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Supplemental Information

Coldness Triggers Northward Flight in Remigrant Monarch Butterflies

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Supplemental Inventory

Supplemental Figures

Figure S1, Related to Figures 1C and 2. Migratory Monarchs Returning Northward from Their Overwintering Sites in Mexico

The remigrants are distinctive because they are faded and tattered. Top, male; bottom, female. Scale bar = 1 cm.

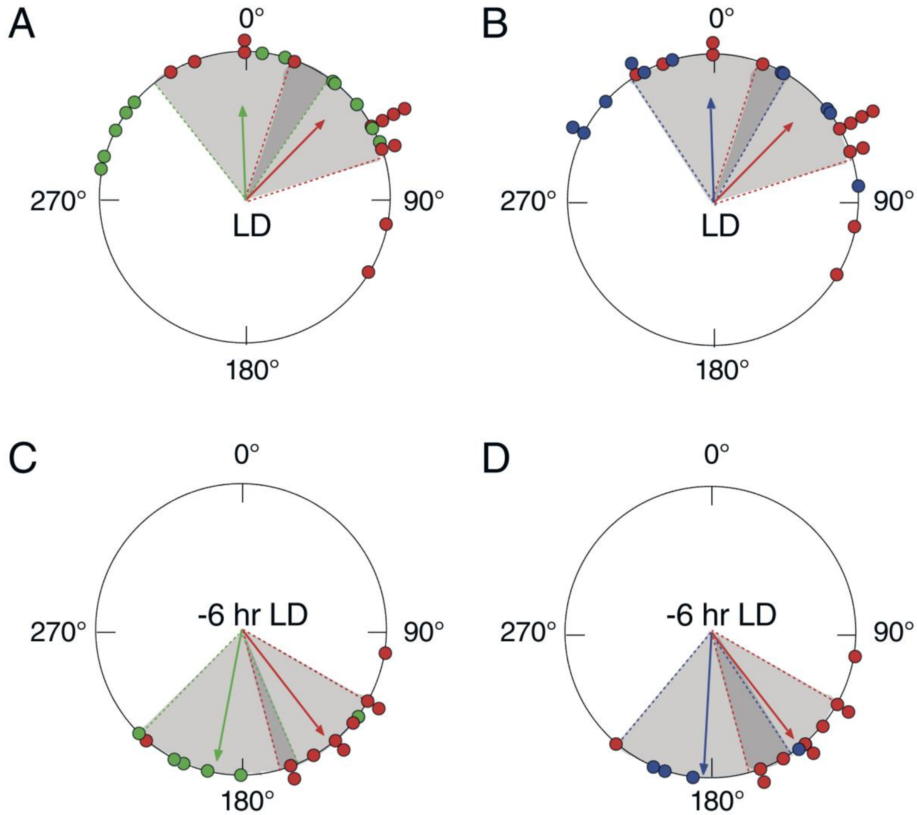

Figure S2, Related to Figures 1C, 3B, and 3C. **Cold-Treated Fall Migrants Orient Their Flight in a Manner Similar to Spring Re-migrants**.

(A) The northward mean flight orientation of fall monarchs subjected to overwintering-like conditions of increasing photoperiod and cold temperatures from Figure 3B (green dots) is not different from the mean orientation of spring remigrant monarchs from Figure 1C, upper panel (red dots) (Watson $U^2_{12,13} = 0.076$, $0.5 > p > 0.2$).

(B) The northward mean flight orientation of fall monarchs subjected to overwintering-like conditions of constant photoperiod and cold temperatures from Figure 3C (blue dots) is not different from the mean orientation of spring remigrant monarchs from Figure 1C, upper panel (red dots) (Watson $U^2_{11,13} = 0.5 > p > 0.2$).

(C) The mean flight orientation of fall monarchs subjected to overwintering-like conditions (increasing photoperiod and cold) and tested a second time after housed in 6-hr delayed lighting (green dots) (α of 191°, n = 6, $r = 0.866$, $p = 0.005$) was not different from the mean orientation of spring monarchs housed under the phase-shifted lighting conditions from Figure 1C, lower panel (red dots) (Watson $U^2_{6,10} = 0.179$, $0.1 > p > 0.05$).

