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Methods for studying the metabolic basis of *Drosophila* development

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Abstract

The field of metabolic research has experienced an unexpected renaissance. While this renewed interest in metabolism largely originated in response to the global increase in diabetes and obesity, studies of metabolic regulation now represent the frontier of many biomedical fields. This trend is especially apparent in developmental biology, where metabolism influences processes ranging from stem cell differentiation and tissue growth to sexual maturation and reproduction. In this regard, the fruit fly *Drosophila melanogaster* has emerged as a powerful tool for dissecting conserved mechanisms that underlie developmental metabolism, often with a level of detail that is simply not possible in other animals. Here we describe why the fly is an ideal system for exploring the relationship between metabolism and development, and outline a basic experimental strategy for conducting these studies.

Graphical Abstract

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INTRODUCTION

As animals progress through successive developmental stages, cellular metabolism must adapt to the changing energetic and biosynthetic needs of growth, differentiation, and maturation. In mice, for example, the transition from fetal to neonatal heart development results in a dramatic metabolic switch that highlights the unique energetic needs of this life stage. While the fetal heart derives most of its energy from glucose, the neonatal heart largely relies on fatty acid β -oxidation.¹ Each of these metabolic programs is ideally suited to satisfy the energetic demands of the corresponding developmental stages. Glycolysis both supports the biosynthetic reactions required for rapid growth and reliably generates ATP in the low oxygen uterine environment.² Meanwhile, β -oxidation allows the neonatal heart to use fat as an energy source, which has a much higher energy content than glucose. Intriguingly, this metabolic transition is largely reversed in diseased and failing hearts, which increasingly rely on glucose for ATP production, and suggests that studies of fetal and neonatal cardiac metabolism can provide essential information regarding heart failure.¹ The correlative relationship between metabolic diseases and developmental metabolism, however, is not limited to the mammalian heart, as metabolic transitions associated with the onset of many human diseases can be effectively studied in the context of animal development.³ Furthermore, studies in developmental systems have a distinct advantage when compared with diseased tissues – unlike the onset of Type 2 diabetes or heart failure, the metabolic changes that occur during development are highly predictable and provide an opportunity to identify the precise changes in gene expression, metabolic flux, and cell signaling that occur before, during, and after these transitions.

While a variety of animals are used to study the links between metabolism and developmental progression, the fruit fly *Drosophila melanogaster* is ideally suited for this endeavor and the fly community has a rich history of conducting metabolic research. Not only was *Drosophila* used in some of the earliest studies of developmental metabolism,^{4, 5} but prior to the advent of modern gene cloning techniques, studies of *rosy* (xanthine dehydrogenase), *rudimentary* (aspartate carbomyltransferase/dihydroorotase), and *Zwischenferment* (glucose-6-phosphate dehydrogenase) were essential in establishing the fields of modern genetics and molecular biology.^{6–8} The appeal of using *Drosophila* to study metabolism, however, extends far beyond classical genetic approaches. The last decade of *Drosophila* research has demonstrated that metabolism is highly conserved across animal phyla. Essential metabolic regulators such as insulin and Tor have similar roles in *Drosophila* and mammals, and studies in the fly have successfully uncovered novel genes involved in human metabolic disease.^{9, 10} Furthermore, recent advances in metabolomics have established *Drosophila* as a powerful model for understanding how gene expression and metabolic flux are coordinated with tissue growth and differentiation. Most importantly, the fly is ideally suited for studying the relationship between metabolism, growth, and development, as the rare combination of easily recognized life stages, tightly regulated developmental timing events, and an abundance of genetic tools allows for the precise dissection of developmental metabolism.^{11, 12} Here we provide a basic experimental strategy for using the fly to study the conserved mechanisms that coordinate growth and metabolism.

EXPERIMENTAL DESIGN

Developmental Staging

Just as the purpose and output of a signal transduction pathway will vary depending on temporal and spatial context, metabolic flux and biosynthesis are tailored to the stage-specific requirements of animal growth and development. Therefore, the metabolic researcher must ensure that all samples contain animals of the same developmental age. Staging is easy to achieve in embryos and pupae, which can be synchronized based on easily recognized developmental events; however, this endeavor is more labor intensive in larvae. Larval studies should begin by collecting embryos for a 4-hour interval on a molasses agar plate that contains a smear of yeast paste on the surface. Eggs can either be hatched on this media and raised through the first two larval stages or transferred to a plate containing standard fly food. Synchronized populations of L1 and L2 larvae can be collected immediately following the respective larval molt, however, many of the assays described below will require a sample population that makes this level of synchronization technically impossible. In these cases, larvae can be raised from eggs that were laid within a 4-hour period and populations checked at key timepoints (such as hatching and molting) to ensure that all animals are developing at the same rate.

