Diet-Induced Insulin Resistance & Exercise in *Drosophila melanogaster* is Highly Influenced by Genotype & Sex

by

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Abstract

Type 2 Diabetes, characterized by a combination of insulin resistance and impaired insulin secretion, is a serious metabolic disorder in humans that not only affects adults, but now young children. The model organism *Drosophila melanogaster* has emerged as an excellent model to study metabolic regulation. Using a high sucrose diet to induce insulin resistance, I used multiple fly lines to explore how genotype and sex interacts with the progression of insulin resistance and how exercise can influence this response. Flies were grouped and placed on one of four conditions: control diet (CD), high sucrose diet (HSD), control diet with exercise (CDEx), or high sucrose diet with exercise (HSDEx). Flies were exercised using a fly treadmill. Weight and various metabolites known to respond to diet and exercise were quantified, along with gene expression of various nodes in the insulin pathway. Twenty-five percent of fly lines on the HSD displayed symptoms of insulin resistance, such as hyperglycemia, glucose intolerance, and/or hyperinsulinemia, as well as patterns in insulin pathway gene expression that is indicative of insulin resistance. Flies experienced physiological changes in response to exercise, which changed 11-13% of the metabolome of each sex, and 20-29% of the metabolome of each line. Strikingly, across all experiments, fly response to diet and exercise was highly genotype and sex-dependent. This study demonstrates the complex nature of insulin resistance and most importantly, the importance of studying disease related states using multiple genetic backgrounds and both sexes.

Keywords

Type 2 Diabetes, insulin resistance, Drosophila melanogaster, exercise, metabolome

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List of Abbreviations

μL: microlitre

AKT: Protein Kinase B

Akt: *Protein Kinase B* gene

ANCOVA: Analysis of co-variance

CD: Control diet

CDEx: Control diet with exercise

dL: decilitre

DGRP: *Drosophila* Genetic Reference Panel

DILP: *Drosophila* insulin-like peptide

Dilp: *Drosophila insulin-like peptide* gene

HSD: High sucrose diet

HSDEx: High sucrose diet with exercise

IIS: Insulin/insulin-like growth factor signaling pathway

InR: Insulin-like receptor protein

InR: *Insulin-like receptor* gene

IRS: Insulin receptor substrate

Chico: *Insulin receptor substrate* gene

IPCs: Insulin producing cells

REQS: Rotating Exercise Quantification System

Tukey's HSD Test: Tukey's Honestly Significant difference test

T2D: Type 2 Diabetes

Chapter 1

1 General Introduction

Type 2 Diabetes (T2D) is a serious human metabolic disorder that develops as a result of insulin resistance and insulin deficiency and can cause serious microvascular and macrovascular complications. T2D affects individuals worldwide and causes significant physical, psychological, and financial stress to both the individual and health care system (Chatterjee *et al*. 2017). This disorder develops from several factors including being overweight or obese, advanced age, family history of diabetes, ethnicity, and level of physical inactivity (Okay *et al*. 2009). The progression of T2D is still poorly understood, and the complexity of the disease does not allow for many aspects to be studied using human subjects, for example, controlling for genetic background and environment, issues that can be overcome with model systems. Fortunately, *Drosophila melanogaster*, commonly known as the fruit fly, can be used as a model organism for various diseases such as Alzheimer's disease, cardiovascular disease, Parkinson's disease, and Diabetes (Pandey and Nichols 2011). *D. melanogaster* is a strong research tool as genetic background, the genetic makeup that influences biological and physiological responses, and diet can easily be controlled, aspects that are essential to disease studies. In addition, the insulin/insulin-like growth factor signaling pathway (IIS) is evolutionarily conserved between fly and mammal (Emlen *et al*. 2012; Fridell 2013) meaning that conclusions from fly studies can be easily related to mammals, including humans. The conservation of IIS pathways allow for *Drosophila* to be used to study complex diseases, in this case T2D. T2D studies using flies have shown that larvae and adult flies

raised on a high sugar diet develop hyperglycemia, insulin resistance, and obesity, as found in humans (Skorupa *et al*. 2008); Morris *et al*. 2012; Chatterjee *et al*. 2017).

Obesity, a chronic disease that is commonly linked to T2D, is clinically defined as having a body mass index (BMI) of greater than or equal to 30 kg/m^2 (Okay *et al.* 2009). Non-invasive methods to combat obesity include lifestyle changes such as incorporation of a healthy diet and exercise. Exercise has been shown to promote changes in skeletal muscle capability (Adams *et al*. 2008) and cardiac performance (Ascensão *et al*. 2007; Kemi *et al*. 2008) in both human and animals. The current challenge in the science of the exercise field is understanding how exercise response, an individual's physiological reaction to exercise, is determined, since it is known that genetic background and sex play a role in its determination. To account for genetic and environmental variability, problems when using human subjects, flies can be used as a model for exercise and diet to further understand how exercise response is determined (Rakshit *et al.* 2013; Sujkowski *et al.* 2015; Mendez *et al.* 2016; Li *et al.* 2019).

In this thesis, I use *D. melanogaster* as a model organism for insulin resistance that is induced by a high sugar diet. Using a suite of *Drosophila* Genetic Reference Panel (DGRP; Mackay 2004) fly lines, a set of 200 inbred and genetically diverse lines, I explore insulin resistance in flies. This study uses multiple genetic backgrounds to explore and understand how genotype and sex interacts with the progression of insulin resistance and how exercise can influence this response.

1.1 Type 2 Diabetes

Diabetes and related metabolic disorders are growing health problems worldwide that pose serious risks to an individual's well-being. T2D, frequently cited as a global pandemic, accounts for 90% of total diabetes cases, which may be due to increased incidence of obesity and sedentary lifestyles (World Health Organization Publications 2014; Graham and Pick 2017). T2D is characterized by a combination of both insulin resistance and insulin secretion defects, which results in hyperglycemia and insulin deficiency (Álvarez-Rendón *et al*. 2018). Insulin resistance and insulin secretion defects are modulated by primary and secondary mechanisms, as well as intrinsic and extrinsic mechanisms respectively. Primary mechanisms of insulin resistance result from impaired insulin-to-insulin-receptor signaling, and secondary mechanisms of insulin resistance, for example, would be impaired glucose uptake or inappropriately elevated glucose production (Alfa and Kim 2016). Intrinsic mechanisms of defects in insulin production/secretion are involved in cellular processes that regulate insulin transcription, translation, or secretion (Alfa and Kim 2016). Extrinsic mechanisms are associated with neuro-hormonal signals that control the steady state of insulin production or secretion (Alfa and Kim 2016).

T2D is multifactorial in nature and both genetic and environmental components play a role in its development. These factors include being overweight or obese, advanced age, family history of diabetes, ethnicity, and physical inactivity (Okay *et al*. 2009). T2D leads to microvascular and macrovascular complications that can cause psychological and physical stress to patients, caretakers, and overwhelm the health-care systems (Chatterjee *et al*. 2017). Despite the financial impact on both the global health economy and individual, long term complications may arise such

as neuropathy, retinopathy, heart disease, and stroke (Seuring *et al*. 2015; Chatterjee *et al*. 2017). The complexity and popularity of T2D worldwide increases the demand for new therapeutic interventions and further understanding molecular mechanisms associated with insulin signaling.

1.2 *Drosophila melanogaster* as a Model Organism for Type 2 Diabetes

Given the rising rates of T2D worldwide, and the severity of the health implications associated with the disease, the demand for further understanding the underlying biology has increased greatly. Since the disease is multifactorial, as the diabetic state can stem from several origins and possess different progressions, the complexity of the disease does not allow for many aspects to be studied using human patients as test subjects. *Drosophila melanogaster*, commonly known as the fruit fly, can be used as an effective model organism for various diseases such as Alzheimer's, Parkinson's, triplet repeat expansion diseases, some types of cancers, cardiovascular disease, and more specifically, Diabetes (Pandey and Nichols 2011). Nearly 75% of disease-related genes found in humans have a functional ortholog in the fly (Reiter *et al*. 2001; Lloyd and Taylor 2010). *D. melanogaster* is a powerful research tool since genetic background and diet can be controlled and manipulated easily. Further, by working with flies we are able to study multiple genotypes in a single study; an advantage over vertebrate models which are generally limited to one or a few genotypes (Guo *et al*. 2018; Petrovic *et al*. 2020). Also, because of their rapid life cycle and high fecundity, flies can produce hundreds of offspring in less than two weeks at 25 ˚C, easily allowing large sample sizes for experiments (Pandey and Nichols 2011). One benefit of working with

multiple genotypes and both sexes is that broader conclusions can be drawn from experiments, as well as analyzing genotype and sex-dependent mechanisms. In flies, genetic variation has been shown to account for a large portion of phenotypic variation, for example, in metabolic syndrome phenotypes, highlighting the importance of studying genotypes separately (Reed *et al.* 2010). Both genotype and genotype-by-diet interactions are found to be a major component of transcriptional and metabolomic variation (Reed *et al.* 2014). In addition to genotype influencing response to various stimuli, sex-dependent mechanisms are also found to be influential in *D. melanogaster*, for example, the sex-specific regulation of the IIS responsible for growth during development (ie. females are typically larger than males) (Shingleton *et al.* 2005; Gronke *et al.* 2010; Testa *et al.* 2013; Rideout *et al.* 2015; Liao *et al.* 2020). Fly sex has been found to influence mating (Kubli 2010), and more recently, both sex and genotype influence mating-induced shifts in dietary choices, and quality and quantity of the chosen diet (Camus *et al.* 2018). The ease of incorporating many genotypes and both sexes into experiments to answer questions pertaining to genotype and sex-dependent mechanisms, make flies an ideal research tool.

Alternatively, there are drawbacks to working with flies such as brain anatomy, cardiovascular and respiration systems differ substantially from humans (Prüßing and Schulz 2013). Another drawback is flies can only be maintained as living cultures as there is no permanent conservation (i.e. frozen stocks) (Prüßing and Schulz 2013). In addition, the fly's immune system is less adaptive and complex than in vertebrates (Prüßing and Schulz 2013). The ethics surrounding each vertebrate and invertebrate models revolve around the three R's (Russell and Burch 1959; Tannenbaum and Bennett 2015): Replacement, Reduction, and Refinement. Using animal models in general involves discomfort and possible pain at the animal's expense which ranges from minor

discomfort (i.e. single blood sampling) to severe discomfort (toxicity testing) (Baumans 2004). The development of protocols for minimizing pain while using invertebrates is challenging since the perception of pain and suffering is not fully understood (Adamo 2016). Working with fruit flies, an invertebrate model, can eliminate many of the ethical issues related to using human subjects or even other vertebrate models, while still controlling for genetic background and environmental factors. Nonetheless, each model species has strengths and weaknesses, but the appropriate model needs to be selected to match the question scientists are trying to ask.

1.3 *Drosophila* Insulin Signaling

Many basic biological, physiological, and neurological properties are conserved between mammals and fly. Although *Drosophila* and humans do not share all the same organs, *Drosophila* contain several organs that correspond to those found in humans such as malpighian tubules and nephrocytes (kidney), dorsal vessel (heart), the fat body (liver and adipose), and the hemolymph (blood and gastrointestinal tract) (Dhar *et al*. 2018). In addition, the *Drosophila* neuroendocrine system contains insulin producing cells (IPCs) located in the *Drosophila* brain, that are functionally equivalent to human pancreatic β-cells (Morris *et al*. 2012). These IPCs are responsible for producing and secreting three of seven *Drosophila* insulin-like peptides (DILPs; 2, 3, and 5) that are circulated in the hemolymph (Morris *et al.* 2012; Fridell 2013; Dhar *et al*. 2018). Of all DILPs, DILP2 has the highest sequence homology to mammalian pre-proinsulin, and is the most highly expressed (Broughton *et al.* 2008). The other DILPs are released from various tissues under different conditions and have diverse roles (Nässel *et al*. 2013; Semaniuk *et al*. 2018). IPC

activity, and release of the DILPs, is nutrient dependent and controlled by multiple factors such as neurotransmitters, neuropeptides, and fat body-derived proteins (Luo *et al*. 2014). Not only do *Drosophila* contain IPCs that resemble human pancreatic β-cells, they also contain cells located in the ring gland surrounding the fly heart, that produce and secrete adipokinetic hormone (AKH), the functional equivalent of human α-cells that secrete glucagon, and counterbalances insulin in humans (Haselton and Fridell 2010; Morris *et al*. 2012).

