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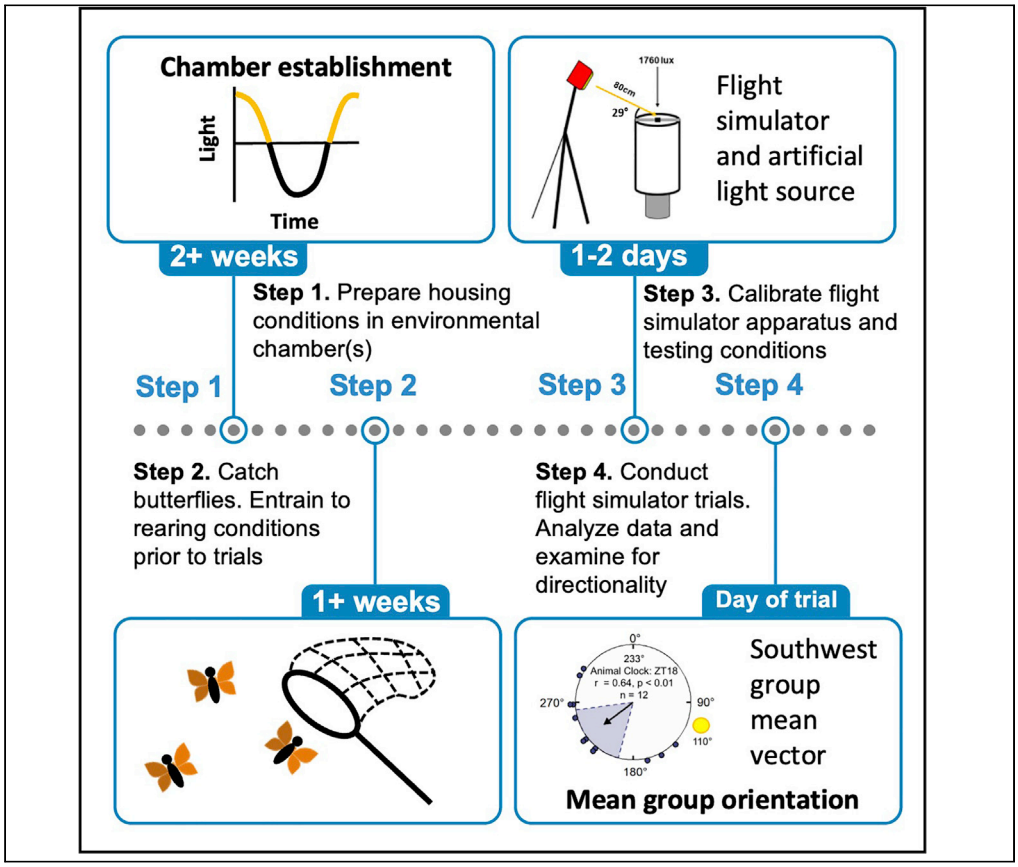
A behavioral assay to test sensory-cue-guided oriented flight in monarch butterflies under controlled conditions



Many animals use sensory cues to guide movement. Testing animals under conditions in which cues can be isolated and manipulated is key for understanding the function of cues. Here, we present a protocol to assess the flight of migratory monarch butterflies (*Danaus plexippus*). We describe procedures to optimize and conduct trials, especially under indoor conditions. This protocol facilitates testing monarchs in various experimental conditions including during their subjective night when they are not normally flying.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Protocol for indoor controlled behavioral flight assays with monarch butterflies

Adaptable to test experimental conditions that are difficult to perform in the field

Procedures to optimize and conduct indoor flight simulator trials

Can be modified to test other flying insects that are similar in size

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Protocol

A behavioral assay to test sensory-cue-guided oriented flight in monarch butterflies under controlled conditions

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SUMMARY

Many animals use sensory cues to guide movement. Testing animals under conditions in which cues can be isolated and manipulated is key for understanding the function of cues. Here, we present a protocol to assess the flight of migratory monarch butterflies (*Danaus plexippus*). We describe procedures to optimize and conduct trials, especially under indoor conditions. This protocol facilitates testing monarchs in various experimental conditions including during their subjective night when they are not normally flying.

For complete details on the use and execution of this protocol, please refer to Parlin et al. (2022).[1]

BEFORE YOU BEGIN

The protocol we describe below has been used to test the orientation behavior (e.g., time-compensated sun compass use during flight) of fall migratory monarch butterflies using a flight simulator under indoor, controlled conditions in response to different sensory cue conditions. This protocol, however, can be used to test the orientation behavior of flying monarchs from other seasonal generations, e.g., spring re-migrant, spring, and summer butterflies. Similarly, this protocol can be used to test the orientation behavior of other lepidopteran species (butterflies and moths), as well as be potentially modified to test other flying insects that are similar in size, e.g., bees and field crickets. When testing individuals from insect species outside of the Lepidoptera, other tethering techniques beyond those that we cite below[2] might have to be used, adapted, or developed.

Institutional permissions

If needed in your area or collection site, obtain the relevant permits that allow for the scientific collection and handling of live specimens prior to acquiring specimens in the field. The requirements and regulations for obtaining a permit can vary between localities and countries. In certain jurisdictions, the collection of protected species will entail stricter permits than for species that are non-regulated, e.g., fewer specimens can be collected if the species is protected. Although the research protocol we outline is non-invasive and permits the release of animals back into the wild after experiments, institutions can vary in their guidelines and regulatory standards for experimentation with live insects. Researchers should consult with their own institutions to determine if specific permissions and handling methods are necessary.

Preparing environmental chambers for housing animals

Timing: Minimum 2 weeks before the start of trials

1. Setup environmental chambers to the desired housing conditions for the animals during the course of the study.
   a. Program or setup environmental chambers to have the desired photoperiod (Light:Dark, L:D light cycle), temperature, and relative humidity conditions during housing.

⚠ CRITICAL: Place environmental chambers on emergency electrical power to prevent the chamber from shutting off in case of loss of power in the building.

Note: We use Percival environmental chambers (Percival model I36LLC8; Perry, IA, USA), but any other appropriate environmental chamber can be used for housing animals so long as the temperature is maintained within $\pm$ 1°C of the target test temperature or temperature range (e.g., cyclic temperature change).

Note: As an example, when we test the migratory flight behavior of fall monarch butterflies, we setup our environmental chambers to have fall-like conditions consistent with that of Cincinnati, OH that consist of a 12L:12D light cycle (for our trials, we have lights come on at 06:00 AM and turn off at 06:00 PM), cycling temperatures of 21°C during the day and 12°C at night, and 70% relative humidity.

Note: For our environmental chambers, it takes approximately 5 min to program them to the appropriate housing conditions. Times will vary for researchers, however, and are dependent on the type of environmental chamber that is used and the ease of programming the chamber.

2. Monitor the environmental chambers for at least one week to see if conditions are reliably stable, prior to placing animals inside of it.

Note: If possible, conditions in environmental chambers should be monitored and recorded electronically. For example, a wired (Ethernet cable) or non-wired (WiFi) connection can be used to connect the chamber to a suitable data-recording device, such as a laptop with the environmental recording software that accompanies the environmental chamber.

3. Periodically spot-check the conditions of the environmental chambers (e.g., once a week) by checking the readout of the settings as indicated by the control panel on the outside of the chamber or check the data collected by the data-recording device used.

Note: It takes us 5 min for each weekly check of each chamber in use for an experiment.

