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Modeling sex as a personalized biological variable using the *Drosophila* metabolome

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A dissertation

submitted in partial fulfillment of the

requirements for the degree of

Doctor of Philosophy

University of Washington

2024

Reading Committee:

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Program Authorized to Offer Degree:

Molecular Medicine and Mechanisms of Disease

University of Washington

Abstract

Modeling sex as a personalized biological variable using the *Drosophila* metabolome

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The research community now routinely considers "Sex as a Biological Variable" (SABV) in medical research and healthcare, because differences between genders/sexes can significantly impact disease prevalence, progression, and responses to treatment. Personalized medicine considers genetics and SABV, but a truly precise approach requires an understanding of how genetics and sex interact (GxS) to influence an individual's internal biochemistry. Currently, empirical research is lacking that assesses the degree to which genetic diversity contributes to variability in SABV. Animal models with robust genetic toolkits, such as *Drosophila melanogaster*, can be used to measure the prevalence of GxS interactions in a population, interactions that are difficult to detect in humans. This dissertation presents the results from two complementary projects measuring GxS interactions in the metabolome of *Drosophila*–a model for natural genetic variation and a model for monogenic, Mendelian genetic variation in sex characteristics (VSC). This work conceptualizes sex differences in metabolite levels as genotype-specific biological effects. The analyses presented here reveal that the effect of sex on the metabolome is far from binary or dichotomous, but rather falls along a continuum, with genotype playing a larger role than sex alone on most metabolite levels. I show that genetic variation in external, measurable dimorphic traits are associated with genetic variation in sex differences at a molecular level, which effectively renders biological sex a variable as unique and diverse as any individual genome. These studies underscore the importance of considering genetic context in research that incorporates SABV, and the potential pitfalls of analyzing sex as a fixed effect in statistical models.

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ACKNOWLEDGEMENTS

I must first, and extra appropriately here, thank my parents Gay and Cornelius Jr. for each gifting me the unique parts of themselves (genes *and* alleles) that gave me the abilities required to complete this dissertation. I couldn't have finished this work without the help of many, many people. Thank you, Kenneth, for your unceasing support and motivational words through the ups and downs of the last two years. You tethered me to sound reason when I most wanted to dramatically burn this dissertation in a dumpster fire. Thank you, Autumn, for being the best active listener and compassionate responder I've ever known. I am especially grateful for my siblings and friends who continued to text me periodically even though I've barely seen them over the past seven years. Thanks to my primary thesis mentors, Daniel Promislow and Whitney Harrington. Daniel, I will always be grateful for the many hours you've put into our discussions, the way you have reflected my excitement during the highs and for trusting me to take space and step back when I needed to. Not many PIs would support a thesis project like this. Whitney, our numerous early conversations were so helpful, a light during the most confusing times. I will never forget how you made space for me to present these ideas in your lab meetings even though it had nothing to do with immunology or microchimerism. Thanks to Ben Harrison for teaching me 99% of what I know about "pushing flies" and *Drosophila* crosses, to the other former graduate students of Promislow lab: Kelly Jin, Ashley Paynter and Jessica Hoffman, and former post-docs: Xiaqing Zhao, Ming Yang and Mitchell Lee; I learned so many practical skills from each of you that show up in this work. Thank you to Lily Hamlin for all your hard work in the last big stages of the DGRP project – seriously, Chapter 2 couldn't exist without you. And of course, thank you thank you to my committee members, M3D program advisors and the T32 and R21 funding that made this work possible. Finally, I will not forget you, Dr. Purrbot, and your dedicated commitment to cuddles and scratches over the past seventeen years. You have worked very hard to attain this PhD.

DEDICATION

This work is dedicated to all who are told they aren’t normal and need to be fixed.

Never forget, being unique is normal.

Chapter 1. Introduction

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Preface

Biological sex, while often categorized simplistically, is a complex trait influenced by numerous genetic and epigenetic factors that vary uniquely from person to person (Ober *et al*., 2008; Prum, 2023; Smiley *et al*., 2024). This genetic diversity contributes to context-specificity and variability in sex as a biological variable (SABV) (Pape *et al*., 2024; Richardson, 2022). Given the role of genetic variation in personalizing health experiences (Scott, 2011; Stenson *et al*., 2014), and the ways in which genetic variation can modify the effect of sex on downstream traits such that these effects fall along a continuum, it is critical to incorporate genetic information in studies of sex differences. Complex clinical outcomes are increasingly being understood through emerging systems biology approaches, such as metabolomics (Chan *et al*., 2010; Gieger *et al*., 2008; Jacob *et al*., 2019; C. H. Johnson *et al*., 2016; Suhre *et al*., 2011). Many human metabolomics studies stratify or adjust results by sex using a simple binary approach (Costanzo *et al*., 2022; Krumsiek *et al*., 2015; Lawton *et al*., 2008; Mittelstrass *et al*., 2011; Tabassum *et al*., 2023; Würtz *et al*., 2015). However, few studies consider that genetic variation in sex chromosomes and other sex-related molecular factors may make biological sex a variable unique to every individual. *Drosophila* and other animal models can help us better understand the degree to which genetic variants—be they mendelian or quantitative in nature— shape phenotypic sexual diversity.

The idea that genetic variation profoundly influences sex is not new. Over 100 years ago, pioneering *Drosophila* geneticist Calvin Bridges argued that the “modern unit” of sex inheritance was the gene, rather than sex chromosomes, lamenting:

“…the subject of sex has been rather separated off from the main body of heredity.”
(Bridges, 1922)

Prior to this, other fruit fly researchers had discovered X-linked inheritance (Morgan, 1910; Sturtevant, 1913), confirming the existence of chromosomes. The discovery of X-linked factors enabled Bridges’ work as well as the first documentations of tumors (Stark, 1919) and mutagenesis (Muller, 1927). These foundational *Drosophila* studies not only revolutionized our understanding of genetic inheritance but also laid the groundwork for modern genetics. By linking sex and genetic traits, early researchers set the stage for future explorations into genetic disorders, cancer biology, and mechanisms of DNA mutation and repair. Yet somehow, the connection that genetic variation and SABV are deeply integrated concepts has eluded contemporary biomedical research.

SABV could have a role in personalized medicine, but its place is yet to be determined. Empirical research is needed to establish a basic understanding of how genetics and sex interact in the bodies of model organisms, and context for the conservation of such interactions. Genotype-by-sex (GxS) interactions occur when the effect of sex on a trait varies depending on a gene variant, leading to sex differences in traits influenced by genetic variants. Despite evidence from both humans and model organisms, most studies assume sex as a fixed effect and do not empirically measure the prevalence of these interactions. In this dissertation, I use the *Drosophila* metabolome, one of countless traits that are influenced by SABV, to model personalized sex. I report the specific results of my analyses in Chapters 2 and 3, but this dissertation is not primarily about *Drosophila*, nor is it about metabolites. Instead, I use *Drosophila* and the metabolome here as tools to expand the traditional scrutiny of SABV. The central hypothesis of this work is that genetic variation leads to significant heterogeneity in sex differences across a population, such that the effect of sex for an individual is dependent on genetic background. By demonstrating this in the *Drosophila* metabolome, I present foundational evidence for the consideration of sex as a personalized variable.

SABV past, present and future

Sex is widely recognized as an important variable in biomedical research, as evidenced by the more than 20 major funding organizations with policies in place for integrating sex, gender, and diversity analysis (SG&DA) into research designs (Hunt *et al*., 2022). Proponents of these mandates claim they address historical biases, enhance scientific rigor and improve the overall value of biomedical science (Arnegard *et al*., 2020). However, the question of how to classify, measure, and report sex-related variables is a topic of active debate (Ritz & Greaves, 2024). Critiques of SABV policies in their current form are largely centered on two main themes—first, the oversimplification of sex as it is currently defined by most research organizations, and second, the neglect of requiring context-specificity in their implementation (DiMarco *et al*., 2022; Eliot & Richardson, 2016; Pape, 2021). In this dissertation, I present two studies that address both aspects. To address oversimplification, I use the metabolome, a measure of the small molecules in an organism that provides many continuous measurements of sex differences (SD). I address context-specificity through stratification by genetic background in Chapter 2, and both genotype and tissue type in Chapter 3.

To start, I provide a summary of key milestones in the modern understanding of sex differences in medicine, recent developments in expanding from binary approaches, and the challenges that remain in sex differences research.

Binary sex differences in medicine

The modern history of biomedical research that is focused on a simplified, binary and non-contextual model of sex starts in the 19th century, when the pathologization of intersex anatomy became popular in medical journals (Mak, 2013). A distinction between gender identity and biological sex became a focal point in the 1950's (Money *et al*., 1957), after which non-consensual surgeries became the recommended treatment for intersex infants and children to fit them into the binary anatomical model (Kessler, 1990). "Sex chromosomes" and "sex hormones" were discovered in the early twentieth century (Ha, 2011), providing the main binary causal factors cited today for biological differences between the sexes. However, in practical terms, "sex differences" were not well studied in humans until the 1990's, due in large part to social norms and policies excluding women from biomedical research, particularly in drug discovery (Merkatz & Junod, 1994). In response, a series of policies evolved over the next 40 years in the United States, Canada and the European Union to incorporate sex and/or gender into research designs (Marts & Keitt, 2004; White *et al*., 2021). In 2009, a compendium of at least 18,000 studies of sex differences was published with topics ranging from "Health and Illness Factors" to "Preferences, Interests, Attitudes, Beliefs, and Intentions" (Wolff & Puts, 2009), establishing seemingly irrefutable evidence that women and men were fundamentally different.

In response, challenges to the universality of these study results were made (Fausto-Sterling, 2010; Jordan-Young & Rumiati, 2012). The "gender similarities hypothesis", introduced by psychologist Janet Hyde, noted that most claims of differences between men and women were overinflated and based on small effect sizes (Hyde, 2005). Intersectionality, a framework originating within legal studies, emphasized that interactions between race and sex/gender lead to unique experiences for Black women that are more complex than a simple sum of Black experiences and female experiences (Crenshaw, 1991). Crenshaw's ideas were adopted by feminist scholars in other fields and have been foundational in contemporary understandings of context-dependency. Indeed, both Fausto-Sterling and Hyde argued early on that many sex/gender differences are age, population and context-specific.

The idea that both sex and gender are cultural, historical and social constructions (Fausto-Sterling, 2008, 2012; Hird, 2000) has also been key in the sex 'differences' debate. These constructions can shape the experimental design and interpretation of biomedical research

(Epstein, 2007; Fine, 2013), in some cases even leading to maladaptive public health policies (Zhao *et al*., 2023). In the past 10 years, visibility has greatly increased for transgender and non-binary individuals whose gender identities do not strictly align with their biological sex, further challenging the binary model (Coleman *et al*., 2022). Scholars have acknowledged the harms of social ignorance in biomedical research, including the over-pathologization of intersexuality and atypical sex/gender characteristics (Jones, 2018; Maldonado *et al*., 2024; Reis, 2007). Now the scientific community increasingly recognizes the need for research that accurately reflects the complexity of sex and gender, moving beyond simplistic binary models to better understand human biology and behavior (Hyde *et al*., 2019; Legato, 2023; Prum, 2023; Richardson, 2022).

Pioneering biomedical research challenging the binary sex model

Empirical research paradigms that expand on the “gender similarities” hypothesis have primarily been developed in the field of neuroscience and neuroendocrinology (Hyde *et al*., 2019), the most prominent being the “brain mosaic hypothesis” (Joel, 2021; Joel *et al*., 2015) and the “steroid/peptide theory of social bonds” (S/P theory) (Anders *et al*., 2011). Joel’s work challenged the binary framework using a multivariate approach, measuring sex differences in various brain regions to establish female-typical and male-typical traits, then evaluating an individual’s likelihood of being in the typical range for their sex. Results showed that it was most common for any individual to have a mosaic of female and male-typical brain traits (Joel *et al*., 2024), with recent studies corroborating these results (Zoubi *et al*., 2022). S/P theory builds on findings that social context, such as relationship status, is predictive of testosterone levels (Dibble *et al*., 2017). S/P theory challenges the concept of sex hormone levels as “fixed” aspects of sex difference, an idea that is strongly perpetuated by the social mythology that testosterone defines “maleness” (Fine, 2017; Jordan-Young & Karkazis, 2019). Findings from experiments in both theories provide evidence that human brain function is significantly individualized by interactions between neurobiological sex and sociocultural gender (Pavlova, 2017).

Novel statistical methodologies

The exploration of novel methodologies is critical in pushing forward sex research. Methodological considerations for studies incorporating SABV depend on the type of difference studied. McCarthy *et al*. (2012) classify sex differences into three categories: qualitative differences, such as sex-limited organs; quantitative differences, where traits differ in distribution but overlap between sexes; and latent differences, which emerge only in specific contexts. The main methods used to measure sex differences include Cohen’s *d*, Mahalanobis’ *D*, indices of overlap, variance ratios

and tail ratios (Giudice, 2022). Regardless of what is measured, there is an emerging consensus that shifting conceptually away from categorical sex and towards sex-related variables is warranted for clinically relevant interpretations (Pape *et al*., 2024).

Continuous measures of sex are an obvious place to start in challenging binary models. The first studies using a continuous metric for a sex-related variable can ultimately be traced back to the Kinsey scale, originally introduced to measure sexual orientation (Kinsey *et al*., 1948), which was followed by the Klein grid for sexual orientation (Klein *et al*., 1985; Weinrich, 2014). Building on this, continuous and multivariate measures of gender or gender difference are now more frequently utilized (Gülgöz *et al*., 2022; Lönnqvist & Ilmarinen, 2021). This practice is still uncommon in the biological sciences, however. In both studies presented here, I utilize continuous measures of SD to compare metabolome profiles. Yang & Rubin (2024) recently introduced a novel method for sex and gender-adapted precision cancer treatment planning by calculating transcriptomic index values from the sex similarities in 8370 transcriptomes across 30 cancer types, identifying sex and gender-skewed extremes in cancer mechanisms. Vosberg *et al*. (2021) developed continuous sex scores based on brain and body traits in adolescents, highlighting within-sex variability and revealing associations with sex hormones and personality traits. These studies demonstrate the utility of continuous metrics for identifying precise biological mechanisms.

<u>Future challenges in sex-related research</u>

Many challenges remain for the integration of SABV in the years to come. Technological advances, particularly in the field of artificial intelligence (AI) will be a major challenge. Algorithms used in some AI applications are potentially entrenched with biases related to sex/gender and race (Marinucci *et al*., 2023). Oversight will be needed to ensure that AI technologies do not inadvertently contribute to health disparities. Another challenging area involves the constant evolution of sex/gender terminology, which can lead to confusion and miscommunication. This can often be addressed by including marginalized groups in conversations about language preferences (Rioux *et al*., 2022). Integrating social scientists into biomedical research labs may help prevent biases from influencing experimental results (Velocci, 2024).

Most importantly, as I point out throughout this dissertation, genetic context must be considered in studies of SD. However, the integration of genetic information may be challenging for non-geneticists. Access to these data can be cumbersome, costly or even impossible to obtain. Additionally, GxS interactions can be complex to analyze, difficult to conceptualize, and different fields conceptualize these topics in different ways. To that end, in the next section of this

introduction, I will discuss some fundamental aspects of thinking in genetics that may perpetuate binary concepts of sex and how we might think about it differently.

Genes, sex, and genetic variation in sex-related traits

A common point of confusion in the subject of genetics is the difference between a gene and an allele. Pashley (1994) identified three common misconceptions: a) genes contain alleles; b) alleles contain genes; c) genes and alleles are the same. To clarify, alleles, also known as genetic variants, are versions of genes. This genetic variation is why individuals are unique, rather than clones of one another. Before we can examine the individualized function of an allele, we must first understand the generalized function of a gene. This gives us an idea of the operational range of that allele, which could vary from no function all the way to hyperfunction, or some alternative function, creating a continuum of effect sizes across a population (Mackay *et al*., 2009). This concept is the fundamental basis of the field of quantitative genetics. Some alleles act like a dimmer knob dialing a trait in by degrees, whereas other alleles in the same gene can turn a trait on and off like a light switch, the classical Mendelian effect.

In *Drosophila*, we have a very detailed understanding of the genes that regulate sex development (e.g., the canalization of primarily dichotomous "female" or "male" forms). I discuss the first of these to be identified, *transformer* (*tra*) (Sturtevant, 1945) and *doublesex* (*dsx*) (Hildreth, 1965), in Chapters 2 and 3. Loss of function (LOF) in either *tra* or *dsx* results in an intersex phenotype, with function-specific protein isoforms primarily transcribed through alternative splicing mechanisms (Nagoshi *et al*., 1988). At least 10 genes are known to interact to develop *Drosophila* sexual anatomies (Saccone, 2022), many of which also regulate the actions of numerous other genes (Cho & Wensink, 1997; Clough *et al*., 2014; Sakashita & Sakamoto, 1994).

Genetic variation can be categorized into two types: mutational variation and segregating "natural" variation (Charlesworth, 2012; Mackay, 2004). Mutational variation arises from new mutations, whereas segregating variation comes from the reshuffling of existing alleles during sexual reproduction. Mutational variation typically occurs at a low rate, whereas segregating variation is a constant process in sexually reproducing populations. Because LOF mutations in major sex development genes lead to intersexuality, which is often accompanied by a reduction in mating and reproduction, those mutations will under normal conditions be lost in a population and are thus rare. Importantly, when the focus of research is how genes get passed on, this categorization effectively assigns lower levels of importance to genetic variants that lead to

intersexuality (such as LOF mutations in *dsx* or *tra*), creating a “binary sex goggles” trap (i.e., the perception that intersexuality is “not natural”). While it is sometimes useful to make these distinctions for studies of population genetics and epidemiology, I argue that they are not useful for translational medicine. All variants were originally mutations, and for personalized medicine, the impact of a variant on quality of life is of greatest importance. A more useful distinction for context-dependent SABV may be between the study of genes that regulate sexual development, and the study of genetic variation in sex-related factors. These studies serve different purposes in advancing our understandings of biology.

Modeling personalized medicine

Here I argue that SABV is inherently personalized because sex cannot be separated from genetics. Personalized medicine, also known as precision medicine, aims to customize healthcare, with medical decisions, treatments, practices, or products being tailored to the individual patient (Scott, 2011). Traditional medical approaches often rely on the "one-size-fits-all" model, which can lead to suboptimal outcomes for many patients. When customization is based on an individual's unique genetic profile, the theorized benefit is targeted therapies with minimal side effects, and an improvement in overall health outcomes. Some argue that accounting for SABV in and of itself is “personalizing” medicine (Iribarren *et al*., 2022; Johnson *et al*., 2023). However, adding one additional dimension— sex— to create a “two-sizes-fit-all” model does nothing to account for individual genetic variation. Care must be taken to ensure that sex/gender stratified results are not taken out of context (Richardson, 2022). A more nuanced framework is needed to recognize that every human (and indeed, every living organism) has a sex as unique as the rest of their genome.

Others argue that the benefits of including SABV in animal models are limited for human health (Richardson *et al*., 2015). The FDA no longer requires the use of animal testing in drug discovery due to extremely low translational success rates (Mak *et al*., 2013; Moutinho, 2023; Wadman, 2023), and genetic differences across species contribute to these failures. The “Tree of Sex” is a comprehensive database cataloging sexual systems across 24,739 eukaryotic species (Tree of Sex Consortium, 2014). It serves as a reminder that variation in sex development is a rule, rather than an exception in biology. Indeed, speciation can be conceptualized as sex-related genetic incompatibilities arising from mutations. The use of SABV in this study is fundamentally different from the traditional animal model that attempts to “mimic” human biology in a non-human organism. Rather than extrapolate the effects of sex in *Drosophila* to humans,

the experiments presented here show that the effect of sex on *Drosophila* metabolism varies so greatly across genotypes that we must not attempt to directly translate any SD result to humans. These findings are of great benefit to human health and would not be possible without the use of an animal model.

In this dissertation, I describe continuous effects of sex on the metabolome using two complementary genetics approaches. In chapter 2, I take a quantitative genetics "dimmer knob" approach to identify novel variants of sexual size dimorphism (SSD), using the *Drosophila* Genetic Reference Panel (DGRP) (Mackay *et al*., 2012). In chapter 3, I use a Mendelian "light switch" approach to remove the function of *dsx* in XX and XY *Drosophila*. My goal with these projects is to inspire others to think critically about the interpretation and use of SABV in biomedical research.

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Chapter 2. Metabolomics demonstrate that genetic context is critical for analysis of sex differences

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Keywords: sex, genetics, GxS, *Drosophila*, metabolomics, SSD, DGRP

Abstract

Biological sex, traditionally understood in binary terms, encompasses a diverse array of morphological, physiological, molecular, and functional traits that shape biological diversity. Advances in genomics and systems biology suggest a more nuanced understanding of sex, emphasizing the importance of considering sex as a complex trait influenced by genetic and environmental factors, such that the effects of sex are best understood along a continuum. One emerging area in the study of sex differences is the metabolome, which reflects the dynamic interactions between an organism's genome and environment and varies significantly between sexes. We hypothesize that variation in the metabolome can capture the effect of genotype-by-sex (GxS) interactions, and potentially reveal their underlying mechanisms. Using the *Drosophila* Genetic Reference Panel (DGRP), which reflects the standing genetic variation found in a population derived from nature, we measured sexual size dimorphism (SSD) and head metabolome profiles in 29 genotypes. Our findings indicate that GxS interactions significantly influence metabolite levels, with genotype playing a larger role than sex alone. We identified TCA metabolites and pathways, including lysine degradation, that are significantly associated with variation in SSD. Genome-wide association results revealed a potential role for *Syncrip* (*Syp*) and *malate dehydrogenase-2* (*Mdh2*) as modulators of sex differences in metabolite levels. This study underscores the importance of considering genetic context to fully understand sex differences in metabolism and their impact on phenotypic diversity.

Introduction

The vast array of morphological, physiological, molecular, and functional traits associated with biological sex underscores its fundamental role in shaping biological diversity (Arbeitman *et al*., 2014; Bachtrog *et al*., 2014). Traditionally, "biological sex" has been understood in binary terms, defined by clear-cut distinctions between what have historically been termed ‘female’ and ‘male’ characteristics. However, advances in genomics and systems biology allow us to reevaluate this binary perspective and add context that opens new avenues of biological inquiry. Sex, while often categorized simplistically, is a complex trait influenced by a multitude of genetic, hormonal, and environmental factors (Pape *et al*., 2024; Smiley *et al*., 2024). This complexity manifests not only in the stark differences observed between the sexes but also in the subtler, often overlapping variation between these groups. Therefore, it is crucial to shift the focus from the binary concept of sex to the effects of sex, examining how sex influences variation in biological processes and phenotypes along a continuum.

One emerging area of systems biology is the metabolome, the complete set of small-molecule metabolites found within a biological sample. These metabolites, which include lipids, amino acids, nucleotides, and other small molecules provide a snapshot of the physiological state of an organism. The metabolome is influenced by both genetic and environmental factors, reflecting the dynamic interactions between an organism's genome and its environment (Fiehn, 2002; Johnson *et al*., 2016). Genetic variation can affect metabolic pathways by altering enzyme activities, receptor functions, and signaling pathways, leading to differences in metabolite levels (Julkunen *et al*., 2023; Karjalainen *et al*., 2024; Raamsdonk *et al*., 2001). Environmental factors, such as diet, lifestyle, and exposure to toxins, also play a significant role in shaping the metabolome (Hernandes *et al*., 2022; Liu *et al*., 2022; Wishart, 2016). The interplay between these genetic and environmental influences results in a highly individual metabolic profile, which can vary significantly between sexes (Buergel *et al*., 2022; Wishart *et al*., 2017). Sex differences are frequently observed in metabolite levels, yet remarkable heterogeneity in the strength and direction of the effect of sex have been observed across metabolome studies in humans (Costanzo *et al*., 2022; Darst *et al*., 2019). Given that sex is comprised of many variables (e.g., sex chromosomes, reproductive organs, etc.), it is imperative to determine which of these variables specifically shapes the metabolome.