(D) Although there were not enough fall monarchs subjected to overwintering-like conditions (constant photoperiod and cold) who flew a second time after being phase-shifted (blue dots: n=4) to be compared statistically with spring monarchs housed under 6-hr shifted LD (red dots from Figure 1C, lower panel), the 95% confidence intervals of both groups overlapped (cold-treated fall migrants: 120-164°; spring re-migrants: 146-220°). These cold-treated, phase-shifted fall monarchs had a mean orientation of 183° (n = 4, r = 0.917, p = 0.022).

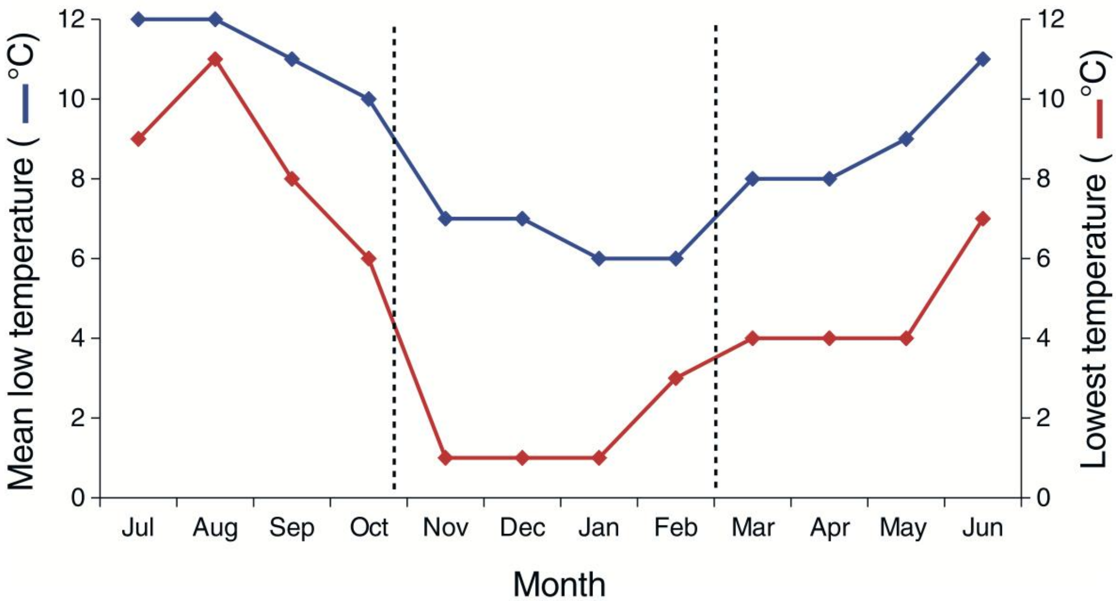

Figure S3, Related to Figure 3A. Overwintering Low Ambient Temperature Patterns for Western North American Migrants

The pattern of low temperatures at Monterey, California, a major overwintering site for western monarchs. Blue line, mean low temperature for a given month; red line, lowest temperature for a given month. Vertical dashed lines, approximate arrival and departure dates. Values are from July 2010 - June 2011 (source: wunderground.com). Although the temperature decrement is less pronounce compared to that in Mexico (Figure 3A), the low temperature trend over the year is similar.

Supplemental Experimental Procedures

Flight Release Trial Experiments

Animal Rearing and Housing

We tested both fall migrant (fall 2011) and spring re-migrant (spring 2011 and 2012) butterflies in outdoor flight release trials (see below). Fall migrant monarchs were captured near St. Paul, Minnesota by Karen Oberhauser and supplied to us on 7 September 2011. Spring re-migrant monarchs were captured at various sites in southeastern Texas in the spring of 2011 and 2012 (Elgin, Texas 30°35'N, 97°37'W; Smithville, Texas 30°00'N, 97°16'W; Cheapside, Texas 29°28'N, 97°40'W; Port Lavaca, Texas 28°61'N, 96°63'W; Seadrift, Texas 28°42'N, 96°71'W). Monarchs were identified as fall migrants returning from overwintering sites in Mexico based upon their wing scale loss and wing tattering relative to next generation butterflies (Figure S1).

