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Field-Based Thermal Physiology Assay: Cold Shock Recovery Under Ambient Conditions

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TITLE:

Field-based Thermal Physiology Assay: Cold Shock Recovery Under Ambient Conditions

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KEYWORDS:

Thermal biology, physiology, cold shock, chill coma, insect, thermal performance

SUMMARY:

Here, a low-cost, accessible protocol is described to evaluate cold shock recovery of butterflies under ambient environmental conditions.

ABSTRACT:

Ecological physiology, particularly of ectotherms, is increasingly important in this changing world as it uses measures of species and environmental traits to explore the interactions between organisms and their surroundings to better understand their survival and fitness. Traditional thermal assays are costly in terms of time, money, and equipment and are therefore often limited to small sample sizes and few species. Presented here is a novel protocol that generates detailed data on individual behavior and physiology of large, volant, terrestrial insects, using the example of butterflies. This paper describes the methods of a cold shock recovery assay that can be performed in the field under ambient environmental conditions and does not require costly laboratory equipment. This method has been used to understand the response and recovery strategy to cold shock of tropical butterflies, generating individual level data across entire butterfly communities. These methods can be employed in both remote field settings and classrooms and can be used to generate ecologically relevant physiological data and as a teaching tool.

INTRODUCTION:

The integration of thermal physiology and ecology in the late 1970s and early 1980s[1,2] launched the field of ecological physiology. Extensive thermal studies conducted on ectotherms highlight ecological–physiological synergies across diverse eco-evolutionary contexts[3–5]. Research on thermal physiology of ectothermic organisms has regained attention recently in the face of climate change and altered thermal landscapes across the world[6,7]. In addition to informing studies in the academic field of ecological physiology, thermal physiology assays can be broadly accessible to researchers and can serve as a hands-on teaching approach for all levels. Components of thermal performance, including thermal limits and effects of temperature shocks, are fundamental to the ecology, behavior, and life history of animals[8,9].

Specifically, ectotherms are used to address questions of physiology, as endothermy dictates an inextricable link between ambient and organismal temperature. The temperature range that organisms can withstand (their critical thermal minimum to maximum—thermal range) and the temperatures at which their behaviors and fitness are maximized (thermal optima) are often rooted in ecological and evolutionary processes. These physiological traits are of increasing importance as temperatures, both means and extremes, are increasing[10]. For example, the abiotic changes, including temperature increases, that accompany habitat destruction and fragmentation has affected communities of ectotherms, including anurans, limiting physiologically fragile species (with narrow thermal tolerance) to small remnant habitat patches[11,12].

Assessing key components of thermal performance can be expensive both in terms of time and resources and traditionally requires laboratory equipment and standardized conditions. Moreover, conventional assays often do not reflect the breadth of ambient conditions experienced in nature by a given animal[13] as temperature in similar physiology experiments is carefully controlled and often unrelated to ambient conditions experienced by an animal. This temperature control can diminish the understanding of variation in individual responses[2,14]. Physiologists have relied on laboratory-based heating and cooling experiments, using programmable water baths to steadily heat or cool an animal's environment to inform thermal performance curves[15].

Typically, animals are placed in vials with a thermocouple, and their ambient temperature is changed steadily by controlling the temperature of the surrounding water bath. Researchers measure the time it takes to achieve an altered physiological state (e.g., chill coma, knockdown) and the temperature at which the status change occurred[16,17]. Starting at a minimum of USD $500, these tools are large, heavy, and require additional technical equipment (e.g., computer, thermocouples). Consequently, the basic tools to carry out classic methods of assessing thermal performance are 1) not economically accessible to all, 2) not suitable for assaying animals too large to be contained in customary vials used for small dipterans, and 3) not portable for use in remote field settings. Adherence to common practice has resulted in limited representation across taxonomy and experimental conditions[18–20].

While complete thermal performance curves can inform species distribution, life history traits, and behavior, among other traits, the quantification of fewer and simpler thermal metrics can be more efficient and still extremely informative. Physiological assays, measuring chill coma onset and subsequent cold shock recovery, cold-hardening, and righting behavior, are effective and executable proxies for the critical thermal minimum of an organism[8]. Described here is a cold shock assay useful for eliciting physiological data from large terrestrial ectothermic insects. The assay is affordable, accessible, and easy to execute under field conditions or in the classroom. Data on cold shock recovery generated by this protocol can be coupled with species or individual-level trait data to pursue questions regarding ecological physiology and/or used to teach students about physiological principles.

PROTOCOL:

1. Identification of species of interest

1.1. Identify species of interest to determine cold shock recovery time. Keep in mind that each group will differ in the time it takes to induce a chill coma (i.e., the point at which the insect is still alive, but is not moving and not responsive). Likewise, based on the organism and use of data, choose different cutoff points at which to stop the experiment if the focal individual(s) do not fly (see section 4).

NOTE: This protocol was designed and developed for use on Lepidoptera. However, it is applicable to large, volant, terrestrial insects, in particular, those that can be stored flat in glassine envelopes restricting movement and damage (e.g., butterflies and dragonflies/damselflies).

2. Conducting a pretrial

2.1. Conduct a pretrial on a small sample of individuals to determine the key parameters. Follow sections 3 through 5 of the protocol below with 5–10 individuals for a pretrial.

2.1.1. Test the time required on ice to induce a chill coma (not moving), but not kill the focal species by following step 5.1 using treatments of 30 min, 60 min, and 90 min.

NOTE: The time required to induce a chill coma will depend on the size, location, and natural history/behavior of the individuals.

2.1.2. Based on results from steps 4.1–4.4 and using knowledge of the ecology of the focal insects, choose a time at which to conclude the trial if a given individual does not make a full recovery. Base this time cutoff on ecology of the species as well, keeping in mind that after many minutes of being incapable of flight, many insects are predated.

NOTE: For example, if most preliminary trials end in flight after 15 min, one could decide to end trials after 25 min to ensure that even outliers have a chance to fully recover (i.e., fly). This protocol is based on a 30 min cutoff time (step 5.4).

2.2 Use parameters from pretrial data to inform data collection for the experiments. Modify the protocol described below based on the needs of the focal organisms, including time in the ice slurry, time at which to call trials to an end, and behaviors documented on the data sheet (e.g., shiver may be an inappropriate behavior for the insect of choice).

2.2.1 Define specific research questions to be answered with these data while refining the parameters.

NOTE: For example, if the researcher is interested in the effect of prolonged exposure on recovery, the time in ice is a key variable to modify. If researchers are interested in differences in physiology between light and dark colored species, they can choose either two distinctly colored species or modify the insect's wing color to measure the effect of wing color on recovery time.

Importantly, this method is highly customizable to the needs and research questions posed (see the discussion section).

