark (LD) conditions prior to testing. As expected, when the fall migrants were released outdoors, they exhibited directional flight to the southwest with a mean disappearance bearing (α) of 209° ($n = 11$, $r = 0.58$, $p < 0.02$, Rayleigh's test) (Figure 1B). When remigrants captured in the spring of 2011 in Texas and housed under spring-like LD conditions were released outdoors, they also exhibited highly directional flight behavior, but to the northeast, with a mean disappearance bearing of 45° ($n = 13$, $r = 0.73$, $p < 0.001$) (Figure 1C, upper panel), similar to that observed in naturally flying remigrants studied during two consecutive years in Texas (spring 2011: $\alpha = 20°$, $n = 10$, $r = 0.86$, $p < 0.0001$; spring 2012: $\alpha = 46°$, $n = 12$, $r = 0.93$, $p < 0.0001$) (Figure 2A); the mean disappearance bearing was not different among the three remigrant groups ($F_{2,32} = 1.851$, $p = 0.173$, Watson-Williams test).

Spring remigrants housed in a 6 hr delayed lighting cycle had an α of 142° ($n = 10$, $r = 0.87$, $p < 0.0001$). There was a difference of 97° between the mean orientation of the remigrants housed in 6 hr delayed lighting compared with the remigrants housed in LD ($F_{1,21} = 30.63$, $p < 0.0001$) (Figure 1C, lower panel). The clockwise direction and magnitude of the shift in the mean flight bearing of the remigrants housed under the 6 hr delayed LD cycle is consistent with that expected of a time-compensated sun compass that has been delayed by 6 hr [4–6].

The remigration north, studied in remigrants in Texas during April 2012, appears to utilize an antenna-dependent sun compass system, like the one used to migrate south in the fall [9, 10]. Accordingly, not only does removal of both antennae disrupt the migration south [9], but bilateral antennal removal also disrupted the remigration north ($n = 15$, $r = 0.13$, $p = 0.781$) (Figure 2B). Curiously, the group orientation of antenna-less remigrants was distributed axially ($r = 0.495$; $p = 0.023$) [11]. A similar bimodal trend was observed in antenna-less fall migrants [9], although it was not statistically significant. One antenna was equally sufficient to maintain the northeasterly flight orientation in remigrants: there was no orientation difference between butterflies with the right versus left antenna clipped (Watson $U^2_{6,6} = 0.048$, $p > 0.5$) (Figure 2C), similar to findings in southbound fall migrants [10]. Milkweed olfactory cues, which may be needed for remigrants to find newly emerged milkweed plants for oviposition [1, 12], are not necessary for the flight north, because remigrants with their antennae coated with clear enamel paint, which blocks odor sensing [9], still flew to the northeast ($\alpha = 40°$, $n = 7$, $r = 0.75$, $p = 0.013$). Taken together, these data strongly suggest that the same anatomical substrates are used by the monarch sun compass system for flight in either direction and that the daylight sky provides the dominant directional cues.

Having established that monarchs possess a bidirectional sun compass, we sought to determine the conditions that trigger the northward switch in flight direction. Monarchs begin to arrive at their overwintering sites in the Transvolcanic Mountains of Michoacán in central Mexico around November 1, and they begin leaving for the return trip north in mid-March [13, 14]. The two environmental features during their stay at the overwintering grounds that we explored were day length and temperature. Day length increases by one hour between December 1 (11 hr of light) and March 15 (12 hr of light) (Figure 3A). This is the most stable environmental feature at the overwintering areas. In addition, a modest, one-hour change