While proper staging is always important, it is essential during L3 development, which is often chosen for metabolic studies due to the relatively large body size and the ease of isolating tissues. L3 larvae experience a series of ecdysone pulses that induce changes in autophagy, insulin signaling, circulating sugars, and a host of other metabolic parameters.¹² As a result, studies of L3 metabolism must be conducted with synchronized populations that have undergone the L2-to-L3 molt within a defined period of time. L3 larvae can also be resynchronized using the *sgs::GFP* reporter, which is only expressed during the later half of L3 development.¹³ In this regard, any mutant lines that exhibit defects in growth rate must be carefully staged to ensure that all samples contain animals of a similar developmental age. For example, mutations that disrupt insulin signaling result in prolonged larval development and small body size.^{14, 15} When analyzing such mutants, researchers must ensure that samples are collected at the same relative developmental timepoint and normalized to the body mass. Although precisely staging animals is tedious, carefully collected samples can reveal unexpected details about both developmental progression and metabolism.

Diet

Metabolism is intimately associated with nutrition and researchers must pay special attention to diet of both the animals that are collected for metabolic analysis and of their parents, as several studies have observed the non-Mendelian inheritance of metabolic phenotypes and embryonic metabolism is entirely dependent of maternally-loaded nutrients.^{16, 17} All fly stocks should be carefully maintained on consistent food and all parents should be of a similar age. Ideally, newly eclosed males and virgin females are separated, maintained at population densities of less than 50 animals per vial, and mated within a week.

While parental diet will influence any developmental stage, studies of post-embryonic metabolism are built upon larval nutrition. The simplest method for collecting larvae is to grow animals on either a molasses or grape agar plate that is partially covered with yeast paste. Larvae will eat at the interface between these two food sources, and animals can be harvested with a short paintbrush. This method works particularly well for isolating homozygous mutant larvae that are interspersed among heterozygous siblings marked with a GFP-labeled balancer chromosome. Alternatively, embryos or larvae can be moved from the egg-collection plates to a plate containing a standard fly media and larvae are then dug out of the food at the appropriate timepoint. This method is useful when analyzing L3 larvae, as these animals burrow into the food and can be difficult to maintain on molasses agar plates. Again, a variety of media are appropriate for these studies, but any of the standard fly foods, as well as variations of a yeast-glucose media,^{18, 19} are well suited for this purpose.

Special Diets—Many metabolic studies are enhanced by the use of diets that allow for the addition or subtraction of specific nutrients. In fact, prior to the advent of modern gene cloning techniques, this approach was essential for determining the molecular nature of *Drosophila* genes such as *vermillion* and *rudimentary*.^{20, 21} The same strategies used in these classic experiments can now be combined with modern pharmaceuticals, small chemical libraries, and the emergence of new diets that are specifically designed for metabolic studies.^{22–24} The simplest dietary experiments involve adding compounds to standard media, however, these experiments should not use yeast paste because yeast will metabolize the supplemented compounds. Rather, compounds should be added to sterile standard media after it has cooled to a temperature of 50°C–60°C. This method can determine if specific metabolites enhance or rescue mutant phenotypes, and provide a means of delivering pharmaceutical and xenobiotic compounds that interfere with normal metabolism. The emergence of chemically-defined (holidic) diets have the potential to further refine metabolic studies;²⁵ however, most of these recipes are designed to study adult metabolism and lack essential nutrients that are required normal larval growth rates.^{25, 26} Studies of developmental metabolism should only use diets that support normal growth, such as a recently described defined diet that uses casein as an amino acid source.²⁷

Nutrient Deprivation—Many metabolic mutants only exhibit larval phenotypes under conditions of stress, such as starvation or amino acid deprivation. An easy method for conducting such experiments is to place two circles of black filter paper in the lid of a 60 mm polypropylene plate, add 2 mL of PBS, replace the bottom, and wrap the plate with parafilm to keep the plate firmly closed.²⁸ Air holes are poked in the side opposite the filter paper using a hot 22-gauge syringe needle, and if necessary, a fine nylon mesh can be glued across the inside of the holes. Control larvae start dying after 24 hours of starvation, and dead carcasses must be removed every 6–12 hours, prior to being cannibalized by siblings. Please note that all experiments must be conducted using carefully staged animals, as the starvation response will vary depending on developmental age. Furthermore, L3 starvation experiments must take critical weight into consideration, as starved pre-critical weight larvae will arrest development, but larvae that have passed this milestone will continue development and enter metamorphosis, regardless of available nutrients.²⁹

High Sugar Diets—*Drosophila* models of metabolic syndrome require a high calorie diet that is capable of inducing obesity, hyperglycemia, and insulin resistance. While several high-sugar diets can be used to analyze adult metabolism, larval studies typically use a modified version of semi-defined medium that omits glucose and contains a high sucrose concentration (51 g/L for control food; 342 g/L for high sugar food).^{30, 31} This food contains all of the essential nutrients required for normal growth, and as a result, metabolic defects can be directly attributed to elevated sucrose levels.³¹ Please note that high-sugar food induces developmental delays and extra precaution should be taken to ensure that samples are collected at the same developmental stage.³¹

Genetic Background

Developmental studies often overlook the presence of *w¹¹¹⁸*, *y¹*, and other commonly used background mutations; however, these mutations can generate metabolic artifacts at any developmental stage. For example, a recent metabolomics analysis demonstrated that *y¹* larvae exhibit an array of metabolic defects.³² Therefore, all studies must use a uniform genetic background, transgenic selection markers, such as *mini-white*, must be analyzed as a potential source of variability, and transgenic strains should be generated using the same phiC31 integration site. These simple precautions will minimize artifacts and allow for the study of subtle, but significant changes in metabolic flux.

