Protocol 2. Semi-high throughput measurement of hemoglobin concentration in *Daphnia*

Materials and equipment

Daphnia cultures as described above
TrisHCl buffer, 0.05 M pH 7.2
Micropipettes 5, 20, 200 ul and tips
Microcentrifuge vials
Centrifuge
384-well plates, flat bottom, transparent
Spectrophotometer Tecan Infinite 200 or similar
Bradford reagent

Methods

1. Place a single Daphnia in a 1.5 microcentrifuge vial with 50 ul of TrisHCl buffer, pH 7.2. Homogenize on ice.
2. Centrifuge at max speed for 6-10 min.
3. Transfer 50 ul of supernatant from each vial into a 384-well plate well
4. Measure absorbance spectrum from 230 to 660 nm at 2 nm intervals for full spectrum data; alternatively measure absorbance at 260 nm (to quantify total tissue about by the proxy of nucleic acid absorbance), and at 414, 500, 540, 560, 576 and 600 nm to quantify absorbance peaks of hemoglobin and background level between peaks (Fig. 3). If the spectrophotometer allows so, measure absorbance by a 3x3 grid of beams, 5 flashes in each.
   Note: it is convenient to initiate absorbance measurement of the first 4 rows of the 384-well plate; the next 4 rows can be pipetted out into a different plate while measurements described in 4 are done. Tecan-i script accomplishing these measurements is available for download:
   http://faculty.etsu.edu/yampolsk/hb/hbspectra.zip.
   Note: Use TrisHCl buffer as the blank. Commercial hemoglobin standards can be used if absolute hemoglobin concentration is needed.
5. Pipette out 5 ul from each well into the next 384-well plate (very large *Daphnia* may require 2.5 ul samples). Add 95 ul of undiluted Bradford reagent and measure absorbance at 595 nm for total protein within 45 min (Fig. 5). It is critical to pipette quickly and measure absorbance immediately to complete the entire 384-well plate within 45 min. Use 0.05, 0.1, 0.2, 0.4, 0.8 and 1.6 mg/ml BSA standards as recommended by Bradford reagent manufacturer.
6. To detect in vitro heat damage to hemoglobin set the spectrophotometer to 40^{o}C with a 30 min delay, if the equipment allows such setting. Alternatively set the plate aside at 40^{o}C in a thermostat for 30 min. Measure the same absorbances as in 4.
7. Subtract blanks from absorbances. Calculate total heme-containing proteins concentration from A414. Calculate relative height of hemoglobin peaks as
   $H_{540} = A_{540} - (A_{520} + A_{560})/2$;
   $H_{576} = A_{576} - (A_{560} + A_{600})/2$;
   Normalize values by total protein concentration measured by Bradford method.

Calculate relative abundance of methemoglobin as

$M = A_{500} / (A_{500} + A_{540})$.

Figures

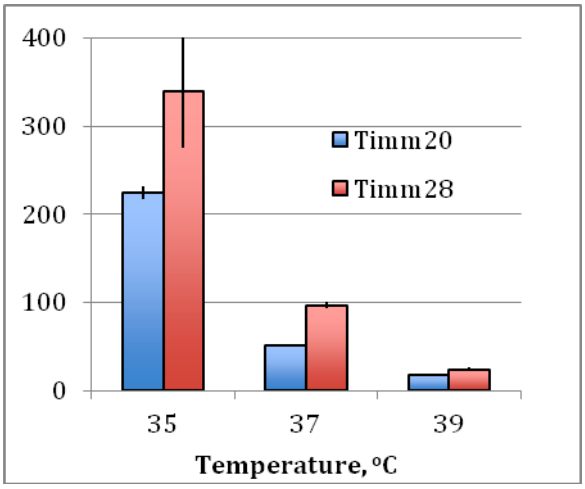


Figure 1. Time until immobilization (Timm) (in minutes) in *Daphnia* acclimated to either 20ºC or 28ºC and exposed to 35º, 37º or 39º C. The value for 28ºC-acclimated *Daphnia* at 35ºC is a lower estimate as the experiment as terminated after 6 hours.

