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Metabolic pools differ among ecologically diverse *Drosophila* species

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ABSTRACT

Studies of the genetic mechanisms underlying metabolic storage have focused on a few model organisms. Although very fruitful, these studies have not allowed for the examination of mechanisms across a phylogenetic spectrum. The exploration of natural patterns of metabolic pool size variation across species will help us to better understand the genetics of metabolic adaptation.

We examined the metabolic pools size (triglyceride, glycogen and protein) at two ages in 12 *Drosophila* species with distinctly different ecologies for which complete genome sequences (for 11 of the 12 species) are known. Overall, there were significant differences across species for all three pools, while age and sex appear to affect some metabolic pools more than others. After correcting for the phylogenetic relatedness of the species used, we observed no association between triglyceride and glycogen content. Although within species these two pools sometimes are correlated, at a larger phylogenetic scale control of triglyceride and glycogen contents may have been shaped independently by natural selection.

1. Introduction

Of invertebrate models for physiological studies, *Drosophila melanogaster* has offered numerous insights into the genetic mechanisms underlying major metabolic pathways and their relationship to stress, nutrition, and aging (Montooth et al., 2003; Merritt et al., 2006; Flowers et al., 2007). Conversely, with few exceptions, little is known about the variation in control of metabolic resources within a species and even less across species. Other *Drosophila* species can differ dramatically from *D. melanogaster* in their geographic distributions and ecologies. The resources utilized by *Drosophila* vary widely: substrates such as tree slime fluxes, fruits, mushrooms, cacti and flowers serve as feeding and breeding sites for different species (Powell, 1997; Markow and O'grady, 2007). This variance in substrate utilization suggests that control of metabolic pathways in *Drosophila* likely has undergone evolutionary changes.

Evidence of evolutionary changes in *Drosophila* metabolic pathways comes from a range of studies, some empirical at the organismal level, others purely bioinformatic via the analysis of sequence differences between species and/or populations and yet others examining ecological stoichiometry. For example, in *D. melanogaster* selected for increased desiccation resistance contain greater glycogen content (Djawdan et al., 1998). The glycogen not only functions as a source of metabolic water it also stores three to five times its mass in water (Gibbs, 2002). The relationship between desiccation resistance and glycogen storage is not universal within *Drosophila*. The desiccation survivorship strategy of desert *Drosophila*, such as *D. mojavensis*, involves a decrease in water loss (Gibbs and Matzkin, 2001) via an overall reduction in metabolic rate (Gibbs et al., 2003) and not increases in glycogen. Furthermore, the mechanism underlying the desiccation-specific metabolic rate reduction in *D. mojavensis* has been narrowed down to the modulation of just four central metabolism genes (Matzkin and Markow, 2009). Using a bioinformatic approach Kunieda et al. (2006) observed that across *Anopheles gambiae*, *D. melanogaster*, and *Apis mellifera* genomes, the number of genes for carbohydrate metabolism has been more evolutionarily labile than observed for lipid metabolism genes. Finally, differences in body nitrogen and phosphorus in ecologically diverse *Drosophila* suggest that species have adapted, metabolically, to the nutritional composition of their hosts (Markow et al., 1999; Jaenike and Markow, 2003).

Now, with entire genome sequences available for 12 ecologically and evolutionarily diverse *Drosophila* species (*Drosophila* 12 Genomes Consortium, 2007), even greater opportunities exist to elucidate the roles of genes, environments and their interactions in metabolic processes. Choosing those *Drosophila* species that will be most informative for larger-scale systems studies of metabolism

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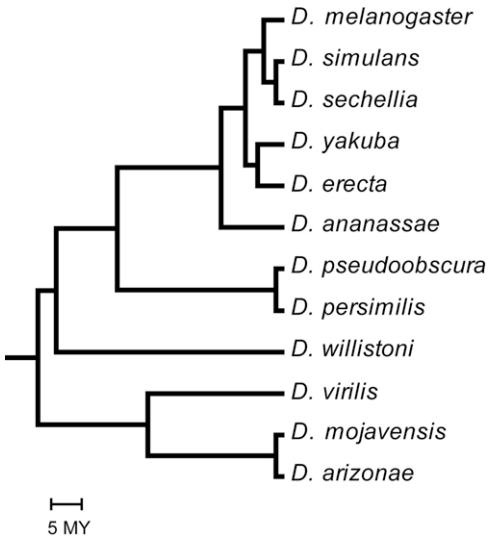

