Metabolomic Studies in *Drosophila*

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ABSTRACT Metabolomic analysis provides a powerful new tool for studies of *Drosophila* physiology. This approach allows investigators to detect thousands of chemical compounds in a single sample, representing the combined contributions of gene expression, enzyme activity, and environmental context. Metabolomics has been used for a wide range of studies in *Drosophila*, often providing new insights into gene function and metabolic state that could not be obtained using any other approach. In this review, we survey the uses of metabolomic analysis since its entry into the field. We also cover the major methods used for metabolomic studies in *Drosophila* and highlight new directions for future research.

KEYWORDS metabolism; homeostasis; physiology; systems biology; Flybook

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"The molecules found in living organisms not only conform to all the familiar physical and chemical principles governing the behavior of all molecules but, in addition, interact with each other in accordance with another set of principles that we shall refer to as the molecular logic of the living state." Albert L. Lehninger

STUDIES of insect metabolism originated from an interest in understanding their remarkable morphological diversity and ecology. In *Drosophila*, these early studies focused on bioenergetics and nutrient metabolism, but quickly expanded in the middle of the 20th century in many directions, facilitated by the advent of biochemical genetics. This landmark discovery by Beadle and Tatum led to the "one gene one enzyme hypothesis" that unified genetics and biochemistry, paving the way for detailed metabolic and physiological studies in *Drosophila* (Beadle and Tatum 1941; Horowitz 1985). As a result, early studies of metabolism in *Drosophila* were often focused on reporting the effects of mutations on specific steps in metabolic pathways. Classic examples of this work include studies of the *rudimentary* locus, which encodes the first three enzymes in the pyrimidine biosynthetic pathway, and the genetics of *alcohol dehydrogenase* (Norby 1973; Rawls and Fristrom 1975; Sofer and Martin 1987). More complex studies of multiple metabolites followed in later work, as exemplified by the classic work of Winifred Doane and colleagues on one of the first obese mutants in *Drosophila*, *adipose*^{60} (Teague *et al.* 1986). All of these studies, however, were limited by the number and type of specific metabolites that could be detected and measured. This situation changed dramatically at the turn of this century with the advent of high-throughput methods for metabolite identification. These approaches relied on nuclear magnetic resonance (NMR), or gas or liquid chromatography (GC or LC) combined with mass spectroscopy (MS). Other methods for metabolite fractionation, such as capillary electrophoresis (CE) have also been implemented (Soga *et al.* 2003). For the first time, large scale studies could be conducted that included accurate measurements of hundreds of metabolites, giving birth to the field of metabolomics (Raamsdonk *et al.* 2001; Fiehn 2002). This technical innovation, combined with other high throughput methods for DNA sequencing, transcriptional profiling, and proteomic profiling, established a foundation for functional analysis that has shed new light on *Drosophila* metabolism and provided an integrated systems-level understanding of its regulation.

In this review we provide an overview of metabolomic studies in *Drosophila*. We start by providing an introduction to this topic, covering specific applications of metabolomics for gaining new insights into different aspects of *Drosophila* biology. We also emphasize the importance of experimental design, genetic background, and environmental conditions in conducting the most accurate metabolomic studies, allowing the investigator to draw clear and reproducible conclusions from the analysis. We survey the major methods used for metabolomics in *Drosophila* and highlight some of the advantages and disadvantages of each approach. Finally, we survey some future directions for the field that hold the promise of providing important new insights into insect physiology.

For studies of metabolism and physiology, a metabolomic profile provides the most complete representation of the phenotype of the animal, revealing the combined contributions of gene expression, enzyme activity, and environmental context. Moreover, the power provided by measuring multiple metabolites and entire pathways can allow the discovery of important aspects of regulation that might be missed otherwise. For example, metabolomic analysis allowed for the identification of coenzyme A as protecting flies against the toxicity of a high sugar diet (Palanker Musselman *et al.* 2016). Metabolomics can also be invaluable for pinpointing the metabolic defects in a mutant. For example, elevated levels of succinate combined with reductions in fumarate and malate demonstrated that two different adult viable mutants impacted distinct steps in the assembly of the succinate dehydrogenase holocomplex (Na *et al.* 2014; Van Vranken *et al.* 2014). In addition, metabolomics has provided a powerful new analytic tool for genetic studies of aging in *Drosophila*. For example, metabolomic studies have shown that the longevity-promoting effects of dietary restriction are associated with specific shifts in metabolic pathways (Laye *et al.* 2015). In addition, metabolomics revealed that methionine metabolism shifts during aging in *Drosophila*, and allowed the identification of S-adenosyl-homocysteine as playing an important role in this process (Parkhitko *et al.* 2016). Discoveries of this nature have only become possible with the ability to detect and quantify hundreds to thousands of metabolites in a single experiment. For these reasons, metabolomics has been established as an essential tool in model organism metabolic studies.

The power of this approach is also apparent when combined with other large-scale high-throughput technologies. Thus, for example, while transcriptional profiling can provide a

genomewide perspective on the effect of a particular genotype or treatment on gene expression, this approach is dependent on the accuracy of gene annotation and the assumption that changes in transcript levels are reflected by similar changes in the encoded protein product. Metabolomics provides a very different perspective, which, when combined with transcriptional profiling, can synergize to allow new discoveries. Thus, combined transcriptional and metabolic profiling studies have provided new insights into the interplay between genetic and environmental factors on larval physiology (Reed *et al.* 2014; Williams *et al.* 2015), cold tolerance (Teets *et al.* 2012; MacMillan *et al.* 2016), the physiological effects of a high fat diet (Heinrichsen *et al.* 2014), the effect of methamphetamine treatment (Sun *et al.* 2011), and the aerobic glycolytic state of *Drosophila* larvae (Tennessen *et al.* 2011).

The sensitivity and breadth of metabolomic studies require special attention to the multiple factors that can impact metabolite levels, including the genotype of the animal, its diet, environment, inherited epigenetic effects, circadian rhythms, and the microbiome within the gut. Each of these factors, alone or in combination, can have a profound effect on metabolite levels, leading to different overall results. In this sense, there is no canonical "metabolome" for a particular strain of *Drosophila* since it can be impacted by so many extrinsic factors. For example, as has been widely studied in mammals, many metabolites display major fluctuations in response to the day-night cycle in flies (Gogna *et al.* 2015). In addition, eye and body color mutations, which are widely used as genetic markers in *Drosophila*, can have a significant effect on metabolite levels. Thus, for example, the inability of *rosy* mutants to synthesize uric acid results in an expected accumulation of purine metabolites such as xanthine and hypoxanthine, as well as changes in more distal metabolites such as tryptophan, kynurenine, and related compounds (Hilliker *et al.* 1992; Kamleh *et al.* 2008). Similarly, *yellow* mutants accumulate phenylalanine, tyrosine and dihydroxyphenylalanine, and display more distal effects on lysine metabolism (Bratty *et al.* 2012). This is consistent with the important role of *yellow* in body color pigmentation through its production of melanin. Superimposed on these genotypic backgrounds are the impact of many environmental factors that can affect metabolism (Reed *et al.* 2014; Williams *et al.* 2015). Some of these, such as diet, have been studied using metabolomic approaches in *Drosophila*, whereas others, such as the effect of epigenetic inheritance or the gut microbiome on the metabolome, remain to be characterized (Heinrichsen *et al.* 2014; Laye *et al.* 2015; Williams *et al.* 2015; Palanker Musselman *et al.* 2016). Thus, close attention needs to be paid to genetic background when designing metabolomic studies of mutants, ideally using multiple control genotypes for internal confirmation. Carefully controlled dietary conditions, fly maintenance, and environmental factors are also critical for accurate metabolomic studies in flies.

Applications of metabolomics in Drosophila

Although the technology required for metabolomic studies has only been available for ~15 years, over 50 studies in *Drosophila* have exploited this technology, covering a wide range of topics (Table 1). These applications have proven particularly valuable for the characterization of tissue- and cell-type specific metabolites, genetic studies of disease models, and the effects of different drug treatments, diets, environmental conditions, or stresses on overall physiology (Table 1). In many cases, this level of analysis has provided insights into metabolism that could not be gained in any other way.

