Modular Evolution of the Drosophila Metabolome

Benjamin R. Harrison*1
Jessica M. Hoffman*2
Ariana Samuelson3
Daniel Raftery4
Daniel E.L. Promislow1,3

1 Department of Lab Medicine & Pathology, University of Washington School of Medicine, Box 357705, 1959 NE Pacific Street, Seattle, Washington, 98195, USA
2 Department of Biology, University of Alabama at Birmingham, 1300 University Blvd CH464, Birmingham, AL 35294, USA
3 Department of Biology, University of Washington, Seattle, Washington 98195, USA
4 Department of Anesthesiology & Pain Medicine, University of Washington School of Medicine, Seattle, WA, 98195 USA

* These authors contributed equally to this work

Corresponding Author:
Daniel Promislow (promislo@uw.edu)
Abstract

Comparative phylogenetic studies offer a powerful approach to study the evolution of complex traits. While much effort has been devoted to the evolution of the genome and to organismal phenotypes, until now relatively little work has been done on the evolution of the metabolome, despite the fact that it is composed of the basic structural and functional building blocks of all organisms. Here we explore variation in metabolite levels across 50 million years of evolution in the genus *Drosophila*, employing a common garden design to measure the metabolome within and among 11 species of *Drosophila*. We find that both sex and age have dramatic and evolutionarily conserved effects on the metabolome. We also find substantial evidence that many metabolite pairs covary after phylogenetic correction, and that such metabolome coevolution is modular. Some of these modules are enriched for specific biochemical pathways and show different evolutionary trajectories, with some showing signs of stabilizing selection. Both observations suggest that functional relationships may ultimately cause such modularity. These coevolutionary patterns also differ between sexes and are affected by age. We explore the relevance of modular evolution to fitness by associating modules with lifespan variation measured in the same common garden. We find several modules associated with lifespan, particularly in the metabolome of older flies. Oxaloacetate levels in older females appear to coevolve with lifespan, and a lifespan-associated module in older females suggests that metabolic associations could underlie 50 million years of lifespan evolution.

Introduction

The development of high-dimensional ‘omics’ methods has had a dramatic impact on the nature of comparative studies. First, genome data enable researchers to derive accurate, high-resolution phylogenies to probe the evolution of gene families and the genomic signatures of selection (e.g. Whitkus, et al. 1992; Clark, et al. 2007). Second, transcriptomics, proteomics and metabolomics allow for the study of adaptation at the molecular level with a breadth not previously possible (e.g. von Mering, et al. 2003; Spirin, et al. 2006; Bedford and Hartl 2009; Brawand, et al. 2011; Gordon and Ruvinsky 2012; Martin and Fraser 2018; Cope, et al. 2020). Metabolomic methods are able to measure
the levels of hundreds to thousands of metabolite features with a high level of accuracy (Jones, et al. 2012). Given the central role that the metabolome plays in organismal structure and function, and the fact that it integrates upstream genetic and environmental variation, it is surprising how little is known about the evolution of metabolite abundance (Flowers, et al. 2007; Noda-Garcia, et al. 2018).

There are just a handful of comparative studies of the animal metabolome (Khaitovich, et al. 2008; Fu, et al. 2011; Park, et al. 2012; Blekhman, et al. 2014; Bozek, et al. 2014; Ma, et al. 2015; Bozek, et al. 2017). Comparative studies are often confounded by lineage-specific environments, phylogenetic non-independence, and measurement error in estimating species-level phenotypes. Lineage-specific environments are particularly relevant in metabolomics studies as environment, genotype, sex, age and tissue/organ can each have dramatic effects on metabolome profiles (Steuer 2006; Hoffman, et al. 2014; Li, et al. 2017; Khrameeva, et al. 2018; Wilinski, et al. 2019). Comparative analysis is also affected by phylogenetic non-independence, although methods to handle phylogenetic confounding, including within systems-level data, are well established (Felsenstein 2008; Dunn, et al. 2018). We address these issues using a common garden design, with multiple strains per species, and phylogenetic correction using a standard approach (Felsenstein 2008).

There is abundant evidence that gene expression within species is modular, where groups of genes covary in their expression, sharing many more such covarying partners within the group (module), than with genes in other groups (Hartwell, et al. 1999; Wagner, et al. 2007). Patterns of covariation are of great interest as they are widely assumed to reveal functional associations (Ge, et al. 2001). In contrast to within species coexpression, phylogenetic patterns of coexpression and modularity are less explored. There is a substantial literature describing comparative analyses of modularity in morphology (Klingenberg 2014), but relatively limited comparative analyses of covariation and modularity in gene content (von Mering, et al. 2003) or in gene expression (von Mering, et al. 2003; Fraser, et al. 2004; Innocenti and Chenoweth 2013; Martin and Fraser 2018; Cope, et al. 2020).

The covariation of cellular traits across species may indicate evolution in pathway activity, functional interactions, or of development. Theories of biochemical pathway
evolution are focused in large part on the evolution of metabolic enzymes, perhaps owing to the relative scarcity of systems level data on metabolite abundance (Noda-Garcia, et al. 2018). Measuring the divergence and covariation among the metabolome can provide such insight. For instance, comparisons of the tissue-level metabolome in just four mammal species highlighted the divergence of the human brain metabolome as a putative hallmark of human evolution (Fu, et al. 2011; Bozek, et al. 2014; Bozek, et al. 2015). Thus, the metabolome may be reshaped in coordination with evolutionary change in organ systems and other organismal phenotypes. All of these possibilities provide a strong rationale for taking a systems-level approach to understand the evolutionary context of natural variation.

The fruit fly genus *Drosophila* offers a powerful model to examine evolutionary dynamics of metabolic pathways. While studies of the classic model species *D. melanogaster* predominate, evolutionary biologists have long appreciated the potential of this diverse clade, with both classic and contemporary studies in *D. pseudoobscura* (e.g. Dobzhansky 1946), the Hawaiian *Drosophila* (Carson and Kaneshiro 1976), and cactophilic *Drosophila* species (Markow, et al. 1982), among many others (Kambysel and Heed 1971; Schnebel and Grossfield 1983; Partridge, et al. 1987; Coyne and Orr 1989; Kellermann, et al. 2009). *Drosophila* have also played a central role in advances in genomics, including comparative work among fully sequenced genomes of numerous species (Ballard 2000; Bai, et al. 2007; Clark, et al. 2007; Stark, et al. 2007).

Here we measure a panel of metabolites across the genus *Drosophila* in both sexes at two ages. We find that patterns of variation in the metabolome are largely consistent with phylogenetic relatedness. Comparing two modes of evolution, we find that the simple Brownian motion model of evolution is a good fit to overall metabolome divergence, though sex and age are significant and conserved contributors to variation. The metabolome also shows evidence of modular coevolution, where groups of metabolites vary in concert across the phylogeny. Interestingly, the patterns of covariation are somewhat specific to each sex and age, highlighting the dynamic nature of metabolomic variation, its potential to explain variation in phenotype over sex and age, as well as the importance of common garden design in comparative studies. We then examine the variation within modules and find they show distinct patterns of evolution, including some
that appear to be under stabilizing selection. Consistent with the idea that the evolution of pathway activity may explain metabolite coevolution, we find that some modules are enriched for specific biological pathways. Additionally, we find evidence for the coevolution of lifespan with metabolite modules, which suggests that lifespan evolution has a conserved molecular basis across a 50-million-year phylogeny. Our hope is that this work might inspire further explorations so that we can begin to understand how, over hundreds of millions of years, evolution has shaped systems of functional and structural building blocks that make up all of life.

Results

We raised one to three wildtype strains from each of 11 species of *Drosophila* in a common environment and collected age-matched samples of each sex at 5 days (young) and 31 days (old) after eclosion. Strain-specific mean lifespans in these experiments ranged from 20.1 to 85.7 days with a grand mean across all species of 50.5 days (Fig S8). Targeted LC-MS/MS metabolome profiles of whole flies were measured at each age and in each sex, along with additional untargeted LC-TOF-MS profiling of young flies, with up to three replicates per strain, sex, age and species. Our metabolomic datasets comprised a panel of 97 targeted metabolites and an untargeted dataset with 4419 features detected in at least one sample. Metabolomic analysis often includes missing values due to the absence of a metabolite in a sample or to limits of detection. All 97 targeted metabolites were detected in all samples, and the untargeted panel detected 590 features present in all or almost all samples, imputing 228 features that were absent in only one sample. Missingness, a measure of the number of missing values within a sample, lacked any evidence of phylogenetic signal ($K = 0.13, P = 0.90$), and was associated with signal intensity ($F_{1,4416} = 578.8, P = 2.78 \times 10^{-120}$), suggesting that many missing values are likely to represent features that fall below the limit of detection.