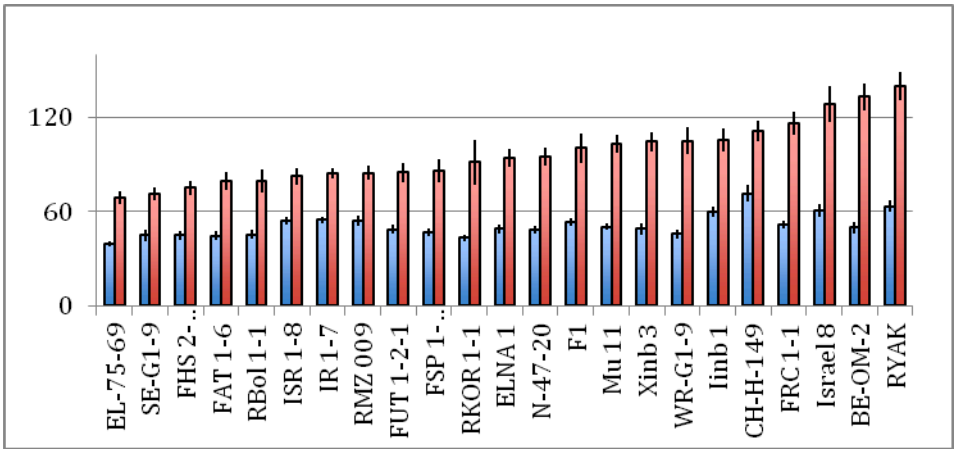


Figure 2. Time until immobilization (min) in *Daphnia* from 23 geographically distinct clones acclimated to either 20ºC or 28ºC (sorted by Timm28). Colors as on Fig. 1.