A number of remarkable discoveries can be attributed to the advent of metabolomic methodology. For example, an elegant early use of this technology was directed at characterizing the *sxe1* locus, which encodes a male-biased cytochrome P450 that is expressed in the sensory cells of the head and required for efficient mating. Lipidomic profiling of isolated heads from *sxe1* mutants provided evidence that it acts as a fatty acid ω-hydroxylase through its effects on a number of specific lipid species (Fujii *et al.* 2008). This study provided a foundation for more focused biochemical studies aimed at identifying the enzymatic activity of the encoded protein product.

Another early use of metabolomics was aimed at characterizing mitochondrial proteins of unknown function that are conserved through evolution from yeast to humans. In this study, specific changes in the levels of pyruvate, tricarboxylic acid (TCA) cycle components, and amino acids in mutant flies suggested that the proteins act at a critical step in mitochondrial pyruvate utilization (Bricker *et al.* 2012). Further experiments showed that these proteins form a complex that acts as the Mitochondrial Pyruvate Carrier (MPC), which couples cytoplasmic glycolysis with mitochondrial pyruvate oxidation. The discovery of this new step in intermediary metabolism provided a basis for subsequent characterization of the role of mitochondrial pyruvate uptake on normal metabolism, metabolic disorders, and cancer (McCommis and Finck 2015; Bender and Martinou 2016).

In an elegant study, Obata *et al.* (2014) used CE-MS metabolomics to analyze the molecular basis for sterile inflammatory responses. They showed that mutants for *dark*, which encodes a critical activator of apoptosis, Apoptotic protease activating factor-1 (Apaf-1), activates a necrotic immune response in the wing. Metabolomic analysis of hemolymph isolated from *dark* mutants revealed elevated levels of circulating sarcosine and reduced S-adenosyl-methionine caused by Toll activation and increased expression of glycine N-methyltransferase (*Gnmt*) in the fat body. This change in expression occurs in response to FOXO activation, leading to a loss of lipid stores and organismal wasting. They conclude that localized immune signaling from wing cells lacking *dark* function leads to a systemic wasting response mediated by remote changes in S-adenosyl-methionine metabolism in the fat body.

A subsequent study by Kwon *et al.* (2015) showed that intestinal cell overproliferation leads to systemic hyperglycemia, reductions in glycogen and triglycerides, and a wasting response. LC-MS metabolomic analysis of these animals revealed reduced

Table 1 Applications of metabolomics to *Drosophila* research

Method	Condition analyzed	Reference
NMR	Effect of heat stress	Malmendal *et al.* (2006)
NMR	Effect of hypoxia	Feala *et al.* (2007)
NMR	Effect of cold shock	Overgaard *et al.* (2007)
NMR	Effect of hypoxia in the heart	Feala *et al.* (2008)
NMR	Effects of hypoxia and age	Coquin *et al.* (2008)
LC/MS	Analysis of *rosy* mutants	Kamleh *et al.* (2008)
LC/MS	Analysis of *sex-specific enzyme 1* (*sxe1*, *cyp4d21*)	Fujii *et al.* (2008)
NMR	Effect of temperature on inbred and outbred lines	Pedersen *et al.* (2008)
NMR	Effect of acute hypoxia	Feala *et al.* (2009)
LC/MS	Analysis of *chocolate* and *maroon-like* mutants	Kamleh *et al.* (2009)
GC/MS	Effects of a plant-derived protease inhibitor: Bowman–Birk inhibitor	Li *et al.* (2010)
GC/MS	Cold tolerance in *Drosophila melanogaster*	Kostal *et al.* (2011)
GC/MS	Effect of methamphetamine treatment	Sun *et al.* (2011)
GC/LC/MS	Effect of infection with *Listeria monocytogenes*	Chambers *et al.* (2012)
GC/MS	Discovery of the mitochondrial pyruvate carrier	Bricker *et al.* (2012)
LC/MS	Analysis of *yellow* mutants	Bratty *et al.* (2012)
GC/MS	Cold tolerance of *Drosophila montana* flies	Vesala *et al.* (2012)
LC/MS	Metabolite profiling of ten tissues in *Drosophila*	Chintapalli *et al.* (2013)
GC/MS	Genetic studies of mitochondrial aconitase	Cheng *et al.* (2013)
NMR	Metabolite response to selection for tolerance to cold, heat, starvation, and desiccation	Malmendal *et al.* (2013)
LC/MS	Effects of a cytosolic superoxide dismutase (cSOD) mutation, paraquat-induced oxidative stress, and the metabolomic profile of four *Drosophila* species	Knee *et al.* (2013)
GC/MS	Changes in metabolites during embryogenesis	An *et al.* (2014)
GC/MS	Effects of a high fat diet	Heinrichsen *et al.* (2014)
GC/MS	Changes in metabolites during embryogenesis	Tennessen *et al.* (2014b)
CE/MS	Analysis of hemolymph from apoptosis-deficient *dark* mutants	Obata *et al.* (2014)
GC/MS	Effects of genetic variation and diet	Reed *et al.* (2014)
LC/MS	Effects of age, sex, and genotype	Hoffman *et al.* (2014)
GC/MS	Effect of Sdhaf4 mutation on SDH activity	Van Vranken *et al.* (2014)
NMR	Effect of cold (0°) on lines selected for fast or slow recovery from chill coma	Williams *et al.* (2014)
LC/MS	Effects of warm (27°) or cool (18°) conditions	Hariharan *et al.* (2014)
GC/MS; LC/MS	Effects of pioglitazone feeding on larvae that express TDP-43 in motor neurons	Joardar *et al.* (2015)
LC/MS	Effects of organ wasting caused by overproliferation	Kwon *et al.* (2015)
GC/MS	Effects of age and dietary restriction	Laye *et al.* (2015)
GC/MS	Effects of genetic variation and diet	Williams *et al.* (2015)
NMR	Circadian regulation of metabolites	Gogna *et al.* (2015)
GC/MS; LC/MS	Effects of permethrin treatment	Brinzer *et al.* (2015)
GC/MS	Investigate the role of TRPA1 in metabolism	Lee *et al.* (2016)
GC/MS	Correlation of metabolite changes with indicators of oxidative stress, dopaminergic neurodegeneration, and behavior	Shukla *et al.* (2016)
GC/MS	Analysis of the protective effect of coenzyme A upon caloric overload	Palanker Musselman *et al.* (2016)
NMR	Young and old flies expressing amyloid β peptide in their brain	Ott *et al.* (2016)
LC/MS	Reprogramming of methionine metabolism in adults of different ages	Parkhitko *et al.* (2016)
LC/MS	Effects of warm (21.5°) or cold (6°) conditions	MacMillan *et al.* (2016)
GC/MS	Effect of freeze tolerance	Kostal *et al.* (2016)
GC/MS; LC/ESI/MS	Effects of cold tolerance on metabolome and lipidome	Colinet *et al.* (2016)
NMR	Effects of infection with *S. aureus*	Bakalov *et al.* (2016)
NMR	Effect of different temperatures during development	Schou *et al.* (2017)
NMR	Long-term effects of repeated mild heat treatment	Sarup *et al.* (2016)

Publications are listed that highlight metabolomic studies in *Drosophila*.

levels of ATP, NADH, and NADPH in the hemolymph. These effects are mediated by the inhibitor of insulin signaling ImpL2, which is expressed in the intestine and reduces systemic levels of DILP signaling that leads to remote wasting of peripheral organs.

Systems level studies have also provided insights through metabolomics that could not be achieved otherwise. Examples of this include papers that use a combination of metabolomics, microarrays, and measurements of stored macromolecules to determine how gene expression and metabolism respond to both nutritional cues and genotype (Reed *et al.* 2014; Williams *et al.* 2015). A key takeaway from these studies is that the transcriptome and the metabolome are differentially

regulated by diet and genetic background and, as a result, changes in gene expression are not easily correlated with changes in metabolic flux. These observations emphasize that metabolomic approaches provide a means of studying diet-induced changes in animal physiology that could not be predicted by gene expression data.

