1 # Environmental, Developmental, and Genetic Conditions Shaping

2 # Monarch Butterfly Migration Behavior

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16   **Abstract**

17   Monarch butterflies in North America migrate south each autumn, but the mechanisms that

18   initiate their migratory flight remain incompletely understood. We investigated environmental,

19   developmental, and genetic factors that contribute to directional flight by testing summer and

20   autumn-generation monarchs in three flight simulators: two at ground level (with and without wind

21   blockage) and a novel balloon-based system that raised butterflies 30 meters into the air.

22   Monarchs reared under autumn-like conditions in a growth chamber during the summer were also

23   tested to explore the influence of developmental cues. Autumn generation monarchs

24   demonstrated significant southwestern flight orientation, observed exclusively in the balloon

25   simulator, underscoring the importance of high-altitude flight for migratory behavior. Summer

26   generation monarchs reared under autumn-like conditions displayed southward orientation, larger

27   wing sizes, and partial reproductive diapause, indicating specific seasonal environmental cues

28   that are sufficient to induce migratory traits. In contrast, a lab line of monarchs reared in captivity

29   since 2016 exhibited diminished wing size and reduced orientation ability, even when raised

30   outdoors in the autumn, consistent with a loss of migratory traits in the absence of migration.

31   Surprisingly, butterflies in the balloon simulator tended to orient upwind, which suggests that wind

32   may also serves as a directional cue during migration. These findings highlight the critical roles of

33   altitude, wind, and environmental cues in monarch migration and validate the balloon flight

34   simulator as a powerful tool for studying migratory behavior. This research advances our

35   understanding of the initiation of monarch migration and informs strategies for conservation

36   efforts amidst environmental change.

37

Main Text

Introduction

Migration is a vital strategy used by many animal species to access seasonal resources or track favorable environmental conditions. This complex behavior depends on the timing of departure, which influences stopover patterns, arrival timing, and ultimately the fitness of migrating individuals (1–3). Understanding the triggers that initiate migration is therefore key to understanding how migrants adapt to their environments (4, 5). The onset of long-distance migration involves two interconnected components: a developmental phase that prepares the organism for migration (6–8) and a flight strategy that enables navigation through varying environmental conditions (9–11). During development, migrants undergo physiological, morphological (12), and metabolic changes (13) that equip them for sustained long-distance travel, with these traits shaped by genetic and environmental factors. In addition, effective flight strategies allow migrants to respond to dynamic environmental cues such as sun position (14), wind conditions (9, 15), and temperature (16, 17), ensuring successful navigation and energy efficiency during migration.

Among migratory species, insects are especially numerous, both in terms of biodiversity and ecological significance, serving as important pollinators and decomposers (18–20). Particularly, the monarch butterfly (*Danaus plexippus*) is a well-known migratory species that is famous for the long-distance migration of the eastern North American population. Unlike many migratory animals, which repeat their migration multiple times, eastern North American monarchs migrate only once in their lifetime. In response to seasonal cues, monarchs eclosing in the autumn embark on a southward journey of up to 4,000 kilometers to their overwintering sites in central Mexico. In spring, these same individuals fly north to the southern United States, where they reproduce and die, with successive generations continuing the northward journey (21–23). This multi-generational migration precludes the possibility of learning the migration route, indicating that this behavior is innate.

Migratory monarchs display distinctive traits, including southern-oriented flight in autumn, relatively large forewings, and reproductive diapause. Previous studies have explored the environmental cues influencing these traits (24–30), with particular attention placed on factors such as decreasing day length and temperature (30). Research has also delved into the neural mechanisms underlying migration (31, 32) and how monarchs utilize flight strategies to navigate environmental conditions, employing methods such as radio tracking (28, 33, 34) and vanishing bearings (35, 36). However, while considerable progress has been made in understanding the

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73   developmental processes that initiate migration, these studies have predominantly focused on the

74   impact of rearing environments, as these are relatively straightforward to manipulate through

75   common garden experiments. In contrast, comprehensive investigations into the interplay

76   between developmental processes and flight strategies—especially how both factors combine to

77   trigger migration—remain scarce due to the challenges of tracking small insects over long

78   distances and the low likelihood of retrieving released butterflies.

79   In this study, we addressed two critical aspects of monarch butterfly migration: (1) how genetic

80   background and environmental conditions during development shape the initiation of migration,

81   and (2) how flight strategies—such as directional orientation, altitude preferences, and wind

82   utilization—interact with environmental conditions to facilitate successful migration. To explore

83   these questions, we reared monarch butterflies from summer and autumn generations under both

84   controlled (growth chamber) and outdoor conditions, assessing migration-related traits under

85   varying flight scenarios. Additionally, we tested monarchs from a long-term lab-reared population

86   to evaluate how intermediate numbers of captive generations affect migratory behavior,

87   particularly the retention or loss of directional orientation. Using a novel balloon-based flight

88   simulator alongside ground-based systems, we examined the contributions of environmental and

89   developmental cues to migratory traits.

90   Our findings emphasize the critical roles of altitude, wind, and seasonal cues in the initiation of

91   migration. They also reveal how migratory behavior diminishes in lab-reared populations over

92   generations. These results provide new insights into the drivers of monarch migration,

93   highlighting the challenges faced by these butterflies and the importance of preserving the

94   environmental conditions essential for their migratory success.

95

96   **Results and Discussion**

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98   To investigate the flight strategies that monarch butterflies use during the initiation of migration,

99   we captured wild North American monarchs and reared them outdoors for both summer and

100   autumn generations (hereafter, "summer field" and "autumn field", Fig S1). We then tested their

101   orientation behavior in the autumn using three different flight simulators that mimicked various

102   flight conditions: (1) ground level open air, representing natural flight conditions, (2) ground level

103   wind block, simulating a controlled environment at ground level, and (3) the balloon method,

104   where butterflies experienced ascent to a 30-meter height with full wind exposure (Fig 1A). The

105   ground-level simulators did not provide a height stimulus, while the transparent wind block

106   allowed butterflies to perceive their surroundings visually but without wind interaction. In the

107 balloon method, butterflies experienced both altitude and wind conditions similar to those in the

108 wild, allowing us to simulate their migration flight more realistically.

109 We recorded each butterfly's average flight direction (0° to 359°) and the consistency of its flight

110 (0 to 1) across all three simulators. In the summer field generation, butterflies exhibited no

111 significant directional orientation across any flight simulator (ground level open air: σ = 86°, n =

112 10, r = 0.092, Rayleigh test, P = 0.19; ground level with wind block: σ = 132°, n = 19, r = 0.072,

113 Rayleigh test, P = 0.15; balloon: σ = 230°, n = 18, r = 0.068, Rayleigh test, P = 0.37, Fig 1B),

114 aligning with previous findings that monarchs do not migrate in summer and thus show no

115 directed flight pattern (24, 37). In contrast, the autumn field generation, presumed to be migratory,

116 showed strong southwest directionality exclusively in the balloon flight simulator (σ = 240°, n =

117 20, r = 0.11, Rayleigh test, P = 0.017, Fig 1C). Ground-level simulators, with and without wind

118 blocking, yielded no significant southern orientation (open-air: σ = 134°, n = 14, r = 0.1, Rayleigh

119 test, P = 0.4; wind block: σ = 67°, n = 20, r = 0.16, Rayleigh test, P = 0.017, Fig 1C). These

120 results support existing evidence that high-altitude flight enables monarchs to integrate visual

121 height cues and directional information from the sun, enhancing migratory orientation (10). Such

122 behavior is analogous to findings in migratory birds, where high-altitude release after visual

123 orientation results in sustained migratory behavior (38).

124 To evaluate environmental effects on orientation, we reared summer-generation butterflies in a

125 growth chamber under autumn-like conditions (hereafter referred to as "autumn growth chamber",

126 Fig S1) and tested their orientation using the balloon simulator. Additionally, we tested butterflies

127 from a lab population reared in captivity since 2016/2017, which we then raised outdoors under

128 conditions similar to field populations (hereafter referred to as "summer lab line" and "autumn lab

129 line", Fig S1). Due to the high mortality rate of the summer lab line population, we were unable to

130 test the orientation behavior of this group.

131 In the balloon test, butterflies from the autumn growth chamber treatment exhibited strong

132 directional orientation (mean vector angle = 214°, n = 23, r = 0.17, Rayleigh test, P < 0.001; Fig.

133 2A), indicating that autumn-like developmental conditions effectively prime migratory behavior.

134 This result has significant implications. While previous studies have struggled to pinpoint the

135 specific developmental conditions that trigger migration in monarch butterflies (24, 27), our

136 findings demonstrate that gradual decreases in day length and temperature, mimicking natural

137 seasonal variation, are sufficient to induce southern flight orientation (39). This highlights the

138 critical role of environmental cues in initiating migratory behavior.

