**NMR Metabolomics Methods for Thermally Challenged Mayfly Larvae**

Control and heat-treated mayfly samples were provided in triplicate and were processed individually. One sample was provided for AD, ControlX, MF control (from a previous study) and MG larvae. Three replicates of the larvae listed above having only a single sample were created. Aliquots of 50-300mg of insect larvae were mixed with degassed 1:1 Acetonitrile:Water solution in a 2 mL snap cap tube at a concentration of 50 mg/mL. Megalopterans were substantially larger than the other larvae and were prepared at 200 mg/mL. Samples were homogenized and then centrifuged at 4⁰C for 5 minutes at 14000rcf. The supernatant was removed and placed into a new tube. Samples were centrifuged again at 4⁰C for 5 minutes at 14000rcf and a volume of the homogenate corresponding to 40 mg insect larvae were taken. After homogenization, the three larval replicates from AD, ControlX, MG and MF control (from a previous study) were pooled and a single sample created for each larval type. There was insufficient Mayfly (heat and control) sample material to create an internal pool, so an external pool was made by combining equal concentration amounts of AD, MG and MF control (from a previous study) pool quality check (QC) samples. Samples were completely dried by vacuum centrifuge and were reconstituted by adding 630 µL of D2O (Aldrich) and 70 µL of Chenomx ISTD solution (Chenomx, Edmonton, Alberta, Canada). The samples were vortexed and centrifuged at 14000rcf for 2 minutes. 650 µL of sample was transferred into 5mm NMR tubes and analyzed by NMR. 300 µL of the returned NMR sample was taken to a new Eppendorf tube and dried by vacuum centrifuge.

1H NMR spectra of urine samples were acquired on a Bruker Avance 950 MHz NMR spectrometer (located at the David H. Murdock Research Institute at Kannapolis, NC, USA) using a 3 mm cryogenically cooled ATMA inverse probe and ambient temperature of 25℃. A 1D NOESY presaturation pulse sequence (noesypr1d, [recycle delay (RD)-90°-t1-90°-tm-90°-acquire free induction decay (FID)]) was used for data acquisition. For each sample 64 transients were collected into 65k data points using a spectral width of 14.01 kHz (20.14 ppm), 2 s relaxation delay, 100 ms mixing time, and an acquisition time of 2.324 s per FID. The water resonance was suppressed using resonance irradiation during the relaxation delay and mixing time. NMR spectra were processed using ACD Software 12.0 (ACD Labs Inc., Toronto, Canada). Spectra were zero filled, and Fourier transformed after exponential multiplication with line broadening factor of 0.5. Phase and baseline of the spectra were manually corrected for each spectrum. Spectra were referenced internally to the DSS signal. The quality of each NMR spectrum was assessed for the level of noise and alignment of identified markers. Spectra were assessed for missing data and underwent quality checks. NMR bins (0.50-9.0 ppm) were made after excluding DSS, water (4.68-4.88 ppm), and Imidazole (7.20-7.28 ppm) using bucket Integration with a 0.04 ppm bucket width. Integrals of each of the bins were normalized to total integral of each of the spectrum.