Recent applications of lipidomic analysis have also provided unique and important insights into the complexity and distribution of different lipid species in *Drosophila*. This is best exemplified by a pioneering study from the Eaton and Shevchenko labs, in which they characterized 250 species of 14 major lipid classes in different tissues at multiple stages of development and under different dietary conditions (Carvalho *et al.* 2012). This study showed that the phospholipid content of the animal is closely linked to its diet and revealed unexpected tissue-specificity in the accumulation of different sterol species within the animal as well as shifts in lipid metabolism during development. A later comprehensive characterization of ecdysteroids in *Drosophila* showed that four different steroid classes are produced overall, each of which is derived from distinct dietary precursors (Lavrynenko *et al.* 2015). These efforts highlight the power of lipidomic analysis and demonstrate that future applications of this methodology can provide new and important insights into *Drosophila* biology.

The widespread impact of metabolomic analysis on model organism research has prompted a formalized endorsement of its utility. This was achieved in July 2015 with the formation of the Metabolomics Society's Model Organism Metabolomes (MOM) task group (Edison *et al.* 2016). This group, consisting of researchers who study a wide range of microbial, animal, and plant model systems, including *Drosophila*, has proposed a "grand challenge to identify and map all metabolites onto metabolic pathways, to develop quantitative metabolic models for many model organisms, and to relate organism metabolic pathways within the context of evolutionary metabolomics (Edison *et al.* 2016)." This cross-species comparison of metabolomic datasets should facilitate interactions between researchers studying metabolism in different systems, allow the development of new bioinformatic tools and metabolomic strategies, and enhance our understanding of the evolution of metabolic networks. Similarly, an initial effort has been made to determine the metabolite composition of individual tissues in adult *Drosophila* using a ZIC-HILIC LC-Orbitrap MS platform (Chintapalli *et al.* 2013). This study included samples of dissected heads, crops, midguts, hindgut, Malpighian tubules, accessory glands, testes, ovaries, and cuticle. This analysis identified 242 polar metabolites, 251 lipids in the positive ion mode, and 61 lipids in the negative ion mode, revealing a range of metabolites that are consistent with tissue function. These included high levels of acylcarnitines in the intestine, ether lipids in the head, and decarboxylated S-adenosylmethionine in the male accessory gland. This analysis provides a valuable framework to take the analysis of *Drosophila* metabolomics to a tissue and, eventually, cellular level, raising the possibility of providing major new insights into metabolic regulation and function.

Methods used for metabolomic analysis in Drosophila

The metabolome is defined as any small molecule (<1500 Da) that an organism ingests, synthesizes, catabolizes, or encounters in the environment (German *et al.* 2005; Wishart *et al.* 2007). While the *Drosophila* metabolome remains undefined, the Human Metabolome Database currently includes entries for over 42,000 small molecules (Wishart *et al.* 2013), which will continue to grow as technology advances. Since many metabolic pathways are highly conserved (Rajan and Perrimon 2013; Padmanabha and Baker 2014), the fly metabolome likely contains a similar diversity of compounds. No one method is capable of measuring all of these molecules, as a protocol optimized for detecting small, highly polar molecules (*e.g.*, pyruvate, lactate, and glycerol) will fail to accurately detect large, hydrophobic molecules, such as very-long chain fatty acids, cuticular hydrocarbons, and insect hormones such as juvenile hormone. Similarly, molecules such as NAD(P)H are unstable and should only be quantified under basic conditions (Wu *et al.* 1986). Therefore, each study must be targeted to measure a specific fraction of the metabolome. Below we outline the common methods and instruments used for metabolomic studies and describe how each provides advantages for detecting specific classes of metabolites.

Instrumentation

Most metabolomic studies are based on three types of instrumentation: Gas Chromatography/Mass Spectrometry (GC-MS), Liquid Chromatography/Mass Spectrometry (LC-MS), and Nuclear Magnetic Resonance (NMR). Each of these approaches has specific advantages and disadvantages (Table 2), and while many metabolomic analyses are dictated by equipment access, understanding the capabilities of each instrument can significantly increase the effectiveness of study design.

GC-MS: Most major *Drosophila* metabolic pathways can be surveyed using GC-MS, which is capable of measuring small molecules with a mass <500 Da. Since this technology requires that a molecule transition between the liquid and gaseous phase at temperatures below 350°, many of the polar compounds associated with intermediate metabolism must be chemically altered or derivatized to render them more volatile (see Figure 1 for example). While there are a variety of different derivatization protocols, a common method for preparing *Drosophila* samples uses a two-step derivatization that modifies the metabolite pool with O-methoxylamine hydrochloride (MOX) and N-methyl-N-trimethylsilyltrifluoracetamide containing 1% TMCS (MSTFA) (Tennessen *et al.* 2014a). The resulting chemical modification both increases volatility and stabilizes heat-sensitive compounds. While these chemical reactions will inevitably result in the loss of sample material, GC-MS is a relatively simple technology that generates highly reproducible data. Furthermore, a number of well-annotated GC-MS compound libraries are available

Table 2 A comparison of common metabolomic techniques

Modality	Sensitivity	Number of observed metabolites	Sample preparation and analysis time	Molecular weight range	Advantages	Disadvantages
LC/MS	High (pmol–nmol)	High (200–1500)	Medium	Broad	Highly sensitive across a broad range of metabolites	Difficult to separate constitutional isomers
GC/MS	Medium (nmol–μmol)	Medium (100–150)	High	Low (<500 Da)	Able to separate constitutional isomers	Intensive sample preparation; limited molecular weight range
NMR	Low (μmol–mmol)	Low (10–50)	Low	Broad	Quantitative; determination of isotopic label position; nondestructive	Limited to high abundance metabolites

The advantages and disadvantages of using LC/MS, GC/MS, and NMR to conduct metabolomic studies.

for metabolite identification and unknown molecules can be identified based on accurate mass measurements (Babushok *et al.* 2007; Kind *et al.* 2009). Current methods allow for the reliable identification of ~150 compounds in the *Drosophila* metabolome, which include most of the amino acids, nearly all of the TCA cycle intermediates, several sugars and glycolytic intermediates, as well as a range of compounds associated with other aspects of intermediate metabolism. This type of survey thus provides an outstanding overview of the metabolic state of the animal.

LC-MS: Perhaps the most versatile technique for metabolomics is LC-MS, which is capable of measuring nearly any component of the *Drosophila* metabolome. Molecules do not need to be derivatized prior to separation, which minimizes sample loss, and state-of-the art instruments are capable of measuring metabolites in relatively small samples. LC-MS-based analysis, however, is more complicated than a GC-MS-based study. Spectral data are not easily comparable between instruments, metabolites are not separated to the same extent as GC-MS, and unknown molecules can be difficult to identify. Nonetheless, regardless of these disadvantages, the ability of LC-MS to measure a broader range of metabolites than GC-MS or NMR makes this the favored technology among many in the metabolomics field. In addition, many of these compounds can provide critical insights into cellular metabolism, including AMP, ADP, ATP, NAD, NADH, NADPH, GSH, and GSSG.

NMR: While most fly metabolomics studies have relied on a MS-based approach, NMR is a powerful technique that allows for rapid, highly reproducible, and quantitative measurements of the metabolome. Furthermore, this technology offers the distinct advantage that samples are not destroyed during analysis and thus can be used in subsequent measurements. In addition, as described below, NMR studies generate positional information during stable isotope tracer experiments that can provide essential insights into metabolic flux that would be more difficult to address using MS. NMR, however, is not as sensitive as MS-based studies and requires a larger sample mass (~50 adult flies per samples). In spite of this shortcoming, however, NMR has been used for a number of studies in the field (Table 1).