While there are clearly main effects of sex in the metabolome, here we hypothesize that the more specific effects of sex vary from individual to individual because of genotype-by-sex (GxS) interactions. GxS interactions occur when the effect of sex on a trait differs depending on a gene variant. Importantly, it also means that genetic variation in a population can cause sex-specific differences to vary. For example, some gene variants might lead to greater differences between the sexes, while other variants might constrain these differences. Still others may change the directionality of difference. GxS interactions have been reported in humans (Bernabeu *et al*., 2021; Ober *et al*., 2008; Rawlik *et al*., 2016) and many model organisms (Huang *et al*., 2020), but few studies set out to empirically measure the prevalence of these interactions. Rather, most traditional studies of sex differences assume sex to be a fixed effect across all individuals in a population.

GxS interactions can manifest in several ways, including polygenic inheritance, epistasis, and dosage compensation. Gene networks that work in tandem to differentiate gonads (Bachtrog *et al*., 2014) are an example of polygenic inheritance, where multiple genes collectively contribute to a single trait. Gene variants in these networks lead to variation in sex characteristics (Reyes *et al*., 2023), including hormone levels. Sex chromosomes contain gene variants that can contribute to epistatic interactions, modifying the effects of one or more other genes (Jobling & Tyler-Smith,

2017; Sayres, 2018; Schaffner, 2004). X-linked genes can be upregulated or inactivated by dosage compensation mechanisms (Sidorenko *et al*., 2019), which may cause some metabolic pathways to be more influenced by sex depending on the genomic location of critical metabolic enzymes. The goal of this study is to measure the prevalence of GxS interactions in the metabolome and explore their connection to downstream phenotypes.

To understand how GxS interactions shape phenotype, we propose a novel framework and methodology linking a continuous sex difference in a trait— sexual size dimorphism (SSD) in *Drosophila melanogaster*, to its underlying biochemistry— the metabolome. Previously, we identified numerous GxS interactions in the *Drosophila* metabolome (Hoffman *et al*., 2014). We also measured significant genetic variation in the female fly metabolome for phenotypes as wide ranging as age and mortality (Zhao *et al*., 2022), peroxide resistance (Harrison *et al*., 2020), and the lifespan response to diet restriction (Jin *et al*., 2020). Here we expand on this work to ask whether we can identify genetic mechanisms underlying variation in SSD in the metabolome. The idea that the metabolome might reflect SSD is supported by work in *Drosophila* that two nutrient signaling pathways, insulin/insulin-like growth factor 1 signaling (IIS) and target of rapamycin (TOR), are considerably involved in shaping SSD (Shingleton & Vea, 2022). Context-dependency in SSD has also been observed, where inhibition of the IIS genes decreases body size more in females, and upregulation of IIS genes increases body size more in males (Millington *et al*., 2021). Moreover, the architecture of genes influencing SSD is complex. For instance, P-element insertions reducing SSD are more commonly observed on autosomal chromosomes. However, insertions affecting body size and those leading to increased SSD are observed similarly in both sexes similarly (Carreira *et al*., 2009).

Despite these insights, large gaps remain in our understanding of the genetic variability of these functional relationships. The *Drosophila* Genetic Reference Panel (DGRP), a tool created to investigate the genetic underpinnings of quantitative phenotypes (Mackay *et al*., 2012), can help address these gaps. Significant GxS interactions have been previously measured in the DGRP for triglyceride levels and mass (Mendez *et al*., 2016) as well as lifespan (Huang *et al*., 2020), sleep (Harbison *et al*., 2013), grooming behavior (Yanagawa *et al*., 2020), cold tolerance (Garcia *et al*., 2020), and the transcriptional response to ethanol exposure (Morozova *et al*., 2022). In this study, we measure SSD and sex differences in the metabolome of 29 *Drosophila* strains from the DGRP. The DGRP consists of inbred lines, meaning that individuals within each genotype are genetically identical except for their sex chromosomes. This high level of genetic uniformity ensures that each autosomal genotype provides a consistent basis for measuring sex-specific differences in body size and metabolite levels, and consequently, for the detection of GxS

interactions. By using the DGRP, we can assess the difference between female and male phenotypes in a common autosomal genetic background. This provides us with the power to understand the effect of GxS interactions in dimorphism that is not possible in a population (like humans) where we are not able to compare XX and XY individuals in a common genetic background.

This study aims to comprehensively assess the relationship between SSD and sex differences in metabolite levels by controlling environmental variables and utilizing a dual analysis of body triglycerides (TAG) and mass in *Drosophila*. While metabolites are the proximate regulators of body size, both metabolite levels and body size are ultimately regulated by genes when environment is controlled, as is the case here. We further limited environmental influence by sampling the metabolome and measuring body size from the same individual flies. We separated heads for metabolomics and measured TAG and mass in bodies. Body mass indicates overall size, encompassing both structural and soft tissues, while TAG represents stored energy in fats, linked to body size. Larger organisms typically have more storage capacity and higher TAG levels. By measuring both, we capture a comprehensive picture of body size, including physical dimensions and energy reserves. We chose to measure the head metabolome as it is closely associated with brain function and neural activity. This region allows for brain-specific links between metabolic changes and functional outcomes due to its direct impact on neural circuits and brain physiology. For example, some TCA cycle metabolites in the *Drosophila* head metabolome are dependent on the serotonin 2A receptor in response to nutrient choice (Lyu *et al*., 2021). The brain's central role in regulating metabolism (Myers & Olson, 2012) also means changes in the head metabolome can indicate broader systemic metabolic shifts such as between fasted and fed states (Wilinski *et al*., 2019).

Our analysis reveals that sex differences in both body size, and the head metabolome, are best described as following along a continuum shaped by the interaction between sex and genetic background. We show that GxS interactions significantly influence metabolite levels, with genotype playing a larger role than sex alone. We identify several metabolites, including oxaloacetate, cadaverine and N6-acetyl-lysine, and at least one pathway, lysine degradation, that are significantly associated with variation in SSD. We identify gene variants in *Syncrip* (*Syp*) and *malate dehydrogenase-2* (*Mdh2*) as candidate modulators of GxS interactions in the metabolome. The structure of inbred lines allows us to model these effects, using large numbers of individuals that are genetically identical at all autosomal genes, differing only in the sex chromosomes. While this is not possible in humans, this work points to the importance, and the challenge, of identifying these complex GxS background effects in real-world populations.

Methods

Drosophila Stock Maintenance

Lines from the *Drosophila* Genetic Reference Panel (DGRP) (Mackay *et al*., 2012) were obtained from the Bloomington *Drosophila* Stock Center. We selected genotypes based on a preliminary screen of the DGRP to ensure that we captured a wide range of sexual dimorphism for TAG levels. We maintained stocks in incubators at 25°C on a 12-hour light/dark cycle with approximately 50% humidity. All stocks were fed the same standard diet: cornmeal (60g, Quaker brand), glucose monohydrate (55g, MP Biomedicals), sucrose (30g, generic), yeast (25g, MP Biomedicals), agar (9g, type II, Genesee Scientific), 100% ethanol (15g, Decon Labs), methylparaben (3g, Genesee Scientific), and propionic acid (3g, Fisher Scientific) per 1.14 L of water. We expanded fly cultures in bottles for several generations to synchronize the timing of eclosion across all genotypes. One generation prior to the experiment, we transferred one- to three-day-old females and males from each genotype to fresh bottles supplemented with dry active yeast to encourage egg output. We allowed flies to mate for two days prior to egg-laying in vials for the experimental generation. For each genotype, we placed three to five mated females in each vial and observed vials periodically to monitor egg density. When egg density reached approximately 30-50 eggs per vial, we discarded maternal flies. Vials were randomized across trays and larvae left to develop undisturbed in the same incubator until eclosion.

We collected experimental flies within four hours of eclosion to prevent mating and separated flies under light CO_2 anesthesia into same-sex vials of 10 flies each, targeting a total of 90 flies per genotype and sex. To identify the sex of the *Drosophila* in our study, we used established methods for distinguishing between female and male flies using anatomical characteristics. However, we know that the flies have XX and XY chromosomes because their genomes are mapped and thoroughly characterized. Each genotype provides independent measures of the difference between the sexes, which can be interpreted as proxies for distinct individuals, each with a unique genome. Flies were allowed to recover from CO_2 exposure for four days and transferred to fresh media on the second day. At age four days, flies were transferred to 1.5mL Eppendorf tubes without anesthesia and immediately snap frozen in liquid nitrogen. We chose four days of age to ensure a complete transition of the larval fat body into its adult form (Aguila *et al*., 2007). We separated heads from bodies in a cold room by sequentially placing the sample tube in liquid nitrogen, vortexing the tube for 10-20 seconds and sifting the contents over two stainless steel mesh sieves with sizes of 710μm and 355μm, respectively (Hogentogler & Co). For every three fly samples collected, these were combined into a single metabolomics

replicate of approximately 20-30 fly heads, for a total of 141 metabolomics samples. Bodies were retained and stored at -80°C until phenotype assays were performed. This design allowed us to measure phenotype and metabolome profiles from pools of the same individuals.

Phenotypic Assays

Triglyceride Assay

Five bodies per sex per genotype were homogenized using a 3.2mm diameter stainless steel bead in 400 µl of 0.1% Tween 20 (Fisher Bioreagents, BP337-100) in 1X PBS, then shaken at 28/s for four minutes (Tissuelyser II) in a 96-well tissue block. The 96-well tissue block was then centrifuged at 3100 rpm for three minutes (Avanti J-15R) to precipitate cellular debris. For each sample, 100 µl of homogenate was transferred to a 96-well PCR plate and heat treated at 70°C for 10 minutes (Bio-Rad T100 Thermal Cycler). A standard curve was prepared by diluting TAG Standard (A Pointe Scientific) in PBST to give concentrations of 0 mg/ml, 0.03 mg/ml, 0.0625 mg/ml, 0.125 mg/ml, 0.25 mg/ml, 0.5 mg/ml, 1 mg/ml, and 2 mg/ml to ensure linearity of measurements. 20 µl of heat-treated homogenate was transferred to a flat-bottom 96-well plate. 150 µl of Infinity reagent (Thermo Scientific, TR22421) was added to all wells except negative controls. Negative controls received PBST in place. Plates were incubated at 37°C for 60 minutes and then absorbance (abs) was read at 540 nm (Thermo Scientific – Multiskan FC Microplate Photometer).

Mass

Masses were measured by weighing frozen fly bodies, without heads, in groups of five on an analytical balance scale (Mettler Toledo, XS105) in a cold room.

Liquid chromatography-mass spectrometry (LC-MS)

Sample Prep

Aqueous metabolites for targeted LC-MS profiling of 141 fly samples were extracted using a protein precipitation method similar to the one described elsewhere (Kurup *et al*., 2021; Mhatre *et al*., 2023). Samples were first homogenized in 200 µL purified deionized water at 4 ˚C, and then 800 µL of cold methanol containing 124 µM 6C13-glucose and 25.9 µM 2C13-glutamate was added (reference internal standards were added to the samples in order to monitor sample preparation). Afterwards samples were vortexed, stored for 30 min at -20 ˚C, sonicated in an ice bath for 10 min, centrifuged for 15 min at 18,000 x g and 4 ˚C, and then 600 µL of supernatant was collected from each sample. Lastly, recovered supernatants were dried on a SpeedVac and

reconstituted in 0.5 mL of LC-matching solvent containing 17.8 µM 2C13-tyrosine and 39.2 3C13-lactate (reference internal standards were added to the reconstituting solvent in order to monitor LC-MS performance). Samples were transferred into LC vials and placed in a temperature controlled autosampler for LC-MS analysis. Samples were randomized prior to LC-MS assay.

LC-MS Assay

Targeted LC-MS metabolite analysis was performed on a duplex-LC-MS system composed of two Shimadzu UPLC pumps, CTC Analytics PAL HTC-xt temperature-controlled auto-sampler and AB Sciex 6500+ Triple Quadrupole MS equipped with ESI ionization source (Mhatre *et al*., 2023). UPLC pumps were connected to the auto-sampler in parallel and were able to perform two chromatography separations independently from each other. Each sample was injected twice on two identical analytical columns (Waters XBridge BEH Amide XP) performing separations in hydrophilic interaction liquid chromatography (HILIC) mode. While one column was performing separation for MS data acquisition in ESI+ ionization mode, the other column was being equilibrated for sample injection, chromatography separation and MS data acquisition in ESI- mode. Each chromatography separation was 18 min (total analysis time per sample was 36 min). MS data acquisition was performed in multiple-reaction-monitoring (MRM) mode. The LC-MS system was controlled using AB Sciex Analyst 1.6.3 software. Measured MS peaks were integrated using AB Sciex MultiQuant 3.0.3 software. The LC-MS assay targeted 361 metabolites (plus 4 spiked reference internal standards). Up to 198 metabolites (plus 4 spiked standards) were measured across the study set, and over 95% of measured metabolites were measured across all the samples. In the addition to the study samples, two sets of quality control (QC) samples were used to monitor the assay performance as well as data reproducibility. One QC [QC(I)] was a pooled human serum sample used to monitor long term system performance and the other QC [QC(S)] consisted of pooled study samples and was used to monitor data reproducibility. Each QC sample was injected once per every 10 study samples. The data were highly reproducible with a median CV of 6.2 % over a 5-day period of non-stop data acquisition.

Quality Assurance and Data Normalization

We included metabolites that had complete measurements across all samples, with a final data set of 185 metabolites for each of 141 samples across 29 DGRP genotypes. Sample sizes ranged from 1-3 samples per sex per genotype (Supplementary Table 1). We performed several steps to ensure quality control in the metabolome data. Metabolome data were log-transformed to stabilize variance and linearize relationships between variables. We found that differences in the sample

preparation process between two technicians led to a difference in tissue content across head sample preps. This affected overall metabolite values such that smaller flies (typically males) of one technician had lower tissue content and thus lower overall metabolite signal across the entire data set. We thus median-centered the data grouped by technician and sex to equalize these differences in tissue content. We subsequently centered and scaled by sample so that each sample had a mean of 0 and a standard deviation of 1. Last, as we found significant batch effects by LC-MS run in the majority of metabolite levels, we corrected our data by LC-MS batch using a linear regression across all metabolites, retaining the model residuals as our final data set for analysis.

Statistical Methods

The statistical analyses for this study were performed using the R programming language (R Core Team, 2023), version 4.3.0. When multiple tests were conducted, we applied a stringent false discovery rate (FDR) correction to all p-values, setting the threshold for significance at 0.01. FDR correction was implemented using the p.adjust function in R, method = "fdr". Adjusted p-values are reported throughout the written manuscript unless otherwise noted. Principal component analysis (PCA) was performed on all samples using the prcomp function in R to observe how well the metabolome reflected GxS interactions in TAG and mass. After performing PCA, we applied the Tracy-Widom test for the significance of PCs, which identified PCs 1 through 24 as statistically informative. We thus limited further analyses to these PCs. Pathway analysis was conducted using the FELLA package in R. For GWAS analysis we used DGRPool, a web-based tool designed to compare and analyze DGRP data (Gardeux *et al*., 2023). DGRP gene variant calls were retrieved from the UCSC Genome Browser (Nassar *et al*., 2022).

Metrics of Sex Difference

The use of "**Δ**" signifies a sex difference, calculated as female - male throughout this manuscript. The subscript "G" signifies that the metric is a genotype-specific mean.

ΔPhenotype. All sex-specific summary statistics for TAG and mass are reported as means across replicates of flies (n=5 flies per replicate). A total of 320 TAG measurements were taken with 2 to 8 replicates per sex and genotype across all 29 genotypes. A total of 239 mass measurements were taken with 1 to 10 replicates per sex and genotype across 25 of the 29 genotypes. SSD metrics (**Δ**TAG and **Δ**Mass) are reported as differences between those means. For each genotype, we calculated a **Δ**Phenotype value:

$$\Delta\text{TAG}_G = \text{mean } \text{F}_G \text{ TAG} - \text{mean } \text{M}_G \text{ TAG} \quad (1)$$

$$\Delta\text{Mass}_G = \text{mean } \text{F}_G \text{ mass} - \text{mean } \text{M}_G \text{ mass} \quad (2)$$

ΔMetabolite Level. The normalization process resulted in a range of negative and positive values for metabolite levels. Thus, for analyses involving sex differences in metabolite levels, we first made all values positive by adding the minimum value to all metabolite levels such that the lowest metabolite level was 0. We then calculated mean normalized metabolite levels first by genotype before subtracting male from female values:

$$\Delta\text{ML}_G = \text{mean } \text{F}_G \ \log ML - \text{mean } \text{M}_G \ \log ML \quad (3)$$

ΔPC. To systematically evaluate the difference between females and males within each genotype across the multivariate space of the metabolome, we calculated this measure for each genotype separately, for each principal component (PC). Initially, we calculated the mean PC scores separately for females and males within each genotype group. This yielded two matrices of the genotype-specific means of females and males in the PC space. Subsequently, we computed the distance between these means for each principal component, to quantify global metabolome differences between sexes among genotypes:

$$\Delta\text{PC}_G = \sqrt{(\text{mean } \text{F}_G \text{ PC} - \text{mean } \text{M}_G \text{ PC})^2} \quad (4)$$

Analysis of Variance and Model Comparison

Akaike Information Criterion (AIC), (Akaike, 1974) was calculated using the AIC function in R for model comparison. To test the effect of genotype (G), sex (S), and their interaction (GxS) on metabolite level or PC (Y), we compare the following linear models:

$$\text{Y} = \mu + \text{S} + \text{G} + (\text{GxS}) + \varepsilon \quad (5)$$

$$\text{Y} = \mu + \text{S} + \text{G} + \varepsilon \quad (6)$$

$$\text{Y} = \mu + \text{S} + \varepsilon \quad (7)$$

$$\text{Y} = \mu + \text{G} + \varepsilon \quad (8)$$

For each metabolite and PC, the model with the lowest AIC value and a delta AIC >2 from the next most parsimonious model was identified and considered to be the best fit. The GxS interaction model (equation 5) was fit to all samples, then subjected to further examination through Type "III" Analysis of Variance (ANOVA) using the 'car' package in R. Regressions were weighted by the number of samples per genotype, as some genotypes were represented by more replicates than other genotypes. Type III ANOVA is designed to provide a more uniform approach by

evaluating each term in the model after accounting for all other terms, including interactions. We chose Type III ANOVA to avoid bias in our results from the choice of term order, as the choice of either "sex" or "genotype" as the first term in ANOVA using base R and other packages can influence the reported effect size and significance of each model term. We report eta squared (η^2) as our measure of effect size, representing the amount of variance explained by each of the model's terms, using the formula:

$$\eta^2 = \frac{SS_{between}}{SS_{total}} \quad (9)$$

Associations between Phenotypes and Metabolites

For all association tests between continuous variables, we employed Spearman's rank correlation coefficient (ρ).

Results

We present our results in four sections. First, we report our observations of genetic variation in *Drosophila* SSD, using measurements of **Δ**TAG and **Δ**Mass. Second, we test various statistical models to determine how well sex, genotype, and their interaction explain observed variation in the *Drosophila* head metabolome (multivariate analysis) and for single metabolites (univariate analysis). Next, we test the hypothesis that SSD is correlated with sex differences in head metabolism. In our final section, we report candidate metabolites and pathways for further study.

Genetic variation for sexual size dimorphism

In concordance with other studies (Vea *et al*., 2023), we found appreciable genetic variation for SSD in the DGRP. We measured both **Δ**TAG and **Δ**Mass at 4 days of age (Figure 1, Supplementary Figure 1). Of the 29 DGRP genotypes that we measured, the pooled standard error for TAG in four genotypes spanned the **Δ**TAG population mean of 0.21 mg/ml. Ten genotypes had **Δ**TAG greater than the population mean and 15 genotypes had **Δ**TAG less than the population mean. Mean **Δ**TAG absorbance ranged from 0.04 mg/ml in genotype 31 (mean F_G TAG = 0.52 mg/ml, mean M_G TAG = 0.48 mg/ml) to 0.45 mg/ml in line 535 (mean F_G TAG = 0.72 mg/ml, mean M_G TAG = 0.27 mg/ml). We also measured **Δ**Mass in 25 genotypes (Supplementary Figure 1), which ranged from 0.49 mg to 1.56 mg. We found a significant correlation between **Δ**Mass and **Δ**TAG (Supplementary Figure 2, ρ = .52, p = .008) across genotypes, as well as

between mean F_G TAG and mean M_G TAG (Figure 1A, $\rho = 0.76$, $p = 5 \times 10^{-6}$) and mass (Supplementary Figure 1A, $\rho = 0.93$, $p = 3 \times 10^{-11}$).

Standard deviation in the TAG phenotype was greater in females than in males ($s_F = 0.16$ mg, $s_M = 0.11$ mg), which was also true for mass ($s_F = 0.59$mg, $s_M = 0.36$mg). We then asked whether the relative contribution of female samples to the formation of the **Δ**TAG and **Δ**Mass phenotypes was also greater. We found a significant correlation between F_G mean TAG and **Δ**TAG (Figure 3C, $\rho = 0.75$, $p = 6 \times 10^{-6}$) that was not observed in the male samples (Figure 3C, $\rho = 0.19$, $p = 0.3$). Correlations between mean mass and **Δ**Mass were significant for both sexes (Figure S3C, $\rho_F = 0.83$, $p_F = 3 \times 10^{-7}$, $\rho_M = 0.6$, $p_M = 0.002$). Overall, we found that variation in female samples contributed more to the overall variation in SSD in both TAG and mass. However, the **Δ**Mass phenotype is more equitably contributed to by both sexes than the **Δ**TAG phenotype, suggesting that multiple biological factors contribute independently to SSD, and that those factors may vary independently by sex.

Effects of genotype are stronger than effects of sex in the head metabolome

We hypothesized that the influence of biological sex on the head metabolome is dependent on genotype, shaping the effects of sex into a metabolic continuum across the population. We measured a panel of targeted metabolites in heads of both sexes from our 29 DGRP lines, chosen to maximize the range of SSD within a genetically heterogeneous population. We summarized AIC results across different linear models including combinations of sex and genotype for each measured metabolite and across the top 24 principal components (PCs). We then reported relative effect sizes and significance of sex and genotype components using the best fit model for each metabolite and PC. By examining which models (e.g., including only sex, only genotype, both sex and genotype) had the lowest AIC values, we inferred the relative contributions of sex and genotype to the global metabolome. For example, in cases where we found more metabolites and PCs where a model including ‘genotype’ has a significantly lower AIC compared to models including ‘sex’, and the effect sizes for ‘genotype’ are stronger and more significant than those of ‘sex’, we accepted our hypothesis. Conversely, if we find that most metabolites and PCs are best fit by a model including only sex, we rejected our hypothesis.

Relative influence of sex and genotype in the metabolome

In our comparison of AIC values across metabolites and PCs (Table 1, Supplementary Table 2 and 3), we found that the 'genotype × sex' (GxS) model was the best fit for the largest number of metabolites (143, 77.3% of all metabolites), and 20 of 24 PCs. The 'genotype + sex' model was best for 23 metabolites (12.4%) and 2 PCs. Models that consider only one factor ('genotype' or 'sex') associated with fewer metabolites, 11 (5.9%) and 5 (2.7%) respectively, and fewer PCs (2 for 'genotype' and 0 for 'sex'). Very little of the metabolome is left unaffected by the influence of genetic variation or sex. Overall, we note that 95% of metabolites have sex effects – however of those metabolites, the sex effects of all but 34 (18.4%) are genotype dependent.