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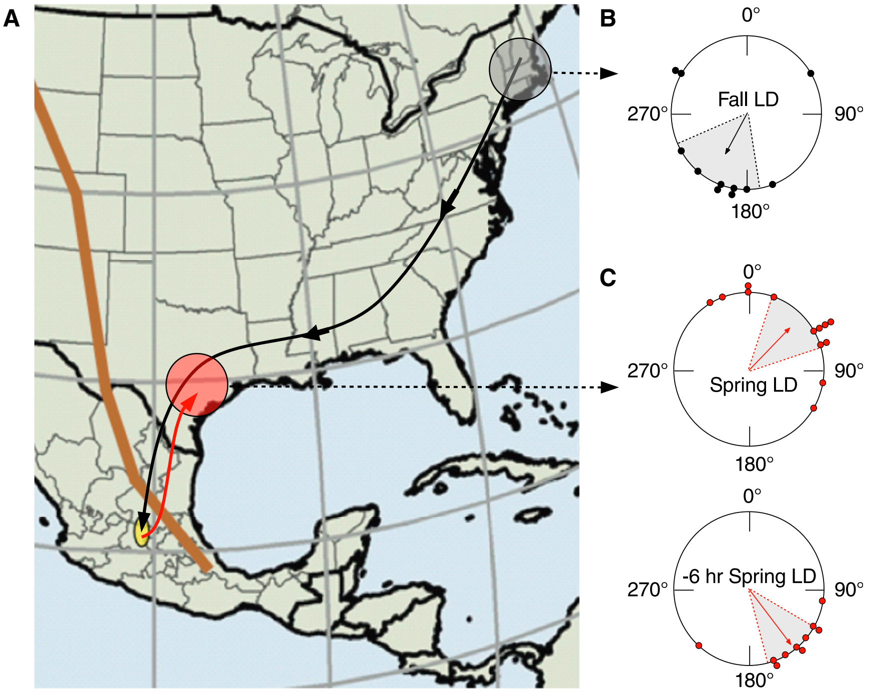

Figure 1. Long-Distance Migration of Eastern North American Monarch Butterflies

(A) Migratory monarch butterflies from the northeastern United States fly southward during the fall (black line) to their overwintering sites in Mexico (yellow oval) and then return northward during the spring (red line). We assayed the orientation behavior of migratory monarchs caught and tested in the fall (Massachusetts; gray circle) and those caught and tested in the spring (Texas; pink circle). The thick brown line demarcates the Rocky Mountains.

(B) Disappearance bearings of migratory monarchs caught during the fall, housed under fall-like conditions (11 hr:13 hr light:dark [LD], with lights-on from 0600 to 1700 Eastern Standard Time [EST]; 21°C during the light phase and 12°C during the dark phase), and tested during the fall in an open field in Shrewsbury, Massachusetts (latitude 42°17′ N, longitude 71°42′ W) on September 21, 2011 (1300–1500 EST). The fall migrants were oriented southwest as a group in release trials (black dots).

(C) Disappearance bearings of returning migratory monarchs caught during the spring (see Figure S1) and tested in an open field in Smithville, Texas (latitude 30°00′ N, longitude 97°16′ W) after being housed under different LD conditions. Upper panel: returning migrants housed under spring-like conditions (12 hr:12 hr LD, with lights-on from 0600 to 1800 Central Standard Time [CST]; 23°C during the light phase and 9°C during the dark phase) showed group orientation to the northeast in release trials performed between April 15 and April 18, 2011 (red dots). Lower panel: the group orientation of returning migratory monarchs entrained to a 6 hr delayed light-dark cycle (lights-on from 1200 to 2400 CST) (red dots).

For each circle diagram, arrow indicates mean group orientation, shaded area is 95% confidence interval, 0° is north, and each colored dot represents the disappearance bearing of a single butterfly. See also Figure S2.

in day length is sufficient in photoperiodic species to be sensed as a change in day length and translated into appropriate biological events (e.g., change in reproductive function in photoperiodic species) [15]. However, the most dramatic environmental feature of the overwintering site is low temperature (Figure 3A). Upon arrival in November, monarchs soon congregate in tightly packed clusters in a few isolated locations in the high-altitude coniferous forests. Both the clustering and the forest cover provide a microenvironment that protects the migrants from environmental extremes [13, 14, 16]. For example, the microenvironment provides temperatures low enough to keep metabolic demands low while overwintering, but not cold enough to cause freezing (and subsequent death).

We thus subjected fall migrants in Massachusetts between September 16 and October 9, 2011 to changes in both photoperiod and temperature over a 24-day period to mimic those at the overwintering sites in Mexico (lighting: graded 1 hr increase in light from 11 hr to 12 hr; temperature: 11°C in light and 4°C in dark); the temperature cycle was based on mean day and night temperatures previously measured in overwintering clusters [13, 14]. We then tracked flight orientation in

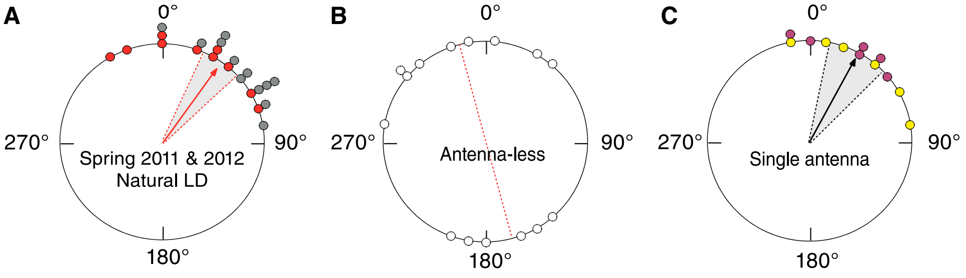

Figure 2. Characteristics of the Time-Compensated Sun Compass Used by Remigrating Monarchs