139   For the autumn lab line, southern directional orientation in the balloon simulator was marginally

140   significant (σ = 200°, n = 21, r = 0.09, Rayleigh test, P = 0.047, Fig 2B). Thus, evidence for

141   southern orientation in this group is limited, likely due to prolonged captive rearing. This

142   diminished orientation behavior, coupled with reduced immune function and survival rates

143   observed in lab populations relative to field populations, may reflect a decline in migratory traits

144   over time.

145   We further examined orientation behavior using ground-level simulators with autumn growth

146   chamber and autumn lab line populations, both of which displayed no significant southern

147   orientation (autumn growth chamber: ground level open-air: σ = 70°, n = 8, r = 0.14, Rayleigh

148   test, P = 0.29; ground level with wind block: σ = 58°, n = 21, r = 0.14, Rayleigh test, P = 0.092,

149   Fig 2A; autumn lab line: ground level open-air: σ = 183°, n = 14, r = 0.12, Rayleigh test, P = 0.18;

150   ground level with wind block: σ = 1°, n = 17, r = 0.26, Rayleigh test, P = 0.008, Fig 2B).

151   Consistent with our results with the autumn field generation, these results demonstrate that

152   butterflies do not exhibit migratory orientation at ground level.

153   Although our study demonstrated that monarchs did not exhibit migratory orientation in the

154   ground-level simulators we tested, it is important to acknowledge that prior research using the

155   traditional fixed-tether monarch flight simulator—a ground-level device enclosed in an opaque

156   cylinder—has shown southern orientation (40, 41). The traditional simulator differs in key ways

157   from the ground-level simulators in our study, which lacked full enclosure and used a free tether

158   design, potentially accounting for the differing results. Notably, the balloon flight simulator

159   employed here had higher participation rates (almost 100%) compared to both the ground-level

160   simulators and the traditional fixed-tether flight simulator, indicating its utility as an improved

161   method for assessing orientation behavior. This enhanced participation, combined with the robust

162   southern orientation observed in the balloon method, highlights its value for studying insect

163   migratory behaviors under natural conditions.

164   Reproductive status and wing size, both migration-linked traits, were assessed after orientation

165   behavioral tests for all groups (summer lab line, summer field, autumn growth chamber, autumn

166   lab line, autumn field). All females from the summer lab line and summer field groups were

167   reproductively mature (summer field: n = 28, summer lab line: n = 7, Fig. 3A), with a mean of 90

168   or more mature oocytes per female (summer field: n = 28, 93.4 ± 9.8 SE; summer lab line: n = 7,

169   148 ± 24 SE, Fig. 3B). In contrast, fewer than 20% of females from the autumn lab line and

170   autumn field groups were reproductively mature (autumn lab line: n = 64, mature ratio = 0.17 ±

171   0.048 SE; autumn field: n = 41, mature ratio = 0.12 ± 0.052 SE, Fig. 3A). Additionally, mature

172   oocyte counts were significantly lower in these groups than in summer butterflies (autumn lab

173   line: n = 64, 5.56 ± 2 SE oocytes; autumn field: n = 41, 1.44 ± 0.76 SE, Mann–Whitney U test, P <

174   0.001, Fig 3B), consistent with previous observations that autumn generation, migratory

175   monarchs are in reproductive diapause (25, 43).

176   Interestingly, butterflies reared in the autumn growth chamber treatment exhibited intermediate

177   reproductive traits compared to summer and autumn field females. Although these butterflies had

178   a higher reproductive maturity ratio than autumn field butterflies (autumn growth chamber: n = 39,

179   mature ratio = 0.85 ± 0.059 SE; autumn field: n = 41, mature ratio = 0.12 ± 0.052 SE, Mann–

180   Whitney U test, P < 0.001, Fig. 3A), they stored fewer oocytes than summer females (autumn

181   growth chamber: n = 39, 31.3 ± 4.9 SE; summer field: n = 28, 93.4 ± 9.8 SE, Mann–Whitney U

182   test, P < 0.001, Fig. 3B). This pattern suggests that reductions in day length and temperature can

183   influence the induction of reproductive diapause. However, the differences between the autumn

184   growth chamber and autumn field butterflies indicate that some aspects of the natural autumn

185   environment that are not replicated in our growth chamber, such as exposure to the position of

186   the sun (42), may contribute to reproductive diapause.

187   We next examined forewing size across all butterfly groups, as larger forewings are typically

188   associated with migratory monarch populations, aiding in long-distance travel (43). Autumn-

189   generation butterflies raised in the field had significantly larger forewing sizes compared to

190   summer-generation individuals (summer field: n = 49, forewing area = 7.58 ± 0.12 cm²; autumn

191   field: n = 72, forewing area = 8.27 ± 0.12 cm², Mann–Whitney U test, P < 0.001, Fig. 3C),

192   consistent with their migratory behavior. Interestingly, butterflies from the summer generation

193   raised in the autumn-like growth chamber exhibited even larger forewing areas than those from

194   the autumn field generation (n = 26, forewing area = 8.97 ± 0.14 cm², Mann–Whitney U test, P <

195   0.001, Fig. 3C). However, the captive lab colony monarchs, regardless of season, displayed

196   smaller forewing sizes compared to wild-derived individuals (summer lab line: n = 7, forewing

197   area = 6.9 ± 0.3 cm², Mann–Whitney U test, P = 0.0013; autumn lab line: n = 114, forewing area

198   = 7.04 ± 0.056 cm², Mann–Whitney U test, P < 0.001, Fig. 3C). These results suggest that both

199   environmental and genetic factors influence forewing size, a key migration-related trait.

200   The reduced forewing size and orientation behavior observed in the lab-line butterflies are

201   consistent with trends documented in commercially bred monarchs (24), which may have been

202   raised in captivity for decades. Commercial populations show smaller wings than wild-derived

203   monarchs and fail to orient south in the autumn, yet they retain phenotypic plasticity related to

204   reproductive diapause. The similarities between these commercial monarchs and our lab line,

205   which has been reared in captivity since 2016/2017, provide insight into the temporal dynamics of

206   migration trait loss under artificial rearing conditions. Furthermore, these trends in captivity

207 parallel the natural loss of migratory behavior in monarch populations around the globe that

208 historically dispersed out of North America (44). Non-migratory populations often exhibit smaller

209 wings while retaining seasonal plasticity in female reproduction (45, 46) (though their directional

210 flight orientation behavior remains unknown). These parallels suggest that evolution in long-term

211 lab and commercial populations may reflect, to some extent, the dynamics of natural migration

212 loss in monarchs. Our results demonstrate that migration-related traits such as orientation

213 behavior and wing size can evolve very rapidly after a population transitions from migratory to

214 sedentary.

215 Given that two of our flight simulators were open-air, we examined how butterflies interacted with

216 wind during their flights. Fine-scale wind data were recorded during each experiment, though a

217 sensor failure limited data collection for the ground-level open-air simulator. To assess the

218 relationship between flight and wind, we compared the difference between each butterfly's flight

219 direction and wind direction. A smaller difference indicated alignment with the wind, while a larger

220 difference indicated upwind flight. Among the autumn growth chamber butterflies tested in the

221 ground-level simulator, the direction differences were uniformly distributed, indicating no

222 significant relationship between flight orientation and wind (skewness = 0.077, n = 8, KS test, P =

223 0.55; Fig. 4A). In contrast, in the balloon-based simulators, the distributions of direction

224 differences were negatively skewed across all treatment groups. This pattern revealed that most

225 butterflies orienting southward in the balloon simulators flew against the wind (autumn lab line:

226 skewness = -0.561, n = 21, KS test, P < 0.001; autumn field generation: skewness = -0.733, n =

227 20, KS test, P < 0.001; autumn growth chamber: skewness = -1.53, n = 23, KS test, P < 0.001;

228 Fig. 4B).

229 Upwind flight is unusual for migratory animals, particularly monarch butterflies, which are

230 generally thought to rely on tailwinds for long-distance travel (34). Furthermore, we observed no

231 significant difference in wind interaction patterns between non-migratory summer field and

232 migratory autumn field generations (summer field: n = 18; autumn field: n = 20, two-sample t-test,

233 P = 0.42, Fig. S2). However, when wind direction and speed were explicitly incorporated into the

234 analysis of butterfly flights in the balloon simulator, no significant directional wind pattern was

235 detected for either the summer or autumn field generations (summer field: σ = 342°, n = 18, r =

236 0.46, Rayleigh test, P = 0.22; autumn field: σ = 0.5°, n = 20, r = 0.49, Rayleigh test, P = 0.16; Fig.

237 S3A). These results suggest that monarchs in the balloon simulator actively selected their flight

238 direction independent of wind conditions, reinforcing the idea that their orientation behavior is not

239 dictated by wind direction.