Sample collection

Accurate metabolomic studies necessitate that samples be efficiently collected, processed, and frozen in liquid nitrogen. Since metabolite turnover can occur at incredible rates, sample collection protocols are designed to stop or quench enzymatic reactions as quickly as possible (Figure 2). We have found that the most efficient method for both quenching metabolism and homogenizing samples is to place flies in a 2 ml screwcap tube with 1.4 mm ceramic beads and destroy the tissue using a bead mill homogenizer (we recommend the Omni Bead Ruptor 24). For embryos, pupa, and adults, animals can simply be placed in a pre-tared tube, the sample mass recorded using an analytical balance capable of accurately

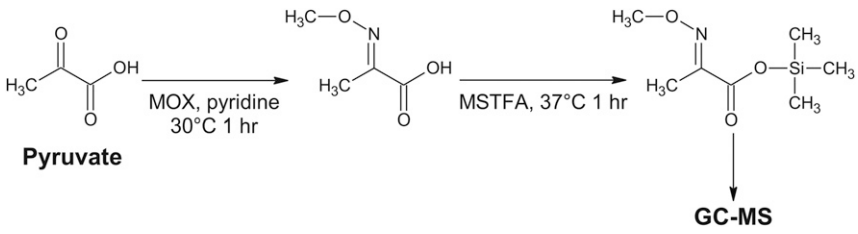

Figure 1 Derivitization of pyruvate for GC-MS analysis. Small polar molecules such as pyruvate must be chemically altered prior to separation by gas chromatography. As shown in this example, the ketone in pyruvate is methoximated with O-methoxylamine hydrochloride (MOX; dissolved in pyridine). Subsequent treatment with N-methyl-N-trimethylsilyltrifluoracetamide (MSTFA) containing 1% TMCS silylates the carboxyl group and increases the volatility of the molecule.

measuring 0.01 mg, and the tightly closed tube immediately dropped into liquid nitrogen. There is no need to dechorionate embryos, as the bead mill will destroy the embryonic cuticle. In contrast, larval samples must first be washed to remove any yeast or debris. This can be achieved by serial rinses with either a cold PBS or NaCl solution in a 1.5 ml microfuge tube. Prior to freezing, the sample should be centrifuged at 3000 × *g* to pellet the larvae and all wash solution must be removed with a 200 μl pipette, as any extra liquid will result in inaccurate mass measurements and PBS will interfere with MS signals. In order to transfer larval samples into bead tubes, remove an individual tube from the liquid nitrogen and dislodge the larval pellet by sharply pounding the top of the tube against a desktop. Pour the pellet into a pre-tared bead tube, quickly record the mass, and immediately return to liquid nitrogen for processing. Frozen samples can be stored at −80° for several months prior to processing and analysis; however, some metabolites, such as L-2-hydroxyglutarate, appear to be unstable even at −80° (Tennessen, unpublished data). We therefore recommend that samples be analyzed within a few months of collection.

Metabolite extraction

The rapid and efficient homogenization of flies in organic solvent is crucial to generating reproducible data. This extraction step not only releases the intracellular metabolites into the solvent for subsequent analysis, but also precipitates proteins and terminates all metabolic reactions. Extraction methods will vary between protocols and instrumentation, but the researcher must ensure that samples remain frozen and that tissues are completely destroyed. In many cases, an inability to generate reproducible data can be traced to inefficient tissue homogenization and metabolite extraction. These steps are often problematic for metabolomics core facilities that focus on cell culture experiments or biofluids, such as blood or urine. The insect cuticle is difficult to homogenize and must be destroyed with sufficient force to ensure the rapid quenching of metabolic pathways. As described above, we have found that a bead mill homogenizer capable of destroying a sample in less than a minute provides the most reliable means of extracting metabolites (Figure 2). Samples that were collected in the presence of ceramic beads can be transferred to a −20° tube caddy. A chilled organic solvent (−20°), such as methanol or acetonitrile, is added to the sample and immediately homogenized using a bead mill. The resulting homogenate is incubated at −20° to precipitate proteins and the sample is centrifuged to clear insoluble debris. The supernatant is then dried and the metabolite containing precipitate can be resuspended in an appropriate solvent.

Internal standards

All samples must be processed and analyzed in the presence of an internal standard(s) to ensure that final metabolite measurements account for sample loss, difference in extraction efficiency, and in the case of GC-MS, incomplete derivitization of metabolites. The initial homogenization step should take place in a solvent that contains a defined concentration of a stable isotope labeled compound, such as succinic-*d*4 acid for GC-MS and negative mode LC-MS, and L-carnitine (trimethyl-*d*9) in positive mode LC-MS. The inclusion of these standards prior to sample homogenization allow for the normalization of metabolite concentration regardless of material lost during any stage of sample preparation (Figure 2). In addition, biological samples contain a complex mixture of compounds that can interfere with ionization efficiency during MS analysis. This so-called "matrix effect" prevents accurate quantification of co-eluting metabolites (Taylor 2005; Buscher *et al.* 2009), however, targeted metabolomics analysis can bypass this phenomenon by adding known concentrations of stable isotope labeled standards, which can be differentiated by MS (see below). Finally, samples that are analyzed by GC-MS can be spiked with a fatty acid methyl esters standard (FAMES) solution just prior to chromatographic separation to normalize retention times. A description of the FAMES solution used in our studies can be found in Tennessen *et al.* (2014a).

Choice of extraction solvent

Just as no single analytical method is capable of fully profiling the metabolome, no single solvent is able to completely extract all metabolites. Polar metabolites involved in central metabolism such as intermediates in the TCA cycle and glycolysis, organic acids, and amino acids, are easily extracted using polar solvents such as acetonitrile, methanol, ethanol, or a combination of these in water or buffer. Little agreement on the type or ratios of these solvents is found in the literature but most methods are capable of efficiently extracting the low molecular weight metabolome (Reed *et al.* 2014; Gogna *et al.* 2015; Schou *et al.* 2017).

While the commonly studied classes of polar molecules described above can be extracted using polar solvents, certain

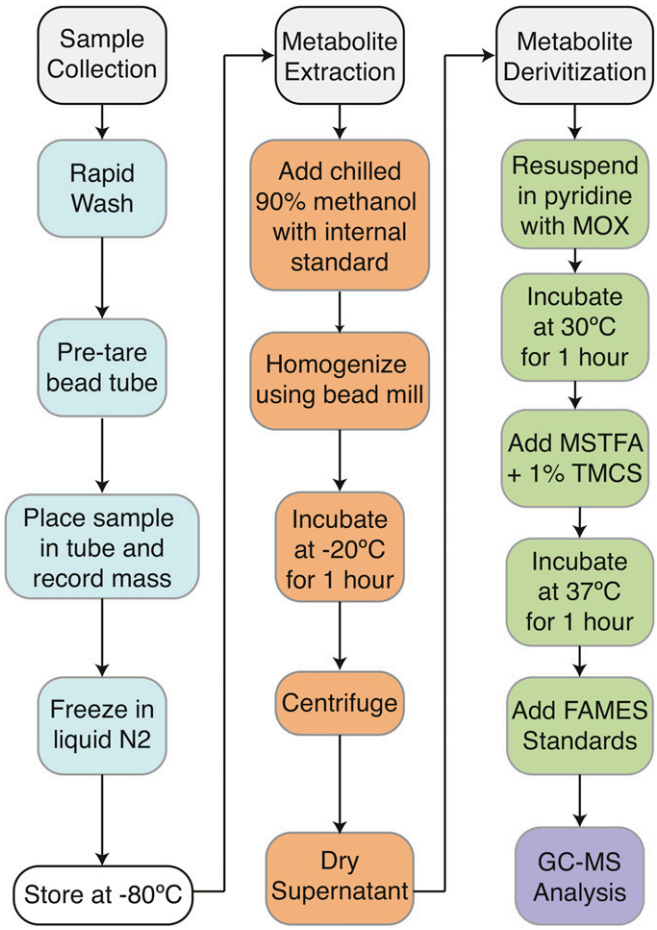

Figure 2 A workflow diagram to prepare samples for GC-MS analysis. *Drosophila* samples can be prepared for GC-MS analysis using a relatively simple protocol. Samples are washed, weighed, and frozen in tubes containing ceramic beads. Metabolites are homogenized in 90% methanol with a stable isotope labeled standard. Following a 1 hr incubation at −20° to precipitate proteins, the samples are centrifuged and the supernatant is moved to a new tube and dried using a vacuum concentrator. Metabolites are sequentially derivatized with MOX dissolved in pyridine and MSTFA + 1% TMCS. A FAMES solution is added immediately prior to GC-MS analysis to serve as a retention time standard.

classes of widely studied metabolites require careful attention to extraction conditions. Both NADH and NADPH are unstable under acidic conditions, GSH and GSSG are oxidized under basic conditions, and maximal recovery of ATP requires the use of perchloric acid as an extraction solvent (Wu *et al.* 1986; Klawitter *et al.* 2007). Similarly, free thiols are problematic, as accurate quantitation of cysteine residues requires their reduction with sodium borohydride, metallic zinc or another reducing reagent (Winther and Thorpe 2014). Overall, these examples emphasize that studies targeting a specific metabolite should carefully analyze the appropriate extraction conditions.