The physiological similarities between fly and mammal extends to the highly conserved IIS pathway, meaning that the most essential metabolic functions have been evolutionarily conserved (Emlen *et al*. 2012; Fridell 2013). Although insulin and insulin growth factor function as two separate activities in humans, the IIS pathway in *Drosophila* is a combined network of metabolism and growth (Figure 1) (Baker and Thummel 2007).

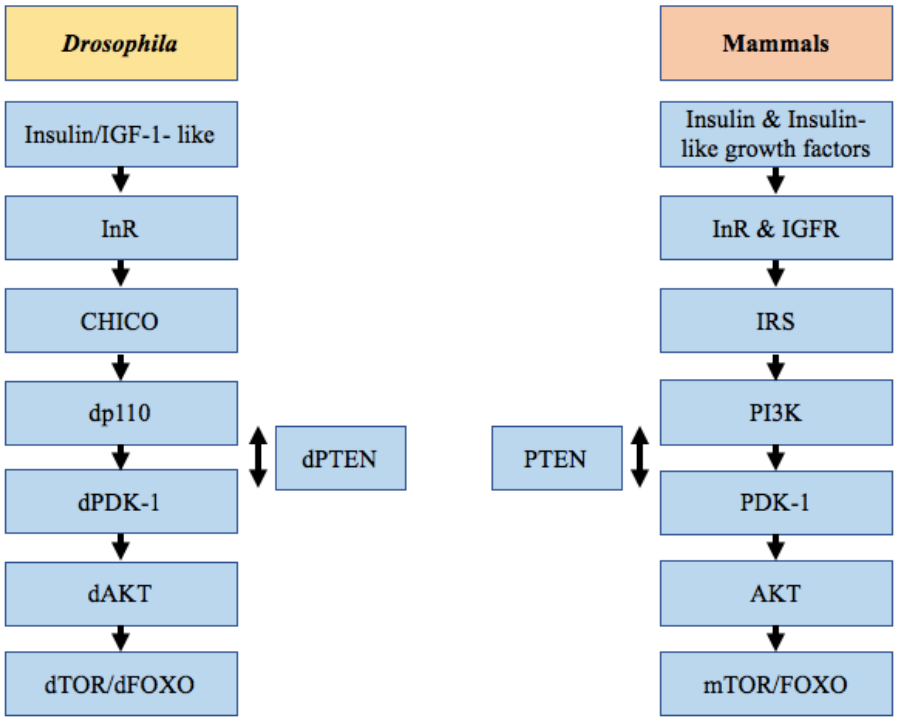

Figure 1. The evolutionary conservation of insulin signaling pathways between *Drosophila* and mammals.

The intracellular cascade is regulated by special protein kinases that transduce signals through phosphorylation (Baker and Thummel 2007). First, DILP protein binds to the single *Drosophila* insulin-like receptor protein (InR) within the cell membrane which triggers the IIS pathway. The stimulation of DILP binding to the InR results in the phosphorylation of CHICO, the homolog of the insulin receptor substrate found in mammals (IRS) (Böhni *et al*. 1999). The downstream targets of CHICO include the activation of dp110 (PI3K in mammals) and protein kinase B (AKT), which modulates the activity of a variety of proteins and signaling pathways, for example the dTOR/dFOXO pathway that regulates protein synthesis (Grewal 2009; Dhar *et al*. 2018). Not only do the IIS pathways share positive regulators, there are many conserved negative regulators

including dPTEN, an antagonist towards dp110, which is the equivalent interaction between PTEN and PI3K in the mammalian IIS pathway (Goberdhan *et al*. 1999). Defects in the IIS pathway, including compromised expression of the InR, can induce common phenotypes of a T2D state (Dhar *et al*. 2018).

1.4 Diet-Induced Insulin Resistance in *Drosophila*

By manipulating the nutrient content in fly diets, we can promote the deregulation of fly metabolism and of insulin signaling (Álvarez-Rendón *et al*. 2018). The diabetic state found in vertebrates is similar to that found in *Drosophila*, in both a diet high in sugar or fat increases initial DILP (2, 3, and 5) expression, where the organism tries to increase insulin production to compensate for the excess nutrients in the diet (Buch *et al*. 2008; Birse *et al*. 2010, Musselman *et al*. 2011). Over time in vertebrates, the deterioration of beta cells leads to the failure of initial insulin compensation (Samuel and Shulman 2016). This phenomenon is also found in overfed flies; as overfed flies age the fat body secondarily reduces its insulin signaling response to increased circulating DILPs, and that this response decreases significantly (Morris *et al*. 2012). A high sugar diet can be fed to either larvae or adult *Drosophila* to study a range of damaging effects on growth, metabolism, glucose homeostasis, cardiac function, and lifespan (Musselman *et al*. 2011; Morris *et al*. 2012; Na *et al*. 2013; Rovenko *et al*. 2015; Dhar *et al*. 2018). Adult flies and larvae that are fed a high sucrose diet develop hyperglycemia, insulin resistance, and obesity, as found in humans (Skorupa *et al*. 2008); Morris *et al*. 2012; Chatterjee *et al*. 2017). In addition, adult flies that are high sugar fed are found to have a significantly shortened lifespan (Skorupa *et*

al. 2008; Na *et al*. 2013; Lushchak et al. 2014). Using flies to try answer complex questions about T2D could provide strong insight to diabetes research and perhaps new therapeutic targets.

1.5 Physical Exercise & Obesity

Physical exercise influences various metabolic, developmental, and behavioral processes (Siddiqui *et al*. 2010). The lack of physical exercise in humans could pose two critical risks: obesity and a decline in mobility, which is associated with advanced age (Piazza *et al*. 2009; Mendez *et al*. 2016). These risks have been linked to human sedentary lifestyles and high caloric diets that have increased the number of incidences of obesity in adults and now young children (Cutler *et al*. 2002). In humans, Obesity has reached epidemic proportions worldwide with approximately 1.9 billion overweight and 650 million obese adults (Ruban *et al*. 2019). Obesity is a chronic disease that is clinically defined as having a BMI of greater than or equal to 30 kg/m^2 (Okay *et al*. 2009). Obesity is linked to a multitude of medical conditions such as hypertension, diabetes mellitus, osteoarthritis, obstructive sleep apnea, and coronary artery disease (Okay *et al*. 2009). Current treatment options include surgical interventions such as gastric bypass and pharmacotherapies, which could pose additional health risks to the individual. A series of non-invasive lifestyle choices can be used to reduce the risk of obesity which include weight-loss through a healthy diet and exercise, although the impact diet and exercise has on an individual is highly influenced by genetic background and sex.

Exercise-training has been found to promote changes in skeletal muscle capability (Adams *et al*. 2008) and cardiac performance (Ascensão *et al*. 2007; Kemi *et al*. 2008) in both human and animals. These functional effects are associated with metabolic remodeling in both human (Flück 2006; Egan and Zierath 2013) and vertebrate models (Bo *et al*. 2010; Cameron *et al*. 2012). The popularity of exercise is not as universally effective as many believe as many factors determine how an individual will respond to an exercise regime (Keith *et al*. 2006; McAllister *et al*. 2009). These factors include genetic variation and sex differences that interact with exercise, although the physiological impact of this interaction is poorly understood (Karoly *et al*. 2012). It is unclear whether single genes, epistatic interactions, epigenetics, or a combination of these factors are responsible for controlling exercise response. Given the popularity of exercise for its health benefits, it is crucial that we improve our understanding of the underlying mechanisms that determine exercise response.

1.6 *Drosophila melanogaster* as an Exercise Model

Physical exercise has long been used as a method for preventing and treating various disease conditions (Mitchell and Barlow 2011), mental health measures (Penedo and Dahn 2005), improving muscle function (Kim *et al*. 2015), and weight-loss (Petridou *et al*. 2019). Although the benefits of exercise have been long known, the mechanistic action remains unclear. The challenge in understanding exercise response comes from the lack of experimental control when using humans. Exercise studies require the control of environmental and genetic factors which can be nearly impossible to accomplish in humans for ethical reasons (Shephard 2002; Ross *et al*. 2019).

Recently, *D. melanogaster* has become a promising model system for exercise studies (Rakshit *et al.* 2013; Sujkowski *et al.* 2015; Mendez *et al.* 2016; Li *et al.* 2019). Environmental and genetic variability factors can easily be controlled. Comparative genetic resources are readily available for *Drosophila*, such as the DGRP. The DGRP lines allow for a multitude of genotypes in a single study and access to Genome Wide Association Studies (GWAS).

Drosophila have behavioral and physiological responses to exercise that have been demonstrated in a series of experiments. Fly exercise experiments generally harness the fly's innate negative geotaxis response, the natural tendency of flies to move in the opposite direction of gravity when agitated, and timed exercise bouts separated by periods of rest. Exercise regimes are generally standardized so that all experimental subjects experience the same level of exercise and exertion. In *Drosophila*, this standardization is often done using unique exercise machines, such as the Power Tower (Piazza *et al*. 2009), the TreadWheel (Mendez *et al.* 2016), the Swing Boat (Berlandi *et al.* 2017), and the Rotating Exercise Quantification System (REQS; Watanabe and Riddle 2017), that all exploit fly negative geotaxis. An early *Drosophila* exercise system, the Power Tower, lifts the flies in their enclosure (vials containing sets of flies), and then drops them repeatedly using a motorized lever system (Piazza *et al.* 2009). The dropping of the enclosures results in the animal being knocked to the bottom of the enclosure, and due to their negative geotaxis instinct, they will attempt to climb back to the top of the enclosure until they are dropped again (Piazza *et al.* 2009). This form of exercise is relatively intense and may result in collateral behavioural or physical stress-related effects (Sujkowski *et al*. 2015). Other lab groups have used partial (Swing Boat; Berlandi *et al.* 2017) or full rotations (TreadWheel; Mendez *et al.* 2016. REQS; Watanabe and Riddle 2017) of the animal enclosures to induce exercise. When the animal enclosures are slowly

rotated, the animals walk along the edge of the enclosure to continuously reach the "up" orientation, which leads the animals to exercise. The Swing Boat and REQS are able to measure activity level of the animals during exercise by recording the number of laser beam crossings, such that each time a fly crosses the centre of the vial, the crossing is recorded automatically by computer (Berlandi et al. 2017; Watanabe and Riddle 2017). These established methods for inducing exercise in *Drosophila* can easily be incorporated by other lab groups to pursue exercise-related questions.

1.7 Objectives & Hypothesis

In this thesis, I build from previous studies by combining a chronic disease, T2D, with exercise and diet and examining how these interact across a small suite of genotypes to determine how each affects the progression of insulin resistance. Previous work has determined that exercising flies regularly is beneficial for cardiac performance (Piazza *et al*. 2009), climbing mobility (Piazza *et al*. 2009), improving mitochondrial biogenesis (Bajracharya and Ballard 2018), and improved survival of Alzheimer's disease (Berlandi *et al*. 2017). Previous work with fly diets has determined that a diet rich in sucrose, mimicking that of an overfed state, induces an insulin resistant state over time in flies, similar to that of human T2D. Flies displaying insulin resistance are found to have hyperglycemia, glucose intolerance, and an initial rescue by increasing *Dilp* expression to compensate for the excess nutrients in the diet (Skorupa *et al*. 2008); Morris *et al*. 2012; Chatterjee *et al*. 2017). The focus of my study is to further understand how insulin resistance progresses and how this progression can be altered by using exercise as a positive factor on the health of the fly.

The first objective of my study is to determine the presence or absence of insulin resistance by using a suite of DGRP lines. I will quantify metabolites that are specific to glucose homeostasis and lipid metabolism including glucose, glycogen, and triglyceride content. I will be monitoring the gene expression of *Dilp2* along with *InR*, *IRS-Chico*, and *Akt* insulin pathway genes to determine the activity of the IIS pathway. While doing this, I am also studying how a high sucrose diet interacts with both sex and genetic background.

