Molecular Ecology Lab Functional Ecology Protocols

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Contents

NOTES ON STANDARDIZATION

These notes are modified from:

Moretti *et al.* (2016), Handbook of protocols for standardized measurement of terrestrial invertebrate functional traits. Functional Ecology. doi:10.1111/1365-2435.12776

Animal and site descriptions

Moretti *et al.* (2016) recommend that results of functional ecology experiments be reported with information on:

- Latitude, longitude, and elevation of the individuals measured (when collecting animals from the field)
- Average minimum, mean, and maximum temperatures, and annual precipitation of the site where the animals have been sampled. This is often unrealistic, but any description of the climate is useful.
- Developmental stage, and sex where possible.

Acclimation

From Moretti *et al*: "Some trait values may depend on the immediate conditions an organism is subjected to at the place or time of collection. In these cases, to achieve standardized trait measurements it is necessary to provide the same conditions for all individuals measured."

- Before measurements, individuals should be acclimated to a common environment for a period of time to minimize effects of prior environmental conditions.
- The period of time will vary among species, but should usually be at least 1 week; for *Nebria* and *Grylloblatta*, we usually use 2 – 4 weeks.
- Moretti *et al* recommend standard conditions of 20°C. This is unrealistic for *Nebria* and *Grylloblatta* but, when preparing a manuscript for publication, keep in mind that reviewers might be expecting a 20°C standard and deviations from this should be explained.
- Report the acclimation treatment, along with the light:dark cycle employed in all publications.
- Static, rather than fluctuating temperatures are recommended for the acclimation treatment.
- Animals should be provided with *ad libitum* food and water throughout the acclimation period.
- **Reporting exactly what was done is the most important thing.**

EQUIPMENT AND SOFTWARE

Water bath and LabWise

Setting up and maintaining the water bath

The water bath is filled with a 1:1 propylene glycol:water solution, to prevent the liquid freezing at low temperatures. It *will* freeze, into one giant ice cube, if there isn't enough propylene glycol. The bath itself holds 5 L, and the ice bath holds about 1 L. It's easiest to make extra solution, and then just top it up as needed. If the liquid accumulates a lot of debris, the water bath should be emptied and the liquid either replaced or filtered to get the debris out.

Creating a program

Open up the program LabWise, once open click programs on the upper left hand corner. Then from the new window that opens up:

File –> New

Programs can have up to 30 segments with different temperatures and ramping rates. Set the temperature using "Target Temp" and, if a temperature ramping is required, set the target rate. On the right hand side, you can change what the program does on completion ("On Program Completion" box). The default setting, "Return to Isothermal" will return the bath to the temperature specified on the controller (see Controlling the bath without the software). Make sure that this is set to an appropriate temperature. With our water bath, the pump speed cannot be changed. See the LabWise manual for more detailed instructions on creating a program.

Running a program

Open up the program LabWise, once open click "Programs" on the upper left hand corner, then:

Bath –> Send to bath

After this, on the first LabWise page go to the lower right hand corner and click on the dropdown menu below the word "Program" and select the name of the program that you previously sent to the bath. This will start the program running.

Controlling the bath without the software

The water bath can be controlled manually via a knob on the center of the circle below the screen (see image below). First, press the grey button to the right of the knob once, then turn the knob to the temperature you want and press the grey button again. This method is helpful for setting a constant temperature in preparation for carrying out a run.