3. Collection of insects

3.1. Collect insects using appropriate methods such as baited traps and entomological nets, (**Supplementary Figure 1**). Upon collection, place each individual in a separate glassine envelope with a unique ID.

3.2. Store animals in a shaded, cool place after being captured and before exposure to the cold shock experiment. Always expose the animal to the experimental treatment within 24 h of being captured, and standardize this time as much as possible across trials.

3.2.1. Although storage conditions may vary, keep the insects out of direct sun. If possible, place them indoors in a cool, dark room.

3.2.2. In the field, ensure that they will be shaded while stored and are protected from wind (blowing away) and other insect predators that may enter the envelopes.

4. Set up the cold shock experiment

4.1. Fill a cooler with ice and water. Ensure that there is sufficient ice to persist for at least one hour, and add ice periodically as needed with the goal to maintain the environment in the water at 0 °C.

4.2. Choose between 1 and 4 focal individuals for a round of experimentation, making sure that each individual is identifiable.

4.2.1 If using multiple species, use only one of each to avoid confusing individuals on the datasheet. If experimenting with only one species, only use individuals that can easily be distinguished, for example by a broken wing or distinct marking.

4.2.2. If the goal of the experiment is unrelated with wing coloration, mark the wings with unique IDs (e.g., numbers) with a fine felt-tipped marker to distinguish individuals.

4.2.3. If the experiments meet neither of the above criteria, conduct the experiment on one individual at a time.

4.3. Populate the rows of the data sheet with the information pertinent to each insect assayed including their unique ID and a useful identifier in the notes such as species name or distinguishing character (**Supplementary Table 1**).

4.4. Place all focal individuals (still in their individually marked envelopes) in a sealed plastic bag with a weight (**Table of Materials**), and place the bag in ice water for 60 min (or until chill

coma has been induced; see discussion) (**Supplementary Figure 2**).

4.4.1. Ensure the weight is heavy (e.g., large coins, large washers, or smooth rocks) and large enough to keep the bag of insects submerged in the ice water and perpendicular to the surface of the water. However, use a weight that does not cause leaks in the sealed plastic bag.

NOTE: While insects are still able to recover if they are exposed directly to the water while submerged, wet envelopes complicate removal of each individual. It is best to maintain the insects dry in their bag.

4.5. Record temperature and light data.

4.5.1. Use a data logger (see the **Table of Materials**) to record ambient temperature and light data.

4.5.1.1. Program the data logger to collect temperature and light data at 10 s intervals, starting at the time that the insects will be released.

4.5.1.2. Base the start time of the data logger on when the insects were placed in the ice water. Ensure that the data logger information (date, time) is synchronized so that data on ambient conditions may later be matched with each individual focal insect.

4.5.2. Use a simple thermometer to record temperature and light data at short intervals by hand (by a second researcher).

4.5.2.1. Decide on the experimental parameters to associate with recovery time that can be measured without a data logger. Use distinct treatments: shade/sun; twilight/mid-day.

4.6. Place a mesh cage for the insects in an appropriate location so that the temperature and light environments are as homogenous as possible within the cage, and so that the base of the cage is elevated and can be tapped by the observer.

4.7. Place the data logger just outside of the cage, or inside the cage so that it will not be knocked over or otherwise affected by small movements inside the cage. If not using a data logger, position a thermometer appropriately and/or set up the cages in the appropriate configuration.

NOTE: The data logger must be placed so that the ambient conditions recorded are as close as possible to those that the insect is experiencing.

5. Start cold shock experiment

5.1. Remove the animals from the ice water bath after 60 min (or time determined appropriate; see above). Immediately remove the insects from the plastic bag, and remove each individual

from its envelope as quickly as possible while minimizing handling (**Supplementary Figure 3**).

5.2. Start the stopwatch as soon as the animals are in the mesh cages (see example data, **Supplementary Table 1**).

5.3. Tap the base of the cage with a pencil to agitate the recovering insects.

NOTE: Providing stimuli during recovery ensures that the focal insects demonstrate recovery status and behaviors as soon as they are physiologically capable **(Supplemental Video**).

5.3.1. Tap frequently and strongly enough to ensure that an animal will respond if possible, but not cause a response.

NOTE: For example, when tapping the cage, if an animal is catapulted into the air and lands upright, but does not move to stand on its own, that is not considered a "stand" behavior, as the organism did not indeed stand on its own.

5.4. Mark the trial as complete once an individual has flown (i.e., shown a full recover). End the trial and consider the insect to have achieved a full recovery if it does not move after 30 min.

5.5. Remove the insects from the mesh cage, and place the individuals back into their labeled glassine envelopes. Liberate the animals or keep them for further data collection (e.g., individual traits of size, weight).

5.6. If using a data logger, stop data logger data collection, and save the file of temperature and light data during the experiment with appropriate date/time information.

6. Data processing

6.1. Enter the data presented in the data sheet into a spreadsheet.

6.2. If using a data logger, add temperature and light data for each response of each individual assayed.

6.2.1. Calculate the mean and standard deviation of the temperature and light for each behavior of each individual.

NOTE: As the data logger logs data every 10 s, if the stand behavior for one animal took 48 s to occur, use the first 5 entries from the data logger for that trial.

6.2.2. Associate each recovery behavior of each individual with abiotic data recorded by the datalogger, rounding up or down to 10 s intervals as necessary.

6.3. Plot and analyze data as desired. Visualize the data, as seen in **Figure 1**, to explore the effects

of temperature and light on cold shock recovery. Compile other relevant data (species traits, regional habitat characteristics) to examine the ecological and evolutionary patterns in physiological traits of the groups tested.

NOTE: **Figure 1** was plotted using the ggplot2 package in R. The level of detail of data on ambient conditions will differ based on the instruments used to measure ambient conditions. If a data logger is used, figures with detail comparable to **Figure 2** can be generated. If a thermometer is used, the researcher will not be able to create a plot informed by ambient light. Likewise, if researchers use categories of light or temperature, these scatterplots can be modified into boxplots or another appropriate template to illustrate these phenomena.

REPRESENTATIVE RESULTS:

The data collected in this protocol allow for examination and partitioning of variables important to organismal physiology. For example, both temperature and light conditions contribute to the recovery of butterflies from cold shock (**Figure 1**). The plot is intended to explore the interaction between ambient conditions and cold shock recover. Using wild-caught butterflies from both traps and netting, 181 species of butterflies demonstrated distinct recovery from chill coma induced by cold shock (**Figure 2**). Data presented in **Figure 2** were collected by three observers over approximately five months (January, February, May–July 2020) in the Colombian Andes. Experiments were always conducted on the morning after butterfly collection. At maximum efficiency, it was possible for two observers to simultaneously observe four butterflies each, repeated seven times (minimum 7.37 hours), resulting in the testing of 56 individuals on a single morning. This allowed for a great deal of data collection across entire butterfly communities while including and considering data on individual variation. As assays can occur under ambient environmental conditions, recovery conditions are representative of their habitats and reflect the natural variation experienced by organisms in nature. **Figure 3** illustrates the overlap between temperature and light conditions of the cold shock recovery experiment and conditions in a pasture from which some tested butterflies were collected.