Effects of GxS interactions on individual metabolites

We identified 143 metabolites that were best fit by a GxS interaction model, indicating that the effects of sex in these metabolite levels vary across genotypes. We next analyzed the results of the interaction model across these metabolites to determine the degree of influence for sex as compared to genotype. Notably, for all metabolites we analyzed, we found a larger effect size ($\eta2$) for either 'genotype' or the GxS interaction term as compared to the main 'sex' term (Supplementary Table 4). The top 10 metabolites with the largest $\eta2$ for GxS (Table 2) included metabolites implicated in energy metabolism (NAD, ATP, ADP), glycolysis (glyceraldehyde) and tryptophan metabolism (tryptophan). For 41 metabolites, the $\eta2$ for GxS was greater than either 'sex' or 'genotype', and for 118 metabolites, $\eta2$ was greatest for 'genotype' by a large order of magnitude. The remaining 12 metabolites had effect sizes that were approximately the same for 'genotype' or the interaction term.

Effects of sex and genotype in multivariate space

Given that most metabolites in our dataset were best fit by the GxS interaction model, we expected to find similar interactions in multivariate space using PCA. Indeed, we found that the first 5 PCs, which cumulatively explain nearly half of the variance in our data (48.6%), were all best fit by the GxS model (Supplementary Table 3). Although PC1 (PVE = 21%) appeared to visually separate the sexes more strongly than other PCs (Figure 2A), the main effect of sex in PC1 was not significant ($p_{sex} = 0.2$), whereas genotype and interaction terms were significant ($p_{geno} = 0.02$, $p_{int} = 3 \times 10^{-4}$). PC1 genotype effect sizes were larger than that of sex as a main effect ($\eta2_{sex} = 0.01$, $\eta2_{geno} = 0.2$, $\eta2_{int} = 0.4$). Across PCs, the effect sizes for genotype far surpassed that of sex except in PC1 and PC3, where we found significant interactions.

This tally across metabolites and PCs suggests that while sex and genotype are highly interdependent, the larger underlying driver of variance in the *Drosophila* head metabolome is genetics. We found that sex differences in most metabolites are dependent on genetic background regardless of how this dependence was quantified (e.g., number of metabolites affected, effect size, significance, proportion of variance explained). This means that the variation seen between females and males in these metabolites are contingent on the specific genetic makeup of the flies. However, we did find a small subset of metabolites that were better fit by a model with 'sex' only (Table 3). This group of metabolites included D vitamins (ergocalciferol ($p = 2 \times 10^{-7}$) and cholecalciferol ($p = 1 \times 10^{-5}$), along with vitamin A (retinol, $p = 0.008$) and 8 acidic molecules. These metabolites were all significantly lower in female samples at an FDR < 0.01, suggesting a more dominant role for sex in vitamin metabolism. These results highlight the importance of measuring genotype both to uncover critical variations in effects of sex and to gain more confident assessments of dominant sex effects once genotype is accounted for.

Genetic structure for SSD is present in the metabolome

For each of our 29 genotypes, we identified independent measures of SSD. We then showed that global sex differences in the metabolome are dependent on genotype. We next wanted to better understand the relationship between SSD and the metabolome taking this genetic context into account. For each genotype, we calculated the PC distance between the sexes (**Δ**PC, see Methods) as an independent measure of overall sex difference in the metabolome. We found that **Δ**PC varied across genotypes and PCs (Figure 2B, Supplementary Table 5). **Δ**PC1 was largest for most genotypes (18 of 29), followed by **Δ**PC2 (7 of 29). However, four genotypes had their largest sex distance captured by **Δ**PC3.

We found that **Δ**PC1 and **Δ**TAG were significantly correlated (Figure 3A, $\rho = 0.7$, $p = 4 \times 10^{-4}$), indicating that top loadings in PC1 space might be reflections of a continuous, rather than categorical, separation of the sexes. We observed that the relationship between **Δ**PC1 and mean sex-specific TAG levels resembled the pattern we had observed in **Δ**TAG with female-biased relative effects (Figure 3B, $\rho_F = 0.5$, $p_F = 0.004$, $\rho_M = 0.08$, $p_M = 0.7$). **Δ**PC1 and **Δ**mass were also significantly correlated (Supplementary Figure 3, $\rho = 0.5$, $p = 0.02$). Of individual metabolites, cadaverine had the highest absolute loading for PC1 (Table 4) and was negatively correlated with both TAG ($\rho = -0.6$, $p = 4 \times 10^{-4}$) and mass ($\rho = -0.6$, $p = 1 \times 10^{-4}$). In our data, **Δ**cadaverine represents the relative levels of cadaverine between females and males across genotypes. Of sex differences across metabolites, **Δ**cadaverine was most strongly correlated with **Δ**PC1 (Figure 3D, $p = 3 \times 10^{-7}$) and **Δ**TAG ($p = 3 \times 10^{-5}$). We found that as **Δ**PC increased, female levels of

cadaverine decreased and male levels increased, resulting in a larger effect size between the sexes as **Δ**PC increased (Figure 3E), a pattern also observed with **Δ**cadaverine at increasing levels of **Δ**TAG. Interestingly, despite this strong relationship to **Δ**TAG, cadaverine was not significantly associated with TAG or mass within females (p_{TAG} = 0.1, p_{MASS} = 0.2) or males (p_{TAG} = 1.0, p_{MASS} = 0.8). The strong relationship between cadaverine levels and overall sex differences in the metabolome, despite the lack of direct association within each sex, suggests a potential regulatory or mediating role of cadaverine in sex-specific metabolic processes.

ΔTAG-associated metabolites are enriched in the lysine degradation pathway

In addition to cadaverine, 30 additional metabolites had a significant correlation between the sex difference in metabolite level and sex difference in TAG at a threshold FDR < 0.01 (Table 5). Of these, we found that sex differences in oxaloacetate and 1-methylnicotinamide (Table 6, "Group A") were nearly perfectly correlated with cadaverine across all genotypes. We found a second group of metabolites, consisting of G6P, G1P/F1P/F6P and erythrose-4-phosphate (Table 6, "Group B"), that were also highly correlated. Associations between **Δ**metabolite levels in Group A and B were not significant.

Pathway analysis of the 31 **Δ**TAG-associated metabolites revealed an enrichment for lysine degradation, tyrosine and porphyrin metabolism (Supplementary Table 6). Cadaverine and N6-acetyl-lysine, both top hits for their relationship to **Δ**TAG, are both direct products of lysine catabolism. Lysine itself had no relationship with TAG in females (p = 0.7) or males (p = 1.0), nor did **Δ**lysine have a relationship to **Δ**TAG (p = 0.4) in our dataset. We next investigated the relationship of **Δ**cadaverine and **Δ**N6-acetyl-lysine levels to one another and discovered that they were anti-correlated in their relationship to **Δ**TAG across all genotypes (Figure 3F), as well as to **Δ**TAG in both females and males (Figure 4A). This interaction between cadaverine and N6-acetyl-lysine levels across genotypes is such that as SSD increases across genotypes, the divergence of lysine catabolites between the sexes also increases. This means that as genotypes increase in their SSD, females have increasingly lower cadaverine levels in relation to their N6-acety-lysine levels, with a reverse relationship in males with increasingly higher cadaverine levels in relation to N6-acetyl-lysine. Figure 4B illustrates how this relationship is masked in males when looking only at TAG levels across genotypes, without accounting for the genotype-specific sex difference in TAG.

Natural genetic variation for *malate dehydrogenase-2* segregates high and low SSD flies

The interaction between lysine catabolism and sex, where sex differences in catabolite levels appear to dynamically switch depending on genetic background, led us to hypothesize that allele variants in one or more genes regulating lysine catabolism may be interacting with sex to modulate relative levels of cadaverine to N6-acetyl-lysine. Previously we identified novel gene variants influencing lifespan in the DGRP by conducting a genome wide association study (GWAS) on relative metabolite levels (Jin *et al.*, 2020). As **Δ**cadaverine was most strongly associated with **Δ**TAG, we used the DGRP genome sequence data to conduct a GWAS on **Δ**cadaverine levels. Interestingly, the top gene hit from these results (Table 7) is associated with a protein-coding single nucleotide polymorphism (SNP) in *malate dehydrogenase-2* (*Mdh2*), an autosomal gene encoding an enzyme in the TCA cycle that converts malate to oxaloacetate. As **Δ**cadaverine and **Δ**oxaloacetate are nearly identical in our data, we were not surprised to find a gene clearly implicated in regulating oxaloacetate levels. The most significant SNP from our GWAS was in an intron of *Syncrip* (*Syp*), which encodes an RNA-binding protein. Other top gene hits included *dopamine-1 like receptor 2* (*Dop1R2*, intron) and β amyloid protein precursor-like (*Appl*, intron), both of which have known functions in cellular signaling and neural processes.

We downloaded the DGRP variant calls for the top SNPs in *Syp*, *Mdh2* and *Dop1R2* to test whether they segregated high and low **Δ**TAG genotypes. For both *Syp* (Figure 4C) and *Mdh2*, the variant alleles were associated with higher **Δ**TAG ($p = 0.001$ and 0.01 respectively, unadjusted), whereas the reference allele was associated with higher **Δ**TAG in *Dop1R2* ($p = 0.01$ unadjusted). Upon further analysis, we found significant GxS interactions between *Syp* alleles and the levels of cadaverine ($p = 1.4 \times 10^{-5}$) and N6-acetyl-lysine ($p = 0.0008$). Notably, when accounting for *Syp* variants, the sex-specific relationships we had previously observed between TAG and metabolite levels were altered in N6-acetyl-lysine and oxaloacetate, an effect clearly visualized in Figure 4D. This highlights the critical importance of accounting for genetic variation to achieve accurate measures of difference between the sexes.

Discussion

In this work we demonstrate that genetic variation for the effect of sex on body size is directly observable through GxS interactions in the metabolome. While previous studies have measured the effects of sex and genotype on the metabolome in the DGRP (Hoffman *et al*., 2014; Zhou *et al*., 2019), our study expands on this literature to connect sex differences in body size to sex differences in metabolite levels. This relationship emphasizes the role of genetic variation in

modulating intermediate metabolic factors that can cause the sexes to differ in body size, but only if causal genetic variants are present. Using a novel sexual dimorphism screen in the DGRP, coupled with continuous measures of sex differences in the metabolome, we identified several candidate genes that may alter the degree of SSD in *Drosophila*, as a proof-of-concept for this approach. Further research is needed to validate our findings and to understand the extent to which GxS interactions in metabolites might act as the underlying causal mechanisms leading to variation in dimorphism of downstream phenotypes.

Our study found that over three-quarters of the metabolites measured in *Drosophila* heads showed significant genotype-by-sex (GxS) interactions, much higher than previous DGRP studies. Previous DGRP studies on the metabolome have shown strong and significant effects of sex, but these studies used whole-body tissue samples, potentially conflating the genetic effects of sex in the metabolome with signals for reproductive organ specificity (Hoffman *et al*., 2014; Zhou *et al*., 2019). Hoffman *et al*. (2014) found that fewer than 1% of metabolites showed such interactions across 15 genotypes, whereas Zhou *et al*. (2019) reported 38% (172 out of 453 metabolites). These differences might be due to the previous studies using whole flies, which included reproductive organs, and so increased the sex signal relative to genotype. There are vastly different tissue-specific metabolome signatures in *Drosophila* (Chintapalli *et al*., 2013). We focused here on the *Drosophila* head metabolome to avoid confounding effects of these gonadal tissues. Also, different genotypes were measured in prior studies, with only six overlapping between our study and Zhou *et al*., with different methods to select populations. As our genotypes were selected to maximize variation in SSD, this finding was not surprising. Principal component analysis (PCA) supported our univariate findings. The first five principal components, explaining nearly half the variance, were best explained by the GxS model. In multivariate space, genotype and interaction terms had larger effect sizes than sex, highlighting the dominant role of genetics. These results emphasize the importance of considering tissue and organ specificity to understand sex differences in metabolism.

We observed that increases in SSD across genotypes are largely the result of an increase in female body sizes, and less associated with changes in male body sizes. This finding is consistent with work measuring SSD in larvae across 196 DGRP genotypes (Vea *et al*., 2023), and earlier studies showing that female-specific weight loss during the larval period contributes significantly to adult SSD (Testa *et al*., 2013). A parsimonious hypothesis to explain this effect is that metabolic regulation in females is fundamentally different than that of males, either through a mechanism that upregulates growth of females, or suppresses growth of males, or both. This line of thinking is supported by empirical findings that X-linked genes *Myc* and *transformer*

influence body size in a female-biased manner (Mathews *et al*., 2017; Rideout *et al*., 2015). The role of *transformer* extends to regulating fat storage via interactions with neuronal Adipokinetic hormone (Akh)-producing cells (APCs) (Wat *et al*., 2021). However, we found that cadaverine, oxaloacetate and N6-acetyl-lysine levels in both females and males contributed to the continuous effect of sex on fat levels (as measured by **Δ**TAG) – a provocative finding. Stratifying by allele variants in the *Syncrip* and *Mdh2* genes underscored the way in which genetic variants contribute to the GxS effect on variance in sex differences of TAG.

Mdh2 has a well-documented role in metabolic regulation and some studies suggest that sex-related factors may interact with its function. *Mdh2* encodes a mitochondrial malate dehydrogenase that is a component of the malate-aspartate shuttle (MAS) crucial for ATP synthesis. *Mdh2* mutations lead to energy deficits and impaired cell death processes in *Drosophila* metamorphosis, resulting in reduced ATP levels and accumulation of late-stage citric acid cycle intermediates (Wang *et al*., 2010). Under high-sugar diets, *Mdh2* expression is down-regulated, leading to hyperglycemia and dyslipidemia in *Drosophila*, mimicking a human type 2 diabetes profile (Loreto *et al*., 2021). Beghelli *et al*. (2022) found sex-specific effects of pterostilbene on lifespan and oxidative stress responses, with *Mdh2* being upregulated in males but not females. The interplay between metabolic genes like *Mdh2* and signaling pathways such as ecdysone-triggered transcriptional cascades (Ihry & Bashirullah, 2014) indicates that hormonal regulation could mediate sex-specific metabolic differences. Disruptions in *Mdh2* could impair energy production, influencing metabolic intermediates like oxaloacetate and consequently affecting SSD.

Less is known about the potential role of *Syncrip* in regulating metabolism, but some evidence suggests that the RNA-binding protein might be involved indirectly. For instance, Chi *et al*. (2021) demonstrated that *Syncrip* plays a critical role in regulating behavioral responses to starvation through alternative splicing mechanisms. *Syncrip*'s regulatory functions begin during *Drosophila* neuronal development, where it controls the translation of key synaptic proteins, impacting synaptic plasticity and growth (McDermott *et al*., 2014). Loss of *Syncrip* disrupts proteome homeostasis, leading to increased protein synthesis, accumulation of misfolded proteins, and endoplasmic reticulum stress (Chavez *et al*., 2023). Differential expression or splicing of *Syncrip* may lead to sex-specific metabolic phenotypes indirectly through its role in neural regulation and signaling.

The broad regulatory roles of RNA-binding proteins (RBPs), including *Syncrip*, in connecting intermediary metabolism and gene expression are an emerging field of study. RBPs are known to interact with metabolic enzymes to form extensive regulatory networks (Castello *et*

al., 2015; Curtis & Jeffery, 2021). Given *Syncrip*'s role in maintaining proteostasis and regulating translation, it could potentially interact with metabolic enzymes such as *Mdh2*. More research is needed to characterize the potential relationships between *Syncrip*, *Mdh2* and genes already known to affect SSD such as *transformer*, discussed previously.

Overall, we found that sex differences in the *Drosophila* head metabolome are highly dependent on genetic background. The conceptual framework that we use here has implications of how we think about sex differences in human populations. Sex-related mechanisms vary greatly across species, including differences in dosage compensation and the copy number and allele composition of sex chromosomes (Furman *et al*., 2020). This, coupled with overall variation in genome organization, creates obvious challenges in translating results from studies of sex difference in model organism to human populations. For example, tryptophan 2,3-dioxygenase (TDO) is an enzyme that catalyzes the first step in tryptophan catabolism leading to downstream kynurenine pathway metabolites (Schober *et al*., 2023). The TDO gene is located on the X chromosome in *Drosophila*, the third chromosome in mice and the fourth chromosome in humans. Thus, one might hypothesize that sex differences in kynurenate levels for *Drosophila* may be more heavily influenced by X-chromosome dosage compensation than in mice or humans. Consequently, a finding that kynurenate levels are higher in one sex of *Drosophila* is unlikely to predict that kynurenate levels might be higher in the same sex in mice or humans. However, genetic variation, shaped by fundamental evolutionary processes, is a fundamental characteristic of all living organisms. In this light, it is reasonable to anticipate that the considerable role of genetic background on shaping the effect of sex on the metabolome is likely at work in other species as well, including humans. A key element in the design of our experiment, where we were able to compare XX and XY flies with identical autosomal backgrounds, and so ask how genotype influences the difference between them, cannot be replicated in human populations. Moreover, societal constructs and gender-segregated environments further affect our ability to measure biological sex differences in humans (Richardson, 2022). One idea for translating our study design is to utilize chromosome transplantation (Grua *et al*., 2024) in human cell lines, where effects of a gene variant could be compared within the context of a controlled genetic background between sex chromosome karyotypes.

<u>Limitations</u>

There are several limitations to the current study to keep in mind. First, our results revealed that TAG- and mass-associated metabolites exhibited stronger and more significant correlations across females compared to males. However, it is possible that allometry in the nature of the

phenotype could play a role in the strength of these associations, leading to Type II errors. Flies of smaller size tend to have lower TAG levels, but we have found that the assay that generates our TAG phenotype is noisier at lower absorbances. Thus, measurements may be less reliable in smaller body sizes, and in *D. melanogaster*, males are typically smaller than females. These issues underscore the complexity of interpreting metabolic data in relation to body size. Second, a significant limitation in our comparison of effect sizes across metabolites is the inherent variability in what constitutes a biologically meaningful difference across pathways. Statistically significant differences in metabolite levels do not necessarily indicate significant differences in physiological functions. Last, our pathway enrichment and GWAS results must also be interpreted cautiously, as our study was underpowered to detect significant associations in 29 lines. Typically, GWAS is conducted on the entire DGRP, using all 200 lines. Our results should be considered preliminary and set the stage for how we might go about asking similar questions in larger-scale studies. Further research is needed to validate candidate genes.

Conclusion

This study shows that effects of biological sex on the metabolome are complex, and not a simple binary. The effects of sex on phenotype will often fall along a continuum, shaped by genetic background. Both sex and genetic context matter when it comes to understanding the role of an organism's unique genome in shaping their internal biochemistry. Future studies considering sex as a biological variable must also consider how genetic variation may impact sex-related factors, which in turn, modulate sex differences. This has profound implications for considering the role of sex in precision medicine, such as determining when and how treatments or dietary recommendations could be tailored based on both genetic and sex-related factors. Animal models for natural genetic variation, such as the DGRP that we have used here, offer one strategy for unraveling these complexities. Further investigation into the role of *Syncrip*, *Mdh2* and relative levels of oxaloacetate and N6-acetyl-lysine on body size could provide deeper insights into the biological basis of SSD in *Drosophila*.

Tables

Table 1. AIC model comparison for sex and genotype terms across metabolites. Each tally represents the number of metabolites or Principal Components that best fit each model and was a significant improvement (delta AIC > 2) in explaining variation in those metabolites over the next most parsimonious model. N = 141 samples per metabolite or PC. Detailed AIC results can be found in Supplementary Table 2 and 3.

Best fitting model	# of Metabolites	# of PCs
genotype × sex	143 (77.3%)	20
genotype + sex	23 (12.4%)	2
sex	11 (5.9%)	0
genotype	5 (2.7%)	2
null	3 (1.6%)	0

Table 2. Top results for metabolites with strong interaction effects of genotype and sex. Top 10 metabolites with largest effect size for genotype × sex (GxS) interaction from Type III ANOVA interaction model. All metabolites FDR <.01 for GxS and GxS was the best fitting model. Detailed ANOVA results for all metabolites in Supplementary Table 4.

	Effect size (η2)			Adjusted p-value (FDR)		
	S	**G**	**GxS**	**S**	**G**	**GxS**
IMP	0.01	0.16	0.6	0.2	0.01	2.18E-11
AMP	0.05	0.12	0.59	2.18E-03	0.08	2.18E-11
GMP	0.02	0.17	0.56	0.04	4.85E-03	6.64E-11
NAD	0.02	0.24	0.53	0.03	8.62E-06	1.44E-11
ADP	0.01	0.23	0.51	0.1	3.68E-04	1.39E-09
5'-Methylthioadenosine	0.001	0.27	0.47	0.7	5.83E-05	1.41E-08
Glyceraldehyde	0.003	0.23	0.46	0.5	2.39E-03	3.22E-07
Tryptophan	0.01	0.33	0.44	0.1	1.58E-08	1.83E-10
Betaine	0.0003	0.19	0.41	0.9	0.1	1.38E-04
Uridine	0.001	0.27	0.41	0.7	6.28E-04	4.80E-06

Table 3. Metabolites with a stronger effect of sex than genotype. Type III ANOVA results for the 11 metabolites best fit by a model with sex alone. Normalized mean metabolite levels were lower in female samples as compared to male samples. Effect sizes are reported as $\eta 2$**.** Detailed ANOVA results for all metabolites in Supplementary Table 4.

	Female mean	**Male mean**	**($\eta 2$)**	**Adjusted p-value (FDR)**
3HBA	-0.07218	0.075316	0.23	1.09E-09
Adenylosuccinate	-0.04349	0.045376	0.18	9.86E-08
Ergocalciferol	-0.04844	0.050543	0.18	1.69E-07
2-Hydroxyphenylacetate	-0.08019	0.083677	0.15	3.27E-06
Hydrocinnamic Acid	-0.04813	0.050221	0.14	5.04E-06
Cholecalciferol	-0.07676	0.080099	0.13	1.43E-05
3-(4-Hydroxyphenyl)Propionate	-0.0882	0.09203	0.12	2.67E-05
D-Leucic Acid	-0.03673	0.038327	0.1	0.0003
Phenylacetic Acid	-0.09076	0.094705	0.07	0.002
Retinol	-0.06978	0.072819	0.05	0.008
Maleic Acid	-0.09626	0.100445	0.05	0.01

Table 4. Top Loadings for PC1. Top metabolites most contributing to variance in PC1.

Metabolite	Loading
Cadaverine	0.15
1-Methylnicotinamide	0.14
Oxalacetate	0.14
Urate	0.14
Choline	0.14
Betaine	0.14
Arginine	0.14
Palmitic Acid	0.14
PEP	-0.14

Table 5. Significant correlations between ΔMetabolite Level and ΔTAG. Metabolites with a significant FDR <.01 are highlighted in yellow, while metabolites with an FDR < .05 and >.01 are highlighted in gray. P-values are from Spearman's rank correlation test for genotype-specific female-male differences. Negatively correlated metabolites (left group) are metabolites where sex differences decrease when sex differences in TAG increase. Positively correlated metabolites (right group) are metabolites where sex differences increase as sex differences in TAG increase.

