molecular systems biology

Effects of diet and development on the Drosophila lipidome

Maria Carvalho, Julio L. Sampaio, Wilhelm Palm, Marko Brankatschk, Suzanne Eaton, Andrej Shevchenko

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 21 March 2012

Thank you again for submitting your work to Molecular Systems Biology. We have now heard back from the three referees who agreed to evaluate your manuscript. As you will see from the reports below, the referees find the topic of your study of potential interest. They raise, however, some important concerns that we would ask you to address in a revision of this work.

Broadly, the reviewers agreed that this work could present an important and novel resource describing the Drosophila lipidome. The most fundamental concern appears to be raised by reviewer #3, who felt that it is essential that the technical reliability of the lipidomic measurements are validated with an orthogonal technology. Given the centrality of these data to this work, the editor feels that this is a vital point.

If you feel you can satisfactorily deal with these points and those listed by the referees, you may wish to submit a revised version of your manuscript. Please attach a covering letter giving details of the way in which you have handled each of the points raised by the referees. A revised manuscript will be once again subject to review and you probably understand that we can give you no guarantee at this stage that the eventual outcome will be favorable.

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Sincerely,

Editor - Molecular Systems Biology
msb@embo.org

Referee reports:

Reviewer #1 (Remarks to the Author):

This paper by Carvalho et al. presents an overview of the effects of diet and development on lipid composition in the fruit fly, Drosophila melanogaster. The authors raised Drosophila on one of four diets and measure the abundance of different lipid species using quantitative shotgun profiling by mass spectrometry. This included 250 species from 14 major classes of lipids, providing a complete catalog of the fly lipidome. The authors characterize the lipid composition of individual larval tissues, including the gut, fat body, salivary glands, wing discs, and brain, and identify several novel and interesting tissue-specific differences. For example, the sphingolipid composition of the Drosophila midgut is different from that of other tissues, perhaps contributing to proper epithelial barrier function. The authors point out that some lipids are enriched in the same manner in specific tissues of Drosophila and mammals, indicating that some of these patterns are conserved through evolution and thus likely reflect a key aspect of organ function. The authors characterize the lipid composition of lipoprotein assemblies and show that these contain mostly DAG, as is known in other insects, along with MCFAs and high PE and sterol levels. Importantly, the authors find that the composition of membrane fatty acids is heavily influenced by the animal's diet, emphasizing the critical link between specific dietary lipid classes and membrane composition, similar to results reported in humans. The authors also examine lipid composition during development in animals maintained on a yeast diet. This results in a number of interesting observations that will be of broad use in the field, including the first evidence that membrane lipids decrease immediately after pupariation, accompanied by an increase in TAG, suggesting that the FFAs released during larval tissue autophagy are being recaptured as triglycerides.

Although essentially a descriptive study, this body of work provides a new and important foundation for future research. The quality of the data is outstanding, as expected from a leading lab in the field of Drosophila lipid metabolic research. The writing is clear and the conclusions are well supported by the data. This study represents a major new landmark for the field that is bound to support significant future studies. This manuscript is essentially ready for publication. I have only a few minor suggestions to offer.

1) The "yeast food" used by the authors is the same as the "Semi-Defined medium" listed on the Bloomington Stock Center web page. They should acknowledge this source in their paper.

2) Is it possible to provide the lipid composition of the diets that are used in this study? This would allow an evaluation of how the distribution of dietary lipids compares to the lipid classes found in different tissues and at different times during development. The authors provide suggestions that this data is available. For example, they write on pg. 10 that "Plant food has longer and less saturated fatty acids than yeast food" and present some numbers. They also include some pie charts showing the proportions of different sterols in the foods. It would be ideal to have this data available for all lipid classes. I am also wondering about the nutrient composition of the diets that are used. It might be helpful to present the kcal distributions of the basic components - carbs, fat, and protein - so that, for example, the high fat levels present in the PF will be clear to the reader.

3) On pg 21, the authors state that "When presented with a mixture of different sterols, Drosophila absorb them into the intestine with different efficiencies." I am not sure they can make this claim without doing labeled uptake assays.

Reviewer #2 (Remarks to the Author):

The authors present a large and complex dataset, detailing tissue specific lipid compositions of Drosophila over different developmental stages and diet. The science is well thought out and likely required substantial effort to collect biological specimens in an accurate and precise manner. The article reports and discusses a large amount of data in the form of numerous histograms and the

authors have made their dataset publicly available for data mining. The article is well written, the language is not overly technical or complex in terms of the biology and should be amenable to a wide audience; there are few typographical errors. This reviewer believes that this article should be accepted for publication after the following minor points are addressed.