FIGURE AND TABLE LEGENDS:

Figure 1: Scatterplots of recovery time (in seconds) of butterflies after cold shock. (**A**) Mean temperature and (**B**) mean LUX (light intensity) during their recovery. Species are organized and colored by Family. Overall, as light and temperature increase, cold shock recovery time decreases, showing variability across taxa.

Figure 2: Example of results from the cold shock recovery assay on 181 species of butterfly from the Colombian Andes. The data represent the number of seconds that elapsed from removing the butterfly from cold and when it was able to fly. Species are organized and colored by Family. This figure demonstrates the taxonomic breadth across which this experiment can be successfully applied, and the variety of cold shock recovery responses across species.

Figure 3: Ambient temperature and LUX during cold shock recovery trials. Plot of ambient temperature (blue) and LUX (light intensity, red) as recorded by data loggers placed in the

pastures where butterfly collection took place (light colors, conditions span entire day) and conditions during cold shock recovery trials (dark colors, only morning hours). The ambient field conditions and experimental conditions plotted show the range of and average conditions experienced by butterflies over one week of field sampling and experimentation. Experiments were only conducted in early hours (07:00–13:00 h), while the dataloggers were deployed in the field for one week (daylight hours, 06:00–18:00 h shown). Shown here is the overlap between experimental conditions and ambient conditions experienced by butterflies, demonstrating the ecological relevancy of conducting physiology assays under ambient conditions.

Supplementary Figure 1: Procedure for collecting focal insects—in this case, butterflies—using baited Van-Someren traps and active netting. Traps were baited with both rotting fish and rotting fruit baits. Trap (without bait) in background, in the foreground is a specimen in its unique envelope against a blue plastic collection box.

Supplementary Figure 2: Bags with up to four individual butterflies submerged in ice water in a cooler. Plastic bags were marked with the time they were placed in the ice water, so that cold shock experiments could be staggered through the morning. Plastic bags should be sealed to prevent specimens from getting wet; however, flooding of the bags and envelope in this case had no measurable effect on the recovery of the butterflies.

Supplementary Figure 3: Two observers collect data in the field. Each mesh cage contains four unique butterflies recovering from cold shock. The polyvinyl chloride T-joint in the cage houses the data logger to prevent direct sun or rain exposure. Each observer has a stopwatch that was started immediately upon butterfly release into the cage. The cages are elevated by benches, permitting observers to agitate the base of the cage to ensure that the butterflies responded behaviorally as quickly as physiologically possible.

Supplementary Table 1: Example data sheet. The sheet shows each butterfly's unique ID as assigned in the field and distinguishing characters (species name, key colors) in notes. Also recorded is the dominant position of the butterfly (i.e., which side of the wing was exposed to the sun) during the recovery period, noted as D (dorsal) or V (ventral).

Supplemental Video 1: Tapping of the cage for cold shock recovery. As butterflies recover, the observer taps the base of the cage gently to induce behaviors as soon as the butterflies are capable.

DISCUSSION:

The study of thermal physiology incorporates measures of species and environmental traits to better understand the interactions between organisms and their surroundings that are key to survival and fitness. While always integral to understanding the natural history and ecology of plants and animals, thermal traits are of increasing importance in the face of landscape and climate change[11,21]. Several groups of ectothermic terrestrial insects, in particular, lepidoptera and odonatan, are relatively large and abundant, exhibit distinct behaviors, and are amenable to manipulation. Outlined here is an efficient and low-cost assay to effectively measure

physiological responses of such insects. This protocol requires a source of healthy organisms to assay, whose handling time prior to the experiment is limited. While flexible in the number of organisms assayed at one time, the number of focal individuals per experiment will vary based on the purpose of data collection and/or number of observers.

For example, this protocol was developed to collect detailed individual data on butterflies across entire communities. As such, the representative results illustrate an effort to maximize the data collection for individuals of as many species as possible and under a variety of conditions relevant to the local environment. Regardless of the number of focal species, it is crucial for the observer to be able to identify each individual in the cage experiencing the recovery. If the goal is to collect data from only one species, then only one or two individuals (if identifiable based on different wing wear or if individually marked) should be assayed at once. The study subjects must be chosen in accordance with a specific research question or plan of study. Based on the question posed and the purpose of data collection (research or classroom, for example), sample size and collection of other traits will differ.

To illustrate the fundamental components of physiology elucidated by this protocol (induction of chill coma, steps of recovery, role of ambient conditions), a classroom instructor may choose two distinct species or morphs of a single species. If the focal individuals differ only in one key trait (e.g., color), a smaller sample size will be necessary, and students can closely study the relationship of that trait and organismal physiology. Researchers interested in ecological physiology may use their experimental data to explore complex ecological and evolutionary questions. Researchers must be sure to carefully choose focal insects that directly address their questions (e.g., based on life stage, age, sex, location), and, based on the number of variables involved, determine the appropriate sample size. Sample sizes for complex models will be larger than those described above.

While collecting behavioral recovery data, it is key that the cage rest above the ground because the observer must be able to tap the bottom of the cage to elicit recovery behaviors. This ensures that the organism responds (stands, flies) as soon as it is physiologically capable of doing so, and the terminal recovery behavior (flight) is documented. Recording ambient conditions during the cold shock recovery is integral to the study of thermal physiology, as this protocol is designed to study and disentangle the role of environment in organismal physiology. Data loggers (see the **Table of Materials**) are useful to record standardized measures of relevant conditions (e.g., temperature, light, and even humidity). However, if these tools are unavailable, relevant conditions can be measured in other ways like with a digital thermometer or by simplifying the variable of environmental conditions and using distinct environments such as shade and sun. This protocol gives the researcher options to measure the conditions during cold shock recovery based on the purpose and scope of the study.

Although this method can be modified to better suit specific taxonomic groups, it is recommended that large, volant insects be used. Flying insects that regain their ability to fly independently may be considered to have accomplished a full recovery. The method, as described, was successfully used on butterflies in the tropics and subtropics. Based on the

thermal trends of a given area (i.e., the range of temperatures experienced at a site that will vary, thus influencing expectations based on elevation, latitude, canopy cover), an organism may require more or less than one hour in an ice water bath to enter a chill coma. The size of the organism may also affect the time necessary to enter a chill coma. It is key to find the time of cold exposure necessary to induce a chill coma (not moving), but not kill focal species. The time required to induce a chill coma will depend on the size, location, and natural history/behavior of the individuals. Based on results from the cold shock experiment described herein and using knowledge of the ecology of the focal insects, choose a time at which to conclude the trial if a given individual does not make a full recovery.