The second objective of my study is to understand how exercise interacts with a fly that displays symptoms of insulin resistance on the high sucrose diet. I want to understand how the progression is changed, if changed, when exercise is added. Flies will be exposed to 10 days of diet and 10 days of exercise. To monitor this, I will be analyzing the same metabolites and genes as previously described.

The third and final objective of my study is to understand how exercise interacts with sex and genotype at a broad metabolomic level using an untargeted metabolomic approach. By identifying changed metabolites and on a larger scale, pathway analysis, it will lead us to a better understanding of how exercise is beneficial and how exercise response is determined. Flies will be exposed to a slightly shorter exercise regime (5 day) to potentially induce changes in the metabolome.

Chapter three is a summary and exploration of future directions of this project. A better understanding of how insulin resistance progresses is needed to provide new therapeutic targets as well as translate this information to diabetes research. Combining insulin resistance with exercise is an interesting step to being able to identify how exercise positively influences T2D as well as

other chronic diseases. Determining how exercise response is regulated is crucial to our understanding of the underlying mechanisms involved and how exercise regimes need to be tailored to each individual's response.

I hypothesize that the high sucrose diet will induce insulin resistance to perhaps one, if not all fly lines and that this response can be potentially prevented by incorporating daily exercise. I do expect there to be sex differences as well as genetic background effects. I expect that flies that display insulin resistant symptoms will have hyperglycemia, glucose intolerance, as well as a downregulation in insulin pathway genes. For the exercise metabolomics, I expect we will find an abundance of changed metabolites and that will lead us to performing pathway analysis for possible mechanisms. This study will provide further insight to the progression of insulin resistance and how exercise positively influences this response, as well as give a better understanding of the regulation of exercise response.

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Chapter 2

2 Diet-Induced Insulin Resistance & Exercise in *Drosophila melanogaster* is Highly Influenced by Genotype & Sex

2.1 Introduction

The rising cases of Type 2 Diabetes (T2D) found in adults, and now young children, worldwide has pushed research to further understand the progression of insulin resistance (Xu and Verre 2018). T2D, frequently cited as a global pandemic, accounts for 90% of total diabetes cases, which may be due to increased incidence of obesity and sedentary lifestyles (World Health Organization Publications 2014; Graham and Pick 2017). T2D is characterized by a combination of insulin resistance and impaired insulin secretion, which results in hyperglycemia and insulin deficiency (Álvarez-Rendón *et al*. 2018; Xu and Verre 2018). Risk factors for T2D include being overweight or obese, advanced age, family history of diabetes, ethnicity, and physical inactivity (Okay *et al*. 2009). The multifactorial nature of T2D does not allow for many aspects to be studied using human patients as test subjects.

Drosophila melanogaster, the common fruit fly, can be used as an effective model organism for diseases such as Parkinson's, Alzheimer's, some types of cancers, cardiovascular disease, and more specifically, Diabetes (Allocca *et al*. 2018). Working with fruit flies, an invertebrate model, eliminates many ethical issues involved in using human or vertebrate models, while still controlling for genetic background and environmental factors. In *Drosophila*, both genetic background and sex have been shown to account for a large portion of phenotypic variation, for example, metabolic syndrome phenotypes and mating-induced shifts in dietary choices, respectively (Reed *et al.* 2010; Camus *et al.* 2018). Genotype and sex-specific responses to various

stimuli, such as diet, highlight the importance of studying genotypes and both sexes separately, as all respond substantially different.

Although *Drosophila* and humans do not share all the same organs, *Drosophila* do have several organs that correspond to those found in humans such as malpighian tubules and nephrocytes (kidney), dorsal vessel (heart), the fat body (liver and adipose), and the hemolymph (blood and gastrointestinal tract) (Dhar *et al*. 2018). In addition, the *Drosophila* neuroendocrine system contains insulin producing cells (IPCs) located in the *Drosophila* brain, that are functionally equivalent to human pancreatic β-cells (Morris *et al*. 2012). The *Drosophila* IPC's are responsible for producing and secreting three of seven *Drosophila* insulin-like peptides (DILPs), that are circulated in the fly hemolymph (Morris *et al*. 2012; Fridell 2013; Dhar *et al*. 2018). DILPs bind to the single insulin receptor found in flies, and this binding activates the insulin/insulin-like growth factor signaling (IIS) pathway (Morris *et al*. 2012). DILP2 is one of the three DILPs secreted from the IPC's, and its binding modulates lifespan and blood sugar in the fly (Post *et al*. 2018). Of all DILPs, DILP2 has the highest sequence homology to mammalian pre-proinsulin, and is the most highly expressed (Broughton *et al.* 2008). In addition to flies sharing many physiological similarities with humans, both fly and human utilize the highly conserved IIS pathway, making *Drosophila* an appropriate model to study Diabetes (Emlen *et al*. 2012; Fridell 2013). Previous studies analyzing transcriptional or translational regulation of the mammalian IIS pathway activity frequently examine various pathway nodes such as insulin, insulin receptor (*InR* or InR), insulin receptor substrate (*IRS* or IRS), or protein kinase B (*Akt* or AKT) (Ducluzeau *et al*. 2001; Hsieh *et al*. 2002; Jolivalt *et al*. 2008). In contrast, the equivalent IIS pathway nodes (*Dilps* or DILPs, *InR* or InR, *Chico* or CHICO, *Akt* or AKT, respectively) are examined in

Drosophila to measure transcriptional or translational IIS pathway regulation in response to various stimuli, for example diet (Morris *et al.* 2012; Naganos *et al*. 2012; Dhar *et al*. 2018; Kim *et al*. 2018; Li *et al.* 2019).

Recently, *Drosophila* has been used to study T2D. Insulin resistant phenotypes found in mammals are similar to the phenotypes found in *Drosophila* (Álvarez-Rendón *et al*. 2018). These phenotypes include hyperglycemia (elevated glucose levels), hyperinsulinemia (elevated insulin levels), insulin resistance, and increased levels of triglycerides and free fatty acids (Musselman *et al*. 2011; Álvarez-Rendón *et al*. 2018). High sucrose diet-induced insulin resistant phenotypes have been found in both larval and adult fly stages (Musselman *et al*. 2011; Morris *et al*. 2012; Pasco and Léopold 2012). In mammals, such as human or rat, fasting glucose and oral glucose tolerance tests have been used to analyze glucose clearing kinetics in suspicion of diabetic-like symptoms (Sornalakshmi *et al*. 2016; Altuve *et al*. 2016). Similarly, in *Drosophila*, fasting glucose and oral glucose tolerance tests have been developed, in which healthy flies remarkably respond with similar glucose clearing kinetics as found in mammals (Haselton *et al*. 2010; Haselton and Fridell 2011). Together, these tests are able to measure responses in glucose disposal patterns and identify glucose intolerance/tolerance in flies (Haselton *et al*. 2010).

In addition to *Drosophila* being an excellent model for diseases, *Drosophila* has recently become a promising model system for exercise studies (Rakshit *et al*. 2013; Sujkowski *et al*. 2015; Mendez *et al*. 2016; Li *et al*. 2019). Physical exercise has long been used as a method to treat various disease conditions such as obesity, although the mechanistic action of exercise remains unclear. Previous work has shown that *Drosophila* have behavioural and physiological responses to

exercise, induced by unique lab created exercise machines such as the Power Tower (Piazza *et al*. 2009), the TreadWheel (Mendez *et al*. 2016), the Swing Boat (Berlandi *et al*. 2017), and the Rotating Exercise Quantification System (REQS; Watanabe and Riddle 2017), that all exploit fly negative geotaxis. These machines use partial (Swing Boat; Berlandi *et al*. 2017) or full rotations (TreadWheel; Mendez *et al*. 2016. REQS; Watanabe and Riddle 2017) of the animal enclosures to induce exercise. Several metabolites have been shown to respond to exercise in other species (Vieira *et al*. 2009; Gordon *et al*. 2014; Hansen *et al*. 2015) and induce significant phenotypic effects in *Drosophila* such as weight, glucose, glycogen, and triglycerides (Mendez *et al*. 2016). In addition to flies responding to exercise, it has been shown that flies may be separated based on motivation, the fly's inherent tendency to remain active, and have been classified by Mendez *et al*. 2016. Flies differing in motivation class are shown to be significantly different in weight and respond differently in glucose and glycerol concentrations in response to exercise (Mendez *et al*. 2016).

The aim of this study is to better understand the progression of insulin resistance and the effect exercise may have on this progression using *Drosophila melanogaster*. Here, using four isogenic fly lines, including both sexes, flies were subjected to a high sucrose diet known to induce classical insulin resistant phenotypes. In addition to measuring fly response to a high sucrose diet, flies were subjected to a 10-day exercise regime on the Flygometer 2.0, a unique fly treadmill, to measure fly response to exercise, and diet and exercise. Flies were subjected to a fasting glucose and oral glucose tolerance test to monitor the presence or absence of glucose intolerance, a phenotype of insulin resistance. These diagnostic tests determined all flies displayed hyperglycemia, but only one line in particular, DGRP 852, displayed glucose intolerance. Weight and various metabolites

(glucose, glycogen, triglycerides, and trehalose) known to respond to diet and exercise were quantified along with transcriptional regulation of IIS pathway genes (*Dilp2*, *InR*, *Chico*, *Akt*). In general, across all experiments, fly response to diet and exercise was heavily influenced by genotype and sex, as all lines and sexes responded substantially different. Strikingly, a diet that is expected to induce characteristics of insulin resistance showed insulin resistance in one of the four lines tested. In particular, DGRP 852 displayed symptoms of classical insulin resistant phenotypes such as hyperglycemia, glucose intolerance, and/or hyperinsulinemia. Overall, these results highlight the importance of studying in multiple genetic backgrounds, including both sexes, for future studies involving insulin resistance.

2.2 Materials and Methods

2.2.1) Fly Stocks and Lines

Fly lines used in this study were obtained from the Bloomington *Drosophila* Stock Centre (Bloomington, USA). Four lines were selected from the *Drosophila* Genetic Reference Panel (DGRP; Mackay 2004) fly lines based on high and low motivation, their inherent tendency for individuals to remain active (Mendez *et al.* 2016; Jumbo-Lucioni *et al.* 2012). Previous work in the *Drosophila* exercise field using these four lines has demonstrated the influence of sex and genotype on physiological response to exercise, and additionally the influence of motivation (Mendez *et al.* 2016). These fly lines represent a diverse group of mitochondrial efficiencies that has been linked to the *Drosophila sallimus (sls)* gene, which encodes a component of the muscle sarcomere (Jumbo-Lucioni *et al.* 2012). The lines selected are DGRP 852 and DGRP 315

representing high motivation, and DGRP 380 and DGRP 189 representing low motivation. I chose these lines to analyze the interactions between diet and exercise on genetic background and sex. I included an additional fly line, BAE 318 since this line has previously shown to have symptoms that mimic human T2D after being fed a high sucrose diet (Morris *et al.* 2012).

All experiments were performed using adult male and female flies aged 1-3 days following eclosion. I chose to explore the effect of diet and exercise in adult flies since the majority of conclusions have been found in the larval stage, therefore little is known about adult fly metabolism. Additionally, since larvae spend most of their developmental stage feeding, exercise is near impossible to achieve. Aged flies were separated into groups and each group was placed on one of four conditions: control diet (CD, typical sucrose) (10% w/v), high sucrose diet (HSD) (30% w/v), high sucrose diet with exercise (HSDEx), or control diet with exercise (CDEx, typical sucrose). A diet rich in sucrose has previously been used to induce symptoms that mimic human T2D in flies (Morris *et al.* 2012). Flies were held on their respective diets for 10 days and, in the exercise groups, induced to exercise once a day for those 10 days. The fly exercise regime was modified from (Mendez *et al.* 2016) which consisted of two hours of exercise per day in time increments of 15 minutes of exercise and five minutes of rest. I exercised the flies from 1:00pm to 3:00pm each day using a fly treadmill, The Flygometer 2.0 (NORSE Industries; Figure 2). When not exercising, flies were housed in an incubator with a 12:12-hr light dark cycle at 25 ˚C. All flies were frozen by liquid nitrogen following each condition and stored at ~80 ˚C until analysis was performed.