Phylogenetic and selection signatures in the metabolome. For targeted metabolome profiles, the first and second principal components (PC1 and PC2) together explain 30.1% of the variance and, by visual inspection, capture latent variation by age and sex respectively (Fig 1A). Along with the age and sex-related variation, we explored the
influence of phylogeny on the multivariate metabolome by plotting the PCs in a phylogenetic context, and by measuring their phylogenetic signal. Visual inspection of PC1 and PC2 as well as statistical tests suggest phylogenetic signal in each PC for young flies, but not necessarily for older flies (Fig 1B and Table 1). We also found that metabolome-derived phylogenies showed significant concordance with the genome-based phylogeny, where branch scores, the sum of the squares of the difference between each branch in the true and deduced trees, ranged from 0.131 to 0.178 for each sex, age, or detection methodology (Kuhner and Felsenstein 1994; Kumar, et al. 2017). In all cases, the scores were significantly closer to the real phylogeny than were permutations of the genome phylogeny ($P \leq 0.017$, Table S1).

Having established a strong phylogenetic signature in the metabolome, we then sought to determine the mode of metabolome evolution. We compared two relatively simple models of evolution, the Brownian motion (BM) and the Ornstein–Uhlenbeck (OU) models. The BM model posits that traits diverge linearly with respect to time in a direction that is independent of the current trait values, whereas the OU model extends the BM model with the addition of a parameter representing stabilizing selection and thus can model limits on the extent of divergence (Cressler, et al. 2015). We find that the BM model is a better fit than the OU model to the divergence of the metabolome, where the $r^2$ of the BM fit ranged from 0.16 to 0.27 across both ages and sexes, and in each case $\Delta$AIC analysis favored BM (Fig S2). Additionally, we fit a linear model, equivalent to BM with all data and tested for effects of age, sex, or their interaction on the metabolome distance and the rate of divergence. The metabolome distance was greater ($\beta_{\text{age}}=0.063$, $P=0.022$), and the divergence rate higher ($\beta_{\text{time} \times \text{age}}=1.80 \times 10^{-3}$, $P=0.012$) in the metabolome of older flies (Fig 1C).

**Evidence of modular coevolution in the *Drosophila* metabolome.** We explored the possibility that the levels of metabolites co-evolve by measuring the pairwise covariance among phylogenetically independent contrast scores (PICs, Methods). PICs remove the confounding effect of phylogeny while preserving the correlations that may exist between traits as they diverge at each node in a phylogeny (Felsenstein 2008), and have been applied to multivariate comparative morphometric data in similar ways (Klingenberg
2014). We find substantial covariance between PICs of metabolite levels in samples of each age and sex. In comparison with randomized data, where species labels are permuted such that the relationships within species are preserved, the original data show a much higher frequency of highly positive and negative pairwise correlations among the PICs of metabolites than expected by chance (Fig S3). Clustering of pairwise PIC correlations among the metabolites indicated a high degree modularity (Fig 2). We evaluate the significance of the modularity in these networks by measuring edge betweenness community detection (Girvan and Newman 2002), and comparing them to rewired networks of the same degree distribution. In each sex and age group, the real network is significantly modular ($P < 0.001$).

To define the metabolites within each module, we used weighted gene correlation analysis (WGCNA), which led to over 88% of the 97 targeted metabolites being placed in a module, with five to six modules in each sex and age group (Figs 2 and S4). The metabolite members of modules in each sex and age are somewhat distinct, where for example, two metabolites might be members of module A in young males, and be members of two different modules in older males, though pairwise comparison between sex and age groups revealed from 2 to 4 modules with significant intersections in each comparison (Fisher’s exact test $P < 0.05$, Fig S5, Table S2).

Rather than metabolite levels evolving under direct selection, we hypothesized that the modularity in metabolite coevolution reflects selection acting more directly on biological pathways (Hartwell, et al. 1999; Wagner, et al. 2007; Noda-Garcia, et al. 2018). In support of this hypothesis, we made two observations. First, we tested the hypothesis that selection may operate at the level of metabolomic module. We compared the fit of the OU and BM models on the divergence of metabolites within each module (Cressler, et al. 2015). Across the 23 modules, we find that eight are better fit by the OU model, suggesting that some modules are evolving under stabilizing selection (Table S3, Fig S6). Second, we found by enrichment analysis that the metabolites within five of the modules, are more connected to subgraphs of the KEGG database than we would expect by chance (Picart-Armada, et al. 2018), with at least one KEGG pathway in each of these five modules showing such enrichment (FDR $\leq 0.2$, Table S5). Thus, there is evidence of extensive modularity in the metabolome at the phylogenetic level, and the patterns of
covariation are consistent with adaptive coevolution of metabolites that may share biological function.

**Sex and age affect interspecific metabolome variation.** To examine the effects of sex and age on the metabolome in a phylogenetic context, we use a Bayesian mixed model (Methods). Among the targeted metabolites, we found 44 of 97 metabolites with significant sex effects, 38 with age effects, and 2 with sex-by-age interactions (FDR<0.05, Fig 3). Analysis of 590 untargeted features at young age found effects of sex for 228 metabolite features at FDR<0.05. Thus, we find evidence that a substantial portion of the metabolome varies with sex and age in ways that have persisted over at least 50 million years.

Along with the conserved effects of sex, we also find evidence for evolution in sexual dimorphism within the metabolome. At a multivariate level, measuring the distance along PCs 1 and 2 for each sex within each species, we see that the nine species of the *Sophophora* subgenus separate with the female samples having consistently higher PC values than the males, whereas in the two species of the *Drosophila* subgenus, the male and female samples remain clustered along this axis (Fig S7).

**Lifespan and the metabolome coevolve.** Lastly, we analyzed the lifespan of the 26 strains with an average of 95.3 flies for each sex and strain (± 30 SD, n=16 to 133, Fig S8). To identify metabolites and modules that covary with lifespan, we took two approaches. First, regression of PICs of lifespan on each targeted metabolite identified an association between lifespan and oxaloacetate (FDR=0.007, $P<1\times10^{-4}$, Fig 4C). The PICs of several other metabolites were significant at less conservative FDR (Fig 5, Table S6). We also regressed lifespan PICs on the eigenmodules within each sex and age and identified module D in the older male metabolome associated with lifespan PICs ($r^2=0.80$, FDR=0.01, Table 2), and module B in the older female metabolome at a more modest FDR of 0.2 ($r^2=0.52$, Table 2). While neither of these modules were enriched for KEGG
pathways after FDR correction, KEGG pathways whose enrichment had a nominal P<0.05 are shown in Table S5.

Discussion

**Evolution of the *Drosophila* metabolome.** Here, we examine the *Drosophila* metabolome and its associations with sex, age and lifespan across 11 species. We find strong phylogenetic signal, with both Blomberg’s K and Pagel’s λ (Revell, et al. 2008) significant in the metabolome of younger flies (Table 1), and concordant with the genome-based phylogeny (Figs 1B and S1). Surprisingly, we see little evidence of selection acting to constrain divergence in overall metabolome variation (Fig 1C). However, there is considerable evidence for selection acting on metabolite levels when we analyze variation within co-evolving modules (Table S3, Fig S6). Some of the co-evolutionary modules enrich biological pathways, and modules in the older male and female metabolome associate with evolution in lifespan.

Before we discuss each of these points below, there are several possible caveats to keep in mind. First, to control for effects of environment, we have used a common garden design. However, it is perhaps inevitable that what is a viable environment for all species in the study might be far from ideal for some species. Second, although this study explores variation within as well as between species, we are capturing a very small snapshot of within-species variation, with only three strains per species. Moreover, the individual strains used are likely to be inbred, which could have a strong impact on phenotypic variation in general, and sexual dimorphism more specifically (Connallon and Clark 2014; Yassin, et al. 2016; Ruzicka, et al. 2019). Third, with only 11 species, we lack the power to fully explore the full evolutionary range of the metabolome, lifespan and sexual dimorphism (Cressler, et al. 2015) within *Drosophila*, let alone more broadly. Finally, using whole body samples likely obscures natural variation that manifests in organs, tissues or cell types—variation that might ultimately drive phenotypic variation (Chintapalli, et al. 2013).