Page 8: The calculations for the average number of carbons and double bonds is straightforward, however the assumption for the average carbon chain length being a multiple of 0.5 for DAGs and 0.33 for TAGs requires justification. It is simple to think of DAGs as having two fatty acids that are equal, but this is not always the case. Similarly, TAGs are not always a combination of three identical fatty acids. This is likely a good method for approximation, however the language the authors use indicate that it is a conclusive method for calculating the average chain length when in actuality it is an assumption. Evidence for this practice in the literature should be cited, or the language should be modified to indicate that it is an approximation.

Page 11: Souza et al. reference is missing the year of publication.

Figures 2, 3 and 5: The selection of fatty acid chain lengths seems inappropriately short. Surely there were other fatty acid chain lengths in these samples as well? What about lyso-forms, which (in mammalian cells at least), are of fairly high abundance? There is no comment on lyso-forms of lipids, other than an acknowledgment on page 9 that they are formed in mammalian cells. Were these forms even present? If so, at what abundance? If they were not present, comments / discussion to this effect would strengthen the manuscript.

Supplementary Data Figures S5 and S6: The labels on these figures are ambiguous. I would guess that "PC32" is phosphocholine with 32 carbons, but "PS1" makes me doubt this assumption. Many of the lipids appear to be labeled with a code that is unhelpful to the reader. Figure S6, panel B has triple quotation marks around each label; if this signifies a unique feature of the plot, it is unclear as to what it is.

Reviewer #3 (Remarks to the Author):

In this manuscript, Carvalho/Sampaio present measurements of the Drosophila lipidome. Especially they determine the lipid composition of a number of tissues, and of entire flies at different developmental stages and under different nutrient conditions. Despite the fact that only a subset of lipids is measured (e.g. cardiolipins are not detected), this manuscript reports a remarkable technical achievement resulting in a great data collection. However, that is also pretty much it. There are little novel insights generated in this paper, and overall the results are highly descriptive. As this paper will provide primarily a resource for scientists (and it is not exactly clear how it may be useful), it should maybe be considered primarily as a resource article. Overall, this is high quality paper that likely will have some impact as a resource.

Since this paper attempts to set the standard for the Drosophila lipidome, it is important to more rigorously establish the reliability of the measurements. Specifically, how confident are the authors to extract all lipids with similar efficiency? How reliable is the quantiation in light of different ionization of the different lipids and derivatives (particularly for the sterols where in addition derivatization efficiency might vary)?. Really, if the authors want to set the reference for Drosophila lipidomics, they need to provide some evidence that their methods are reliable. For example they should provide some data using e.g. enzymatic assays on the abundance of lipids and compare extraction methods at least on total flies. Since the authors mention that an analysis takes 10min such experiments should be straightforward.

Reviewer's critique is typeset in italics

***Reviewer #1**:*

This paper by Carvalho et al. presents an overview of the effects of diet and development on lipid composition in the fruit fly, Drosophila melanogaster. The authors raised Drosophila on one of four diets and measure the abundance of different lipid species using quantitative shotgun profiling by mass spectrometry. This included 250 species from 14 major classes of lipids, providing a complete catalog of the fly lipidome. The authors characterize the lipid composition of individual larval tissues, including the gut, fat body, salivary glands, wing discs, and brain, and identify several novel and interesting tissue-specific differences. For example, the sphingolipid composition of the Drosophila midgut is different from that of other tissues, perhaps contributing to proper epithelial barrier function. The authors point out that some lipids are enriched in the same manner in specific tissues of Drosophila and mammals, indicating that some of these patterns are conserved through evolution and thus likely reflect a key aspect of organ function. The authors characterize the lipid composition of lipoprotein assemblies and show that these contain mostly DAG, as is known in other insects, along with MCFAs and high PE and sterol levels. Importantly, the authors find that the composition of membrane fatty acids is heavily influenced by the animal's diet, emphasizing the critical link between specific dietary lipid classes and membrane composition, similar to results reported in humans. The authors also examine lipid composition during development in animals maintained on a yeast diet. This results in a number of interesting observations that will be of broad use in the field, including the first evidence that membrane lipids decrease immediately after pupariation, accompanied by an increase in TAG, suggesting that the FFAs released during larval tissue autophagy are being recaptured as triglycerides.

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1) The "yeast food" used by the authors is the same as the "Semi-Defined medium" listed on the Bloomington Stock Center web page. They should acknowledge this source in their paper.

