14 Metabolomics: State-of-the-Art Technologies and Applications on *Drosophila melanogaster*

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Abstract

Metabolomics is one of the latest "omics" technology concerned with the high-throughput identification and quantification of metabolites, the final products of cellular processes. The revealed data provide an instantaneous snapshot of an organism's metabolic pathways, which can be used to explain its phenotype or physiology. On the other hand, *Drosophila* has shown its power in studying metabolism and related diseases. At this stage, we have the state-of-the-art knowledge in place: a potential candidate to study cellular metabolism (*Drosophila melanogaster*) and a powerful methodology for metabolic network decipherer (metabolomics). Yet missing is advanced metabolomics technologies like isotope-assisted metabolomics optimized for *Drosophila*. In this chapter, we will discuss on the current status and future perspectives in technologies and applications of *Drosophila* metabolomics.

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Keywords

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14.1 Introduction

14.1.1 Metabolomics

Metabolomics is a rapidly emerging field of the high-throughput identification and quantification of the small molecule metabolites (Putri et al. 2013) (Fig. 14.1). The complete set of metabolites within an organism, cells, or tissues is called metabolome. As metabolites are the substrates and products of metabolism, the changes in the metabolome will reflect the effects of genetic, pathophysiological, developmental, and/or environmental factors (Fukusaki 2014). The dramatic breakthrough in the field of metabolomics within the past decade offers valuable insights in the correlation of metabolism with phenotype. Since metabolites are the final products of cellular processes, metabolomics is the puzzle piece fitting in the current central dogma. In the concept of system biology, metabolomics together with genomics, transcriptomics, and proteomics can give a more complete picture of the living organisms, cells, or tissues physiology (Nielsen 2017).

Same as other "omics" fields, the ideal condition for metabolomics is studying the whole metabolome in biological samples under certain

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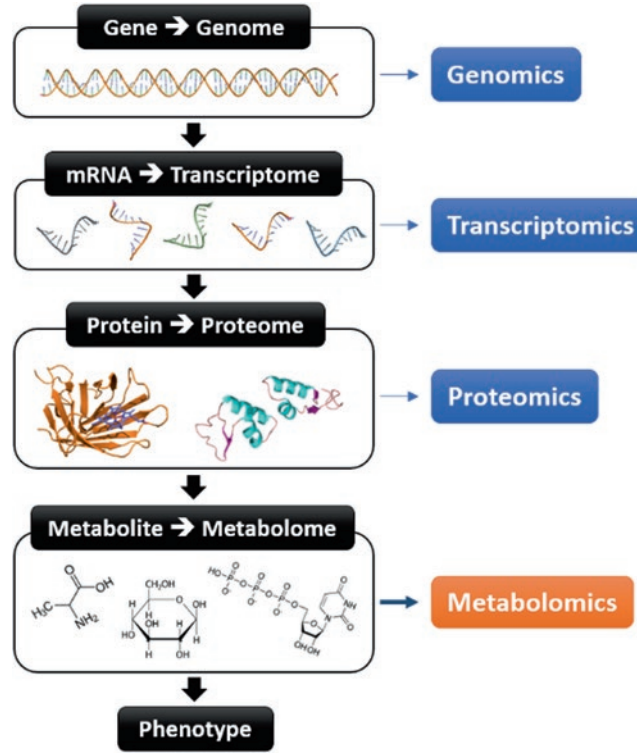

Fig. 14.1 The central dogma of biology referring the "omics" cascade

conditions. However, many metabolites are still unknown, and not any analytical platforms can cover all metabolites. Each metabolite has different characteristics that make the identification and quantification of all detected metabolites very complicated. Thus, the following approaches are now commonly used in the metabolomics field (Fukusaki 2014; Fiehn 2002):

(1) *Metabolic profiling* is an approach focusing on predefined biochemical pathways or specific classes of compounds. The strategy will be developed based on biochemical questions or hypothesis that motivates the research. With the development of analytical platforms, targeted metabolomics becomes one of the most powerful and rapid strategies for metabolic profiling.
(2) *Metabolic fingerprinting* is used to find the differences among the samples caused by their biological relevance. In this strategy, it is not necessary to give the detailed metabolite information. First, the nontargeted metabolomics method is useful to screen as many metabolites as possible without preference. Then, only the metabolites that show significant differences will undergo further investigations. This method provides a broader coverage, which has great potential to give insights into fundamental biological processes.
(3) *Metabolic footprinting (exometabolomics)* focuses on extracellular metabolites in cell culture media before and after culturing to provide a reflection of metabolite excretion or uptake by cells (Silva and Northen 2015).

14.1.2 *Drosophila* as a Model to Study Metabolism

Drosophila has been well established as one of the most tractable multicellular organisms for researches in areas of developmental biology, cell biology, and neurobiology (St Johnston 2002). Thanks to the development of robust analytical methods to evaluate cellular metabolism and the extent knowledge on which organ systems

have functional analogues to vertebrate counterparts, *Drosophila* applications have been expanded to study metabolism in the past decade (Hoffmann et al. 2013; Rajan and Perrimon 2013; Graham and Pick 2017; Herranz and Cohen 2017).

Drosophila organs responsible for absorptions and storage of nutrients share similar structures and functions to those of mammals including the midgut (works like the intestine and stomach) (Pitsouli and Perrimon 2008), the fat body (stores nutrients and functions as a nutrient sensor) (Rajan and Perrimon 2013), the oenocytes (take part in cycle and storage energy) (Gutierrez et al. 2007), and the Malpighian tubules (perform basic functions as kidneys) (Dow and Davies 2006). The energy homeostasis, including carbohydrate and lipid metabolisms, is highly conserved between *Drosophila* and mammalians (Mattila and Hietakangas 2017; Bharucha 2009). Thus, *Drosophila* model has great contributions to widen the knowledge on Type 1/Type 2 diabetes, obesity, metabolic syndrome, and insulin resistance diseases (Hoffmann et al. 2013; Rajan and Perrimon 2013; Graham and Pick 2017).

Besides, the cardiovascular system and tracheal system work independently in *Drosophila*, allowing the investigation on various aspects of metabolic dysfunction and cardiac dysfunction without compromising viability (Rajan and Perrimon 2013). Flies' cardiovascular system is an open circulatory system essential for the circulation of nutrients and immune cells, while the tracheal system is responsible for oxygen delivery (Choma et al. 2011). As cardiac dysfunction is usually a consequence of metabolic disorder, *Drosophila* has recently drawn a lot of attentions as a powerful paradigm to provide insights into high-fat- and sugar-induced cardiovascular disease (Na et al. 2013; Birse et al. 2010).