(A) Disappearance bearing of remigrants observed remigrating northward in the field during spring 2011 (red dots) and spring 2012 (gray dots). There was no difference in orientation direction between years (Watson's $U^2_{10,12} = 0.096$, $p < 0.5$), and the pooled disappearance bearing shown is 35° ($n = 22$, $r = 0.876$, $p < 0.000005$).

(B) Disappearance bearing of remigrants with both antennae amputated and maintained under spring-like conditions (open dots). The antenna-less remigrants were no longer oriented northeast as a group but were instead distributed bimodally along an axis running between 165° and 345° (dotted red line).

(C) Disappearance bearings of remigrants entrained to spring-like conditions but that had one antenna removed (magenta dots, right antenna; yellow dots, left antenna). The pooled group orientation direction of the single-antenna remigrants was 28° ($n = 12$, $r = 0.896$, $p < 0.0001$).

For each circle diagram, arrow indicates mean group orientation, shaded area is 95% confidence interval, 0° is north, and each colored dot represents the disappearance bearing of a single butterfly. See also Figure S1.

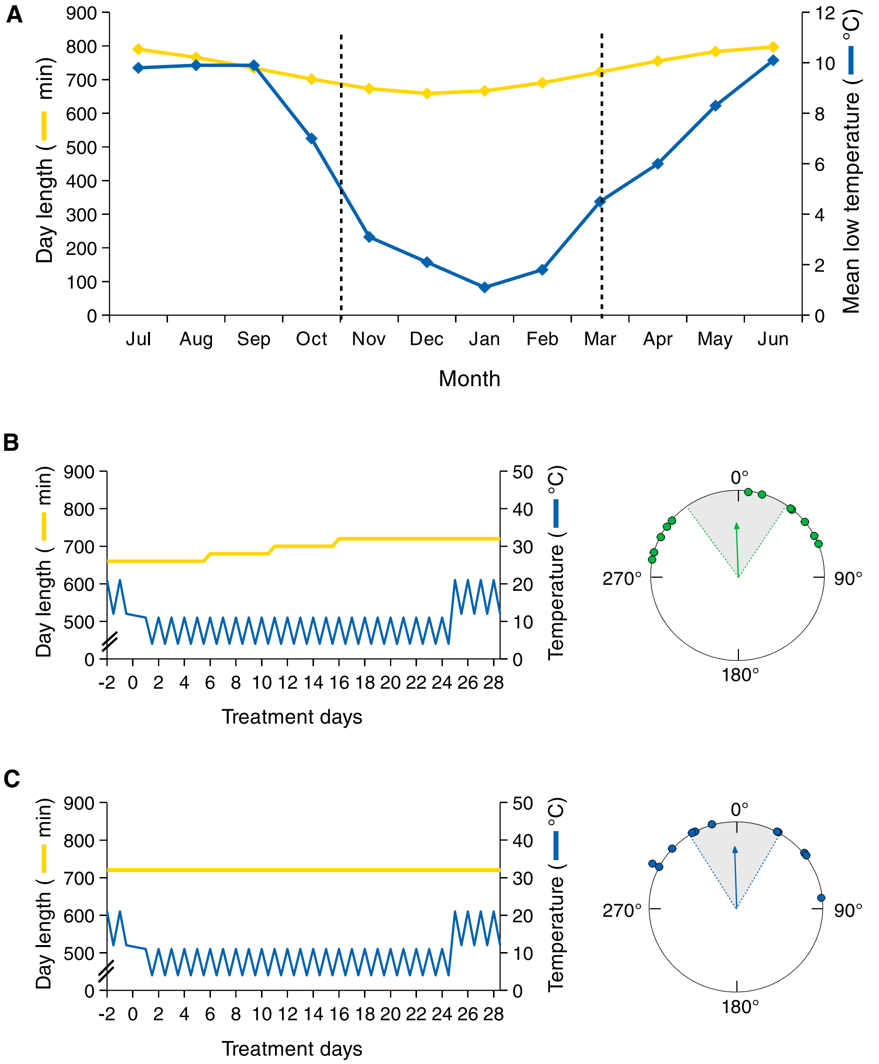

Figure 3. Coldness Shifts Flight Orientation Northward in Fall Migrants

(A) Mean monthly day lengths (yellow) and mean monthly low temperatures (blue) at the overwintering sites. Day length values were obtained from http://www.timeanddate.com/ and temperatures from Journey North (http://www.learner.org/jnorth/). Black vertical dashed lines indicate the beginning and end of the time period when migratory monarchs are at the overwintering sites. See also Figure S3.