ΔMetabolite	ρ ΔTAG	p-value ΔTAG
Cadaverine	-0.7	3.21E-05
1-Methylnicotinamide	-0.7	7.24E-05
Oxaloacetate	-0.7	8.78E-05
Tyrosine	-0.6	2.19E-04
3-(4-Hydroxyphenyl)Propionate	-0.6	2.82E-04
FAD	-0.6	4.40E-04
Citraconic Acid	-0.6	9.06E-04
Arginine	-0.6	1.03E-03
Betaine	-0.6	1.92E-03
S-Methylcysteine	-0.5	3.57E-03
Palmitic Acid	-0.5	4.06E-03
Histamine	-0.5	4.69E-03
Taurine	-0.5	6.04E-03
Urate	-0.5	6.16E-03
Ergocalciferol	-0.5	7.45E-03

ΔMetabolite	ρ ΔTAG	p-value ΔTAG
Uridine	0.6	2.53E-04
SAH	0.6	3.24E-04
N6-Acetyl-Lysine	0.6	4.63E-04
N6-Trimethyllysine	0.6	4.69E-04
Cystine	0.6	1.16E-03
N-Ac-Arginine	0.6	1.24E-03
Succinylcarnitine	0.6	1.36E-03
Oxobutanoic Acid	0.6	1.68E-03
SAM	0.6	1.72E-03
6-Methyladenosine	0.6	2.32E-03
Aminolevulinate	0.5	3.86E-03
DCDP	0.5	4.51E-03
L-Kynurenine	0.5	4.60E-03
1/3-Methylhistidine	0.5	4.83E-03
Ethanolamine	0.5	6.50E-03
Homoarginine	0.5	6.50E-03

Table 6. ΔMetabolite sex difference correlation groups. Correlation coefficients and p-values are from Spearman's rank correlation test for ΔMetabolite Level, the measure of sex difference between each genotype. ΔG6P and Δoxaloacetate are not significantly correlated (ρ = -0.5).

Group	ΔMetabolite A	ΔMetabolite B	*ρ*	Adjusted p-value (FDR)
A	Cadaverine	Oxaloacetate	0.99	3.15E-22
A	Cadaverine	1-Methylnicotinamide	0.99	5.72E-18
A	Oxaloacetate	1-Methylnicotinamide	0.99	8.00E-19
B	G6P	G1P/F1P/F6P	0.99	4.71E-18
B	G6P	Erythrose-4-Phosphate	0.98	1.51E-18
B	G1P/F1P/F6P	Erythrose-4-Phosphate	0.98	5.88E-18

Table 7. Top GWAS gene hits for ΔCadaverine. GWAS results from DGRPool. Intergenic SNPs with no associated genes are not listed.

Chrom	SNP	p-value	FDR	Type	Gene	Gene name
3R	16595999	2.03E-07	0.4	Intron	*Syp*	*Syncrip*
3R	14053915	5.54E-07	0.5	Syn. Coding	*Mdh2*	*Malate dehydrogenase 2*
3R	25470323	3.30E-06	0.8	Intron	*Dop1R2*	*Dopamine-1 like receptor 2*
2R	17660172	5.60E-06	0.8	Upstream	*CG4021*	
3R	14119500	6.47E-06	0.8	Intron	*l_3_05822*	*lethal (3) 05822*
2L	7722775	8.36E-06	0.8	UTR 5 PRIME	*CG13794*	
X	441947	1.25E-05	0.9	Intron	*Appl*	*β amyloid protein precursor-like*

Figures

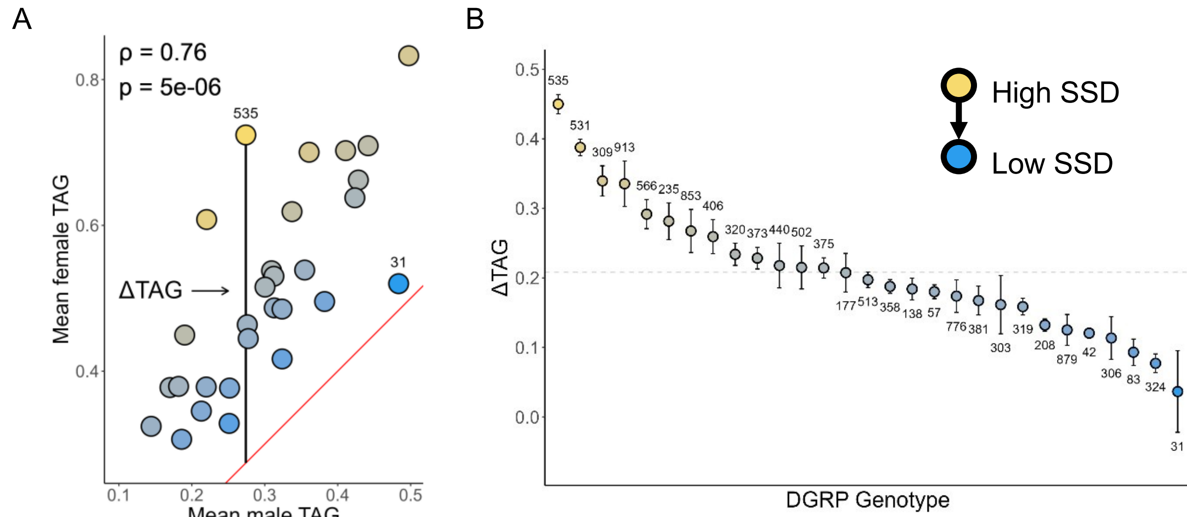


Figure 1. GxS interactions in total triglyceride levels across the DGRP. (A) Relationship between female and male measurements of total body triglycerides (absorbance per 5 flies, n = 320 measurements). Points represent mean TAG absorbance values for each genotype, colored by degree of dimorphism. Red line marks the line of unity between females and males. Black line indicates **Δ**TAG for line 535, the genotype with the largest sex difference in TAG. Line 31 is the genotype with the smallest sex difference. (B) Variation in **Δ**TAG across the 29 DGRP genotypes used in this study, plotted from high to low SSD. A value of 0 represents no difference between female and male mean TAG. Dotted gray line represents the **Δ**TAG population mean value. Error bars represent the pooled standard error between female and male samples. Coefficient and p-value is generated from Spearman's rank correlation test.

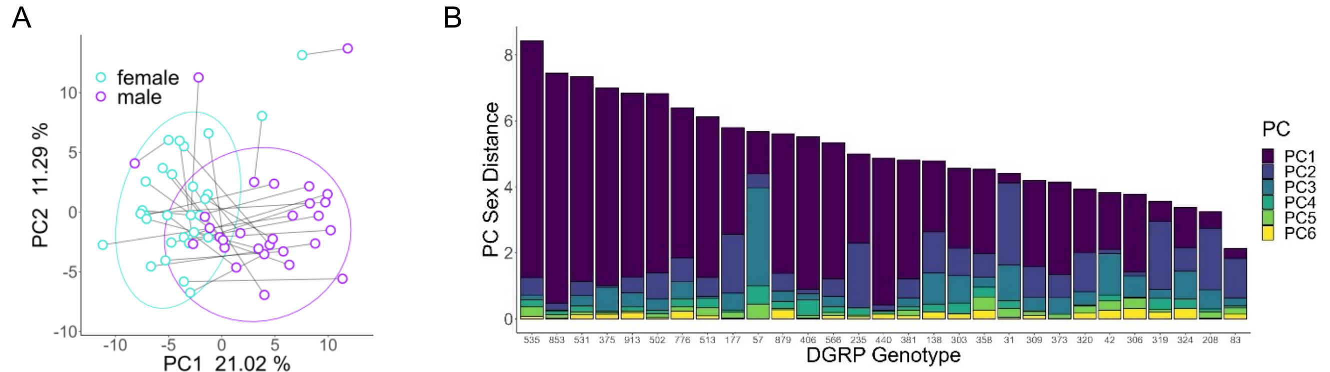


Figure 2. Sex variation in the *Drosophila* head metabolome. (A) PCA of all samples using metabolite profiles colored by sex. Points are mean PC scores for each sex/genotype combination. Gray lines connect the female and male means by genotype. Ellipses represent a confidence level of 90% for all samples grouped by sex. (B) Distances between female and male mean PC scores for the top 6 components across genotypes. Plotted PC distances are weighted by proportion variance explained for each PC and arranged from highest total distance to lowest.

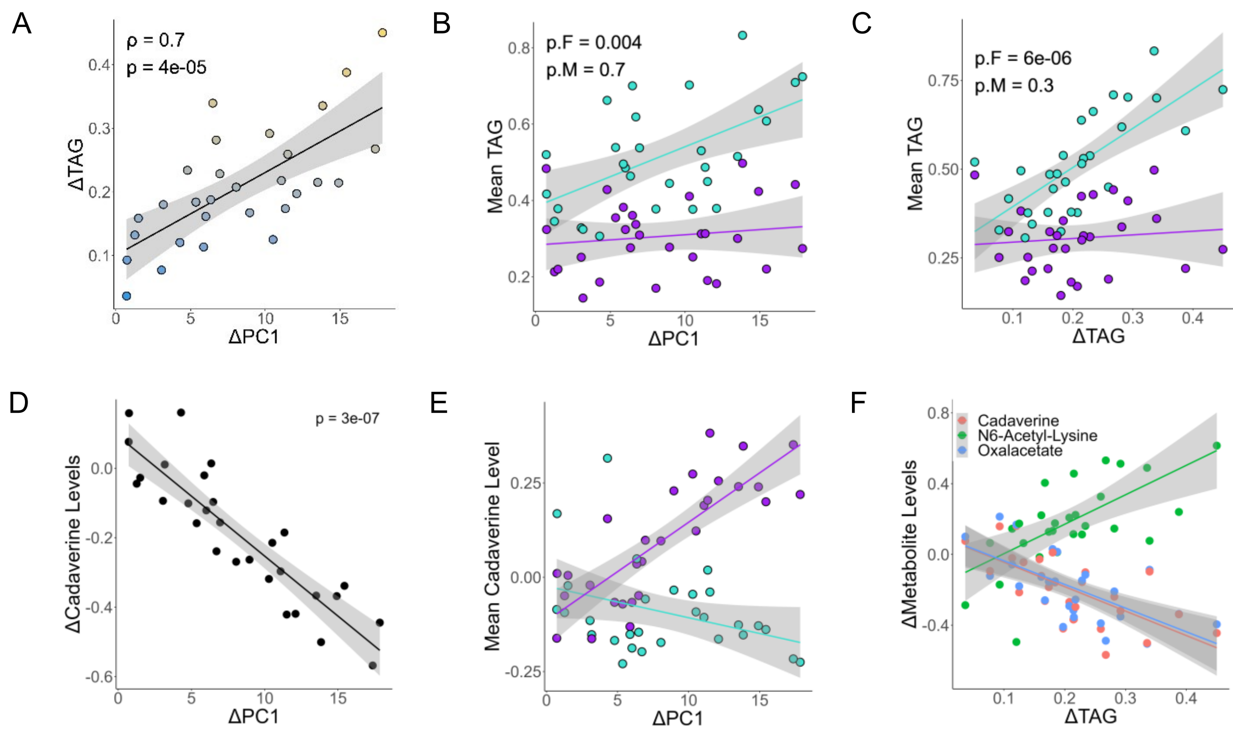


Figure 3. Genetic variation for sexual size dimorphism in the head metabolome.

(A) Correlation between **Δ**PC1 and **Δ**TAG. (B-C) Correlation between mean sex-specific TAG and **Δ**PC1. (B) and **Δ**TAG (ρ_F = 0.75, ρ_M = 0.19) (C). Each point represents a genotype-specific mean. (D) **Δ**Cadaverine is significantly associated with **Δ**PC1 ($p = 3 \times 10^{-7}$). (E) Male cadaverine levels increase with SSD in relation to female cadaverine levels. (F) **Δ**N6-acetyl-lysine is significantly anti-correlated with **Δ**cadaverine ($p = 9 \times 10^{-8}$) and **Δ**oxaloacetate ($p = 1 \times 10^{-8}$) in relation to **Δ**TAG. Female means are depicted in turquoise, male means in purple. Coefficients and p-values are generated from Spearman's rank correlation tests.

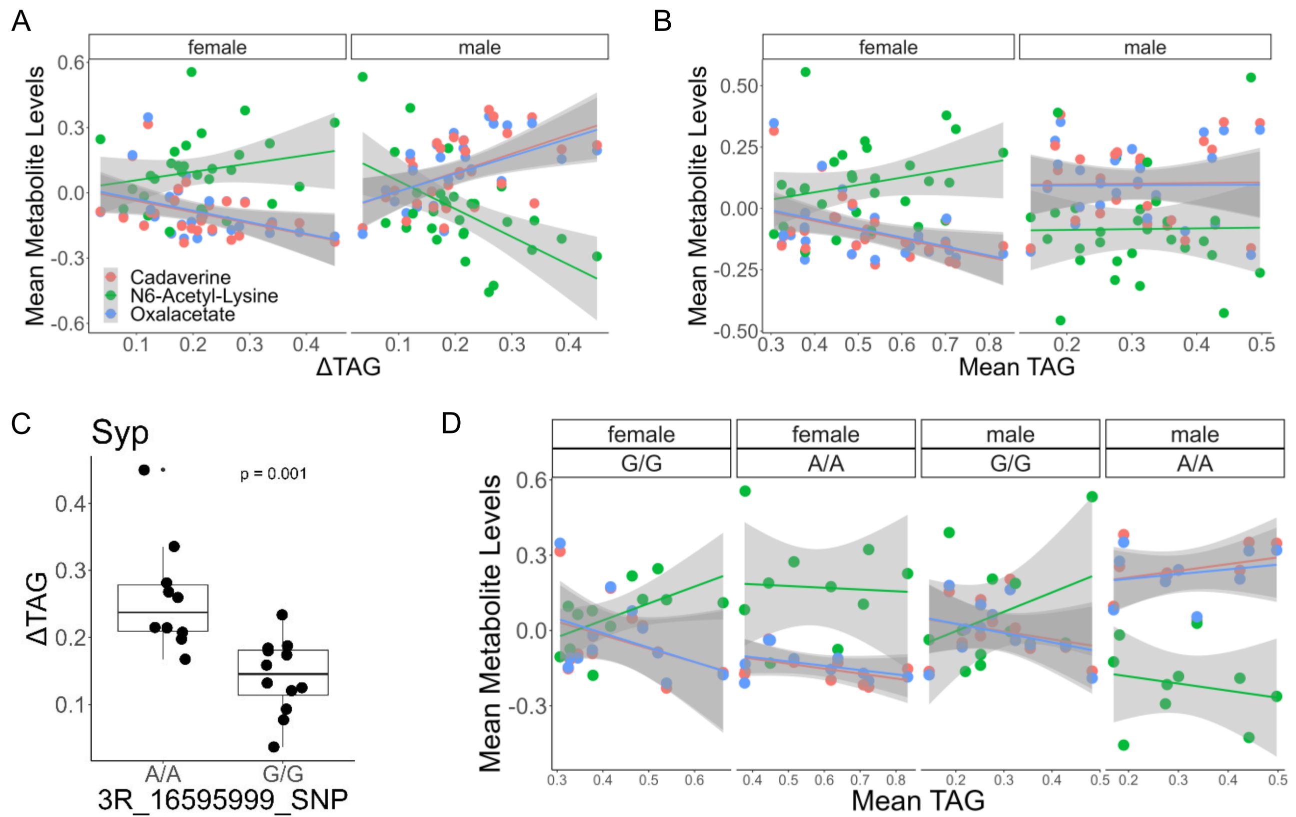


Figure 4. GxS interactions in *Syp* and ΔTAG-associated metabolites. (A-B) Sex-specific correlations between mean metabolite levels and ΔTAG (A) and TAG (B). (C) *Syp* SNP 3R_16595999 segregates high and low TAG flies (p = 0.001). (D) *Syp* alleles disrupt sex-specific relationships between metabolites and TAG. Pink dots represent mean cadaverine, green dots represent mean N6-acetyl-lysine and blue dots represent mean oxaloacetate.

Supplementary Tables

Supplementary Table 1. Head metabolome sample counts by DGRP genotype and sex.

Genotype	N samples	Female N	Male N
31	4	2	2
42	2	1	1
57	6	3	3
83	5	3	2
138	5	3	2
177	6	3	3
208	4	2	2
235	6	3	3
303	6	3	3
306	4	2	2
309	6	3	3
319	5	3	2
320	6	3	3
324	4	2	2
358	3	2	1
373	6	3	3
375	2	1	1
381	6	3	3
406	6	3	3
440	6	3	3
502	4	2	2
513	6	3	3
531	5	2	3
535	4	2	2
566	6	3	3
776	4	2	2
853	6	3	3
879	2	1	1
913	6	3	3

Supplementary Table 2. AIC analysis results by metabolite. The minimum AIC value is shaded in gray for each metabolite. Δ AIC values <2 highlighted in yellow.

	AIC					Δ AIC	Best fitting model
	null	S	G	G+S	GxS		
1-Methyladenosine	-225	-253	-261	-321	-352	31	GxS
1-Methylnicotinamide	-53	-101	-38	-106	-115	9	GxS
1/3-Methylhistidine	-145	-222	-125	-239	-292	52	GxS
2'-Deoxycytidine	-81	-79	-163	-161	-159	81	G
2-Aminoadipate	-50	-48	-232	-230	-254	24	GxS
2-Aminoisobutyric Acid	-302	-330	-338	-396	-412	16	GxS
2-Hydroxyglutarate	-106	-105	-146	-146	-158	12	GxS
2-Hydroxyisobutyrate	-135	-141	-155	-167	-154	11	G+S
2-Hydroxyphenylacetate	68	59	105	95	66	9	S
2-isoPropylmalic Acid	-108	-114	-152	-168	-177	9	GxS
3-(4-Hydroxyphenyl)Propionate	-74	-114	-55	-111	-92	40	S
3-Aminoisobutyrate	-301	-317	-336	-366	-392	26	GxS
3-Hydroxyisovaleric Acid	-25	-64	-2	-52	-78	25	GxS
3-Hydroxypropionic Acid	-89	-115	-120	-173	-246	73	GxS
3-Methyl-3-Hydroxyglutaric Acid	-235	-238	-280	-287	-295	8	GxS
3HBA	-72	-91	-63	-92	-76	1	S
4-Guanidinobutanoate	90	15	63	-121	-190	70	GxS
4-Hydroxybenzoic Acid	-62	-66	-102	-111	-126	15	GxS
4-Pyridoxic Acid	-56	-64	-102	-116	-138	22	GxS
5'-Methylthioadenosine	-207	-208	-233	-235	-326	91	GxS
5-Methylcytidine	-145	-143	-241	-240	-235	96	G
5,6-Dihydrouracil	-407	-406	-424	-425	-460	35	GxS
6-Methyladenosine	-82	-91	-90	-105	-115	10	GxS
7-Methylguanine	-77	-75	-266	-265	-270	6	GxS
Acetylcarnitine	-117	-142	-156	-213	-244	31	GxS
Acetylcholine	19	21	36	37	47		*null*
Adenine	-53	-61	-152	-191	-186	39	G+S
Adenosine	42	41	-36	-47	-67	20	GxS
Adenylosuccinate	-241	-271	-224	-265	-261	30	S
Adipic Acid	85	82	114	109	15	94	GxS
ADP	-66	-65	-104	-105	-207	102	GxS
Agmatine	-399	-397	-401	-400	-409	10	GxS
Alanine	-347	-386	-366	-440	-458	19	GxS
Allantoin	18	20	9	10	4	6	GxS
Alpha-Ketoglutaric Acid	44	34	-1	-25	-25	24	G+S
Aminolevulinate	-2	-1	-114	-113	-185	72	GxS
AMP	39	36	-3	-11	-133	122	GxS
Anthranilic Acid	35	28	28	16	41	12	G+S
Arabitol/Xylitol	-199	-197	-282	-282	-275	84	G
Arginine	-331	-387	-321	-407	-434	27	GxS

Argininosuccinate	-122	-124	-249	-257	-272	16	GxS
Asparagine	-80	-149	-94	-234	-269	36	GxS
Aspartic Acid	31	23	28	14	19	9	G+S
Azelaic Acid	89	81	120	110	47	63	GxS
betaAlanine	-147	-152	-167	-177	-171	10	G+S
Betaine	-4	-36	22	-20	-66	46	GxS
Biopterin	-192	-217	-226	-279	-319	40	GxS
Cadaverine	-36	-83	-18	-83	-100	17	GxS
cAMP	122	106	102	69	52	18	GxS
Carnitine	-280	-282	-390	-407	-446	40	GxS
Carnosine	-18	-18	-91	-96	-102	6	GxS
cGMP	-3	-17	-16	-44	-74	31	GxS
Cholecalciferol	15	2	55	39	8	13	S
Cholesteryl Sulfate	-11	-12	-1	-3	14	1	*null*
Choline	-247	-344	-235	-405	-433	29	GxS
Citraconic Acid	38	29	50	36	28	9	GxS
Citrulline	-225	-231	-287	-302	-311	9	GxS
Creatine	15	15	31	31	20		*null*
Cystathionine	-56	-63	-97	-112	-176	65	GxS
Cysteinyl-Glycine (Cys-Gly)	-50	-59	-123	-154	-191	37	GxS
Cystine	44	36	8	-6	-31	25	GxS
Cytidine	-180	-186	-196	-209	-270	61	GxS
D-Leucic Acid	-145	-152	-120	-129	-147	6	S
DCDP	-145	-145	-162	-164	-184	20	GxS
Deoxycarnitine	21	-29	-15	-137	-178	41	GxS
Deoxyguanosine	-140	-188	-134	-210	-246	36	GxS
DHAP/D-GA3P	72	50	55	18	40	32	G+S
Dimethylarginine (A/SDMA)	-32	-33	-221	-235	-246	11	GxS
Dimethylglycine	15	-7	-51	-112	-136	23	GxS
Ergocalciferol	-150	-168	-118	-140	-128	18	S
Erythrose-4-Phosphate	94	38	71	-49	-76	27	GxS
Ethanolamine	-107	-133	-110	-156	-188	31	GxS
FAD	-175	-208	-221	-299	-329	30	GxS
Fructose	-87	-128	-78	-140	-152	12	GxS
G1P/F1P/F6P	77	25	43	-77	-99	22	GxS
G6P	82	31	49	-66	-94	28	GxS
gamma-Aminobutyrate	-203	-207	-223	-232	-258	26	GxS
Glucoronate	-156	-169	-225	-256	-285	30	GxS
Glucosamine	-223	-275	-211	-291	-339	49	GxS
Glucose	-218	-274	-231	-343	-383	41	GxS
Glutamic acid	-314	-313	-344	-343	-372	29	GxS
Glutamine	-41	-55	-99	-134	-135	1	G+S
Glutaric Acid	-61	-68	-27	-36	-92	55	GxS
Glyceraldehyde	-174	-235	-165	-264	-341	77	GxS
Glycerol-3-P	65	66	-1	0	13	66	G
Glycerophosphocholine	-31	-30	-49	-48	-81	34	GxS