Fig. 1. Phylogenetic relationships of the 12 *Drosophila* species examined. Tree is based on *Drosophila* 12 Genomes Consortium (2007), Machado et al. (2007) and Matzkin (2008).

will depend upon the nature of the metabolic differences among them. The first step in selecting species for detailed metabolomic studies is to determine how species and sexes differ at adult emergence in their basic metabolic pools, and how these pools change as adult males and females mature. We take this first step with 12 ecologically diverse *Drosophila* species for which fully sequenced genomes are available for 11 (*Drosophila* 12 Genomes Consortium, 2007) (see Fig. 1). Thus, in the present study, we ask the following questions: (1) How do *Drosophila* species, when reared on identical larval diets, differ in their adult protein, triglyceride and glycogen contents? (2) What are the influences of sex and age on levels of these metabolic components?

2. Materials and methods

2.1. Drosophila strains and culture conditions

Stocks were obtained from the Tucson *Drosophila* Species Stock Center now located at the University of California at San Diego (https://stockcenter.ucsd.edu). Eleven of the strains chosen correspond to those used in the genome sequencing projects and the twelfth, *D. arizonae*, has a BAC library. The *Drosophila* species chosen belong to two subgenera, *Sophophora* (*D. melanogaster*, *D. simulans*, *D. sechellia*, *D. yakuba*, *D. erecta*, *D. ananassae*, *D. pseudoobscura*, *D. persimilis* and *D. willistoni*) and *Drosophila* (*D. virilis*, *D. mojavensis* and *D. arizonae*). All flies were reared using standard *Drosophila* Stock Center banana/*Opuntia* media in all phases of the experiment. We chose to rear all species on an identical diet, a standard laboratory *Drosophila* medium, in order to reveal how the natural histories of these ecologically diverse species have been shaped to deal with the same food. For example, *D. melanogaster* and its relatives naturally breed in carbohydrate-rich decaying fruits, and others such as *D. virilis*, breed in tree slime fluxes or *D. mojavensis* and *D. arizonae*, in cacti, the latter of which are comparatively low in carbohydrates. If adaptation to their natural diets has modified the metabolic processes underlying the sizes of metabolic pools, we reasoned that such adaptations would be reflected in the flies reared on a standardized diet.

Virgin flies were collected and sexed within 24 h of eclosion and placed in 8-dram food vials (20 flies per vial). Half of the newly eclosed flies were frozen (−80 °C) in groups of five flies per sex (0-day treatment). We also were interested in whether, after emergence, metabolic pools change as adult flies age. Thus the remaining flies were kept in male-only or female-only vials for 8 days, transferring them into fresh food vials once at day four. At the end of the 8-day period flies were frozen (−80 °C) in groups of five flies per sex (8-day treatment). We chose 8 days because by this age the majority of adults of all species are sexually mature (Markow and O'grady, 2005).

2.2. Metabolic pool assays

Prior to measuring metabolic pools the dry mass of the flies was measured. Flies were placed in a 50 °C oven for 3 days. Mass was recorded before and after the drying using a Cahn Model C-31 microbalance. Dried flies were homogenized in 1 ml of phosphate buffer (25 mM KH_2PO_4, pH 7.4) and then centrifuged for 3.5 min at 12,500 rpm. A total of 850 μl of supernatant were removed and frozen.

Glycogen content was measured using the PGO Enzymes (Sigma–Aldrich) kit with the addition of 0.1 U of amyloglucosidase (Sigma–Aldrich) per ml of reaction buffer. Samples (40 μl of homogenate + 200 μl of reaction buffer) were incubated at 37 °C for 3 h and absorbance was measured at 445 nm. Triglyceride content in each sample was determined using 100 μl of Triglyceride Reagent Set (Pointe Scientific Inc.) and 20 μl of homogenate, incubated at 37 °C for 30 min and then absorbance was read at 540 nm. Triglyceride content instead of total lipid content, which includes such things as cuticle lipids, was examined since the focus of this study was on energy storage compounds. Soluble protein concentration was determined using a 50:1 solution of bicinchoninic acid (BCA) and 4% $CuSO_4$, respectively. A total of 200 μl of BCA/$CuSO_4$ solution were added to 20 μl of sample and incubated for 30 min at 37 °C. Absorbance was measured at 562 nm. The metabolic pools per sample were measured in triplicate and means were used for the analysis.