240   In contrast, significant northward wind patterns were recorded during experiments with the

241   autumn growth chamber and autumn lab line butterflies (autumn growth chamber: $\sigma = 2°$, n = 23,

242   r = 1.1, Rayleigh test, P < 0.001; autumn lab line: $\sigma = 341°$, n = 21, r = 0.72, Rayleigh test, P =

243   0.0028; Fig. S3B). Long-term wind data from the European Centre for Medium-Range Weather

244   Forecasts (ECMWF) corroborated these findings, revealing that prevailing winds in the Midwest

245   during September—the peak migration period—blow predominantly from the south (Fig. S4). This

246   aligns with previous studies reporting northward winds at migration starting points in southern

247   Canada and the northern United States (47). These data suggest that migratory monarchs, like

248   those in our balloon experiments, may initially fly upwind to orient themselves or to initiate

249   migration. Such upwind behavior could serve as a mechanism for navigation, with prevailing wind

250   patterns acting as important directional cues during the early stages of migration.

251   These findings raise intriguing questions about how monarchs detect and eventually exploit

252   favorable winds to sustain their southward journey. Understanding the mechanisms by which

253   migratory butterflies adjust their flight strategies in response to wind direction and strength could

254   yield valuable insights into their navigation and energy conservation strategies. Further research

255   is needed to explore how monarchs compensate for adverse winds and maintain their migratory

256   trajectory en route to overwintering sites.

257   In summary, our findings highlight the complex interplay between environmental cues,

258   physiological traits, and flight strategies in shaping monarch butterfly migration. By employing

259   innovative flight simulator methods, we demonstrated that high-altitude flight conditions elicit

260   strong migratory orientation, suggesting a critical role for visual and wind-related cues at these

261   altitudes. Our results also underscore the influence of developmental conditions on migratory

262   behavior and associated traits, such as forewing size and reproductive diapause, while

263   emphasizing the rapidity with which migratory characteristics are lost in sedentary populations.

264   These insights not only advance our understanding of monarch migration but also provide a

265   framework for investigating broader questions about the ecological and evolutionary factors

266   driving animal migration (48). Future research should focus on unraveling the mechanisms

267   underlying wind cue detection, the physiological adaptations enabling energy-efficient flight, and

268   the potential genetic basis of migration-related traits. Such efforts will be pivotal for informing

269   conservation strategies as migratory species face increasing environmental challenges (5).

270   **Materials and Methods**
271

272   **Animal Husbandry**

273   We collected monarch butterfly caterpillars from the field near Chicago in early June 2023 and

274   housed them in 30.5 cm³ mesh pop-up cages with access to their host plant, *Asclepias syriaca*

275   (common milkweed). To prevent *Ophryocystis elektroscirrha* (OE) infection, we collected fresh

276   milkweed daily and cleaned the plants with a 1:19 bleach solution. Once adults eclosed, we

277   screened each individual for OE spores using adhesive tape on their abdomens, and any infected

278   butterflies were immediately frozen. OE-free butterflies were housed in medium-sized (91.5 cm ×

279   30.5 cm²) mesh cages, separated by sex. This group constituted the "wild-caught" generation.

280   To produce the first captive-bred generation, we hand-paired males and females from the wild-

281   caught generation. Mated females were placed individually in medium-sized cages with a host

282   plant for egg-laying. Half of the eggs from each family were placed in an environmental chamber

283   that simulated autumn conditions, including decreasing day length and temperature (see details

284   below), to produce the "autumn growth chamber" treatment. The remaining eggs were reared

285   outdoors at a garden on the University of Chicago campus, under natural summer conditions,

286   generating the "summer field generation."

287   Butterflies from the outdoor-reared summer generation were then hand-paired to produce the

288   "autumn field generation," which was reared outdoors at the University of Chicago's Warren

289   Woods Ecological Field Station. Throughout the process, all adult butterflies were individually

290   labeled and fed Birds Choice butterfly nectar twice daily.

291   **Autumn-like Environmental Chamber**

292   To replicate autumn-like environmental conditions, we recorded daily temperatures at the field

293   station from August 5 to October 1, 2022, using a temperature sensor. Daytime and nighttime

294   temperatures were calculated as averages for their respective periods. These data informed the

295   settings of a growth chamber from July 5 to August 31, 2023, which simulated the recorded

296   temperatures and day lengths from the 2022 field measurements. After October 1, 2022, the

297   average temperature dropped below 10 °C, a threshold that could negatively affect butterfly

298   survival or behavior. To address this, starting September 1, 2023, we adjusted the chamber to

299   maintain autumn-like conditions conducive to butterfly survival. The adjusted daytime and

300   nighttime temperatures were set to 20.5 °C and 15.7 °C, respectively, reflecting the September

301   2022 monthly averages, while day length continued to decrease incrementally to mimic the

302   natural seasonal transition. The growth chamber used dual fluorescent lamps with fixed on/off

303   timing that matched the programmed light schedule. These settings provided a controlled

304   environment closely approximating the autumn conditions monarchs experience in the wild.

305   **Experiments with monarch lab lines**

306   We conducted experiments using two separate lab colonies of monarch butterflies that have been
307   maintained at Iowa State University since 2016 and 2017, respectively. From 2016/2017 to 2021,
308   both colonies were reared in growth chambers set at 26.6°C with a 16-hour photoperiod. After
309   2021, the colonies were moved to classrooms without windows for educational purposes, where
310   the butterflies were reared at ambient room temperature and artificial light conditions (15h dark:
311   9 h light). In July 2023, we received batches of eggs from both colonies, which were raised
312   outdoors as separate families at our experimental location in Chicago. We used the same rearing
313   protocol as for wild-caught caterpillars, feeding the caterpillars freshly cut and bleached *Asclepias*
314   *syriaca* (common milkweed) daily. The adults from these caterpillars were combined to form the
315   "summer lab line" experimental population.

316   Unfortunately, many individuals from the summer lab line died before flight tests could be
317   conducted, preventing us from collecting orientation behavior data from this group. A small
318   number of surviving adults were paired to generate an autumn generation, which was
319   supplemented by a second batch of eggs from the 2016 and 2017 colonies, received in August
320   2023. The adults that emerged from this second round of caterpillars were combined with
321   offspring from the summer lab line generation to form the "autumn lab line" population.

322   **Flight Simulator Methods**

323   We used three different flight simulators to assess the directional flight orientation of individual
324   butterflies under varying wind and altitude conditions. To ensure secure tethering, we first
325   removed hair from the thorax of each butterfly using transparent tape, then applied a bandage to
326   make the surface rougher for better attachment (49). A loose fishing line, attached to the bandage
327   with UV glue, provided stability and allowed for more natural flight while also increasing survival
328   rates. Butterflies were kept in mesh cages for at least one day to recover from the tethering
329   process. Prior to testing, all butterflies were placed outdoors for at least one hour.

330   The first simulator method, the "ground level open air," involved tethering the butterfly with a
331   fishing line to a horizontal rope aligned with true north in an open area without any wind
332   obstruction. The second method, the "ground level wind block," was similar but included a large,
333   30 cm diameter transparent PVC cylinder to block wind, ensuring that the butterfly could fly
334   without wind interference. A GoPro camera placed on a table directly beneath the butterfly
335   recorded flight duration and direction for both methods.

336   For the "balloon method," each butterfly was tethered with a fishing line beneath a 2.1-meter
337   balloon, which was released to a height of 30 meters. This setup allowed the butterfly to fly at a
338   high altitude while interacting with natural wind conditions (50). A GoPro camera and an LI-550F

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339 TriSonica weather sensor, positioned below the butterfly, recorded flight behavior alongside

340 environmental variables, including wind speed, direction, temperature, and air pressure. A

341 Raspberry Pi 4 connected to the weather sensor served as a data logger for weather metrics.

342 Each test lasted 10 minutes and 30 seconds; however, if a butterfly stopped flying for more than

343 15 seconds, the test was terminated. To ensure accurate analysis, the first 30 seconds of each

344 video were excluded, allowing the butterfly time to ascend and stabilize its flight. All experiments

345 were conducted on sunny days between early September and October 2023 at the Warren

346 Woods Ecological Field Station, a well-preserved natural site with minimal human disturbance.

347 **Flight Orientation Data Analysis**

348 We used DeepLabCut version 2.2, a ResNET-50-based network, to train a tracking model to

349 analyze butterfly heading directions. Five body parts—head, thorax, right forewing, left forewing,

350 and abdomen tip—were labeled in the training videos. The output of the DeepLabCut model

351 provided the coordinates of the five labeled body parts for each frame. To calculate the butterfly's

352 heading direction per second during the balloon flight simulator trials, we sampled every 60th

353 frame, corresponding to the 60 fps GoPro framerate. For the two ground-level flight simulators,

354 we sampled every 60th or 30th frame depending on the GoPro's framerate. Only the head and

355 thorax coordinates were used to determine the butterfly's heading direction by subtracting the

356 thorax coordinates from the head coordinates to create the flight vector.

357 For the two ground-level flight simulators, the butterfly was tethered to a white line oriented

358 toward true north, which served as the reference for the north vector. The butterfly's heading

359 direction was determined by measuring the angle between its flight vector and the north vector. In

360 the balloon flight simulator, heading direction was recorded using the LI-550F TriSonica weather

361 station attached to the balloon, as the balloon could rotate while floating in the sky. A GoPro

362 camera, aligned with the weather station's reference direction, captured the butterfly's flight

363 vector. To calculate the butterfly's heading direction, we first measured the angle between its

364 flight vector and the long axis of the GoPro's field of view (represented as the vector (0,1)). This

365 angle was then added to the heading direction recorded by the TriSonica at the corresponding

366 time frame, yielding the butterfly's final heading direction.