Obtaining animals from the field

Timing: At least 1 week before the start of trials

Timing: 4 min per butterfly captured (for step 6)

4. If needed, prepare insect nets for capturing animals.

Note: Our collapsible nets with interchangeable handles take less than 30 s to setup, but setup times will vary with the type or brand of net used.

Note: Nets should consist of soft netting with small mesh hole size (e.g., 1.2 mm), as this prevents animals from getting injured or stuck, when being caught and held in the insect net, respectively.

a. Prior to capture, inspect netting for large holes or tears (approximately 1 min per net).

Note: Specimens can escape through such holes, even after being caught. Similarly, specimens can enter through these holes but get stuck, increasing their chance of getting injured.

Note: Although holes in the mesh of the net can be repaired, e.g., with duct tape or similar tape, repair methods should avoid creating points in the net in which animals can get stuck and injured. For example, animals can get their tarsi, wings, or antennae stuck to the adhesive backing of tape if parts of the tape backing are exposed.

5. Capture monarch butterflies outdoors under natural conditions (wild-caught).

Note: We have identified locations in the Greater Cincinnati Area at which we can reliably collect wild monarch butterflies, e.g., during the summer and fall field seasons. You will need to find areas at which monarchs can be found at your location. If you are having issues with seeing monarchs, you should consult with members of local or state wildlife, conservation, entomology, or naturalist organizations for advice on where to look for monarchs in your specific area.

6. House each butterfly in its own housing immediately upon capture, e.g., each butterfly in its own glassine envelope.
   a. After placing the captured butterfly in its own glassine envelope, record the day, date, time, and location of capture of the butterfly on the glassine envelope.
   b. Record the sex of the captured animal on the glassine envelope.
   c. Record other relevant information, e.g., weather conditions during capture and person that captured the butterfly, either in a field book, lab notebook, or electronically.
   d. Attach an individualized or unique alar tag to one of the hind wings of each butterfly captured to facilitate identification.

Note: We use glassine envelopes to house butterflies individually, but any other container appropriate to individuals of a species can be used as well.

Note: We prefer to immediately house individual monarchs each in its own glassine envelope, rather than housing in a communal cage, during collection days. Animal interactions, such as fighting or mating, can occur while housed in the communal cage, which can affect the status of animals prior to their testing in trials.

Note: Although animals are constrained in glassine envelopes, our own previous work[1,2] and those of other researchers[3,4] have found no negative effect on monarch behavior. Housing and storing butterflies in glassine envelopes during experimental studies involving monarch behavior has been a common and long-standing practice in the literature,[5,6] with some researchers using glassine envelopes since the late 1970s.[7] For example, we have kept fall migrant monarchs in glassine envelopes under laboratory conditions, from the time they were caught in the fall to the time they were tested in flight simulator trials in the following spring, and monarchs exhibited normal migratory flight behavior when tested.[8]

Note: Suitable alar tags can be purchased from Monarch Watch (https://www.monarchwatch.org), with tags part of their annual community science outreach program for monitoring the

yearly fall monarch migration in North America. Additionally, writing directly on the hind wings with a permanent marker is also suitable.

7. After capture from the field, place animals in the appropriate environmental chamber for trials for at least one week.

Note: This one-week long period allows animals to be acclimated and entrained to the desired conditions for trials. This one-week long period also ensures that animals are standardized to the same conditions prior to the start of testing.

Maintaining animals captured from the field

Timing: throughout the course of an experiment

In this part of the protocol, we describe how to feed animals while housed under controlled conditions. We describe in the following sections, 1) how to make the food for the butterflies, 2) how to feed each butterfly individually, and 3) an alternative method of feeding butterflies communally.

Section 1: How to make butterfly food

Timing: 2 min

8. Create a 25% honey solution that will be the food for the monarchs.
   a. Place 12.5 mL honey in a Falcon tube and dilute with tap water by filling the tube with water until 50 mL.
   b. Shake the Falcon tube until the honey has become a diluted solution with the water.

Note: We use 25% honey solution, but any other appropriate food for the butterflies can be used as well, if questions about diet composition and caloric intake are of interest to the researcher.

Note: We make fresh 25% honey solution for each feeding day, but any excess food can be stored in the refrigerator; however, if food is left out for an extended period of time (e.g., days), discard and make a new batch.

Section 2: How to feed butterflies individually

Timing: 1–2 min (for step 9)

Timing: 5–10 min per individual butterfly (frequency of feeding: 3 times per week) (for step 10)

9. Clean the surface, e.g., table or counter, upon which butterflies will be fed.
   a. Wipe down the area with 10% bleach solution and let dry.
   b. Wipe down the area with Alconox solution and let dry.
   c. Wipe down the area with ethanol (70% minimum) and let dry
   d. Wipe down the area with water and wipe dry using a paper towel.
   e. Wipe down the area with ethanol (70% minimum) and let dry.

⚠ CRITICAL: A clean surface is required in order to allow deposited honey solution to form a droplet from which a butterfly feeds.

10. Feed each butterfly individually by hand.

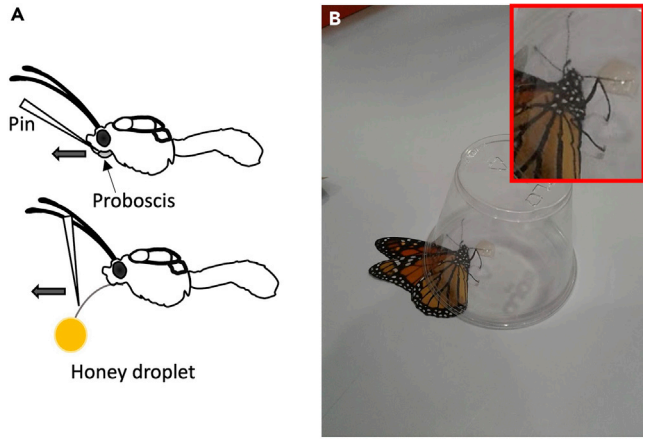

Figure 1. Demonstration of proboscis extension for feeding individual butterflies

(A) Cartoon elaboration on use of pin to extend butterfly proboscis into honey droplet. The pin should be inserted into the middle of the curled proboscis and gently extended towards the honey droplet. It is recommended to hold the proboscis in place until the butterfly stops moving and begins feeding.

(B) Photograph of monarch butterfly underneath a plastic cup with the proboscis. Red inset photograph shows a zoomed in view of the proboscis in the honey droplet on the table.

a. Deposit a drop of honey solution onto the clean surface using a pipette (e.g., 25 mL with pipette pump).
b. Remove a butterfly from its glassine envelope by hand by holding the butterfly by its wings (both forewings and hindwings).

Note: We usually use a "pinch" method in which we hold the wings of the butterfly by the thumb on one side and the index and middle fingers on the other.

c. Place the butterfly on its side on the table.
d. Gently extend the butterfly's proboscis using a pin and place the proboscis into the drop of honey solution (Figure 1A).

Note: See troubleshooting – problem 1 on how to address monarchs that do not want to feed.

e. Restrain the butterfly on its side during feeding by positioning a small plastic cup on its wings (Figure 1B).

Note: Restrain the butterfly to prevent it from escaping while feeding (see troubleshooting – problem 2 on how to keep monarchs from escaping this feeding position). When feeding, butterflies will remain quiescent, keeping their proboscis in the honey solution.

