7.2 Sample Collection

The amount of material required for a successful metabolomics experiment varies depending on the developmental stage and type of analysis. For a basic survey of small, polar metabolites using GC/MS, samples should contain at least 300 embryos, 25 second instar larvae, and 20 mature adult males. All samples, except for larvae, are collected in screw-cap tubes that contain 1.4 mm ceramic beads (MoBio; 13113-50) and flash frozen in liquid nitrogen. These tubes are designed for the Omni Bead Ruptor, described below. Embryos do not need to be dechorionated prior to collection, but should be washed gently with a paintbrush on a piece of Whatman filter paper soaked in PBS. Larvae are collected in a 1.5 ml tube, repeatedly washed with ice-cold PBS, and flash frozen in liquid nitrogen. The frozen pellet is then dislodged by gently flicking the tube and transferred into a pre-chilled screw cap tube with ceramic beads.

7.3 Sample Processing for GC-MS

All samples should be stored at -80ºC until processing, at which point they are transferred to an enzyme-type carrier caddy (Nunc Lab-Top cooler) that has been chilled to -20ºC. Add 800 µl of prechilled 90% methanol containing 1.25 µg/ml succinic-*d*4 acid (Sigma-Aldrich; 293075) and 6.25 µg/ml U-13C, U-15N amino acid mix (Cambridge Isotope; CDNLM-6784) to each tube with a positive displacement pipette. These stable-isotope labeled internal standards provide a means to normalize samples, provide quality control, and allow for the monitoring of instrument efficiency across batches. Additionally, negative controls that contain no fly tissue should be prepared to detect chemical contamination and false-positive peaks during the subsequent GC/MS analysis.

The hard cuticle of the fly is difficult to homogenize and requires a strong physical disruption to efficiency release metabolites. We have found that the Omni Bead Ruptor 24 homogenizer (Omni International) is ideally suited to rapidly and efficiently disrupt fly tissue, with samples homogenized for 30 seconds at 6.45 m/sec. While a variety of bead-filled tubes can be used for this purpose, screw-cap tubes containing 1.4 mm ceramic beads are optimal for removing extraction solvent after processing. Homogenized samples are incubated at -20ºC for one hour to enhance protein precipitation and centrifuged at 20,000 x g for 5 minutes at 4ºC to remove the resulting

precipitate. The supernatant is transferred to a 1.5 ml microfuge tube and the solvent removed with a Speed-Vac (Genevac).

7.4 GC-MS analysis

We perform GC-MS analysis with a Waters GCT Premier mass spectrometer fitted with an Agilent 6890 gas chromatograph and a Gerstel MPS2 autosampler. Dried samples are suspended in 40 μl of 40 mg/ml O-methoxylamine hydrochloride (MOX) (MP Biomedicals; 155405**)** in pyridine solution (EMD Millipore; PX2012-7) and incubated for one hour at 30°C. Samples are then centrifuged for 5 minutes at 20,000 x g to remove particulate matter, and 25 μl of the supernatant is placed in an autosampler vial (Agilent; 8010-0172 and 5181-1215) with a 250 μl deactivated glass microvolume liner (Agilent**;** 5183-2086**)**. Forty microliters of N-methyl-N-trimethylsilyltrifluoracetamide containing 1% TMCS (MSTFA) (Thermo Scientific; TS-48915**)** is added automatically via a Gerstal autosampler and the samples are incubated for 60 minutes at 37°C with shaking. Following incubation, 3 μl of a fatty acid methyl ester standard solution (FAMES; Table 2) is added via the autosampler and 1 μl of the prepared sample is injected to the gas chromatograph inlet at a 10:1 split ratio with the inlet temperature held at 250°C. Fatty acid methyl esters do not occur naturally, but will elute in a highly reproducible manner across the entire chromatogram. The retention time curve of FAMES solution, therefore, allows for the building of reliable retention time libraries of metabolites. Furthermore, this solution is used to assess column quality because a degraded column will exhibit peak tailings of the FAMES standards.

The gas chromatograph is set to an initial temperature of 95°C for one minute followed by a 40°C/min ramp to 110°C and a hold time of 2 minutes. This is followed by a 5°C/min ramp to 250°C with no hold time and a third ramp at 25ºC/min to 330°C with a final hold time of 3 minutes. A 30 meter Phenomex ZB5-5 MSi column (0.25 um film thickness and 0.255 mm internal diameter) with a 5 meter long guard column is employed for chromatographic separation. For each analysis, instrument performance is assessed by analyzing the negative control samples, which only contain the internal standard. Experimental samples are only processed if the instrument passes a preset

sensitivity and peak shape criteria. Finally, samples are run in a randomized order, except for quality control samples, which are analyzed every nine samples.

7.5 Data Analysis

An initial dataset is prepared using a targeted approach to identify known metabolites. Chromatograms are analyzed using the MassLynx utility QuanLynx, and metabolites are identified based on known retention times and mass fragmentation patterns. The peak area for each metabolite is recorded, and the data is exported to Excel. For our analyses, metabolite identity in QuanLynx is experimentally established using pure, purchased standards, and in limited cases by the commercially available NIST library (National Institute of Standards and Technology; version 11). MarkerLynx is used for peak identification during a second, non-targeted analysis of the chromatograms, and the formatted data is transferred to SIMCA-P+ for principle component analysis (PCA) and partial least squares-discriminate (PLS-DA) analysis. If the PCA analysis identifies significant separation between the experimental groups, PLS-DA analysis is employed to detect significantly altered metabolites. The peaks of unknown metabolites are quantified and the resulting data is exported to Excel. Statistical analysis can be performed with any number of software packages that are available for uni- and multivariate analysis, including JMP and Statistica.