Measuring temperature

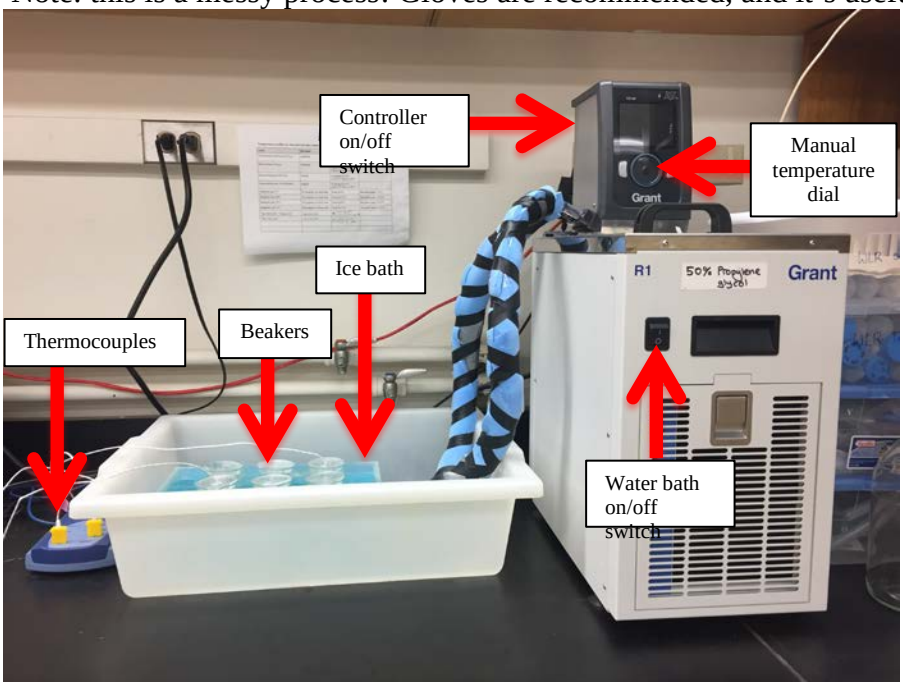
Temperature is measured by two methods. The ice bath screen will display a temperature that is the same as the one in the LabWise program.

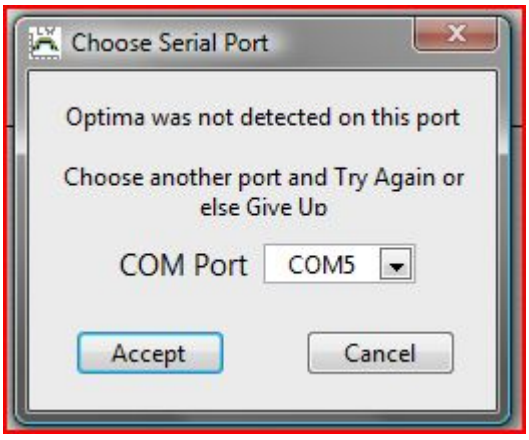
However for a more accurate temperature, two thermocouples are placed inside of two beakers (see image below). These thermocouples are what input the data for the Picolog recorder.

Changing the attachments

Most of the time, the water bath will be attached to the ice bath. The water bath can be detached from the ice bath completely and be used as a self-contained system (useful for measuring supercooling point), or be attached to something different. First, disconnect the inflow/outflow hoses at the ice bath end. Make sure that the ice bath is inside a tub with at least 6 L capacity. Let the ice bath fully drain into the tub, and let fluid drain out of the water bath until it stops. Once there is no fluid running through the hoses, these can be disconnected. If you want to use the water bath on its own, swap the connector plate (where the hoses were attached) for the solid plate (without hose attachment points). Otherwise, attach new hoses and whatever apparatus you want to attach, then pour the propylene glycol back into the water bath.

*Note: this is a messy process! Gloves are recommended, and it's useful to have a spare tub nearby.





*Note: sometimes if the water bath is not turned on before LabWise is turned on, this message appears. If this is the case, you can exit out of LabWise, turn on the bath and then reopen it.