Based on the specific questions of the researcher, this method can be employed either in the field or the laboratory to allow for both natural environmental variation and control for important variables, respectively. This assay is simple and inexpensive and helps to fill existing gaps in the field of thermal physiology. The ease of this protocol makes it accessible to employ for a diverse array of taxa, opening the field to more than lab-friendly organisms. The novelty of performing a standardized yet ambient thermal assay fills the gap between laboratory and field results[22]. Leveraging ambient conditions for organism recovery will help researchers partition the role of environmental and species factors in physiology[14,22]. Finally, because of its low cost and lack of required materials, this protocol can be used in remote locations in the field with little equipment—ideal for many field biologists—as well as in classrooms to allow young students a hands-on learning experience.

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DISCLOSURES:

The author has no competing financial interests or other conflicts of interests. .

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487

Figure 1

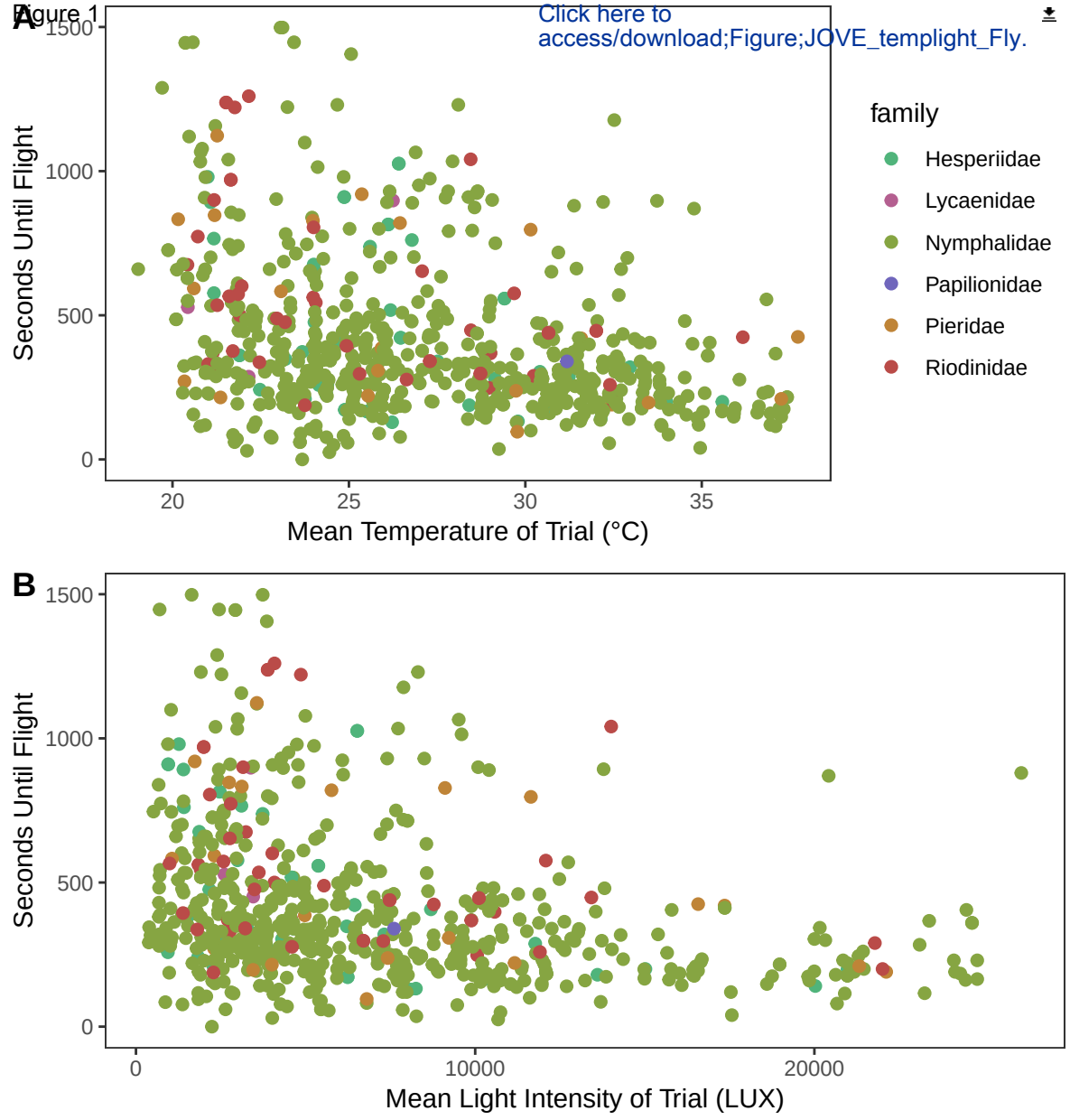


Figure 2

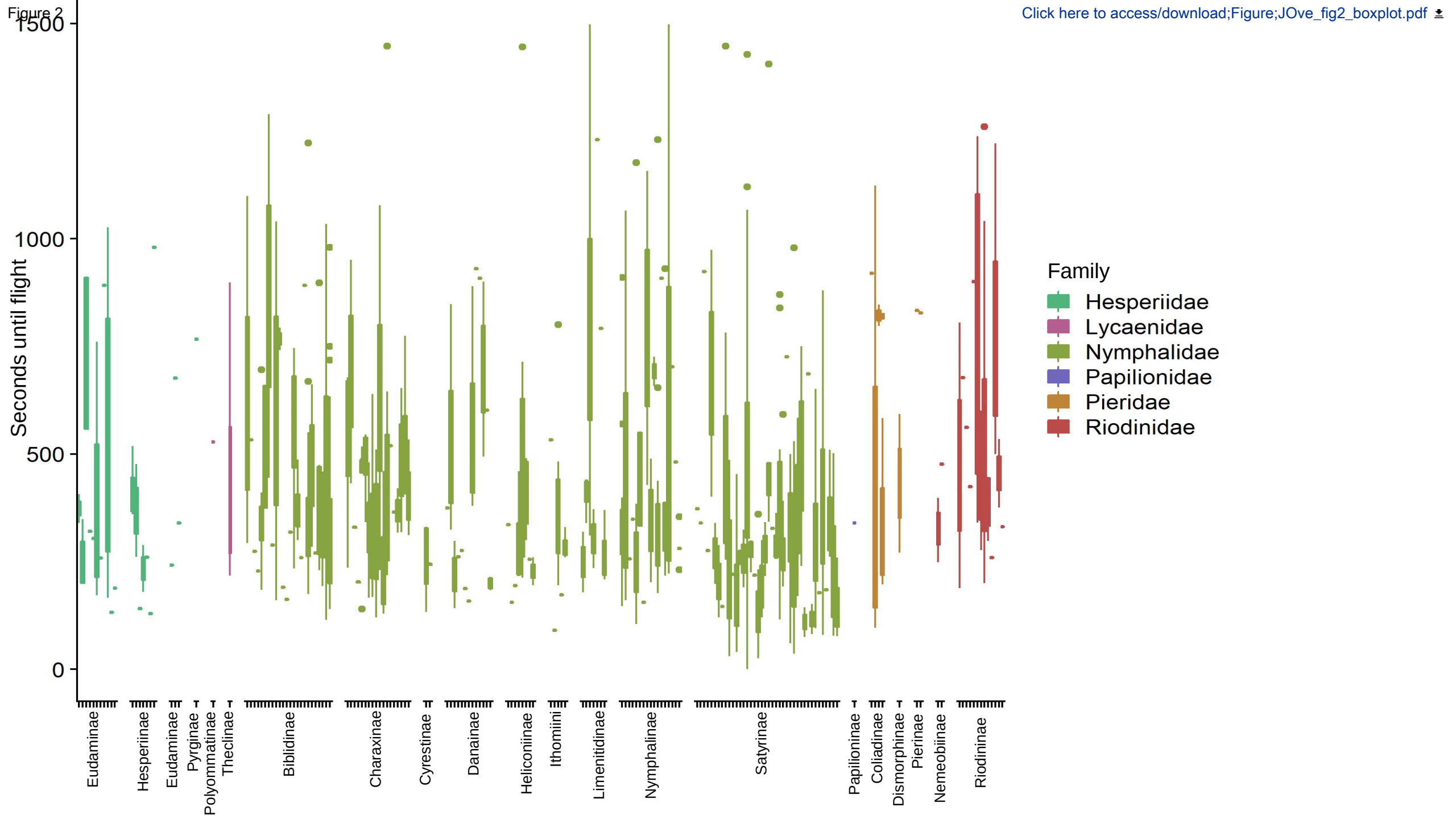
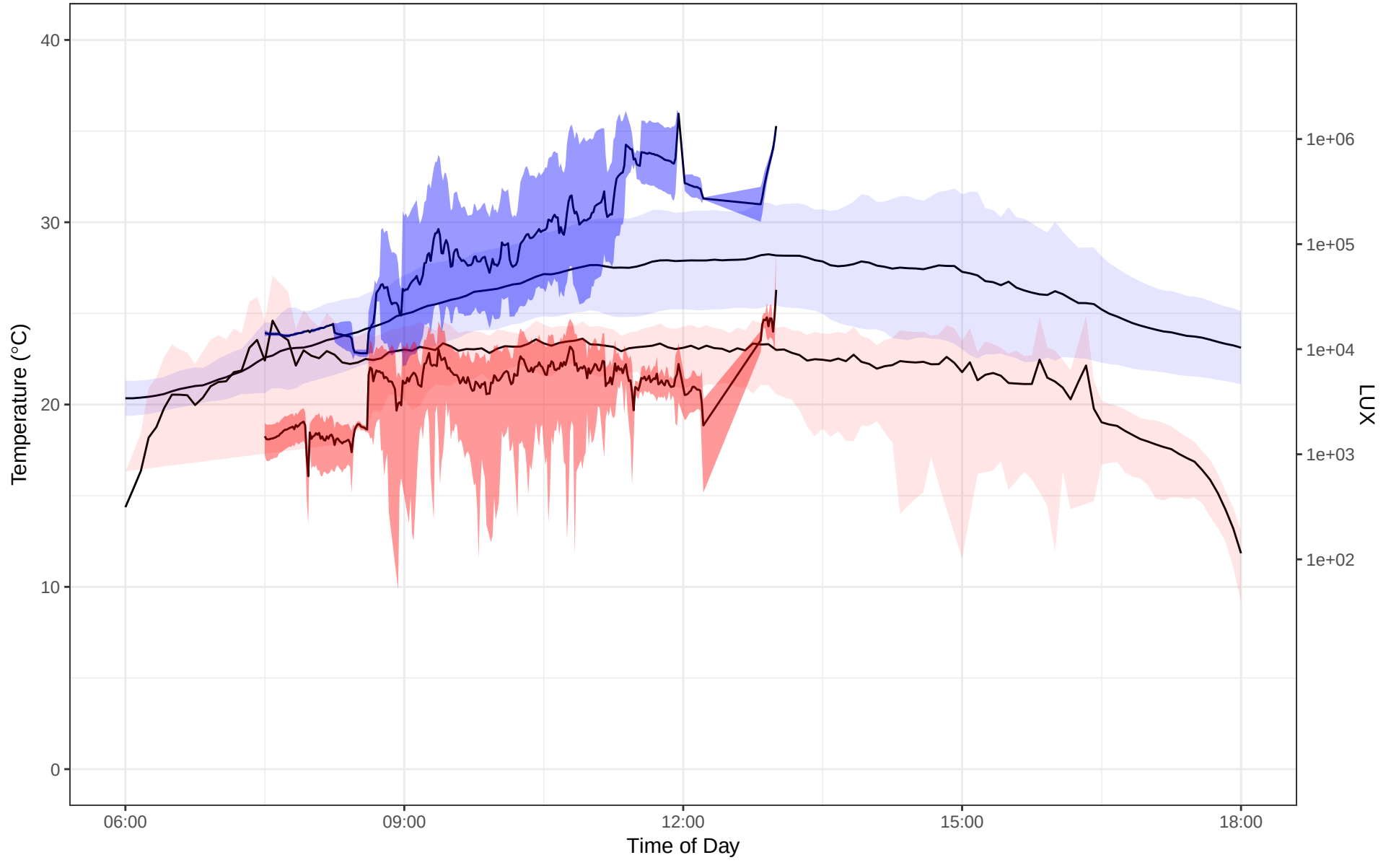


Figure 3

Ambient Conditions in a Pasture and During Experimentation



Name of Material/ Equipment	Company	Catalog Number	Comments/Description
24 x 24 x 36" Popup Rearing & Observation Cage	Bioquip	1466PB	Ensure that the cage is slightly elevated from the ground to be able to tap the floor of the cage during experiments.
Cooler	Any	NA	
Glassine envelopes	Bioquip	1130B	
HOBO Pendant Temperature/Light 8K Data Logger	Onset	UA-002-08	If a datalogger is not accessible, researchers may choose to use a digital thermometer to record ambient temperatures at regular intervals. See protocol step 4.5 for additional information.
HOBO Optic USB Base Station	Onset	Base-U-1	
Ice water	NA	NA	
Insects (focal taxa)	NA	Any	Collect sufficient samples to test, ensuring replication of experimental groups (e.g. species, sampling location)
PVC T-joint	Any	Any	
Sealable plastic bag	Any	NA	
Stopwatch/timer	Any	NA	
Weight	Any	NA	Large coins or small rocks to weigh down the plastic bags will ensure that specimens are submerged in ice water. A standardized weight is ideal.