Finally, metabolomics analysis of lipids (lipidomics) requires the use of an apolar solvent. Lipid analysis in *Drosophila* commonly relies on the Bligh-Dyer method, which employs a chloroform:methanol:water liquid extraction protocol (Guan *et al.* 2013); however, a less toxic MTBE: methanol:water procedure has also been introduced for general lipidomics analysis (Matyash *et al.* 2008). An advantage of this later procedure is that the lipid containing hydrophobic layer is the upper phase in the biphasic system, which allows for facile removal by freezing the bottom aqueous layer in dry ice and pouring off the lipid layer into a new tube. The implementation of both the Bligh-Dyer and the MTBE method has allowed for the simultaneous extraction of both polar and apolar metabolites from the same sample (Chen *et al.* 2013), and is therefore a good method to consider if one desires a global metabolomics/lipidomics analysis.

GC-MS analysis

Our laboratories use a GC-MS method that has been optimized for the unambiguous separation of amino acids, TCA intermediates, sugar isomers, and phosphorylated glycolytic intermediates. A variety of instruments can be used for this analysis, and our labs have used a Waters GCT Premier, Agilent 7200 GC-QToF, and an Agilent 5977B GC-MSD – all fit with either Gerstel or CTC auto samplers for automated sample derivatization and injection. Completely dried fly samples are suspended in 40 μl of a 40 mg/ml O-methoxylamine hydrochloride (MOX) in pyridine and incubated for 1 hr at 30°. 25 μl of this solution is transferred to vials and placed onto the robotic autosampler. The auto sampler adds 40 μl of N-methyl-N-trimethylsilyltrifluoracetamide (MSTFA) and incubates each sample for 60 min at 37° with shaking. After incubation, the autosampler adds 3 μl of a FAMES solution and 1 μl of the prepared sample is injected to the gas chromatograph inlet in the split mode with the inlet temperature held at 250° (a 10:1 split ratio is used for initial analysis). A 30 m Phenomex ZB5-5 MSi column with a 5 m long guard column is employed for chromatographic separation and helium is used as the carrier gas (1 ml/min). The GC is set to an initial temperature of 95° for 1 min, followed by a 40°/min ramp to 110°, and a hold time of 2 min. The GC then undergoes a second 5°/min ramp to 250°, a third ramp of 25°/min to 350°, then a final hold time of 3 min. Due to detector saturating amounts of signal from several metabolites, including proline, lactic acid, glucose and glutamine, each sample is analyzed once again at a 100:1 split ratio with a faster ramp rate. This protocol generates highly reproducible data across a variety of platforms and provides a rapid means of surveying of *Drosophila* intermediate metabolism.

LC-MS analysis

Several studies have used LC-MS to analyze the polar metabolome in *Drosophila* (Table 1). The University of Utah Metabolomics Core Facility uses hydrophilic interaction chromatography (HILIC), originally described by Kamleh *et al.* (2009), to analyze polar metabolites such as NTPs, NAD(P)H, and glutathione, all of which are not observable by GC-MS. This method, however, is slightly modified with regard to the buffer composition and the column dimensions

due to differences in LC-MS instrumentation. We perform HILIC based LC-MS analysis using an Agilent 6550 QToF with a 1290 UPLC system, with samples analyzed in both the positive and negative mode, and a SeQuant ZIC-HILIC column (2.1 × 100 mm) employed for chromatography. A binary gradient is used with solvent A = 10 mM ammonium acetate and solvent B = acetonitrile. The initial gradient condition is 10% A at a flow rate of 0.3 ml/min followed by a 20 min ramp to 70% A. These conditions are held for 1 min and followed by a 2 min ramp back to 10% A. The column is equilibrated for 7 min at a flow rate of 0.4 ml/min, as this extended equilibration time is crucial for repeatable data in HILIC chromatography. Source conditions are as follows: gas temperature 250°, drying gas flow rate 17 l/min, nebulizer 20 psig, sheath gas temperature 380°, sheath gas flow 10 liter/min, capillary voltage 3000 V, and nozzle voltage 2000 V. The scan rate is five per second with a mass range of 63–1700 m/z. Pooled samples that contain ~10% of every sample are both intermixed in the sample queue and at the end of the sample run for quality control. Data are collected without fragmentation for all samples, but exhaustive MS/MS is performed on the QC samples for downstream metabolite identification. This is achieved by doing Top 10 MS/MS on the QC sample using three collision energies (10, 20, and 40 eV) followed by excluding the metabolites fragmented in the first pass analysis to perform MS/MS on low abundance metabolites. This approach allows for both targeted and non-targeted analysis using MS1 data while ensuring the identification of metabolites using the MS/MS data. While specific details regarding LC-MS analysis will vary depending on instrumentation, this general protocol provides a powerful method for assaying hundreds of compounds within the *Drosophila* metabolome.

Targeted vs. untargeted metabolomics

In general, metabolomic analysis falls into two types – targeted (quantitative) and non-targeted (chemometric). Each of these methods has advantages and disadvantages, with the choice between the two depending on the desired end goal and available instrumentation. Non-targeted metabolomics, also known as discovery metabolomics, is often used as a global approach for biomarker discovery and hypothesis generation. This method uses a combination of instrumentation and software that are capable of recording and searching all of the chemical signals recorded by the analytical instrument, allowing for the detection of hundreds to thousands of molecular features. As a result, data mining techniques, such as principle component analysis (PCA), partial least squares-discriminant analysis (PLS-DA) and volcano plots, are necessary to prioritize chemical signals of interest based on differences between the experimental groups (see below). One disadvantage of this approach, however, is that the instrumentation used in global analysis, such Time-of-Flight (ToF) and Orbitrap analyzers, are often less sensitive than those used in targeted analyses and can miss subtle changes in metabolite abundance. In contrast, more targeted studies are usually used to examine the metabolic effects of a gene deletion or dietary treatment on specific metabolites or pathways. Triple quadrupole instruments are often used for this type of targeted analysis because they offer greater sensitivity to low abundance metabolites by increasing the signal to noise ratio and eliminating data mining, as targeted approaches rely on detecting known metabolites. Quantitative approaches, however, are limited to detecting ~100–200 compounds (depending on instrumentation) and are not appropriate for biomarker discovery.

Despite key differences between quantitative and chemometric analyses, these two approaches are not mutually exclusive and both can be used for data acquisition and analysis. Ideally, sample sets are analyzed using instruments that allow for non-targeted analysis, such as ToF or Orbitrap, which provide a comprehensive evaluation of the data. First, a non-targeted data analysis is used to identify metabolic alterations within the data. If the altered metabolites are already known and part of the targeted analysis list, then no further action is taken. However, if the chemical features of interest are unknown, then their identity can be interrogated using accurate mass and MS/MS data. Once a putative identity has been assigned to a previously unknown feature, the metabolite can be added to the target list and the appropriate standard is purchased to confirm its identity (assuming that a standard is commercially available). In many instances, the identity of an altered metabolite will remain ambiguous, however, the retention time and accurate mass measurement (RT_m/z) of the unknown compound can still be added to the quantitation list. The data can then be reanalyzed using a targeted approach to quantitate both known and unknown metabolites. This powerful approach ensures that most metabolic alterations are included in the final analysis and is especially important when profiling organisms such as *Drosophila*, which has a relatively poorly annotated metabolome.