Programs currently used for thermal tolerance and other assays

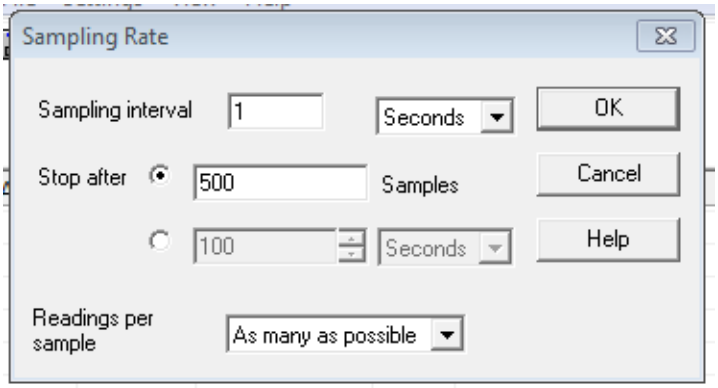
Assay	Program file name	Temperature profile	Notes
Nebria & ice crawler cold tolerance (chill coma, CT_{min})	coldstress	10 min @ 4°C, ↓ @ 0.2°C/min to -15°C	
Nebria & ice crawler heat tolerance (CT_{max})	heatstress	10 min @ 4°C, ↑ @ 0.5 °C/min to 20°C, ↑ @ 0.2 °C/min to 40°C	
Supercooling point (*Nebria*)	scpneb	10 min @ 4°C, ↓ @ 0.2°C/min to -15°C	
Supercooling point (*Grylloblatta*)	scpgryl	10 min @ 4°C, ↓ @ 0.2°C/min to -10°C	
Metabolic rate 5°C	No program; set from bath.	Fixed @ 0°C	Recorded temp = 5.8°C
Metabolic rate 10°C	No program; set from bath.	Fixed @ 5.5°C	Recorded temp = 10.2°C
Metabolic rate 12°C	No program; set from bath.	Fixed @ 9.5°	Recorded temp = 12.5°C
Metabolic rate 15°C	No program; set from bath.	Fixed @ 12°C	Recorded temp = 15.0°C
Termite cold tolerance	termiteCold	10 min @ 20°C, ↓ @ 0.2°C/min to -15°C	
Termite SCP	termiteSCP	10 min @ 20°C, ↓ @ 0.5°C/min to 10°C, ↓ @ 0.2°C/min to -15°C	

Picolog

Sampling settings

Open PLW Recorder. To change the sampling settings:

Settings –> Sampling

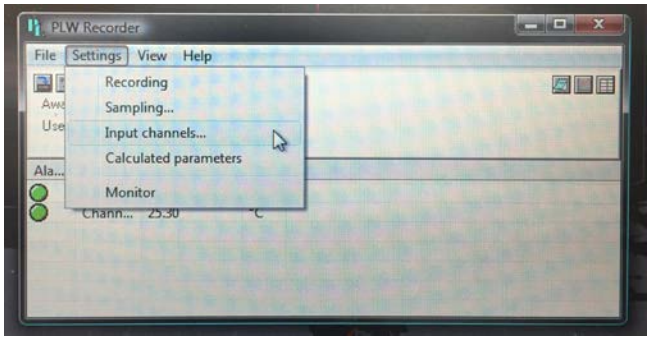


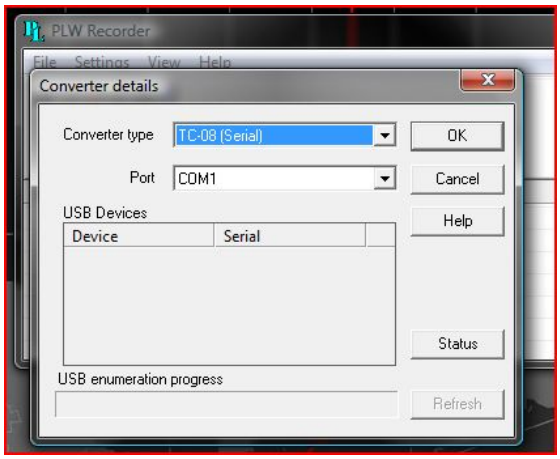
Set the sampling interval (usually 1 second) and the number of samples to record (usually set to 10000). Click "OK" to save the sampling settings.

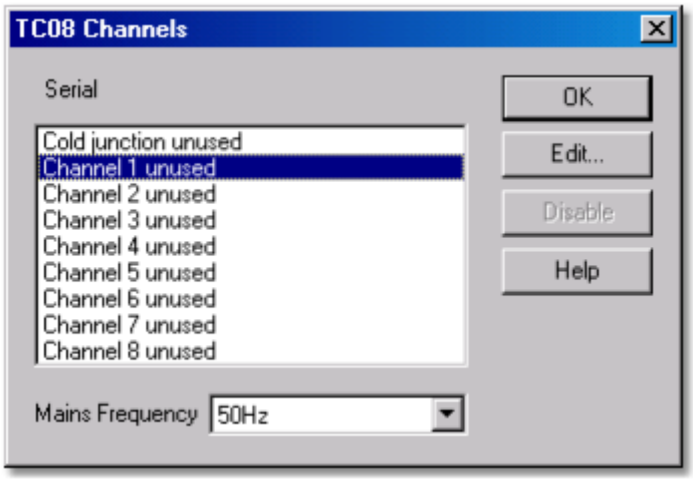
To change the input channels (depends on which channels you have thermocouples attached to):