2.3. Statistical analysis

Metabolic pool concentrations were standardized by dry mass and analyzed independently using a two-way ANOVA (sex, age and species as factors). To remove the possible correlation associated with phylogenetic relatedness (Felsenstein, 1985) we calculated phylogenetically independent contrasts of size-independent measurements of glycogen and triglyceride content. Phylogenetically independent contrasts were calculated using the relationship of *Drosophila* species shown in Fig. 1 and the CAIC v. 2.6.9 software (Purvis and Rambaut, 1995). The relationships between the phylogenetic independent contrast of glycogen and triglyceride content were examined by calculating the product–moment coefficients of "positivized" contrasts through the origin as suggested by Garland et al. (1992). All statistical analyses (ANOVA, regression and correlations) were performed using the JMP ver. 7 software.

3. Results

3.1. Metabolic pools

The means and standard errors for all three metabolic pools and dry mass are available online in Supplementary Table S1. Due to insufficient replicate number some species were omitted from the statistical analysis: *D. persimilis* for triglyceride content, *D. yakuba* for glycogen content, and *D. erecta* for protein content. Dry mass was used to standardize all metabolic pools. All three factors (sex, age and species) plus their interactions significantly affect the dry mass of the 12 *Drosophila* species examined (Table S2).

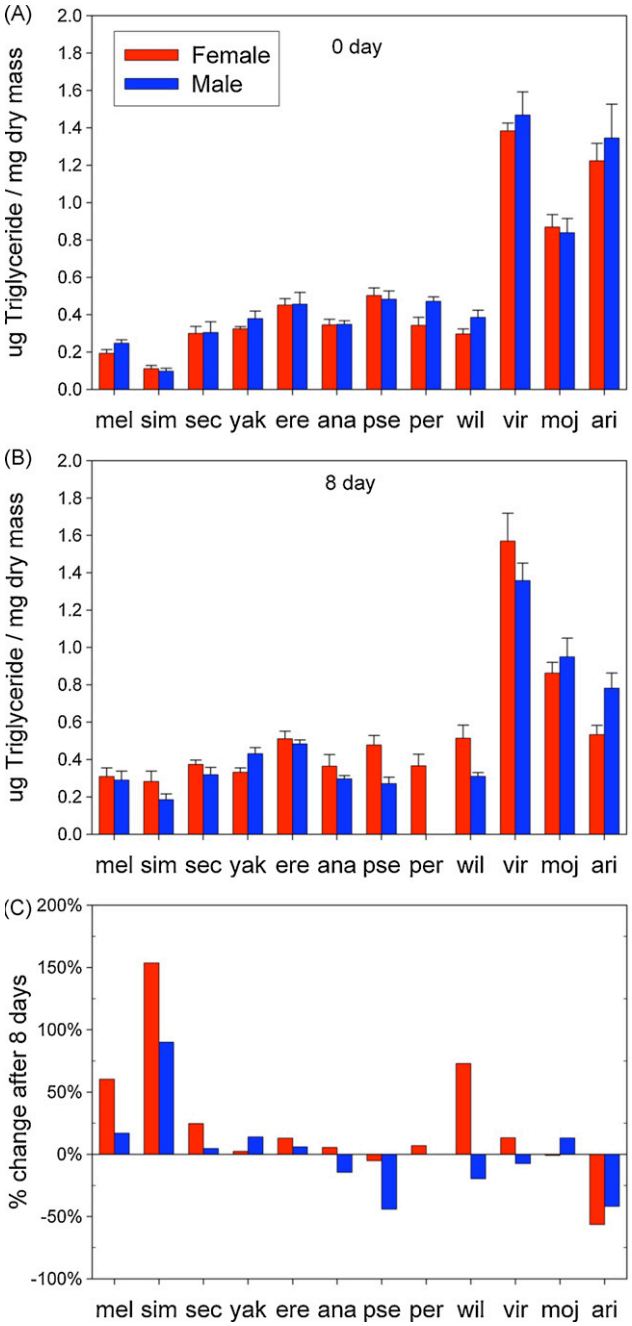

Fig. 2. Triglyceride content per mg of dry mass for females and males of all 12 species at (A) 0 day and (B) 8 days posteclosion. Percent change in triglyceride content over time is shown in (C). Species are ordered according to their phylogenetic relationships (Fig. 1). The first nine species belong to the *Sophophora* subgenus and the last three to the *Drosophila* subgenus.