After capture, each monarch was housed in a glassine envelope and placed in an environmentally-controlled incubator. Fall migrants were housed in a Percival incubator in which the temperature was maintained at 21 ºC during the light phase and at 12 ºC during the dark phase, to coincide with typical fall conditions [1]. Spring re-migrants were housed in a Tritech Research incubator, where the temperature was maintained at 23 ºC during the light period and at 9°C during the dark period, to coincide with typical spring conditions. Prior to release trials, butterflies were entrained to their respective LD cycle for at least five days. The incubator of fall migrants from September 2011 had its lighting regime set to coincide with prevailing fall light conditions, with lights on from 0600 to 1700 hours Eastern Standard Time (EST). Spring re-migrant monarchs from April 2011 were placed in one of two incubators with a 12hr:12hr light:dark cycle (LD), in which the LD cycle of one was timed to prevailing light conditions, with lights on from 0600-1800 hrs Central Standard Time (CST). The other group was housed under a 6-hr delayed lighting cycle, with lights on from 1200-2400 hrs. Spring re-migrant monarchs from April 2012 having either no antennae, a single antenna, or both antennae painted clear were treated as described previously prior to testing [2, 3]. All monarchs were removed from their envelopes and fed 25% honey every third day.

Flight Release Trials and Analysis

In contrast to prior work that used a flight simulator to assess the flight orientation of monarchs [1-3], we were unable to use a flight simulator as temperature (mean temperature: 31 ± 0.8 °C, n = 5, range: 29-33 °C) conditions in Texas during spring 2011 precluded its consistent use. Therefore, we performed trials in a manner similar to that of [4] and [5] to assess the flight orientation of the butterflies in spring 2011; as spring 2012 temperature conditions were similar to those of spring 2011, and to keep a consistent protocol in our behavioral assay, we also performed release trials during fall 2011 and spring 2012 using the same method.

Prior to trials, butterflies were entrained to their respective LD cycle for at least five days. On the day of trials, monarchs from the incubators were housed together in an outdoor holding cage to acclimate to outdoor conditions, for 30 minutes. For spring monarchs, although each monarch possessed an arbitrary number written on one of its hindwings using a permanent marker for recording purposes, the observer did not know from which specific incubator a monarch came from during a trial. We conducted all trials outdoors, between the hours of 1230 and 1500 hrs CST, under sunny skies in a field. Prior to each trial, we recorded the ambient wind speed; if wind conditions were non-conducive for trials, i.e., wind gusts >3 m/s, we waited for the winds to calm before commencing a trial. For a given trial, a single, randomly chosen monarch was removed from the holding cage and was placed in a covered cage for five minutes. The cover was then removed, and the monarch was free to leave the cage; monarchs would typically leave the cage between 30-45 seconds after cover removal.

Once a monarch left the cage and was flying, we used a hand-held compass (Spring 2011: Silva Starter 1-2-3 base plate compass; Fall 2011 and Spring 2012: Silva Guide Model 426 sighting compass) to estimate the butterfly's disappearance bearing (i.e., the compass direction when the monarch disappeared from sight) [4, 5]. We found no difference in the readings given between the two compass types. For example, when observing the mean disappearance bearing of fall 2011 migrants, the disappearance bearings obtained with either the sighting ($\alpha = 209°$, $n = 11$, $r = 0.577$, $p = 0.022$) or base plate compass ($\alpha = 212°$, $n = 11$, $r = 0.608$, $p = 0.014$) were similar ($R_{0.05,\ 11} = 0.357$, $p > 0.05$, Moore's test). Although our estimates of disappearance bearing will contain a degree of error (i.e., we made measurements to the nearest 5°), any systematic bias on orientation measurements would not have been introduced using this method [6].

For our analysis, we excluded monarchs that did not fly or that did not exhibit sustained flight to disappearance. We had a 35% success rate of butterflies that exhibited sustained flight, as 23 out of 66 monarchs used yielded stable flight data; this number is similar to the number of fliers for migrants during the fall tested in a flight simulator (31% success rate, as 34 out of 110 monarchs flew continuously for five minutes during their first attempt in a flight simulator during Fall 2010). As a natural control, we estimated the disappearance bearings of naturally flying monarchs observed at a separate field in the same manner as our release trials.