Settings –> Input channels

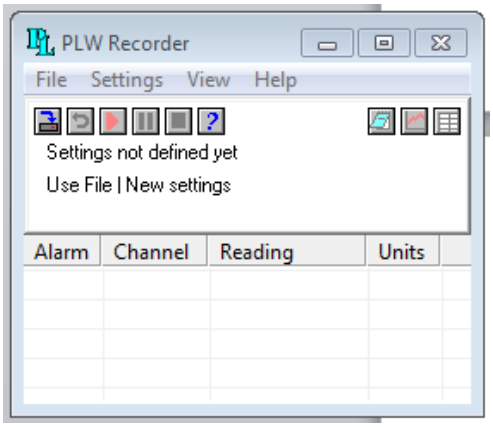
Click "OK" on the next screen. Select the first channel that you are going to use and click "Edit", then "OK" if using a k-type thermocouple, or modify as needed if using another type of thermocouple. Repeat with any other channels that you want to use. De-select channels that you aren't using by selecting the channel and clicking "Disable".







Recording samples



In order to record at exactly what time you remove an insect, you should have a running list of the recorded samples. This means that at the beginning of every trial, one should create a new file to keep track of the recorded data. To create a new file:

File –> New data

Give the file a new name and click “Save”. Press the red “Play” button to start recording.

Viewing recordings

To view the temperature recordings as a spreadsheet, click the grid icon on the far right-hand side of the PLW window. The spreadsheet displays the time and the temperatures for each of the thermocouples. A graph of the recordings can also be viewed by clicking on the “Graph” icon.

Thermal camera and FLIR Tools

Thermal camera

The thermal camera is accessed through the mobile application FlirOne, to use this application one must plug the FLIR camera attachment into the side of the phone. Once plugged in, make sure to press the power button on the FLIR attachment. Then, navigate to the FlirOne application and use the camera icon on the screen to take a photo. Photos are automatically stored in a library on the phone.

Loading files into FLIR Tools

Using a USB cord to connect the phone to a computer and then from the memory of the phone, one can download the photos to the computer. Open FLIR Tools and click “Add” in the top left corner to add a folder of images.

Measuring temperature

To measure the temperature of a spot in the image, open the image and click on the “Spot” icon on the left hand side of the screen. Click the spot you want to measure. To measure additional points, keep repeating this procedure.

The temperature profile (e.g. maximum, minimum, mean) of a line or area within the image can also be measured. See the FLIR Tools manual for more information.

Outputting temperature data

Right click anywhere on the image and click “Export to csv”. On the “Export” dropdown menu, choose “Measurements” and click “Export”.

FirestingO_2 and Oxygen Logger

Oxygen sensor calibration

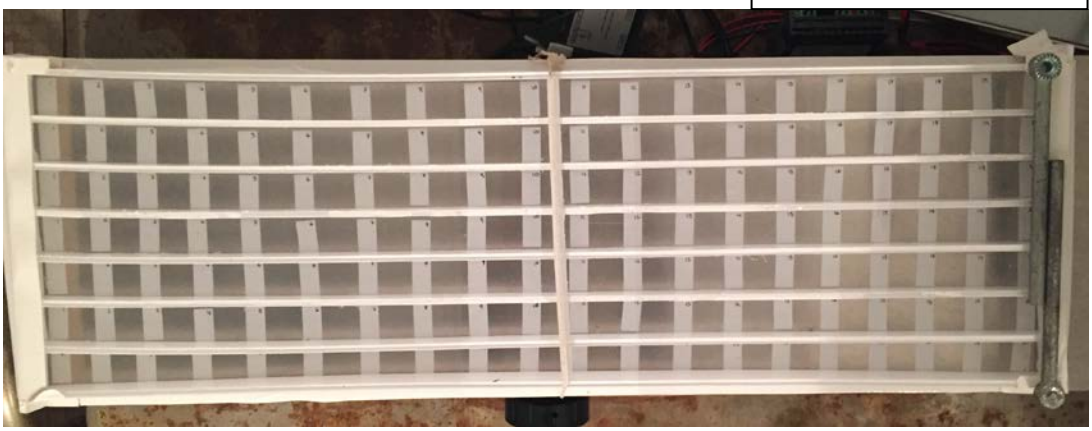
The oxygen sensor needs to be calibrated before using the oxygen vials for the first time, or if the sensor gets disconnected from the vial. Attach the sensor to the oxygen vial, remove the lid from the vial and put the vial next to the FirestingO_2. Leave it for at least 10 minutes so that the inside of the vial can equilibrate to normal air conditions.