The advantages of *Drosophila* model in studying metabolism are attributable to the conservation in the signaling pathways controlling cell growth, proliferation, and death. Many reports have shown that the insulin, TOR, and JNK signaling pathways not only controlled normal cellular metabolism but also related to tumor formation and aging process (Herranz and Cohen 2017; Owusu-Ansah and Perrimon 2014; Newgard and Pessin 2014). Otherwise, the cellular metabolites such as sugar and free amino acids and metabolites in purine/pyrimidine metabolism could also regulate the cell signaling (Ben-Sahra and Manning 2017; Pavlova and Thompson 2016). As a result, the cellular metabolism has recently been recognized as a hallmark of cancer and aging (Pavlova and Thompson 2016; López-Otín et al. 2013). Therefore, *Drosophila melanogaster* is emerging as a valuable model to study multiple aspects of the connection between cellular metabolism and signaling pathways.

14.2 General Workflow for *Drosophila* Metabolomics Studies

As a high-throughput approach, metabolomics study required the integration of analytical chemistry, biology, mathematics, and informatics to interpret the data and unravel the biological insights. The Metabolomics Society conceived the Metabolomics Standards Initiative (MSI) focusing on community-agreed reporting standards, which allow data from different research institutes to be shared, integrated, and interpreted (Fiehn et al. 2007). In metabolomics field, MSI has been wildly used as the basic requirement for publication on peer-reviewed journals. In this chapter, the metabolomics approaches for *Drosophila* will be introduced based on the general workflow of metabolomics matched with MSI standard:

- **Experimental design**: As with any scientific studies, the design of a metabolomics experiment is the most curial step and depends on the scientific question under consideration. Though the whole *Drosophila* metabolome still remains unclear, many metabolic pathways are remarkably well conserved between *Drosophila* and mammals (Graham and Pick 2017; Alfa and Kim 2016). Researchers can choose the metabolomics approach (metabolic profiling, metabolic fingerprinting, or

metabolic footprinting) that suites their research objectives, sample types, and working conditions. Several applications of metabolomics established on *Drosophila* model can be used as references (Table 14.1), though not any methods have been reported for *Drosophila* metabolic footprinting.

- ***Drosophila* growing conditions**: Various food types are now utilizing to rear *Drosophila* including "homemade" food or commercial instant food. As the diet will directly affect the metabolism of an organism, the consistent diet throughout research is crucial to avoid experimental errors. Active yeast (*S. cerevisiae* or *S. carlsbergensis*) has been routinely supplemented to *Drosophila* food to mediate attraction, oviposition, and development (Becher et al. 2012). However, the use of yeast as supplement is not recommended in metabolomics study to prevent the alteration in fly's metabolism caused by the host-microbe interaction. Besides, the growing temperature throughout the study should also be controlled strictly. Many methods to control gene expression levels in *Drosophila* require switching temperature from permissive condition (18–22 °C) to restrictive condition (28–30 °C) such as temperature-conditional mutations, GAL4/GAL80ts/UAS system, and FLP/FRT system (Theodosiou and Xu 1998; Duffy 2002). Previous studies show that changes in rearing temperature had a dramatic effect on the metabolic profiles of *Drosophila* even in non-stressed conditions (22–29 °C) (Hariharan et al. 2014; An et al. 2017a; An 2017). Thus, flies in control and experimental test should be reared in exactly same temperature.
- **Sample preparation**: Several procedures for sample collection, quenching, extraction, and storage methods have been developed for every developmental stages of *Drosophila* (Table 14.1). While collecting sample, anesthesia/euthanasia methods (Colinet and Renault 2012; Overmyer et al. 2015), genetic backgrounds (An et al. 2017a), and genders (An 2017) are important factors to consider to prevent unexpected sample variations. Samples are immediately quenched in liquid N_2 and sometimes subsequently lyophilized to inhibit enzyme activities, avoid metabolite turnover, and capture the instant snapshot of the metabolic profile. The selection of extract solvent depends on the metabolite of interest (e.g., hydrophobic, hydrophilic) and the analytical platforms. It is recommended not to leave the samples sitting at room temperature long time before storage or analysis (Stringer et al. 2015). During sample preparation, the measurement of the pooled quality control/quality assurance (QC/QA) samples is highly recommended throughout the analysis to evaluate the stability and reproducibility of the analytical system (Dunn et al. 2012).
- **Metabolome analysis**: To date, the two most commonly used analytical platforms for metabolomics studies are nuclear magnetic resonance (NMR) spectroscopy and mass spectroscopy (MS) (Alonso et al. 2015). Each method has its own features, and both have been applied for *Drosophila*; the choice of analytical technique will be depending on characteristic of sample and target pathways (Emwas et al. 2013; Wang et al. 2015). Basically, NMR is a nondestructive method with simple sample preparation and high analytical reproducibility but relatively low sensitivity compared to MS. NMR is accepted as the gold standard to elucidate metabolite structural. Otherwise, MS-based metabolomics (mainly gas chromatography (GC) and liquid chromatography (LC) coupled with MS) has the advantages of high sensitivity, high selectivity, high throughput, and deep coverage. However, it usually requires more than one analytical method to wide range of molecules, requiring more optimization steps (extraction, derivatization, ionization, etc.). As a destructive method, MS-based metabolomics has more complicated data acquisition and metabolite identification.
- **Data acquisition**: Data acquisition (baseline correction, noise filtering, peak detection, peak alignment, normalization, and scaling) is used to archive accurate identification and quantification of detected metabolites (Alonso et al. 2015). Data acquired from metabolomics