Triglyceride pools at emergence and at 8 days of age are presented in Fig. 2A and B, respectively. ANOVA (Table 1) revealed significant species and sex × age interactions. Triglyceride levels were much higher at both ages in species of subgenus *Drosophila*, especially in *D. virilis*, which contained several times the levels seen in the *Sophophoran* species. Over time, however, the increase in triglycerides was greater in some of the *Sophophoran* species and in some *Drosophila* subgenus species it actually decreased (Fig. 2C).

Glycogen levels for adults at emergence (Fig. 3A) and at 8 days of age (Fig. 3B) reflect an increase in this metabolite with age. The greatest percentage increase was in species of the melanogaster group of the subgenus *Sophophora*, but did not include the two obscura group species *D. pseudoobscura* and *D. persimilis* (Fig. 3C).

Table 1
ANOVA for triglycerides content.

Source	DF	Sum of squares	*F* ratio	Prob > *F*
Species	10	29.5887	151.43	<0.001
Age	1	0.0338	1.73	0.19
Sex	1	0.0011	0.06	0.81
Species × age	10	2.1783	11.15	<0.001
Species × sex	10	0.3259	1.67	0.09
Age × sex	1	0.0711	3.64	0.06
Species × age × sex	10	0.2506	1.28	0.24
Error	171	3.3414		

Table 2
ANOVA for glycogen content.

Source	DF	Sum of squares	*F* ratio	Prob > *F*
Species	10	0.0107	13.39	<0.001
Age	1	0.0142	177.16	<0.001
Sex	1	0.0003	3.94	0.049
Species × age	10	0.0164	20.45	<0.001
Species × sex	10	0.0024	3.05	0.001
Age × sex	1	0.0001	1.29	0.29
Species × age × sex	10	0.0014	1.77	0.07
Error	164	0.0131		

The ANOVA was significant for all three main effects and for the species × age and species × sex interaction terms (Table 2).

Protein pool levels are shown in Fig. 4A and B for adults at the time of emergence and at 8 days of age, respectively. Species differences, up to twofold, existed at both ages. When there was a sex difference, females had higher levels of protein. Protein was lost with age in all cases with the single exception of *D. willistoni* females (Fig. 4C). All main effects and two-way interactions were significant (Table 3).

3.2. Phylogenetic analyses

The two *Drosophila* subgenera studied differ in their triglyceride content. This was observed for both sexes at day 0 ($F_{1,58} = 268.2$, $P < 0.001$ and $F_{1,56} = 151.8$, $P < 0.001$, females and males, respectively) and day 8 ($F_{1,56} = 53.9$, $P < 0.001$ and $F_{1,51} = 149.0$, $P < 0.001$, females and males, respectively). Additionally, differences between the two subgenera were found in glycogen for 0-day females ($F_{1,53} = 4.93$, $P = 0.03$), and for protein content in males ($F_{1,58} = 5.4$, $P = 0.02$ and $F_{1,52} = 18.1$, $P < 0.001$, 0-day and 8-day, respectively). Our aim is to examine the correlation between triglyceride and glycogen content across the species analyzed, but the close phylogenetic distance between some species can bias the correlation. Species that are closely related will tend to have more comparable concentrations of the metabolic pools because of similarity of their genomes and ecology. Given the phylogenetic correlation between the species analyzed, we examined the relationship between glycogen and triglyceride content using independent contrasts. The correlation between these two pools was not significant at day 0 ($r = 0.33$, $P = 0.32$ and $r = 0.09$, $P = 0.81$

Table 3
ANOVA for protein content.

Source	DF	Sum of squares	*F* ratio	Prob > *F*
Species	10	0.1129	16.97	<0.001
Age	1	0.0724	108.81	<0.001
Sex	1	0.0426	64.04	<0.001
Species × age	10	0.0236	3.55	<0.001
Species × sex	10	0.0275	4.13	<0.001
Age × sex	1	0.0056	8.37	0.004
Species × age × sex	10	0.0061	0.91	0.52
Error	169	0.1124		