We now acknowledge the recipe in the Materials and Methods section (see page 23 line 19).

2) Is it possible to provide the lipid composition of the diets that are used in this study? This would allow an evaluation of how the distribution of dietary lipids compares to the lipid classes found in different tissues and at different times during development. The authors provide suggestions that this data is available. For example, they write on pg. 10 that "Plant food has longer and less saturated fatty acids than yeast food" and present some numbers. They also include some pie charts showing the proportions of different sterols in the foods. It would be ideal to have this data available for all lipid classes.

The exact lipidome composition of all three diets used in this study was provided in the Dataset spreadsheet (worksheet 1), which provided source data for sterol composition of yeast food (pie chart K3 in Figure 8) and average fatty acid length and saturation (Table SI in Supplementary Materials). We now explicitly refer the reader to this spreadsheet in Materials and Methods (see page 24 line 2) and Results section (see page 10 line 10).

I am also wondering about the nutrient composition of the diets that are used. It might be helpful to present the kcal distributions of the basic components - carbs, fat, and protein - so that, for example, the high fat levels present in the PF will be clear to the reader.

We now include this information in the Materials and Methods (see page 23, section *Drosophila* diets).

3) On pg 21, the authors state that "When presented with a mixture of different sterols, Drosophila absorb them into the intestine with different efficiencies." I am not sure they can make this claim without doing labeled uptake assays.

Yes, we agree that we should not be so specific about the mechanism. We now state that this could reflect either absorption or efflux (see page 13 line 10) and we changed the text where we refer to absorption to accumulation (see page 13 lines 16 and 23; page 21 line 29) or uptake (see page 17, line 1).

Reviewer #2:

The authors present a large and complex dataset, detailing tissue specific lipid compositions of Drosophila over different developmental stages and diet. The science is well thought out and likely required substantial effort to collect biological specimens in an accurate and precise manner. The article reports and discusses a large amount of data in the form of numerous histograms and the authors have made their dataset publicly available for data mining. The article is well written, the language is not overly technical or complex in terms of the biology and should be amenable to a wide audience; there are few typographical errors. This reviewer believes that this article should be accepted for publication after the following minor points are addressed.

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We fully agree with the Reviewer on this point. Abundance-weighted averaging the number of carbons / double bonds in fatty acid moieties is not even an approximation, but is only a convenient way of illustrating global changes in the fatty acid composition using a single intuitive number and of course we have never assumed the fatty acid moieties in any lipid species are necessarily equal. These averaged numbers may only reflect the average property of a given molecular population, yet they bear no relevance to any individual lipid molecule. We have now emphasized this point it the text to avoid confusion (see page 8 line 24). Although it is easier to survey global compositional differences this way, we always refer readers to the Supplementary materials containing the actual lipid composition.

Page 11: Souza et al. reference is missing the year of publication.

The typo has been corrected. (see page 11 line 26)

Figures 2, 3 and 5: The selection of fatty acid chain lengths seems inappropriately short. Surely there were other fatty acid chain lengths in these samples as well? What about lyso-forms, which (in mammalian cells at least), are of fairly high abundance? There is no comment on lyso-forms of lipids, other than an acknowledgment on page 9 that they are formed in mammalian cells. Were these forms even present? If so, at what abundance? If they were not present, comments / discussion to this effect would strengthen the manuscript.

This is clearly a misunderstanding: we did not select for any particular lipid classes, or for lipid species with particular fatty acid combinations. We report all lipids we could identify that were present at levels sufficient for reliable quantification. These and other figures were computed from all identified lipids reported in Supplementary materials, datasets 2 and 3.

Please note that, in general, *Drosophila* lipids do contain shorter fatty acids moieties, compared to mammals (see de Renobales and Blomquist, 1984; Rietveld et al. 1999), so such a distribution was expected.

With respect to the more minor lipid classes, there were several that we detected but excluded from the quantitative analysis based on their low abundance and resulting difficulties in quantification (for example lyso-lipids and sterol esters). We now state explicitly in the results section which lipids we detected but did not quantify. (see page 5 line 22)

Supplementary Data Figures S5 and S6: The labels on these figures are ambiguous. I would guess that "PC32" is phosphocholine with 32 carbons, but "PS1" makes me doubt this assumption. Many of the lipids appear to be labeled with a code that is unhelpful to the reader. Figure S6, panel B has triple quotation marks around each label; if this signifies a unique feature of the plot, it is unclear as to what it is.

We introduced a color code for double bonds to visually distinguish them from total fatty acid chain length (see Figures S5 and S6).