Before starting the calibration, check that the correct sensor code has been entered in the Settings. The sensor code is on the black zip-lock bag that the each oxygen vial is stored in.

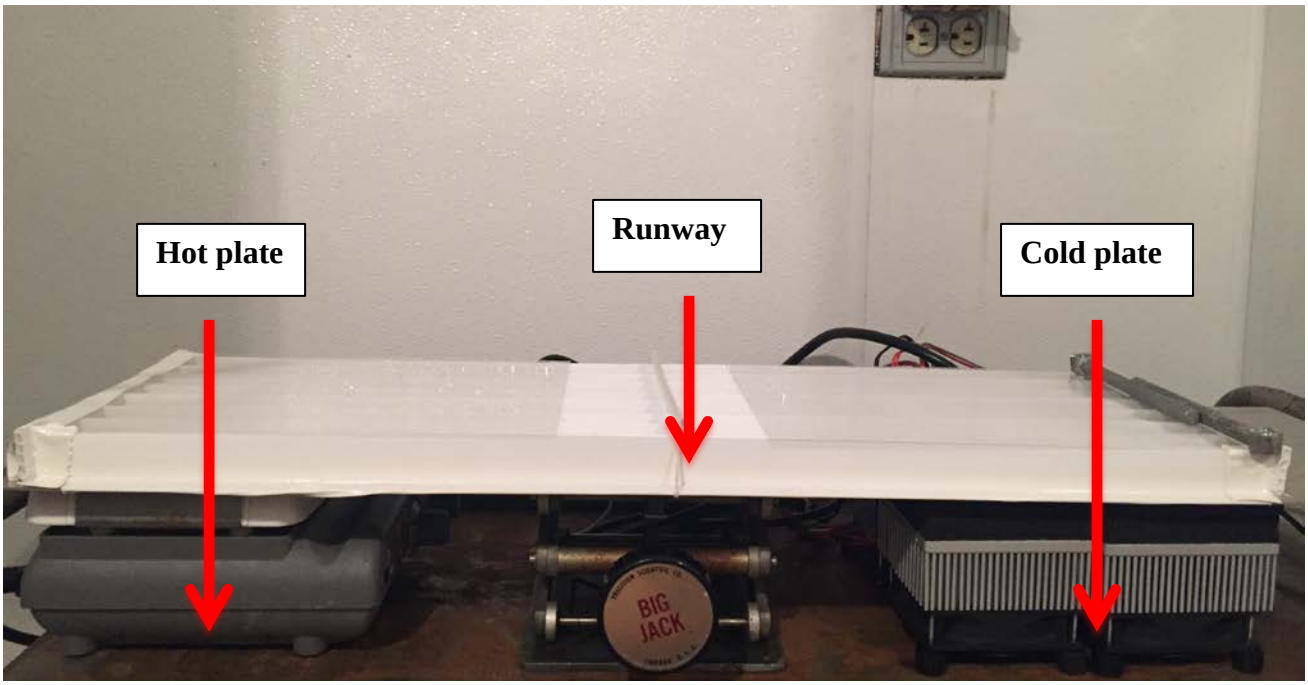
Click “Calibrate” to open up a new window for calibration. Select “1 Point in Ambient Air” from the options on the left. Wait for the Oxygen Concentration in the graph on the right to stabilize, and then click “Set Air”. Click “Finish” to close the window.

Thermal preference runway

Components



**The runway has white labels for two reasons. First, as position markers, to make it easy to record the position of the animals. Second, the aluminium is reflective, and the thermal camera images are not accurate for the aluminium parts. When getting temperatures off the thermal camera, make sure to only select areas of the image that correspond to the white labels.



