TITLE: Breathing in the dark: interactive influence of intrinsic and extrinsic factors on stygofauna metabolic rate

APPENDIX 1 - METHODOLOGY

Organisms collection

Specimens were collected using a simple trapping method. Three weighted buckets were placed horizontally in the water with its opening facing the deeper part of the cave. Approximately 10 g of chicken liver bait was placed inside, attracting *S. bottazzii* to feed and remain in the bucket. By quickly lifting the buckets into a vertical position, the specimens were trapped. The trap was checked after 24 hours, and the captured animals were collected. *S. bottazzii* was the only species observed and collected at this site using this method. After field collection, the specimens were transported in an insulated thermos container and placed in plastic trays with field water to minimize potential osmoregulatory stress.

Given the unknown IUCN status of the species, additional precautions by conducting three repeated sampling events at the same location in the weeks following the initial collection were taken. During these subsequent samplings, the captured specimens of *S. bottazzii* were rapidly counted and released. In each case, the observed number of individuals was similar to or even greater than the original sample, leading to conclude that specimen collection for the experiments did not significantly impact the local population.

Maintenance in laboratory

Plastic trays measuring 23 x 35 x 10 cm were used to house the animals throughout the experiment (ca. 18 individuals for tray), except for the specimens designated for growth rate measurements. The trays were inspected weekly to ensure both food and water were available. During the inspection, excreta and any remaining organic residues were removed using a pipette to keep the trays clean. The water level was maintained at a fixed mark, with the trays filled using a mixture of deionized and field water to contrast evaporation and keep the salinity consistently around 2. For feeding, the animals received a weekly ration consisting of 37 mg per capita of dry fish food containing 36% spirulina (Sera© Nature Micron) diluted into field water. Additionally, stones collected from the field were provided as environmental enrichment, offering natural hiding spots or resting areas.

Respirometry Measurements

CVR assays were conducted by using two 4-channel fiber-optic oxygen meters (FireSting O2, PyroScience GmbH, Regensburg, Germany) equipped with sensor spots attached to the inner walls of 0.8 mL (V) respiration chambers. The eight respiration chambers used were filled with artificial water at the target salinity. Six chambers contained the specimens undergoing measurements, while one chamber for each of the two oxygen meters served as a control. The sensors were calibrated at 0% and 100% oxygen saturation before each respiration trial. A submersible temperature sensor for the FireSting O2 system was placed in the thermostatic water bath to ensure a stable temperature throughout the experiment.

Oxygen concentration was recorded every second for at least 60 minutes, excluding the initial 10 minutes when a rapid decline in oxygen levels typically occurs due to sensor temperature equilibration. To maintain constant water mixing, the respiration chambers were gently shaken by hand every 15 minutes. The specimens Respiration Rate (RR, mg h^{-1}) was calculated from the slope (*b*) of the linear regression oxygen concentration in the respiration chamber (mg ml^{-1}) over time (sec), so that RR = (b*V*3600). The obtained values were converted in Standard Metabolic Rate (SMR, J day^{-1}), using an oxy-joule equivalent of 14.06 J mgO_2^{-1} (Gnaiger, 1983). After measurements, each individual's Dry Weight (DW, mg) was determined by drying specimens at 60°C for 48 hours in an oven and weighing them using an analytical balance.

Growth Rate Measurements

The specimens were placed in a transparent plastic glass 6 cm in diameter. The bottom of the containers were transparent and placed on a sheet of graph paper. Photographs of the individuals were taken at different time intervals: once per week initially, then every two weeks. All images were taken using an iPhone SE (2022), ensuring that the device was perpendicular to the container by using the built-in level to prevent image distortion. The growth rate was assessed by measuring the body length, defined as the distance from the anterior margin of the carapace to the posterior margin of the telson without spines (Ariani & Wittmann, 2010), in each image using ImageJ (version 1.54g), with graph paper as reference scale. As the measurements are still ongoing, none of the specimens was sacrificed to determine their sex and body mass.

REFERENCES

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