Dear Dr. Iyer,

Thank you and the two anonymous reviewers for helpful comments and edits to my manuscript. I have made significant edits, as seen in the manuscript file, that address these comments. I am confident that this version of the manuscript and protocol are improved from the original, and I appreciate your reconsideration of this paper.

Below you will find responses to reviewer comments explaining my action (or inaction) based on the suggestions, as well as the edited components of the manuscript that address reviewer concerns.

Thank you again for your time and reconsideration of this manuscript.

Best regards,

Emily Khazan

Please note that the reviewers raised some significant concerns regarding your method and your manuscript. Please revise the manuscript to thoroughly address these concerns. Additionally, please describe the changes that have been made or provide explanations if the comment is not addressed in a rebuttal letter. We may send the revised manuscript and the rebuttal letter back to peer review.

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

The manuscript has been proofread by the author and several colleagues to check for and correct grammar and spelling issues.

2. Please sort the Materials Table alphabetically by the name of the material.

The items are now in alphabetical order.

3. Please avoid the use of personal pronouns (I, etc.).

Personal pronouns have been removed.

4. For in-text formatting, corresponding reference numbers should appear as numbered superscripts after the appropriate statement(s).

I have changed the citation format throughout the manuscript.

5. What insects are used in the experiment. Please present a specific experiment with specific insects.

The summary statement has been modified to state that butterflies were use. I have edited the introduction and discussion to communicate that butterflies were used in the experiment described for which I have obtained representative results. I have attempted to be clearer throughout the manuscript, that while these experiments were performed on butterflies, other large, terrestrial, flying insects (e.g. dragonflies, large beetles, etc.) are appropriate test subjects for this protocol.

6. Please highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

The appropriate steps most integral to the success of this protocol have been highlighted.

7. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.

8. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

9. The color codes for Hesperidae and Nymphalidae are not very distinct. One of these could be changed for improved clarity.

I have changed the color palette which should help – I chose a palette which should be distinguishable by people with color blindness.

10. Please check the labels on the X-axis of Figure 1. Some labels are not legible. Please edit them.

These labels have been edited for legibility.

11. Figure 3: A color-coded legend for temperature and LUX could be included on the graph itself for additional clarity.

The figure legend has been edited to the following to make this figure clearer: "Ambient Temperature (blue) and LUX (light intensity, red) during cold shock recovery trials (dark colors, only morning hours) and daily conditions from a pasture from which butterflies were collected (light colors). recorded from the datalogger in the butterfly recovery enclosure (dark colors) and in a pasture from which butterflies were collected (lighter color). Experiments were only conducted in early hours (07:00-13:00), while the dataloggers were deployed in the field for one week (daylight hours, 06:00-18:00 shown). Shown here is the overlap between experimental conditions and ambient conditions experienced by butterflies, demonstrating the ecological relevancy of conducting physiology assays under ambient conditions. Environmental data in the field were collected for other studies. "

12. In the Disclosures section, please include a statement providing information regarding the authors' competing financial interests or other conflicts of interest. If authors have no competing financial interests, then a statement indicating no competing financial interests must be included.

The disclosure section has been edited to incorporate this statement.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

In this manuscript a method for assaying cold shock recovery for insects is described. It requires only simple tools and equipment, and is thus suitable for field-work, class-rooms and modestly equipped laboratories. Overall I like this method, but do have a number of suggestions for the author to consider.

Major Concerns:

My first major concern regards placing the butterflies in glassine envelopes for up to 24 hours. In my hands butterflies suffer when kept like this, and I wonder if this may lead to undue stress that could affect the subsequent cold recovery process and would recommend that:
1) The envelopes are replaced with small net-covered jars, where the animals can sit in a natural position.

Based on my experience and that of my collaborators, butterflies and odonates survive and fare much better when stored flat and with their motion restricted by glassine envelopes. They do not struggle and therefore damage their wings less and lose fewer scales than if they were able to fly. The survival rate over the 24 hour period when stored in envelopes in a cool, shady place was extremely high for all groups, with skippers having the lowest survivorship (likely due to their morphology and highly active and strong flight as well as their higher propensity to struggle within the envelope).

2) That the effect of the envelope is tested against a comparable group not in an envelope (does it affect the chill coma recover time?)

As all individuals are placed in an envelope, any effect of the envelope is shared across all tested individuals. While the envelope likely has a slight insulating effect around the focal insect, the goal of the cold shock, i.e. onset of chill coma, is met. Removing the envelope, or other protective barrier from the water, would result in the insects being submerged under water which would pose additional and distinct physiological distress as gas exchange would be more limited.

3) Or at the very least, that time in the envelope is added as a confounding factor to any statistical analysis (as a covariate)

This variable could definitely be explored, for example by testing individuals immediately in the field, after 12 hours in an envelope, 24 hours, etc. As written, the protocol allows for that time to be flexible, but suggests standardization, thus inviting experiments such as that suggested by this reviewer. I have added this phrase to the protocol (1.1.i.1) to clarify that the time required for onset of a chill coma will vary, and the experimenter must take this into account when deciding for how long to keep the insects cold. "The time required to induce a chill coma will depend on the size, location, and natural history/behavior of the individuals."

My second concern is the temperature of the ice-slurry. My experience is that ice-water can vary somewhat in temperature, and since this is the main determinant of cold treatment, it would be good to know what the exact temperature is. My recommendation is that the temp-logger is placed into a plastic bag and added to the ice slurry. Perhaps it can even be moved with the samples to the cage, upon removal. The true temperature can be added as covariate to the statistical model.

The goal of the cold exposure is to induce chill coma on the insects, and to apply cold as consistently and equally as possible across all individual data points.

My third major concern is large variation in temperature and light that seems to influence the recovery time. This finding in itself is no surprise, and while I like the idea of letting them recover in ambient conditions, the large variation seen in the response variable (in figures 1 and 2) that seems to be partly the consequence of ambient recovery conditions, seems to override the among-species differences (the large variation might also be due to the first two concerns). Thus the method requires very large sample sizes in order for any general conclusions to be drawn. As such, it is not very effective. I wonder if it would be better to at least shade the cage to minimize variation in ambient light conditions, for a more stable surrounding. It seems from Figure 3 that the cage actually elevates temperature above the ambient. Even better would be to do it indoors, or in an AC-controlled room, but I understand this is not easily done in the field (but certainly in a classroom).