Figure 2. Fly Treadmill 'Flygometer 2.0' used to induce gentle fly exercise. Flies were exercised in their vials for two hours a day in 15-minute time increments with five minutes of rest. The fly treadmill rotates vials along their long axis and induces the fly walking because of flies' behavioural negative geotaxis instinct.

2.2.2) Fly Wet Weight

Flies were weighed using a Mettler Toledo microbalance MX5 to the nearest 0.01 mg. Fly weight was taken to standardize differences in fly size which effects metabolite concentrations. Weight was used as a covariate in analyses of covariance (ANCOVA).

2.2.3) Fly Homogenization

Assays were performed using whole fly homogenates created by homogenizing five flies in grinding buffer (100 mM Tris-HCl, 0.15 mM $NADP^+$, pH 7.4) at a concentration of one fly per 100 μL of buffer. Flies were centrifuged at 13, 000 RPM for 10 minutes at 4 ˚C to pellet all solids. Homogenate supernatant was collected and transferred to a 96-well plate at a volume of 300 μL. Aliquots of sample were taken from this master plate for each particular assay. All assays were performed using three biological replicates and two technical replicates per sample, for each line, sex, and condition.

2.2.4) Glucose Content

To quantify glucose, I measured the production of NADH at 340 nm using 20 μl of fly homogenate from the master plate to 100 μl of glucose assay reagent (Sekisui #235-60). The glucose assay reagent contains Hexokinase which converts glucose into Glucose-6-Phosphate and then is further converted to 6-phosphogluconate and NADH by Glucose-6-phosphate dehydrogenase. Each assay was incubated at 37 ˚C for 10 minutes and measured spectrophotometrically at 340 nm. Each sample was assayed twice, and the mean was used for the analysis. Results are reported as milligram per decilitre of glucose.

2.2.5) Total Carbohydrate Content

I quantified total carbohydrate content as previously described (Merritt *et al.* 2006). Briefly, complex carbohydrates were converted to glucose using a digestion cocktail that contained 10 µl of fly homogenate sample and 2 µl of amyloglucosidase (Sigma Aldrich, St Louis, MO, A1602) at a concentration of 1 unit/sample in 2.0 M sodium acetate buffer (pH 5.7). Samples were digested at 55 ˚C for 45 minutes. Following digestion, total glucose was measured using a commercially available kit (Genzyme, Cambridge, MA, Catalog No. 235- 17). Digested homogenate was added with 200 µl of glucose reagent and incubated at 37 ˚C for 10 minutes. Sample absorbance was measured at 340 nm and total carbohydrate concentration was determined by comparison with a glycogen standard (Sigma Aldrich, St Louis, MO, Catalog No. G0885). I assayed each sample was assayed twice and used the mean for the analysis. Results are reported as milligrams per liter.

2.2.6) Soluble Triglyceride Content

I quantified soluble triglyceride using a commercially available kit (Triglyceride-SL Assay, Pointe Scientific, Canton, MI, Catalog No. T7531) following the manufacturer's protocol. Briefly, assays contained 10 µl homogenate and 100 µl reagent and were incubated at 37 ˚C for 10 minutes. Sample absorbance was measured at 500 nm and total soluble triglyceride concentrations were determined by comparison with a commercially available standard (Pointe Scientific, Canton, MI, Catalog No. T7532). Each sample was assayed twice, and the mean used in analysis. Results are reported as millimole per litre of triglyceride.

2.2.7) Trehalose Content

I quantified trehalose content as previously described (Tennessen *et al.* 2014). Flies were homogenized in trehalase buffer (5 mM Tris pH 6.6, 137 mM NaCl, 2.7 mM KCl) and the supernatant was heated for 10 minutes at 70 ˚C to halt any potential enzymatic breakdown of glycogen and trehalose into free glucose. Samples were diluted 1:4 in trehalase buffer and mixed with 30 µl of trehalase buffer in one Eppendorf tube, to determine free glucose, and an additional 30 µl of sample was mixed with trehalase stock (Sigma #T8778), to digest trehalose into free glucose. All samples were incubated at 37 ˚C for 18-24 hours. Free glucose was determined by measuring NADH at 340 nm using 20 µl of fly homogenate to 100 µl of glucose assay reagent (Sekisui #235-60). The glucose assay reagent contains Hexokinase which converts glucose into Glucose-6-Phosphate and then is further converted to 6-phosphogluconate and NADH by Glucose-6-phosphate dehydrogenase. Each assay was incubated at 37 ˚C for 10 minutes and measured spectrophotometrically at 340 nm. Each sample was assayed twice, and the mean was used for the analysis. Trehalose was calculated by subtracting the amount of free glucose from the amount of glucose measured after trehalase digestion. Glucose results are reported as milligram per decilitre and trehalose results are reported as milligram per decilitre.

2.2.8) Quantification of Food Intake

I quantified food consumption of the four DGRP lines and BAE 318 using a Smurf assay modified from Wong *et al.* (2009). I made CD and HSD medium that contained a blue food dye (FD & C Dye no.1) at a concentration of 2.5% (w/v) and flies were allowed to consume the food for a total

of 30 minutes before being transferred to Eppendorf tubes and snap frozen in liquid nitrogen. Groups of five flies were then homogenized in 200 µl of distilled water and a further 800 µl of distilled water was added. The homogenate was then passed through a 0.22 µm Millex filter (Millipore Corporation, Bedford) to remove debris and lipids. The absorbance of the homogenate was measured at 629 nm (Molecular Devices SpectraMax 384 Plus). Flies exposed to non-dyed food were used as the baseline during spectrophotometry and the amount of labelled food in the fly was calculated from a standard curved produced by a serial dilution of blue food dye. Each sample was assayed twice, and the mean was used for the analysis. Results are reported in milligram per millilitre.

2.2.9) RNA Extraction and Quantitative RT-PCR

I used quantitative real time quantitative PCR, qPCR, to quantify expression levels of four genes: *Dilp2*, *InR*, *Chico*, and *Akt*. Total RNA was isolated from three groups of ten flies using the RNeasy Kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions, and was stored at -80 ˚C until needed for reverse transcription. Total RNA concentration was quantified using the Qubit RNA Broad-Range assay kit (Invitrogen) with the Qubit 2.0 Fluorometer. Each sample was reverse transcribed using the High Capacity cDNA Reverse Transcription Kits (Applied Biosystem). Total cDNA concentration was quantified using the Qubit ssDNA assay kit (Invitrogen) with the Qubit 2.0 Fluorometer. Reverse transcriptase qPCR was performed to monitor the changing expression levels of *Dilp2*, as well as changes in expression of *InR* and *Chico*. These genes were analyzed to monitor insulin to insulin receptor binding, and the presence or absence of hyperinsulinemia

(*Dilp2*) in response to the conditions. *Akt* gene expression was quantified to monitor downstream IIS pathway activity. A list of primers can be found in Table 1. All qPCR reactions consisted of 5 µl of diluted cDNA, 0.4 µM of each primer, and 10 µl of PowerUp SYBR Green Master Mix (Applied Biosystems); a total reaction volume of 20 µl. Reaction conditions were followed according to the manufacturer's instructions (Applied Biosystems). No-template controls lacking reverse transcriptase for each sample were used as a negative control to assess the level of genomic DNA contamination and overall contamination. Three biological replicates were used along with four technical replicates in which the mean was used for analysis. Samples were normalized to *RpL39* and are shown as relative to control using the ΔΔCT method (Livak and Schmittgen 2001).

Gene	**Forward Primer (5'-3')**	**Reverse Primer (5'-3')**
Dilp2 (Bauer *et al.* 2007)	AGC AAG CCT TTG TCC TTC ATC TC	ACA CCA TAC TCA GCA CCT CGT TG
InR (Roth *et al.* 2018)	GCC AAT TCA ATA GCG GGA TAC	CTC GCA TAG AAC GGA TTC ACC
Chico (Mohr *et al.* 2014)	ACA TCA ATC GCC GTT TGG ACA	GAG AAC GAT GCC GAA TCC AC
Akt (Li *et al.* 2019)	TTT CGT TCC GTC CTC TGG TG	GCA CTG CGG TTT GTT TTC CT
RpL39 (Mohr *et al.* 2014)	CTG CAC ACA AGT CGT TCA GA	GGG AAC GGA TCT GTT CTG CT

Table 1. Primers used in RT-qPCR experiments. All primers were diluted to 0.4 μM and mixed in a 20 μL reaction volume with PowerUp SYBR Green Master Mix and diluted cDNA.

2.2.10) Fasting & Oral Glucose Tolerance Tests

To test whether flies displayed symptoms of insulin resistance, I subjected flies to fasting and oral glucose tolerance tests to determine the presence of hyperglycemia and/or glucose intolerance. I separated flies based on genetic background and sex and placed them on one of four conditions for 10 days: CD, HSD, CDEx, and HSDEx. Our fly treadmills only accommodate 14 vials per treadmill therefore flies were separated into two groups and exercised at two independent times; group 1 (10:00am-12:00pm), and group 2 (12:00pm-2:00pm). On the 10^{th} day of the exercise regime, flies in each group were exercised during their respective times and then samples from group 1 and group 2 were pooled together after the second group's final exercise event to avoid the block effect during further experiments. Flies were starved on a 2% (w/v) agar solution for 12-16 hours to decrease insulin signaling (Haselton and Fridell 2011). Following starvation, a subset of flies were immediately flash frozen in liquid nitrogen to be assayed for a fasting glucose concentration. The remaining flies were refed on a 10% glucose-soaked filter paper for one hour and then allowed to recover for 15, 30, and 45-minute time points to analyze glucose clearing kinetics (Haselton *et al.* 2010). At each time point, flies were immediately flash frozen in liquid nitrogen to be assayed for glucose concentration. Each time point had a minimum of three biological replicates containing five flies per sample. Each sample was assayed twice for glucose determination and the mean was used for analysis.

2.2.11) Elucidation of Metabolites by UPLC-QTof-MS

I quantified the broad metabolomic effect of exercise using liquid chromatography – mass spectrometry generally following Doran *et al.* 2017. I used the same set of fly lines to analyze the comparison between exercise versus no exercise flies and how this response is influenced by genetic background and sex. Flies were reared on a standard cornmeal-yeast-agar-corn syrup diet. Metabolomic experiments were performed using adult male and female flies aged 3-5 days. Aged flies were separated into two groups and each group was held on their respective condition for five days: control (no exercise) and exercise. When not exercising, flies were housed in an incubator with a 12:12-hr light dark cycle at 25 ˚C.

Metabolites were extracted from seven groups containing at least 10 flies each that were collected and snap frozen in liquid nitrogen following their respective treatments. Samples were weighed to the nearest 0.01 mg then homogenized with 85:15 of ACN:H_2O as the extraction solvent using a TissueLyser (Qiagen) and steel beads. The volume of extraction solvent used for each sample was normalized by the mass of the sample by pipetting 8 µl per milligram of fly. Samples were centrifuged at 13,000 RPM to remove debris and then transferred to new screw top vials tubes and left at -20 ˚C for one hour to promote protein precipitation. Samples then underwent a series of centrifugation steps to ensure no large particles entered the LC-MS. The samples were then transferred to glass inserts (Thermo Scientific, Catalogue no. C4012-529) for loading onto the LC-MS. Quality controls were prepared by mixing 5 µl from each sample into a glass insert. All samples were stored at -20 ˚C until further analysis.

Briefly, LC-MS analysis was performed on an Acquity Class I UPLC Xevo G2-XS Quadrupole Time-of-Flight- Mass Spectrometer (Waters). Two microliters of each sample was injected onto a 150 mm × 2.1 mm × 1.7 μm UPLC BEH Amide column with 1.7 μm BEH Amide Vanguard column attachment (Acquity). The column temperature was held at 40 ˚C. Samples were kept at 4 ˚C until loading to avoid metabolite degradation. Metabolites were separated using a 15:85 solvent gradient consisting of solvent A (20 mM ammonium formate in LCMS-H_2O) and solvent B (0.1% formic acid in ACN). The solvent flow rate was 0.4 mL/min and the gradients were as follows: 85%B at (0-3 min), 40%B (5 min), 85%B (10-18 min). The MS was operated in positive ionization mode and was calibrated using sodium formate (1M NaOH, 90% 2-propanol in H_2O, 20% formic acid in H_2O). The ionization source working parameters, optimized for detection of a wide mass range of metabolites, were as follows: capillary voltage 1 kV, source temperature 150 ˚C, desolvation temperature 500 ˚C, and desolvation gas 1000 L/h. To achieve accurate mass calibration, I used the leucine enkephalin background ion (556.27) as a lock mass. The scan range was 50-1500 *m/z*. Data was retrieved in acidic positive ionization mode.