Note: Position the plastic cup on the butterfly such that it will not be able to move so much that body parts other than the proboscis, e.g., tarsi or wings, come into contact with the honey solution. Contact with the honey solution can injure (e.g., tarsi become sticky and adhere to surfaces) or degrade (e.g., lost of scales on wings) body parts over time.

f. Monitor the butterfly during feeding to see when it has finished.

Note: Butterflies are done feeding when either the honey solution has been fully consumed or when the butterfly retracts its proboscis and starts moving its legs rapidly.

g. Return the butterfly to its individual housing (glassine envelope) after it has finished feeding.
h. Return the butterfly to its incubator.
i. Record in a data log (hard copy or electronic) that the butterfly has been fed individually (record the day, date, and time of feeding).

Note: We have found that fall monarch butterflies, kept in an incubator set to fall-like conditions for Cincinnati, OH (photoperiod of 12L:12D; cycling temperature of 21°C during the day and 12°C during the night) can be fed using this hand feeding method three times a week with long term survival in housing (up to six months).[2]

Note: We have found that monarchs can become overfed while captive and therefore should be monitored during the course of an experiment. We have seen that overfed monarchs, e.g., often observed with bloated or distended abdomens, can have low flight motivation when tested in flight simulator trials.

Note: Overfed monarchs can still be kept for experiments. To reduce or prevent overfeeding, you can adjust the feeding schedule to feeding once a week. We have found no ill effects on the behavior or survivorship of butterflies with this reduced feeding schedule.

Note: The flight motivation of previously overfed butterflies can be increased by periodic bouts of exercise when held captive. Periodically, e.g., once every other day when housed indoors, remove butterflies from their glassine envelope housing and incubator, and place them in a large insect cage or mesh terrarium to allow for flight and exercise. If you are allowing butterflies to exercise communally, i.e., in a single large cage, make sure that each butterfly is individually marked for proper identification (see Obtaining animals from the field, section 6.d). We typically give monarchs 60 min in a communal cage for exercise.

Section 3: Alternative method – How to feed butterflies communally

Timing: 20–30 min (frequency of feeding: 3 times per week)

11. Setup the communal cage for feeding.
    a. Soak a sponge in honey solution.
    b. Place sponge onto a Petri dish.
    c. Place Petri dish with sponge in the middle of the cage.
    d. Remove butterflies from their glassine envelopes and introduce butterflies into the communal feeding cage.
    e. Leave butterflies together in the communal cage to feed for 20 min.
    f. After 20 min, remove all butterflies from the communal cage and return each to their own glassine envelope.
    g. Return all butterflies to their incubator.
    h. Record in a data log (hard copy or electronic) that the butterflies have been fed communally (record the day, date, and time of feeding).

Note: Butterflies will feed from the sponge. Animals fed in this manner need to have individual markers for proper identification when held together in a cage.

Note: An alternative to the sponge is to drill small holes into a mini petri dish and allow the butterflies to stick their proboscis into the fluid beneath.

Note: Replace food from the communal feeding station every day to prevent mold from growing.

Calibrating the flight simulator and trial conditions

Timing: At least 1 day before the start of trials

Timing: 60–90 min (for step 12)

Timing: 30–60 min (for step 16)

12. Select and prepare a dedicated darkroom for experiments.

Note: If a dedicated darkroom is unavailable, improvise a darkroom by turning off or covering existing lighting using materials such as blackout or shade cloth.

Note: We conduct flight simulator trials in a darkroom that is away from very strong magnetic fields, e.g., a room adjacent to an MRI machine (see troubleshooting – problem 3 for potential solutions for controlling magnetic field interference).

Note: We do not conduct flight simulator trials when strong sound or vibration is occurring, e.g., such as during construction in an adjacent room or floor or during other times in which noise and vibratory cues can be sensed by monarchs during trials. If this is a recurring problem for your experimental room, you will need to relocate to another suitable location for trials.

13. Position the flight simulator at the desired location of the darkroom used for experiments.
    a. Position the flight simulator within the room such that no landmarks are visible to the butterfly when in the flight simulator during trials.
    b. Using a compass, position the flight simulator (Figure 2[1]) in the room such that north in the flight simulator (i.e., the position of the butterfly at the beginning of a trial) is aligned with geomagnetic north during trials.

Note: If possible, it is preferable to have the flight simulator in the center of the room, equidistant from the four walls.

Note: We use a flight simulator based on the design of Mouritsen and Frost[9] with modifications from Reppert et al.[10] Our flight simulator consists of an open-topped, cylindrical, white, translucent, UV-transmitting plastic container (Figure 2A). At the opening at the top of the container sits the data encoder (mounted on a clear plastic crossbar in order to sit at the top of the container; Figure 2B), at which a tethered monarch is attached. Here, the butterfly can rotate fully in a frictionless manner. The data encoder collects the orientation bearings (from the possible 360° of orientation, sampled at a desired rate) of the flying monarch during a trial with these data sent to a data acquisition device via an encoder cable. The data acquisition device is itself linked to a computer for storing the data. These orientation data are then analyzed statistically with circular statistics, e.g., test if an individual butterfly is flying consistently and with a mean orientation heading (see quantification and statistical analysis section).

Note: At the bottom of the flight simulator container, we mount a small camera that allows us to monitor the flight behavior of a butterfly during trials without disturbing or being near the butterfly (Figure 2A).

Note: We use a small fan at the bottom of the flight simulator container to produce laminar airflow by blowing air through hundreds of plastic straws (airflow < 3 m s^{-1}), as this can help the butterfly have sustained flight (Figure 2C).

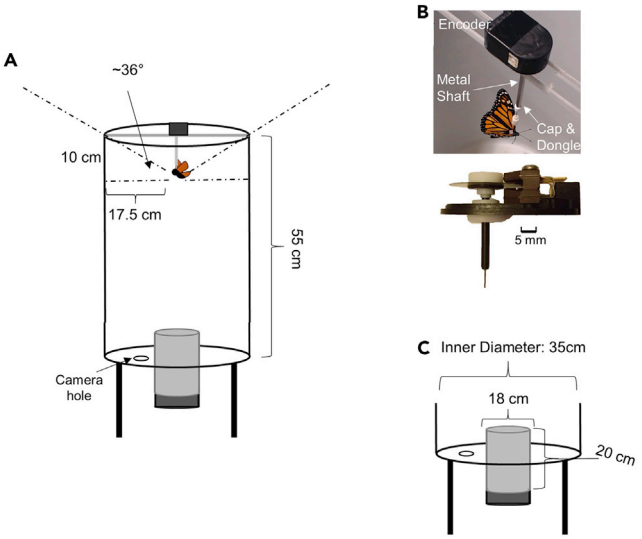

Figure 2. Flight simulator apparatus used for testing the flight behavior of butterflies

We use a flight simulator based on the original design by Mouritsen and Frost[9] that has been modified according to Reppert et al.[10]

(A) Schematic of the open-topped flight simulator for testing butterfly flight behavior. Dashed lines at the top of the flight simulator indicate the approximate field-of-view of a butterfly during trials (butterfly can see above ~36° based on how it is tethered 10 cm from the top of the flight simulator), allowing it to only see celestial cues from above (e.g., the sun).

(B) Close-up view at the top of the flight simulator of the data encoder and tethering apparatus of a monarch used during trials.