Table 14.1 Metabolomics on studies using *Drosophila**

Application field		Analytical platform	Sample type	Important metabolites/pathways	References
Cold tolerance		GC-MS LC-MS NMR	Adult fly	Increase in gluconeogenesis, amino acid synthesis, and cryoprotective polyol synthesis	Hariharan et al. (2014), Teets et al. (2012), MacMillan et al. (2016), Koštál et al. (2012), Colinet et al. (2012), Colinet et al. (2013), Olsson et al. (2016), Kostal et al. (2011) and Colinet et al. (2016)
Heat stress		NMR	Adult fly	Alterations in the levels of free amino acids, maltose, galactoside, and 3-hydroxykynurenine	Malmendal et al. (2006), Pedersen et al. (2008) and Sarup et al. (2016)
Hypoxia tolerance		NMR	Adult fly	Flexibility in energy metabolism supports hypoxia tolerance in *Drosophila* flight muscle and in correlation with aging	Feala et al. (2007) and Coquin et al. (2008)
Oxidative stress		LC-MS	Adult fly	The metabolic response, especially glutamine level, to oxidative stress (superoxide, paraquat, and allopurinol)	Knee et al. (2013) and Al Bratty et al. (2011)
Infection	*Staphylococcus aureus* infection	NMR	Adult fly	Sepsis survivors had a metabolic signature characterized with decreased glucose, tyrosine, beta-alanine, and succinate	Bakalov et al. (2016)
	Listeria monocytogenes infection	GC-MS LC-MS	Adult fly	Lose both energy stores, triglycerides and glycogen, and show decreases in intermediate metabolites for beta-oxidation and glycolysis	Chambers et al. (2012)
Insecticide	Insecticidal activity (Bowman-Birk inhibitors)	GC-MS	Adult fly	Increase in F6P and decrease in citric acid/isocitric acid levels	Li et al. (2010)
Fundamental research	Effects of CO_2 anesthesia	GC-MS	Adult fly	The most important metabolic changes were the accumulation of succinate and G6P	Colinet and Renault (2012)
	Metabolic profile throughout life cycle	GC-MS	All stages	Over all metabolic transitions during *Drosophila* developmental stages	An et al. (2017a), An (2017), Tennessen et al. (2014a) and An et al. (2014)
	Tissue-specific metabolomes	LC-MS	Adult fly	A baseline tissue map of *Drosophila* for polar metabolites and for a range of lipids	Chintapalli et al. (2013)
	The influences of light cycles and temperatures	NMR	Adult fly	Alterations in the levels of free amino acids	Gogna et al. (2015)

(continued)

Table 14.1 (continued)

Application field		Analytical platform	Sample type	Important metabolites/pathways	References
	Rosy, *y* mutation	MS	Adult fly	Metabolomics profiling of *Drosophila*	Kamleh et al. (2008) and Bratty et al. (2012)
	Effect of storage temperature of quiescent larvae	GC-FID LC-MS	Larvae	Over all metabolic transitions	Tennessen et al. (2011)
	Different among wild-type strains	MS	Adult fly	Differences in genotype caused the differences in metabolome	Kamleh et al. (2009) and Reed et al. (2014)
	Effect of genetic background and growing	GC-MS	Pupae	Purine and pyrimidine metabolisms were altered between CS and OR	An (2017)
	temperature	LC-MS		Alterations in the metabolic profile in nonstress temperatures (22–29 °C)	
	Estrogen-related receptor	GC-MS	Larvae	Abnormally high levels of circulating sugar and diminished concentrations of ATP and triacylglycerides	Tennessen et al. (2011)
	Transient receptor potential TRPA1	GC-MS	Adult fly	Downregulation of intermediates in the methionine salvation pathway, in contrast to the synchronized upregulation of a range of free fatty acids	Lee et al. (2016)
	Effects of diet and development	FTMS	Larvae, pupae	The overall changes of lipidome	Carvalho et al. (2012)
	dG9a function in starvation tolerance	GC-MS LC-MS	Adult fly	dG9a-controlled energy reservoirs including amino acid, trehalose, glycogen, and triacylglycerol levels	An et al. (2017b)
Aging	Effect of diet, age, sex, and genotype on aging	LC-MS	Adult fly	Pathways involving sugar and glycerophospholipid metabolism, neurotransmitters, amino acids, and the carnitine shuttle were affected	Hoffman et al. (2014), Laye et al. (2015), Sarup et al. (2012) and Parkhitko et al. (2016)
Diseases	Charcot-Marie-Tooth disease (gene GDAP1)	NMR	Adult fly	Alterations in the levels of free amino acids and carbohydrate metabolism	López del Amo et al. (2017)
	Obesity	GC-MS LC-MS	Larvae	CoA is required to support fatty acid esterification and to protect against the toxicity of high sugar diets.	Palanker Musselman et al. (2016) and Heinrichsen et al. (2014)
	m-Aconitase deficiency	LC-MS	Adult fly	Reduced triacylglyceride and increased acetyl-CoA	Cheng et al. (2013)
	Alzheimer's disease	NMR	Adult fly	Metabolomic changes may lead to the age-relatedtoxicity of the amyloid beta (Aβ) peptide	Ott et al. (2016)

(continued)

Table 14.1 (continued)

Application field		Analytical platform	Sample type	Important metabolites/pathways	References
	Parkinson-like model (paraquat exposure)	GC-MS	Adult fly	Alteration in 24 metabolites, including amino acids, carbohydrates, as well as fatty acids	Shukla et al. (2016)
	Drug efficacy test	LC-MS	Adult fly	The alterations in these metabolites were associated with perturbations in amino acid and fatty acid metabolism, in response to insomnia through immune and nervous system	Yang et al. (2012)

**Adapted and updated from An PNT* (An 2017)

study could be from different batches or analytical platforms. Hence, appropriate methods for merging or comparing data should be applied to avoid experimental errors. In MSI standard, it is recommended to publish the raw data together with the paper or to other open sources such as MetaboLights (Haug et al. 2013).

- **Metabolite identification**: This is one of the major challenges of high-throughput metabolomics analysis. For the "known" metabolites, whose identities are already cataloged in accessible databases, the identification can be very accurate and efficient. The available metabolite library from different analytical techniques is growing continuously (Table 14.2). Since the differences in spectra comparing to structural isomers can be very small or not present at all, it is necessary to compare the library search results with a reference spectrum of the standard and desirably by chromatographic retention of the standard (Dettmer et al. 2007). In opposition, the identification of "unknown" metabolites is quite challenging due to the lack of commercial standard compounds. In MSI, the identification of metabolites is classified into four levels:
  Level 1 – Identified metabolites: metabolite identification is verified by analyzing the authentic chemical standard in the same condition with experimental data acquired.
  Level 2 – Putatively annotated compounds: metabolite identification is acquired by comparing with in-house database or online database.
  Level 3 – Putatively characterized compound classes: the metabolite cannot be identified but can be classified based on the functional groups.
  Level 4 – Unknown metabolites: the use of "identified" or "annotated" metabolites is very much different; it is important to clarify the level of metabolite identification in the publication.
- **Statistical analysis**: In metabolomics, chemometric methods including multiple univariate analysis (UVA) and multivariate analysis (MVA) are critical part to deal with big dataset (Madsen et al. 2010). MVA using non-supervised methods (e.g., HCA, PCA, and SOMs) can be used first to achieve a general view of the dataset. Then, MVA using supervised methods (e.g., PLS, PLS-DA, and OPLS-DA) will be utilized to explore the differences in the metabolic profiles among samples and reveal the important metabolites. While utilizing MVA supervised methods, cross-validation test is always required to prove the model is not overfitting or overprediction (Eriksson et al. 2003). UVA (e.g., student's *t*-test and ANOVA) will be used simultaneously for the validation of candidate metabolite credentials. Details of these