367 To calculate the average heading direction for each trial, the butterfly's heading was recorded

368 every 10 seconds. These angular data (in degrees) were converted into Cartesian coordinates

369 using the "Circular" package in RStudio 1.3.959. The mean heading direction (0–359°) and flight

370 consistency (r = 0–1, where higher values indicate greater consistency) were then computed for

371 each butterfly. To determine whether butterflies within each group exhibited significant directional

372   flight, we weighted each individual's mean flight direction by its consistency score and conducted

373   a Rayleigh test using the "astropy.stats.rayleightest" package in Python.

Ovary and Wing Analyses

375   To assess reproductive status, we froze all butterflies post-experiment and dissected female

376   ovaries. The abdomens of the female butterflies were removed, and a longitudinal cut was made

377   to open the abdomen. We classified an oocyte as mature if it had surface ridges typical of those

378   about to be laid. We counted the number of mature oocytes inside the abdomen, and individuals

379   with more than one mature oocyte were considered sexually mature. Those without were

380   classified as being in reproductive diapause.

381   After flight tests, we removed and analyzed the right forewing of each butterfly to measure its

382   area. Photographs of the wings were taken, and the images were converted to binary form. The

383   software ImageJ was then used to calculate wing area.

384   We compared physiological and morphological traits across different generations and treatment

385   groups using the Mann-Whitney U test. For reproductive traits, we assessed binary data

386   indicating whether individuals had reached maturity as well as the number of mature oocytes.

387   Wing variables were also analyzed using the Mann-Whitney U test to evaluate differences across

388   groups.

Wind Data Extraction

390   Real-time wind data were recorded during each flight experiment using two instruments: a

391   TriSonica weather station mounted on the balloon and a HOBO by Onset S-WCG-M003

392   Ultrasonic Wind Smart Sensor at ground level. Both sensors collected wind speed and direction

393   at one-second intervals throughout the flight tests. To analyze the butterflies' flight behavior in

394   relation to wind, we calculated the difference between the wind direction and the butterflies'

395   heading direction on a per-second basis. The distribution of these differences was plotted, and

396   skewness was calculated to assess wind alignment. A negatively skewed distribution (skewness

397   < 0) indicated upwind flight, while a positively skewed distribution (skewness > 0) suggested

398   downwind flight. To evaluate whether the data distribution deviated from uniformity, we applied

399   the Kolmogorov-Smirnov (KS) test. Additionally, to determine whether wind conditions during

400   each test were significantly directional, wind direction was weighted by wind speed and analyzed

401   using the Rayleigh test, following the same method applied to the butterflies' flight direction.

402 We also obtained long-term wind data using the ERA5-Land reanalysis dataset from the

403 European Centre for Medium-Range Weather Forecasts (ECMWF). To represent wind conditions

404 across the Midwest, we selected six locations: the university garden (Lat: 41.80, Long: -87.59)

405 and the field station (Lat: 41.84, Long: -86.62) where monarch butterflies were reared, along with

406 four additional locations reported in the JourneyNorth program for monarch migration

407 observations (Lat: 43.77, Long: -86.44; Lat: 41.57, Long: -88.49; Lat: 42.66, Long: -88.6; Lat:

408 41.56, Long: -87.51). Hourly wind speed and direction data for the month of September were

409 extracted for each of these locations.

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546 **Figures and Tables**

547
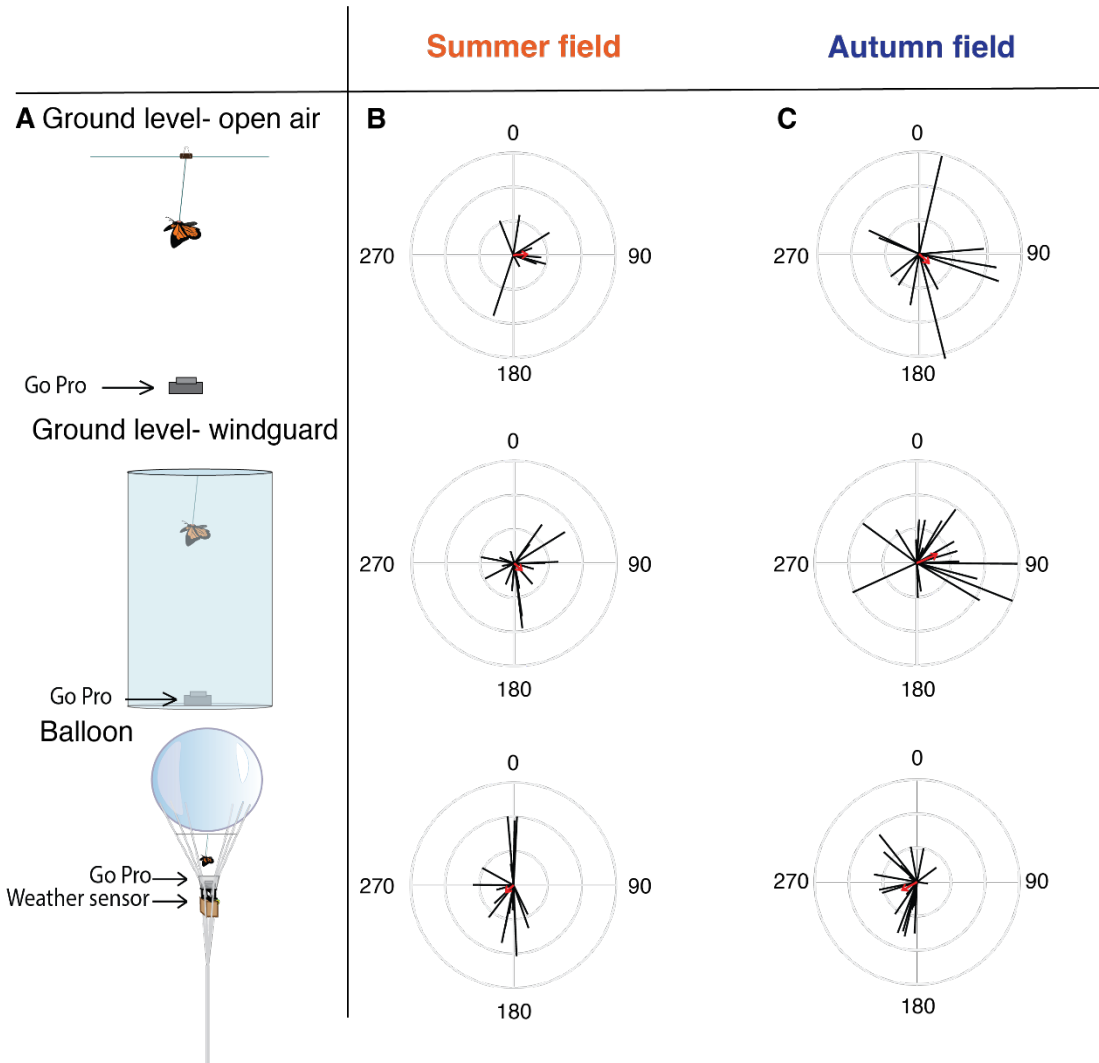
Figure 1. Monarch butterfly orientation behavior in field-reared populations across flight simulators. (A) Schematic diagram of the three flight simulators: two open-air ground-level simulators, one with a wind guard, and the balloon flight simulator. The GoPro camera beneath the butterfly records orientation behavior during flight. (B, C) Orientation plots for Eastern North American monarchs reared in the field, comparing the Summer (B, ground level open air: n = 10, Rayleigh test, P = 0.19; ground level with wind block: n = 19, Rayleigh test, P = 0.15; balloon: n = 18, Rayleigh test, P = 0.37) and Autumn generations (C, ground level open-air: n = 14, Rayleigh test, P = 0.4; ground level with wind block: n = 20, Rayleigh test, P = 0.017, balloon: n = 20, Rayleigh test, P = 0.017) across the three simulators. Each black line indicates the mean flight direction of an individual butterfly (0°–359°), with line length proportional to the strength of directional preference (0–1). The red arrow represents the mean group direction. Directions are plotted relative to 0° (north) and 180° (south). Autumn-generation butterflies oriented south only in the balloon flight simulator.