(C) Schematic of the bottom of the flight simulator showing where the PVC pipe (with plastic straws glued inside to produce laminar air flow from the fan; grey section of cylinder) and fan (dark section of cylinder) is located. The camera is placed at the camera hole to monitor the monarch from below during trials. Figure modified and used with permission from Parlin et al.[2] Copyright 2021 British Ecological Society.

Note: We recommend checking that the components of the flight simulator apparatus are connected and working prior to each experimental session. This takes 5 min.

14. Remove any sources of other sensory noise or cues that can be used for orientation by the animals from the room, e.g., equipment generating noise, vibration, or strong magnetic fields.
15. Set the desired sampling rate for the capture of orientation data by the flight simulator apparatus during trials.
    a. Verify that the desired sampling rate has been set correctly by turning on the flight simulator data acquisition system and examining the captured dummy orientation data.

Note: To facilitate direct comparisons across trials and within a specific experiment, we set the sampling rate such that it is always the same. For our studies, we use a 200 ms sampling rate during a 5–10 min trial.

16. Position the lighting to be used in trials in the appropriate or designated location in the darkroom as needed for specific experimental trial or control conditions.

Note: For indoor experiments, we use an artificial light source such as a shop light or lamp to simulate the sun. For this experiment, we used a shop light (250W 4-in-1 Work Light, LG Sourcing, Inc., N. Wilkesboro, NC, USA) that is powered by a full spectrum halogen bulb (120V 250W R7S, 4,000 lumens; Feit Electric Company, Inc., Pico Rivera, CA, USA), that is mounted on an adjustable tripod positioned 80 cm away (at various angles off of the horizontal to simulate different sun elevations and at different positions around the flight simulator to

simulate different sun azimuthal positions) relative to the position of the butterfly in the flight simulator.

17. Calibrate the light emitted by the light source for the specific trial or experiment.

Note: We use a spectrometer (Ocean Optics Inc., Dunedin, FL, USA) and an optic fiber (QP230-1-XSR, 235 microns; Ocean Optics Inc.) with cosine corrector (CC-3-UV-S; Ocean Optics Inc.) to measure and calibrate our light source for trials.

Note: For this experiment, we use a light source that produces a photon flux intensity of 7.45×10^{15} photons s^{-1} cm^{-2} which although is orders of magnitude less bright than the sun is sufficiently bright enough to elicit normal flight behavior in monarchs when it is the only source of light during trials.[1,11] Although we use a full-spectrum light in this study, full-spectrum light is not necessary to examine monarch flight behavior, as monarchs will fly normally even when exposed to non-full-spectrum light during trials. Non-full-spectrum light sources or the use of specialized light wavelength filters can test the role of specific wavelengths on oriented flight and the use of various compass mechanisms. For example, light filters blocking out UVA/UVB light wavelengths (long-wavelength filters E420 and E380; Gentex Corporation, Zeeland, MI, USA) from a full-spectrum light source can test magnetic compass use in monarchs,[11] as monarchs need to be exposed to these wavelengths in order for their magnetic sense to be activated.

18. Measure the temperature when the light is on.
    a. Measure the temperature in the flight simulator at the position of the butterfly during trials with a thermometer.
    b. Measure the overall temperature of the room away from the flight simulator, e.g., 1–2 meters away, using a thermometer.

Note: For more accurate readings, we use a digital thermometer to measure temperatures. Monitoring the temperature takes 1 min for each measurement.

Note: Although the shop light that we use can increase the temperature around the flight simulator during testing, we found no negative effects of our light source on the monarchs that we tested in any of our previous work[1,11] and the heat emitted from the light did not affect the airflow around the flight simulator.

Note: If you wish to specifically control or to directly test the effects of heat on monarch flight behavior in indoor flight simulator trials, you might consider using appropriate LED lights.

19. Measure the relative humidity of the room.
    a. Use a hygrometer to measure the relative humidity of the room.

Note: We use a digital hygrometer to acquire an accurate measurement of the room's relative humidity. Monitoring the relative humidity takes 1 min.

Note: The temperature and relative humidity of the experimental room should be set to the desired trial conditions and periodically monitored for consistency during trials. Our experimental rooms are controlled centrally by our institution's Facilities department, and we contact Facilities to set conditions. You will need to consult with your institution's Facilities department if conditions are set centrally or you will need to manually set the conditions yourself.

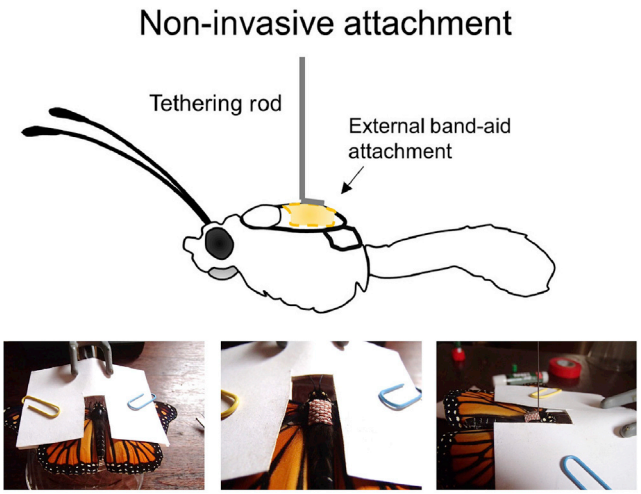

Figure 3. Non-invasive tethering method for butterflies

(Top) Schematic outlining how the tether (tethering rod) is attached to the butterfly via adhesive and an external band-aid attachment. (Bottom) Left picture shows restrained butterfly (held by the index card and paperclips) with scales from the thorax removed for attaching the bandage. Middle picture shows where the bandage is placed on the thorax of the butterfly. Right picture shows where the tethering rod is attached on the bandage. This non-invasive procedure does not harm the butterfly, does not require anaesthetization (e.g., cold temperature or carbon dioxide), and does not involve any surgical procedure. The tethering rod and bandage can be removed after experiments without harming the butterfly. Figure modified and used with permission from Parlin et al.[2] Copyright 2021 British Ecological Society.

Tethering butterflies for flight simulator trials

Timing: 5–7 min per individual butterfly

20. Tether butterfly using non-invasive tethering procedure[2] for flight simulator trials.
    a. Remove a monarch from its glassine envelope housing using the same manner as if it was being removed for feeding (see Maintaining animals captured from the field, sections 10.b).
    b. Restrain the butterfly using a folded notecard (7.6 × 12.7 cm) (or any other type of sturdy cardboard) with a small piece removed (width: 10 mm; length: 30 mm) (Figure 3, bottom left).
    c. Hold the butterfly gently but firmly in place within the notecard using paperclips (one on each side of the butterfly), to prevent the butterfly from moving such that it does not damage its wings or injure itself (Figure 3, bottom left).
    d. Hold the butterfly within the notecard in place by using a clamp that is attached to a support stand (Figure 3, bottom left).
    e. Position the butterfly so that it sits and stays on a plastic cup (e.g., plastic cup used for feeding; Figure 1B), to prevent the flight response from initiating (e.g., flying insects will initiate flight if their feet are not in contact with a surface).
    f. Expose the thorax of the butterfly via the small opening in the notecard (Figure 3, bottom middle).
    g. Remove the scales from this region of the thorax carefully by using any standard razor blade.
    h. Use a Q-tip with one of its ends coated with double-sided tape to wipe this region of the thorax to produce a dry surface free from any scales.