Raw, untargeted metabolite data was subjected to automatic intra-run calibration with sodium formate and putative metabolites were identified using Progenesis QI, a LC-MS software package that allows the identification of small molecules, lipids, and proteins. Males and females were run on the LC-MS on different days, because of the large number of samples in the experiment and limitations on numbers of samples that could be included in a single day's analysis and data from these two sets analyzed independently.

2.2.12) Data Analysis

All experiments included three biological replicates and a minimum of two technical replicates for assays. Data is presented as the sample mean and error bars indicate ± standard error. ANCOVA and Tukey's honesty significant difference (HSD) multiple comparison tests were performed using JMP 14 Software (SAS Institute) to determine if there was significance in glucose, glycogen, triglyceride, trehalose, or gene expression using wet weight as a covariate.

2.3 Results

2.3.1) Fasting Glucose & Oral Glucose Tolerance Tests

Averaged across all lines and both sexes, I did not detect an overall trend of insulin resistance under any conditions. Flies did have overall elevated glucose levels, commonly referred to as hyperglycemia, on both diet and exercise conditions, but ultimately responded with typical glucose clearing kinetics. Each fly line responded to diet and exercise differently and our inability to detect glucose tolerance/intolerance across the lines suggests that the phenotype is highly influenced by genetic background. This said, one fly line in particular, DGRP 852, responded with what I would expect from classic hyperglycemia and glucose intolerance, which are characteristic of insulin resistance.

Across the complete experiment, both diet and exercise significantly impacted fasting glucose levels (Figure 3A,B). Previous research has shown that a diet rich in sucrose significantly impacts fasting glucose levels (Haselton *et al*. 2010 and Musselman *et al*. 2011), and I found that same effect (Figure 3A). There were also the expected significant differences in fasting glucose levels

between sexes (Figure 3C) and indicates that I can resolve differences in glucose levels when they do exist. Similarly, as anticipated, low motivation flies had significantly lower fasting glucose levels than high motivation flies (Figure 3D). As with the other phenotypes (below), response in fasting glucose levels to diet and exercise was substantially different across the four lines (Figure 3E,F), indicating that the phenotype is genotype and sex specific.

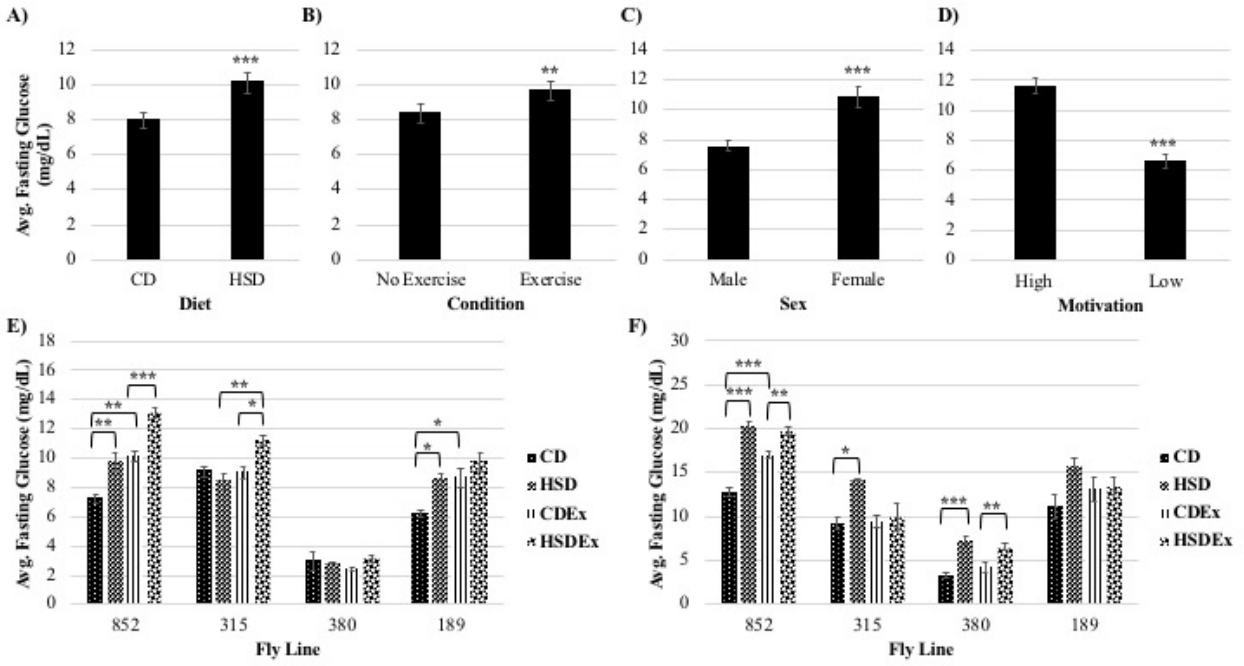

Figure 3. Fasting glucose content represented across different groups. Fasting glucose averaged across all genotypes, both sexes, and separated by (A) CD and HSD conditions, inclusive of exercise conditions, (B) no exercise versus exercise, inclusive of diet conditions, (C) sex, (D) high or low motivation. (E) Fasting glucose from female genotypes. Comparisons are between conditions within a genotype. (F) Fasting glucose from male genotypes. Comparisons are between

conditions within a genotype. A Student's t-test was used to determine significant differences in fasting glucose between diet and diet with exercise conditions, as well as sex and motivational groups in Figures (A)-(D). An ANCOVA test was used to determine significant differences within the data set and a Tukey Honesty Test was used to determine significant differences in fasting glucose between diet/exercise conditions within genotypes in Figures (E) and (F). Error bars represent SE±. Asterisks denote the following: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Across the entire experiment, neither diet nor exercise significantly impacted glucose clearing kinetics in the oral glucose tolerance test, i.e. I did not induce glucose intolerance (Figure 4A,B). As expected, there were significant differences in glucose levels over time between sexes and glucose tolerance in both females and males (Figure 4C). Further, when I examined the effect of HSD and exercise on glucose clearing kinetics of fly's differing in motivation, there were significant differences in glucose levels over time and typical glucose clearing kinetics (Figure 4D).

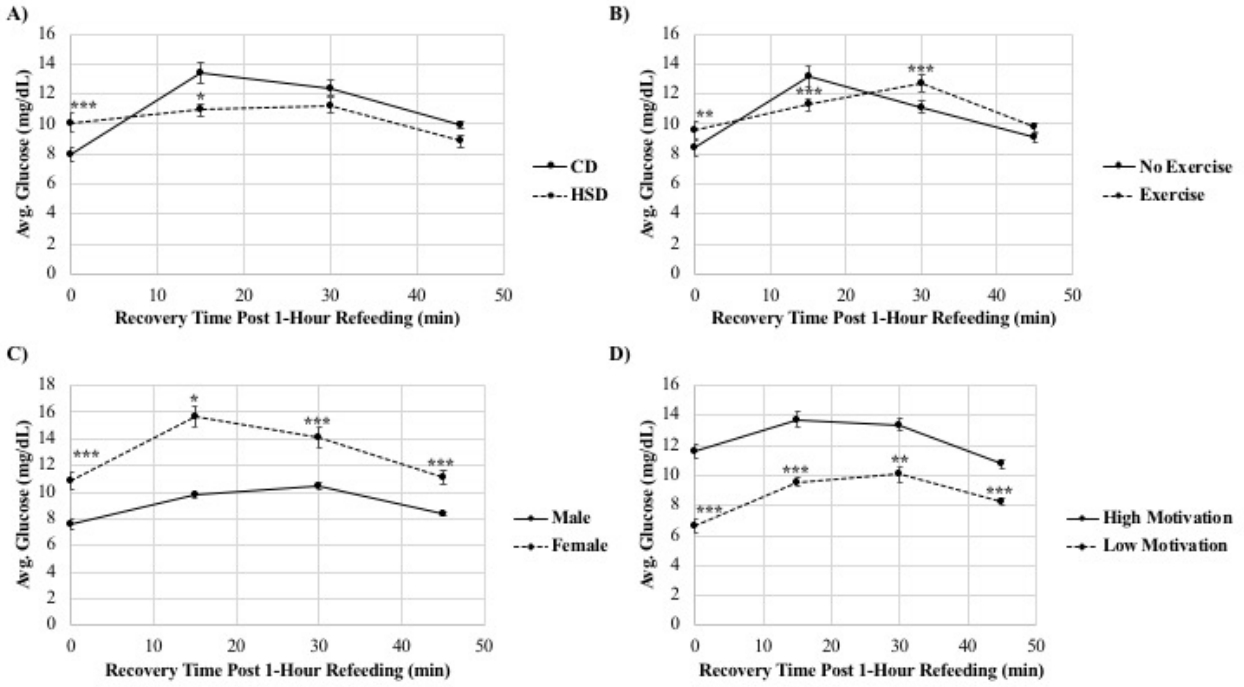

Figure 4. Glucose tolerance depicted amongst different groups. Glucose content averaged for each respective time point across all conditions, genotypes, both sexes, and separated by (A) CD and HSD conditions, inclusive of exercise conditions, (B) no exercise versus exercise, inclusive of diet conditions, (C) sex, (D) high or low motivation. A Student's t-test was used to determine significant differences between glucose levels for each time point for diet, exercise, sex, and motivation groupings. Error bars represent SE±. Asterisks denote the following: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

The inability to resolve differences in glucose clearing kinetics on the HSD is most likely due to diet and exercise being strongly influenced by genotype and sex. For this reason, I examined glucose clearing kinetics separately for females and males of each line, including only diet

conditions. One fly line in particular, DGRP 852, displayed hyperglycemia and glucose intolerance in both males and females, by displaying higher glucose over time than controls (Figure 5A,C). A diet rich in sucrose is expected to lead to characteristics of insulin resistance (Morris *et al*. 2012), but interestingly, this phenomenon was only observed in 25% of our fly lines. When I analyzed diet and exercise conditions together, I found complicated responses in both sexes, but ultimately, all conditions had similar glucose levels at the end of our observations (Figure 5B,D).