BASIC METABOLIC CHARACTERIZATION

Any metabolic study should start by analyzing the major pools of stored energy. For the purpose of *Drosophila* development, the most important metabolic parameters are lipids in the form of triacylglycerides and cholesterol, the major carbohydrates pools of glucose, trehalose, and glycogen, and ATP levels. Detailed methods are available for analyzing all of these metabolites.³³ We will not reiterate the specifics of these protocols, but rather discuss these metabolites in the context of developmental metabolism.

Most assays are based on similar biochemical principles. Samples are homogenized in assay buffer, and if necessary, the macromolecule of interest is broken down into monomeric components. The processed samples are then subjected to an enzymatic reaction that is coupled to either the production of a colorimetric dye or changes in NADH concentration. The most important task for a developmental study is to define the sample size required to measure a compound within the linear range of an assay, which will vary depending on both developmental stage and metabolite. Optimal sample size can be determined by collecting a series of control and mutant samples that contain increasing numbers of animals, and comparing the metabolite concentration in these samples with a standard curve. All samples should give readings within the linear range of the standard curve, and if a mutation induces a dramatic change in metabolite concentration, the sample may need to be diluted or concentrated prior to analysis. The optimal sample size will vary for each developmental stage, however, 300 embryos, 25 L2s, 10 L3s, or 10 pupae can be used as a starting point for most metabolic assays.

Ideally, animals should be collected on ice in a 1.5 mL centrifuge tube, washed with cold PBS, and processed immediately, but such rapid analysis is often impractical when isolating

mutant animals from a population of mixed genotypes. In these cases, washed samples can be frozen in liquid nitrogen and stored at -80°C . While this method works well for larval and pupal samples, embryonic samples need special preparation. Many metabolic assays require a large number of embryos that must be collected in a time-sensitive manner. Therefore, we recommend using a bead mill, such as the OmniRuptor 24 (Omni International), for metabolic studies in embryos. Synchronized embryos are moved onto two pieces of black filter paper that have been placed in 60 x 15 mm cell culture dish and moistened with 2 mL of PBS. Slowly pipette an additional 1 mL of PBS onto the embryos and use a paintbrush to gently remove any large debris. Tilt the plate such that the embryos remain in place and the PBS drains to the side. Remove the excess PBS with a pipette and repeat if necessary. The washed embryos are placed into a 2 mL screwcap tube with an appropriate amount of assay buffer and 1.4 mm ceramic beads (MoBio; 13113-50). The tubes are frozen in liquid nitrogen and can be either stored at -80°C or immediately homogenized using a bead mill. Samples should remain frozen prior to homogenization and there is no need to dechorionate the embryos.

Carbohydrates

Carbohydrate metabolism undergoes striking changes throughout the fly life cycle (Figure 1). Stored carbohydrates in the form of glycogen are maternally loaded into the embryo and slowly decline during the course of embryogenesis.³⁴ This trend is reversed upon hatching, as larvae begin to rely on a unique form of carbohydrate metabolism known as aerobic glycolysis, which is ideally suited for biomass production.³⁵ As a result, larvae exhibit high levels of glycolytic flux, lactate production, and a significant increase in glycogen synthesis.^{35, 36} Once growth is complete, glycolysis is transcriptionally down-regulated and glycogen levels decline throughout the remainder of development.^{36, 37} In contrast, concentrations of the disaccharide trehalose increase during the course of embryogenesis, reach a maximal level in larvae, and then decline during metamorphosis.^{36, 38} Finally, free glucose levels also increase during embryogenesis,^{34, 38} but only represent a minor component of larval sugars.³³ Since sugar metabolism is so closely coordinated with developmental progression, understanding these general trends in carbohydrate storage is essential for identifying the cause of a metabolic defect.

Glucose can be measured directly using a Hexokinase- or Glucose Oxidase (GO)-based assay (Sigma), but trehalose and glycogen must be enzymatically broken down into glucose prior to quantification. Detailed protocols for these assays have been previously described;³³ however, there are a few precautions to consider while using these reagents (see Figure 2). Porcine trehalase is acutely sensitive to buffer conditions and all assays must verify that this enzyme is efficiently degrading the trehalose standards (Figure 2A). In addition, glycogen will rapidly degrade in samples that are inefficiently processed, not stored at -80°C , or exposed to repeated freeze-thaw cycles. The most reliable method for preventing nonspecific glycogen degradation is to immediately homogenize samples after collection, heat treat at 70°C for five minutes to denature endogenous enzymes, and drop freeze the sample tubes in liquid nitrogen. Samples are stored at -80°C and thawed immediately prior to analysis.