Note: If a Q-tip coated with double-sided tape is unavailable, you can use a fine paint brush to gently sweep and remove scales from the surface.

i. Take a small piece of bandage and cut a piece out of it that is custom fit to the size of the butterfly being tethered (Figure 3, bottom middle).
j. Apply the custom fit piece of bandage to the dry, scale-free area of the thorax (Figure 3, bottom middle) using fine-forceps, with the backing of the bandage removed via another pair of fine-forceps.
k. Using wire cutters, cut a piece of tungsten rod (approximately 40 mm in length) that will serve as the tether for the monarch.
l. Use pliers to bend 4–5 mm of one end of the tungsten rod to form a 90° angle.
m. Attach the bent end of the tungsten rod tether perpendicular to the bandage on the monarch via a light coating of Krazy Glue (Figure 3, bottom right).

⚠ CRITICAL: We give the tether between 15–30 s to fully adhere to the bandage via the Krazy Glue.

Note: Butterflies can be tethered (Figure 3) a day or more before the trial to ease the removal from individual housing containers and placement into the flight simulator.

Note: We recommend that butterflies have a minimum of 24 h with the tethering attachment prior to being tested in flight simulator trials if it is non-invasive, and at least 48 h if it is surgically implanted or glued to ensure no complications from attachment.

Note: We have found that with practice and experience, the time it takes to tether a butterfly can decrease (e.g., down to 5 min per butterfly).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental models: Organisms/strains		
Eastern North American monarch butterflies (*Danaus plexippus*)	Wild-caught animals of any seasonal population	Adult butterflies of any age and of either sex.
Software and algorithms		
R Project for Statistical Computing (version 3.6.0)	R Core Team	Resource Identification Portal: RRID_SCR_001905
Other		
Collapsible insect net (45.72 cm diameter) with white mesh and 12.7 cm long handle	BioQuip	N/A
Collapsible insect net handle extension (30.48 cm long)	BioQuip	N/A
Side-opening glassine envelopes (size: 7.94 × 10.32 cm)	BioQuip	N/A
Percival environmental chamber	Percival Scientific, Inc.	Model I36LLC8
Fine-tip forceps	Fine Science Tools	Dumont #5 Forceps (Foster City, CA, USA)
Cotton-weave bandages	Johnson & Johnson	Band-Aid® brand Tough-Strips
Strong, fast-acting adhesive (super glue)	Krazy Glue	Precision tip, all purpose Krazy Glue
Standard pliers	Grainger	N/A
Standard wire cutter	Grainger	N/A
3-prong clamp	Thomas Scientific	ECPVC1
Support stand	Thomas Scientific	54101
Artificial light source – Shop light	Home Depot	250 W 4-in-1 Work Light, LG Sourcing, Inc. (N. Wilkesboro, NC, USA)
Artificial light source – Bulb	Home Depot	120 V, 250 W R7S, Feit Electric Company, Inc. (Pico Rivera, CA, USA)
Flight simulator barrel (white, translucent, polypropylene cylindrical container)	Thermo Fisher Scientific	2210-0130 or 2318-0130
PVC pipe	Lowe's	Charlotte pipe (18 cm outside diameter)
Axial fan (18 cm diameter)	Dayton Electric Manufacturing	Fan: 60/50 Hz; AMPS 023/0.22; Watts 27/25

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Camera	Adafruit	Raspberry pi zero W unit with camera (8 megapixel; WiFi/Bluetooth capability)
Optical encoder	US Digital	E5 series
Encoder cable	US Digital	CAT#: CA-FC10-SH-NC-30
Data acquisition device	US Digital	USB4 Encoder
Tungsten rod	A-M Systems, Inc.	CAT#: 718000, Lot: 554525

MATERIALS AND EQUIPMENT

Alternatives: This protocol uses a flight simulator system whose design is based on flight simulators used previously[3,9] to assay the orientation behavior of tethered monarchs. Other flight simulator or flight treadmill designs (e.g., other enclosed or isolated circular arena-type setups) which a) allow for the collection of orientation data preferably in an automated manner, b) provide continuous monitoring of the butterfly during a trial (e.g., flight observed via a video monitor), and c) which permit the control of what visual cues flying animals can perceive can also be used.

Alternatives: This protocol uses the non-invasive tethering method that we developed[2] for tethering various species of Lepidoptera for testing in flight simulator trials. Other tethering methods, such as the use of surgical implantation of tethers into an animal can be used as well. If animals are to be released back into the wild unharmed (e.g., for conservation purposes) or if they are to be used in other long-term studies (e.g., animals are required to live as long as possible for multiple or longitudinal experiments), we recommend the use of the non-invasive tethering technique.

Alternatives: Although this protocol uses the R Project statistical package to analyze data (e.g., circular statistics to analyze orientation data[12]), other suitable circular statistics programs can be used, such as Oriana (Kovach Computing Services, Anglesey, Wales, UK).

STEP-BY-STEP METHOD DETAILS

Prepare for indoor flight simulator trials – Day of experiment

Timing: 1–2 h before trials

In this section of the protocol, we describe how to prepare the workspace for conducting flight simulator trials under indoor, controlled testing conditions. This process consists of two major steps that are to be done on each day of experiments prior to conducting any trials: 1) preparation of the experimental room for the specific treatment or control conditions for trials to be conducted; 2) checking that all aspects of the flight simulator apparatus are in working order.

Preparing the flight simulator and treatment conditions for indoor trials

Timing: 2 min (for steps 1a and 1b)

Timing: 5 min (for step 1c)

Timing: 1–2 min (for step 1d)

Timing: 5 min (for step 2)

Note: This protocol summarizes the indoor, controlled testing of butterflies, such as migratory monarchs, during their subjective nighttime in response to nighttime light pollution stimuli as conducted by Parlin et al.[1] Tests subjecting butterflies to other stimuli during their subjective night can use this protocol. Similarly, if animals are to be tested during their subjective daytime under indoor, controlled conditions in response to daytime occurring stimuli, the same protocol can be used.

1. Adjust (if necessary) and record ambient testing conditions.
   a. Set the temperature of the room at the desired level for trials.

   Note: We have found that temperature settings between 20°C–22°C work well for flight simulator trials. For reference, if temperatures are too cool (e.g., below 10°C) or too hot (>32°C), monarch flight motivation is decreased to the point where they do not fly consistently during trials. See Calibrating the flight simulator and trial conditions, sections 18.a and 18.b for setup and checking of temperature conditions.

   b. Check that the relative humidity level of the testing room is neither too dry (e.g., < 10% humidity) nor too humid (e.g., > 80% humidity).

   Note: See Calibrating the flight simulator and trial conditions, section 19.a for setup and checking of relative humidity conditions.

   c. Check that the artificial lighting to be used for trials are set to the appropriate testing conditions (see Calibrating the flight simulator and trial conditions, sections 16 and 17).

   ⚠ CRITICAL: Check that the position of the light source(s), the intensity of the light source(s), and the wavelengths of light (spectral properties) emitted by the light source(s) are set to the specific conditions to be tested for trials.

   d. Check that any magnetic, acoustic, and vibratory sensory cues are controlled or eliminated during trials.

   ⚠ CRITICAL: When selecting a suitable testing room, the initial calibration process to control for these conditions in the experimental room can take 60 min. On each day of trials, each pre-experiment check takes 1–2 min (see Calibrating the flight simulator and trial conditions, sections 12 and 14).