Data preprocessing and statistical analysis

The raw data generated by a metabolomics experiment must be processed, normalized, and statistically analyzed. A detailed description of these topics is beyond the scope of this review and we direct interested readers to recent comprehensive descriptions of these processes (Worley and Powers 2013; Saccenti *et al.* 2014; Yi *et al.* 2016). Instead, we provide here a brief overview of the strategy used to process and analyze metabolomics datasets. Data preprocessing involves the extraction of useful information from the raw instrumental data and must be performed prior to statistical analysis. For our studies of *Drosophila* samples, we use the software programs Profinder (Agilent) and MetAlign (https://www.wur.nl/en/show/MetAlign-1.htm; Lommen 2009) to identify chromatographic peaks, align those peaks between samples, estimate missing values, and filter the data to eliminate baseline noise. However, a number of highly integrated software programs can also be used for this purpose, including the web-based platforms XCMS and Metaboanalyst (Smith *et al.* 2006; Xia *et al.* 2009; Tautenhahn *et al.* 2012; Xia *et al.* 2012; Gowda *et al.* 2014; Saccenti *et al.* 2014; Siuzdak 2014; Xia *et al.* 2015). Both of these programs are

capable of uploading raw data files (NetCDF, mzXML, or mzDATA file format) and both use the open source XCMS algorithm to perform peak alignment, matching, and identification of the uploaded chromatographic data.

After peak extraction, a number of optional steps can be implemented including data filtering to eliminate noise from low abundance chemical features and missing value imputation. The data should then be normalized using both the internal standards that were added prior to homogenization and the sample mass (or animal number). Other normalization methods can also be employed, such as using a pooled sample, reference sample, or the sum of the rows for each sample. In addition, log or cube root transformation can be employed to reduce the impact of high abundance features, and Pareto or range scaling can be used to convert the data into a more Gaussian distribution. These normalization and transformation steps generate a dataset in a matrix format that is ready for either chemometric or quantitative analysis.

As described above, chemometric analysis has traditionally been employed in metabolomics studies to detect altered metabolites without prior knowledge of identity. This approach was necessary due to the lack of complete electronic data sets for the identification of detected metabolites and an in-depth discussion of these methods can be found elsewhere (Wishart *et al.* 2007; Xia and Wishart 2011; Worley and Powers 2013). The two most commonly used chemometric methods for data visualization are PCA and PLS-DA. These methods search for the highest inter-sample group variability while minimizing the intra-sample group variability. PCA is an unsupervised clustering method that refines complex data sets by decomposing them to simple components. While this method is excellent at determining if valid differences are present between sample groups, PCA of complex datasets has difficulties determining which metabolites are responsible for these differences. Instead, PLS-DA is often used to search for those components that contribute to creating distinct groups. This supervised method adds an additional matrix of class labels (Y matrix) on top of the multivariate data (X matrix) used in PCA analysis. The additional Y matrix forces groupings and is valuable in determining which chemical features are most relevant to class separation through the use of Variable Importance in Projection (VIP). In Metaboanalyst, the preprocessed data are first visualized using a PCA plot to give a general overview of the data and identify outliers. PLS-DA is then performed and VIP scores are used to identify chemical features that contribute to the class separation. As discussed above, these alterations can be identified for further interrogation using univariate statistics, such as the Student's *t*-test, fold change analysis, and a combination of these using a volcano plot. We typically analyze targeted metabolomics data using Metaboanalyst or custom R-script, and use a 1.5-fold change cut-off and a *P*-value of 0.05 as thresholds for identifying altered metabolites.

Confirming changes in metabolite abundance

Metabolic systems are inherently noisy and thus any dataset will contain false-positive results. The most reliable method for confirming a change in metabolite abundance is to conduct additional rounds of analysis using distinct biological samples that were collected independently of those analyzed in the first dataset. We routinely use six samples per experiment for each condition and then repeat the entire experiment two to three times. Alternatively, many institutions maintain basic NMR and GC-MS instruments as part of a chemistry core facility. If the metabolite of interest can be detected using one of these methods, then *Drosophila* samples and the appropriate chemical standards can be analyzed using a targeted approach. Finally, changes in metabolite concentration can be verified using enzyme-based assays. Commercially available assay kits, however, are expensive and must be optimized for *Drosophila* studies to ensure that they accurately reflect metabolite levels.

Data interpretation

The first step in understanding metabolomics data are to determine if significant metabolite changes are associated with a common metabolic pathway. The Metabolomics Society maintains a list of online databases that can be used for data analysis. Many of these databases, such as Metacyc (www.metacyc.org), Reactome (www.reactome.org), and Metaboanalyst (www.metaboanalyst.ca), contain both general and *Drosophila*-specific metabolic information (Xia *et al.* 2015; Caspi *et al.* 2016; Fabregat *et al.* 2016; Xia and Wishart 2016). Perhaps the easiest starting point for the novice user, however, is the map of *Drosophila* metabolism available at The Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa *et al.* 2017). This database provides a graphical user interface for exploring *Drosophila* metabolism and thus allows the user to quickly identify the *Drosophila* gene(s) that are either predicted or known to be associated with a given metabolic reaction.

In the simplest case, a metabolomics analysis will reveal the disruption of a specific enzymatic reaction or set of sequential reactions, which results in an accumulation of substrate molecules and a decrease in reaction products. This classic profile is expected when a mutation or environmental treatment disrupts the activity of a specific metabolic process, and is often the first clue toward elucidating the function of an unknown gene. For example, metabolomics studies of the *Drosophila* SDHAF4 homolog (*dSdhaf4*) revealed that *dSdhaf4* mutants harbor elevated levels of succinate and decreased fumarate and malate concentrations – a metabolic profile that led to the discovery that the SDHAF4 protein family promotes succinate dehydrogenase complex assembly (Figure 3) (Van Vranken *et al.* 2014). Similarly, a metabolomic analysis of *Drosophila Estrogen Related Receptor* (*dERR*) mutants uncovered elevated monosaccharide levels and decreased lactate and pyruvate concentrations, which, when considered in the context of gene expression data, demonstrated that dERR is a master regulator of aerobic glycolysis (Tennessen *et al.* 2011). Therefore, the first priority for analyzing any dataset is to identify potential metabolic bottlenecks. A first step toward identifying these bottlenecks is to

use the Pathway Analysis tool in Metaboanalyst. This tool has 21 species-specific databases, including *Drosophila*, in which alterations in specific pathways are ranked by *P*-values from pathway enrichment analysis. This tool is used in our laboratories to determine possible pathways affected by a genetic mutation or dietary treatment.

Metabolomics datasets can also highlight compensatory mechanisms that allow animals to maintain homeostasis despite severe metabolic disruption. This metabolomic signature is often manifested as the shunting of metabolites into related metabolic pathways. For example, mutations in the *Drosophila MPC* result in elevated glycine and serine levels (Bricker *et al.* 2012). Since *dMPC* mutants are unable to transport pyruvate into the mitochondria, the shuttling of glycolytic intermediates into amino acid production allows the cell to maintain adequate levels of glycolysis in the absence of glucose oxidation. Since many metabolic mutants exhibit unexpectedly mild phenotypes, identifying these compensatory pathways is key for understanding the metabolic plasticity that underlies animal physiology.

Finally, metabolic enzymes are not as specific as we are led to believe in undergraduate biochemistry. Rather, many enzymes will act on a range of similar substrate molecules (Khersonsky and Tawfik 2010). The promiscuous activity of common metabolic enzymes, however, remains poorly understood and can lead to unexpected metabolomic profiles. For example, a recent analysis of *Drosophila Lactate Dehydrogenase* (*dLdh*) mutants uncovered a significant decrease in both lactate and L-2-hydroxyglutarate (L-2HG) (Li *et al.* 2017). While the changes in lactate were expected, the link between L-2HG and *dLdh* revealed a unique mechanism by which dLDH activity directly synthesizes L-2HG from α-ketoglutarate – a compound that shares structural similarities with pyruvate. In all likelihood, most enzymes can act on a variety of similar substrates and metabolomic studies of individual enzymes should be carefully analyzed for possible enzymatic infidelity.

Stable isotope tracer analysis

A standard metabolomics dataset provides a steady state measurement of metabolite pools, allowing the user to identify changes in overall abundance of a specific compound. These data, however, do not provide information about rate or direction of metabolic flux and should not be used to infer the metabolic source of a specific compound. Instead, questions regarding the origin of a metabolite or the rate at which it is produced must to be addressed using stable isotopes.

Nearly all of the elements that compose biological systems exist as multiple isotopes. While biologists are most familiar with radioactive isotopes, uncommon stable isotopes of hydrogen (^{2}H; deuterium), carbon (^{13}C), and nitrogen (^{15}N) will be key for future metabolomics studies. The presence of a stable isotope within a compound can be detected using either MS or NMR. In the case of MS analyses, the addition of a single isotope will increase the mass of a molecule by one atomic mass unit (amu; represented as m+1). When an animal is given a source of stable-isotope labeled compound, the movement of these isotopes into specific metabolic pathways can be traced based on the increased mass of downstream metabolites. In addition, the rate of metabolic flux can be determined by collecting samples at regular intervals following the introduction of a labeled compound.