The linear metabolome-wide divergence pattern that we see suggests that the *Drosophila* metabolome is not broadly constrained by stabilizing selection. The linearity
we see contrasts with the plateau that some (Bedford and Hartl 2009; Ma, et al. 2018), but not all (Khaitovich, et al. 2004), have observed in transcriptome divergence among the Drosophila. There may be some difference in the nature of metabolome evolution compared to the transcriptome, and our results are similar to an analysis of metabolome divergence in primates (Bozek, et al. 2014). While the whole metabolome may lack evidence of selective constraint, we also consider if selection acts on subsets of the metabolome differently and/or with varying strength. Our observation of better fits for the OU model to the divergence of some modules is consistent with this idea. Selection that affects subsets of the metabolome is an expectation of metabolic adaptation and the evolution of biochemical pathways (Hartwell, et al. 1999; Flowers, et al. 2007; Wagner, et al. 2007; Wagner 2009).

Several theories predict that covarying or coevolving traits will tend to include components of functional modules, either in development, cellular interactions, or other biological processes (Cheverud 1984; Hartwell, et al. 1999; Wagner, et al. 2007; Wagner and Zhang 2011; Collet, et al. 2018). To our knowledge this is the first study to examine the co-evolution of the metabolome. Most studies of modularity in the evolution of endophenotypes have compared gene content, where the evolutionary persistence of homologs is an indication of evolutionary conservation (von Mering, et al. 2003; Snel and Huynen 2004; Li, et al. 2014). Comparative analyses of gene co-expression have also detected modular structure, either in the genes encoding interacting proteins, or among members of biological pathways (Martin and Fraser 2018; Cope, et al. 2020), and others find conservation of within-species gene co-expression across taxa (Stuart, et al. 2003; Oldham, et al. 2006). There is evidence that gene co-expression within Drosophila species can predict the axes of variation between Drosophila species (Innocenti and Chenoweth 2013). However, Martin and Fraser (2018) find no evidence that genes that covary across environmental or genetic backgrounds within species also covary over evolutionary time. These results indicate that such analyses are complementary ways to gain insight into patterns of endophenotypic covariation (Dunn, et al. 2018), and ultimately, both approaches may shed light on the functional interrelations among genes, their products, and organismal phenotypes. Similar to comparative analysis of gene co-
expression, our study sought to identify covariation in metabolite levels across the Drosophila to shed light on the nature of evolutionary change in this phylogeny.

**Effects of sex on metabolome profiles.** In addition to the modular nature of co-variation in metabolite levels, we also find two interesting patterns of sex-specific variation. These include sex differences in coevolutionary patterns among metabolites (Fig 1B), and the difference in metabolome sexual dimorphism between the Sophophora and Drosophila subgenera (Fig S7). Given that metabolites are the building blocks of downstream traits, the relative lack of dimorphism in these metabolomic principal components for the Sophophora subgenus might reflect a downstream trait or set of traits that are sexually monomorphic in this lineage but not the other. Further work will be needed to determine what that might be. There are several examples of evolved sexual dimorphism in organismal traits in the Drosophila (Kopp, et al. 2000; Luo, et al. 2019), and our results suggest that LC-MS techniques could provide mechanistic insights into such evolutionary change. In using principal components to identify dimorphic phenotypes, we are biased toward detecting the largest sources of latent variation and so we do not suggest that sexual dimorphism is not present in the metabolome of the Sophophora. We also note that in sampling whole flies for metabolomics analysis, at least for some metabolites we are likely detecting variation that reflects sex differences in reproductive structures of the abdomen. We took care to sample only virgins in our analysis to avoid the more profound effects of egg development in inseminated females; however, a future analysis of tissues without obvious sexual dimorphism would allow us to investigate sexual dimorphism in the metabolome while minimizing the influence of structural morphology.

**Age, lifespan and the metabolome.** The evolutionary forces shaping phenotypic variation across the lifespan are central to theories of aging (Medawar 1946; Williams 1957; Hamilton 1966). We find that the metabolite composition of coevolutionary modules differs by age (Fig S5), implying that metabolites that are within a coevolving module in the context of a young Drosophila might be a part of a separate coevolutionary module in older Drosophila. We emphasize that selection is most likely acting on the biological pathways, even on the activity of single enzymes, and not the level of individual
metabolites per se, so it is not surprising to see metabolites whose levels reflect different evolutionary patterns in flies at different ages. Interestingly, we see evidence of coevolution of lifespan and metabolic modules in the older metabolome of both sexes, while we fail to detect such association in the younger metabolome (Table 2).

We also find larger between-species divergence in the whole metabolome of old flies when compared to that of young flies (Fig 1C). This pattern mirrors the increase in metabolome divergence with age observed within each species seen in mammals (Ivanisevic, et al. 2016; Dansereau, et al. 2019), and is consistent with predictions from evolutionary theory that age-related genetic variation should increase with age (Charlesworth and Hughes 1996; Moorad and Promislow 2009).

Most of what we know about the molecular mechanisms of aging is derived from lab studies of inbred lines in single species. In recent years, comparative studies have begun to probe evidence for genes and metabolites associated with inter-specific variation in lifespan (Ma, et al. 2015; Ma, et al. 2018; Cui, et al. 2019; Kowalczyk, et al. 2020). Previous work across a broad mammalian phylogeny identified metabolites associated with lifespan, albeit among samples from dissimilar environments and with lifespans estimated in other studies (Ma, et al. 2015). Our work is the first to use metabolomic approaches to study correlates of lifespan in a common garden design, where animal rearing is done together in a controlled environment and samples are taken from the same population in which lifespan is measured.

Here we detect metabolites that coevolve with lifespan, suggesting the potential of metabolomics to identify longevity-regulating pathways that are conserved across species. A similar comparative analysis of lifespan and the transcriptome in 14 Drosophila species identified few individual genes of strong effect, but provided evidence that sets of genes with marginally significant coevolutionary association with lifespan might be enriched for a small number of biological pathways (Ma, et al. 2018). Ad hoc comparison of the lifespan-associated pathways identified by (Ma, et al. 2018) and those identified here finds little evidence of overlap, indicating either that the study designs were different enough to obscure commonality that might exist between the metabolome and transcriptome, or possibly that lifespan coevolves with sets of co-expressed genes and metabolites that are somehow involved in non-overlapping processes. Regardless, the
coevolution of metabolite modules and lifespan supports the theory that lifespan variation in diverse species is influenced at least in part by common biological pathways. Our results in no way exclude the possibility that species-specific mechanisms also shape the evolution of life history traits (Martin, et al. 1996; Partridge and Gems 2002).

We see clues to conserved mechanisms of lifespan regulation in the relative enrichment of KEGG pathways (Table S5). With the caveat that FDR correction indicates that none of the pathways are enriched, in the metabolome of older females, the pathway dme04213, annotated as a ‘longevity regulating pathway – multiple species’ reaches an empirical P value of 0.0093 (FDR=0.911) and includes insulin-like signaling, mTOR signaling, and superoxide dismutase expression (Table S5). In addition to the modular analysis, lifespan associates with oxaloacetate in the older female metabolome. There is no direct connection between oxaloacetate and lifespan in Drosophila that we are aware of. However, oxaloacetate is part of the TCA cycle which has previously been implicated in lifespan variation in Drosophila melanogaster (Talbert, et al. 2015; Jin, et al. 2020). Interestingly, oxaloacetate supplementation extends the lifespan of Caenorhabditis elegans (Williams, et al. 2009). The association of lifespan variation and oxaloacetate levels, and a module that enriches known lifespan regulating pathways in various species, suggest that lifespan evolution may have common underlying mechanisms that are reflected in the metabolome.

Conclusion

Overall, we have shown that evolution in Drosophila cooccurs with interconnected effects on the metabolome. We find both broadly conserved effects of sex and age, as well as dynamic and phylogenetically independent correlated evolutionary change. The modular nature of coevolution among the metabolites measured here points not only to the individual metabolites, but also the broader biological processes, that underlie evolution of organismal phenotypes.

The metabolome describes a system of the basic structural and functional building blocks of all organisms on the planet. In this light, it is surprising how little about the patterns and process that underlie its evolution over billions of years. The work we present
here, while focused on questions related to the evolution of aging, is also an effort to stimulate a much deeper and broader exploration of metabolome evolution.