Previously, methods of measuring lepidopteran flight orientation in release trials have been criticized [7]. One concern is the potential of an escape response, in which butterflies will fly erratically upon leaving the holding cage during release trials. However, we found that it was easy to distinguish escape behaviors or erratic flight from the natural flight of monarchs that were flying in a migratory manner. In our release trials, monarchs exhibited their natural migratory flight pattern when observed using our method. Individuals that did demonstrate an escape response were excluded from our analysis. Importantly, the r value for the mean disappearance bearing of monarchs in release trials (e.g., Spring 2011: $r = 0.73$, $n = 13$) was virtually identical to the r value seen in experiments with tethered fall migrants whose flight orientation was monitored in a flight simulator ($r = 0.67$, $n = 10$ [2]; $r = 0.714$, $n = 12$ [1]). Thus, our release data provide an excellent assessment of flight direction.

Flight Simulator Experiments

Animal Rearing and Housing

Fall migratory monarch butterflies were captured by Fred Gagnon (between 13 September 2011 and 27 September 2011; near Greenfield, Massachusettts [latitude 42°59'N, longitude 72°60'W]) and David Cook (29 October 2011; near St. Marks, Florida [latitude 30°9'N, longitude 84°12'W]). After capture, migrants were housed indoors in glassine envelopes in a Percival incubator (set at 70% humidity), and were removed from their envelopes every third day and fed a 25% honey solution.

To test monarchs subjected to lighting (source: www.timeanddate.com) and temperature (source: www.learner.org/jnorth/) conditions similar to those found at roosts at the overwintering sites in Mexico (Figure 3A), monarchs were placed in a Percival where the photoperiod increased from an 11hr:13hr LD (lights on between 0600 and 1700 EST) to a 12hr:12hr LD (lights on between 0600 and 1800 EST), where the photoperiod increased in length every 5 days (Figure 3B). In addition, during this photoperiod increase and for 4 days after (24 days total), monarchs were kept at 11 °C during the light phase and at 4 °C during the dark phase (Figure 3B). After this period, to facilitate testing in the flight simulator (see below), the temperature conditions were changed to 21 °C during lights on and 12 °C during lights off (Figure 3B). As a control for changing photoperiod conditions, other monarchs were housed in another Percival with constant 12hr:12hr LD conditions (lights on between 0600 and 1800 EST) (Figure 3C). Monarchs in this different Percival received the same 24-day period of cold conditions as described above, followed by the same change to a warmer temperature regime after this period (Figure 3C). Other fall migrants were kept in fall-like control conditions, where the Percival was set with a constant 11hr:13hr LD (lights on between 0600 and 1700 EST), and temperature conditions consisting of 21 °C during lights on and 12 °C during lights off (Figure 4A and B).

Flight Simulator Trials and Analysis
Monarchs of mixed sex were tethered for flight trials as previously described and their flight behavior was assayed with a flight simulator outdoors [1-3]. Monarchs housed under overwintering-like photoperiod and temperature conditions, and those housed under overwintering-like temperature conditions alone, were tethered and tested in the flight simulator after receiving the full 24-day temperature treatment period. Monarchs housed under fall-like control conditions were tested after at least 10 days after housed under fall-like conditions.

Flight simulator trials were conducted under sunny skies in Shrewsbury, Massachusetts (latitude 42°17'N, longitude 71°42'W), during a time when the sun could be seen from the butterflies' position in the flight simulator. Flight trials were conducted between 1300 and 1500 hours EST. Monarch butterflies that were tested in the flight simulator a second time (i.e., monarchs from a Percival with either photoperiod and temperature conditions similar to the overwintering sites or to these temperatures alone) were placed in another Percival with a 6-hr delayed lighting cycle, and were tested at least 5 days after being switched to this shifted light cycle. Only butterflies that flew continuously for a period of at least 5 min and that presented a directional flight path (Z-scores > 500) were considered for all analyses [1-3]. We determined the significance of flight orientation and the mean direction of monarchs tested in the flight simulator using circular statistics in Oriana (Kovach Computing Services).

Supplemental References

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