1 Methods

1.1.1 Sample Collection and Storage

Haemolymph was collected over a 5-week period from 27th of October to the 30th of November 2017. On each occasion 200µL of haemolymph was collected per animal, haemolymph was drawn from the ventral sinus of each crayfish using a 21G needle and 1mL syringe inserted into the soft tissue at the base of the 5th pereopod (Leland & Furse, 2012). All haemolymph collections for all animals occurred at the same time of day and the animals were handled in the same order on each of the four collection dates. Haemolymph (200µL) was added to 2mL Eppendorf tubes preloaded with 600µL LC-MS grade acetonitrile (Optima, Thermo Fisher Scientific, AUS) containing deuterated internal standards (*d8*-valine, *d9* –trimethylamine-N-oxide (TMAO) , *d3*-leucine, *d6-trans*-cinnamic acid, *d5*-tryptophan, 1µg/mL) from Cambridge Isotope Laboratories (Cambridge, MA, USA) and stored on ice. At the time of haemolymph extraction each Eppendorf tube was placed on an analytical balance (Sartorius BP210S) and 0.200 grams (+/- 10%) of haemolymph was added. Tubes were capped and shaken immediately to prevent clotting and then placed on ice. Samples were mixed at 1400rpm (Thermal Mixer, Thermo Fisher Scientific, AUS) for 60 seconds at 4°C, then centrifuged (Heraeus Megafuge 8R, Thermo Fisher Scientific, AUS) for 20 minutes (1800 × g) at 4°C. After centrifuging, 100µL of supernatant was aliquoted into five separate vials. The samples were then dried using a SpeedVac™ centrifugal vacuum concentrator (Thermo Fisher Scientific, AUS). The dried samples were stored at -80°C for subsequent metabolomics analysis.

1.1.2 Sample Preparation for Metabolomic Analysis

The dried haemolymph samples were reconstituted using 100µL of LC-MS water containing 0.1% Formic acid. Samples were manually swirled, then placed in a thermomixer for 2 minutes at 4°C, before being centrifuged at (1800 × g) for 5 minutes at 4°C. Next, 40µL of the supernatant was transferred into LC-MS amber vials with inserts and placed in the autosampler kept at 6°C. The order in which samples were analysed was randomised to avoid any potential instrument bias. A pooled quality control (QC) sample was prepared by adding 40 µL from each reconstituted sample to a single Eppendorf tube, which was then mixed to homogenise in a thermal mixer and centrifuged as above. This pooled sample was aliquoted (40µL) into LC-MS amber vials to create 16 QC samples and placed into the autosampler tray kept at 6°C ready for analysis. Samples were analysed within 24 hours from preparation. At the start of the analytical batch, a solvent blank, matrix blank, and ten conditioning

QC samples were analysed (Broadhurst et al., 2018). QC samples were then injected after every fifth marron haemolymph sample with two QCs analysed at the end of the batch, following the standard protocols for metabolomics (Broadhurst et al., 2018).

1.1.3 Liquid chromatography-mass spectrometry

All samples were analysed using an Ultra High-Pressure Liquid Chromatography pump (Dionex UltiMate 3000 RS) coupled to an Orbitrap Q-Exactive mass spectrometer (Thermo Fisher Scientific) fitted with a heated electrospray ionisation probe (HESI). Metabolites were separated on a reversed phase Hypersil GOLD C18 column (100 × 2.1 mm, 1.9 μm particle size; Thermo Fisher Scientific) with an in-line filter. Sample analysis in both positive and negative ionization modes was performed using 0.1% formic acid in LC-MS water (solvent A) and 0.1% formic acid in LC-MS acetonitrile (solvent B). The elution gradient was as follows: isocratic at 99% solvent A for 1 min, followed by an increase to 50% solvent B (1-2 min) then a linear increase to 99% solvent B over 7 min, which was maintained at 99% solvent B for 2 min. Initial conditions were returned over 2 min and then held at 100% solvent A to equilibrate for 3 min. The flow rate was 0.3 ml/min for positive and negative; injection volume was 10 μL and column oven temperature was 45°C.

Full scans with data-dependent tandem mass spectrometry were acquired on the Orbitrap mass analyzer. Full scans were acquired at a resolution of 70,000 at mass-to-charge ratio (*m/z*) 200 over the *m/z* range 70–1000 with the ESI conditions as follows: source heater = 350°C, sheath gas = 35 (arbitrary units), auxiliary gas = 10 (arbitrary units), capillary temperature 350°C, ion spray voltage = 3.0 kV (positive ion mode) and 2.5 kV (negative ion mode), S-lens 50%, and automatic gain control = 1×10^{-6}. Tandem mass spectrometry experiments were performed at a resolution of 17,500 at *m/z* 200 on each sample with the higher energy collisional dissociation energy set at 20 eV. Data acquisition was carried out using Xcalibur software (Thermo Fisher Scientific). Before analysis, the Orbitrap was externally calibrated using ready-made calibration solutions (ESI-negative ion calibration and ESI-positive ion calibration solutions) obtained from Thermo Fisher Scientific.

1.1.4 Data preprocessing

Raw spectral data were preprocessed by Compound Discoverer 3.0 software (Thermo Fisher Scientific) using the standard untargeted metabolomic workflow. Compound Discoverer was setup to align total ion chromatograms along retention time using an adaptive curve, with a maximum shift of 0.5 min and 5 ppm mass tolerance. Detected features with an intensity of no less than 1,000,000 and a signal-

to- noise ratio greater than 5 in each set of data were extracted and merged into components according to ion adducts. Compounds detected in the blank samples were deleted from the final data matrices. Metabolite data from both positive and negative ionization modes were combined into a single data matrix. For each metabolite, relative standard deviations were calculated for the pooled QC injections (RSD_{QC}), and for the total sample variance (RSD_{Sample}), following standard protocols (Broadhurst et al., 2018) metabolites with RSD_{QC} >20%, or a ratio RSD_{QC} /RSD_{Sample} > 30%, were considered to be below accepted quantification precision and removed from further statistical analyses.

1.1.5 Metabolite identification

Before statistical analyses, metabolites were annotated by matching accurate mass and mass spectrum fragmentation patterns to an in-house MS/MS spectral database and the mzCloud online spectral library (https://www.mzcloud.org/) and scored according to the Metabolomics Standards Initiative (MSI) reporting protocol (Sumner et al., 2007). A definitive match (MSI level 1) indicates that identification was confirmed to an in-house authentic standard using retention time. A putative match (MSI level 2) indicates that the mass spectra were matched to mzCloud online MS/MS database but without in-house authentication. An MSI level 3 match implies most probable physiochemical class and name, based on reported molecular weight and retention time.