**Methods**

**Species and Strains**

This study included eleven species of *Drosophila*: *D. melanogaster*, *D. simulans*, *D. sechellia*, *D. ananassae*, *D. erecta*, *D. yakuba*, *D. willistoni*, *D. pseudoobscura*, *D. persimilis*, *D. mojavensis*, and *D. virilis*. We obtained all species from the Drosophila Species Stock Center then at the University of California San Diego, except for *D. mojavensis* lines, which were provided as a gift from Luciano Matzkin (University of Arizona). We obtained three wild type lines from each species with the exception of *D. yakuba* and *D. erecta*, for which we obtained one wild type strain (Table S7). This design enabled us to study intra- and inter-species variation in metabolite levels and lifespan.

**Media and Fly Culture**

To limit the effect of diet on metabolome profiles all lines were reared on the same diet, banana medium, consisting of 14% peeled banana, 4% molasses, 4.75% corn syrup, 2.75% brewer's yeast, 1% methylparaben and 3% ethanol, solidified in 1.4% agar. For survival assays and metabolomics, flies were placed on banana medium in bottles and allowed to mate and lay eggs for 48 hours at 24°C and 50 to 60%RH, after which adults were removed, and offspring were allowed to develop. We collected virgin males and females into vials with banana medium under light CO₂ anesthesia within 8 hours of eclosion, except *D. virilis*, which was collected within 12 hours of eclosion.

**Metabolomics Assay and Data Normalization**

**Targeted metabolomics:** For targeted metabolomic analysis, *Drosophila* were reared under the conditions described above, and three flies from each sex/strain combination at 5 and 31 days of age were flash frozen and stored at -80°C. One to three samples were collected per sex for each species at each age, for a total of 93 targeted LC-MS/MS samples. Metabolites were extracted by homogenizing samples in 200uL
HPLC water (Sigma) using a TissueLyser II (Qiagen) for 6 minutes at 25 Hz at 4°C. We then added 800uL methanol and incubated for 30 minutes on dry ice. The suspension was homogenized again for 10 minutes at 25Hz, then spun at 14,000rcf for 10 minutes at 4°C. The supernatant was transferred to a new tube and dried in a Speed-Vac at 30°C.

LC-MS/MS experiments were performed on an Agilent 1260 LC (Agilent Technologies, Santa Clara, CA)-AB Sciex QTrap 5500 mass spectrometer (AB Sciex, Toronto, ON, Canada) system at the University of Washington Northwest Metabolomics Research Center (UWNMRC). Each sample was injected twice, 10 µL for analysis using negative ionization mode and 2 µL for analysis using positive ionization mode. Both chromatographic separations were performed in hydrophilic interaction chromatography (HILIC) mode on two Waters XBridge BEH Amide columns (150 x 2.1 mm, 2.5 µm particle size, Waters Corporation, Milford, MA) connected in parallel. The flow rate was 0.300 mL/min, auto-sampler temperature was kept at 4 °C, and the column compartment was set at 40 °C. The mobile phase was composed of Solvents A (5 mM ammonium acetate in 90%H₂O/ 10% acetonitrile + 0.2% acetic acid) and B (5 mM ammonium acetate in 90%acetonitrile/ 10% H₂O + 0.2% acetic acid). After the initial 2 min isocratic elution of 90% B, the percentage of Solvent B decreased to 50% at t=5 min. The composition of Solvent B maintained at 50% for 4 min (t=9 min), and then the percentage of B gradually went back to 90%, to prepare for the next injection. Targeted data acquisition was performed in multiple-reaction-monitoring (MRM) mode. The LC-MS system was controlled by Analyst 1.5 software (AB Sciex). The extracted MRM peaks were integrated using MultiQuant 2.1 software (AB Sciex). Samples were spiked with 13C internal standards, and two types of LC-MS/MS quality control (QC) samples were run at 11 evenly-spaced intervals throughout the run to track potential drift in the assay. One QC sample was a pool of 10 fly samples, and the other was a sample of human serum. The CV for these QCs was 8.2% and 7.7% respectively. Detailed LC-MS methods and data are available on www.metabolomicsworkbench.org/ [study ID pending approval].

**Untargeted metabolomics:** Flies were raised as above and at five days old, 1-3 biological replicates of 10 flies from each strain and sex were flash frozen in 1.5 mL tubes
in liquid N\textsubscript{2} and stored at -80°C. This gave a total of 168 samples, with 3 replicates for 53 of the 58 strain and sex combinations. Metabolite extraction was identical to the procedure for targeted LC-MS-MS described above. Untargeted analysis was completed using an Agilent 1200 SL LC-6520 Quadrupole-Time of Flight (TOF) MS system (Agilent Technologies) at the UWNMRC. The separation conditions for the LC-TOF-MS experiments were the same as those for the LC-MS/MS described above. The ESI voltage was 3.8 kV, and the m/z scan range was 60-1000. The LC-TOF-MS data were extracted using Agilent MassHunter Qualitative Analysis (version B.07.00), Quantitative Analysis (version B.07.01), and Mass Profiler Professional (MPP, version B.13.00) software. The absolute intensity threshold for the LC-TOF-MS data extraction was 1000, and the mass accuracy limit was set to 10 ppm. Missingness was assigned to peaks below 4,500 counts per second. Detailed methods and data are available at www.metabolomicsworkbench.org/ [study ID pending approval].

All statistical analyses were conducted in R version 4.0.3 (R Core Team 2018) unless otherwise stated. The targeted and untargeted metabolome data were analyzed separately. All targeted metabolites and untargeted features were log\textsubscript{e}-normalized and the data from each sample were centered by subtracting the sample mean. For the untargeted LC-TOF-MS, the positive and negative mode data were combined, giving 4419 features. Only 362 features were detected in every strain (n=1 to 3 for each sex). In the 228 features with a single missing observation among all strains, missing values were imputed by 10-nearest-neighbor imputation, resulting in 590 complete features. Principal components analysis (PCA) was performed using prcomp on the sample-centered and scaled log-abundance of 590 untargeted metabolite features, and on the 97 targeted metabolites.

Drosophila Phylogeny

We use the topology, branch lengths, and estimated divergence time of the current consensus tree available at [http://www.timetree.org/](http://www.timetree.org/) (Kumar, et al. 2017). The Newick format is available in Supplementary Materials.
Phylogenetic Signal and Multivariate Clustering

To measure the phylogenetic signal in the metabolomic profile, we estimated Pagel’s $\lambda$ and Blomberg’s $K$, using the `phylosig` function within the `phytools` R package (Revell 2012). We used the strain-level data (n=1 to 3) to estimate the standard error of the species-level means and used these errors as a measure of intraspecies variance (Ives, et al. 2007; Revell 2012). For the two species with only a single strain, *D. erecta* and *D. yakuba*, we used the maximum standard error among the remaining species as the standard error. The significance of $K$ was determined by $10^5$ randomizations, and of $\lambda$ using the likelihood ratio test.

For hierarchical clustering of the metabolome, we separately analyze the sexes and, for the targeted data, both ages as well. In all cases, the species means (n=1 to 3) of each metabolite feature were used. We constructed trees using the complete linkage method of hierarchical clustering in R and evaluated node support by bootstrapping 1000 times. We also compare each tree to the genome-based phylogeny by the branch score method (Kuhner and Felsenstein 1994) with the `dist.topo` function of the `ape` R package, using relative branch lengths of the phylogenies which were calculated by dividing branch lengths of each tree by the respective total branch lengths (Kuhner and Felsenstein 1994). We used a permutation approach to test the significance of each comparison by calculating the branch scores of $10^5$ randomized phylogenies, made using the `rtree` function of the `ape` package, in comparison to the real phylogeny.

Modeling Metabolome Evolution

We modeled the divergence of the metabolome using a similar method used for divergence in the transcriptome (Bedford and Hartl 2009; Ma, et al. 2018), by measuring the metabolome distance ($y$) as the squared difference between each log$_e$-metabolite among pairs of strains from the same age and sex. We first fit a linear model, analogous to the BM model, and to evaluate the effect of sex and age—young or old as a categorical variable—on the rate of metabolome-wide divergence:

$$y \sim a + \delta^2 x + \beta_{age} + \beta_{sex} + \beta_{x \times age} + \beta_{x \times sex} + \beta_{x \times age \times sex} + \varepsilon$$  (1)

where $y$ is the metabolome distance, $a$ is the intercept, $\delta^2$ is analogous to the force of mutation/drift in the BM model, $x$ is the evolutionary divergence time, $\varepsilon$ is the residual
error, and the separate $\beta$ values represent the main or interaction effects of each model term, which were tested for significance by ANOVA.