**There are two cold plates at the cold end of the gradient, with the one at the very end set to a colder temperature than the one closer to the centre. Adjust the temperatures as need to get a good gradient.

Cold and heat tolerance

Definition

In insects, cold and heat tolerance are usually quantified by measuring the onset of chill coma (a.k.a. critical thermal minimum, CT_{min}) and the heat movement threshold (a.k.a. heat knockdown temperature, critical thermal maximum, CT_{max}). These endpoints mark the loss of coordinated muscle function and are assessed by checking for righting ability – if an insect is turned onto its back, it should be able to right itself in a reasonable amount of time (a few seconds) and, if not, it is deemed to have reached its lower or upper thermal limit.

Thermal tolerance is strongly dependent on the assay method, and so it is important to keep conditions as similar as possible when comparing among species. Exposure time is usually inversely related with tolerance (Chown *et al.* 2009). Thermal tolerance can be measured using static or dynamic assays, with the latter being preferred because they are more ecologically relevant and more widely used. In these methods, the temperature is gradually changed from an initial non-stressful temperature.

See Sinclair *et al.* (2015) for a review of cold tolerance measurement methods.

Materials

Grant water bath & software, PicoLog recorder two channels utilized, beaker in water bath with 2 thermocouples, spreadsheet, forceps. The water bath should be filled with a 1:1 propylene glycol/water solution.

Duration

Cold- (shorter) 10 min @4°C, decrease 0.2°C /min to -15°C

Heat- (longer) 10 min @ 4°C, increase @ 0.5°C /min to 20°C, increase @.2°C /min to 40°C

Directions

1. Turn on water bath (front & back buttons).
2. Let the machine acclimate to 4°C degrees.
3. Click on PicoLog recorder (icon on desktop) and make a new file for the trial.
4. Make sure that the two channels show up on the PicoLog recorder and that the ends of the temperature probes are placed in beakers 3 & 4.
5. Place one insect in each of the six beakers of the water bath .
6. Click on Labwise 9 (icon on desktop), make sure temperature trajectory looks correct and send either the coldstress program or heatstress program to the bath.
7. Click the start button on the PicoLog recorder.
8. Move the insects every couple of minutes with a tweezers in order to observe how their motor skills are holding up.
9. When insect loses coordination make sure to record the time that it lost coordination on the spreadsheet.

Extra notes

- The initial temperature used during the ramping should be the same as the acclimation temperature.

- For any new species, it's a good idea to do a couple of test runs before recording data. Every species has a different response to cold and heat, and will have different indicators that they are approaching their CT_{min}/CT_{max}. Getting a good idea for what these are, and for the approximate CT_{min}/CT_{max} will make your life a lot easier and the determination of endpoints more consistent once you come to running the experiment.

Thermal preference

Definition

Many ectotherms use behavior to control their body temperature within a range much narrower than their thermal tolerance. The temperature than an individual regulates their body temperature to, given choice, is referred to as their "thermal preference" (T_{pref}), "preferred body temperature" (T_p), "thermal preferendum" or "selected temperature". Thermal preference is often lower than the thermal optimum (T_o) and this is thought to be because of imperfect thermoregulation and an asymmetric temperature-fitness curve that is typical for ectotherms, such that total fitness (long-term) is maximized by centering thermal preferences over a body temperature below that which maximizes instantaneous performance (short-term) (Martin & Huey 2008). Thermal preferences and thermal optima are usually closely and positively correlated, but this could depend on the particular traits being considered for measures of optima (Haupt *et al.* 2017).

Materials

Headlamp with red light, stopwatch, phone recorder with apps (Tape-a-Talk, or any other voice-recording app; FLIROne), thermal preference runway.

Duration

~ 1 hour

Directions

1. Turn on the thermal preference runway 30 minutes beforehand, to allow the temperature gradient to stabilize.
2. Place an insect in the center of each of the 7 lanes and let acclimate for ~15 minutes.
3. Record 4 trials total, during each trial spend 2.5 minutes on each of lanes 1-7.
4. Begin recording at lane 1, as the insect moves say the number that it is on.
5. If an insect sits for more than 1 minute in its lane, can skip it and move on.
6. Take a photo of the runway at the end of each trial, making sure to capture the whole runway (you will probably need to take 2 photos)

Alternative method

*This method has less data-processing involved, but does not allow an estimate of how much movement there was or the distribution of temperatures used. It is most suited to animals that don't move a lot (like ice crawlers).

