

Techniques and Protocol(s)

Protocol 1. Measurement of time until immobilization and time until the loss of heartbeat during acute exposure to high temperature in *Daphnia*

Materials and equipment

ADAM, COMBO or similar artificial freshwater medium (or filtered natural water)

Daphnia culture

Scenedesmus, *Ankistrodesmus* or a similar green algae culture as food

100- and 400-mL jars for housing groups of *Daphnia*

Temperature controlled incubators

Tubes and pipettes for handling *Daphnia*

50-mL cell culture flasks

Dissecting microscope

Water boiler

Large forceps

Water bath with forced circulation, must maintain 37°C +/- 1 ° within the temperature-controlled area, equipped with rubber hose separators allowing at least 5 mm gap between culture flasks

Electronic heating block with 24 nests for 1.5 ml vials

1.5-mL centrifuge vials

Lab timer

Methods

I. *Daphnia* acclimation

20 - 30 day prior to the experiments:

Prepare a mass culture of the desired *Daphnia* clone(s). If the experimental design includes different acclimation temperatures, place neonate *Daphnia* into 400-mL jars stored in constant temperature incubators at desired acclimation temperatures. Add food to a desired concentration daily, replace water twice weekly.

10 – 20 days prior to the experiment: as females reach maturity, collect neonates <24 h after hatching and transfer to fresh jars, 20 ind. to a 400-mL jar. Continue until the 2nd – 4th clutch is laid.

II. Time until immobilization

1. Up to 3 h prior to the experiment transfer individual adult females into 50-mL cell culture flask containing either regular or degassed water at the acclimation temperature.
2. Place flask into the water bath adjusted to the desired temperature. Randomize!
Note: at 35C most clones acclimated to 28°C will retain mobility for over 6 hours; at 39C most clones acclimated to either 20°C or 28°C will be immobilized in less than 30 min (Fig. 1). At 37 °C most of genotypes tested retained mobility for 1 – 2 hours (Fig. 2), which allows for sufficient temporal resolution.

3. Monitor *Daphnia* swimming activity every 1-2 minutes thereafter (starting 15 minutes after increasing the temp. to 37°C). A convenient criterion of irreversible immobilization is side-wise position on the bottom of the flask, with or without antennae movement but with no vertical movement of the whole body. Up to 50 flasks can be simultaneously monitored by a single observer. Runs may be staggered, depending on the treatment (for example a majority of 20°C acclimated individuals will be immobilized before any of the 28°C-acclimated ones are).
4. Record time until immobilization, transfer *Daphnia* under a dissecting microscope, measure body length (top of the head to the base of the spine), record number of eggs, as these are important covariables affecting heat tolerance.

III. Loss of heartbeat.

1. Place individual adult female *Daphnia* in a 1.5 ml centrifuge vial and insert the vial into Eppendorf (or similar) electronic heating block adjusted to the desired temperature.
2. Monitor mobility during the first 10 min of exposure. Transfer immobilized *Daphnia* under dissecting microscope to detect heartbeat.

IV. Preparation of degassed water the experiments in anoxic conditions

1. Place 50-mL cell culture flasks held together in blocks of 5x4 flasks by wire, into a 5-L boiler, filled with the same water used in the experiments and bring to boiling. Shake the flask blocks periodically to remove air bubbles. Boil for 30 min until all bubbles disappeared.
2. Using forceps place the screw tops onto the flasks without removing the flasks or tops from water. Tighten.
3. Cool to room temperature
4. After placing a *Daphnia* into the flask immediately return the flask and the top under water and screw on the top without retaining an air bubble.

Notes: This procedure allows maintaining *Daphnia* at <0.01 of oxygen saturation. Degassed water without heat shock has no acute (>24hours) effect on *Daphnia* survival at 20 and 28°C.. At 37°C degassing has a moderate effect on heat tolerance (Fig. 3).