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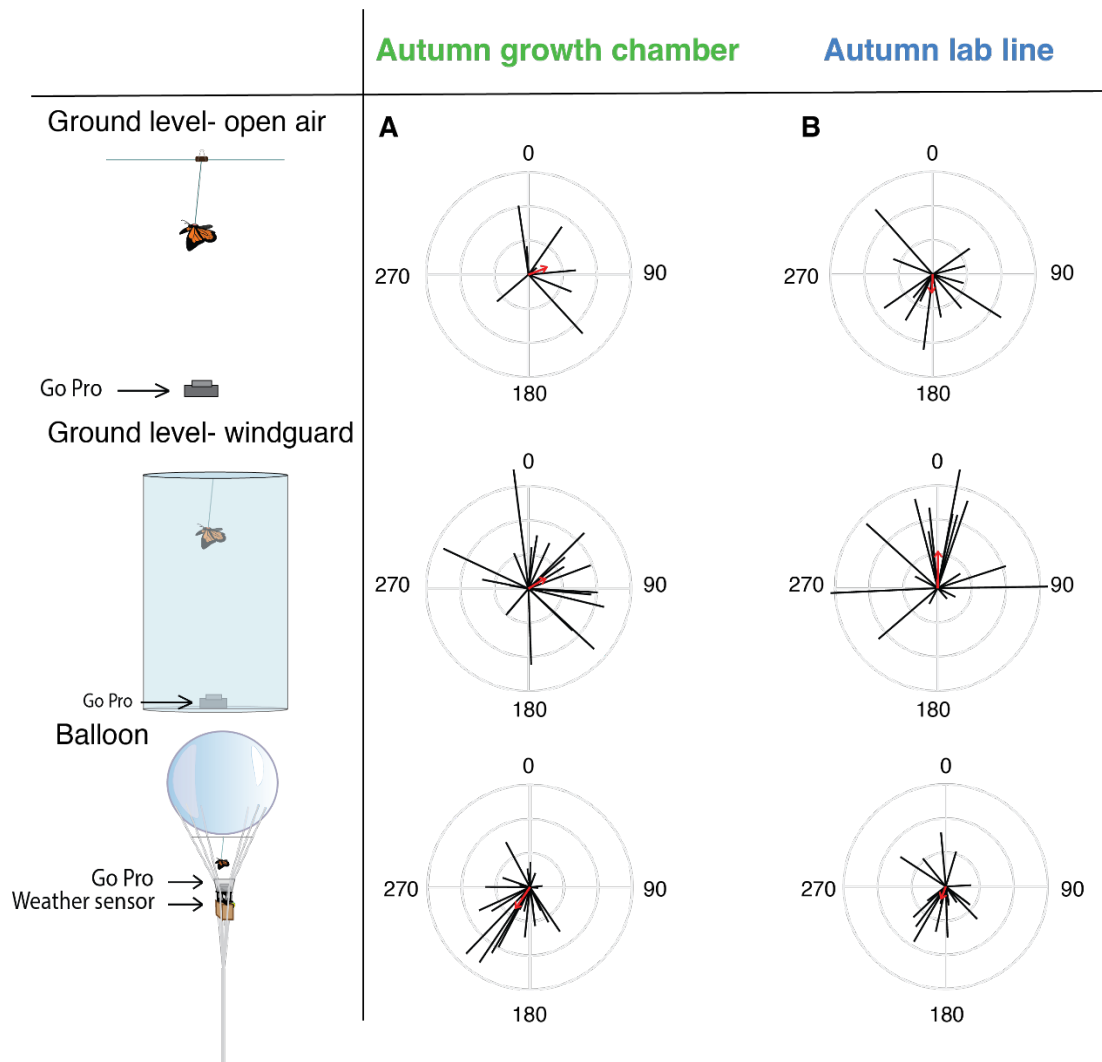
Figure 2. Orientation behavior of monarch butterflies reared in autumn-like conditions and the laboratory-maintained autumn generation. (A) Orientation plots of summer generation monarchs reared in a autumn-like growth chamber, tested across three flight simulators. Butterflies oriented south only in the balloon flight simulator, with no significant southern orientation in the ground-level simulators (ground level open-air: n = 8, Rayleigh test, P = 0.29; ground level with wind block: n = 21, Rayleigh test, P = 0.092; balloon: n = 23, Rayleigh test, P < 0.001). (B) Orientation plots of autumn lab line monarch butterflies, which showed only marginally significant southern orientation behavior in the balloon flight simulator (ground level open-air: n = 14, Rayleigh test, P = 0.18; ground level with wind block: n = 17, Rayleigh test, P = 0.008; balloon: n = 21, Rayleigh test, P = 0.047). Each black line represents the mean flight direction of an individual butterfly (0°–359°), with line length proportional to the strength of directional preference (0–1). The red arrow indicates the mean group direction. Orientation is plotted relative to 0° (north) and 180° (south).

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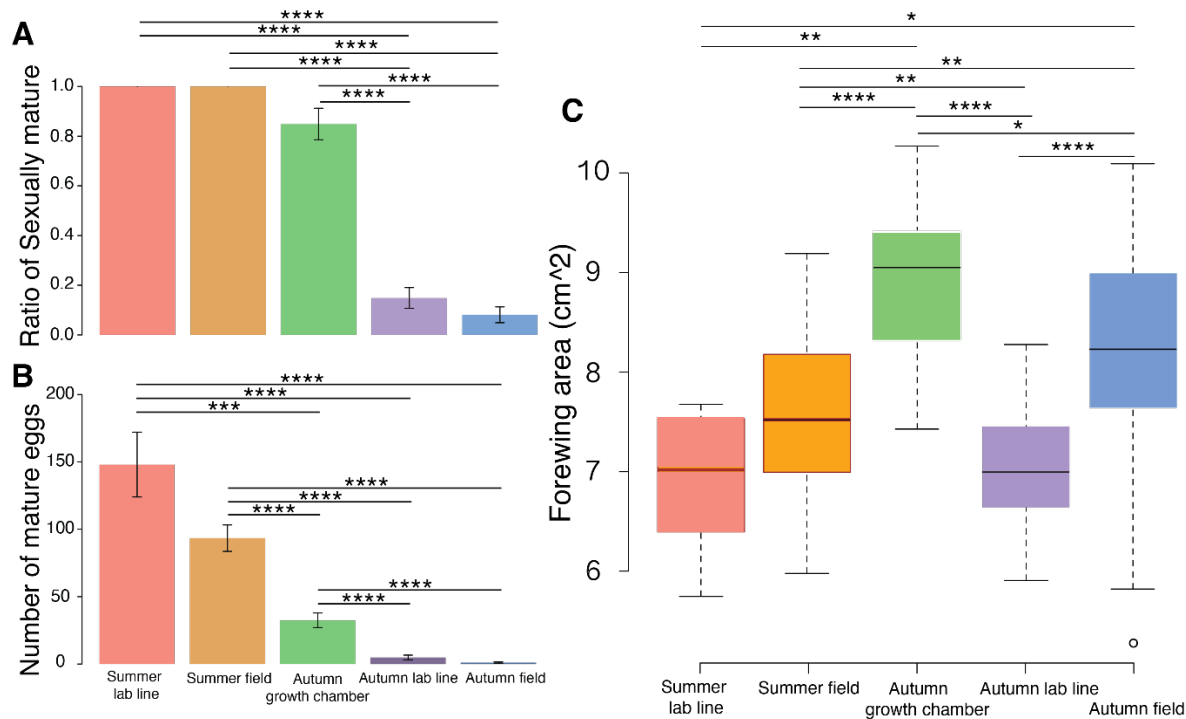
Figure 3. Reproductive status and forewing size differ among monarch butterfly treatment groups. (A) Proportion of sexually mature females (summer lab line: n=7; summer field: n = 28; autumn growth chamber: n=39; autumn lab line: n = 64; autumn field: n = 41). and (B) number of mature oocytes in the ovaries of females across five treatment groups: summer lab line, summer field, autumn growth chamber, autumn lab line, and autumn field generation (summer lab line: n=7; summer field: n = 28; autumn growth chamber: n=39; autumn lab line: n = 64; autumn field: n = 41). (C) Forewing area of butterflies across the five treatment groups, with autumn field and autumn growth chamber butterflies generally exhibiting larger forewing areas (summer lab line: n=7; summer field: n = 49; autumn growth chamber: n=26; autumn lab line: n = 114; autumn field: n = 72). The standard error was presented as the error bar. The number of asterisks indicates the level of statistical significance: *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