Reviewer #3 (Remarks to the Author):

In this manuscript, Carvalho/Sampaio present measurements of the Drosophila lipidome. Especially they determine the lipid composition of a number of tissues, and of entire flies at different developmental stages and under different nutrient conditions. Despite the fact that only a subset of lipids is measured (e.g. cardiolipins are not detected), this manuscript reports a remarkable technical achievement resulting in a great data collection. However, that is also pretty much it. There are little novel insights generated in this paper, and overall the results are highly descriptive. As this paper will provide primarily a resource for scientists (and it is not exactly clear how it may be useful), it should maybe be considered primarily as a resource article. Overall, this is high quality paper that likely will have some impact as a resource.

Although we are happy that the reviewer believes this work to be a remarkable technical achievement, we disagree with his/her opinion that our paper lacks novel insights. In our opinion, the value of this work is not just that it produced a sheer knowledge base (with lipid species catalogue as just one “resource” element) for use in forthcoming “hypothesis-driven” studies, although this is also an important aspect. Several surprising findings with important biological implications emerge from this work. For example, we have discovered that the fatty acid composition of the diet directly influences the fatty acid composition of phospholipids in tissues throughout the body. This is surprising, given the ability of cells to autonomously synthesize fatty acids, and the importance of biophysical membrane properties for cell behavior and signaling. It raises the important question as to how (or whether) these properties are preserved, and how the diet impinges on these properties and, eventually, on health beyond notable accumulation of fat.

Our study of lipidomic changes during development suggested unexpected metabolic shifts occurring in larval and pupal life. Furthermore, very interesting selectivity and tissue specificity in sterol trafficking is indicated by our tissue-specific lipidomic analysis. We have now modified the abstract of the paper to emphasize the novel biological insights emerging from this work, in addition to its value as a resource (see page 2 lines 10-16).

Since this paper attempts to set the standard for the Drosophila lipidome, it is important to more rigorously establish the reliability of the measurements. Specifically, how confident are the authors to extract all lipids with similar efficiency? How reliable is the quantiation in light of different ionization of the different lipids and derivatives (particularly for the sterols where in addition derivatization efficiency might vary)?. Really, if the authors want to set the reference for Drosophila lipidomics, they need to provide some evidence that their methods are reliable. For example they should provide some data using e.g. enzymatic assays on the abundance of lipids and compare extraction methods at least on total flies.

The mass-spec-based measurement we report here for sterols (8.3 mol%) in whole third instar larvae fed on yeast food is remarkably consistent with the value of 8 mol% we obtained previously from enzymatic and chemical assays (Carvalho et al., 2010). In this previous study, we used an enzymatic assay (Cholesterol/Cholesteryl Ester Quantitation Kit K603-100 from Biovision, Mountain View, CA) to quantify sterol and a phosphate assay to quantify phospholipids from third instar larvae fed with yeast. Note that our current determinations by mass spectrometry delivered much lower standard deviation compared to the enzymatic assays and allowed us to quantify individual sterol species.

For our current sterol analyses, we adapted the method described in (Sandhoff *et al*, 2009) using D7-cholesterol as the internal standard. We determined correction coefficients for the abundances in experiments with structurally related standards that were available in quantities sufficient for making stock solutions with precisely known concentrations (Supplementary material, Figure S9). We noted that correction coefficients for ergosterol (in respect to cholesterol) reported by Sandhoff *et al* and independently determined by us (Figure S9), were consistent. We had not included these data in the previous version of the manuscript since it is an established analytical method. To address reviewer's critique, now we present sterol calibrations in the Supplementary material Figure S9 and provide full experimental details, including the correction factors, in the Material and Methods section (p.26, line 22).

As the reviewer correctly pointed out, different lipids may be extracted and ionized with different efficiencies. To control for these differences, we added standards to the samples before extraction. This means that any differences in extraction and ionization efficiencies are already taken into account by this quantification method. Internal standards have been convincingly shown to control for the different ionization efficiencies of the different lipid classes. Importantly, fatty acid moieties of different lipid species within one class do not affect ionization efficiency (Koivusalo *et al*., 2001; Zacarias *et al*., 2002). Moreover, we validated the approach of internal standards specifically for our techniques (Ejsing *et al*, 2009; Ejsing *et al*, 2006; Matyash *et al*, 2008, and reference list below).