(B) Flight orientation of fall migrants in a flight simulator subjected to an increasing photoperiod (yellow line) and oscillating temperature conditions (11°C during lights-on, 4°C during lights-off; blue line) consistent with those found at the overwintering sites. Left: environmental conditions; right: orientation data (green dots represent individual monarchs tested between October 8 and October 24, 2011). See also Figure S2.

(C) Flight orientation of fall migrants subjected to a control constant photoperiod and the same oscillating temperature conditions as in (B) prior to testing (blue dots). Left: environmental conditions; right: orientation data. See also Figure S2. The temperature regime for both (B) and (C) is consistent with the mean high and mean low temperature values found inside the center of an overwintering roost measured over a 40-day period (January 19–February 28, 1979) [13] and from February 2 to February 5, 2008 [14]. For each circle diagram, arrow indicates mean group orientation, shaded area is 95% confidence interval, 0° is north, and each colored dot represents the flight orientation of a single butterfly.

a flight simulator, after equilibration to fall temperature conditions (21°C light, 12°C dark); monarch butterflies need temperatures of >15°C to initiate flight [13]. After the 24-day treatment, we found that the fall migrants now flew north with a mean orientation of 358° ($n = 12$, $r = 0.606$, $p = 0.009$) (Figure 3B). The northward mean orientation of fall monarchs subjected to overwintering-like conditions of increasing photoperiod and cold temperatures was not different from the mean orientation of spring remigrant monarchs (Watson $U^2_{12,13} = 0.076$, $0.5 > p > 0.2$) (Figure S2A).

When photoperiod remained constant from capture (12 hr:12 hr light:dark) but only temperature was decreased over the 24-day period (11°C light, 4°C dark) in a second group of migrants (treatment period occurring between September 28 and October 23, 2011), those butterflies also flew north with a mean orientation of 358° ($n = 11$, $r = 0.675$, $p = 0.004$) (Figure 3C), indicating that it is low temperature, not changing photoperiod, that causes the northward change in flight orientation. Again, the northward mean orientation of fall monarchs subjected to overwintering-like conditions of constant photoperiod and cold temperatures was not different from the mean orientation of spring remigrant monarchs (Watson $U^2_{11,13} = 0.087$, $0.5 > p > 0.2$) (Figure S2B).

We also showed that the northerly orientation of fall monarchs subjected to overwintering-like conditions (increasing photoperiod and cold, or cold alone) could be shifted by a 6 hr delayed lighting cycle (Figures S2C and S2D). These results indicate that coldness had in fact shifted the directionality of a time-compensated sun compass in the fall migrants. Although a 24-day exposure to overwintering-like cold conditions was sufficient to reverse flight direction in fall migrants, we do not yet know the minimum number of cold days necessary to induce the change in orientation. Our results raise the interesting possibility that an unseasonal cold spell in the north during the journey south could disrupt sun compass orientation in migrants en route in that area.

Importantly, fall migrants maintained in fall-like conditions (11 hr light at 21°C:13 hr dark at 12°C) from capture until after the overwintering-mimicking experiments were completed (i.e., they were never subjected to coldness) still maintained the expected southwesterly mean orientation direction of 227° ($n = 6$, $r = 0.928$, $p = 0.001$). This result shows that it was indeed cold exposure that shifted the orientation of fall migrants northward, and not some peculiarity of the group of fall migrants tested. Remarkably, fall migrants collected from roosts in the wild in the southern United States in late October 2011 and maintained in the laboratory (in Massachusetts) under fall conditions (11 hr light at 21°C:13 hr dark at 12°C) over 4 months from capture through mid-March 2012 (when remigrants in the wild begin to move north) (Figure 4A) continued