Difference between the butterfly and wind vector
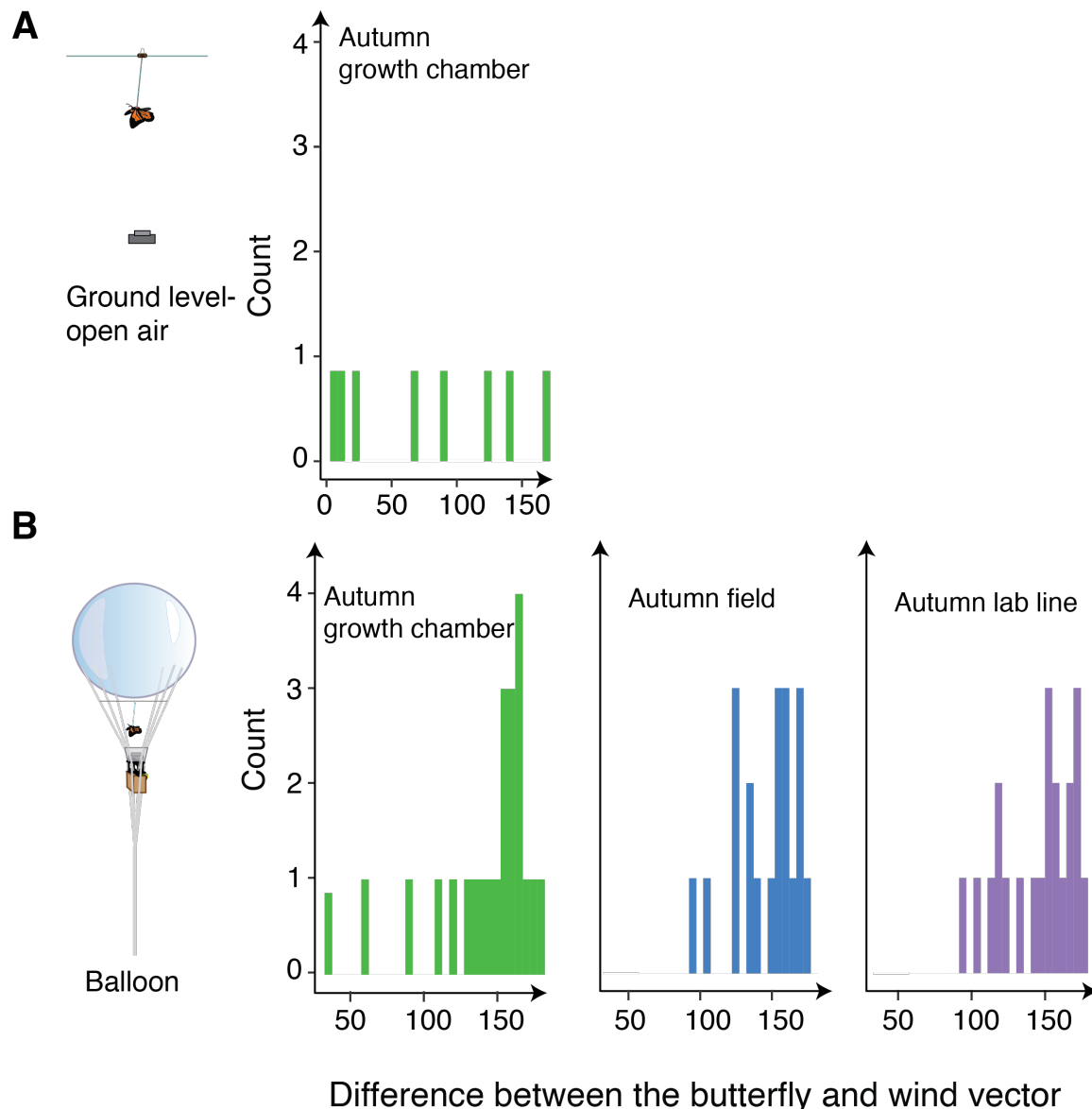
Figure 4. Interaction of migratory butterflies with wind across flight simulators.
(A) In the open-air flight simulator at ground level, migratory butterflies showed no significant interaction with wind direction. (n = 8, P = 0.55). (B) In the balloon flight simulator, migratory butterflies consistently oriented upwind, flying against the wind (autumn growth chamber: n = 23, P < 0.001; autumn field generation: n = 20, P < 0.001; autumn lab line: n = 21, P < 0.001). The interaction between butterfly and wind direction was quantified as the difference between the butterfly's mean heading and the wind direction during flight tests. Larger differences indicate more pronounced upwind flight, while smaller differences suggest alignment with the wind (tailwind).
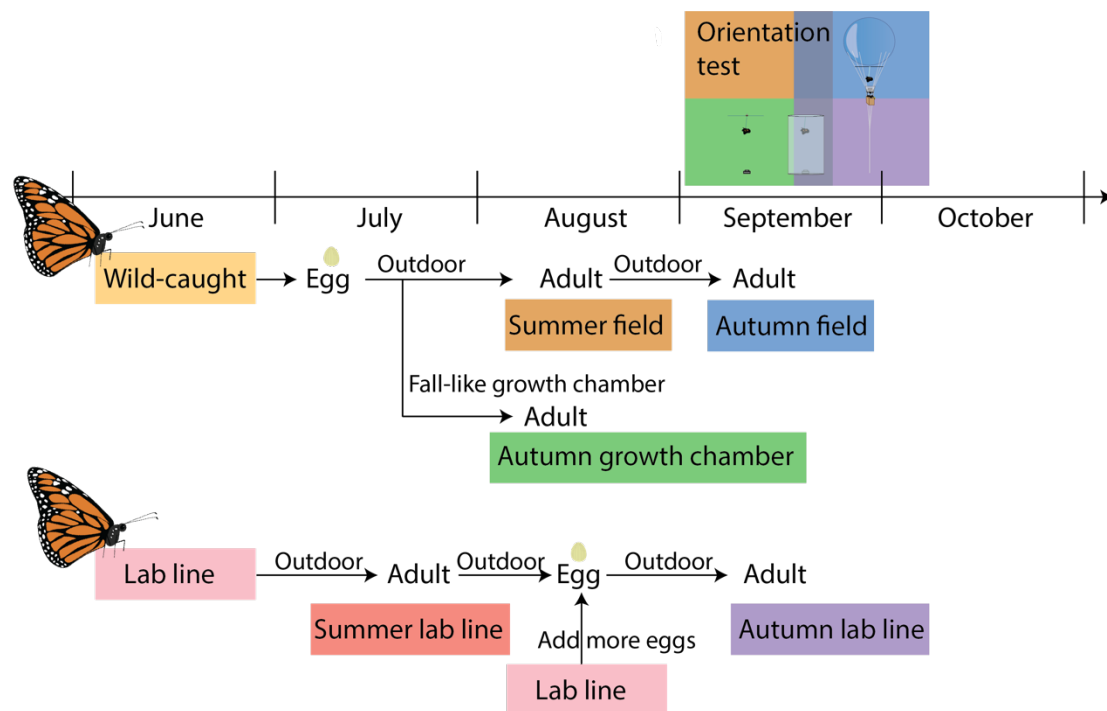
Supplementary Figure 1. Schematic of rearing protocols and flight experiment timeline.
Monarch butterflies were captured in the field in June and designated as the "wild-caught" generation. Eggs laid by wild-caught butterflies were split into two rearing conditions. Half were raised in an autumn-like growth chamber, and the resulting adults were classified as the "autumn growth chamber" group. The other half were reared outdoors, producing adults classified as the "summer field" group. Eggs laid by the summer field generation were reared outdoors, and their eclosed adults were classified as the "autumn field" group. Separately, monarch eggs obtained from a laboratory stock maintained at the Iowa State University since 2016/2017 were reared outdoors, with the resulting adults classified as the "summer lab line" group. Eggs laid by the summer lab line adults, along with a second batch of laboratory eggs, were also reared outdoors, producing adults classified as the "autumn lab line" group. Flight experiments were conducted on adults from the summer field and autumn growth chamber groups from early to mid-September, while adults from the autumn field and autumn lab line groups were tested from mid-September to early October.
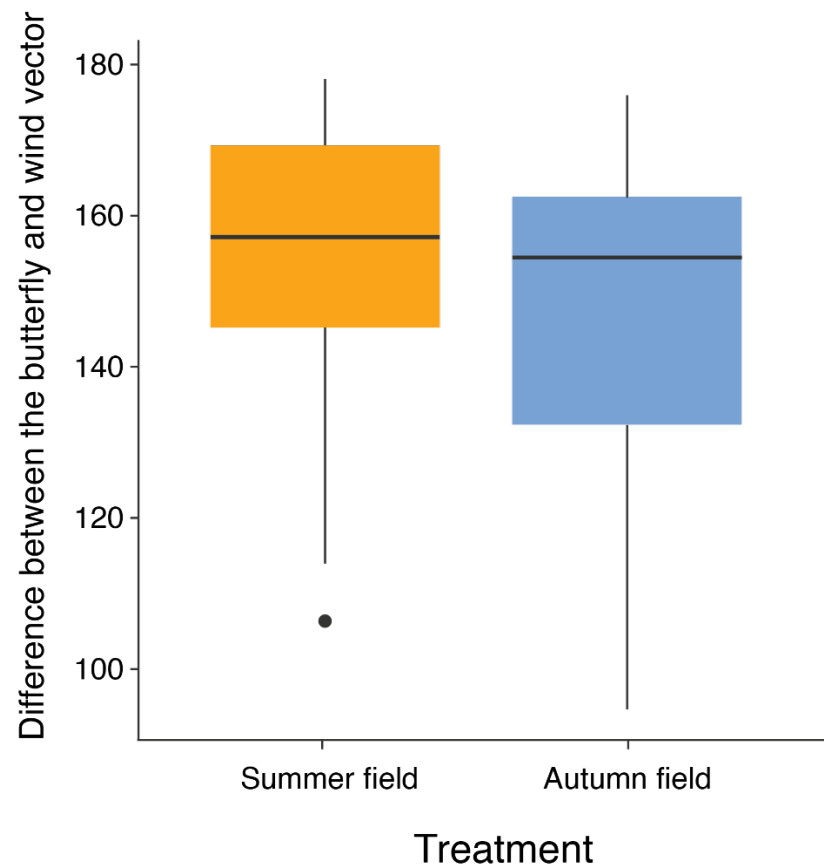
Supplementary Figure 2. Comparison of wind interaction between Summer field and Autumn field groups. The difference between wind direction and butterfly flight direction was not significantly different between the summer field group (n = 18) and the autumn field group (n = 20) (two-sample t-test, P = 0.42). Error bars represent standard error.