Glycine	-288	-290	-325	-332	-331	7	G+S
GMP	-13	-12	-58	-58	-175	116	GxS
Guanine	-152	-191	-150	-214	-258	44	GxS
Guanosine	-171	-171	-318	-324	-331	7	GxS
Histamine	-214	-228	-243	-276	-270	34	G+S
Histidine	-198	-205	-305	-341	-402	61	GxS
Homoarginine	63	30	-5	-105	-155	50	GxS
Homocitrulline	61	60	17	14	37	2	G+S
Hydrocinnamic Acid	-26	-31	-3	-11	-21	5	S
Hypotaurine	-130	-128	-223	-222	-240	18	GxS
Hypoxanthine	108	87	53	-6	-40	34	GxS
Imidazoleacetic Acid	-132	-170	-116	-170	-175	6	GxS
IMP	122	120	93	88	-34	122	GxS
Indole-2-Carboxylic Acid	-129	-147	-150	-184	-184	34	G+S
Indole-3-Carboxylic Acid	-107	-134	-116	-164	-166	2	G+S
Inosine	-239	-239	-268	-270	-281	12	GxS
iso-Leucine	-216	-234	-238	-274	-282	8	GxS
isoValeric Acid	-59	-64	-65	-74	-67	8	G+S
Kynurenic Acid	28	-2	-67	-216	-227	11	GxS
L-2-Aminobutyric Acid	47	35	-36	-67	-84	17	GxS
L-Kynurenine	70	35	80	32	24	8	GxS
Lactate	-212	-220	-220	-233	-219	13	G+S
Lactose/Trehalose	42	40	-118	-130	-168	39	GxS
Leucine	-175	-173	-197	-195	-233	39	GxS
Linoleic Acid	-237	-283	-239	-321	-320	37	G+S
Linolenic Acid	-197	-230	-202	-258	-285	27	GxS
Lysine	-99	-135	-168	-275	-332	57	GxS
Maleic Acid	15	-7	45	17	8	21	S
Margaric Acid	-199	-217	-215	-248	-255	6	GxS
Methionine	-79	-93	-89	-112	-157	46	GxS
Myristic Acid	-306	-335	-301	-343	-354	11	GxS
N-Ac-Alanine	-223	-234	-287	-314	-333	20	GxS
N-Ac-Arginine	100	97	-125	-160	-174	14	GxS
N-Ac-L-Glutamine	-116	-117	-164	-167	-213	45	GxS
N-Ac-Phenylalanine	-91	-99	-183	-210	-205	26	G+S
N-Acetyl-Aspartate (NAA)	187	188	57	59	48	10	GxS
N-AcetylGlycine	-89	-88	-70	-69	-155	86	GxS
n-Glycylproline	-166	-168	-205	-213	-255	42	GxS
N2,N2-Dimethylguanosine	-208	-218	-230	-251	-268	17	GxS
N6-Acetyl-Lysine	0	-40	-5	-71	-114	43	GxS
N6-Trimethyllysine	107	58	58	-67	-128	61	GxS
NAD	-75	-74	-126	-127	-255	128	GxS
NADH	-187	-189	-225	-228	-279	51	GxS
Neopterin	-181	-199	-215	-251	-259	9	GxS
Niacinamide	-30	-42	-26	-46	-64	18	GxS
Nicotinic Acid	-270	-269	-340	-340	-339	1	G+S

o-Phosphoethanolamine	-117	-128	-252	-302	-321	19	GxS
Ornithine	-260	-259	-242	-241	-266	24	GxS
Orotate	-211	-212	-267	-272	-282	10	GxS
Oxalacetate	-44	-87	-26	-87	-102	15	GxS
Oxidized Glutathione	113	114	72	72	-14	85	GxS
Palmitic Acid	-215	-267	-212	-293	-290	26	G+S
Pantothenate	-151	-173	-170	-218	-272	54	GxS
PEP	203	151	211	131	118	13	GxS
Phenylacetic Acid	-25	-53	2	-35	-32	29	S
Phenylalanine	-205	-206	-194	-196	-219	24	GxS
Phosphorylcholine	-151	-150	-234	-234	-257	23	GxS
Phosphoserine	-230	-229	-361	-362	-384	22	GxS
Phosphotyrosine	-91	-91	-95	-95	-78	4	G
Pipecolate	-53	-53	-133	-138	-169	31	GxS
Proline	-219	-226	-320	-339	-355	16	GxS
Pseudouridine	-70	-70	-257	-263	-236	6	G+S
Putrescine	-40	-53	-115	-151	-259	109	GxS
Pyridoxamine	-100	-132	-95	-146	-166	20	GxS
Pyroglutamic Acid	-123	-121	-278	-277	-291	14	GxS
Pyruvate	-3	-17	-17	-42	-49	6	GxS
Reduced Glutathione	67	63	35	28	35	7	G+S
Retinol	-76	-99	-49	-76	-85	22	S
Riboflavin	-29	-39	-141	-180	-197	17	GxS
Ribose-5-P	-59	-79	-107	-153	-176	23	GxS
Ribulose 5-Phosphate	56	25	50	2	3	23	G+S
S-Adenosylmethionine (SAM)	-14	-35	-23	-61	-111	50	GxS
S-Methylcysteine	-39	-66	-48	-96	-102	7	GxS
SAH	-94	-101	-116	-130	-172	42	GxS
Sarcosine	227	136	221	34	-38	73	GxS
Sedoheptulose 7-Phosphate	-100	-113	-116	-139	-137	23	G+S
Serine	-304	-307	-381	-389	-391	3	GxS
Sorbitol	-132	-135	-206	-217	-236	19	GxS
Spermidine	-137	-141	-123	-129	-149	20	GxS
Suberic Acid	98	92	127	119	11	108	GxS
Succinate	-192	-193	-210	-213	-235	23	GxS
Succinylcarnitine	-6	-39	-7	-66	-103	37	GxS
Sucrose	49	44	15	3	-36	39	GxS
Taurine	-322	-383	-319	-423	-475	52	GxS
Thiamine	-178	-177	-266	-265	-294	29	GxS
Threonine	-69	-109	-78	-149	-250	101	GxS
Trigonelline	-83	-82	-123	-121	-141	20	GxS
Tryptophan	-205	-210	-233	-243	-355	112	GxS
Tyrosine	-34	-67	-39	-95	-102	7	GxS
UDP-GlcNAc	-113	-117	-136	-147	-197	50	GxS
UDP-Glucose	-44	-44	-49	-50	-53	3	GxS
UMP	16	16	-123	-127	-153	26	GxS

Uracil	-288	-317	-319	-372	-403	31	GxS
Urate	-149	-209	-134	-223	-262	39	GxS
Uridine	-53	-59	-56	-67	-131	64	GxS
Valine	-147	-156	-197	-220	-272	53	GxS
Xanthine	-86	-116	-154	-254	-267	13	GxS
Xanthosine	-158	-157	-290	-292	-303	11	GxS
Xanthurenic Acid	26	28	-264	-275	-293	18	GxS

Supplementary Table 3. AIC analysis results by PC. The minimum AIC value is shaded in gray for each PC.

	AIC					Δ AIC	Best fitting model
	null	S	G	G+S	GxS		
PC1	921	845	942	834	791	43	GxS
PC2	825	821	799	792	747	45	GxS
PC3	786	762	781	740	631	109	GxS
PC4	714	702	601	553	502	51	GxS
PC5	681	680	614	609	578	31	GxS
PC6	674	674	613	609	614	3	G+S
PC7	643	643	504	496	482	14	GxS
PC8	640	642	490	491	491	150	G
PC9	609	611	469	470	439	31	GxS
PC10	604	606	536	538	536	69	G
PC11	600	601	433	434	417	16	GxS
PC12	595	596	502	501	498	3	GxS
PC13	573	567	441	408	420	33	G+S
PC14	572	574	511	513	493	20	GxS
PC15	554	554	445	441	411	30	GxS
PC16	552	554	446	448	420	28	GxS
PC17	533	533	437	436	429	7	GxS
PC18	540	542	432	434	430	4	GxS
PC19	521	522	431	428	418	10	GxS
PC20	516	513	445	432	386	46	GxS
PC21	496	497	402	402	375	26	GxS
PC22	497	499	392	394	381	13	GxS
PC23	496	498	411	413	378	35	GxS
PC24	466	468	457	458	449	10	GxS

Supplementary Table 4. ANOVA results by metabolite. Metabolites are sorted by GxS effect size (η^2). Terms with significant p-values at FDR <.01 are shaded in green. The term with the largest effect size is shaded in orange for each metabolite.

			Term p-value			Term η^2		
	Model	**R^2**	**S**	**G**	**GxS**	**S**	**G**	**GxS**
IMP	GxS	0.85	0.1	0.01	3.53E-13	0.01	0.16	0.60
AMP	GxS	0.87	8.26E-05	0.1	3.32E-13	0.05	0.12	0.59
GMP	GxS	0.86	0.004	0.003	1.44E-12	0.02	0.17	0.56
NAD	GxS	0.88	0.002	3.87E-06	7.77E-14	0.02	0.24	0.53
ADP	GxS	0.84	0.03	0.0002	6.03E-11	0.01	0.23	0.51
5'-Methylthioadenosine	GxS	0.81	0.5	2.93E-05	8.37E-10	0.00	0.27	0.47
Glyceraldehyde	GxS	0.86	0.4	0.002	2.44E-08	0.00	0.23	0.46
Tryptophan	GxS	0.85	0.03	4.79E-09	4.96E-12	0.01	0.33	0.44
Betaine	GxS	0.71	0.8	0.1	2.69E-05	0.00	0.19	0.41
Uridine	GxS	0.74	0.6	0.0004	5.19E-07	0.00	0.27	0.41
Suberic Acid	GxS	0.76	0.1	2.41E-11	1.24E-11	0.00	0.40	0.41
N-AcetylGlycine	GxS	0.72	0.1	3.26E-08	2.68E-09	0.01	0.36	0.40
Adipic Acid	GxS	0.73	0.1	5.42E-10	3.65E-10	0.01	0.39	0.39
Glutaric Acid	GxS	0.64	0.2	0.001	3.73E-06	0.01	0.28	0.39
Cytidine	GxS	0.77	0.3	4.35E-05	1.07E-06	0.00	0.31	0.39
Glycerophosphocholine	GxS	0.69	0.1	0.3	0.0004	0.02	0.17	0.38
Glucosamine	GxS	0.80	0.04	0.005	1.69E-05	0.02	0.25	0.38
Oxidized Glutathione	GxS	0.82	0.1	2.31E-09	3.37E-09	0.01	0.38	0.38
Cystathionine	GxS	0.81	0.01	3.11E-06	4.56E-07	0.03	0.33	0.37
Azelaic Acid	GxS	0.67	0.2	1.25E-06	6.05E-07	0.01	0.35	0.36
Guanine	GxS	0.79	0.2	0.001	4.46E-05	0.01	0.28	0.36
Putrescine	GxS	0.91	0.1	3.30E-15	1.08E-11	0.01	0.48	0.35
Niacinamide	GxS	0.65	0.8	0.8	0.01	0.00	0.14	0.35
S-Adenosylmethionine (SAM)	GxS	0.78	0.9	9.31E-05	1.20E-05	0.00	0.31	0.35
Taurine	GxS	0.85	0.2	1.33E-05	8.12E-06	0.01	0.34	0.35
Methionine	GxS	0.74	0.7	4.39E-05	3.02E-05	0.00	0.33	0.34
3-Hydroxyisovaleric Acid	GxS	0.69	0.4	0.1	0.002	0.00	0.20	0.34
1/3-Methylhistidine	GxS	0.84	0.1	6.55E-06	7.18E-06	0.01	0.34	0.34
Urate	GxS	0.80	0.1	0.004	0.0001	0.02	0.25	0.34
Ornithine	GxS	0.57	0.0003	0.4	0.002	0.07	0.15	0.34
Deoxyguanosine	GxS	0.79	0.1	0.002	0.0002	0.01	0.29	0.33
SAH	GxS	0.74	0.8	6.81E-05	6.91E-05	0.00	0.33	0.33
Leucine	GxS	0.70	0.4	0.0002	0.0001	0.00	0.32	0.33
Acetylcarnitine	GxS	0.82	0.03	0.004	0.001	0.02	0.28	0.32
Spermidine	GxS	0.59	0.8	0.1	0.005	0.00	0.23	0.32
Succinate	GxS	0.67	0.3	0.04	0.003	0.01	0.24	0.32
Ethanolamine	GxS	0.75	0.001	0.01	0.001	0.05	0.24	0.32
DCDP	GxS	0.66	0.9	0.05	0.004	0.00	0.24	0.32
Phenylalanine	GxS	0.60	0.1	0.02	0.002	0.02	0.25	0.32
Glucose	GxS	0.86	0.03	3.66E-05	8.38E-05	0.02	0.33	0.32
gamma-Aminobutyrate	GxS	0.70	0.3	0.006	0.002	0.01	0.28	0.32
Cadaverine	GxS	0.72	0.2	0.4	0.01	0.01	0.17	0.31
Glutamic acid	GxS	0.71	0.02	0.003	0.001	0.02	0.29	0.31
Arginine	GxS	0.79	0.3	0.003	0.001	0.01	0.29	0.31
Citraconic Acid	GxS	0.59	1.0	0.6	0.03	0.00	0.16	0.31
5,6-Dihydrouracil	GxS	0.70	3.81E-07	0.01	0.0003	0.12	0.23	0.31
Oxalacetate	GxS	0.70	0.3	0.3	0.01	0.01	0.18	0.31
NADH	GxS	0.77	0.03	3.07E-07	1.04E-05	0.02	0.37	0.30
N6-Acetyl-Lysine	GxS	0.80	0.2	1.13E-06	5.63E-05	0.01	0.38	0.30

Cystine	GxS	0.74	0.4	0.003	0.002	0.00	0.30	0.30
Threonine	GxS	0.88	1.62E-05	6.25E-17	6.78E-11	0.04	0.51	0.30
cGMP	GxS	0.73	0.1	0.0002	0.0006	0.01	0.33	0.30
n-Glycylproline	GxS	0.76	0.5	6.35E-07	6.16E-05	0.00	0.39	0.30
PEP	GxS	0.76	0.3	0.1	0.01	0.01	0.23	0.29
Pantothenate	GxS	0.81	0.04	1.08E-09	5.40E-06	0.01	0.44	0.28
Biopterin	GxS	0.82	0.7	2.15E-07	0.0001	0.00	0.41	0.28
UDP-GlcNAc	GxS	0.76	0.2	9.31E-09	1.19E-05	0.00	0.41	0.28
Pyridoxamine	GxS	0.72	0.1	0.004	0.005	0.02	0.29	0.28
1-Methylnicotinamide	GxS	0.71	0.2	0.3	0.03	0.01	0.19	0.28
6-Methyladenosine	GxS	0.65	0.4	0.1	0.02	0.00	0.23	0.28
Valine	GxS	0.82	0.01	2.28E-10	6.77E-06	0.02	0.46	0.28
Ribose-5-P	GxS	0.81	0.3	0.0001	0.003	0.01	0.35	0.28
Succinylcarnitine	GxS	0.78	0.9	5.88E-08	0.0002	0.00	0.44	0.27
3-Hydroxypropionic Acid	GxS	0.85	0.0002	6.74E-15	6.26E-08	0.03	0.52	0.27
4-Pyridoxic Acid	GxS	0.75	0.1	0.0002	0.003	0.01	0.33	0.27
2-isoPropylmalic Acid	GxS	0.73	1.0	0.01	0.03	0.00	0.29	0.26
Creatine	*null*	0.54	0.5	0.003	0.02	0.00	0.31	0.26
N2,N2-Dimethylguanosine	GxS	0.71	1.0	0.0001	0.01	0.00	0.36	0.25
Sucrose	GxS	0.76	0.1	1.98E-09	0.0001	0.01	0.47	0.25
UDP-Glucose	GxS	0.58	0.1	0.1	0.1	0.01	0.24	0.25
L-Kynurenine	GxS	0.68	0.2	0.1	0.03	0.01	0.23	0.25
Trigonelline	GxS	0.70	0.4	1.51E-05	0.005	0.00	0.39	0.25
cAMP	GxS	0.73	0.1	4.39E-05	0.01	0.01	0.38	0.25
N-Ac-L-Glutamine	GxS	0.77	0.04	3.25E-11	3.32E-05	0.01	0.50	0.25
Agmatine	GxS	0.58	0.2	0.002	0.03	0.01	0.33	0.25
Pyruvate	GxS	0.68	0.6	0.007	0.04	0.00	0.31	0.25
1-Methyladenosine	GxS	0.82	0.2	2.61E-08	0.0006	0.01	0.45	0.25
Myristic Acid	GxS	0.68	0.01	0.007	0.02	0.03	0.28	0.24
3-Aminoisobutyrate	GxS	0.77	0.2	6.04E-07	0.002	0.01	0.41	0.24
2-Hydroxyglutarate	GxS	0.69	0.01	0.0004	0.02	0.03	0.34	0.24
Uracil	GxS	0.80	0.01	9.96E-08	0.0006	0.02	0.41	0.24
Alanine	GxS	0.80	0.2	8.43E-05	0.01	0.01	0.34	0.24
N-Ac-Alanine	GxS	0.80	0.03	1.41E-05	0.005	0.02	0.37	0.24
Aminolevulinate	GxS	0.88	0.1	1.06E-17	8.40E-08	0.01	0.59	0.24
Inosine	GxS	0.67	0.2	0.0001	0.02	0.01	0.37	0.24
Hypoxanthine	GxS	0.84	0.01	1.41E-09	0.0003	0.02	0.46	0.23
Fructose	GxS	0.72	0.1	2.50E-05	0.02	0.01	0.39	0.23
Imidazoleacetic Acid	GxS	0.67	0.05	0.006	0.05	0.02	0.29	0.23
iso-Leucine	GxS	0.72	0.1	0.0001	0.03	0.01	0.37	0.22
Adenosine	GxS	0.79	0.3	6.17E-08	0.004	0.00	0.46	0.22
Choline	GxS	0.88	0.03	1.03E-08	0.001	0.02	0.44	0.22
Pipecolate	GxS	0.80	0.2	1.90E-10	0.0006	0.01	0.51	0.22
2-Aminoisobutyric Acid	GxS	0.80	0.1	2.39E-06	0.01	0.01	0.40	0.22
N6-Trimethyllysine	GxS	0.92	0.002	6.14E-17	1.10E-06	0.02	0.58	0.22
FAD	GxS	0.85	0.3	1.54E-10	0.0007	0.00	0.51	0.22
Allantoin	GxS	0.60	0.4	8.10E-05	0.04	0.00	0.39	0.22
Tyrosine	GxS	0.72	0.04	0.0002	0.04	0.02	0.36	0.22
Linolenic Acid	GxS	0.76	0.0002	2.92E-07	0.001	0.05	0.38	0.22
Sarcosine	GxS	0.93	2.18E-05	3.28E-19	7.44E-08	0.04	0.59	0.22
S-Methylcysteine	GxS	0.72	0.05	9.70E-05	0.04	0.02	0.37	0.21
Sorbitol	GxS	0.79	0.005	5.51E-08	0.01	0.03	0.45	0.21
Erythrose-4-Phosphate	GxS	0.87	0.01	7.77E-09	0.001	0.02	0.44	0.21
Neopterin	GxS	0.74	0.03	2.05E-05	0.03	0.02	0.39	0.21
Carnitine	GxS	0.86	0.03	2.44E-13	0.0001	0.01	0.55	0.21
G6P	GxS	0.87	0.01	2.37E-09	0.001	0.03	0.44	0.21

Acetylcholine	*null*	0.46	0.4	0.1	0.3	0.00	0.26	0.21
Margaric Acid	GxS	0.70	0.8	9.32E-06	0.04	0.00	0.42	0.20
Thiamine	GxS	0.80	0.02	8.94E-11	0.0009	0.02	0.50	0.20
4-Guanidinobutanoate	GxS	0.94	4.89E-08	7.70E-19	1.41E-07	0.06	0.56	0.20
Histidine	GxS	0.90	0.6	4.35E-19	1.12E-06	0.00	0.64	0.20
Asparagine	GxS	0.88	0.001	9.53E-13	0.0002	0.03	0.54	0.20
4-Hydroxybenzoic Acid	GxS	0.72	0.9	9.81E-09	0.01	0.00	0.50	0.20
Glucoronate	GxS	0.82	0.02	6.40E-12	0.0007	0.01	0.52	0.19
Orotate	GxS	0.73	0.2	1.17E-07	0.03	0.01	0.47	0.19
G1P/F1P/F6P	GxS	0.87	0.01	1.78E-09	0.003	0.02	0.46	0.19
Cysteinyl-Glycine (Cys-Gly)	GxS	0.84	0.3	2.89E-14	0.0002	0.00	0.57	0.19
3-Methyl-3-Hydroxyglutaric Acid	GxS	0.71	0.2	1.84E-07	0.03	0.01	0.47	0.19
Deoxycarnitine	GxS	0.89	2.13E-05	2.10E-15	8.42E-05	0.05	0.57	0.19
Homoarginine	GxS	0.90	0.7	8.82E-19	1.33E-05	0.00	0.65	0.18
Phosphorylcholine	GxS	0.79	0.2	1.48E-12	0.003	0.01	0.57	0.18
Hypotaurine	GxS	0.80	0.2	1.60E-11	0.01	0.01	0.56	0.18
Citrulline	GxS	0.76	0.005	8.75E-08	0.03	0.03	0.43	0.17
Lysine	GxS	0.91	0.01	1.93E-21	2.43E-06	0.01	0.66	0.17
Cholesteryl Sulfate	*null*	0.46	0.3	0.1	0.6	0.01	0.29	0.17
Serine	GxS	0.76	0.01	4.70E-08	0.1	0.02	0.49	0.17
Carnosine	GxS	0.75	0.6	2.43E-09	0.05	0.00	0.52	0.16
Dimethylglycine	GxS	0.85	0.1	1.87E-15	0.003	0.01	0.63	0.15
Proline	GxS	0.83	0.9	9.64E-14	0.01	0.00	0.60	0.15
Phosphoserine	GxS	0.85	0.1	7.67E-16	0.003	0.01	0.64	0.15
Riboflavin	GxS	0.87	0.01	4.72E-13	0.01	0.02	0.55	0.15
Xanthine	GxS	0.88	0.01	2.69E-13	0.01	0.02	0.58	0.14
UMP	GxS	0.87	0.02	6.37E-16	0.002	0.01	0.57	0.14
N-Acetyl-Aspartate (NAA)	GxS	0.83	0.1	1.31E-13	0.02	0.01	0.61	0.14
2-Aminoadipate	GxS	0.90	0.1	1.80E-18	0.002	0.00	0.68	0.13
o-Phosphoethanolamine	GxS	0.90	0.04	1.65E-17	0.01	0.01	0.67	0.13
Argininosuccinate	GxS	0.85	0.01	3.35E-16	0.01	0.02	0.64	0.13
Pyroglutamic Acid	GxS	0.86	0.04	1.45E-16	0.01	0.01	0.66	0.13
Xanthosine	GxS	0.84	0.1	2.79E-15	0.02	0.01	0.62	0.13
L-2-Aminobutyric Acid	GxS	0.82	0.9	3.55E-11	0.01	0.00	0.39	0.13
Lactose/Trehalose	GxS	0.90	0.001	1.90E-21	0.0001	0.02	0.63	0.12
Guanosine	GxS	0.86	0.03	5.41E-16	0.04	0.01	0.64	0.11
Dimethylarginine (A/SDMA)	GxS	0.90	0.3	1.60E-20	0.02	0.00	0.71	0.10
Kynurenic Acid	GxS	0.93	0.1	8.58E-23	0.02	0.00	0.76	0.09
N-Ac-Arginine	GxS	0.94	0.04	3.25E-25	0.01	0.01	0.77	0.08
Xanthurenic Acid	GxS	0.95	0.8	5.54E-33	0.01	0.00	0.85	0.06
7-Methylguanine	GxS	0.89	0.002	7.22E-23	0.05	0.01	0.46	0.05
Pseudouridine	G+S	0.83	0.01	7.25E-31		0.01	0.78	
Adenine	G+S	0.75	1.63E-08	1.38E-20		0.08	0.67	
N-Ac-Phenylalanine	G+S	0.71	2.45E-06	8.67E-18		0.06	0.63	
5-Methylcytidine	G	0.66		1.07E-15			0.63	
Arabitol/Xylitol	G	0.63		8.68E-14			0.63	
2'-Deoxycytidine	G	0.62		1.84E-13			0.61	
Glycerol-3-P	G	0.58		3.58E-11			0.58	
Nicotinic Acid	G+S	0.60	0.2	8.03E-12		0.01	0.58	
Glutamine	G+S	0.66	9.48E-08	6.54E-13		0.10	0.55	
Homocitrulline	G+S	0.52	0.1	2.90E-08		0.02	0.51	
Alpha-Ketoglutaric Acid	G+S	0.59	6.26E-06	4.53E-10		0.08	0.50	
Glycine	G+S	0.52	0.01	1.22E-07		0.03	0.48	
Histamine	G+S	0.57	1.22E-07	1.60E-08		0.12	0.46	
Reduced Glutathione	G+S	0.50	0.01	7.53E-07		0.03	0.46	
Linoleic Acid	G+S	0.64	5.08E-16	3.89E-07		0.29	0.34	