1. Turn on the thermal preference runway 30 minutes beforehand, to allow the temperature gradient to stabilize.
2. Place an insect in the center of each of the 7 lanes and let acclimate for ~15 minutes.
3. Record the position of each insect within its lane.
4. Every 10 minutes, check and record the position of each insect. Repeat this until you have 7 position recordings (i.e. 1 hour).
5. Take a photo of the runway at the end of each trial, making sure to capture the whole runway (you will probably need to take 2 photos)

Extra notes

- Every couple weeks, re-apply a line of Fluon to the upper part of the walls of each lane.
- The data extraction from voice recordings and thermal camera images takes a long time!

Desiccation rate and resistance

Definition

Invertebrates lose water primarily through excretion, cuticular transpiration, and respiration (Gibbs *et al.* 2003). Desiccation rate refers to the rate at which an animal loses water. Desiccation resistance refers to the amount of time that dry conditions can be tolerated, or the amount of water loss that can be tolerated, by an organism (Addo-Bediako *et al.* 2001). It is the survival time (hours, minutes) of an organism when exposed to dry air conditions and is a function of both temperature and humidity.

Materials

RH can be controlled using different chemicals.

~ 0% RH: use Drierite. A 1 cm layer in the bottom of the desiccation box should be sufficient.

~11% RH: use a saturated LiCl solution. Make this solution by dissolving LiCl in distilled water over a gently heated stirrer. Stir until all of the LiCl is dissolved, then add more. Repeat until no more crystals are dissolving – this means that the solution is saturated. Allow it to stir for 10 minutes, then pour into the desiccation box. Allow the solution to cool before putting the lid on the box to prevent condensation. There should be enough solution to fill the desiccation box about 1 cm deep.

~34% RH: use a saturated $MgCl_2$ solution. Same directions as above.

~76% RH: use a saturated NaCl solution. Same directions as above.

Duration

24 – 48 hours, checking in every 6 – 12 hours. Desiccation resistance measurements might take several days.

Directions

1. Pre-treatment: 24-hours before the experiment, move the animals to a container with wet cotton (to induce a high RH) and no food. This procedure is designed to replenish water content and induce animals to empty their gut, to minimize body mass changes during the experiment that are unrelated to water loss.
2. Prepare the desiccation box: At least 2 hours before the experiment, prepare the desiccation box. Add the desired RH control agent, and place the box in the incubator at the temperature that will be used for the experiments. The temperature should be around the organism's optimum temperature. For *Nebria*, this could be between 2°C and 10°C; for *Grylloblatta*, this could be between 0°C and 5°C.
3. Weigh all the individuals.
4. Place each individual into a … ml tube, covered by a square of cotton secured with an elastic band. Place these tubes into the desiccation box.
5. For desiccation rate measurements:
    a. At intervals of 6 – 12 hours, weigh each individual again.
    b. Repeat for 24 – 48 hours.
6. For desiccation resistance:
    a. Check movement of individuals at intervals of 6 – 12 hours. Do not check more frequently than this, because every time the desiccation chamber is opened, the RH shoots up.
    b. Weigh any individuals that are no longer able to right themselves.

Alternative method for desiccation resistance

*This method is useful because responses can be checked more frequently, without changing the RH conditions.

1. Instead of using a single desiccation box, containing multiple individuals, set up individual desiccation chambers using 50 mL Falcon tubes and ~ 1 Tbsp Drierite in each tube. Put the insect in a 5 mL tube, covered by a cotton square secured with an elastic band. Put this tube inside the Falcon tube and close the lid tightly.
2. Check movement of individuals at regular intervals, *without removing the lids* of the Falcon tubes. If an individual looks to have lost coordination, open the Falcon tube and check for a righting response.

Extra notes

- As temperature can be a stress factor in itself, estimates of desiccation resistance should be made around the organism's optimum temperature, using a low air RH.
- There is currently no consensus in the literature about the RH that animals should be exposed to.