Stable isotope tracers analysis can answer questions that are difficult or impossible to address with steady state measurements. For example, consider a hypothetical study that finds increased levels of the citric acid cycle intermediate α-ketoglutarate (α-KG) with no decrease in succinate,

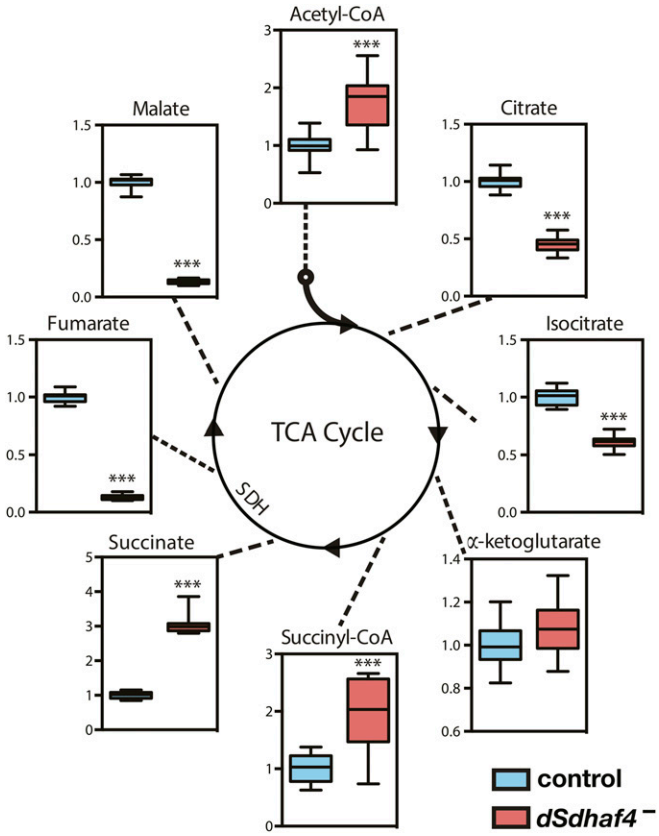

Figure 3 GC/MS analysis of *dSdhaf4* mutants. GC/MS was used to measure the abundance of metabolites in transheterozygous *dSdhaf4*$^{1/3}$ mutants (red boxes) compared to genetically matched *dSdhaf4*$^{+/Ex32+}$ controls (blue boxes) (Van Vranken *et al.* 2014). Loss of *dSdhaf4* results in disrupted assembly of the succinate dehydrogenase (SDH) complex, leading to an accumulation of its precursor metabolite succinate and a reduction in downstream metabolites, including fumarate and malate. Oxaloacetate, which lies downstream from malate and was not detectable in this experiment, is presumably also reduced in these mutants. This would account for the accumulation of acetyl-CoA and reduced levels of citrate because oxaloacetate is required to convert acetyl-CoA into citrate for its entry into the TCA cycle. TCA cycle intermediates are rescued in the mutant at the level of α-ketoglutarate, presumably due to anapleurotic input. The data are depicted in box plot format, with the box representing the lower and upper quartiles, the horizontal line representing the median, and the bars representing the minimum and maximum data points. All data are shown as fold-change relative to the metabolite levels in controls. 8–12 biological replicates from two independent experiments were combined per genotype. ***$P < 0.001$. Figure is reprinted with permission from Van Vranken *et al.* (2014).

fumarate, or malate. Elevated α-KG levels might result from an increase in glycolytic flux, decreased export of citrate from the mitochondria, up-regulation of anaplerotic reactions, such as the deanimation of glutamate, or a block in the electron transport chain. While this phenotype could be studied using a series of genetic experiments and steady-state metabolite measurements, a stable isotope tracer approach would quickly determine the origin of this defect. For this purpose, animals could be fed compounds that are only composed of ^{13}C isotopes, such as glucose, glutamine and/or proline. The catabolism of fully-labeled glucose results in the production m+3 pyruvate, which is transported into the mitochondria and can be incorporated in the TCA cycle by either the pyruvate dehydrogenase complex or pyruvate carboxylase. Regardless of the enzymatic mechanism, α-KG will gain two carbons from glucose (Figure 4). Therefore, if the elevated levels of α-KG are synthesized from glucose catabolism, an MS analysis would find that the α-KG isotopologue pool contains primarily m+2 species. In contrast, if the elevated α-KG levels are derived from the activity of glutamate dehydrogenase, all of the carbons present within labeled glutamine or proline will be used to synthesize α-KG, which will be recognized as m+5 when using MS (Figure 5).

The stable isotope distribution in this hypothetical mutant, however, would not be limited to identifying the metabolic origin of α-KG, but would also allow for the detection of defects that are invisible to steady state analysis. In this hypothetical example, levels of the TCA cycle intermediates succinate, fumarate, and malate appear normal, however, a steady state analysis takes an instantaneous metabolite measurement and provides no information regarding the turnover of those metabolite pools. As a result, the concentration of a single compound might remain unchanged despite a significant increase or decrease in the movement of atoms through that metabolite pool. Such defects in metabolic flux can be uncovered by collecting samples at defined intervals following the introduction of the labeled compound, which allows for measurements of isotope incorporation as a function of time. In our example, if a timecourse analysis revealed that citrate, isocitrate, and α-KG were labeled at a normal rate, but isotope incorporation into succinate, fumarate, and malate was delayed, subsequent experiments might look for a defect in succinate dehydrogenase activity.

While the use of fully labeled compounds are adequate for many experiments, a number of partially labeled compound are available for testing specific hypotheses. The use of partially labeled tracers is particularly powerful when used in combination with NMR, which not only recognizes the presence of a stable isotope but also provides positional information for that isotope. A classic example of this technique was used to map the fates of glucose-derived carbons in adult flies (Eisenreich *et al.* 2004). The resulting study demonstrated that the nonoxidative branch of the pentose phosphate pathway plays a prominent role in reshuffling carbons from glucose. This type of tracer study will be essential for understanding how dietary compounds are metabolized by the fly and is likely to be widely adopted by the *Drosophila* community in future studies.

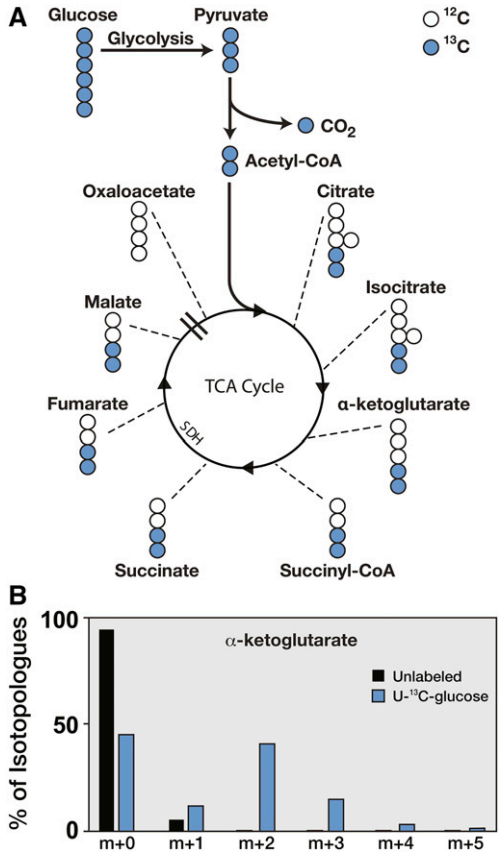

Figure 4 The production of stable isotope labeled α-ketoglutarate from U-^{13}C-glucose. (A) The catabolism of ^{13}C labeled glucose (U-^{13}C-glucose) will result in m+3 pyruvate. The decarboxylation of pyruvate in the mitochondria produces m+2 acetyl-CoA, which is then incorporated into m+2 citrate. As a result, all downstream TCA cycle intermediates will have a mass of m+2. (B) A hypothetical α−ketoglutarate isotopologue distribution following U-^{13}C-glucose feeding. Note that m+2 is the most abundant isotopologue produced by this experiment.