Table 14.2 Available spectral database*

Database	Spectral data	Website	Information	References
HMDB	MS/NMR	http://www.hmdb.ca	114,100 metabolite entries including both water-soluble and lipid-soluble metabolites	Wishart et al. (2013)
LMSD	MS	http://www.lipidmaps.org	37,500 lipid structures with MS/MS spectra	Sud et al. (2007)
METLIN	MS	http://metlin.scripps.edu	961,829 molecules (lipids, steroids, plant and bacterial metabolites, small peptides, carbohydrates, exogenous drugs/metabolites, central carbon metabolites, and toxicants). Over 14,000 metabolites have been individually analyzed, and another 200,000 has in silico MS/MS data	Tautenhahn et al. (2012)
IsoMETLIN	MS	https://isometlin.scripps.edu	All computed isotopologues (>1 million) derived from METLIN on the basis of *m/z* values and specified isotopes of interest (^{13}C or ^{15}N)	Cho et al. (2014)
NIST	MS/NMR	http://chemdata.nist.gov/	Reference mass spectra for GC/MS, LC-MS/MS, NMR, and gas phase retention indices for GC	Simón-Manso et al. (2013) and Babushok et al. (2007)
PRIME	MS/NMR	http://prime.psc.riken.jp/	Standard spectrum of standard compounds generated by GC/MS, LC-MS, CE/MS, and NMR	Akiyama et al. (2008) and Sakurai et al. (2013)
TOCCATA COLMAR	NMR	http://spin.ccic.ohio-state.edu	Multiple spectral NMR datasets: ^{1}H- and ^{13}C-NMR, 2D ^{13}C–^{13}C TOCSY (n = 463), 2D ^{1}H–^{1}H TOCSY and ^{13}C–^{1}H HSQC-TOCSY (n = 475), and 2D ^{13}C–^{1}H HSQC (n = 555)	Robinette et al. (2008), Bingol et al. (2015), Bingol et al. (2014) and Bingol et al. (2012)
MassBank	MS	http://www.massbank.jp	Shared public repository of mass spectral data with 41,092 spectra (LC-MS, GC-MS …)	Horai et al. (2010)
Golm Metabolome	GC-MS	http://gmd.mpimp-golm.mpg.de	2019 metabolites with GC-MS spectra and retention time indices	Hummel et al. (2008)
BMRB	NMR	http://www.bmrb.wisc.edu	9841 biomolecules with ^{1}H, ^{13}C, or ^{15}N spectra	Ulrich et al. (2008)
Madison	NMR/MS	http://mmcd.nmrfam.wisc.edu	794 compounds with NMR spectra (e.g., ^{1}H, ^{13}C, ^{1}H–^{1}H, ^{1}H–^{13}C) and calculated masses for different monoisotopic compositions ($^{12}C^{14}N$, $^{13}C^{14}N$, $^{12}C^{15}N$, $^{13}C^{15}N$)	Cui et al. (2008)
NMRShiftDB	NMR	http://nmrshiftdb.nmr.uni-koeln.de	42,840 structures and 50,897 measured spectra	Steinbeck et al. (2003)
Birmingham Metabolite Library	NMR	http://www.bml-nmr.org	208 metabolites and 3328 1D- and 2D-NMR spectra	Ludwig et al. (2012)

**Adapted and updated from An PNT* (An 2017)

analyses must be reported to show the objective and unbiased data analysis of one study.

- **Data interpretation**: Up to now, there is still a large knowledge gap exists in the translation from changes in the metabolite concentrations to the actual physiological interpretation in an organism. Many informative metabolic databases are available for *Drosophila* such as KEGG (Kanehisa et al. 2012), MetaboAnalyst (Xia et al. 2015), MetaCyc (Caspi et al. 2008), Reactome (Fabregat et al. 2016), and WikiPathways (Kelder et al. 2012). As mentioned above, many metabolic pathways are well conserved between *Drosophila* and mammals (Graham and Pick 2017; Alfa and Kim 2016); the database for other organisms can be used as cross-references. From these databases, we can map the metabolites of interest to the metabolic pathways, find related genes/proteins, search for active cellular processes (DNA repair, cell cycle, or programmed cell death), and compare the metabolic pathways of interest among different organisms.
- **Additional validations for the changes in *Drosophila* metabolic profiles**:
  (1) *Using quantitative metabolomics*: In general, metabolite abundance in metabolomics is measured in relative or absolute quantification (Lei et al. 2011). In relative quantification, the signal intensity of metabolites in the samples and in standard solutions (for calibration curves) will be normalized to the signal intensity of an internal standard or another relative metabolite. In absolute quantification, external standards or internal isotopically labeled standards are utilized.
  (2) *Using assays to study the metabolism* (Tennessen et al. 2014b): Since not any analytical methods can cover all the metabolites, many assays are now available to study the metabolism such as measurements of total proteins, triglycerides, cholesterol, glucose, trehalose, and glycogen. This information can support the hypothesis raised by metabolomics studies.
  (3) *Testing gene, mRNA, and/or protein levels*: As an advantage of high-throughput study, a hypothesis generated from metabolomics is usually not limited at one metabolite but metabolic pathways. Therefore, additional information on the related gene, mRNA, and/or protein abundances will provide more evidences if these pathways are suppressed or upregulated.

14.3 Isotope-Assisted Metabolomics: State of the Art and Potentials for *Drosophila* Studies

Up to now, the available database on metabolic networks of *Drosophila* like KEGG was developed based on genome data. If researchers are interested in the information on the turnover of a metabolite and/or its functions in the metabolic networks, additional analyses should be done. For that purpose, isotope-assisted metabolomics approaches, including metabolite turnover analysis and isotope-assisted absolute quantification, are the state-of-the-art methodologies (You et al. 2014).