2. Prepare flight simulator apparatus for trials.
   a. Check that the flight simulator is positioned as desired in the room (see Calibrating the flight simulator and trial conditions, sections 13.a and 13.b).
   b. Check that all cables that connect equipment are connected.
   c. Check that any relevant switches, e.g., on button, or gain knobs or intensity controls, are on or placed at the appropriate setting for trials, respectively.

Prepare animals for indoor flight simulator trials – Day of experiment

Timing: Immediately before an individual trial

In this section of the protocol, we describe how to prepare animals that will be tested in indoor, controlled flight simulator experiments. This process consists of the steps of 1) transporting the animals from where they are housed to the testing location under darkness and 2) acclimating the animals to the specific conditions that they will be tested at during trials.

Preparing animals to be tested in the flight simulator

Note: This protocol summarizes the indoor controlled testing of migratory monarchs in response to exposure to an acute stimulus simulating outdoor nighttime light pollution (i.e., light trespass) that occurs in urbanized settings during the subjective night of the butterflies. This protocol can also be used for testing monarchs at night when exposed to other types of nighttime light pollution (e.g., over-illumination) or other types of sensory cues or pollution that occur at night. This indoor testing protocol allows researchers to specifically isolate the potential effect of nighttime light pollution stimuli, while controlling other sensory and environmental variables that are either difficult or impossible to control, e.g., outdoor ambient temperature and noise, during experiments conducted under outdoor field conditions.

Note: For trials during the subjective day of the butterflies or in response to other treatment/control conditions, this protocol can also be used to facilitate the performance of controlled experiments. Just make sure to omit portions of the protocol where the animal is kept in the dark (e.g., during handling from the incubator to the flight simulator).

3. Bring monarchs to be tested from where they are housed to the flight simulator testing location.
   a. Make sure that monarchs are not inadvertently exposed to light prior to a trial, by handling all monarchs in the dark before trials.
   b. Use red-light, e.g., a headlamp equipped with red-light, in order to be able to quickly and safely transfer the butterflies to the experimental room while in the dark.

⚠ CRITICAL: Do not expose monarchs to light (e.g., full-spectrum) during their subjective night phase, as light exposure might negatively affect their circadian clock and induce unwanted circadian phase shifting (see troubleshooting – problem 4 for ways to deal with accidental light exposure at night).

Note: Ideally, the room in which monarchs are held (incubator room) is not that far from the experimental room. If necessary, transport butterflies in a dark box that cannot have light penetrate.

Note: If the room in which butterflies are held (incubator room) is a far distance away from the experimental room for indoor flight simulator trials, butterflies can be transported when contained in their individual housing (in a container preventing light exposure or under darkness) to the experimental room or an adjacent room, prior to trials.

Optional: Butterflies can be held under darkness an hour before trials, to facilitate keeping each test animal in the dark until its trial has started.

Conducting flight simulator trials – Day of experiment

◷ Timing: During an individual trial

In this section of the protocol, we outline the procedure for conducting a flight simulator trial with an animal. This process consists of three major steps: 1) introducing and attaching the tethered butterfly into the flight simulator apparatus under darkness; 2) conducting the trial and ensuring that data (orientation data from flight simulator trials) are collected; and 3) returning the animal back to its housing conditions after a trial has been completed.

Conducting flight simulator trials (e.g., light trespass and other nighttime light pollution conditions).

4. While in the dark, remove a butterfly from its glassine envelope housing and install the tethered monarch inside the flight simulator.

   Note: In our setup, this handling process takes approximately 30 s to complete.

5. Once the monarch is tethered, turn on the light according to the parameters of the stimulus to be tested.

   Note: For light trespass conditions, the light is immediately turned on after the monarch is tethered in the flight simulator. The latency for when the light should be turned on during a trial will vary with the type of stimulus conditions occurring during a trial, however.

6. Conduct flight trial for the desired trial duration.

   Note: For flight trials with monarchs, a trial of 5–10 min is suitable to assay the orientation behavior of individuals.[1,2,8]

7. Make sure that orientation data are collected by the flight simulator system prior to removing the monarch from the simulator.

   Note: Unless otherwise indicated by a specific testing protocol, we only test monarchs and obtain orientation data for that butterfly once per day.

8. At the end of a trial, immediately turn the light off.
9. Remove the butterfly from the flight simulator and immediately return it to its glassine envelope.

   Note: Aim to have this process be of similar duration as at the setup of trials, i.e., when placing the tethered butterfly within the simulator (approximately 30 s).

10. Return the butterfly to its incubator while in the dark.

    Note: Use the same process for transporting butterflies in the dark, e.g., transport the butterflies in a box that does not let light penetrate.

EXPECTED OUTCOMES

A successful flight simulator trial will produce orientation data for the animal tested during trial conditions, i.e., a measure of flight directionality of the tethered flying butterfly exhibited during a trial. Data captured during a 5–10 min trial will demonstrate if the monarch is flying directionally or non-directionally. If directionally, the monarch is interpreted as flying with a significant mean vector or orientation bearing. If non-directionally, the monarch is interpreted as flying without any mean vector or orientation bearing, and is flying in a random, uniform, or non-oriented manner. Across different types of flight simulator experiments, we can achieve a 39.0% success rate[1,2,8,11] for monarchs tested in the flight simulator that yield data that can be included in analyses testing for a directional response. The data for individuals can then be aggregated and analyzed to see if monarchs subjected to the same conditions fly directionally or non-directionally as a group (Figure 4). Please see below for how orientation data are analyzed to determine individual and group butterfly directionality in response to different testing conditions (see quantification and statistical analysis section and Table 1).

QUANTIFICATION AND STATISTICAL ANALYSIS

The following summarizes the quantification and analysis of orientation data from flight simulator trials as conducted in previous studies with monarchs.[1,2,13] These data are examined and statistically

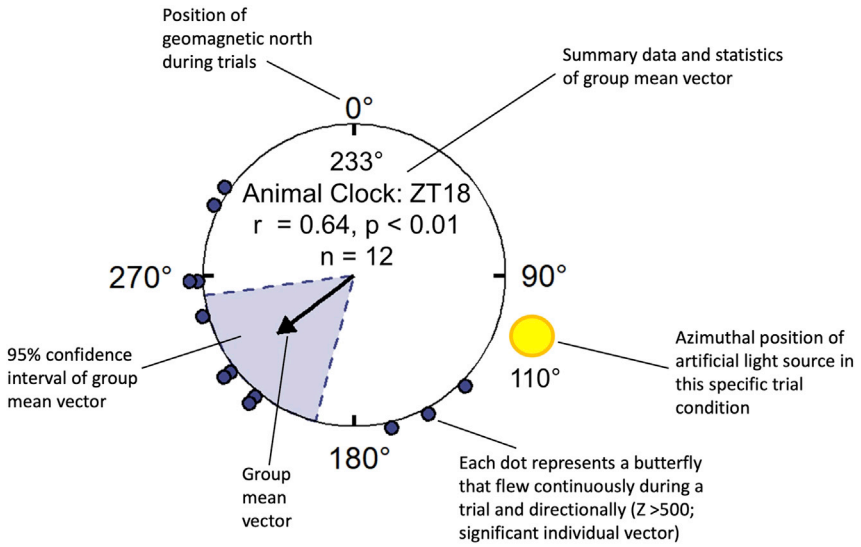

Figure 4. Circle diagram used to show flight orientation data produced during flight simulator trials that demonstrate group directionality (mean vector) of butterflies in response to stimulus conditions