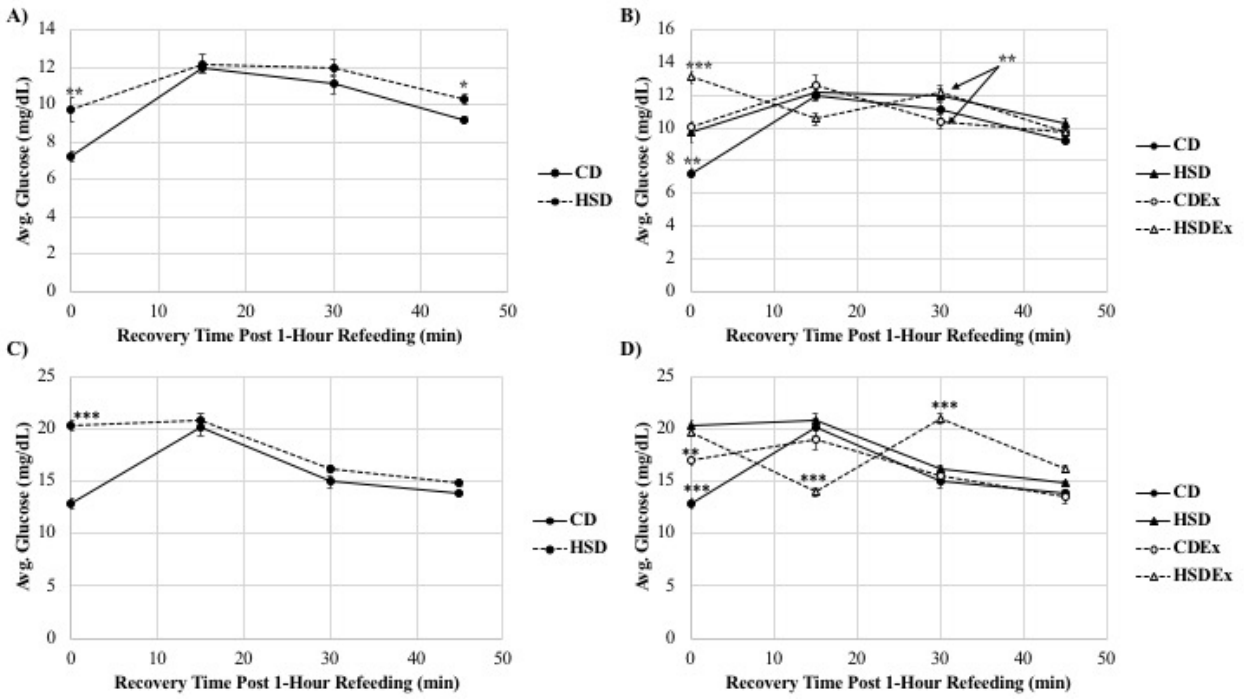

Figure 5. DGRP 852 displays signs of glucose intolerance. (A) Male DGRP 852 glucose content separated by CD and HSD conditions for each respective time point. (B) Male DGRP 852 glucose content separated by CD, HSD, CDEx, and HSDEx conditions for each respective time point. (C) Female DGRP 852 glucose content separated by CD and HSD conditions for each respective time

point. (D) Female DGRP 852 glucose content separated by CD, HSD, CDEx, and HSDEx conditions for each respective time point. A Student's t-test was used to determine significant differences between glucose levels for each time point in Figures (A) and (C). An ANCOVA test was used to determine significant differences within the data set and a Tukey Honesty Test was used to determine significant differences in glucose levels between diet/exercise conditions for each time point in Figures (B) and (D). Error bars represent SE±. Asterisks denote the following: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

2.3.2) Food Consumption

Flies subjected to a high sucrose diet consumed significantly less food than the control food. Although food consumption varied by genotype, I was still able to determine patterns in diet.

When I examined the complete experiment, including both diets, both exercise and control, all four genotypes, and both sexes, diet (Figure 6A) but not exercise (Figure 6B) had a significant impact on food consumption. Flies consumed significantly less of the HSD, which contains roughly twice the number of calories than the CD (Supplemental Table 1-2). There were no significant differences in food consumption between males and females or between high and low motivation flies (Figure 6C,D). However, I also examined food consumption separately in females and males of each line, and surprisingly, lines and sexes show very different food consumption patterns (Figure 6E,F). Despite the variation found in food consumption within the lines and between the sexes, patterns in diet were still observed.

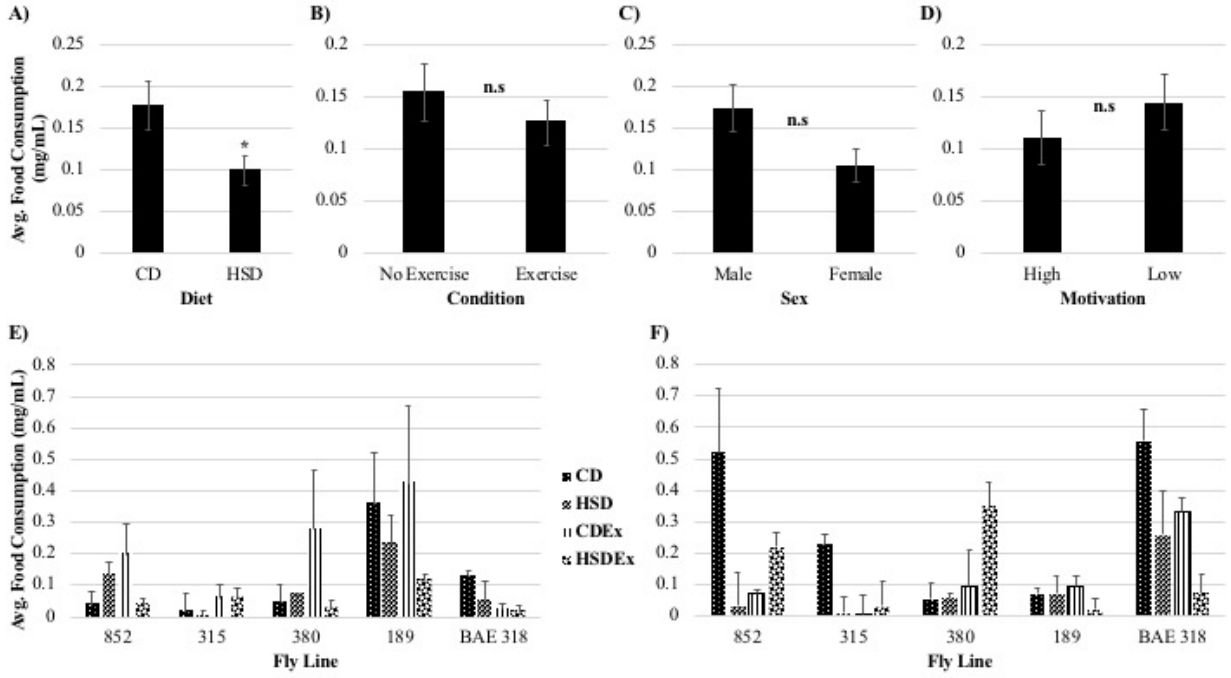

Figure 6. Fly food consumption is decreased on HSD. Food consumption averaged across all genotypes, both sexes, and separated by (A) CD and HSD conditions, inclusive of exercise conditions, (B) no exercise versus exercise, inclusive of diet conditions, (C) sex, (D) high or low motivation. (E) Food consumption from female genotypes. Comparisons are between conditions within a genotype. (F) Food consumption from male genotypes. Comparisons are between conditions within a genotype. A Student's t-test was used to determine significant differences in food consumption between diet and diet with exercise conditions, as well as sex and motivational groups in Figures (A)-(D). An ANCOVA test was used to determine significant differences within the data set and a Tukey Honesty Test was used to determine significant differences in food consumption between diet/exercise conditions within genotypes in Figures (E) and (F). Error bars represent SE±. Asterisk denotes * $p<0.05$.

2.3.3) Exercise Metabolomics

To verify that our exercise regime was affecting the metabolism of the flies, I took a broad approach and quantified the metabolome using mass spectrometry-based metabolomics. There were substantial and significant changes in the metabolome of both male and female flies in response to increased physical activity. By exercising flies for two hours a day for five days, I detected a variety of physiological changes in the metabolome of exercise flies compared to non-exercise flies.

Using an untargeted metabolomic approach, I detected a total of 23,604 metabolites in males and 28,068 metabolites in females (Table 2). Difference between the sexes in the number of metabolites detected likely reflects day to day variation in the analytical runs and not sex-specific differences in metabolism. Using all detected metabolites and separating those metabolites by sex, 12.6 % of the metabolome differed in females in response to exercise, while 10.7 % of the metabolome differed in males in response to exercise (Table 2). Changes in the metabolomes of each sex as a result of exercise can be clearly seen in Figure 7. With respect to sex and motivation, 16.4 % of the metabolome changed in response to exercise in high motivation females, while 15.1 % of the metabolome changed in high motivation males (Table 2). Alternatively, exercise had a greater impact on the metabolomes of flies classified as low motivation (females-17.7 %; males-18.4 %).

Females- 28,068 total compounds				Males- 23,604 total compounds			
	Filter	**Number of Compounds**	**% of Total**		**Filter**	**Number of Compounds**	**% of Total**
	Fold change >2	3539	12.6		Fold change >2	2522	10.7
	P <0.05	780	2.8		P <0.05	648	2.7
	Fold change >2, P <0.05	301	1.1		Fold change >2, P <0.05	175	0.7
Motivation	**Filter**	**Number of Compounds**	**% of Total**	**Motivation**	**Filter**	**Number of Compounds**	**% of Total**
High	Fold change >2	4597	16.4	High	Fold change >2	3568	15.1
	P <0.05	1375	4.9		P <0.05	443	1.9
	Fold change >2, P <0.05	591	2.1		Fold change >2, P <0.05	210	0.9
Low	Fold change >2	4980	17.7	Low	Fold change >2	4342	18.4
	P <0.05	938	3.3		P <0.05	1199	5.1
	Fold change >2, P <0.05	322	1.1		Fold change >2, P <0.05	518	2.2

Table 2. An overview of detected metabolites from mass spectrometry-based metabolomics. All metabolites, with respect to sex, were separated using three different filters to give an overview of changed compounds in both sexes and high and low motivation flies, in response to exercise. Percentages reflect the change in metabolome in response to exercise with respect to the filter used.

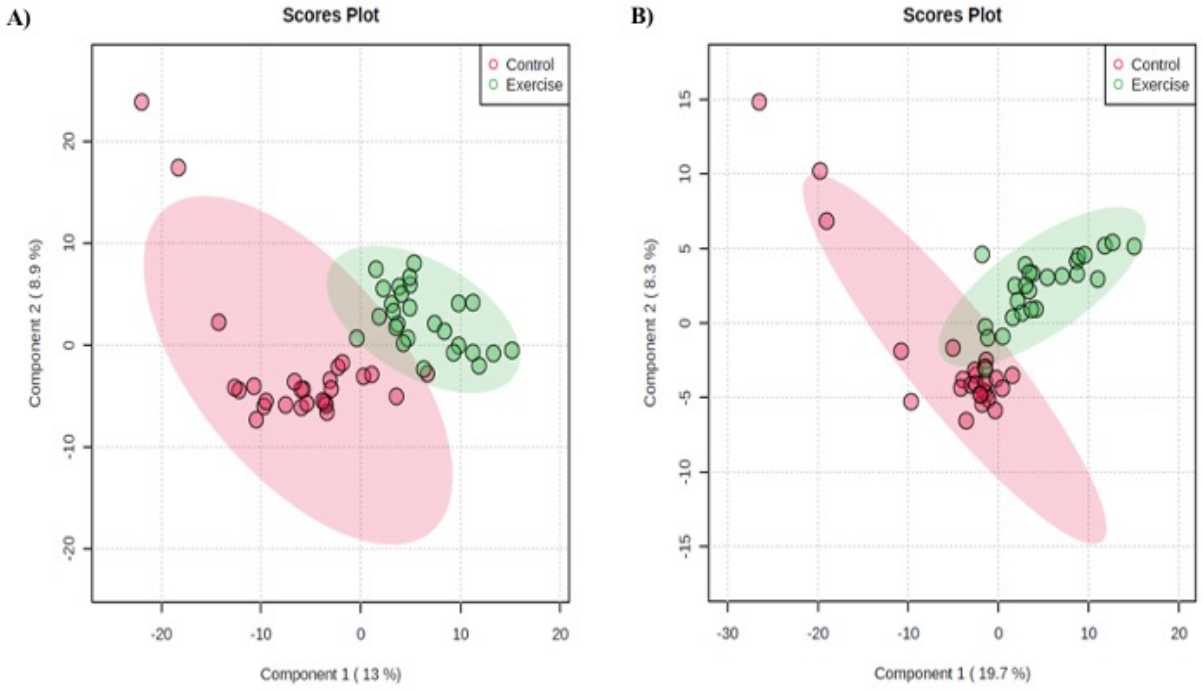

Figure 7. Overview of the effects of a five-day exercise regime on both sexes. Data represents metabolomes of non-exercised flies, represented in pink, versus exercised flies, represented in green, for (A) all female flies, and (B) all male flies. Each point represents a score from a biological replicate.

Much like the glucose diagnostic tests, when I analyze the impact of exercise on the metabolome of each sex of each line, I found that each line's respective metabolome responded differently to exercise (Table 3; Females- 21.2-27.3 %, Males- 20.0-28.8 %). Metabolomic changes with respect to sex and line can be observed in Figure 8. The clear changes in metabolome in response to exercise across both sexes, motivation, and all four genotypes suggests the effectiveness of our exercise regime.