For model comparison, the following BM model was fit by maximum likelihood using the \texttt{nlreg} R package:

\[ y = a + \delta^2 x \]  

(2)

Where $\delta^2$, the slope, is analogous to the force of mutation/drift; and $a$ is the intercept. For the OU model we used the formula of Ma et al., (2018):

\[ y = \frac{\delta^2}{2a} (1 - e^{-2ax}) \]  

(3)

The selection ($\alpha$) and drift ($\delta^2$) parameters were estimated using maximum likelihood. Because the OU model is undefined where divergence time ($t$) is zero, the intraspecies variance, we excluded data from $t=0$ while fitting both BM and OU. The BM and OU models were compared using the Akaike information criterion (AIC).

**Metabolome Coevolution and Modularity**

The evolutionary relationship between metabolites was measured as the Pearson correlation among PICs for each metabolite pair. Within each sex and age group, PICs were estimated for each metabolite using the \texttt{pic.ortho} function in the \texttt{ape} package using multiple measurements per species (Felsenstein 2008). We compared the distribution of correlations between PICs to the distributions among 100 permutations of the species labels, which maintained the relationships between metabolites within each species, and effectively randomized the trait values across the phylogeny. We measured modularity by first defining networks of coevolving metabolites, where edges correspond to metabolite pairs whose PICs correlated at $r > 0.7$, and then identified modules using the \texttt{edge.betweenness.community} function in the \texttt{iGraph} package (Girvan and Newman 2002; Csardi and Nepusz 2006). Modularity was measured using the modularity function and was then compared to 1000 rewired networks with the same degree distribution, and empirical P values were calculated.

To identify coevolutionary modules, we used the \texttt{WGCNA} package to find co-varying PICs within each sex and age group (Langfelder and Horvath 2008). A topological overlap matrix for network construction was made using the \texttt{TOMsimilarity} function on a matrix of pairwise Pearson correlations between all metabolite PICs raised to the power
of 7. Modules were identified by the `cutreeDynamic` function using a deepSplit of 3 and a minimum module size of 8. The first principal component of the PICs of each module (eigenmodule) was used as module-level vector in regression models.

**Enrichment Analysis**

The 97 targeted metabolites measured here are distributed broadly over many metabolic pathways and thus we lack power to detect enrichment of the majority of individual pathways using hypergeometric testing. Instead, we used the network diffusion-based analysis in the FELLA package to evaluate enrichment within the *D. melanogaster* KEGG Release 97.0: 128 pathways, 165 modules, 749 enzymes, 5417 reactions and 3961 compounds (Kanehisa and Goto 2000; Picart-Armada, et al. 2018). To evaluate the significance of the enrichment we ran $10^5$ iterations where the significant metabolites were permuted over the full set of 97 metabolites. We used this analysis to evaluate both metabolites with effects of sex, age or their interaction, as well as the enrichment among metabolites of each coevolving module in each sex and age. Multiple comparisons within each node type (pathway or KEGG module) were corrected using the Benjamini-Hochberg FDR method (Benjamini and Hochberg 1995).

**Mixed Model Analysis**

To determine the effects of sex and age on individual metabolites, we used the MCMCglmm package to analyze a phylogenetic mixed model, with sex and age and their interaction as fixed effects, and random effects of species and strain (Hadfield 2010):

$$ \text{metabolite} \sim \beta_{\text{sex}} + \beta_{\text{age}} + \beta_{\text{sex} \times \text{age}} + \text{species} + \text{strain} + \Sigma^{-1} + \varepsilon $$

where $\beta$ are the fixed effects of sex and age and their interaction, and $\Sigma^{-1}$, the inverse of the phylogenetic correlation matrix, was calculated using the *Drosophila* phylogeny, after resolving the *D. virilis*, *D. mojavensis*, *D. willistoni* polytomy at the root by adding $1 \times 10^{-4}$ MY to all edges, which adds a trivial distance to the *D. willistoni* edge. Errors were assumed to be Gaussian, priors were set at $V=1$ and $v=0.02$, and $\geq 5 \times 10^5$ iterations were run to estimate posterior effects.
Lifespan Analysis

At the time of virgin collection (see Media and Fly Culture, above), approximately 20 flies of each sex/genotype were placed in individual vials (2 to 7 replicates, mean=4.8). Flies were transferred onto new food three times over a seven-day period before the start of the longevity assay. Deaths during this period were not recorded as they may simply be due to extrinsic mortality from handling. On the seventh day, flies were transferred into randomized vials and the vials were censused three times per week thereafter while transferring flies to fresh food vials. Data were recorded using the dLife software (Linford, et al. 2013), and recording continued until all flies in all vials were dead. There were no censorship events during the experiments, so mean lifespan for each strain was simply the arithmetic mean of age at death for all individuals in that strain.

Coevolution between lifespan and metabolites was evaluated by major axis regression forced through the origin in the smatr package, where PICs of lifespan were regressed either on PICs of individual metabolites, or on the eigenmodules of the coevolutionary modules identified previously (see Metabolome Coevolution and Modularity). The metabolome at the two ages was compared to the same mean lifespan for that strain and sex. Therefore, multiple comparisons were handled by controlling FDR for tests of all predictors at both ages; n=194 metabolites for the univariate analysis, and n=10-11 eigenmodules for multivariate analysis.

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**Supplemental Materials**

*Drosophila* phylogeny - Newick format for the phylogeny used in this study (source [http://www.timetree.org/](http://www.timetree.org/)), and also available on GitHub ([https://github.com/ben6uw/HarrisonBR_HoffmanJM_2021](https://github.com/ben6uw/HarrisonBR_HoffmanJM_2021)): fly.species.list.nwk: ((mojavensis:31.05320286,virilis:31.05320286)'14':19.04679714,(willistoni:50.10000000,(persimilis:3.37033600,pseudoobscura:3.37033600)'13':33.81424400,((sechellia:3.31811200,simulans:3.31811200)'11':2.55700891,melanogaster:5.87512091)'10':5.56597642,(erecta:8.18063000,yakuba:8.18063000)'19':3.26046733)'9':22.42252867,ananassae:33.86362600)'22':3.32095400)'8':12.91542000)'6':0.00000000);

**Data Availability**

The data underlying this article and the code used in the analysis are available on GitHub, at [https://github.com/ben6uw/HarrisonBR_HoffmanJM_2021](https://github.com/ben6uw/HarrisonBR_HoffmanJM_2021). The LC-MS data and detailed methods are available on: [ftp://www.metabolomicsworkbench.org](ftp://www.metabolomicsworkbench.org) [study ID pending approval].

**References:**

Bai Y, Casola C, Feschotte C, Betrán E. 2007. Comparative genomics reveals a constant rate of origination and convergent acquisition of functional retrogenes in Drosophila. Genome biology 8:R11–R11.

Ballard JWO. 2000. Comparative Genomics of Mitochondrial DNA in Members of the Drosophila melanogaster Subgroup. Journal of Molecular Evolution 51:48–63.

Bedford T, Hartl DL. 2009. Optimization of gene expression by natural selection. Proc Natl Acad Sci U S A 106:1133–1138.

Benjamini Y, Hochberg Y. 1995. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. Journal of the Royal Statistical Society. Series B (Methodological) 57:289–300.
Bleichman R, Perry GH, Shahbaz S, Fiehn O, Clark AG, Gilad Y. 2014. Comparative metabolomics in primates reveals the effects of diet and gene regulatory variation on metabolic divergence. Sci Rep 4:5809.

Bozek K, Khrameeva EE, Reznick J, Omerbasic D, Bennett NC, Lewin GR, Azpurua J, Gorbunova V, Seluanov A, Regnard P, et al. 2017. Lipidome determinants of maximal lifespan in mammals. Sci Rep 7:5.

Bozek K, Wei Y, Yan Z, Liu X, Xiong J, Sugimoto M, Tomita M, Paabo S, Pieszek R, Sherwood CC, et al. 2014. Exceptional evolutionary divergence of human muscle and brain metabolomes parallels human cognitive and physical uniqueness. PLoS Biol 12:e1001871.