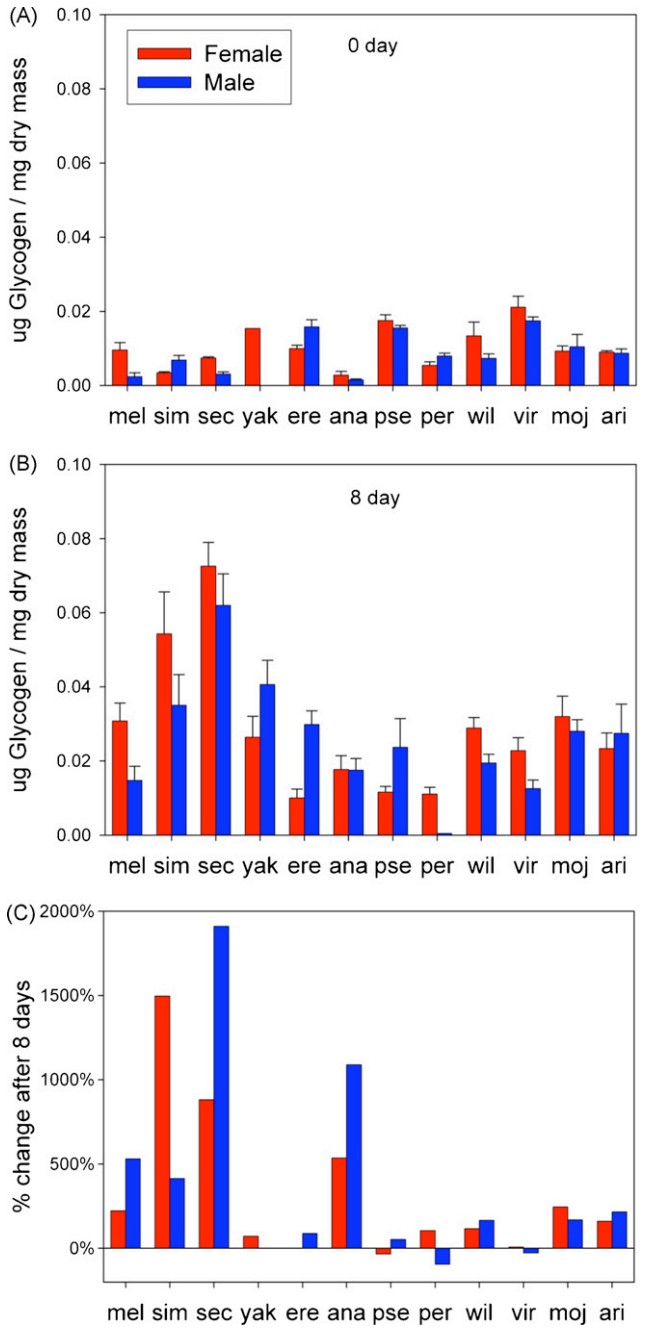

Fig. 3. Glycogen content per mg of dry mass for females and males of all 12 species at (A) 0 day and (B) 8 days posteclosion. Percent change in glycogen content over time is shown in (C). Species are ordered according to their phylogenetic relationships (Fig. 1). The first nine species belong to the *Sophophora* subgenus and the last three to the *Drosophila* subgenus.