Females- 28,068 total compounds				Males- 23,604 total compounds			
Fly Line	**Filter**	**Number of Compounds**	**% of Total**	**Fly Line**	**Filter**	**Number of Compounds**	**% of Total**
852	Fold change >2	7657	27.3	852	Fold change >2	6175	26.2
	P <0.05	2371	8.4		P <0.05	1110	4.7
	Fold change >2, P <0.05	1454	5.2		Fold change >2, P <0.05	682	2.9
315	Fold change >2	5955	21.2	315	Fold change >2	5635	23.9
	P <0.05	1255	4.5		P <0.05	936	3.9
	Fold change >2, P <0.05	674	2.4		Fold change >2, P <0.05	612	2.6
380	Fold change >2	7057	25.1	380	Fold change >2	4719	20.0
	P <0.05	2947	10.5		P <0.05	1789	7.6
	Fold change >2, P <0.05	1214	4.3		Fold change >2, P <0.05	658	2.8
189	Fold change >2	6189	22.1	189	Fold change >2	6802	28.8
	P <0.05	959	3.4		P <0.05	2466	10.4
	Fold change >2, P <0.05	532	1.9		Fold change >2, P <0.05	1488	6.3

Table 3. An overview of detected metabolites from mass-spectrometry based metabolomics in four different lines. All metabolites, with respect to sex, were separated using three different filters to give an overview of changed compounds in four different lines in response to exercise. Percentages reflect the change in metabolome in response to exercise with respect to the filter used.

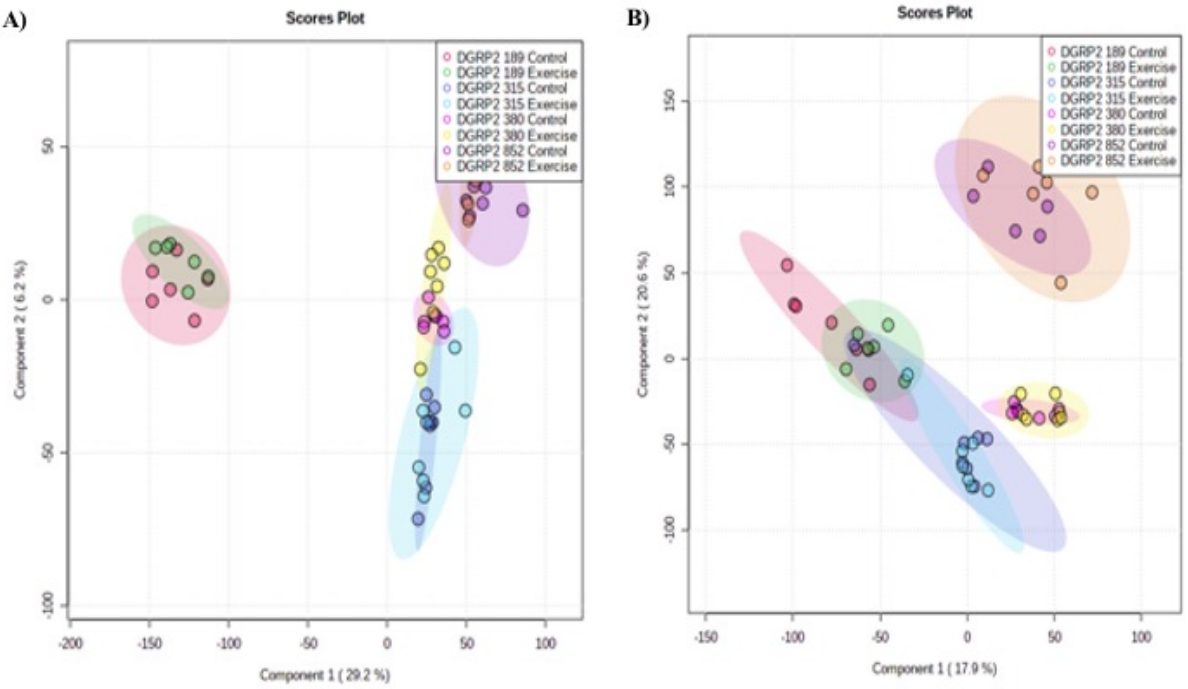

Figure 8. Overview of the effects of a five-day exercise regime on different fly lines. Data represents metabolomes of non-exercised flies versus exercised flies with respect to fly line for (A) female flies, and (B) male flies. Each point represents a score from a biological replicate. Sample treatments are represented by different colours for each line.

2.3.4) Weight & Metabolites

I also examined the effects of diet and exercise on the physiology and metabolism of the flies by quantifying effects on a series of discrete phenotypes. Surprisingly, diet and exercise had limited overall effects on fly weight or metabolite concentration, possibly because, like the earlier glucose diagnostic tests (fasting glucose and oral glucose tolerance tests), there were substantially different responses across the fly lines I used.

When I examine the complete experiment, including both diets, both exercise and control, all four genotypes, and both sexes, neither diet nor exercise had significant impacts on fly weight (Figure 9A,B). In contrast, I could resolve the expected significant differences in weight between sexes (Figure 9C), indicating that, like the glucose diagnostic tests, I could resolve differences when they were present. Previous research has also shown that fly motivational state, as the fly's inherent tendency to remain active, significantly impacts fly weight (Mendez *et al*. 2016) and I found that same significant effect (Figure 9D). I also examined the effect of HSD and exercise separately for females and males of each line (Figure 9E,F, respectively), and, strikingly, lines and sexes show very different responses. The lack of an obvious uniform response likely explains the lack of an overall significant response and is one of the major findings of this study; fly response to diet and exercise is highly genotype and sex specific.

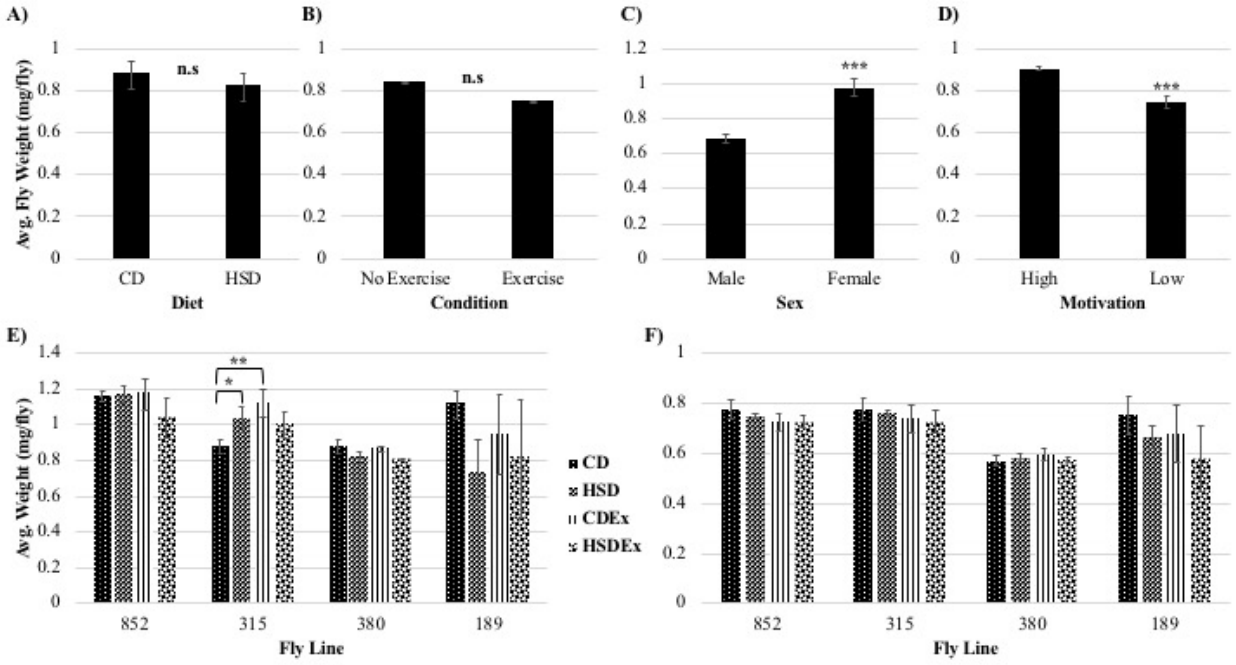

Figure 9. Fly weight represented across different groups. Fly weight averaged across all genotypes, both sexes, and separated by (A) CD and HSD conditions, inclusive of exercise conditions, (B) no exercise versus exercise, inclusive of diet conditions, (C) sex, (D) high or low motivation. (E) Fly weight from female genotypes. Comparisons are between conditions within a genotype. (F) Fly weight from male genotypes. Comparisons are between conditions within a genotype. A Student's t-test was used to determine significant differences in weight between diet and diet with exercise conditions, as well as sex and motivational groups in Figures (A)-(D). An ANCOVA test was used to determine significant differences within the data set and a Tukey Honesty Test was used to determine significant differences in weight between diet/exercise conditions within genotypes in Figures (E) and (F). Error bars represent SE±. Asterisks denote the following: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

To determine if diet or exercise were having small effects that were being lost in the larger analysis, I also separately analyzed flies that only differed in diet or exercise condition (Figure 10). Limiting our analysis to flies on one exercise condition, there was no significant effect of diet (Figure 10A,C) and similarly, limiting our analysis to one diet, there was no significant effect of exercise (Figure 10B,D).

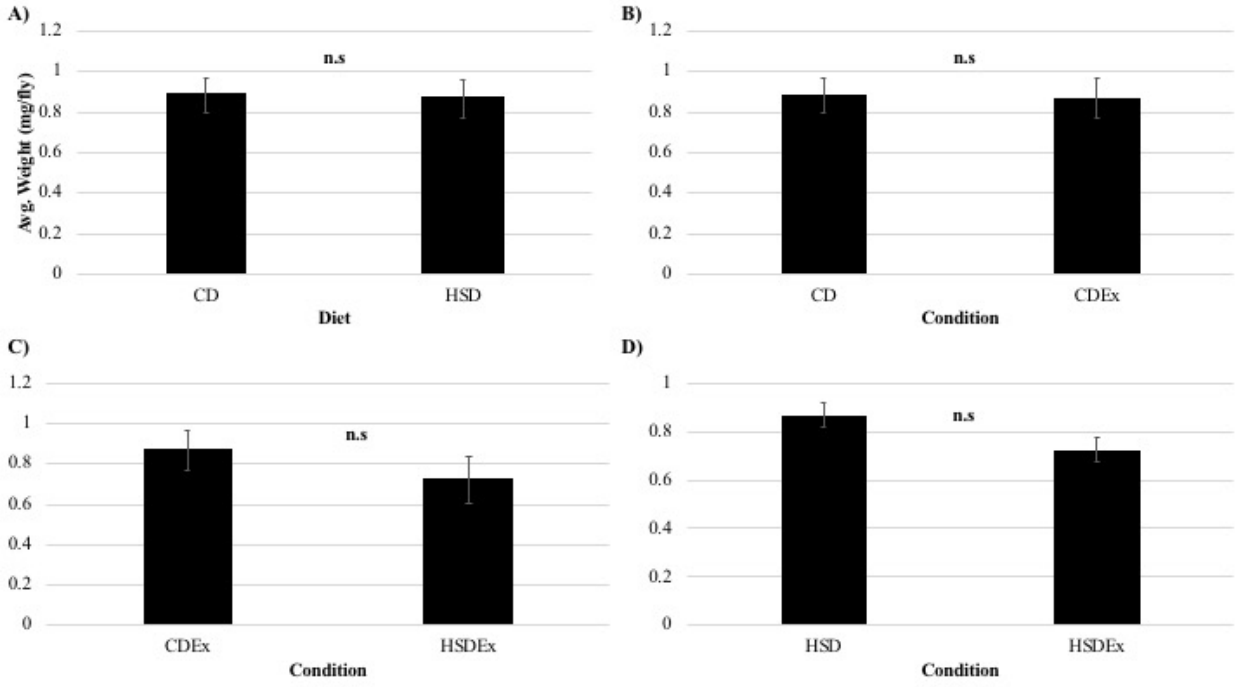

Figure 10. Effect of diet and exercise on fly weight. Fly weight averaged across all genotypes, both sexes, and separated by (A) CD and HSD conditions, exclusive of exercise conditions, (B) CD and CDEx conditions, exclusive of diet conditions, (C) CDEx and HSDEx conditions, (D) HSD and HSDEx conditions. A Student's t-test was used to determine significant differences in weight between diet and diet with exercise conditions in Figures (A)-(D).