Glycogen degradation can also interfere with trehalose measurements (Figure 2B), as this assay simply measures the free glucose concentration and will not distinguish between glucose derived from glycogen or trehalose. Therefore, all samples must include an enzyme-free negative control to identify samples that have experienced excessive glycogen degradation (Figure 2B). Additionally, these assays have a limited linear range and all samples must be properly diluted for accurate measurements (Figure 2B). Finally, the GO assay is inhibited by an unknown compound in pupal extracts and is not appropriate for use with this developmental stage.³³ Understanding the limitations and pitfalls of these assays are key to generating reproducible carbohydrate measurements.

Lipids

Drosophila development is directly influenced by the accumulation and utilization of cholesterol and triacylglyceride (TAG). Cholesterol is used to synthesize the steroid hormone 20-hydroxyecdysone,³⁹ which gates all major developmental events in the fly life cycle. Similarly, TAG represents the major energy store in the fat body, which is the tissue that coordinates larval growth rate with internal nutrient stores. Since both cholesterol and TAG are closely associated with developmental progression, most metabolic studies start by measuring these lipids.

TAG represents the major pool of neutral lipids in *Drosophila*, and like glycogen, the concentration of this molecules undergo dramatic but predictable changes throughout the fly lifecycle (Figure 1). While TAG levels decline during the course of embryogenesis and metamorphosis,^{34, 36, 40} larval metabolism is geared towards synthesizing and storing large quantities of this molecule, much of which is deposited in the fat body for use during metamorphosis and early adulthood.⁴⁰ Since TAG levels are affected by the combined regulation of lipid absorption, biosynthetic flux through glycolysis and the TCA cycle, and catabolic processes such as β -oxidation, TAG measurements can be used as sensitive readout for several metabolic processes. The most common method for quantifying TAG involves adding a lipoprotein lipase to whole animal extracts and measuring the resulting free glycerol concentration with a colorimetric assay.³³ However, several caveats are associated with measuring enzymatically-derived free glycerol, and changes in TAG levels should be verified using other methods, such as thin layer chromatography (TLC) or staining with lipophilic dyes (see below).³³

Similar to TAG, free cholesterol levels are also measured using a commercially available assay and a modified version of this protocol can be used to quantify cholesterol esters.³³ Since *Drosophila* is a cholesterol auxotroph, changes in cholesterol concentrations often stem from absorption and trafficking defects. There are three logical experiments to conduct if a mutant exhibits a developmental delay coupled with decreased cholesterol levels.^{41–43} (1) Stain larvae with the dye filipin (see below) to determine if intestinal cells can properly absorb cholesterol. (2) Raise mutant larvae on a lipid depleted media. Mutants with cholesterol uptake defects are sensitive to low sterol conditions. (3) Determine if adding excess cholesterol and ecdysone to the larval diet can alleviate the mutant phenotype. Since decreased cholesterol levels interfere with ecdysone biosynthesis, providing larvae with an ectopic source of this hormone often rescue developmental defects.

ATP

All metabolic mutants should be analyzed for defects in energy production using a luciferase-based assay.^{33, 44} Decreased ATP production is indicative of metabolic defects, but negative results are somewhat difficult to interpret. This assay does not measure ADP or AMP, and increased concentrations of either nucleotide are also associated with decreased energy production. Therefore, if a mutant strain is suspected to be defective in energy generation, but ATP levels appear normal, samples can be analyzed using LC-MS to simultaneously measure the relative concentrations of ATP, ADP, and AMP. Furthermore, the ATP assay fails to account changes in phosphagen abundance. Ecdysozoans use arginine instead of creatine-phosphate to store high-energy phosphates;⁴⁵ therefore, measurements of creatine-phosphate are meaningless in the context of *Drosophila* metabolism. Unfortunately, arginine phosphate metabolism remains poorly understood in *Drosophila* and the lack of a standard assay represents a major deficit in fly metabolic studies.

Other Metabolites

Commercial kits are now available for compounds ranging from intermediate metabolites to amino acids and fatty acids. These products are designed to measure a variety of individual compounds, but they are often expensive and must be verified to work with *Drosophila* homogenates. In most cases, a metabolomics approach is more accurate and comparable in overall cost.

Normalization

Many metabolic mutants exhibit significant changes in body size, which complicates the direct comparison of metabolite concentrations; therefore, all studies should normalize metabolic data to an intrinsic factor that directly reflects body mass.³³ The most common method to account for differences in body size is to normalize experimental data to the amount of soluble protein present within a homogenized sample, which can be determined using a Bradford assay. This method, however, is not appropriate for mutants that exhibit significant changes in protein translation (such as mutations that disrupt Tor signaling). An alternative approach is to normalize a metabolic measurement to the sample mass, which requires that a sample be washed to remove extraneous debris from the food, the wash buffer completely removed, and the sample mass measured using an analytical balance capable of accurately measuring 0.01 mg. This method requires that samples contain enough animals to generate accurate mass data, and since measurements of body mass do not account for variations in homogenization efficiency, we recommend that samples normalized using this method be processed using bead mill to ensure uniform homogenization. In general, either normalization method is appropriate for generating quantitative measurements in spite of growth defects, but all studies must clearly state which method was used.