14.3.1 Metabolite Turnover Analysis for Metabolic Network Exploration

Metabolite turnover analysis (stable isotope tracer analysis) is a method allowing researcher to trace the fate of a metabolite in metabolic networks (Fig. 14.2) (You et al. 2014; Chokkathukalam et al. 2014). The cells or animals will be exposed to a fully labeled isotope substance of interest, and then samples are collected over time. The changes of labeling in downstream metabolites can be detected by both MS and NMR. Once a single isotope exists, the mass of a molecule will increase one atomic mass unit (amu). Notably, MS/MS-based platform is a very powerful tool to examine the exact location of the stable isotope in the molecule. As a result,

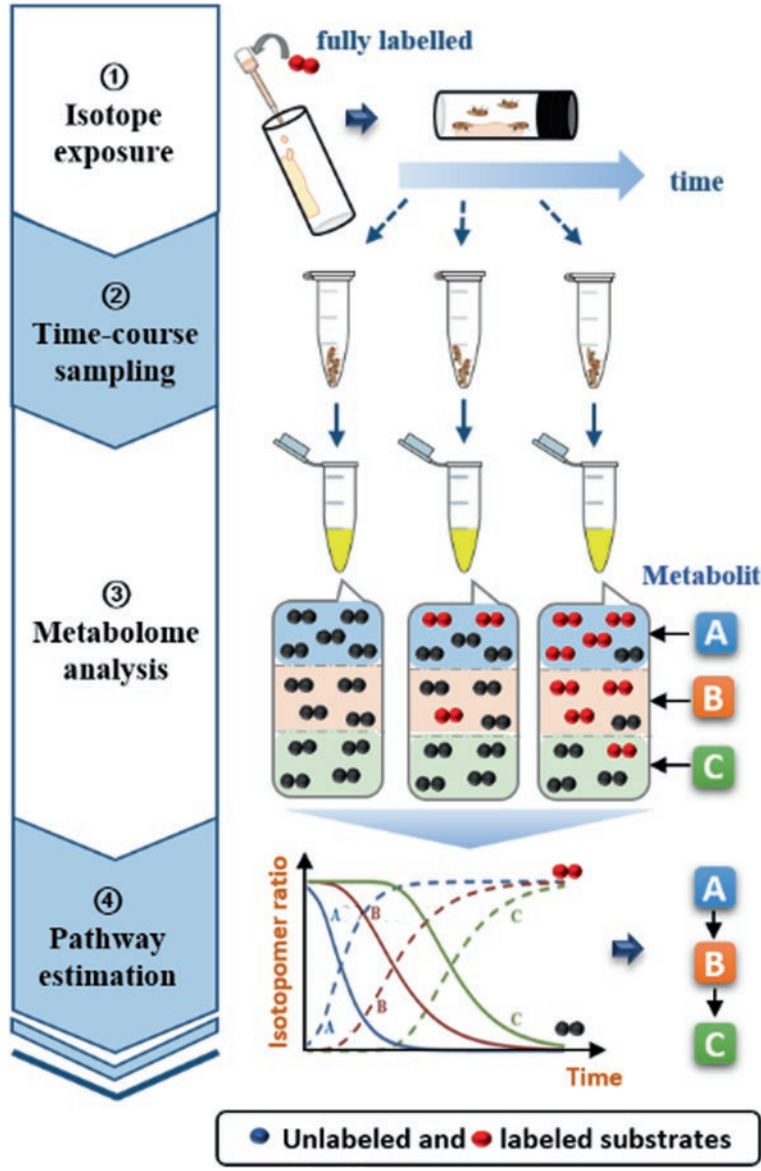

Fig. 14.2 Workflow of metabolite turnover analysis

an overview of which pathways are involved in the turnover of the target metabolite will be revealed. The stable isotopes of hydrogen (^{2}H; deuterium), carbon (^{13}C), and nitrogen (^{15}N) are commonly used in metabolomics fields.

The most important point to set up this experiment is the culture/medium components should be well defined, which makes it easy to modify only the target metabolite to stable isotope. That is the reason why the main application field of this method is on microorganism and cells, while only a few studies applied on *Drosophila* (Coquin et al. 2008; Nicolay et al. 2013). One remarkable case study example is from Dyson group in 2013; the authors applied metabolite turnover analysis on *Drosophila* larvae (Nicolay et al. 2013). By tracing the fate of glutamine, they found that inactivation of the retinoblastoma tumor suppressor (RBF1) increased the flux of glutamine toward glutathione synthesis, apparently reducing oxidative stress. Recently, a "holidic medium" for *Drosophila melanogaster* has been developed

by Piper et al. in 2014 (Piper et al. 2014). Even though it has not been applied for metabolomics study, holidic medium could be very useful if researchers want to strictly control and manually modify the diet.

14.3.2 Isotope Ratio-Based Approach for Absolute Quantification of Metabolites

Isotope-assisted absolute quantification in metabolomics is an advanced method allowing the accurate measurement of all detected metabolite abundances (Bennett et al. 2008). The main obstacle to quantify all metabolites is each metabolite's signal intensity which can be influenced by many factors such as its concentration, its structure, and its matrix effects. Using only one internal standard is not ideal for normalization of all metabolites with different characteristics. To overcome these challenges, one of the best solutions is using isotope internal standard mixtures which have similar profile to sample's metabolome (Chokkathukalam et al. 2014). The isotope compounds are able to account for sample processing variations and matrix effects encountered during analysis because they behave identically to their unlabeled equivalents in sample extracts. Fully labeled ^{13}C-metabolites are the most commonly used; they are however quite expensive. The isotope internal standard mixtures are usually generated by growing the cells with fully labeled substrates to yield exclusively labeled intracellular metabolites (Bennett et al. 2008). The internal standard mixtures will be added and extracted together with samples and standard solutions. After analysis, absolute quantitation of the metabolite levels is calculated using $^{12}C/^{13}C$ ratio-based calibration curves.

Even though isotope-assisted absolute quantification is the most accurate method to measure metabolite levels, it was applied on *Drosophila* only when a small number of metabolites needed to be quantified (Kostal et al. 2011). Hence, if a proper method to yield exclusively labeled intracellular metabolites is developed for *Drosophila*, it will be advantageous to expand applications of metabolomics on *Drosophila* model.

14.4 Central Carbon Metabolism of *Drosophila* Throughout the Life Cycle

The full genome of *Drosophila melanogaster* was successfully sequenced in 2000 (Adams et al. 2000; Fortini et al. 2000). The mRNA expression levels throughout the life cycle (Graveley et al. 2011) and in different organs of adult flies (Brown et al. 2014) were published in 2011 and 2014, respectively. Many projects to explore *Drosophila* proteome are now ongoing with 21,973 protein entries which can be accessed on UniProt (http://www.uniprot.org). Thus, *Drosophila* metabolome information is a viable counterpart to gain deeper understanding on *Drosophila* and expand its applications as a model organism. We recently reported *Drosophila* metabolic profiles at different developmental stages using the combination of GC-MS and ion-paired LC-MS/MS (An et al. 2017a; An 2017; An et al. 2014). Here, we will discuss the changes metabolic profile throughout *Drosophila* life cycle focusing on the central metabolic pathways (amino acids, sugars, and organic acids, as well as intermediates of central metabolism, such as sugar phosphates and cofactors).