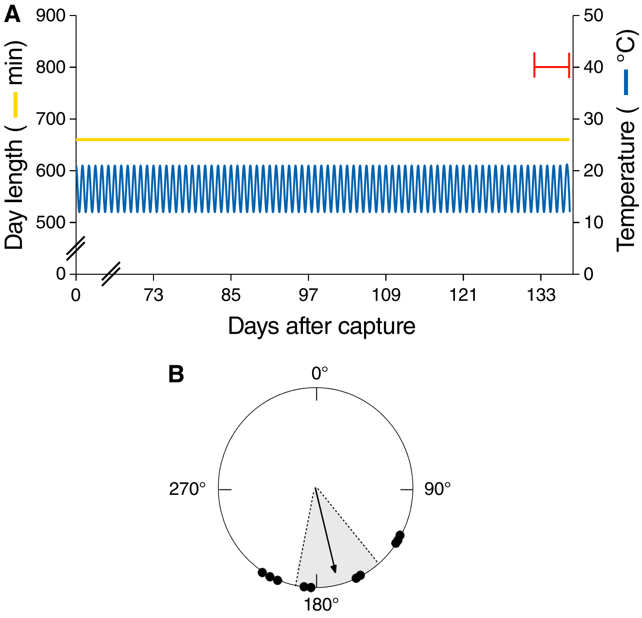

Figure 4. Prolonged Exposure of Fall Migrants to Fall Conditions Does Not Alter Southward Orientation

(A) Fall migrant monarchs were housed under control fall-like conditions in the laboratory from October 29, 2011 to March 14, 2012 and then flown in a flight simulator. Horizontal red line indicates time of testing.

(B) Flight orientation of the aged migrants (black dots). Arrow indicates mean group orientation, shaded area is 95% confidence interval, 0° is north, and each colored dot represents the flight orientation of a single butterfly.

to show a mean orientation in the flight simulator to the south (α = 166°, n = 10, r = 0.826, p = 0.002) (Figure 4B); the more southeasterly orientation direction has been found before in aged migrants [9]. This result rules out the involvement of an interval or annual timer in the directional shift of the sun compass at the overwintering site. That is, without exposure to coldness, the butterflies would continue to fly south; they would not be able to reverse their flight direction, and the annual migration cycle would be disrupted.

Notably, of the aged fall migrants that continued to orient south (Figure 4B), six were female, and five of the six were ripe with mature eggs, showing that increased reproductive function does not influence flight orientation direction, as shown previously in early fall migrants in which reproductive function was prematurely activated [17, 18]. In addition, although untested in the flight simulator, five other females that were of similar advanced age were also found to be ripe with mature eggs.

Conclusions

Bidirectional orientation has been observed in other migratory butterflies [19–21] and in migratory moths [22, 23], because they also reverse their orientation direction between the fall and spring. However, whether this reversal occurs at the individual level or in different generations, and what type of compass is used to navigate in these other species, has not been established. Our results, on the other hand, clearly show that individual monarch butterflies use a time-compensated sun compass to direct their migration both southward and northward. Moreover, the sun compass mechanism is modulated primarily by cold exposure during overwintering to flip the butterflies' orientation direction from south (in the fall) to north (in the spring).

The data show, unexpectedly, that the thermal microenvironment at the overwintering site is critical for successful completion of the migration cycle. Without coldness, eastern North American migrants cannot reverse their flight direction, and the migration cannot progress. Based on yearly low temperature plots at Monterey, California (Figure S3), a major overwintering site for western North American monarchs, coldness may also be involved more widely in flight reversal in western migrants. Knowledge of the "cold trigger" in the migration of the monarch butterfly has broad-ranging implications for conservation strategies related to this endangered biological phenomenon [24] and underscores how vulnerable the migration may be to global warming and overall global climate change. Furthermore, we can now dissect the molecular mechanism by which low temperature causes the switch in flight direction [25] and the location of the relevant temperature sensor.

Even though the migrants are migrating south in the fall to escape frigid temperatures in the northern part of their range, the eastern North American migrants end up overwintering in an environment in which the temperature hovers above freezing. Most conservation concerns have been directed at deforestation and habitat degradation at the overwintering sites in Mexico, which can result in monarchs facing lethal colder temperatures due to the loss of temperature buffering provided by the microclimatic conditions at the overwintering sites [26]. In addition to this concern, we now show that coldness itself is needed to reverse flight direction. There appears to be a fine temperature balance at the overwintering sites that is critical for successful migration but vulnerable to external influences.

Experimental Procedures

Complete experimental procedures, including animal rearing and housing, flight release trials, and flight simulator trials can be found in the Supplemental Experimental Procedures.

Supplemental Information

Supplemental Information includes three figures and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2013.01.052.

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