Glycine	G+S	0.52	0.01	1.22E-07	0.03	0.48
Homocitrulline	G+S	0.52	0.1	2.90E-08	0.02	0.51
Histamine	G+S	0.57	1.22E-07	1.60E-08	0.12	0.46
Alpha-Ketoglutaric Acid	G+S	0.59	6.26E-06	4.53E-10	0.08	0.50
Glycerol-3-P	G	0.58		3.58E-11		0.58
Nicotinic Acid	G+S	0.60	0.2	8.03E-12	0.01	0.58
Glutamine	G+S	0.66	9.48E-08	6.54E-13	0.10	0.55
2'-Deoxycytidine	G	0.62		1.84E-13		0.61
Arabitol/Xylitol	G	0.63		8.68E-14		0.63
5-Methylcytidine	G	0.66		1.07E-15		0.63
N-Ac-Phenylalanine	G+S	0.71	2.45E-06	8.67E-18	0.06	0.63
Adenine	G+S	0.75	1.63E-08	1.38E-20	0.08	0.67
Pseudouridine	G+S	0.83	0.01	7.25E-31	0.01	0.78
Hydrocinnamic Acid	S	0.05	0.008		0.05	
D-Leucic Acid	S	0.06	0.005		0.05	
2-Hydroxyphenylacetate	S	0.07	0.001		0.07	
Cholecalciferol	S	0.10	0.0001		0.10	
Ergocalciferol	S	0.13	9.51E-06		0.12	
3HBA	S	0.14	4.94E-06		0.13	
Maleic Acid	S	0.15	1.64E-06		0.14	
Retinol	S	0.16	1.02E-06		0.15	
Phenylacetic Acid	S	0.19	4.29E-08		0.18	
Adenylosuccinate	S	0.20	2.35E-08		0.18	
3-(4-Hydroxyphenyl)Propionate	S	0.26	1.53E-10		0.23	

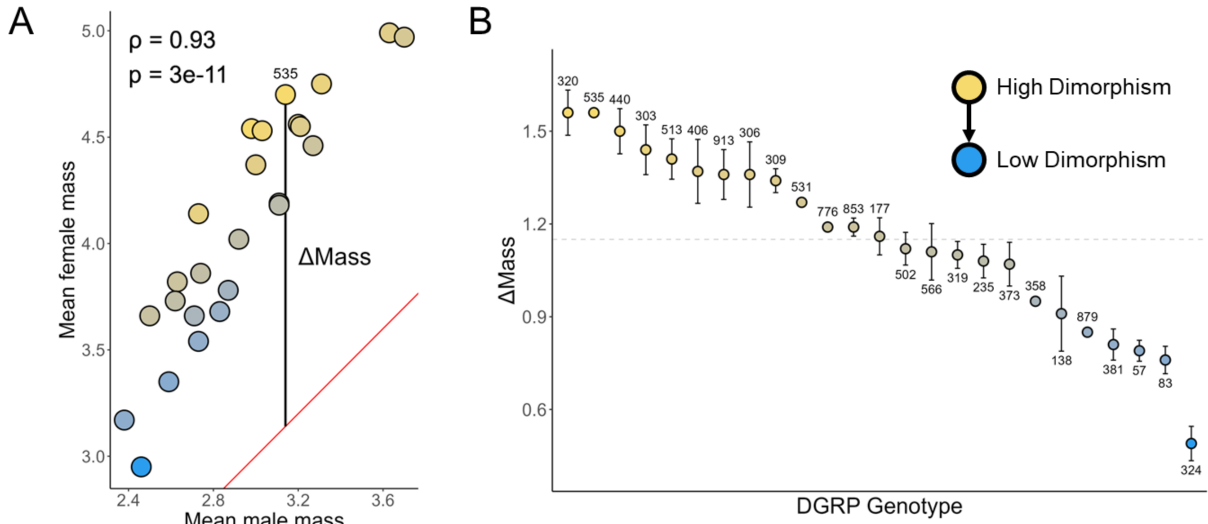
Supplementary Table 5. ΔPC across genotypes. PC distance between the sexes for the top 6 principal components. The largest distance for each genotype is shaded in gray. Genotypes are arranged from highest to lowest ΔPC1 value.

Genotype	ΔPC1	ΔPC2	ΔPC3	ΔPC4	ΔPC5	ΔPC6
535	17.86	2.45	1.01	2.27	3.88	1.18
853	17.37	0.95	0.12	1.22	1.40	0.47
531	15.44	1.92	2.19	2.06	0.98	1.96
375	14.92	0.24	4.89	0.69	0.46	1.99
913	13.85	2.24	2.80	1.51	0.67	2.72
502	13.51	3.61	2.38	1.21	1.54	0.57
513	12.11	2.66	0.39	3.20	3.29	1.31
406	11.50	0.60	1.30	5.34	1.03	0.29
776	11.35	3.32	3.44	2.82	1.71	3.32
440	11.09	0.76	0.13	1.00	0.02	2.03
879	10.52	2.49	2.15	2.38	0.54	3.89
566	10.29	2.43	2.16	1.88	1.29	1.41
381	8.96	2.73	1.70	1.25	2.28	1.34
177	8.05	8.24	3.43	0.82	2.26	0.44
373	6.96	3.22	3.39	0.01	1.78	0.14
235	6.71	9.07	0.10	2.43	0.67	0.93
309	6.49	4.37	2.81	0.13	1.52	1.54
358	6.35	3.34	2.01	3.29	5.35	3.82
303	6.02	3.89	5.56	3.62	0.22	2.08
306	5.88	0.54	4.38	0.35	4.19	4.45
138	5.36	5.78	6.42	2.51	0.08	2.97
320	4.79	5.50	2.79	0.34	2.88	2.42
42	4.29	0.56	8.49	2.03	3.74	3.74
57	3.18	1.96	20.02	6.34	5.89	0.12
324	3.06	3.26	5.73	3.16	0.16	4.38
319	1.51	9.57	1.81	3.85	1.07	2.83
208	1.27	8.64	3.92	0.96	2.64	0.28
83	0.74	5.54	1.63	0.75	2.48	2.06
31	0.72	11.52	7.28	2.68	3.65	0.63

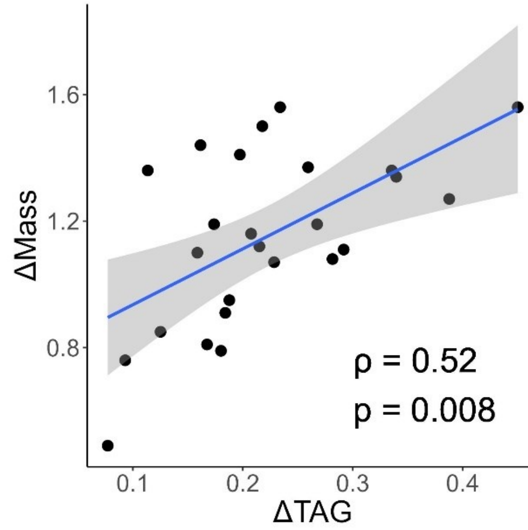
Supplementary Table 6. KEGG pathways and modules overrepresented by ΔTAG-associated metabolites.

Pathways	KEGG ID	p-value
Lysine degradation - *Drosophila* melanogaster	dme00310	0.009
Tyrosine metabolism - *Drosophila* melanogaster	dme00350	0.002
Porphyrin metabolism - *Drosophila* melanogaster	dme00860	0.005
Modules		
Catecholamine biosynthesis, tyrosine => dopamine => noradrenaline => adrenaline	M00042	0.007
Heme biosynthesis, plants and bacteria, glutamate => heme	M00121	0.01
Siroheme biosynthesis, glutamyl-tRNA => siroheme	M00846	0.003
Heme biosynthesis, animals and fungi, glycine => heme	M00868	0.008
Heme biosynthesis, bacteria, glutamyl-tRNA => coproporphyrin III => heme	M00926	0.007

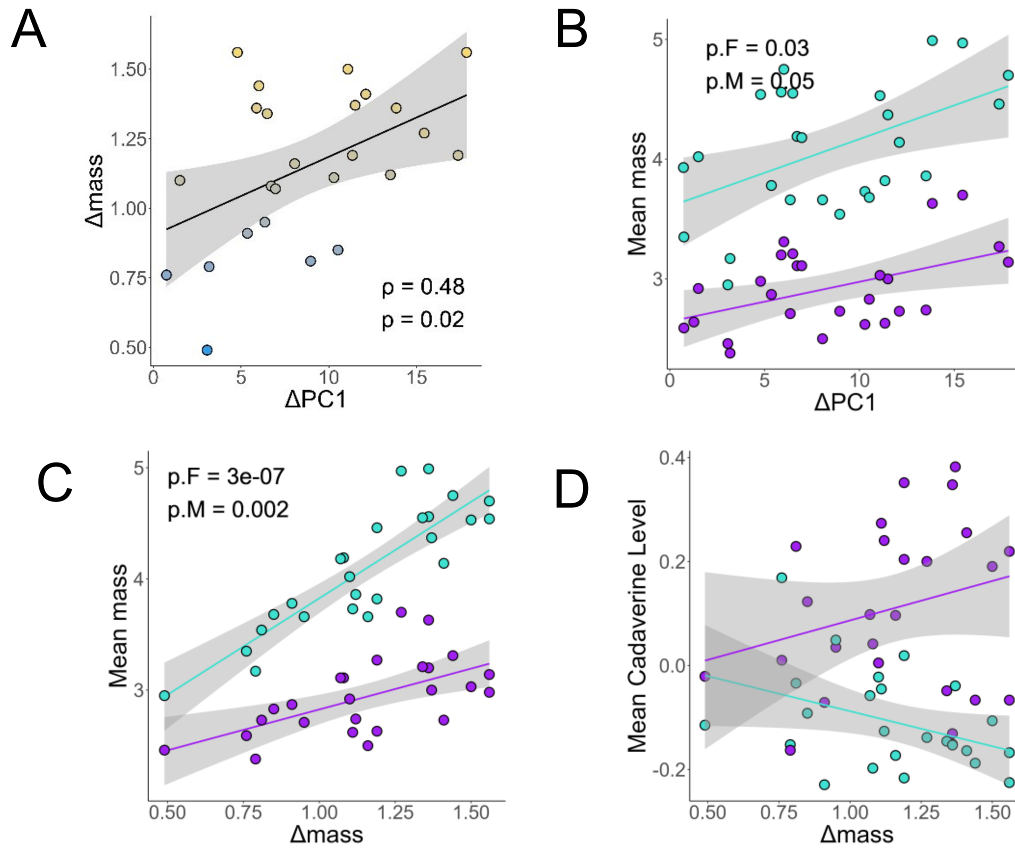
Supplementary Figures



Supplementary Figure 1. GxS interactions in body mass across the DGRP. (A) Relationship between female and male measurements of body mass (mg per 5 flies, n=239 measurements). Points represent mean mass values for each genotype, colored by degree of dimorphism. Red line marks the line of unity between females and males. Black line indicates **Δ**mass for line 535, the genotype with the largest sex difference in mass (**Δ**mass equal to line 320). Line 324, the genotype with the smallest sex difference is colored bright blue. (B) Variation in **Δ**mass across 25 of the 29 DGRP genotypes used in this study, plotted from high to low dimorphism. A value of 0 represents no difference between female and male mean mass. Dotted gray line represents the **Δ**mass population mean value. Error bars represent the pooled standard error between female and male samples. Coefficient and p-value is generated from Spearman's rank correlation test.



Supplementary Figure 2. Relationship between measures of SSD. Relationship between ΔTAG and Δmass across 25 of the 29 DGRP genotypes used in this study. Coefficient and p-value is generated from Spearman's rank correlation test.



Supplementary Figure 3. Genetic variation for mass dimorphism in the head metabolome. (A) Correlation between **Δ**PC1 and **Δ**mass. (B-C) Correlation between mean sex-specific mass and **Δ**PC1 (B) and **Δ**mass ($\rho_F = 0.44$, $\rho_M = 0.40$) (C). Each point represents a genotype-specific mean. (D) Cadaverine, the metabolite with the greatest influence on **Δ**PC1. Correlation between **Δ**cadaverine and **Δ**mass. Female means are depicted in turquoise, male means in purple. Coefficients and p-values are generated from Spearman's rank correlation tests.

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Chapter 3. Tissue-specific metabolomic signatures for a *doublesex* model of reduced sexual dimorphism

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Keywords: variation in sex characteristics, *doublesex*, dimorphism, *Drosophila*, metabolomics

Abstract

Sex has a major effect on the metabolome. This suggests the metabolome has potential to explain and predict molecular mechanisms underlying sex differences in biological outcomes. However, we do not yet understand the degree to which these quantitative sex differences in metabolism are modulated by variations in sex characteristics (VSC), or by proximity to sex-specific tissues. In the fruit fly, *Drosophila melanogaster*, knocking out the *doublesex* (*dsx*) gene gives rise to adults with intermediate sex characteristics. Here we sought to determine the degree to which this key node in sexual development regulates sex differences in the fly metabolome. We measured 91 LC-MS targeted metabolomic features across three tissue types in *Drosophila*, comparing the differences between distinctly sex-dimorphic flies (here referred to as "3G") with those of reduced sexual dimorphism-- *doublesex* (*dsx*) null flies. Notably, in the reduced dimorphism flies, we observed a sex difference in only 1 of 91 metabolites, kynurenate, whereas 52% of metabolites (47/91) were significantly different between 3G XX and XY flies in at least one tissue, suggesting that a *dsx*-dependent mechanism plays a major role in sex differences in fly metabolism. Kynurenate was consistently higher in XX flies in both the presence and absence of functioning *dsx*. We observed unique signatures of knocking out *dsx* in all three tissues, with eight metabolites sharing a similar pattern across all tissues. These metabolites were significantly enriched in metabolites associated with branch chain amino acid metabolism, the mTOR pathway, and arginine metabolism. Our results suggest that VSC mediated by *dsx* may contribute to heterogeneity observed in metabolomic studies of sex differences in *Drosophila*. This highlights the importance of considering variation in genes that cause anatomical sex differences when analyzing metabolic profiles and interpreting their biological significance.

Introduction

Sex differences in biological processes are pervasive and have far-reaching implications for health and disease (Klein & Flanagan, 2016; Mauvais-Jarvis *et al*., 2020; Ober *et al*., 2008). As such, policies emphasize the inclusion of both sexes in biomedical research, acknowledging that sex differences impact a wide array of health outcomes (Clayton & Collins, 2014). These differences extend beyond reproductive functions, influencing bone (Ortona *et al*., 2023), muscle (Landen *et al*., 2023), and fat metabolism (Mauvais-Jarvis *et al*., 2017). However, most research on sex differences in metabolism fail to account for intersex individuals, who present phenotypic traits that do not conform strictly to female or male categories. Currently, there is a significant gap in our understanding of how intersexuality impacts sex differences in metabolism.

Intersex is an umbrella term encompassing many predominantly monogenic conditions that disrupt sexual development such that an individual's sex characteristics do not fit typical binary notions of female or male bodies (McElreavey & Bashamboo, 2023). It is commonly replaced by "variations in sex characteristics" (VSC) or "differences in sex development" (DSD) due to varying preferences and evolving language (Crocetti *et al*., 2020; Reis, 2007). VSC acknowledges the existence of a full range of natural bodily variations in sex characteristics. It includes differences in chromosomal patterns (e.g., XXY, XYY), gonadal structures (e.g., ovotestes), and external genitalia that may not conform to typical female or male patterns. In humans, examples of VSC include conditions such as Androgen Insensitivity Syndrome (AIS), Congenital Adrenal Hyperplasia (CAH), and Turner Syndrome (45,X) (Baetens *et al*., 2019; Conway, 2022). Measuring the impact of VSC on metabolic processes can help us understand how genetic and environmental factors influence the degree of dimorphism in the metabolome. However, the impact of these variations on metabolomic studies has not been thoroughly investigated.

Different tissues in an organism perform specialized functions, and their metabolic needs and activities are tailored to support these functions. For example, muscle tissue requires energy for contraction and movement, while liver tissue is involved in detoxification and glucose metabolism. Sex-limited tissues such as ovaries or testes have their own unique energetic demands due to their specialized functions related to reproduction. The production of sperm and eggs is highly energy-intensive, requiring substantial ATP (adenosine triphosphate) production for extensive cell division, differentiation, and maturation (Blerkom, 2004; Rato *et al*., 2012). Further, proximity to sex-limited tissues such as ovaries or testes can influence the overall metabolic landscape of other tissues in the body. For example, crosstalk between white adipose tissue and ovarian tissues affects the quality of human oocytes (Anaya *et al*., 2024). While VSC can arise from diverse genetic mechanisms, a common theme for most conditions is a reduction in sexual dimorphism for one or several tissues. Despite the recognized importance of these factors, there is a notable gap in the research addressing how tissue-specific VSC might affect observed sex differences in metabolism.

This study aims to address this gap by measuring the tissue-specific metabolome in a *Drosophila* model of VSC. The *Drosophila* sexual development pathway is well characterized (Saccone, 2022), providing an excellent system for modeling VSC. In *Drosophila*, the *doublesex* (*dsx*) gene acts in a global alternative splicing cascade, which is essential for determining the sexual fate of cells throughout the organism (Coschigano & Wensink, 1993). Knockout of *dsx* disrupts this cascade, leading to altered or incomplete sexual development (Hildreth, 1965). *Dsx*

is part of the DMRT (*doublesex*/mab-3 related) family of transcription factors, which plays a crucial role in shaping sexual dimorphism across diverse animal lineages, including mammals, insects, and nematodes (Kopp, 2012). DMRT genes, including *dsx* in *Drosophila*, function as tissue-specific developmental regulators (Clough *et al*., 2014; Rice *et al*., 2019). These transcription factors integrate information about sex, position, and time to direct specific populations of cells toward sex-specific fates. This makes *dsx* an exceptionally powerful gene for unraveling the tissue-specific metabolic consequences of one of many forms of VSC.

In many studies of model organisms, including *Drosophila*, “wild type" implies a standard or "normal" genotype and phenotype from which deviations are measured. This assumption can be misleading because it suggests that the wild type represents a perfect or ideal form, ignoring natural genetic and phenotypic diversity within populations. For that reason, here we replace the term “wild type” with “3G”, a nomenclature adopted from Joel (2012) referring to individuals with consistent genetic-gonadal-genital (3G) sex. In flies, an XX chromosome karyotype (genetics) is typically accompanied by ovaries (gonads) and female genital disc (genitals), while an XY chromosome karyotype is typically accompanied by testis and male genital disc (Salz & Erickson, 2010; Sánchez & Guerrero, 2001). These three major aspects of biological sex are consistent in 99% of individuals. Using this 3G nomenclature leaves room for complexity in sex traits without pathologizing intersexuality, a natural phenomenon in our biological world. In contrast to 3G flies, we refer to *dsx* null flies, which have an intersexual phenotype, as “VSC”. Lastly, here we use sex chromosome karyotype (e.g., XX or XY) instead of “female” or “male” as a more precise terminology. This primary focus of this analysis is to evaluate how sex differences in the metabolome vary between 3G and VSC flies (termed SD groups), rather than the differences between the sexes.

In this study, we aim to compare the metabolome profiles of distinctly sex-dimorphic flies (3G) with those of reduced sexual dimorphism *dsx* null flies (VSC) across head, thorax and abdominal tissues using LC-MS targeted metabolomic analysis. We hypothesized that reduced dimorphism in VSC flies across tissues would cause a reduction in sex differences observed in the metabolome. By measuring 91 features, we seek to identify the extent to which *dsx* contributes to global sex differences in the fly metabolome. Our findings reveal that sex differences in the metabolome are greatly impacted by VSC. Only 1 of 91 metabolites showed sex differences in reduced-dimorphism flies, whereas 52% of metabolites differed significantly between 3G XX and XY flies in at least one tissue, and 9% (8/91 metabolites) showed a common signature across all three tissues. Unique signatures of *dsx* knockout were observed for each tissue, with significant enrichment in BCAA and mTOR pathways across all tissues. These results highlight the

importance of considering natural variation in sex characteristics in metabolomic studies, contributing to a better understanding of sex differences in health and disease.

Methods

Fly husbandry, tissue collection and metabolite extraction

Experimental flies were raised on a banana-based medium and housed in an incubator on a 12-hour light-dark cycle. Lines were created for four experimental groups: (XX-3G, XX-VSC, XY-3G and XY-VSC). We achieved a fly model for VSC by knocking out the gene *dsx* in our experimental group. All samples were collected from a single experiment. Indiana stock center flies *dsx*f00683-d07058 [66710] were backcrossed to a w^{1118}/Bar[S] (Bar-Stone) lab strain for five generations and then crossed to *dsx*f01649-9625 to create *dsx* null trans-heterozygous XX and XY offspring. Offspring of the same cross heterozygous for the *dsx*f01649-9625 null allele were also collected for XX and XY 3G comparisons (w-/Bar[S], *CyO*/+, *dsx*-/+). Isogenicity of the second chromosome was achieved by discarding *CyO* flies.

XX-3G flies: (w-/w-, , *dsx*-/+) XY-3G flies: (w-/Bar[S], , *dsx*-/+)

XX-VSC flies: (w-/w-, , *dsx*-/*dsx*-) XY-VSC flies: (w-/Bar[S], , *dsx*-/*dsx*-)

All flies were collected 3 days post-eclosion and sorted with a two-minute timed exposure to CO2 into four groups. Flies were then allowed to recover for 24 hours on fresh media before being flash frozen in liquid nitrogen. Individual flies were sectioned over dry ice into head, thorax and abdomen samples using a chilled razor blade and sorted into 1.5mL Eppendorf tubes for storage. Each sample consisted of tissue from 10 individual flies, with at least four biological replicates per tissue per experimental group (XX-3G flies had one additional biological replicate per tissue type). Samples were stored at -80 before and after processing for metabolomics. One 5mm zirconium oxide bead was added to each Eppendorf tube before placing them in a frozen Qiagen Tissuelyser block. The tissue was pulverized at 30Hz for two minutes on a Tissuelyser II. Samples were then suspended in 1 mL of an 8:2 Methanol:H20 solution, after which each sample was vortexed for 10 seconds to lyse any remaining cells. Tubes were then centrifuged at 14,000 rpm for 15 minutes at 4˚C. 600 µL of supernatant was transferred to a new Eppendorf tube and dried under vacuum at 30˚C.

Liquid chromatography-mass spectrometry (LC- MS)

Targeted mass spectrometry was carried out as described previously (Zhao *et al.*, 2022), providing measures of 91 metabolites with no missing data.

One replicate sample of XX-3G/abdomen was reported by LC-MS staff as having aberrant values and investigated as a potential outlier. 10,000 data permutations were conducted to quantify the null distribution of metabolite outliers per sample and compared to the observed number of outliers in each sample. Outliers in this test were determined using the 'boxplot.stats' function in R. The reported XX-3G/abdomen sample met a p-value of < .005 and thus was removed from statistical analysis (Supplementary Figure 1). A total of 50 metabolome samples were thus included in our analysis (17 head, 17 thorax and 16 abdomen).