Tissue-specific analysis

Quantitative measurements of *Drosophila* metabolites are usually derived from whole animal homogenates. While these measurements are essential, any study must ultimately determine the tissue-specific origin of a given phenotype. With the exception of L3 larvae, where dissected tissues can be used to conduct some metabolic assays, most developmental studies

will need to use a dye to qualitatively assess tissue-specific macromolecule stores. There are well-established protocols for staining glycogen (periodic acid/Schiff staining) and neutral lipids in both fixed tissues (Sudan Black, Oil Red O) and living cells (Nile Red).³³ Cholesterol localization can be visualized in fixed tissues using the antifungal compound filipin,⁴² which binds to 3- β -hydroxysterols. Additionally, some metabolic analyses will benefit by using dyes to visualize autophagy (lysotracker) and mitochondria (mitotrackers; see below).^{46–48} Many of these stains are well suited for mosaic analysis, where changes in metabolite abundance and localization can be easily compared between mutant and control cells in the same tissue, but comparisons between animals must be carefully controlled. All samples must be simultaneously fixed and stained in the same 9-well glass dish, and the resulting slides should be scored blindly to eliminate confirmation bias.

METABOLIC ENZYME ACTIVITY

The field of developmental metabolism is firmly rooted in a series of decades-old studies that examined the developmental regulation of *Drosophila* enzyme activity (e.g. glycerol-3-phosphate dehydrogenase and lactate dehydrogenase).⁴⁹ These classic publications emphasize that the activity, abundance, and assembly of metabolic enzyme complexes change during the course of *Drosophila* development. While the field of developmental metabolism tends to rely on measurements of gene expression and metabolite abundance, future studies should make a concerted effort to understand how changes in enzyme activity contribute to metabolic phenotypes. Fortunately, many of the enzymes in intermediate metabolism can be directly assayed using two basic approaches.

Spectroscopic analysis

Metabolic enzymes that use the cofactors NAD⁺ /NADH or NADP⁺ /NADPH can be directly assayed from whole animal extracts. Staged samples are homogenized in the appropriate reaction buffer and centrifuged to clear large debris. The supernatant is incubated with the enzymatic substrates of interest and enzyme activity is measured as a function of NADH (or NADPH) production or reduction (as quantified by absorbance at 340 nm). Enzymes that do not use a dinucleotide cofactor will require a more complex assay, but reaction conditions have been optimized for many *Drosophila* enzymes.⁵⁰

Mass Spectrometry (MS) and Nuclear Magnetic Resonance (NMR) based analysis

MS and NMR can be used to detect enzymatic activity if the spectroscopic analysis is not feasible. NMR has a distinct advantage in that it can be used to monitor the progress of enzymatic reaction because it does not destroy the sample during detection. In contrast, MS analysis can be used to conduct an endpoint analysis of an enzymatic reaction. The reaction is quenched using an acid or base at a proper time point (within the linear reaction range), and standards are used to measure the accurate concentrations of the substrate and product.

MITOCHONDRIA

Mitochondria are the focal point of eukaryotic metabolism, as most metabolic processes either converge on the mitochondria or are influenced by a mitochondrial-derived

metabolite. Analysis of mitochondrial function is particularly important during development, where stage-specific roles for mitochondria are key for controlling growth and the rate of developmental progression. Notably, several ecdysone biosynthetic reactions occur in the mitochondria,⁵¹ indicating that this organelle controls developmental timing and adult body size. Furthermore, each developmental stage appears uniquely sensitive to a subset of mitochondrial processes. For example, an antimorphic mutation in the β -oxidation gene *scully* leads to both an embryonic and pupal lethal phase,⁵² loss-of-function mutations in the citrate synthase gene *knockdown* induce delayed larval growth,⁵³ and *malate dehydrogenase 2* mutants have no obvious larval phenotype, but rather arrest at the onset of metamorphosis.⁵⁴ These observations emphasize that any mitochondrial defect must be interpreted within a stage-specific context. While there are many methods that can be used to examine mitochondria, below we describe two simple approaches that can quickly identify defects in mitochondrial metabolism.