It is necessary to report:

- Temperature
- RH
- Body mass at the start end of the experiment, because body mass is an important determinant of water content.

Standard metabolic rate

Definition

Standard metabolic rate (SMR) is the minimal energy necessary for an inactive animal to sustain itself, and encompasses the uptake, transformation, and allocation of energy of animals at rest, per unit time (Withers 1992). The reaction norm of SMR with temperature is important. It can, for example, be related to a species' susceptibility to desiccation, as a large proportion of water loss happens via respiration (Chown 2002).

SMR can be measured either by direct or indirect calorimetry. We use an indirect calorimetry method, which measures O_2 consumption in a closed system.

Materials

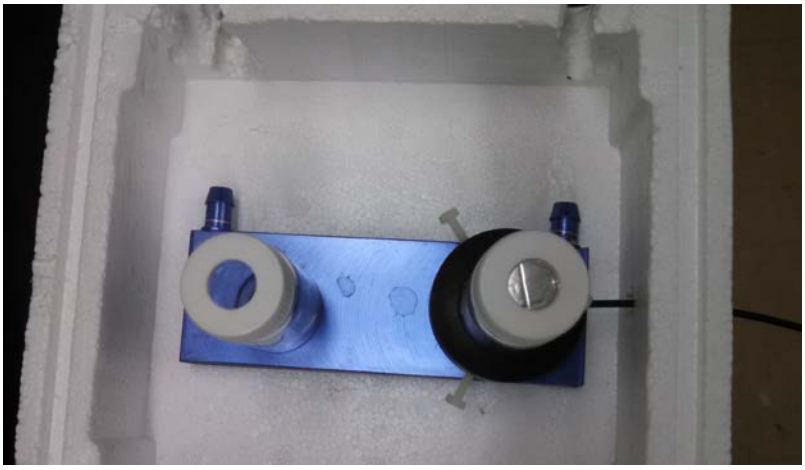
FireStingO_2, 20 mL respiration vial with optical fibre attached, 20 mL respiration vial for temperature measurement, FireSting temperature probe, cooling block, water bath, small styrofoam box with holes cut for optical fibre, temperature probe, and water bath tubes, computer, forceps.

Duration

1.5 hours, plus ~ 30 minutes for the water bath to cool down before the first trial.

Directions

1. 24 hours before the experiment, remove food from individuals that are going to be tested.
2. Set up the FireStingO_2 and respiration vials.
    - The cooling block needs to sit inside the styrofoam box, and the respiration vials sit on top of the cooling block. The styrofoam box serves 2 purposes: temperature insulation, and keeping the system dark.
    - Connect the cooling block to the water bath, and turn the water bath on to the chosen temperature.
    - Attach the optical fibre to one respiration vial and to the FireSting.
    - Attach the temperature probe to the FireSting and place inside the other vial.
    - Leave the lids of the vials off while the system is cooling to the right temperature.
3. Connect the FireSting to the computer and open the Oxygen Logger software. See equipment instructions for details about calibration and settings.
4. When the temperature has stabilized at the correct temperature, place an insect inside the respiration vial and close the lid. Close the lid of the styrofoam box.
5. Wait 10 minutes to allow the insect to acclimate to the system conditions.
6. Open the raw data window and start recording with the Oxygen Logger software. The following settings are good:
    - Continuous sampling
    - 1 second sampling interval
    - 3 second data smoothing
7. Continue recording for 1 hour, then stop the data recording and remove the insect from the vial.
8. Weigh every individual measured.



Extra notes

SMR is highly temperature-sensitive. It is therefore necessary to establish standard conditions if metabolic rates are being compared among species. A non-stressful temperature should be used for single-temperature comparisons. Make sure to keep the vials clean (using distilled water and gently wiping with a Kimwipe) to avoid any O_2 consumption due to microbial respiration.

It is necessary to report:

1. The temperature used
2. Body mass of the individual. Analyses should use mass as a covariate because mass has a significant effect on SMR.
3. Conditions (incl. time since feeding)

*There are functions available on https://github.com/mrsparklesaur/data-functions for processing the data output by the Oxygen Logger software.

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