Extraction efficiency for major lipid classes by chloroform / methanol (Folch *et al*, 1957; Bligh and Dyer, 1959) and by methyl-*tert*-butyl ether (Matyash *et al*, 2008) is the same within experimental error (Matyash *et al*, 2008; Ejsing *et al* , 2009). For each lipid class, it is independent of the fatty acid moieties: both methods produced coherent molecular species profiles from complex biological samples ranging from yeast through human cells to nematode worms.

Furthermore, we have previously shown that shotgun method provides coherent lipid quantification when performed with different mass spectrometry platforms (Herzog *et al*, 2011; Herzog *et al*, 2012). Multiple lipid classes can be quantified in parallel and linear dynamic range (the ratio of the most abundant to least abundant signal ensuring the linear dependence of analyte concentration) is over 1,000 –fold (Schuhmann *et al*, 2011; Schuhmann *et al*, 2012; Ejsing *et al*, 2009; Ejsing *et al*, 2006). High mass resolution (> 80,000 full width at half maximum, FWHM) and low-ppm mass accuracy ensure unequivocal assignment of lipid species in total extracts (Schwudke *et al*. 2007; Schwudke *et al*, 2011; Schuhmann *et al*, 2011).

We now have drawn the reader's attention to the previous technical validation by citing the most relevant references in the revised manuscript (see page 25, line 17).

Please find below a comprehensive list of references to the extensive technical validation underlying the methods we use.

Bligh, EG; Dyer, WJ, A rapid method of total lipid extraction and purification. Can J Biochem Physiol 1959; 37: 911-7.

Ejsing, CS; Duchoslav, E; Sampaio, J; Simons, K; Bonner, R; Thiele, C; Ekroos, K; Shevchenko, A, Automated identification and quantification of glycerophospholipid molecular species by multiple precursor ion scanning. Anal Chem 2006; 78: 6202-14.

Ejsing, CS; Sampaio, JL; Surendranath, V; Duchoslav, E; Ekroos, K; Klemm, RW; Simons, K; Shevchenko, A, Global analysis of the yeast lipidome by quantitative shotgun mass spectrometry. Proc Natl Acad Sci U S A 2009; 106: 2136-41.

Folch, JM, Lees, M., Sloane-Stanley, G. H., A simple method for the isolation and purification of total lipids from animal tissue. J. Biol. Chem. 1957; 226: 497-509.

Herzog, R; Schwudke, D; Schuhmann, K; Sampaio, JL; Bornstein, SR; Schroeder, M; Shevchenko, A, A novel informatics concept for high-throughput shotgun lipidomics based on the molecular fragmentation query language. Genome Biol 2011; 12: R8.

Herzog, R; Schuhmann, K; Schwudke, D; Sampaio, JL; Bornstein, SR; Schroeder, M; Shevchenko, A, LipidXplorer: a software for consensual cross-platform lipidomics. PLoS One 2012; 7: e29851.

Koivusalo M, Haimi P, Heikinheimo L, Kostiainen R, Somerharju P. Quantitative determination of phospholipid compositions by ESI-MS: effects of acyl chain length, unsaturation, and lipid concentration on instrument response. J Lip Res 2001;42:663-672

Matyash, V; Liebisch, G; Kurzchalia, TV; Shevchenko, A; Schwudke, D, Lipid extraction by methyl-tert-butyl ether for high-throughput lipidomics. J Lipid Res 2008; 49: 1137-46.

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Schwudke, D; Hannich, JT; Surendranath, V; Grimard, V; Moehring, T; Burton, L; Kurzchalia, T; Shevchenko, A, Top-down lipidomic screens by multivariate analysis of high-resolution survey mass spectra. Anal Chem 2007; 79: 4083-93.

Zacarias A, Bolanowski D, Bhatnagar A. Comparative measurements of multicomponent phospholipid mixtures by electrospray mass spectroscopy: relating ion intensity to concentration. Anal Biochem. 2002;308:152-9

Acceptance letter 25 June 2012

Thank you again for sending us your revised manuscript. The reviewers agreed that the revisions made to this work had addressed their main concerns, and I am pleased to inform you that your paper has been accepted for publication.

Thank you very much for submitting your work to Molecular Systems Biology.

Sincerely,

Editor - Molecular Systems Biology
msb@embo.org

Reviewer #2 (Remarks to the Author):

The authors have adequately addressed all of the reviewer comments. Manuscript is ready for publication.

Reviewer #3 (Remarks to the Author):

The authors have well addressed the technical comments I had made during the first round of this paper's submission.
While I continue to believe that this paper offers little conceptual advance, I also am sure it will make a fantastic resource for many future studies to come and therefore should be published.