Statistical Methods

All statistical analysis was performed using R (version 4.3.0) open-source statistical software. Metabolite data were stratified by tissue (head, thorax, abdomen), then log-transformed, centered and scaled to have a mean = 0 and SD = 1 by sample. Principal component analysis (PCA) was performed using the 'prcomp' function in R to observe the degree to which 3G and VSC XX and XY flies could be distinguished by the metabolome.

Univariate Analysis

One-way ANOVA was conducted on each metabolite within each tissue type (head, thorax, abdomen) followed by post-hoc analysis using the TukeyHSD function in R to assess mean group sex differences and 95% family-wise confidence levels in 3G as compared to VSC groups. Standard errors were derived from confidence intervals using the following equation:

$$SE = (\text{upper limit} - \text{lower limit}) / 3.92.$$

This was followed by a Benjamini-Hochberg FDR correction for multiple comparisons (Benjamini & Hochberg, 1995). Metabolites that met an FDR of 5% were considered significantly different between the sexes in one or both SD groups (XX-3G vs XY-3G, and XX-VSC vs XY-VSC). To determine whether there was a significant reduction in sex effects on the metabolome in the VSC group as compared to the 3G group, effect sizes between XY and XX, generated from the Tukey HSD function in R, were compared between SD groups by fitting a linear model to the following equation for each tissue:

$$Y = \log10 \mid \text{Effect Size} \mid \sim \text{SD group}$$

To compare the distribution of sex bias between XX and XY groups across all three tissues we fit a generalized linear model (GLM) with a binomial distribution to the number of metabolites

significantly higher in each group. We then calculated the least-squares means for each tissue and performed pairwise comparisons using the 'lsmeans' package in R.

Metabolite Enrichment

Pathway analysis was conducted using the 'FELLA' package in R (Picart-Armada *et al*., 2018), which utilizes a network diffusion method to detect enrichment in small groups of metabolites such as the 91 targeted metabolites measured in this study. Within FELLA, we accessed the *Drosophila melanogaster* KEGG Genes Database Release 106.0+: pathways:130, modules:175, enzymes:764, reactions:5,664, compounds:4,141 (05-19, May 23). We estimated p-values for pathway enrichment by simulation, permuting the data for all 91 metabolites 10,000 times and enriching for the 8 SD^{dsx} metabolites common to all three tissues.

Results

We used LC-MS to measure metabolite concentrations in head, thorax, and abdomen tissues of four types of flies in a w^{1118} genetic background: XX-3G, XX-VSC (XX-*dsx* null), XY-VSC (XY-*dsx* null) and XY-3G flies. We first report sex differences observed in the global metabolome between SD groups (3G flies as compared to VSC flies). Next, we report the tissue-specificity of these sex differences. Last, we report pathway analysis results for notable metabolites.

Reduction in sexual dimorphism is accompanied by a reduction in global sex differences in the metabolome

The primary purpose of this study was to query the degree to which VSC might impact sex differences in metabolism. We hypothesized that global sex differences in the metabolome of intersex flies would be significantly reduced from that of 3G flies. Principal component analysis (PCA) of all samples strongly separated tissues from one another, with PC1 segregating abdomen samples from both head and thorax, and PC2 discriminating head from thorax (Supplementary Figure 2). We thus separated tissues for further PCA and for univariate analyses. Within each tissue, PCA separated all 4 groups from each other, with VSC metabolome profiles largely intermediate to those of 3G flies on PC1 in the thorax and abdomen and in PC2 for head tissue (Figure 1). Notably, there was little overlap between all four groups of flies, though PC1 explained slightly less variance in thorax tissue (35%) as compared to the head (37.1%) or abdomen (41.4%).

We next analyzed sex differences between XX and XY flies in each SD group for each metabolite using ANOVA. As expected, we observed strong sex differences in the metabolome between 3G XX and XY flies, with 47 metabolites (52%) meeting an FDR significance threshold of 5% in at least one tissue (Table 1, Supplementary Table 1). We hereafter refer to these as "SD" metabolites. We compared the absolute values of sex differences within each SD group for SD metabolites (Figure 2A) and found that sex differences were significantly reduced in the head (p=0.002), thorax (p=0.0005), and abdomen (p=7.52E-08). Including all metabolites in the analysis, the reduction was less significant; head (p= 0.007), thorax (p= 0.02), abdomen (p= 0.0002), (Supplementary Figure 3).

Of all SD metabolites, only one metabolite, kynurenate, maintained a significant sex difference between the intersex XX and XY flies (Figure 2B, denoted in triangle). No other metabolites were different between XX and XY flies in the VSC group. We stratified SD metabolites based on whether their concentrations were higher in XX or XY flies within the 3G-XX or 3G-XY groups (Figures 2B and 2C). We found no indication of a bias in terms of which sex karyotype (XX vs XY) had higher metabolite levels, except for kynurenate, which was consistently elevated in XX flies regardless of the SD group. We also evaluated the proportions of metabolites that were higher in XX vs XY across tissues and found no significant difference across tissues (chi-square statistic = 0.6 with 2 degrees of freedom). As our VSC flies are the result of *dsx* knockout, this suggests that the elevated levels of kynurenate in XX flies are independent of the sexual dimorphism typically mediated by *dsx*.

Dsx influences sex differences in the metabolome in a tissue-specific manner

Across tissues, the number and identity of SD metabolites varied (head = 23, thorax = 33, abdomen = 30), with nine of these metabolites showing a consistent effect of sex across tissues in 3G flies. The distribution of SD metabolites across head, thorax, and abdomen tissues is pictured in Figure 3A. Thorax tissue shared more SD metabolites with both the head and abdomen than the head and abdomen shared with each other. Of the nine metabolites, sex differences in all but kynurenate were greatly reduced in all tissues in *dsx* flies. (Figure 3B). These features included tyrosine, deoxycarnitine, phosphorylcholine (aka phosphocholine), glutamate, leucine, isoleucine, valine, and N, N-dimethyl-arginine (aka ADMA). We designated these eight metabolites, excluding kynurenate, as "SD^{dsx}".

Each tissue exhibited a unique set of SD metabolites with sex differences specific to that tissue. SD metabolites unique to the head included 3-nitro-L-tyrosine, gamma-aminobutyrate, and nicotinamide mononucleotide. The thorax tissue had four unique SD metabolites not found in the

other tissues: amino-isobutyrate, pantothenate, putrescine, and histamine. Abdomen tissue had the highest number of unique SD metabolites, including 4-imidazoleacetate, cytidine, hippurate, hypoxanthine, kynurenine, L-carnitine, O-acetylcarnitine, ophthalmate, proline, and spermidine. The higher abundance of unique SD metabolites in the abdomen was not surprising, given the critical function of reproduction in abdominal tissues, and previous findings of sexual dimorphism in the *Drosophila* gut-gonad axis (Ahmed *et al*., 2020; Hudry *et al*., 2019; Regan *et al*., 2016). These findings demonstrate that the influence of *dsx* on sex differences in the metabolome is highly tissue-specific, with certain metabolites showing sex differences consistently across all tissues while others are unique to specific tissues. This tissue-specificity could provide insights into how metabolic sex differences contribute to the diverse physiological and behavioral traits observed between sexes.

Dsx-influenced metabolites are enriched in BCAA and mTOR pathways

We next conducted pathway enrichment analysis for the 8 metabolites most influenced by *dsx* across all three tissues, previously termed "SD^{dsx}" (Table 2). The most significant pathways included those involved in amino acid metabolism, signaling pathways, and transport systems. One of the most significantly enriched pathways was the "Valine, leucine and isoleucine degradation" (KEGG ID: dme00280, FDR < 0.005). This pathway, along with its biosynthesis counterpart (KEGG ID: dme00290), suggests a critical role of *dsx* in sex-specific metabolic regulation of branched-chain amino acids. The enrichment of "Aminoacyl-tRNA biosynthesis" (KEGG ID: dme00970) further emphasizes the importance of protein synthesis and turnover in these metabolic differences. Additionally, the "mTOR signaling pathway" (KEGG ID: dme04150) was significantly affected. The mTOR signaling pathway, known for its role in cell growth, proliferation, and metabolism, suggests that sex differences modulated by the *dsx* gene include fundamental aspects of cellular and organismal physiology across all tissues.

Discussion

The present study used a *Drosophila* model to investigate the extent to which variations in sex characteristics (VSC) impact sex differences in the metabolome. We measured 91 targeted metabolomic features across three tissue types, comparing distinctly sex-dimorphic flies (3G) with *doublesex* (*dsx*) null flies, which exhibit reduced sexual dimorphism. Our findings underscore the importance of considering genetic mechanisms underlying sexual dimorphism when analyzing metabolic profiles.

The significant reduction in sex differences in metabolites observed in *dsx* null flies compared to 3G flies is a key finding of this study. The *dsx* gene plays a pivotal role in regulating sexual dimorphism at the molecular level, directing sex-specific gene expression. In the absence of functional *dsx*, this regulation is disrupted, leading to a convergence of metabolic profiles between XX and XY flies. This finding underscores the crucial role of *dsx* in mediating sex-specific metabolic pathways and highlights the importance of considering genetic mechanisms underlying sexual dimorphism in metabolomic studies. By demonstrating that over 50% of metabolites showed significant sex differences in 3G flies, while only 1 of 91 differed between XX and XY flies in all tissues in *dsx* null flies, the study emphasizes how VSC can significantly influence the metabolome.

One notable exception to the trend of reduced sex differences in *dsx* null flies was kynurenate, a metabolite that consistently displayed higher levels in XX flies, regardless of the presence or absence of *dsx*. This suggests that sex-specific patterns of kynurenate are regulated by mechanisms independent of *dsx*. Two key genes in the *Drosophila* tryptophan-kynurenine (Tryp-Kyn) degradation pathway are *vermilion* and *white* (Green, 1949; Mackenzie *et al*., 1999; Rizki, 1964). Both genes are on the X chromosome (Levis *et al*., 1982; Walker *et al*., 1986), indicating a significant genetic control point for sex-linked metabolic regulation in line with our results. This supports the argument that sex chromosome composition directly influences the metabolism of tryptophan and its derivatives, such as kynurenate, potentially contributing to the observed sex differences independent of *dsx*. Although sex differences in kynurenate levels are not well understood, there is some evidence from human studies suggesting sex-specific variations. For example, tryptophan metabolism is often impaired in various neurological and psychiatric disorders (Gostner *et al*., 2020), which frequently manifest in a sex-specific manner (Wickens *et al*., 2018). However, studies on sex differences in kynurenine metabolites have yielded inconsistent results, with some reporting lower plasma levels of kynurenate in women, while others find no significant differences between sexes (Pais *et al*., 2023). Kynurenate and a high kynurenate-to-kynurenine ratio were associated with poorer clinical outcomes in male COVID-19 patients (Cai *et al*., 2021), indicating a sex-related metabolic and immune interaction. More research is needed to better understand whether relationships between sex-related factors and Tryp-Kyn pathway functions might be conserved across species.

The variation in tissue-specific sex differences between 3G and VSC flies were another critical finding of this study. Different tissues exhibited unique sets of SD^{dsx} metabolites, indicating that *dsx* influences sex differences in metabolism in a highly tissue-specific manner. Clough *et al*. (2014) identified that *DSX*F and *DSX*M bind thousands of the same targets across multiple tissues

but result in sex- and tissue-specific functions. This implies that the same *dsx* gene can direct different developmental and metabolic outcomes depending on the tissue context, which aligns with our observation of tissue-specific SD^{dsx} metabolites. Rice *et al*. (2019) later demonstrated that the expression of *dsx* in *Drosophila* is controlled by separate modular enhancers responsible for sex-specific traits in different organs, suggesting that the regulation of *dsx* is finely tuned and varies across different tissues. This modular regulation likely contributes to the distinct metabolic profiles observed in the head, thorax, and abdomen of the flies. These findings support the idea that sex differences in metabolism are driven by highly localized genetic controls. The ability of *dsx* to modulate the degree of sex difference in metabolic profiles through tissue-specific regulation underscores how VSC, such as those caused by differences in the *dsx* gene, can significantly influence the metabolome and contribute to diversity in sex-related traits.

We observed that the thorax tissue, which houses much of the fat body, shared more SD^{dsx} metabolites with both head and abdomen than head and abdomen shared with each other. This could reflect the fat body's role as a key organ for interorgan communication, regulating metabolism and developmental processes by releasing adipokines in response to nutritional and hormonal signals (Meschi & Delanoue, 2021). *Transformer* (*tra*) is a gene that both regulates the sex-specific splicing of *dsx* pre-mRNA (Hoshijima *et al*., 1991) and acts in Adipokinetic hormone (Akh)-producing cells to regulate sex-specific body fat levels (Wat *et al*., 2021). While it's unclear in which organ specifically *dsx* is acting to create metabolic distinctions between sexes, Clough *et al*. (2014), identified 25 genes in the fat body that were differently regulated by *DSX*F and *DSX*M isoforms. Further experiments are needed to distinguish the roles of *dsx* and downstream genes on metabolites in the fat body and other organs. As sex differences in the metabolome were reduced but not completely depleted in *dsx* null flies, this also implies that the indirect regulatory effects of *tra* on the metabolome are not entirely mediated through *dsx*. These findings illustrate that sex differences in metabolite levels can result from the polygenic effects of multiple sex-related genes, creating a continuum of metabolic distinctions.

Pathway enrichment for the 8 SD^{dsx} metabolites that were shared across all three tissues revealed a significant enrichment of branched-chain amino acid (BCAA) synthesis, degradation and TOR pathways. Sex differences in all three BCAAs (leucine, isoleucine and valine) were significantly reduced in our VSC flies. BCAAs are potent activators of TOR signaling (Juricic *et al*., 2019), which is crucial for regulating various metabolic processes. The significant reduction in BCAA-related metabolites in our VSC flies suggests that *dsx* plays a pivotal role in maintaining sex differences in BCAAs and, possibly, TOR pathway activation as a result. This reduction in BCAA sex differences in the absence of *dsx* indicates that sex-specific regulation of the TOR

pathway is partly mediated by *dsx* or one of its downstream targets. The TOR pathway also mediates the longevity effects of dietary restriction (Katewa & Kapahi, 2011), which has sex-specific outcomes in *Drosophila* (Magwere *et al*., 2004). In mice, restricting dietary BCAAs increased lifespan and metabolic health in males but not females (Richardson *et al*., 2021). This supports the idea that sex-specific regulation of nutrient metabolism, including BCAAs, contributes to the differential effects of dietary restriction observed between sexes. Further research on the role of *dsx* in regulating BCAAs may provide insights into mechanisms regulating sex-specific outcomes in *Drosophila* longevity interventions.

<u>Limitations</u>

This study has several limitations that should be considered. First, our fly lines were generated in a w^{1118} background. While commonly used in *Drosophila* research, this genetic background may have specific metabolic characteristics that could influence the results, making the findings less universally applicable to all *Drosophila* strains or other species. Second, the mini-white gene, used to mark the *dsx* null allele, might influence the metabolic profile, introducing another potential confounding factor. Both factors are especially important to consider in the context of our findings on Tryp-Kyn metabolites, as discussed earlier, as the *white* gene is a key component of this pathway. Third, our crossing scheme utilized the Bar-stone Y-linked marker to distinguish XX and XY flies, as *dsx* null XX and XY flies are otherwise indistinguishable by eye. While effective for identifying sex chromosomes, its presence could potentially affect metabolic processes in a sex-specific manner. Lastly, the study examined head, thorax, and abdomen tissues, each of which includes multiple organs. Our tissue-specific results cannot be attributed to any single organ.

<u>Conclusion</u>

The findings from this study have several important implications for our understanding of variation in and regulation of physiological sex differences. First, they underscore the necessity of accounting for genetic mechanisms underlying sexual dimorphism, such as the role of the *doublesex* gene in *Drosophila*. The absence of *dsx* leads to a significant reduction in sex differences in the metabolome, indicating that genetic context is crucial for understanding and interpreting sex differences in metabolic profiles. Second, this work highlights the importance of considering variations in sex characteristics when analyzing metabolic data. Traditional binary classifications of sex may overlook significant metabolic variations associated with forms of VSC, leading to potential misinterpretations. Including intersex individuals in metabolomic analyses can provide a more accurate and nuanced understanding of sex-specific metabolic pathways. Third,

the tissue-specific nature of *dsx* influence on the metabolome suggests that metabolomic studies should analyze multiple tissues to capture the full spectrum of sex differences. Different tissues have unique metabolic demands and are differentially influenced by sex-specific genetic regulation. Comprehensive tissue-specific analyses can reveal insights into how sex differences manifest at the organ and systemic levels.

While this study is conducted in *Drosophila*, the implications extend to human health research. The findings suggest that similar genetic mechanisms may influence sex differences in human metabolism. Researchers should consider genetic and phenotypic variations in sex characteristics when designing and interpreting metabolomic studies in humans, especially for conditions related to metabolic disorders, endocrine functions, and reproductive health. Recognizing the role of VSC and genetic regulation in metabolism can enhance the precision of personalized medicine approaches. By tailoring metabolic analyses and interventions to account for individual genetic and sex-specific factors, healthcare providers can improve the accuracy and effectiveness of treatments. In conclusion, this study highlights the critical need for metabolomics research to incorporate genetic and phenotypic diversity related to sex characteristics. By doing so, researchers can gain deeper insights into the metabolic underpinnings of sex differences, ultimately advancing our understanding of health and disease.

Tables

Table 1. Summary of results for sex effect sizes in metabolite level by tissue. Metabolites with a significant effect of sex (referred to as SD metabolites) at FDR <.05. Effect size (ES) is calculated using TukeyHSD function in R.

		N Metabolites FDR <.05	% Metabolites FDR <.05	Mean ES Metabolites FDR <.05	Mean ES All Metabolites
3G	Head	23	25%	0.16	0.13
	Thorax	33	36%	0.14	0.10
	Abdomen	30	33%	0.22	0.15
VSC	Head	1	1%	0.10	0.10
	Thorax	1	1%	0.07	0.06
	Abdomen	1	1%	0.08	0.08

Table 2. Tissue-specific sex effect sizes for metabolites enriched for in FELLA analysis. Effect size is calculated between XX and XY flies using TukeyHSD function in R. A positive effect size indicates values are higher in XY flies.

Head	Effect Size		Adjusted p-value (FDR)	
	3G	VSC	3G	VSC
Deoxycarnitine	-0.2	0.1	2.52E-05	4.19E-01
Glutamate	0.1	0.0	8.87E-03	1.00E+00
Isoleucine	-0.3	-0.1	5.19E-07	6.67E-02
Leucine	-0.3	-0.1	4.43E-06	2.52E-01
ADMA	-0.2	0.0	1.30E-03	1.00E+00
Phosphorylcholine	0.1	0.0	4.92E-02	1.00E+00
Tyrosine	-0.2	0.0	4.34E-02	1.00E+00
Valine	-0.2	-0.1	1.18E-05	2.22E-01
Thorax	**Effect Size**		**Adjusted p-value (FDR)**	
	3G	VSC	3G	VSC
Deoxycarnitine	-0.2	0.1	1.06E-04	1.50E-01
Glutamate	0.1	0.0	1.88E-02	1.00E+00
Isoleucine	-0.3	-0.1	1.49E-05	8.54E-02
Leucine	-0.3	-0.1	1.25E-05	8.54E-02
ADMA	-0.3	0.0	2.90E-04	1.00E+00
Phosphorylcholine	0.1	0.0	3.71E-03	1.00E+00
Tyrosine	-0.1	0.0	2.00E-04	8.89E-01
Valine	-0.2	-0.1	1.50E-05	1.00E-01
Abdomen	**Effect Size**		**Adjusted p-value (FDR)**	
	3G	VSC	3G	VSC
Deoxycarnitine	-0.2	0.1	1.14E-02	1.50E-01
Glutamate	0.1	0.0	4.77E-02	1.00E+00
Isoleucine	-0.2	-0.1	7.47E-04	7.18E-01
Leucine	-0.2	0.0	8.15E-04	1.00E+00
ADMA	-0.3	-0.1	7.35E-04	1.00E+00
Phosphorylcholine	0.1	0.0	4.95E-02	1.00E+00
Tyrosine	-0.1	0.0	5.95E-03	6.96E-01
Valine	-0.2	0.0	1.06E-04	6.90E-01

Figures

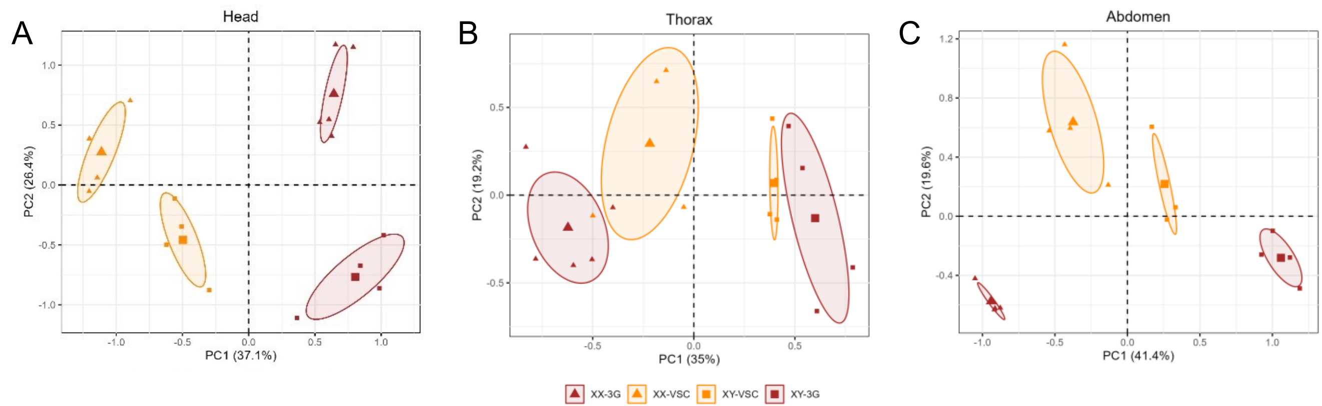


Figure 1. PCA of metabolome samples by tissue. PCA for (A) head tissue, (B) thorax tissue and (C) abdomen tissue. VSC flies are colored in orange, 3G flies are colored in brown. Triangles represent XX and squares represent XY fly samples.