During *Drosophila* embryogenesis (Fig. 14.3), free amino acids (FAAs) were proposed to be essential during *Drosophila* embryogenesis, and different amino acids appear to play distinct roles in different developmental stages of the embryo. High levels of aspartic acid, methionine, and glutamic acid were detected at the first 4 h after egg laying, which includes the rapid nuclear division cycles of *Drosophila* embryos. As aspartic acid, methionine and glutamic acid related to purine and pyrimidine synthesis (Bender 2012), high levels of these amino acids might be a crucial for supplying substrates and energy for DNA replication during the of early *Drosophila* embryogenesis. The changes in purine and pyrimidine metabolism also matched with this hypothesis. On the other hand, essential amino acids

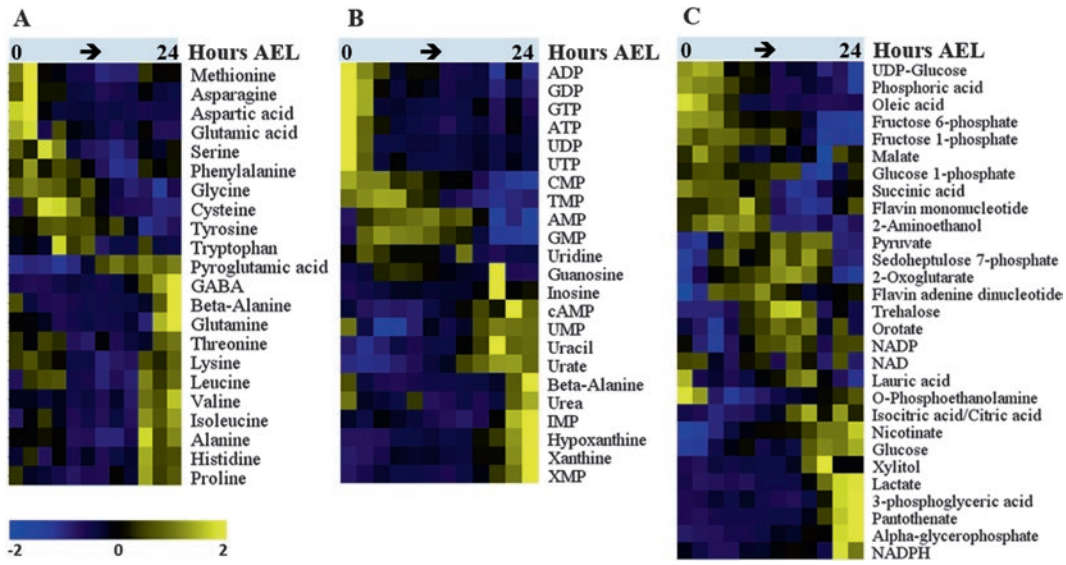

Fig. 14.3 Metabolic profiles of Canton S during embryogenesis. (**a**) Free amino acids. (**b**) purine and pyrimidine metabolism. and (**c**) glycolysis, TCA cycle, fatty acids, and cofactors. Embryos of Canton S were incubated at 25 °C in which samples were collected every 2 h from 0 to 24 h AEL (h after egg laying). The color scale is plotted at the bottom left of the figure

(leucine, isoleucine, threonine, valine, lysine, and histidine) increased significantly at the end of embryogenesis. The only source for essential amino acids in *Drosophila* embryo was from protein degradation, and insects do not carry out gluconeogenesis from lipid substrates (Rockstein 2012). Thus, the embryo must be endowed with an abundance of maternally supplied products, and these amino acids possibly provide another pathway to control energy production during embryogenesis.

Metabolites in sugars metabolism changed drastically during the development of embryos. The level of UDP-glucose decreased, while the level of glucose increased throughout embryogenesis. Interestingly, trehalose was found to be accumulated in an abundant level during gastrulation. Previous transcriptome study reported that *Tret 1–1* and *Tret 1–2* (encoding for trehalose transporters) were highly expressed during gastrulation, while *Treh* (encoding for trehalase that converts trehalose into glucose) was expressed throughout embryogenesis (Fisher et al. 2012). Therefore, trehalose was proposed as the energy source for glycolysis to supply glucose for the cells during embryogenesis. Unlike in larval stage that glucose in the fat body is utilized to generate trehalose (Elbein et al. 2003; Chen and Haddad 2004), trehalose used in embryogenesis must be generated from other sources (properly from the yolk) because the level of glucose is quite low in the early stage. Consistent with this hypothesis, a study from Tennessen et al. showed that the level of triacylglycerol and glycogen decreased during *Drosophila* embryogenesis (Tennessen et al. 2014a).

In larval stage, first, second, and third instar larvae had distinct metabolic profiles (Fig. 14.4). High-abundance metabolites in amino acid, purine, and pyrimidine metabolism were detected in first instar larvae than other larval stages. At the early stage of larval development, these metabolites likely came from their diet since the flies started to uptake food and obtain amino acids from their food by ingesting protein (Nation 2008). The major events during larval stage are rapid growth and proliferation of imaginal discs as well as endoreplication of other tissues. Therefore, high levels of metabolites in purine and pyrimidine metabolism, materials for cell division and cell growth, were detected. Second- and third-instar larvae stages are the extensive