Mitochondrial Genome Abundance

Perhaps the most rapid method for identifying mitochondrial defects is to measure mitochondrial genome number, which can be indicative of changes in abundance and physiology. Total DNA is isolated using either phenol/chloroform extraction or a commercial kit, and the ratio of mitochondrial DNA to genomic DNA is measured by quantitative PCR. A common method for measuring this ratio involves amplifying a region of the mitochondrial genome (*mt:lrRNA* [5'-AAAAAGATTGCGACCTCGAT-3' and 5'-AAACCAACCTGGCTTACACC-3'] or *mt:CoI* [5'-TGCTCCTGATATAGCATTCCCACGA-3' and 5'-TCCACCATGAGCAATTCCAGCGG-3']) and the *Rp132* genomic locus (5'-AGGCCCAAGATCGTGAAGAA-3' and 5'-TGTGCACCAGGAACTTCTTGAA-3'; also known as *rp49*).^{34, 35} This method has been successfully used to characterize a range of mitochondrial mutants; however, a negative result does not exclude the possibility of a defect.

Visualization

Two widely used genetic reagents that allow for the visualization of mitochondria in living cells are a ubiquitously expressed sqh-EYFP-mito construct and a UAS-mitoGFP transgene.^{57, 58} These fluorescent reporters have been used to examine mitochondrial morphology, localization, and transport in a variety of tissues. Since fluorescent mitochondrial proteins can be visualized in living cells, there is no need to worry about fixation artifacts or tissue permeability; however, neither reporter can be used to infer about defects in membrane potential or mitochondrial metabolism.

MitoTracker dyes (Life Technologies) can also be used to stain mitochondria in living tissues. MitoTracker Deep Red and MitoTracker Green stain bulk mitochondria and allow for the visualization of mitochondrial abundance and morphology. Meanwhile, both MitoTracker Red and MitoTracker Orange CMTMRos only stain polarized mitochondria and are used to assess membrane potential; however, both of these dyes form covalent bonds with the proteins of polarized mitochondria and will not be released upon membrane depolarization.^{59, 60} Experiments that depend on the ability to observe changes in membrane

depolarization should instead use a dye such as tetramethylrhodamine methylester (TMRM) or tetramethylrhodamine ethyl ester (TMRE). Both of TMRM and TMRE have been used in *Drosophila* and will be released from the mitochondria upon membrane depolarization.^{61–63}

METABOLOMIC ANALYSIS

Metabolomics has emerged as a key tool for studying the metabolism of *Drosophila* development. Metabolomic approaches allow for the simultaneous analysis of diverse metabolic pathways^{64–66} and can be used to complement any of the methods described above. Typically, a metabolomic analysis measures small molecule metabolites (MW < 1500 Da) and most studies in *Drosophila* have focused on intermediate metabolism. A standard metabolomic analysis involves appropriate sample preparation, metabolite detection, data analysis and interpretation.^{70–72} This approach is often key for unraveling metabolic defects, as the resulting data can identify the origin of complex phenotypes, such as changes in TAG or glycogen concentration.

Several methods are used for conducting metabolomics studies in the fly;^{34, 73–75} however, we will not provide detailed protocols, as most labs will rely on a core facility for such analysis. Instead, we will briefly describe the advantages and disadvantages of individual approaches (Table 1). This overview is meant to serve as a starting point for planning metabolomic studies, and the occasional user should use a University core facility, private company, or one of the recently opened NIH metabolomics centers (Table 2).

Sample Collection

The key for generating reproducible metabolomics data is to carefully design a collection protocol that minimizes variability between samples and ensures sample integrity. In this regard, the most common pitfalls associated with conducting metabolomics experiments have been well summarized and we direct the interested reader to a few key reviews.^{67–69} The first concern for a metabolomics study is to collect samples in a manner that allows for the normalization of data across sample sets. Therefore, all samples should contain the same number of animals and the sample mass recorded using an analytical balance capable of accurately measuring 0.01 mg. In addition, the sample should be spiked with an easily detected internal standard, such as *d4*-succinic acid, which allows the final data to be normalized despite variability in the sample preparation process, such as sample loss and differences in derivitization efficiency.

The second pitfall of collecting metabolomics samples is a failure to quench metabolism, which involves stopping endogenous metabolic pathways and stabilizing metabolite pools. In order to quench *Drosophila* metabolism, samples must be kept at on ice while being collected and all wash steps must be performed 4 °C. Immediately after washing, the sample mass is recorded, the tube is dropped into liquid nitrogen to halt all metabolic processes, and the sample is then stored at –80°C. Stored samples should only be moved on dry ice until the metabolites have been extracted using an organic solvent, such as methanol or acetonitrile, which denatures metabolic enzymes and permanently halts metabolic flux. Overall, careful attention to experimental detail is key for generating reproducible data and

we encourage the reader to outline and practice their collection protocol prior to conducting a metabolomics experiment with valuable sample material.

Gas Chromatography-Mass Spectrometry (GC-MS)

GC-MS is an efficient method for measuring small metabolites that are volatile below 300 °C. Although relatively few metabolic compounds will enter the vapor phase below this temperature, small polar molecules can be modified with other chemical groups, which not only increases their volatility, but also improves the stability of thermally-labile compounds and enhances the detectability of some molecules. As a result, GC-MS can be used to quantify nearly all of the amino acids, as well as many of the compounds associated with carbohydrate metabolism, the TCA cycle, and secondary metabolic pathways such as the pentose phosphate shunt. *Drosophila* samples can be prepared using a two-step derivatization protocol that stabilizes carbonyl groups and replaces active hydrogens with trimethylsilyl groups.³³ This method has been successfully used to study several fly mutants and generates highly reproducible datasets.