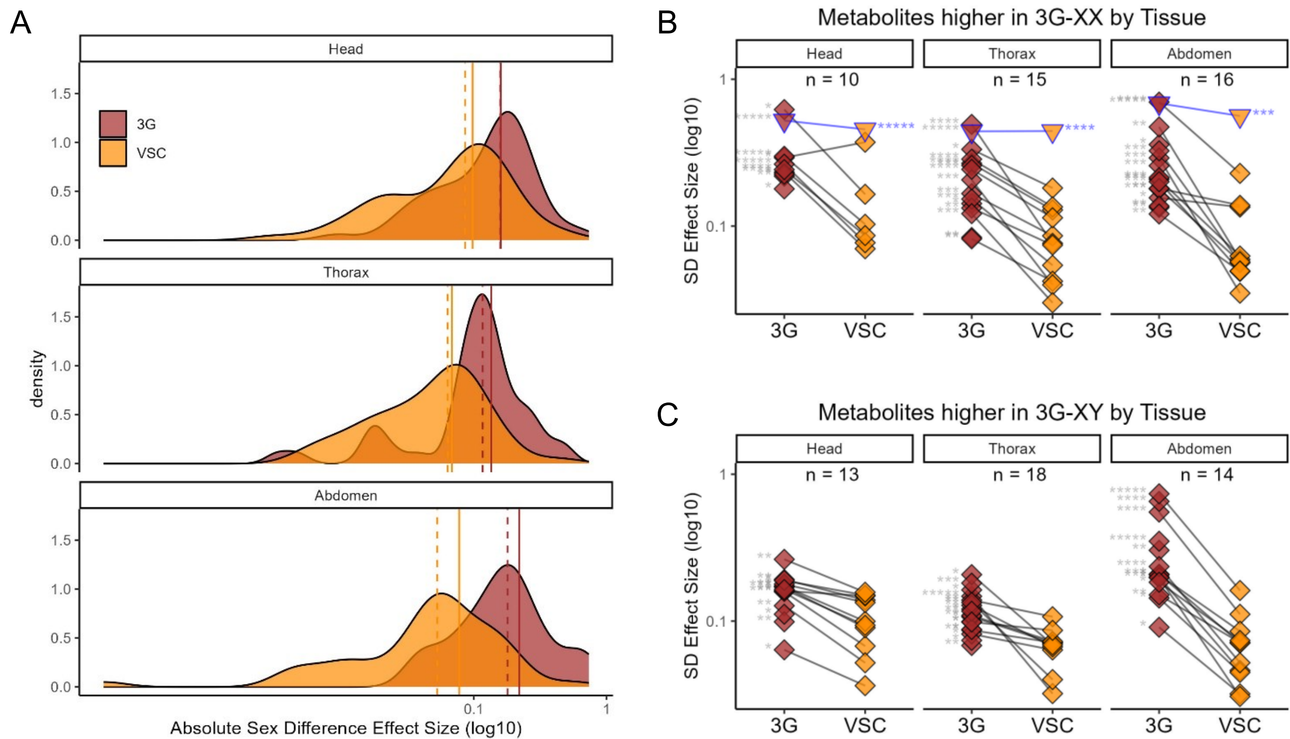


Figure 2. Effect sizes for metabolites with significant sex effects in 3G and VSC groups. (A) Density plot for absolute sex difference effect sizes in 3G and VSC flies. Dashed line marks the median effect size across SD metabolites for each group, solid line marks the mean value for effect size across SD metabolites for each group. (B-C) Effect sizes for SD metabolites, stratified by sex bias in metabolite level. SD metabolites higher in XX flies (B) include kynurenate, denoted in triangle, the one metabolite significantly different between XX and XY flies in both 3G and VSC groups. (C) SD metabolites higher in XY flies. No metabolites other than kynurenate were significantly different between VSC XX and XY flies. Effect sizes are plotted on a log10 scale.

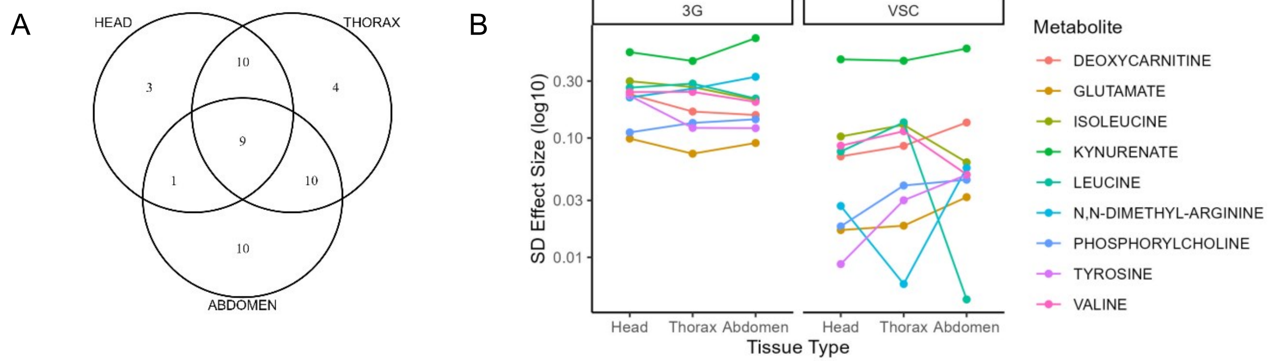


Figure 3. Tissue specificity of SD metabolites. (A) Venn diagram of SD metabolites across head, thorax and abdomen tissues. (B) Sex effect sizes for 9 SD metabolites significantly affected in all tissues, as a function of tissue type for 3G and VSC flies. Effect sizes are calculated from the TukeyHSD function in R. Kynurenate, in green, is the one metabolite that maintains a significant sex difference in both SD groups.

Supplementary Tables

Supplementary Table 1. Tissue-specific TukeyHSD results for all metabolites. Effect size (ES) is calculated between XX and XY flies using TukeyHSD function in R. A positive effect size indicates values are higher in XY flies. Significant effect sizes (<.05 FDR) are highlighted in green.

Head	Effect Size		Adjusted p-value (FDR)	
	3G	VSC	3G	VSC
1-METHYL-L-HISTIDINE	0.2	0.1	2.90E-04	3.41E-01
3-NITRO-L-TYROSINE	-0.3	0.4	1.79E-02	6.66E-02
4-IMIDAZOLEACETATE	0.1	0.0	9.36E-01	1.00E+00
6-CARBOXYHEXANOATE	0.2	0.1	5.66E-04	2.26E-01
ADENINE	0.2	0.1	6.98E-02	5.66E-01
AGMATINE SULFATE	-0.1	0.0	3.25E-01	1.00E+00
AMINOADIPATE	0.2	0.1	9.08E-03	2.59E-01
AMINOISOBUTANOATE	0.0	0.1	1.00E+00	1.00E+00
ARGININE	0.1	0.0	1.53E-02	5.93E-01
AZELATE	0.2	0.1	3.14E-03	3.38E-01
BENZOATE	0.2	0.1	3.41E-02	4.19E-01
CADAVERINE	-0.6	-0.2	1.14E-02	1.00E+00
CITICOLINE	0.1	0.1	9.03E-03	8.72E-01
CYTIDINE	0.2	0.1	3.10E-01	1.00E+00
DEOXYCARNITINE	-0.2	0.1	2.52E-05	4.19E-01
DEOXYGUANOSINE	-0.2	-0.3	9.02E-02	1.15E-01
GAMMA-AMINOBUTYRATE	0.2	0.1	6.97E-03	1.15E-01
GLUTAMATE	0.1	0.0	8.87E-03	1.00E+00
GUANINE	-0.1	-0.2	2.02E-01	4.46E-01
GUANOSINE	-0.1	-0.1	3.25E-01	1.00E+00
HIPPURATE	0.1	0.1	2.10E-01	7.62E-01
HISTAMINE	0.0	-0.2	1.00E+00	5.68E-01
HYPOXANTHINE	0.0	0.0	1.00E+00	1.00E+00
ISOLEUCINE	-0.3	-0.1	5.19E-07	6.67E-02
KYNURENATE	-0.5	-0.5	4.74E-09	5.73E-08
KYNURENINE	0.1	-0.1	1.00E+00	1.00E+00
L-CARNITINE	0.0	0.1	4.89E-01	2.26E-01
LEUCINE	-0.3	-0.1	4.43E-06	2.52E-01
N-ACETYLPUTRESCINE	0.1	0.0	3.40E-01	1.00E+00
N-METHYLGLUTAMATE	0.2	0.2	1.08E-02	2.26E-01
N,N-DIMETHYL-ARGININE	-0.2	0.0	1.30E-03	1.00E+00
N,N,N-TRIMETHYLLYSINE	-0.2	0.0	4.25E-02	1.00E+00
N1-ACETYLSPERMINE	-0.1	-0.2	9.50E-01	6.09E-01
NICOTINAMIDE MONONUCLEOTIDE	0.3	0.2	6.65E-03	4.15E-01
O-ACETYLCARNITINE	0.0	0.1	9.36E-01	7.18E-01
OPHTHALMATE	0.1	0.1	7.78E-01	1.00E+00
PANTOTHENATE	0.0	-0.1	7.22E-01	1.50E-01
PHENYLALANINE	0.0	0.0	4.53E-01	1.00E+00
PHENYLETHANOLAMINE	0.0	0.0	1.36E-01	1.00E+00
PHOSPHORYLCHOLINE	0.1	0.0	4.92E-02	1.00E+00
PROLINE	0.2	0.1	2.68E-01	1.00E+00

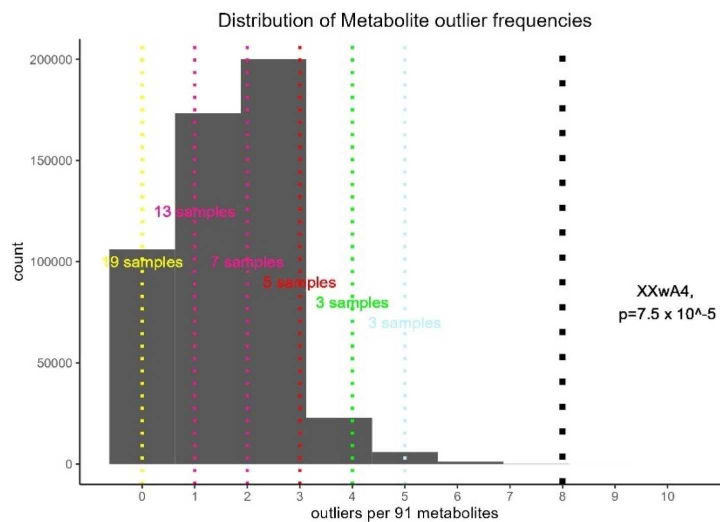
PUTRESCINE	-0.3	0.0	8.05E-02	1.00E+00
S-ADENOSYLMETHIONINE	0.0	0.0	1.00E+00	1.00E+00
SEBACATE	0.2	0.1	1.70E-02	6.09E-01
SPERMIDINE	-0.2	0.0	6.14E-01	1.00E+00
TYROSINE	-0.2	0.0	4.34E-02	1.00E+00
VALINE	-0.2	-0.1	1.18E-05	2.22E-01

Thorax	**Effect Size**		**Adjusted p-value (FDR)**	
	3G	**VSC**	**3G**	**VSC**
1-METHYL-L-HISTIDINE	0.1	0.0	8.33E-06	6.09E-01
3-NITRO-L-TYROSINE	0.0	0.2	1.00E+00	1.00E+00
4-IMIDAZOLEACETATE	0.0	0.0	9.65E-01	1.00E+00
6-CARBOXYHEXANOATE	0.1	0.1	9.03E-03	1.97E-01
ADENINE	0.1	0.1	8.87E-03	2.26E-01
AGMATINE SULFATE	0.1	0.0	1.69E-02	1.00E+00
AMINOADIPATE	0.1	0.1	3.85E-02	5.93E-01
AMINOISOBUTANOATE	-0.1	0.0	1.53E-02	1.00E+00
ARGININE	0.1	0.0	1.28E-03	1.00E+00
AZELATE	0.1	0.1	4.37E-02	4.26E-01
BENZOATE	0.1	0.1	1.57E-03	1.91E-01
CADAVERINE	-0.3	-0.2	4.70E-02	8.36E-01
CITICOLINE	0.1	0.0	3.71E-02	1.00E+00
CYTIDINE	0.0	-0.1	1.00E+00	1.00E+00
DEOXYCARNITINE	-0.2	0.1	1.06E-04	1.50E-01
DEOXYGUANOSINE	-0.5	-0.1	2.91E-05	1.00E+00
GAMMA-AMINOBUTYRATE	0.0	0.1	1.00E+00	7.28E-01
GLUTAMATE	0.1	0.0	1.88E-02	1.00E+00
GUANINE	-0.1	-0.1	5.20E-03	4.19E-01
GUANOSINE	-0.2	-0.1	2.90E-04	5.45E-01
HIPPURATE	0.1	0.0	4.76E-01	1.00E+00
HISTAMINE	0.2	0.0	1.35E-02	1.00E+00
HYPOXANTHINE	0.0	0.1	1.00E+00	1.00E+00
ISOLEUCINE	-0.3	-0.1	1.49E-05	8.54E-02
KYNURENATE	-0.4	-0.4	4.43E-06	2.30E-05
KYNURENINE	-0.1	-0.1	6.03E-01	1.00E+00
L-CARNITINE	0.0	0.0	1.00E+00	9.63E-01
LEUCINE	-0.3	-0.1	1.25E-05	8.54E-02
N-ACETYLPUTRESCINE	0.1	0.0	2.48E-02	1.00E+00
N-METHYLGLUTAMATE	0.1	0.1	2.96E-03	4.19E-01
N,N-DIMETHYL-ARGININE	-0.3	0.0	2.90E-04	1.00E+00
N,N,N-TRIMETHYLLYSINE	-0.1	0.0	1.23E-01	1.00E+00
N1-ACETYLSPERMINE	0.2	-0.1	1.38E-02	1.00E+00
NICOTINAMIDE MONONUCLEOTIDE	0.1	0.0	2.20E-01	1.00E+00
O-ACETYLCARNITINE	0.0	0.1	1.00E+00	1.00E+00
OPHTHALMATE	0.1	0.1	2.20E-01	2.84E-01
PANTOTHENATE	0.2	0.0	7.88E-04	1.00E+00
PHENYLALANINE	-0.1	0.0	1.30E-03	8.36E-01
PHENYLETHANOLAMINE	-0.1	0.0	1.58E-03	3.75E-01
PHOSPHORYLCHOLINE	0.1	0.0	3.71E-03	1.00E+00
PROLINE	0.1	0.1	5.10E-02	2.78E-01
PUTRESCINE	-0.2	0.0	1.06E-04	1.00E+00
S-ADENOSYLMETHIONINE	0.1	0.0	7.53E-03	1.00E+00
SEBACATE	0.1	0.1	2.73E-02	2.53E-01

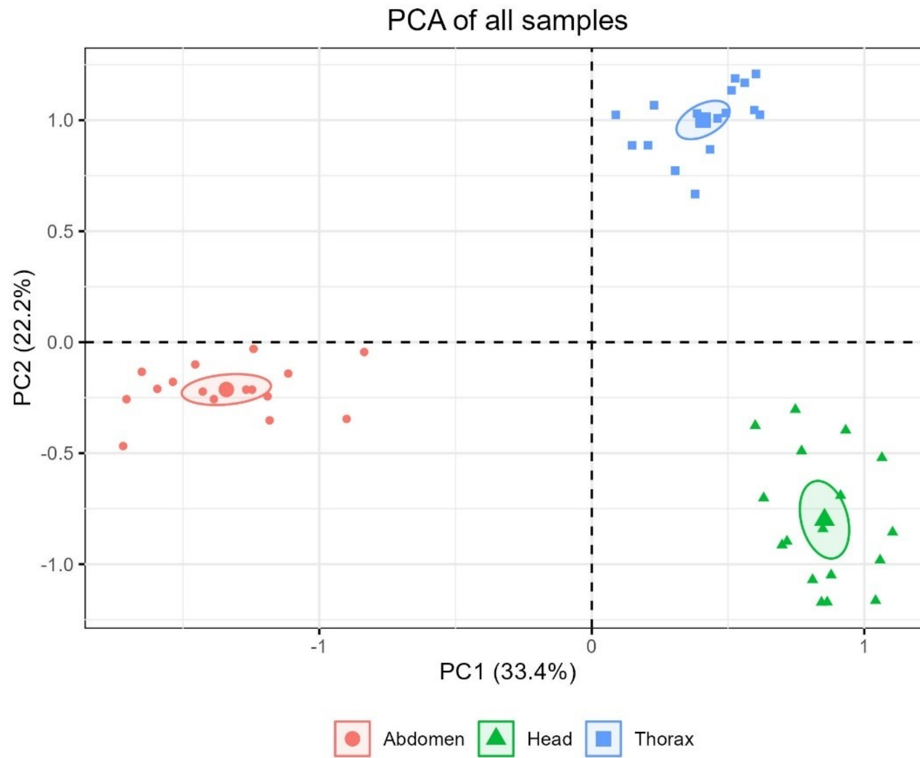
SPERMIDINE	0.0	0.0	9.39E-01	1.00E+00
TYROSINE	-0.1	0.0	2.00E-04	8.89E-01
VALINE	-0.2	-0.1	1.50E-05	1.00E-01

Abdomen	Effect Size		Adjusted p-value (FDR)	
	3G	VSC		3G
1-METHYL-L-HISTIDINE	0.1	0.1	6.98E-02	5.68E-01
3-NITRO-L-TYROSINE	0.0	0.2	1.00E+00	1.00E+00
4-IMIDAZOLEACETATE	0.2	0.1	2.83E-02	1.00E+00
6-CARBOXYHEXANOATE	0.1	0.1	6.15E-01	2.26E-01
ADENINE	0.6	0.1	1.20E-05	6.29E-01
AGMATINE SULFATE	0.2	0.0	8.69E-04	1.00E+00
AMINOADIPATE	0.1	0.0	1.96E-01	1.00E+00
AMINOISOBUTANOATE	-0.2	0.1	1.33E-01	1.00E+00
ARGININE	0.0	0.0	1.29E-01	1.00E+00
AZELATE	0.1	0.1	5.28E-01	5.45E-01
BENZOATE	0.1	0.2	3.25E-01	2.59E-01
CADAVERINE	0.0	0.0	9.36E-01	1.00E+00
CITICOLINE	-0.1	0.0	7.02E-01	1.00E+00
CYTIDINE	-0.4	0.0	1.79E-02	1.00E+00
DEOXYCARNITINE	-0.2	0.1	1.14E-02	1.50E-01
DEOXYGUANOSINE	-0.5	-0.1	1.54E-03	1.00E+00
GAMMA-AMINOBUTYRATE	0.0	0.1	1.00E+00	1.00E+00
GLUTAMATE	0.1	0.0	4.77E-02	1.00E+00
GUANINE	-0.3	0.0	1.06E-04	1.00E+00
GUANOSINE	-0.3	0.0	2.90E-04	1.00E+00
HIPPURATE	0.3	0.0	1.14E-03	1.00E+00
HISTAMINE	0.2	0.0	1.02E-01	1.00E+00
HYPOXANTHINE	0.7	0.0	2.37E-05	1.00E+00
ISOLEUCINE	-0.2	-0.1	7.47E-04	7.18E-01
KYNURENATE	-0.7	-0.6	6.19E-06	1.30E-04
KYNURENINE	-0.7	-0.2	1.49E-05	2.26E-01
L-CARNITINE	0.2	0.1	8.44E-04	4.19E-01
LEUCINE	-0.2	0.0	8.15E-04	1.00E+00
N-ACETYLPUTRESCINE	0.3	0.0	5.20E-08	1.00E+00
N-METHYLGLUTAMATE	0.1	0.1	9.15E-02	6.96E-01
N,N-DIMETHYL-ARGININE	-0.3	-0.1	7.35E-04	1.00E+00
N,N,N-TRIMETHYLLYSINE	-0.2	-0.1	8.15E-04	6.84E-01
N1-ACETYLSPERMINE	0.7	0.2	1.05E-06	2.53E-01
NICOTINAMIDE MONONUCLEOTIDE	0.1	0.1	7.55E-01	1.00E+00
O-ACETYLCARNITINE	0.2	0.0	1.08E-02	1.00E+00
OPHTHALMATE	-0.2	-0.1	5.35E-03	1.50E-01
PANTOTHENATE	0.0	0.0	9.36E-01	1.00E+00
PHENYLALANINE	-0.1	0.0	1.57E-03	1.00E+00
PHENYLETHANOLAMINE	-0.1	0.0	1.30E-03	1.00E+00
PHOSPHORYLCHOLINE	0.1	0.0	4.95E-02	1.00E+00
PROLINE	0.2	0.1	8.87E-03	6.09E-01
PUTRESCINE	0.1	0.0	1.27E-01	1.00E+00
S-ADENOSYLMETHIONINE	0.2	0.1	1.18E-05	5.18E-01
SEBACATE	0.1	0.2	8.02E-01	3.41E-01
SPERMIDINE	0.2	0.1	4.77E-02	1.00E+00
TYROSINE	-0.1	0.0	5.95E-03	6.96E-01
VALINE	-0.2	0.0	1.06E-04	6.90E-01

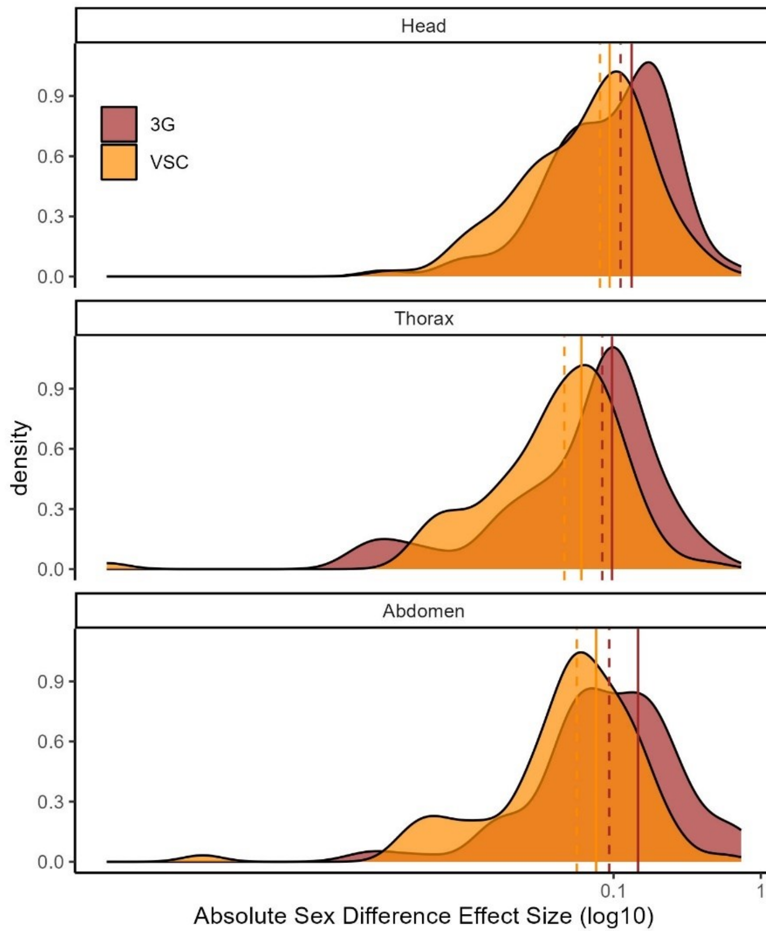
Supplementary Figures



Supplementary Figure 1. Outlier test for aberrant sample. Null distribution of metabolite outlier frequencies across samples after 10,000 permutations. All samples except XXwA4 had fewer than 7 metabolite outliers, whereas sample XXwA4 had 8 outliers. Sample XXwA4 was removed prior to analyses.



Supplementary Figure 2. PCA of all samples by tissue type. Abdomen samples are colored in pink, head samples in green and thorax samples in blue.



Supplementary Figure 3. Effect sizes for all metabolites in 3G and VSC groups. Density plot for absolute sex difference effect sizes in 3G and VSC flies. Dashed line marks the median effect size across all metabolites for each group, solid line marks the mean value for effect size across all metabolites for each group. Effect sizes are plotted on a log10 scale.

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VITA

Rene Coig was born and raised in Louisiana. Being a different sort of child, Rene spent a lot of time wondering why some people are different. After completing a B.A. in Sociology at Louisiana State University, he moved to Seattle, WA where he has lived for the past 22 years. During his time in Washington state, he has worked in the game industry, run a freelance animation business and raised money for LGBTQ mental health. In 2017, after considering it for two years, he decided to get a PhD in biomedical research even though he had not taken biology or advanced math since high school. After seeing an animated video of DNA polymerase, he was hooked. In 2020 he completed his B.S. in Molecular, Cellular, and Developmental Biology at the University of Washington and was accepted into the Molecular Medicine and Mechanisms of Disease PhD program. Now he spends a lot more time thinking about what makes people the same.