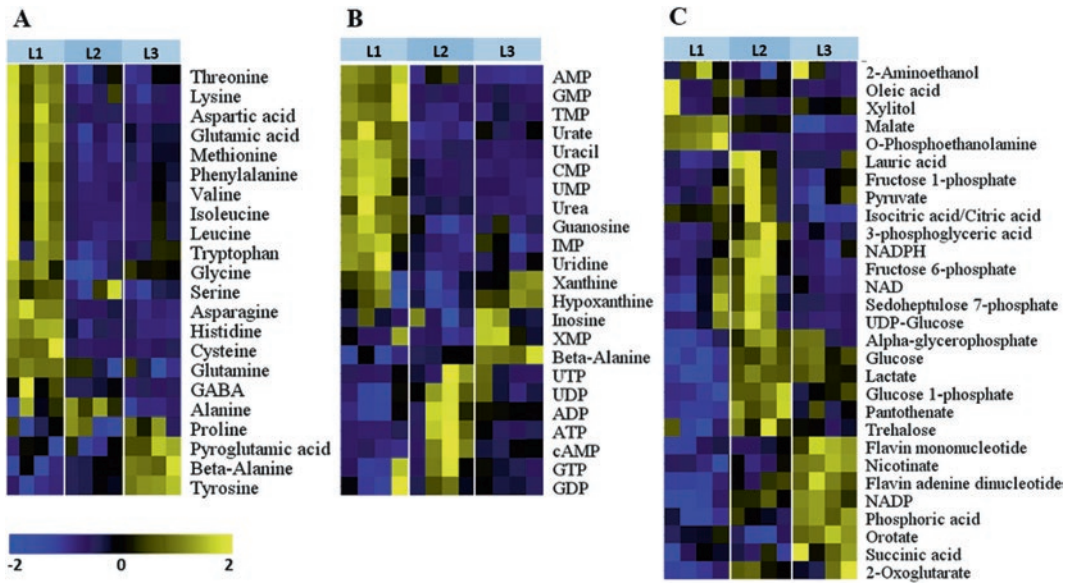

Fig. 14.4 Metabolic profiles of Canton S during larval stage. (**a**) Free amino acids. (**b**) purine and pyrimidine metabolism. and (**c**) glycolysis, TCA cycle, fatty acids, and cofactors. The color scale is plotted at the bottom left of the figure. L1, first instar larvae; L2, second instar larvae; L3, third instar larvae

feeding stage; larvae uptake nutrients not only to fuel developmental reorganization but also to survive during metamorphosis and early adult stage (Church and Robertson 1966). Thus, metabolites related to energy metabolism including some FAAs, sugars, and TCA intermediates were detected in high abundances during the late stage of larval development.

The metabolic profiles of *Drosophila* during metamorphosis were grouped into two main groups including 0–6 h AWP (prepupal period marked by pupariation) and 12–90 h AWP (main pupal period) (Fig. 14.5). Throughout metamorphic processes, the adult progenitor cells such as imaginal discs undergo cell proliferation, differentiation, and organogenesis to give rise to the adult structures, while most larval tissues undergo autophagy and cell death (Aguila et al. 2007). The most significant change was the increase of FAA levels, which was matched with the changes of urea abundance. Further investigation found that the total protein amount in flies decreased throughout metamorphosis. These results suggested that the cells broke down proteins intensively to recycle the amino acids. As flies cannot uptake food from pupal stage until 8 h after eclosion (Chiang and Tactic 1963), these FAAs were likely used as material to construct proteins or to produce energy via gluconeogenesis. Moreover, insects always have to maintain a high hemolymph level (2.9–23.4 mg/ml) comparing to most of vertebrates (0.5 mg/ml) (Gilbert and Schneiderman 1961; Wigglesworth 2012). FAAs were also reported as the most abundant metabolites in ten different tissues of adult flies (Chintapalli et al. 2013). Therefore, the regulation of FAA levels not only maintains the normal development of *Drosophila* pupae but also has to keep a high abundant level of FAAs in the body of adult flies at the end of development. Moreover, the metabolites in purine, pyrimidine, and energy metabolisms (fatty acids, sugars, and TCA intermediates) also changed drastically during the main pupal stage. Autophagy and apoptosis are the important processes for the degradation and turnover components in the cells of an organism (Mariño et al. 2014). These results suggested that in the nutrient-limited environment, the pupae used the materials from cell histolysis for the differentiation of the tissues as well as to generate energy for cellular activities.