Liquid Chromatography-Mass Spectrometry (LC-MS)

While GC-MS provides a rapid and efficient means of analyzing intermediate metabolism, most metabolites are not volatile below 300 °C. Compounds that are either highly polar or larger than a few hundred daltons can be analyzed using a LC-MS-based method. For example, metabolomic analysis of compounds such as NADP⁺, NAD⁺, ATP, and coenzyme A derivatives are primarily measured using LC-MS. There is often no need to derivatize the metabolites of interest. While this simplifies the sample preparation, the resulting analysis must be optimized for factors such as solvent flow rate, pH, and column type. Several LC-MS-based metabolomics studies have been described in the *Drosophila* literature,^{76, 77} but perhaps the best example of this approach can be found in a recent analysis that used hydrophilic interaction chromatography (HILIC) to analyze individual fly tissues. This study identified over 500 metabolites and provided the first metabolomic analysis of tissue-specific metabolic flux⁷⁸. Overall, a LC-MS metabolomics approach can analyze a broader range of compounds when compared to a GC-MS analysis; however, LC-MS data is less reproducible between instruments and there is no spectral library for compound identification.

NMR

Metabolomic analyses can also be conducted using NMR, which in theory, is capable of detecting a broad range organic molecules. However, NMR is less sensitive than GC-MS and LC-MS and limited by peak overlap, and as a result, NMR-based studies are usually limited to measuring highly abundant metabolites, such as amino acids and organic acids involved in glycolysis and the TCA cycle. Nonetheless, this method is popular among *Drosophila* researchers and offers a few distinct advantages.^{79, 80} First, NMR data is very accurate and highly reproducible, which allows for the comparison of datasets between sample sets. Additionally, NMR does not destroy the sample, which can be stored and used for subsequent analysis, and high-resolution magic-angle-spinning (HR-MAS) NMR spectroscopy can detect metabolic profiling of intact tissues without extraction.⁸¹ Finally, NMR is a powerful method for conducting stable-isotope tracer analysis, as it not only

detects the presence of a labeled metabolite, but also can determine the exact position of an isotope within a molecule.

Stable Isotope Tracer Analysis

A basic metabolomic analysis provides a snapshot of cellular metabolism; however, metabolism is not static, and neither the direction nor rate of metabolic flux can be inferred from a single measurement. This problem is exacerbated during development, where many pathways are used for both energy production and biosynthesis, and metabolic flux changes in stage-specific manner. Stable isotope tracer analysis addresses these problems by allowing for the direct measurement of metabolic flux. Compounds that contain stable-isotopes of common elements, such as ^2H , ^{13}C , and ^{15}N , can be directly added to the larval food (but not living yeast). The labeled compound is allowed to metabolize for a defined-periods of time, which results in the incorporation of stable isotopes into intermediate metabolites, and isotope distribution is analyzed using GC-MS, LC-MS, or NMR. For example, if the larvae are fed with U- ^{13}C -glucose, then ^{13}C will incorporate into the carbon backbone of the intermediates in glycolysis and TCA cycle, and change the distribution of isotopologues, which are characterized with different m/z values in mass spectroscopy (Figure 3). The protocol and the principles underlying the calculation of metabolic flux ratios were well summarized previously.^{82–84} The metabolic flux analysis is gaining traction in *Drosophila* studies, and has been used to analyze fat synthesis, diet-induced obesity, and glucose oxidation.^{31, 85}

CONCLUSION AND FUTURE DIRECTIONS

The metabolism of developing animals must adapt to the biosynthetic and energetic demands of cell proliferation, tissue growth, maturation, and reproduction. The rare combination of genetic tools and easily recognized developmental transitions make *Drosophila* the ideal system for studying the conserved metabolic mechanism that coordinate metabolism and development. Here we outlined a basic method for characterizing *Drosophila* metabolism and demonstrate the ease of conducting these studies in the fly. But more importantly, our review emphasizes that current technologies have matured to the point where a few key advances would establish *Drosophila* development as the premier system for exploring the mechanistic links between metabolism and growth, development, and maturation.

Much of our knowledge regarding metabolic regulation stems from studies of large differentiated tissues isolated from adult organisms, such as mammalian liver, brain, and muscle. In most cases, however, these classic studies did not or could not determine how metabolic flux varies depending on developmental stage, tissue type, diet, genotype, and the instantaneous biosynthetic and energetic needs of an organism. In this regard, our ability to control and manipulate these variables during *Drosophila* development provides a means to study metabolic regulation with unprecedented detail. In addition, while the enzymes involved in intermediate metabolism have been the subjects of intense biochemical studies, many of metabolic enzymes encoded within metazoan genomes are unstudied and their function is simply inferred based on homology. These enzymes appear to have specialized

functions, as they are often expressed in specific tissues or during specific developmental stages, suggesting that any assumption about their function based on homology is naïve and interferes with our ability to identify novel metabolic mechanisms. The fly community is uniquely positioned to address all of these problems. The *Drosophila* genome is well annotated, we can quickly mutate and modify genes, and all stages of fly development are easily analyzed using any of the metabolomics approaches described above. Furthermore, the fly community has a history of generating, analyzing, and annotating extremely large datasets in a meaningful way.