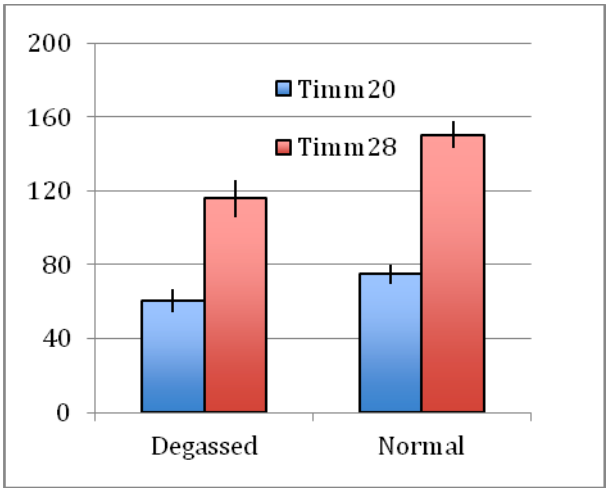
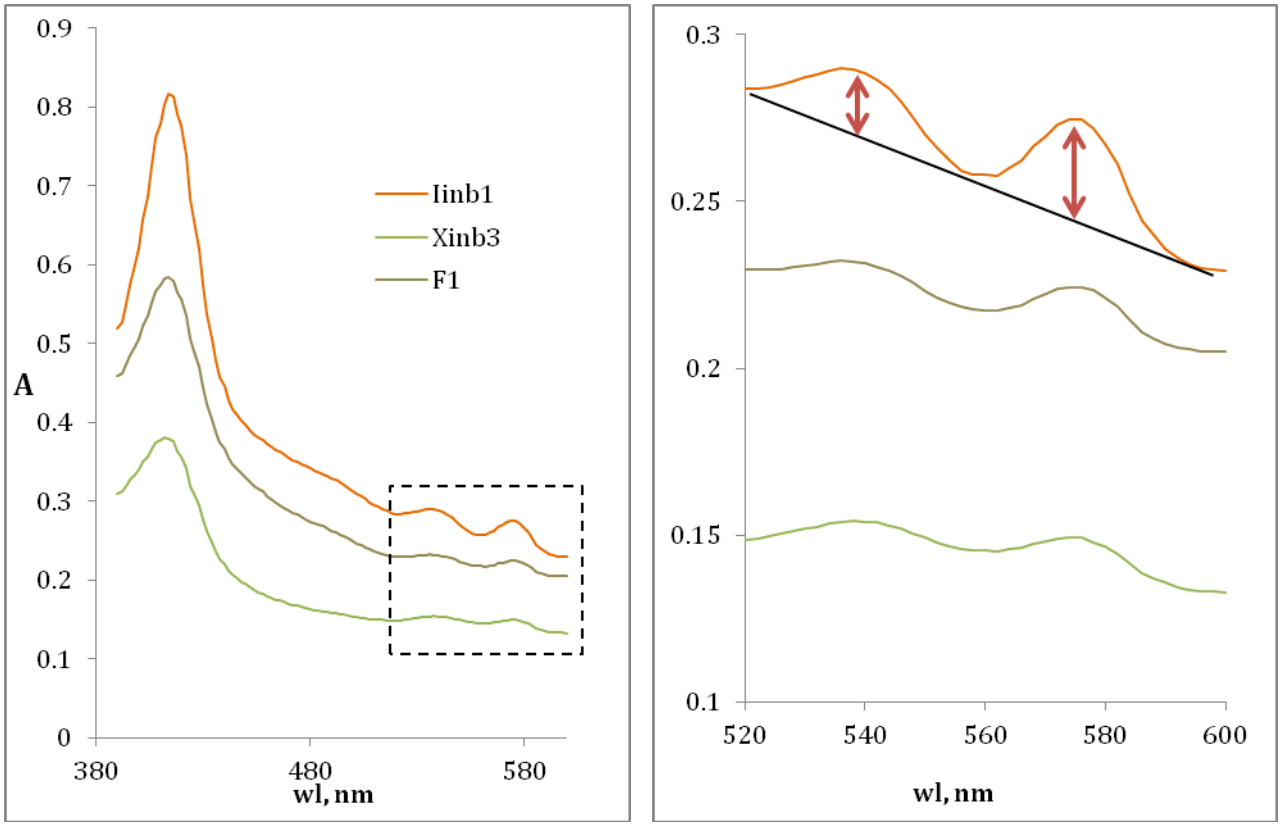


Figure 3. Time until immobilization (Timm) (in minutes) in normal and degassed water. Both gas saturation and acclimation temperature show significant effects, but the magnitude of the anoxia effect is relatively small, indicating that the acclimation effect is different and independent from anoxia tolerance.



1.

Figure 4. Hemoglobin absorption spectra of two QTL progenitor lines (Iinb1 and Xinb3) and their hybrid (F1). Left: the entire spectrum; right: squared area magnified, peak height determination shown.

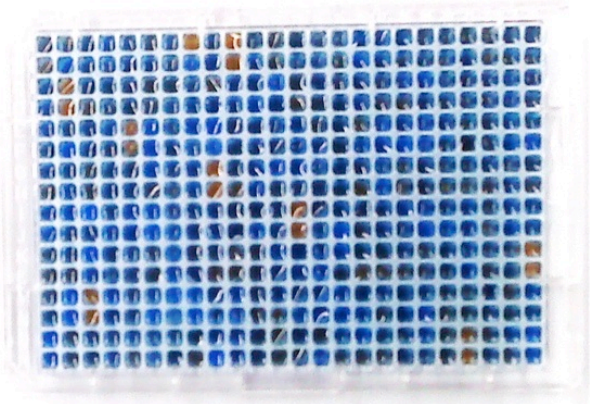


Figure 5. An example of a 385-well plate ready for Bradford absorption measurement. Brown cells are blanks.