Bozek K, Wei Y, Yan Z, Liu X, Xiong J, Sugimoto M, Tomita M, Paabo S, Sherwood CC, Hof PR, et al. 2015. Organization and evolution of brain lipidome revealed by large-scale analysis of human, chimpanzee, macaque, and mouse tissues. Neuron 85:695–702.

Brawand D, Soumillion M, Necsulea A, Julien P, Csardi G, Harrigan P, Weier M, Liechti A, Aximu-Petri A, Kircher M, et al. 2011. The evolution of gene expression levels in mammalian organs. Nature 478:343–7.

Carson HL, Kaneshiro KY. 1976. Drosophila of Hawaii: Systematics and ecological genetics. Annual Review of Ecology and Systematics 7:311–345.

Charlesworth B, Hughes KA. 1996. Age–specific inbreeding depression and components of genetic variance in relation to the evolution of senescence. Proceedings of the National Academy of Sciences of the United States of America 93:6140–6145.

Cheverud JM. 1984. Quantitative genetics and developmental constraints on evolution by selection. J Theor Biol 110:155–171.

Chintapalli VR, Al Bratty M, Korzekwa D, Watson DG, Dow JA. 2013. Mapping an atlas of tissue–specific Drosophila melanogaster metabolomes by high resolution mass spectrometry. PLoS One 8:e78066.

Clark AG, Eisen MB, Smith DR, Bergman CM, Oliver B, Markow TA, Kaufman TC, Kellis M, Gelbart W, Iyer VN, et al. 2007. Evolution of genes and genomes on the Drosophila phylogeny. Nature 450:203–218.

Collet JM, McGuigan K, Allen SL, Chenoweth SF, Blows MW. 2018. Mutational Pleiotropy and the Strength of Stabilizing Selection Within and Between Functional Modules of Gene Expression. Genetics 208:1601–1616.

Connallon T, Clark AG. 2014. Balancing selection in species with separate sexes: insights from Fisher’s geometric model. Genetics 197:991–1006.

Cope AL, O’Meara BC, Gilchrist MA. 2020. Gene expression of functionally–related genes coevolves across fungal species: detecting coevolution of gene expression using phylogenetic comparative methods. BMC Genomics 21:370.

Coyne JA, Orr HA. 1989. Patterns of Speciation in Drosophila. Evolution 43:362–381.
Cressler CE, Butler MA, King AA. 2015. Detecting Adaptive Evolution in Phylogenetic Comparative Analysis Using the Ornstein–Uhlenbeck Model. Syst Biol 64: 953–968.

Csardi G, Nepusz T. 2006. The igraph software package for complex network research. InterJournal Complex Systems:1695.

Cui R, Medeiros T, Willemsen D, Iasi LNM, Collier GE, Graef M, Reichard M, Valenzano DR. 2019. Relaxed Selection Limits Lifespan by Increasing Mutation Load. Cell 178: 385–399. e320.

Dansereau G, Wey TW, Legault V, Brunet MA, Kemnitz JW, Ferrucci L, Cohen AA. 2019. Conservation of physiological dysregulation signatures of aging across primates. Aging Cell 18. e12925.

Dobzhansky T. 1946. Genetics of natural populations; recombination and variability in populations of Drosophila pseudoobscura. Genetics 31: 269–290.

Dunn CW, Zapata F, Munro C, Siebert S, Hejnol A. 2018. Pairwise comparisons across species are problematic when analyzing functional genomic data. Proceedings of the National Academy of Sciences 115: E409.

Felsenstein J. 2008. Comparative methods with sampling error and within–species variation: contrasts revisited and revised. Am Nat 171: 713–725.

Flowers JM, Sezgin E, Kumagai S, Duvernell DD, Matzkin LM, Schmidt PS, Eanes WF. 2007. Adaptive evolution of metabolic pathways in Drosophila. Mol Biol Evol 24: 1347–1354.

Fraser HB, Hirsh AE, Wall DP, Eisen MB. 2004. Coevolution of gene expression among interacting proteins. Proceedings of the National Academy of Sciences 101: 9033.

Fu X, Giavalisco P, Liu X, Catchpole G, Fu N, Ning ZB, Guo S, Yan Z, Somel M, Paabo S, et al. 2011. Rapid metabolic evolution in human prefrontal cortex. Proc Natl Acad Sci U S A 108: 6181–6186.

Ge H, Liu Z, Church GM, Vidal M. 2001. Correlation between transcriptome and interactome mapping data from Saccharomyces cerevisiae. Nature Genetics 29: 482–486.

Girvan M, Newman MEJ. 2002. Community structure in social and biological networks. Proceedings of the National Academy of Sciences 99: 7821.

Gordon KL, Ruvinsky I. 2012. Tempo and mode in evolution of transcriptional regulation. PLoS Genet 8: e1002432.

Hadfield JD. 2010. MCMC Methods for Multi–Response Generalized Linear Mixed Models: The MCMCglmm R Package. Journal of Statistical Software 33: 1–22.

Hamilton WD. 1966. The moulding of senescence by natural selection. Journal of Theoretical Biology 12: 12–45.

Hartwell LH, Hopfield JJ, Leibler S, Murray AW. 1999. From molecular to modular cell biology. Nature 402: C47–C52.
Hoffman JM, Soltow QA, Li S, Sidik A, Jones DP, Promislow DE. 2014. Effects of age, sex, and genotype on high-sensitivity metabolomic profiles in the fruit fly, Drosophila melanogaster. Aging cell 13:596–604.

Innocenti P, Chenoweth SF. 2013. Interspecific divergence of transcription networks along lines of genetic variance in Drosophila: dimensionality, evolvability, and constraint. Mol Biol Evol 30:1358–1367.

Ivanisevic J, Stauch KL, Petrascheck M, Benton HP, Epstein AA, Fang M, Gorantla S, Tran M, Hoang L, Kurczy ME, et al. 2016. Metabolic drift in the aging brain. Aging (Albany NY) 8:1000–1020.

Ives AR, Midford PE, Garland T, Jr. 2007. Within-species variation and measurement error in phylogenetic comparative methods. Syst Biol 56:252–270.

Jin K, Wilson KA, Beck JN, Nelson CS, Brownridge GW, 3rd, Harrison BR, Djukovic D, Raftery D, Brem RB, Yu S, et al. 2020. Genetic and metabolomic architecture of variation in diet restriction mediated lifespan extension in Drosophila. PLoS Genet 16:e1008835.

Jones DP, Park Y, Ziegler TR. 2012. Nutritional metabolomics: progress in addressing complexity in diet and health. Annu Rev Nutr 32:183–202.

Kambysel MP, Heed WB. 1971. Studies of Oogenesis in Natural Populations of Drosophilidae. 1. Relation of Ovarian Development and Ecological Habitats of Hawaiian Species. American Naturalist 105:31–&.

Kanehisa M, Goto S. 2000. KEGG: kyoto encyclopedia of genes and genomes. Nucleic Acids Res 28:27–30.

Kellermann V, van Heerwaarden B, Sgro CM, Hoffmann AA. 2009. Fundamental evolutionary limits in ecological traits drive Drosophila species distributions. Science 325:1244–1246.

Khaitovich P, Lockstone HE, Wayland MT, Tsang TM, Jayatilaka SD, Guo AJ, Zhou J, Somel M, Harris LW, Holmes E, et al. 2008. Metabolic changes in schizophrenia and human brain evolution. Genome Biol 9:R124.

Khaitovich P, Weiss G, Lachmann M, Hellmann I, Enard W, Muetzel B, Wirkner U, Ansorge W, Paabo S. 2004. A neutral model of transcriptome evolution. PLoS Biol 2:E132.

Khrameeva E, Kurochkin I, Bozek G, Giavalisco P, Khaitovich P. 2018. Lipidome Evolution in Mammalian Tissues. Mol Biol Evol 35:1947–1957.

Klingenberg CP. 2014. Studying morphological integration and modularity at multiple levels: concepts and analysis. Philosophical transactions of the Royal Society of London. Series B, Biological sciences 369:20130249–20130249.