The future of Drosophila metabolomics

While metabolomics has revolutionized *Drosophila* metabolic research, the technology remains immature and most studies in the fly rely on basic techniques. In many ways, the current state of *Drosophila* metabolomics mirrors the adoption of microarrays at the turn of the millennium, which were limited by instrumentation, computational power, lack of access, and incomplete genomic coverage. The metabolomics community is experiencing similar limitations – but just as advances in genomic technologies were driven by user demand and advanced instrumentation, the field of metabolomics will inevitably mature. In closing, we highlight a few specific advances in metabolomics research that will allow the *Drosophila* community to better realize the potential of this emerging field.

Untargeted stable isotope tracer analysis

Stable isotope tracer experiments represent an exciting direction for future studies of *Drosophila* metabolism. While the importance of this technique is evident in recent targeted analyses, the manner in which stable isotopes disperse throughout

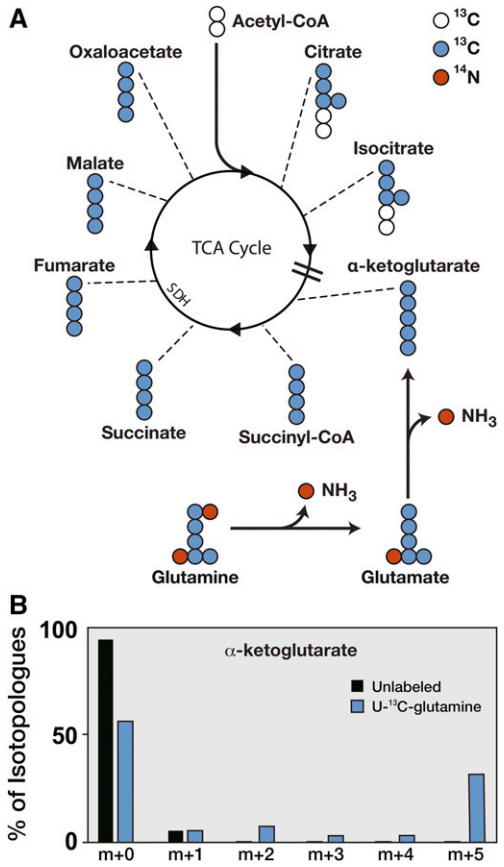

Figure 5 The production of stable isotope labeled α-ketoglutarate from U-^{13}C-glutamine. *Drosophila* metabolism can use the amino acids glutamine and proline to generate α−ketoglutarate. (A) Glutamine can be enzymatically converted to α−ketoglutarate via glutamate. If the glutamine source is completely labeled with ^{13}C (U-^{13}C-glutamine), the resulting α−ketoglutarate molecule will have a mass of m+5. As a result, a single labeling event will result in production m+4 isotopologues in subsequent steps of the TCA cycle. (B) A hypothetical α−ketoglutarate isotopologue distribution following U-^{13}C-glutamine feeding. Note that m+5 is the most abundant isotopologue produced by this experiment.

the metabolome lends itself to an untargeted approach. Simply supplementing fly food with a labeled compound, such as U-^{13}C-glucose, would allow an unbiased discovery of how dietary nutrients are digested, absorbed, and metabolized. Considering that metabolism is the ultimate readout of a biological system, the incredible amount of information garnered from an unbiased flux experiment could be invaluable for any genetic study.

The use of stable isotopes in untargeted experiments, however, remains rare. A recent study using a variation of the XCMS software platform demonstrated that this technique is capable of discovering metabolic changes that are invisible to approaches that only allow steady state measurements (Huang *et al.* 2014). While the *Drosophila* metabolism community has made significant advances in using conventional metabolomics, we lack several tools that are required to realize the full potential of untargeted stable isotope tracer experiments. In particular, we must make a concerted effort to both annotate the *Drosophila* metabolome and understand how metabolism is controlled in a tissue-specific manner. These advances would allow the fly community to conduct experiments that would be too expensive to pursue in larger animals, placing *Drosophila* at the forefront of metabolomic research.

Analyzing the metabolome in space and time

Most *Drosophila* metabolomic studies measure metabolite abundance using whole animal homogenates. While this approach has proven quite successful, the metabolism of *Drosophila*, like that of any metazoan, is compartmentalized into specific tissues. As a result, the lack of tissue-specific metabolic information hinders the discovery of metabolic crosstalk between cells and organs. The importance of moving beyond the whole animal level has been demonstrated by a series of metabolomic analyses in dissected tissues, which not surprisingly have revealed that nearly all metabolites accumulate in a tissue-specific manner (Carvalho *et al.* 2012; Chintapalli *et al.* 2013). For example, every amino acid except proline exhibits some sort of tissue-specific enrichment (Chintapalli *et al.* 2013). Similarly, a shotgun lipidomics approach revealed that hexosyl ceramides are only present within the gut (Carvalho *et al.* 2012). While some of these observations were expected based on previous surveys of tissue-specific gene expression and historic studies of fly metabolism (*e.g.*, the enrichment of tryptophan catabolism in the Malpighian tubules, high levels of tyrosine in the cuticle, etc.), such approaches will be key for understanding how the disruption of individual metabolic pathways affects whole animal physiology. The widespread adoption of tissue-specific metabolomics, however, has been slow due to technical difficulties, as isolating cells and tissues will induce rapid changes in cellular metabolism that can result in aberrant measurements. Therefore, this approach requires that tissues be collected as rapidly as possible and the metabolic reactions present therein be immediately quenched. Such requirements inevitably result in small sample sizes and largely restrict these analyses to sensitive LC-MS instruments. Regardless, metabolomic methods are rapidly advancing and future *Drosophila* studies need to consider a more tissue-centric approach.

While tissue-specific metabolomics represents an emerging tool, the ultimate goal of *Drosophila* metabolic researchers should be to integrate metabolomics with the unparalleled collection of cell-specific genetic tools that are available to the fly community, such as MARCM and the wide collection of GAL4 driver lines (Wu and Luo 2006; Gramates *et al.* 2017). This technical advance would establish *Drosophila* as the premier organism for studying intercellular metabolic signaling. This approach, however, is limited by current technology. In some cases, cell-sorting methods might generate a sufficient mass of cells, but disrupting cell adhesion could lead to rapid changes in metabolic flux that affect the results in a non-physiological manner. At present, the most promising technology for cell-specific metabolomics is matrix-assisted laser desorption ionization coupled with mass spectrometry imaging (MALDI-IMS), which can visualize the spatial distribution of small molecules within intact tissue samples (Zaima *et al.* 2010). Although current techniques

are limited to a small number of abundant compounds, MALDI-IMS has the potential to revolutionize metabolic research.

Just as metabolic flux varies between cells and tissues, so too does the metabolome change during the course of an organism's life. Many of the studies described above emphasize that metabolism must adapt to the energetic and biosynthetic demands of each developmental stage. Furthermore, the metabolome continues to evolve in adults, both in response to gamete production and to the physiological changes associated with aging (Sowell *et al.* 2007; Sarup *et al.* 2012; Hoffman *et al.* 2014; Sieber and Spradling 2015; Sieber *et al.* 2016). Therefore, the *Drosophila* metabolomics community should make a concerted effort to annotate the metabolic changes that occur during the life-cycle. Such a resource could be used to better understand how dietary restriction extends both lifespan and healthspan or determine how metabolic flux influences the timing of metamorphosis. A combined metabolomics/lipidomics project that mirrors the modENCODE developmental gene expression profile would provide a powerful resource for anyone interested in developmental biology, physiology, aging, and models of metabolic disease (Graveley *et al.* 2011).

Since the advent of metabolomics at the turn of the 21st century, this technology has provided important new insights into *Drosophila* metabolism that could not be achieved using other approaches. These discoveries will continue to have a major impact on the field, particularly with the gradual implementation of new and powerful applications such as those described above. This emerging technology, combined with the ability to define evolutionarily conserved mechanisms of metabolic regulation using *Drosophila* as a model system, hold the promise of providing significant new insights into insect physiology as well as a better understanding of the molecular pathways that underlie human disease.

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