We propose that the *Drosophila* community should make a concerted effort to annotate changes in the metabolome in both space and time. In this regard, we believe there are three major questions that should be addressed using a metabolomics approach. (1) How does metabolic flux change during the course of the *Drosophila* development? (2) How are metabolic processes partitioned into distinct tissues? (3) What are the metabolic functions of the unstudied enzymes within the fly genome? Each of these questions is within reach of the *Drosophila* community and answering any of them would significantly advance our understanding of animal metabolism.

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FURTHER READING

86. Tennessen JM, Barry WE, Cox J, Thummel CS. Methods for studying metabolism in *Drosophila*. *Methods.* 2014; 68:105–115. [PubMed: 24631891]
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Figure 1. A schematic representation of changes in metabolite abundance during *Drosophila* development

Trehalose, glycogen, and triglycerides undergo dramatic but predictable changes during the *Drosophila* life cycle. Any metabolic experiment must be interpreted in the context of these developmental trends.

Figure 2. A diagram illustrating common problems associated with using the glucose oxidase (GO) method for measuring larval trehalose
The GO assay oxidizes free glucose to generate a red product. The intensity of the colored product is measured by absorbance at 540 nm. (A) All trehalose assays must include a standard curve for both glucose and trehalose. An acceptable assay will display a linear increase in absorbance that reflects the increasing concentrations of both sugars. Failure to observe a concentration dependent increase in trehalose measurements is the result of inactive trehalase, which can stem from problems with either the buffer or enzyme stock. Expired GO reagent will result in abnormally low readings for both the glucose and trehalose standard cure. (B) Problems associated with measuring trehalose in both a control strain and a mutant strain with a hypertrehalosic phenotype. Improperly prepared samples will result in glycogen degradation, which leads to abnormally high free glucose levels in the no trehalase control (–) of both strains (free glucose levels are normally very low in larval measurements). Trehalose concentrations that fall beyond the linear range of the GO assay generate a purple color, which alters the absorption spectra of the sample and generates inaccurate measurements. Properly prepared samples will exhibit a red hue.

Figure 3. A schematic diagram illustrating the basic principles of stable isotope tracer analysis U- ^{13}C -labeled glucose is six atomic mass units (amu) heavier than unlabeled ^{12}C glucose. The catabolism of labeled glucose will result in the incorporation of ^{13}C into other metabolites, which can be measured using either MS or NMR. For example, lactate and pyruvate molecules derived from labeled carbon will be 3 amu heavier, or m+3, than unlabeled molecules. Pyruvate derived from labeled glucose can enter the TCA cycle via formation of acetyl-CoA, which will result in m+2 citrate. As labeled carbons move throughout the cycle, the mass of intermediates will become more complex, resulting in m+3 and m+4 labeled compounds. The end result is represented by an isotopologue distribution.

Table 1

Comparison of MS and NMR-based methods for metabolomic analysis

	GC-MS	LC-MS	NMR
Sample preparation	Extraction; Derivatization	Extraction; Desalting; Filtration	No sample preparation or simple extraction
Sensitivity	pM- μ M	pM- μ M	μ M-mM
Quantification	Standard required; Matrix and ionization dependent response	Standard required (isotope labelled standard); Matrix and ionization dependent response	No standard required; Linear response
Specific advantages	Sensitivity; Standard library is available for identification	Sensitivity; High number of detectable metabolites	Non-destructive detection; Good replication; Structure information
Specific disadvantage	Complex sample preparation; Destructive detection; Not suitable for heat-labile and very high boiling point metabolites	Ion depression effect; No structure information; Destructive detection	Low sensitivity; Peak overlap

Table 2

Six regional comprehensive metabolomics resource cores funded by NIH

Metabolomics Resource Cores	Website
The West Coast Metabolomics Center at UC Davis	http://metabolomics.ucdavis.edu/
The Michigan Regional Comprehensive Metabolomics Resource Core	http://mrc2.umich.edu/index.php
Eastern Regional Comprehensive Metabolomics Resource Core	http://www.rti.org/page.cfm/Metabolomics_Research
The Southeast Center for Integrated Metabolomics	http://secim.ufl.edu/
The Resource Center for Stable Isotope-Resolved Metabolomics	http://bioinformatics.cesb.uky.edu/bin/view/RCSIRM/
The Mayo Clinic Metabolomics Resource Core	http://www.mayo.edu/research/centers-programs/metabolomics-resource-core/overview