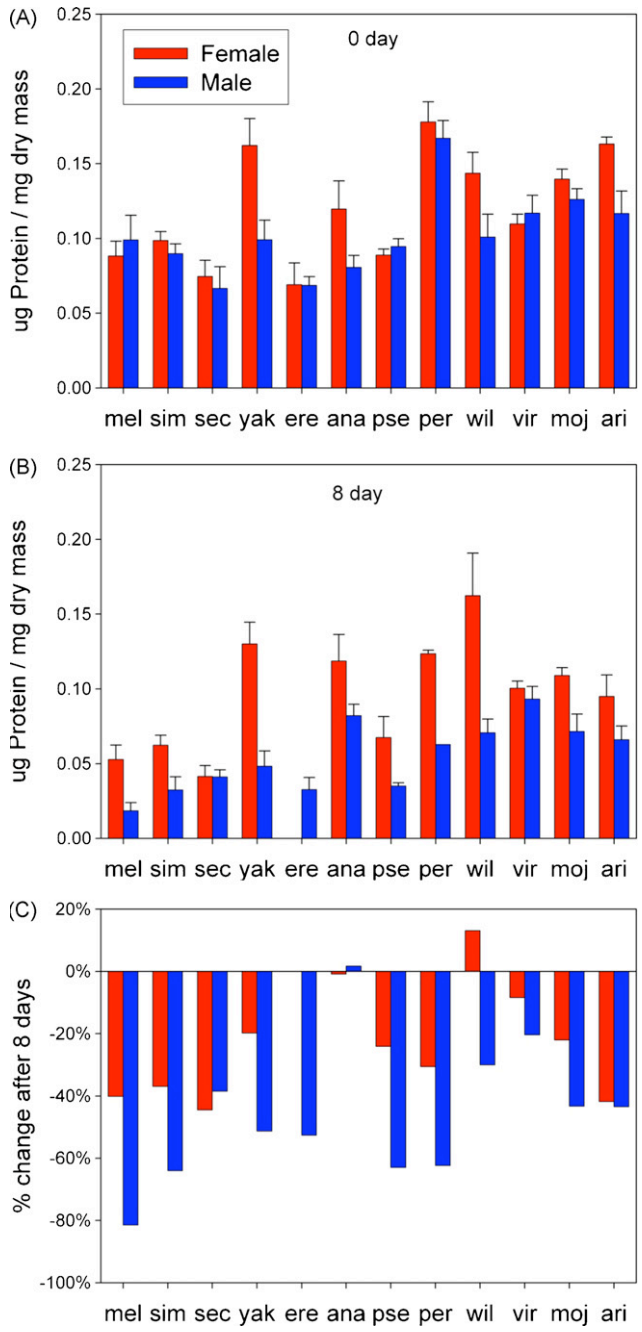

Fig. 4. Protein content per mg of dry mass for females and males of all 12 species at (A) 0 day and (B) 8 days posteclosion. Percent change in soluble protein content over time is shown in (C). Species are ordered according to their phylogenetic relationships (Fig. 1). The first nine species belong to the *Sophophora* subgenus and the last three to the *Drosophila* subgenus.

for females and males, respectively) and day 8 ($r = -0.17$, $P = 0.61$ and $r = -0.05$, $P = 0.90$ for females and males, respectively).

4. Discussion

The 12 species examined here, despite being reared on the identical diet, exhibited strikingly different levels of triglycerides, protein, and glycogen. In the current study all species were reared on banana medium, despite the qualitative differences in the resources they consume in nature (Markow and O'grady, 2005, 2007). For example, species of the subgenus *Sophophora* tend to be fruit breeders, while the *Drosophila* subgenus flies more often feed on decaying plants. Given their contrasting ecological histories, it would be surprising if they had turned out to have similar macronutrient pool sizes. Some metabolic pools, however, display stronger evolutionary signals than others. The most striking example is that of the triglycerides, in which the three species of the subgenus *Drosophila* were characterized by very high levels relative to the other species.

What kinds of mechanisms might account for these species differences? The observed metabolic pools could reflect processes at the behavioral as well as metabolic levels. In some species the machinery to metabolize various nutrients may not be able to deal with the levels present in the experimental food, resulting in a

build-up or reduction of some metabolites. Other species might consume larger quantities of laboratory media to obtain sufficient quantities of a particular micro- or macronutrient found in their natural host. If so, their metabolic pathways will be forced to deal with the excess of the other things they have consumed.

Correlations between glycogen and triglyceride content (Djawdan et al., 1998) and their shared control (Bharucha et al., 2008) have been previously observed in *D. melanogaster*. The lack of correlation between glycogen and triglycerides for the 12 phylogenetically distant species in this study indicates an uncoupling of these two metabolic pools. The 12 *Drosophila* species sampled in this study are the result of a total of 192 million years of evolution (see Fig. 1). Although these pools might be under similar control within a species, their control appears to be evolutionarily plastic, allowing for the perturbation of pools (and their respective control) to match the nutritional and environmental needs of a species.

What are ways in which the species, sex, and treatment differences might be related to features of the flies' ecology other than diet? Ecological differences among these species also include their geographic distribution, from deserts to tropical rainforests, and thus also their needs to resist different environmental stressors such as starvation and desiccation resistance. *Drosophila melanogaster* triglyceride content and glycogen content have been correlated to increases in starvation and desiccation resistance, respectively (Graves et al., 1992; Djawdan et al., 1998; Schmidt et al., 2005). Although it has been shown that under desiccating conditions *D. melanogaster* metabolizes carbohydrates (Gibbs, 2002), when examined in a broad phylogenetic context, water loss rate and not glycogen metabolism, was the major factor explaining the difference in desiccation resistance across multiple *Drosophila* species (Gibbs and Matzkin, 2001; Gibbs et al., 2003). The starvation resistance of *D. mojavensis* and *D. arizonae* is significantly greater than of any other *Sophophoran* species (Matzkin et al., 2009), and their level of triglyceride content shows the same pattern (this study). Although not directly tested in a phylogenetically independent context, the correlation between starvation and triglyceride content appears to be stable across a large phylogenetic scale (Van Herrewege and David, 1997), while the association between desiccation and glycogen content clearly is not (Gibbs and Matzkin, 2001; Gibbs et al., 2003). Glycogen and triglyceride content may be uncorrelated when examined across multiple species owing to the roles of distinct ecological pressures facing each species in reshaping the control of these two pools.