Each dot in the circle diagram represents the individual mean vector of a tested butterfly that flew continuously for the entire trial and that had a Z-score >500. The arrow indicates the group mean vector that quantifies the directionality of butterflies as a group during test conditions. Shaded area within dashed lines indicates the 95% confidence interval of the mean vector. 0°–359° around the circle diagram represent the range of possible orientations that butterflies can display either as individuals or as a group during trials. 0° indicates position of geomagnetic north. Summary data and statistics are contained within the center of the circle diagram, e.g., as related to the Rayleigh's test (*r* value measuring the length of the group mean vector, p value of the Rayleigh's test, sample size). Yellow dot represents the azimuthal location of the artificial light source testing the effect of exposure to light trespass during indoor controlled nighttime light pollution tests. Figure modified from Parlin et al.[1]

analyzed in R Program software (see key resources table) using a circular statistics R package.[12] Flight simulator trials produce data that can be classified into different groups (Table 1). First, butterflies that do not fly at all when placed in the flight simulator or that fly inconsistently during a trial (e.g., the butterfly makes a substantially long pause during its trial, e.g., a pause greater than 15 s, or the butterfly completely stops flying and does not resume flight before the trial has ended) are excluded from analyses on directionality (see troubleshooting – problem 5 on potential solutions to increase flight motivation in the monarchs).

Next, for each butterfly that does fly consistently during a trial, a Z-score is first produced from the butterfly's orientation data. These orientation data consist of the headings of a butterfly measured with a specific sampling rate (200 ms), during the duration of the trial (5–10 min). Each individual sampled data point will consist of an orientation heading ranging between 0°-359°. Previous work from other researchers[13] and our own studies[1,2,11] that have tested monarch flight directionality and that have reconstructed the flight paths from orientation trials have shown that Z-scores provide a more rigorous and conservative assessment of individual monarch flight orientation. A monarch's Z-score measures how consistent it flew in the mean direction and gauges the level of variability occurring in the directionality in the flight of the butterfly during a trial. That is, although a significant p-value from a Rayleigh's test (circular statistical test that measures directionality) can indicate that the monarch flew with an overall significant mean heading during a trial, Z-scores measure how consistently during the entire trial period that butterfly flew with that mean heading. A Z-score below 500 indicates that the butterfly was flying completely non-directionally during a trial, e.g., the butterfly was flying in continuous circles or in a haphazard manner, producing a uniform distribution of circular orientation data during a trial. In contrast, a Z-score greater than 500 shows that the butterfly is exhibiting a consistent directional flight response, in which it reliably maintained a specific mean

Table 1. Types of animal flight data generated in flight simulator trials

Type of directionality	Description of data	Interpretation and uses of data
Individual	Butterfly does not fly continuously for the entire trial	Data can be used in calculations of trial success rate for a given population of butterflies that are tested. Directionality cannot be calculated since the animal did not fly during the entire trial period.
Individual	Butterfly flies continuously for the entire trial but has a Z-score <500	Data can be used in calculations that measure the percentage of monarchs that display oriented flight during a specific treatment or control condition. The data are not useable for further analysis as the butterfly is flying in a non-directional manner.
Individual	Butterfly flies continuously for the entire trial and has a Z-score >500	The data for the butterfly can be used to calculate an individual mean vector as the butterfly displays directional flight (via Rayleigh's test). The mean vector can then be pooled with other mean vectors of other butterflies to calculate a group mean vector in response to specific test conditions.
Group	Group of butterflies that each flew continuously for the entire trial and had a Z-score >500, but there is no significant group directionality	Group data can be used to calculate group directionality in response to specific test conditions (e.g., via Rayleigh's test). A non-significant mean vector shows that as a group, the butterflies do not share a mean orientation heading during test conditions and are instead flying in a non-oriented manner. These group data cannot be compared with group data that display significant group directionality.
Group	Group of butterflies that each flew continuously for the entire trial and had a Z-score >500, and there is significant group directionality, i.e., the group is oriented with a specific mean bearing	Group data can be used to calculate group directionality in response to specific test conditions (e.g., via Rayleigh's test). A significant mean vector shows that the group displays oriented flight with specific directionality during test conditions. Significant group mean vectors can be compared with other significant group mean vectors to compare directional responses across different treatment conditions and testing regimes (e.g., paired versus unpaired experimental designs).

heading while performing tethered flight during the trial. High Z-scores (greater than 500) indicate that not only did the monarch fly with a significant mean heading during a trial, but that it maintained flying in that specific heading very consistently throughout the trial. In contrast, monarchs with lower Z-scores (still greater than 500) did fly with a significant mean heading during the trial, but there was more directional variability (e.g., the monarch might fly in circles or in different directions a few times) in its flight. For each butterfly with a Z-score greater than 500, the individual mean vector of each butterfly is then calculated using the Rayleigh's test. These individual mean vectors are then aggregated to calculate the group mean vector for butterflies tested under the same conditions using the Rayleigh's test (measure of group directionality). To compare the mean vectors of different groups of butterflies tested in different conditions, e.g., daytime versus nighttime trials, analyses for circular data such as the Mardia-Watson-Wheeler test can be used. We apply Bonferroni correction for multiple comparisons when necessary. See the supplemental in previous work[2] for visualization of differing Z-scores and their corresponding virtual flight paths.

LIMITATIONS

Although the flight simulator method produces data that assess the flight consistency and directionality of monarchs in response to specific conditions, it is an open-loop assay whether conducted indoors or outdoors. Here, each butterfly is stationary and only uses similar or current external sensory information cues in a feedback loop. The butterfly does not benefit from receiving direct information or sensory input derived from its own actual movement to guide its behavior, as it would otherwise be able to when freely flying under natural conditions. Closed-loop conditions can have direct consequences on how an animal perceives stimuli or cues, and how these stimuli or cues will then be subsequently perceived once the animal is behaving. To supplement open-loop approaches, outdoor closed-loop assays examining freely flying butterflies under natural conditions, such as disappearance bearing trials, can also be performed with the same animals after they are tested in indoor, controlled trials. Please see the catch-test-release method outlined in Parlin et al.[1] for more details on incorporating open-loop (indoor, tethered butterflies) and closed-loop (outdoor, freely flying butterflies) behavioral assays.

TROUBLESHOOTING

Problem 1

Refer to "section 2: how to feed butterflies individually, step 10.d".

A butterfly will not keep its proboscis extended or continues to retract their proboscis out of the drop of honey solution during feeding.

Potential solution

During individual feeding, some butterflies will not keep their proboscis extended and in the drop of honey solution. We have found that by extending and holding the butterfly's proboscis (using a pin) in the drop of honey solution for a brief period (30–60 s), the butterfly will then keep its proboscis in the honey solution. If the butterfly will not feed at all that day, we then return the butterfly to its glassine envelope in order to minimize any handling stress that the animal might experience. From our experience, a monarch that will not feed on a specific day will feed at the next feeding period, depending on the length of time between feeding intervals. For example, a monarch that will not consume the honey solution will readily feed after three days of that attempt.

Problem 2

Refer to "section 2: how to feed butterflies individually, step 10.e".

Butterflies escape from under the plastic holding cup and fly away or get trapped under the cup during feeding.