Kopp A, Duncan I, Godt D, Carroll SB. 2000. Genetic control and evolution of sexually dimorphic characters in Drosophila. Nature 408:553–559.
Kowalczyk A, Partha R, Clark NL, Chikina M. 2020. Pan-mammalian analysis of molecular constraints underlying extended lifespan. eLife 9: e51089.
Kuhner MK, Felsenstein J. 1994. A simulation comparison of phylogeny algorithms under equal and unequal evolutionary rates. Mol Biol Evol 11: 459–468.
Kumar S, Stecher G, Suleski M, Hedges SB. 2017. TimeTree: A Resource for Timelines, Timetrees, and Divergence Times. Mol Biol Evol 34: 1812–1819.
Langfelder P, Horvath S. 2008. WGCNA: an R package for weighted correlation network analysis. BMC Bioinformatics 9: 559.
Li Q, Bozek K, Xu C, Guo Y, Sun J, Paabo S, Sherwood CC, Hof PR, Ely JJ, Li Y, et al. 2017. Changes in Lipidome Composition during Brain Development in Humans, Chimpanzees, and Macaque Monkeys. Mol Biol Evol 34: 1155–1166.
Li Y, Calvo SE, Gutman R, Liu JS, Mootha VK. 2014. Expansion of biological pathways based on evolutionary inference. Cell 158: 213–225.
Linford NJ, Bilgir C, Ro J, Pletcher SD. 2013. Measurement of Lifespan in Drosophila melanogaster. Jove-Journal of Visualized Experiments.
Luo Y, Zhang Y, Farine JP, Ferveur JF, Ramírez S, Kopp A. 2019. Evolution of sexually dimorphic pheromone profiles coincides with increased number of male-specific chemosensory organs in Drosophila prolongata. Ecol Evol 9: 13608–13618.
Ma S, Avanesov AS, Porter E, Lee BC, Mariotti M, Zemskaya N, Guigo R, Moskalev AA, Gladyshev VN. 2018. Comparative transcriptomics across 14 Drosophila species reveals signatures of longevity. Aging Cell; e12740.
Ma S, Yim SH, Lee SG, Kim EB, Lee SR, Chang KT, Buffenstein R, Lewis KN, Park TJ, Miller RA, et al. 2015. Organization of the Mammalian Metabolome according to Organ Function, Lineage Specialization, and Longevity. Cell Metab 22: 332–343.
Markow TA, Fogleman JC, Heed WB. 1982. Reproductive isolation in Sonoran desert Drosophila. Evolution 37: 649–652.
Martin GM, Austad SN, Johnson TE. 1996. Genetic analysis of ageing: role of oxidative damage and environmental stresses. Nature Genetics 13: 25–34.
Martin T, Fraser HB. 2018. Comparative expression profiling reveals widespread coordinated evolution of gene expression across eukaryotes. Nature Communications 9: 4963.
Medawar PB. 1946. Old Age and Natural Death. Modern Quarterly 1: 30–56.
Moorad JA, Promislow DE. 2009. What can genetic variation tell us about the evolution of senescence? Proc Biol Sci 276: 2271–2278.
Noda-Garcia L, Liebermeister W, Tawfik DS. 2018. Metabolite-Enzyme Coevolution: From Single Enzymes to Metabolic Pathways and Networks. Annu Rev Biochem 87: 187–216.
Oldham MC, Horvath S, Geschwind DH. 2006. Conservation and evolution of gene coexpression networks in human and chimpanzee brains. Proceedings of the National Academy of Sciences 103:17973.

Park YH, Lee K, Soltow QA, Strobel FH, Brigham KL, Parker RE, Wilson ME, Sutliff RL, Mansfield KG, Wachtman LM, et al. 2012. High-performance metabolic profiling of plasma from seven mammalian species for simultaneous environmental chemical surveillance and bioeffect monitoring. Toxicol Lett 205:47–55.

Partridge L, Gems D. 2002. Mechanisms of aging: public or private? Nature Reviews Genetics 3:165–175.

Partridge L, Hoffmann A, Jones JS. 1987. Male Size and Mating Success in Drosophila Melanogaster and Drosophila Pseudoobscura under Field Conditions. Animal Behaviour 35:468–476.

Picart-Armada S, Fernández-Albert F, Vinaixa M, Yanes O, Perera-Lluna A. 2018. FELLA: an R package to enrich metabolomics data. BMC bioinformatics 19:538.

R Core Team. 2018. R: A Language and Environment for Statistical Computing. Vienna, Austria: R Foundation for Statistical Computing.

Revell LJ. 2012. phytools: an R package for phylogenetic comparative biology (and other things). Methods in Ecology and Evolution 3:217–223.

Revell LJ, Harmon LJ, Collar DC. 2008. Phylogenetic signal, evolutionary process, and rate. Syst Biol 57:591–601.

Ruzicka F, Hill MS, Pennell TM, Flis I, Ingleby FC, Mott R, Fowler K, Morrow EH, Reuter M. 2019. Genome-wide sexually antagonistic variants reveal long-standing constraints on sexual dimorphism in fruit flies. PLoS Biol 17:e3000244.

Schnebel EM, Grossfield J. 1983. A Comparison of Life-Span Characteristics in Drosophila. Experimental Gerontology 18:325–337.

Snel B, Huynen MA. 2004. Quantifying Modularity in the Evolution of Biomolecular Systems. Genome Research 14:391–397.

Spirin V, Gelfand MS, Mironov AA, Mirny LA. 2006. A metabolic network in the evolutionary context: Multiscale structure and modularity. Proceedings of the National Academy of Sciences 103:8774–8779.

Stark A, Lin MF, Kheradpour P, Pedersen JS, Parts L, Carlson JW, Crosby MA, Rasmussen MD, Roy S, Deoras AN, et al. 2007. Discovery of functional elements in 12 Drosophila genomes using evolutionary signatures. Nature 450:219–232.

Steuer R. 2006. Review: on the analysis and interpretation of correlations in metabolomic data. Brief Bioinform 7:151–158.
Stuart JM, Segal E, Koller D, Kim SK. 2003. A gene coexpression network for global discovery of conserved genetic modules. Science 302: 249–255.
Talbert ME, Barnett B, Hoff R, Amella M, Kuczynski K, Lavington E, Koury S, Brud E, Eanes WF. 2015. Genetic perturbation of key central metabolic genes extends lifespan in Drosophila and affects response to dietary restriction. Proceedings. Biological sciences 282: 20151646.
von Mering C, Zdobnov EM, Tsoka S, Ciccarelli FD, Pereira-Leal JB, Ouzounis CA, Bork P. 2003. Genome evolution reveals biochemical networks and functional modules. Proceedings of the National Academy of Sciences of the United States of America 100: 15428–15433.
Wagner A. 2009. Evolutionary constraints permeate large metabolic networks. BMC Evol Biol 9: 231.
Wagner GP, Pavlicev M, Cheverud JM. 2007. The road to modularity. Nat Rev Genet 8: 921–931.
Wagner GP, Zhang J. 2011. The pleiotropic structure of the genotype–phenotype map: the evolvability of complex organisms. Nat Rev Genet 12: 204–213.
Whitkus R, Doebley J, Lee M. 1992. Comparative genome mapping of Sorghum and maize. Genetics 132: 1119–1130.
Wilinski D, Winzeler J, Duren W, Persons JL, Holme KJ, Mosquera J, Khabiri M, Kinchen JM, Freddolino PL, Karnovsky A, et al. 2019. Rapid metabolic shifts occur during the transition between hunger and satiety in Drosophila melanogaster. Nature Communications 10: 4052.
Williams DS, Cash A, Hamadani L, Diemer T. 2009. Oxaloacetate supplementation increases lifespan in Caenorhabditis elegans through an AMPK/FOXO-dependent pathway. Aging cell 8: 765–768.
Williams GC. 1957. Pleiotropy, Natural Selection, and the Evolution of Senescence. Evolution 11: 398–411.
Yassin A, Bastide H, Chung H, Veuille M, David JR, Pool JE. 2016. Ancient balancing selection at tan underlies female colour dimorphism in Drosophila erecta. Nature Communications 7: 10400.
Figure 1 Evolution of the *Drosophila* Metabolome [color figure]
Figure 2 Modular Evolution of the Metabolome [color figure]
Figure 3 Metabolite Variation by Sex and Age [color figure]
Figure 4 Lifespan and Metabolite Coevolution [color figure]
Figure Legends

**Figure 1 Evolution of the *Drosophila* Metabolome** (A) Samples from each strain at each age (young 5-day, or old 31-day) and sex are plotted along principal components 1 and 2 of the mean-centered and scaled log-abundance of 97 targeted metabolites. Each sample is mapped by lines to the centroid for each sex and age group. The colored legend at bottom right refers to the sex and age groups in each figure. (B) The mean of PC1 (left) or PC2 (right) of each species is plotted along the phylogeny (Kumar et al., 2017). (C) The pairwise metabolome distance between all samples within each age and sex is plotted over the divergence time between species pairs. Intraspecies sample distances are plotted over time=0. The metabolome distance was calculated as the average squared difference of the log-metabolite abundance for the 97 targeted metabolites between all pairs of samples. The lines in (C) represent the fit to an ordinary least squares linear model: metabolome divergence ~ divergence time within each group and are equivalent to the Brownian motion model of trait evolution.