In addition to the nutritional composition of the food resources of the 12 *Drosophila* species, differences in the abiotic environments of these species could be another major factor in their metabolic pool variation. For example, some species' habitats include temperate forests, where surviving the freezing temperatures of winter might be a strong selective force. Other's, such as *D. mojavensis* and *D. arizonae*, reside in deserts where temperatures can rise as much as 50 °C inside necrotic rots (L.M. Matzkin, unpublished). In some insects both winter diapause and cold-hardening involve a series of metabolic and transcriptional changes (Denlinger, 2002; Michaud and Denlinger, 2007). *Drosophila melanogaster*, a species originally from sub-Saharan Africa, has adapted to the temperate habitats it finds itself in today by undergoing a winter diapause, which is associated with an increase in triglyceride content (Schmidt et al., 2005).

Sex differences and differences among sexes of each species are likely to reflect their particular reproductive biologies. For example, lipids and carbohydrates are stored in the *Drosophila* fat body (Bharucha et al., 2008). For females, during later phases of oogenesis, large amounts of carbohydrates are taken up by ovarian oocytes, and stored primarily as glycogen (Gutzeit et al., 1994). Oogenesis proceeds at different rates in different *Drosophila* species (Kambysellis, 1968), and these differences in ovarian maturation are likely to contribute to the interspecific differences in glycogen observed among females as they advance from emergence to 8 days of age.

Perhaps most surprising is the almost uniform loss of protein content (per mg of dry mass) with age. One possible explanation is that since protein (as well as triglyceride and glycogen) is analyzed per mg of dry mass, mass increases over time and therefore the protein/mg measurement decreases. Dry mass appears to be positively influenced by age (Tables S1 and S2). Furthermore, protein content per fly appears to decrease with age ($F_{1,169} = 8.12$, $P = 0.005$) although it is not uniform across all species given a significant age × species interaction ($F_{10,169} = 6.89$, $P < 0.001$). The question then is what is possibly making the flies heavier. We observed that glycogen content increases with age (Fig. 3) and for most species, so does triglyceride content (Fig. 2). Furthermore, it is possible some other types of compounds we did not measure (cuticle lipid for example) increases with age. The observed decrease in protein content with age could be an artifact of an increase in mass with age.

This group of 12 *Drosophila* species offers an excellent opportunity to better examine the genetic basis of metabolic pools storage. Not only does metabolic pool variation exists among these species, but for 11 of them we have a complete genome sequences and three pairs (*D. mojavensis*/*D. arizonae*, *D. pseudoobscura*/*D. persimilis* and *D. sechellia*/*D. simulans*) can hybridize in the lab. The possibility of performing hybrid crosses will allow for the determination of genetic factors influencing the storage and metabolism of pools.

The effect of dietary shifts on gene expression have been examined within species in *D. melanogaster* (Carsten et al., 2005) and *D. mojavensis* (Matzkin et al., 2006), but in these cases, the dietary shifts were not large and the metabolic pools were not measured. Minor changes were observed in expression of genes involved in carbohydrate, lipid, and protein metabolism with dietary shifts, but the latter study was performed prior to the availability of full genome sequences and the ability to create full genome oligonucleotide arrays. However, the role of species differences in metabolism related to energy storage has not yet been investigated. The availability of fully sequenced genomes for 11 of these species will allow comparative and functional genomic approaches to reveal the relative contributions of structural versus transcriptomic changes in the genomes of species showing significant differences in one or more metabolic pools as well as sex and age differences. Computational analyses of the sequences should detect existing sequence and copy number differences in candidate genes. The sequences also will permit design of full genome expression arrays for examining the age- and sex-dependent variation in expression on standard as well as ecologically specific diets. Given the interspecific variability in their metabolic pools, these species clearly offer a promising path to understanding the range of possible metabolic strategies for energy storage and use.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jinsphys.2009.08.008.

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