A   Summer field

Autumn field

B   Autumn growth chamber

Autumn lab line
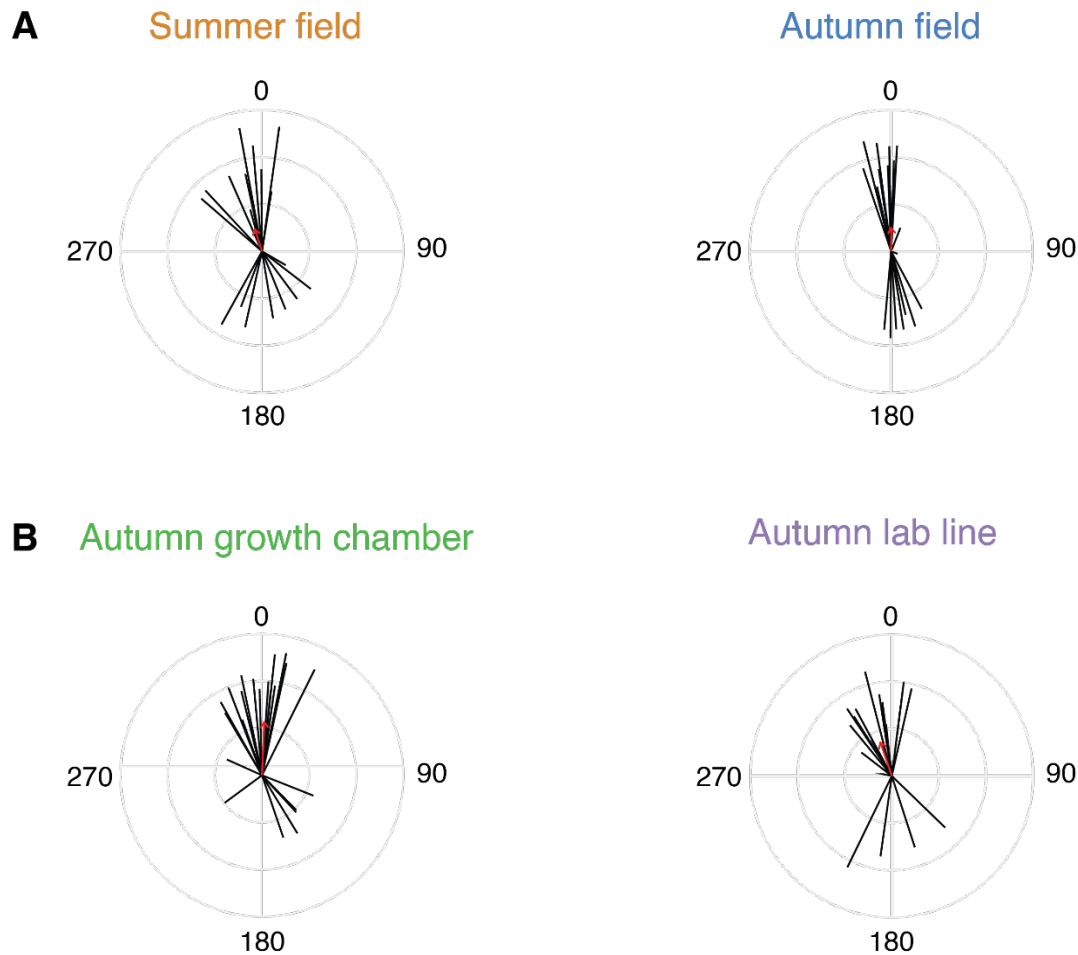
Supplementary Figure 3. Variability in wind patterns during flight tests across treatment groups. Circular plots illustrate wind conditions experienced by butterflies during flight tests for (A) summer and autumn field generation groups (summer field: n = 18, Rayleigh test, P = 0.22; autumn field: n = 20, Rayleigh test, P = 0.16). and (B) autumn growth chamber and autumn lab line groups (autumn growth chamber: n = 23, Rayleigh test, P < 0.001; autumn lab line: n = 21, Rayleigh test, P = 0.0028). Each black line represents the mean wind direction for an individual test (0° to 359°), with line length indicating wind speed. The red arrow denotes the overall mean wind direction across tests for each treatment. 0° represents north, and 180° represents south.

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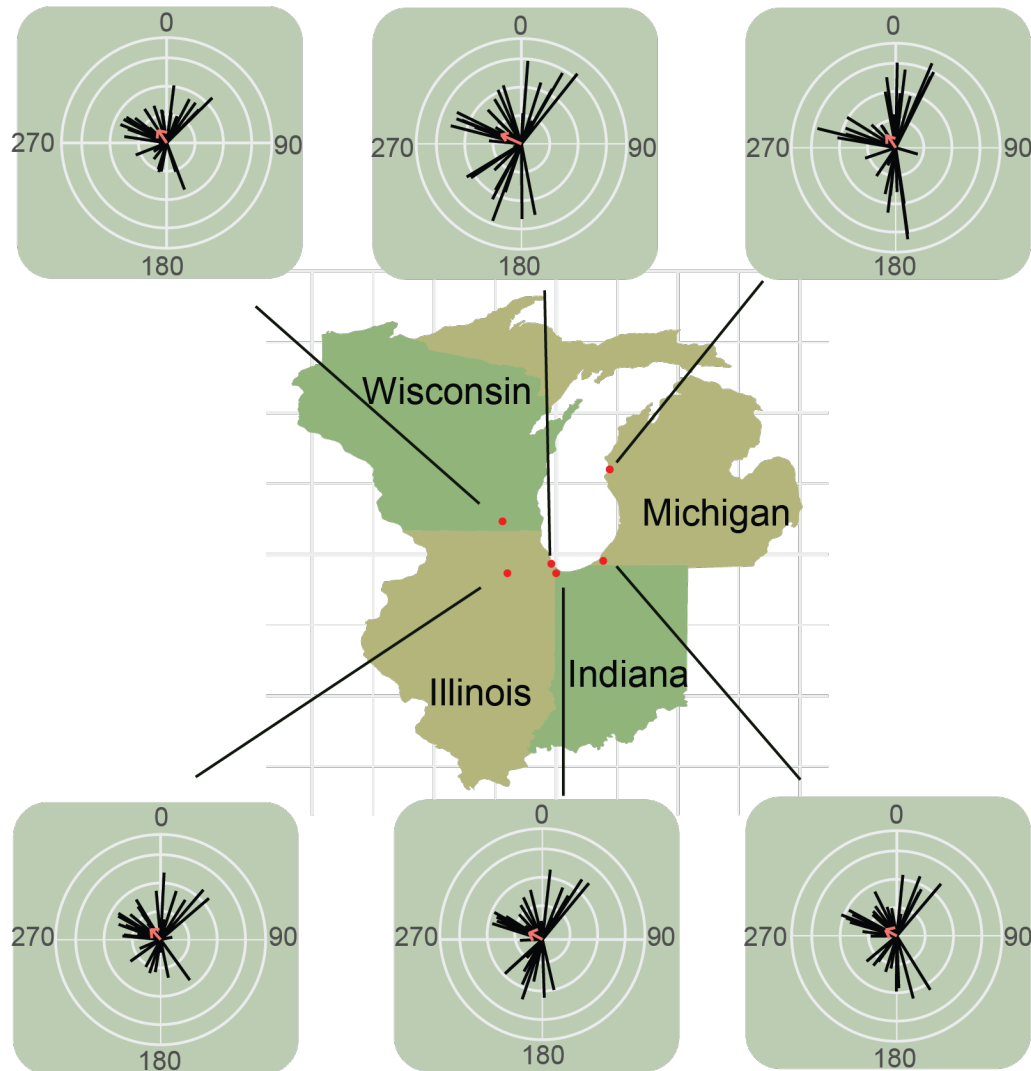
Supplementary Figure 4. Prevailing wind patterns in the Northern Midwest during September. Circular plots show daily average wind direction and speed across six locations in September. Each black line represents the mean wind direction for a single day (0° to 359°), with line length corresponding to wind speed. The red arrow indicates the overall mean wind direction for the month at each location. Wind predominantly blew to the north during this period.