This method's required sample size depends on the base research question being posed by the experimenter. The data shown here are representative data across butterfly communities, however a researcher may choose to compare only two or three different species with vastly distinct morphologies or life histories. The following has been added to the protocol (1.2.1, 1.2.2) to highlight the flexibility and utility of this protocol to tackle a variety of ecological, behavioral, and physiological research questions: "1.2.1 Define specific research questions to be answered with these data while refining parameters. For

example, if the researcher is interested in the effect of prolonged exposure on recovery, the time in ice is a key variable to modify.

1.21.2.2 If researchers are interested in differences in physiology between light and dark colored species, for example, they can choose either two distinctly colored species, or modify the insect's wing color to measure the effect of wing color on recovery time. Importantly, this method is highly customizable to the needs and research questions posed."

As stated throughout the protocol, the procedure is extremely flexible to meet the needs of the question posed. Therefore, if a researcher chooses to test the difference in recovery time under ambient conditions and shaded conditions, that is easily done in accordance with the methods outlined. Here I propose the collection of several variables in order to place the physiological trait data obtained into ecological context. Researchers may choose to use the data to directly compare single metrics (e.g. recovery time, time between recovery behaviors), or to create more complex statistical models to explore the interplay between environmental and species traits (e.g. association between ambient temperature and wing area).

Minor Concerns:
Throughout there are some minor issues with reference formatting. Please check carefully.
On line 52 it is said that classical thermal performance methods are not suitable for studying animals of varying sizes. I find this a strange statement - we use them to study animals between 1 mg and 1 kg. What is this statement based on?

Thank you for pointing out the lack of clarity in this sentence. The purpose was to communicate that many methods use small vials in which behaviors can be observed for small organisms like certain beetles and flies, but which would be unsuitable for organisms like butterflies and odonates. The phrase has been altered to "2) are not suitable for assaying animals of varying size too large to be contained in customary vials used for small dipterans,".

On line 58 consider replacing first "is" with "can be"

This change was made.

On line 79 chill coma is first mentioned. Since this is not a standard term, I strongly recommend that you introduce the term and define it clearly, already in the introduction as the trait you will study here.

The following has been added in the first step of the protocol to define chill coma: Each group will differ in the time it takes to induce a chill coma (i.e. the point at which the insect is still alive, but is not moving and not responsive).

On lines 117-119 I can suggest marking the wings with a permanent marker. Works wonders in most lepidopterans, with very little negative consequences for performance.

The following has been added: 3.2.3 If the goal of the experiment is unrelated with wing coloration, the researcher can mark wings with unique IDs (e.g. numbers, dots) with a fine felt-tipped marker to distinguish individuals.

On line 138 the logger is introduced for the first time. Please expand a bit and describe what type of logger you recommend using (i.e. a temp and light logger).
On line 185 Figure 3 is introduced as the first of the proper figures. Please reorganize so the figures appear in the correct order (1, 2, 3)
Figure 1, please add axis legend to y-axis and re-structure so taxa are visible

This has been adjusted as described above.

Reviewer #2:
Manuscript Summary:
The manuscript "Field-Based Thermal Physiology Assay: Cold Shock Recovery Under Ambient Conditions" submitted by Emily Kazhan aims to describe a genereral protocol to study the physiology upon cold shock in terrestrial insects. While the goal was set to be a general protocol, the manuscript mainly describes a study on more than 180 species of butterflies. My main concern is, that while the methods itself are rather well described and certainly useful for researchers doing similar work, they cannot be easily generalized. Therefore, I would suggest to tone done the claim to provide a protocol for all insects possible into a more specific one. Below I give some more suggestions that hopefully help the author to carve out a clearer manuscript.

Major concerns:
1)
Abstract
In the abstract, the author describes the protocol as consisting of methods that can generate detailed data on individual behavior and physiology of "terrestrial ectothermic insects". After reading the manuscript, I would definitely not go such far. The described methods are to my understanding probably well suited for larger insects capable of flying such as species from the Lepidoptera, Odonata or Mecoptera, for example. The protocol cannot, in my opinion, directly be adapted for smaller insects capable of flying (e.g. Diptera such as Drosophila or Anopheles) or insects that are not capable of flying or do not do so regularly. Therefore, I suggest to be clearer with the promise of the study and restrict to specific orders instead of claiming it works for basically all terrestrial insects.

I agree that this protocol best works with larger insects with more visible, obvious behaviors (hence highlighting odonatan and lepidoptera throughout). The abstract has been edited to read that the protocol focuses on "large, volant, terrestrial insects". After conducting many of these experiments, as well as using more classical methods of small insects (e.g. mosquitoes) in vials in a water bath, I am confident that a researcher could apply this method to small insects as well. This would require a smaller cage and, perhaps, a keener eye!

2)
Introduction
In the introduction, the author outlines a comparison of laboratory-based analyses to gain insight into the physiology of insects via complete thermal performance curves and the quantification of fewer and simpler thermal metrics. Specifically, a cold shock assay is described that can be performed in the field with few, inexpensive equipment. For a reader who is not directly from the field, the introduction reads already very specific. I would ask the author to be clearer: How is the "thermal performance" defined? Which equipment is "always" used making it expensive? What are "classic methods" of assessing thermal performance? What are "basic tools"?
I appreciate the attempt to provide a protocol that works in the field, but the introduction could be clearer.

Thank you for highlighting some gaps in the introduction and ways to make this more accessible to those less familiar with ecological physiology. I have edited the penultimate paragraph of the introduction to contain more information about traditional water-bath physiology experiments. I have also added several citations in this paragraph which direct discuss specific aspects of thermal physiology of ectotherms to enrich the reader's understanding.

3)
Protocol
General comment:
I would be very careful with the experimental design depending on the details of the research question. I think the protocol could well be useful for teaching purposes and I would make clear which individual features have to be taken care of BEFORE the start of the experiment (e.g. size of individual, stage/age, gender...). In addition, how many replicates, what are the replicates (individuals, groups of individuals),

what affects the experiment? (different days, observers...).
I could also imagine to divide the protocol in 2 sections:
a) General instructions (basically the protocol as it is with some adaptations)
b) Recommendations on how the protocol could be adapted for teaching purposes. I consider the possibility of having a protocol for the classroom at hand as very appealing. However, I assume that researchers interested in the protocol ask for different details as students aiming to gain practice. In addition, the number of individuals to assess is probably different in both cases.

The following has been added to the first step of the protocol to more clearly outline this important point: "1.2.1.1. To illustrate the fundamental components of physiology elucidated by this protocol (induction of chill coma, steps of recovery, role of ambient conditions), a classroom instructor may choose two distinct species or morphs of a single species. If the focal individuals differ only in one key trait (e.g. color), a smaller sample size will be necessary, and students can closely study the relationship of that trait and organismal physiology.