**Figure 2 Modular Evolution of the Metabolome** Heatmaps showing the pairwise absolute correlation between PICs for the 97 metabolites in each sex and age group (young 5-day, or old 31-day). The modules identified by WGCNA are shown on the left axis and partitions are included in the heatmap corresponding to these modules. Metabolites not fitting the criteria for modularity are in the ‘grey’ category. Hierarchical clustering was done in WGCNA (Methods) and is shown in Fig S4.

**Figure 3 Metabolite Variation by Sex and Age** (A) The effects ($\beta$) of sex, age or their interaction on metabolite levels in *Drosophila* estimated in a phylogenetic mixed model. For sex, metabolites that are more abundant in males have a positive $\beta$. The effects are clustered by metabolite (row). (A) The number of metabolites effected by sex, age or their interaction (n=97, FDR≤0.05).

**Figure 4 Lifespan and Metabolite Coevolution** Mean lifespans of females (A) and males (B) from 11 *Drosophila* species are mapped on the phylogeny and a color scale is
added based on estimated rates of continuous trait evolution. Means are from 16 to 378 individuals from each of 1 to 3 strains per species per sex. At each sex and age, PICs of mean lifespan for each species were regressed on PICs for 97 targeted metabolites using major axis regression forced through the origin. Plots of the regression for the three lowest \( P \) values from females (C) and males (D) of each age group, and the FDR of each association is show inside the plots. The regression data for all metabolites are shown in Table S6.

**Figure S1 The Metabolome Reflects Phylogeny**  (A) This consensus phylogeny is based on DNA sequence analysis among the 11 species in this study (Kumar et al., 2017). The phylogeny is estimated from the average pairwise distances of each species based on the mean level of 2367 or 2083 LC-MS features in 5-day female (B) or male (C) samples, respectively. The extent of bootstrap support at each node is shown as grey pie sections (n=1000).

**Figure S2 Brownian Evolution is a better fit to Metabolome-Wide Divergence than the Ornstein–Uhlenbeck model**  (A) Metabolome distance between all strain pairs of each group (5-day-old females and males, and 31-day-old females and males), was measured as the mean of the squared difference between log-metabolite levels and plotted over the divergence time estimated from the genome-based phylogeny. Maximum likelihood fits of the BM (solid line) and OU (dashed line) models are shown and the residuals of both models are plotted to the right of each group. (B) Model comparisons were made and the \( r^2 \) of each model and the \( \Delta AIC \) are shown. For \( \Delta AIC \), positive values indicate support for OU and negative values indicates support for BM.

**Figure S3 Metabolome Evolution is Highly Correlated**  (A) Here we compare density plots of the pairwise Pearson correlation coefficients (r) among the features in each group (bold line) to the distributions of correlations from 20 permutations of the species labels prior to PIC calculation and correlation (grey lines). Data in (A) are the 4656 pairwise combinations of the 97 targeted metabolites measured in each sex and age. (B) the mean
of the top 5% absolute r by group (colored points), compared to the means of the top 5% from 100 permutations (empirical $P < 0.01$).

**Figure S4 Coevolution in the Metabolome** Dendrograms depicting the hierarchical clustering of the pairwise distance (1 - topological overlap matrix) between PICs of each metabolite in each sex and age group. Modules identified by WCNGA are shown below each plot. Metabolites not fitting the module criteria are in the ‘grey’ module.

**Figure S5 Intersections in Module Membership for each Sex and Age Group.** Each heatmap shows the pairwise intersection between modules. The color range is associated with the proportion of intersection between modules (from a minimum of 0 to a maximum of 1, in the case of identical modules). Grey bars along the margins show the number of metabolites in the module of one group that are found among all modules of the other group. Note that metabolites in the non-modular class are not shown and so the marginal totals from each map are not necessarily equal. Modules are named A-F during the WGCNA process according to their size without regard to cross-group similarity. Asterisks indicate intersections that are significant by Fisher’s exact test ($P \leq 0.05$).

**Figure S6 Evolutionary Patterns by Module** (A) Examples from the 31-day female metabolome show instances where (A) the OU model (dashed line) was a better fit (module F), and (B) where the BM model (solid line) was a better fit (module B). The table shows the $r^2$ of the models, along with the $\Delta$AIC (negative values indicate a better fit of the BM model).

**Figure S7 Evolution of Sexual Dimorphism in the Metabolome** Projection of PC1 and PC2 of 589 LC-MS/MS features measured in each sex. Each point is the mean of 1 to 3 replicates per strain from females (red), and males (blue). Each panel indicates the position of the 1 to 3 strains of each sex in each species. When more than one strain is measured, those points are connected by red (female) or blue (male) lines, and grey dashed lines connect the mean PC1/PC2 projection of each sex within each species.
**Figure S8 Lifespans of Eleven *Drosophila* Species** Kaplan-Meier plots of survivorship for each strain, and sex by species. Female survival is shown in solid lines and male survival in dashed line, sample sizes averaged 95.25 flies +/-30 (SD) per sex and strain. A histogram of the lifespans of all individuals in the study (n=4953).
Tables

Table 1 Phylogenetic Signal in the Drosophila Metabolome Phylogenetic signal in the first two metabolome PCs at each sex and age (group) was measured, either as Pagel's λ or Blomberg's K, and tested by the likelihood ratio test, or by $1 \times 10^5$ simulations respectively. * $P<0.05$, ** $P<0.005$

| PC | group            | Pagel's λ | Blomberg's K |
|----|------------------|-----------|--------------|
| 1  | young female     | 0.890*    | 1.378**      |
| 1  | young male       | 0.754*    | 0.861*       |
| 1  | old female       | 0.586     | 0.586        |
| 1  | old male         | 0.313     | 0.529        |
| 2  | young female     | 1.062**   | 2.031**      |
| 2  | young male       | 0.977     | 1.123*       |
| 2  | old female       | 0.773     | 0.983        |
| 2  | old male         | 0.000     | 0.387
Table 2 Associations between Metabolite Coevolutionary Modules and PICs of Mean Lifespan

At each age and sex (group), major axis regression through the origin was used to test the association between PICs of mean lifespan and the eigenmodules of the metabolite PICs, as well as the remaining non-modular metabolite PICs (non-modular). The model r-squared ($r^{2}$) and $P$ value ($P$ value) are shown, and multiple comparisons were FDR corrected (FDR).

| group          | module | $r^{2}$ | $P$ value | FDR |
|----------------|--------|---------|-----------|-----|
| young female   | E      | 0.204   | 0.1637    | 1   |
| young female   | D      | 0.123   | 0.2902    | 1   |
| young female   | C      | 0.091   | 0.3678    | 1   |
| young female   | A      | 0       | 0.9962    | 1   |
| young female   | B      | 0       | 0.9502    | 1   |
| old female     | B      | 0.52    | 0.0186    | 0.2 |
| old female     | F      | 0.227   | 0.1641    | 0.9 |
| old female     | E      | 0.142   | 0.2824    | 1   |
| old female     | A      | 0.06    | 0.4944    | 1   |
| old female     | C      | 0.023   | 0.6751    | 1   |
| old female     | D      | 0.004   | 0.8706    | 1   |
| young male     | C      | 0.391   | 0.0395    | 0.47|
| young male     | B      | 0.184   | 0.1877    | 0.96|
| young male     | D      | 0.149   | 0.2405    | 0.96|
| young male     | A      | 0.075   | 0.4167    | 1   |
| young male     | E      | 0.022   | 0.6606    | 1   |
| young male     | F      | 0.015   | 0.7189    | 1   |
| old male       | D      | 0.798   | 0.0005    | 0.01|
| old male       | E      | 0.412   | 0.0454    | 0.27|
| old male       | C      | 0.177   | 0.2262    | 0.68|
| old male       | B      | 0.046   | 0.5528    | 1   |
| old male       | A      | 0.042   | 0.5707    | 1   |
| old male       | F      | 0.005   | 0.8444    | 1   |