Potential solution

Some monarchs are strong enough to push off the plastic cup that is holding them during feeding, enabling them to escape and fly away. In contrast, some monarchs will push the plastic cup off of them or slip from under the part of the cup that is holding them in place, slip fully under the cup, and effectively become trapped. When trapped under the cup, some butterflies will rapidly move in an attempt to fly away, causing them to splash the honey solution all over their body. Researchers should endeavor to prevent honey solution from getting on the butterflies, since it can cause them to become extremely sticky. For example, if honey solution gets on the wings, the wings can get stuck together when the honey solution dries, potentially damaging their wings (e.g., wing tearing or significant loss of wing scales) and making the butterflies less effective at flying during flight behavioral trials. To prevent these issues, we have placed either small metal washers or small rubber test tube stoppers on top of the cups to weigh them down when holding the butterflies in position during feeding.

Problem 3

Refer to "calibrating the flight simulator and trial conditions, step 12".

There might be magnetic interference (e.g., artificial magnetic fields generated by electrical equipment) in or near the testing room that can potentially affect the orientation behavior of monarchs (monarchs can use a magnetic compass to guide flight orientation) when tested during indoor flight simulator trials.

Potential solution

To control for magnetic interference from other electrical equipment during trials, we turn off all unnecessary electrical equipment. If the room is large enough, we also use extension cords such that the flight simulator is isolated within the testing room and other devices (e.g., external monitor, computer) are as far as possible from the flight simulator during a trial. When performing trials that directly assess magnetosensation during flight, we use Helmholtz coils positioned around the flight simulator to generate the desired magnetic field conditions for trials and that offset or cancel any interference. Our Helmholtz coils were custom-built at the University of Massachusetts Chan Medical

School Machine Shop to fit our specific flight simulator[11]; coils can be designed for other apparatus as desired or purchased commercially from a variety of specialized commercial vendors. The artificial magnetic field generated by the Helmholtz coils can be calibrated using a magnetometer (we use an Applied Physics Systems tri-axial fluxgate magnetometer, model 520A).

Problem 4

Refer to "prepare animals for indoor flight simulator trials – day of experiment, steps 3a and 3b".

Monarchs have been inadvertently exposed to light during their subjective night phase during the course of an experiment.

Potential solution

If monarchs have been inadvertently exposed to light during their subjective night phase, we highly recommend that monarchs be given at least 5 days from the time of light exposure to re-entrain to the proper or desired light regime (previous work has shown that 5 days allows for the circadian clock to re-entrain after a light perturbation). Re-entraining monarchs is particularly important when examining how monarchs use their time-compensated sun compass during migratory flight, which relies on properly functioning circadian clocks for correct orientation behavior.

Problem 5

Refer to "quantification and statistical analysis" section.

The butterfly has low motivation to fly during a trial causing it to not fly at all or to not fly for the entire duration of the testing period.

Potential solution

Low flight motivation in butterflies can occur if the animals have been recently fed. For example, a butterfly fed on the day of testing before its trial can have low flight motivation since the motivation to fly and forage can be decreased. To enhance the motivation to fly and to maximize continuous flight by butterflies during trials, we test monarchs on the days that they have not been fed or test them before their scheduled feeding if feeding and trials fall on the same day. To maintain flight motivation and propensity while butterflies are housed indoors in incubators, butterflies can be allowed time to freely fly during captivity. For instance, a butterfly can be released for a certain time (e.g., 1 h) in an indoor mesh holding cage every other day, to allow it to fly and freely move. We have found that both activities increase the number of butterflies that fly continuously and robustly when tested in the flight simulator, particularly for trials conducted under indoor testing conditions.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources should be directed to and will be fulfilled by the lead contact, Patrick A. Guerra (patrick.guerra@uc.edu).

Materials availability

This study did not generate any new or unique materials or reagents.

Data and code availability

This study and the associated published article[1] did not generate any new code. This study did not generate any new datasets.

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AUTHOR CONTRIBUTIONS

Conceptualization, A.F.P., S.M.S., P.A.G.; Methodology, A.F.P., S.M.S., P.A.G.; Resources, A.F.P., S.M.S., P.A.G.; Writing – Original draft, P.A.G.; Writing – Review & editing, A.F.P., S.M.S., P.A.G.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

1. Parlin, A.F., Stratton, S.M., and Guerra, P.A. (2022). Oriented migratory flight at night: consequences of nighttime light pollution for monarch butterflies. iScience *25*, 104310. https://doi.org/10.1016/j.sci.2022.104310.

2. Parlin, A.F., Stratton, S.M., and Guerra, P.A. (2021). Assaying lepidopteran flight directionality with non-invasive methods that permit repeated use and release after testing. Methods Ecol. Evol. *12*, 1699–1704. https://doi.org/10.1111/2041-210X.13648.

3. Froy, O., Gotter, A.L., Casselman, A.L., and Reppert, S.M. (2003). Illuminating the circadian clock in monarch butterfly migration. Science *300*, 1303–1305. https://doi.org/10.1126/science.1084874.

4. Tenger-Trolander, A., Lu, W., Noyes, M., and Kronforst, M.R. (2019). Contemporary loss of migration in monarch butterflies. Proc. Natl. Acad. Sci. USA *116*, 14671–14676. https://doi.org/10.1073/pnas.1904690116.

5. Oberhauser, K.S. (1988). Male monarch butterfly spermatophore mass and mating strategies. Anim. Behav. *36*, 1384–1388. https://doi.org/10.1016/S0003-3472(88)80208-2.

6. Altizer, S.M., and Oberhauser, K.S. (1999). Effects of the protozoan parasite *Ophryocystis elektroscirrha* on the fitness of monarch butterflies (*Danaus plexippus*). J. Invertebr. Pathol. *74*, 76–88. https://doi.org/10.1006/jipa.1999.4853.

7. Herman, W.S., and Barker, J.F. (1977). Effect of mating on Monarch butterfly oogenesis. Experientia *33*, 688–689. https://doi.org/10.1007/BF01946578.

8. Guerra, P.A., and Reppert, S.M. (2013). Coldness triggers northward flight in remigrant monarch butterflies. Curr. Biol. *23*, 419–423. https://doi.org/10.1016/j.cub.2013.01.052.

9. Mouritsen, H., and Frost, B.J. (2002). Virtual migration in tethered flying monarch butterflies reveals their orientation mechanisms. Proc. Natl. Acad. Sci. USA *99*, 10162–10166. https://doi.org/10.1073/pnas.152137299.

10. Reppert, S.M., Zhu, H., and White, R.H. (2004). Polarized light helps monarch butterflies navigate. Curr. Biol. *14*, 155–158. https://doi.org/10.1016/j.cub.2003.12.034.

11. Guerra, P.A., Gegear, R.J., and Reppert, S.M. (2014). A magnetic compass aids monarch butterfly migration. Nat. Commun. *5*, 4164. https://doi.org/10.1038/ncomms5164.

12. Agostinelli, C., and Lund, U. (2017). R package 'circular': circular statistics (version 0.4-93). https://r-forge-.r-project.org/projects/circular/.

13. Zhu, H., Gegear, R.J., Casselman, A., Kanginakudru, S., and Reppert, S.M. (2009). Defining behavioral and molecular differences between summer and migratory monarch butterflies. BMC Biol. *7*, 14. https://doi.org/10.1186/1741-7007-7-14.