1.2.1.2. Researchers interested in ecological physiology may use their experimental data to explore complex ecological and evolutionary questions. Researchers must be sure to carefully chose focal insects that directly address their questions (e.g. based on life stage, age, sex, location), and, based on the number of variables involved, determine the appropriate sample size. Sample sizes for complex models will be larger than those described above."

line 71: I agree that the protocol can be applied for insects that 'can be stored flat in glassine envelopes', but I would not go such far to include all 'terrestrial insects'. I suggest to focus on Lepidoptera and similar-sized insects capable of flying and make this clear in the protocol. It would still be very interesting for other scientists but would not promise too much.

I agree and have changed the text to focus on large, volant insects (e.g. lepidoptera, odonatan) for which I am confident this method works.

line 138: I am not entirely sure what a 'data logger' is. Could the author describe it a bit more or include a picture?

I have included information on the data logger (that it measures ambient light and temperature conditions at pre-determined intervals) throughout the manuscript. Additionally, I added an option to not use a data logger, and instead use just a thermometer, or distinct categories of experimental conditions. The purpose of this protocol is for it to 1) be accessible to researchers and students anywhere and 2) generate data on physiological traits including cold shock recovery under ambient conditions relevant to the ecology of an organism. That is, the method generates ecologically relevant data by way of an accessible, affordable approach. As some schools and researchers may not have access to data loggers, I add an option to generate similarly relevant data using more rudimentary tools.

"4.5 Option 1: Program the data logger (see list of materials) to collect ambient temperature and light data at 10 second intervals, starting at the time that the insects will be released.

4.5.1 Base start time of the data logger on when the insects were placed in the ice water.

Ensure that the data logger information (date, time) are synchronized so that data on ambient conditions may later be matched with each individual focal insect.

Option 2: If not using a data logger to record temperature and light data, the researcher may choose to test insects in two distinct locations (e.g. shaded, unshaded).

4.5.2 Decide on the experimental parameters to associate with recovery time that can be measured without a data logger. A simple thermometer may be used to record ambient temperature at short intervals by hand (by a second researcher) during the experiment, or distinct treatments could be used (e.g. shade/sun; twilight/mid-day). "

This issue is further broached in the discussion, "Recording ambient conditions during the cold shock recovery is integral to the study of thermal physiology, as this protocol is designed to study and disentangle the role of environment in organismal physiology. Data loggers (see list of materials) are useful to record standardized measures of relevant conditions (e.g. temperature, light, and even humidity). However, if these tools are unavailable, relevant conditions can be measured in other ways like with a digital thermometer, or by simplifying the variable of environmental conditions and using distinct environments like shade and sun. This protocol gives the researcher options as to how to measure the conditions during cold shock recovery based on the purpose and scope of the study."

line 184: I think, instructions to plot and visualize data can also be an example in a general protocol like the one provided. However, I would appreciate a more detailed description of the figures in the results and perhaps a supplement on the source code for the figures. Were they done using R or Excel or anything different?

I have added the following to the protocol under step 6 to address this concern

"6.3.1.1 This figure was made using the ggplot2 package in R. Scatterplots like that shown can be made in excel, or even by hand (with young school children, for example). The plot is intended to explore the interaction between ambient conditions and cold shock recover. The level of detail of data on ambient conditions will be differ based on the instruments used to measure ambient conditions. If a data logger that measures ambient temperature and light is used, figures with detail comparable to Figure 2 can be generated. If a thermometer is used, the researcher will not be able to create a plot informed by ambient light. Likewise, if researchers use categories of light or temperature, these scatterplots can be modified into boxplots, or another appropriate template to illustrate these phenomena."

Representative results
General questions:
How many individuals were measured? What are the take-home messages of the presented figures? My guess would be:
Fig 1: Great variation, but no significant difference between species - were there any statistical tests?

The data shown here are representative data across butterfly communities and were used in model selection analyses in a forthcoming paper. As such, this figure serves as a representation of the taxonomic breadth for which this protocol was used, and the degree of variation (or lack thereof) of col shock recovery within species. I have modified the figure caption to better explain the purpose of the figure: "Example of results from the cold shock recovery assay on 181 species of butterfly from the Colombian Andes demonstrating the number of seconds that elapsed from removing the butterfly from cold and when it was able to fly. Species are organized and colored by Family. This demonstrates the taxonomic breadth on which this experiment can be successfully applied, and the variety of cold shock recovery responses across species."

A researcher may choose to compare only two or three different species with vastly distinct morphologies or life histories. The following has been added to the protocol (1.2.1, 1.2.2) to highlight the flexibility and utility of this protocol to tackle a variety of ecological, behavioral, and physiological research questions: "1.2.1 Define specific research questions to be answered with these data while refining parameters. For example, if the researcher is interested in the effect of prolonged exposure on recovery, the time in ice is a key variable to modify.

1.21.2.2 If researchers are interested in differences in physiology between light and dark colored species, for example, they can choose either two distinctly colored species, or modify the insect's wing color to measure the effect of wing color on recovery time. Importantly, this method is highly customizable to the needs and research questions posed."

Fig 2: I do not really understand what is shown here. A negative correlation between temperature and light condition with respect to recovery?

This figure shows the raw cold shock recovery per species (colored by family) data within the context of ambient light and temperature conditions. The figure illustrates roughly the role of temperature and light in the recovery of butterflies. If, as stated earlier, the researcher were to use two species with distinct coloration, for example, they may find strong patterns in the effect of light on recovery time.

Fig 3: Main take-home: It is getting warmer and lighter during the morning until mid-day?
I would suggest to describe the results a bit more and discuss (briefly) what they mean. I guess for a protocol like this it is okay to be very broad, but I would appreciate to have a useful set of examples what can be achieved by using the protocol at hand.

The purpose of this figure is to demonstrate the ecological relevancy of the method – i.e. the animals are being tested/recovering from a cold shock under ambient conditions that they would experience in their natural environment. This differs from traditional assays which measure recovery under standardized lab conditions that rarely, if ever, reflect natural ambient conditions relevant to the study organism. The figure legend has been modified to facilitate understanding of the figure (see above)

Supplemental Figure 1

Click here to access/download;Supplemental File (Figures, Permissions, etc.);Jove supplement fig 1.jpg

Supplemental Figure 2

Click here to access/download;Supplemental File (Figures, Permissions, etc.);jove supp fig 2.jpg

Supplemental Figure 3

Click here to access/download;Supplemental File (Figures, Permissions, etc.);jove sup fig 3.jpg

Supplementary Table 1

Date	ID	Data logger	First movement	Stand	Open Wing	Shiver	Fly	Posture (D/V)	Note
									D. plexippus
21/7/2020	1	A1	32	58	1:12	-	3:10	D	Heavy wing wear
21/7/2020	2	A1	3	27	-	-	1:15	V	Fiery Skipper

Supplemental Video 1

Click here to access/download
Supplemental Coding Files
IMG_1712.MOV