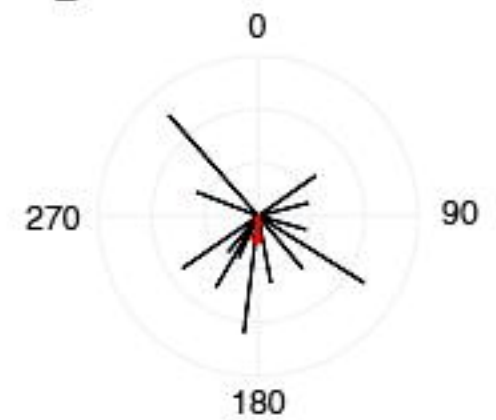**A** Ground level- open air　**B**　　　**C**

Go Pro →

Ground level- windguard

Go Pro →

Balloon
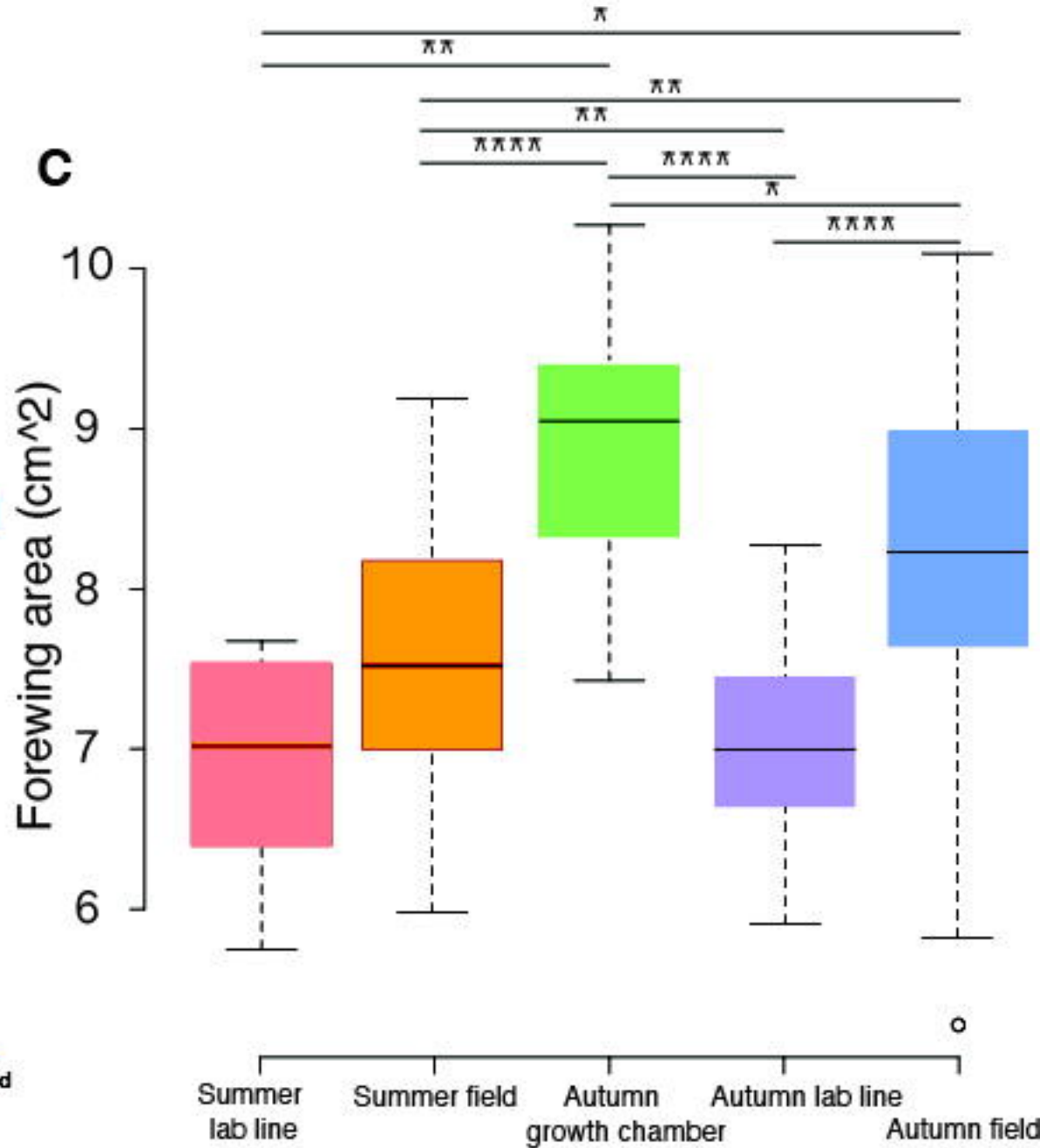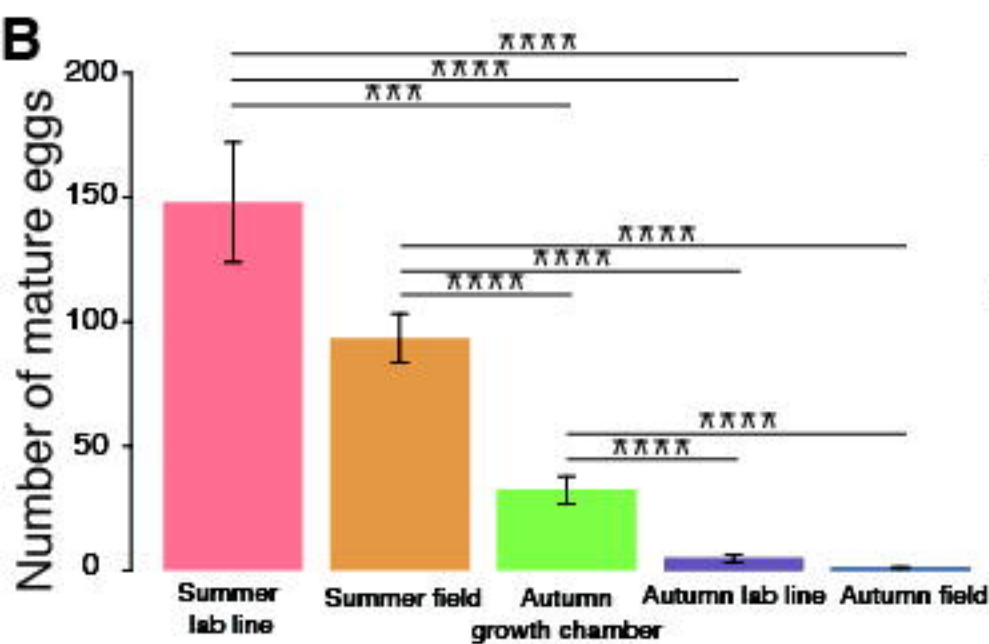
Go Pro →
Weather sensor →

Ground level- open air

Go Pro →

Ground level- windguard

Go Pro →

Balloon
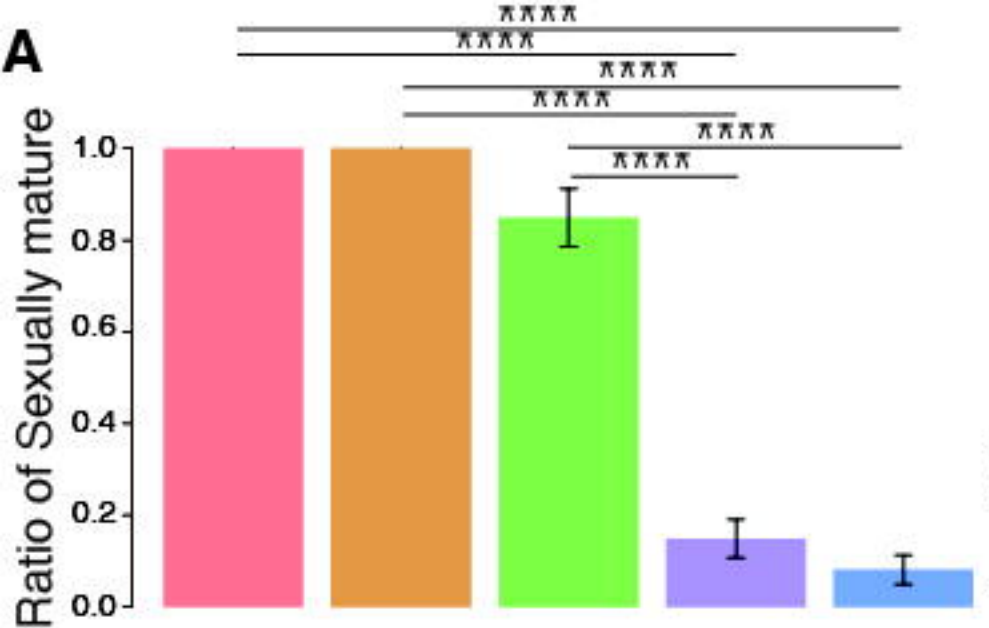
Go Pro →
Weather sensor →

Autumn growth chamber　　　**Autumn lab line**

A　　　　　　　　　　　　B

A

Ground level-open air

Autumn growth chamber

B

Balloon

Autumn growth chamber

Autumn field

Autumn lab line

Difference between the butterfly and wind vector