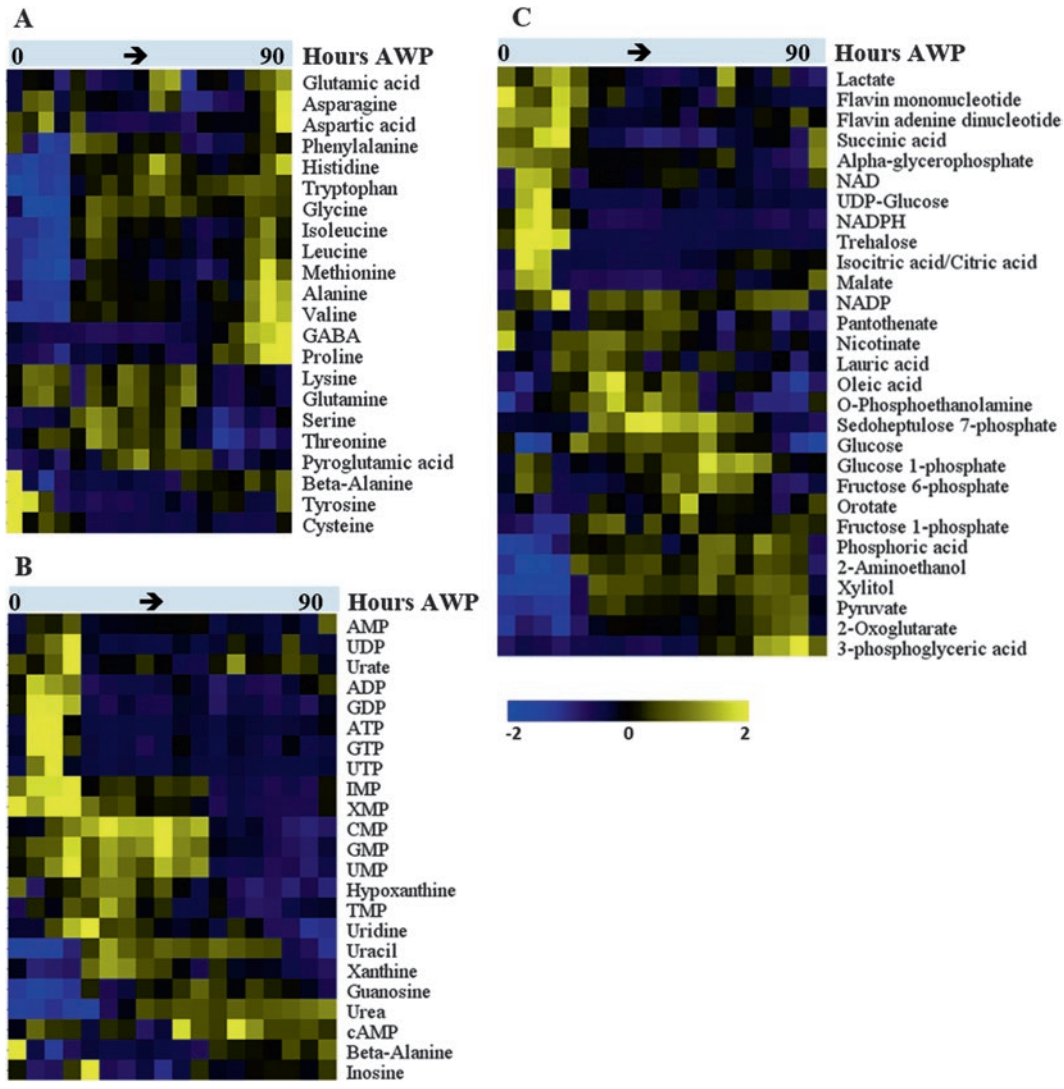

Fig. 14.5 Metabolic profiles of Canton S during metamorphosis. (**a**) Free amino acids. (**b**) purine and pyrimidine metabolism. and (**c**) glycolysis, TCA cycle, fatty acids, and cofactors. At this stage, the time course sampling started when the animals reached white pupal stage. The samples were collected from 0 to 90 h AWP (h after white pupae). The color scale is plotted at the bottom left of the figure

In adult stage, male and female flies appeared to have distinct metabolic profiles especially in purine and pyrimidine metabolism (Fig. 14.6). These data were supported by a previous study showing that female flies required higher DNA biosynthesis and RNA transcription to produce eggs from germ cells (Rong et al. 2014). Moreover, the levels of UDP-glucose, glucose 1-phosphate, fructose 1-phosphate, and fructose 6-phosphate were significantly higher in female flies. Since adult flies can uptake nutrients directly from food, these results indicated that female *Drosophila* had higher energy demand. This finding matched with previous data suggested that female flies had relatively bigger fat body and more storage lipid to maintain the reservoirs the reproductive organs and eggs (Scheitz et al. 2013; Parisi et al. 2011). A transcriptome study in male and female *Drosophila* found that the major differences in gene expression were

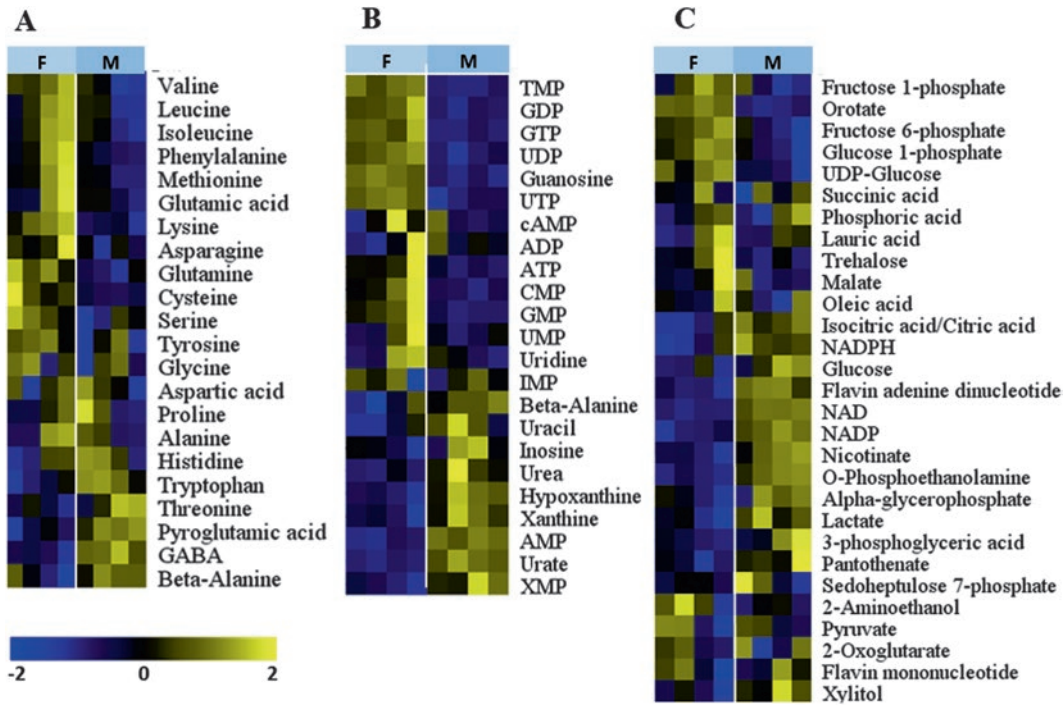

Fig. 14.6 Metabolic profiles of Canton S during metamorphosis. (**a**) Free amino acids. (**b**) purine and pyrimidine metabolism. and (**c**) glycolysis, TCA cycle, fatty acids, and cofactors. For adult stage, the 5-day-old virgin files of each gender were collected after emerging. The color scale is plotted at the bottom left of the figure. *F*, female, *M*, male

attributable to the germ cells (Parisi et al. 2004). Therefore, the differences in the metabolic profiles discovered in this study were likely due to the reproductive systems. Even though the full mechanism was not clearly understood, the metabolism was sex biased in *Drosophila*.

14.5 Future Perspectives of *Drosophila* Metabolomics

In general, *Drosophila* metabolomics is still immature comparing to other application fields of metabolomics. However, it is helping us to gain more insights into *Drosophila* metabolism than any other technologies could have done. Current workflow in *Drosophila* metabolomics is mainly adopted from other organisms. The lack of optimum methods specifically for *Drosophila* is one of the biggest bottlenecks interfering the use of advanced metabolomics technologies such as turnover analysis or absolute quantification. As mention above, *Drosophila* "holidic medium" proposed by Piper et al. could be the key to solve this problem. Besides, expanding the sample types can so widen the applications of *Drosophila* metabolomics. Most of the reports used whole body extract, and only a few studies focus on specific tissues. The current metabolomics technologies can access to the metabolic profile of not only large tissues but also cell lines (in cell culture) (Muschet et al. 2016), single cell (Emara et al. 2017), and even subcellular organelles (Chen et al. 2016; Dietz 2017). Obviously, *Drosophila* cell culture has been using as a quick screening system for basic researches in molecular and cellular biology (Baum and Cherbas 2008). Moreover, the use of *Drosophila* imaginal discs has been providing new insights into a number of discoveries in developmental and cellular biology (Beira and Paro 2016). Therefore, if we can take advantage of metabolomics technologies

and apply to various *Drosophila* experimental systems, *Drosophila* metabolomics will give huge impact on achieving new insights into the cell phenotype and physiology.

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