Although weight was not impacted by diet, glucose and glycogen showed small but statistically significant increases in overall concentration on the HSD (Figure 11A; Figure 12A). Overall in my study, I found no significant effect of exercise on glucose, glycogen, triglycerides, or trehalose concentrations (Figure 11B; Figure 12B; Figure 13B; Figure 14B). Previous studies found a similar lack of effect on glucose levels but did find significant reduction in glycogen levels with exercise (Mendez *et al*. 2016). Similar to weight, there were significant differences in glucose, glycogen, trehalose, and triglycerides between male and female flies (Figure 11C; Figure 12C; Figure 13C; Figure 14C). Further, flies from the low motivation lines were not only significantly lighter than high motivation line flies, but they also had significantly lower glucose, glycogen, trehalose, and triglyceride levels (Figure 11D; Figure 12D; Figure 13D; Figure 14D). When I examined the effect of HSD and exercise separately for all female and male lines, each line and sex responded distinctively in glucose, glycogen, triglycerides, and trehalose metabolites (Figure 11E,F; Figure 12E,F, Figure 13E,F; Figure 14E,F). As with the other phenotypes above, the absence of an overall, average, response likely reflects each line and sex behaving independently for each metabolite. Interestingly, the fly line, DGRP 852, that displayed symptoms of insulin resistance, showed increases in glucose, glycogen, and triglycerides but not trehalose in both male and female flies, consistent with the same phenotypes found using different lines in Morris *et al*. 2012 and Musselman *et al*. 2011 (Figure 11E,F; Figure 12E,F, Figure 13E,F; Figure 14E,F).

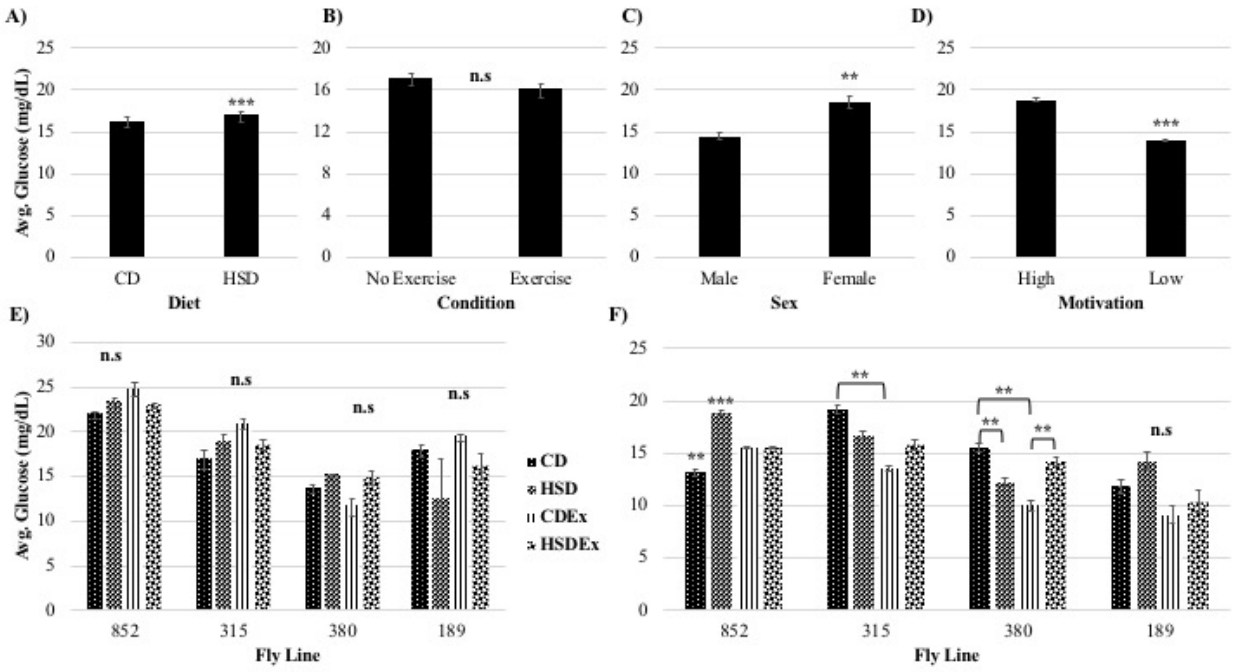

Figure 11. Glucose content represented across different groups. Glucose averaged across all genotypes, both sexes, and separated by (A) CD and HSD conditions, inclusive of exercise conditions, (B) no exercise versus exercise, inclusive of diet conditions, (C) sex, (D) high or low motivation. (E) Glucose from female genotypes. Comparisons are between conditions within a genotype. (F) Glucose from male genotypes. Comparisons are between conditions within a genotype. A Student's t-test was used to determine significant differences in glucose between diet and diet with exercise conditions, as well as sex and motivational groups in Figures (A)-(D). An ANCOVA test was used to determine significant differences within the data set and a Tukey Honesty Test was used to determine significant differences in glucose between diet/exercise conditions within genotypes in Figures (E) and (F). Error bars represent SE±. Asterisks denote the following: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

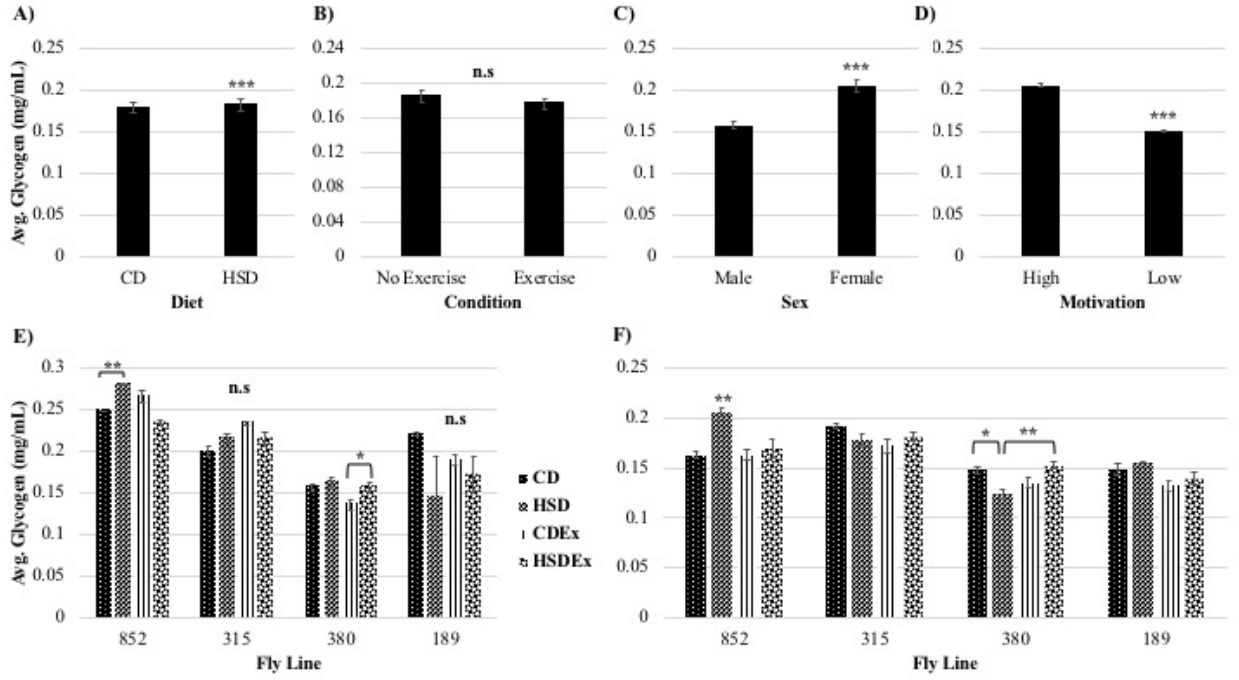

Figure 12. Glycogen content represented across different groups. Glycogen averaged across all genotypes, both sexes, and separated by (A) CD and HSD conditions, inclusive of exercise conditions, (B) no exercise versus exercise, inclusive of diet conditions, (C) sex, (D) high or low motivation. (E) Glycogen from female genotypes. Comparisons are between conditions within a genotype. (F) Glycogen from male genotypes. Comparisons are between conditions within a genotype. A Student's t-test was used to determine significant differences in glycogen between diet and diet with exercise conditions, as well as sex and motivational groups in Figures (A)-(D). An ANCOVA test was used to determine significant differences within the data set and a Tukey Honesty Test was used to determine significant differences in glycogen between diet/exercise conditions within genotypes in Figures (E) and (F). Error bars represent SE±. Asterisks denote the following: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

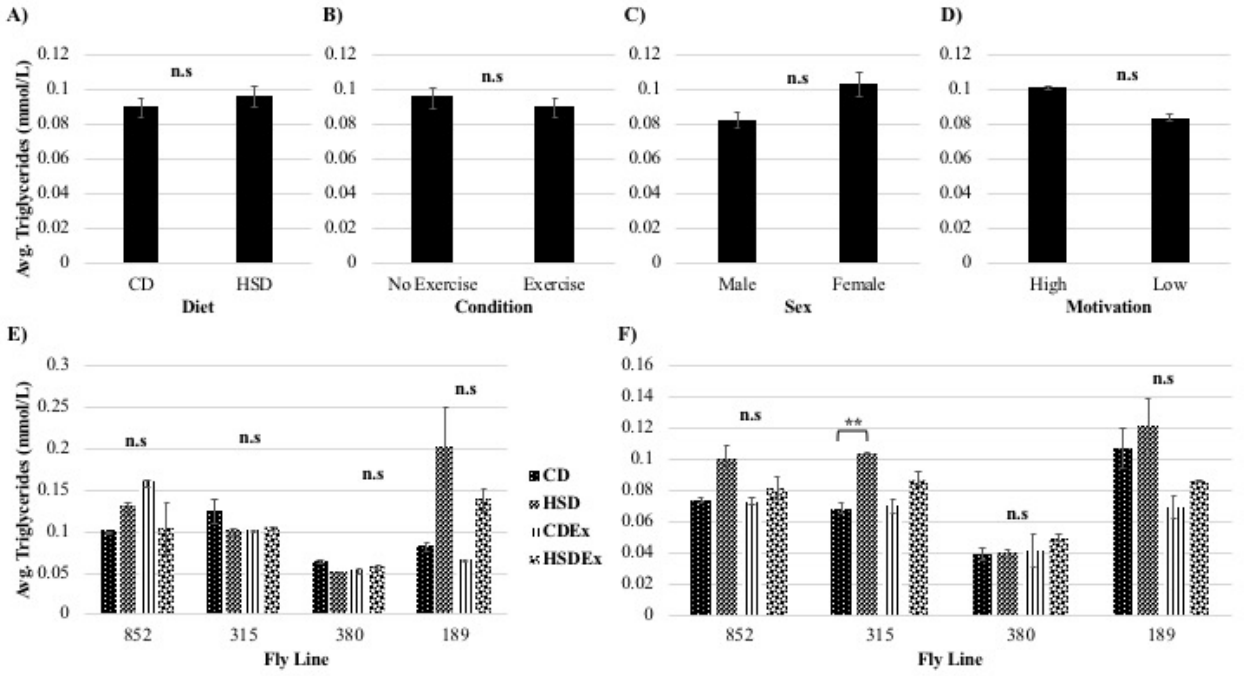

Figure 13. Triglyceride content represented across different groups. Triglycerides averaged across all genotypes, both sexes, and separated by (A) CD and HSD conditions, inclusive of exercise conditions, (B) no exercise versus exercise, inclusive of diet conditions, (C) sex, (D) high or low motivation. (E) Triglycerides from female genotypes. Comparisons are between conditions within a genotype. (F) Triglycerides from male genotypes. Comparisons are between conditions within a genotype. A Student's t-test was used to determine significant differences in triglycerides between diet and diet with exercise conditions, as well as sex and motivational groups in Figures (A)-(D). An ANCOVA test was used to determine significant differences within the data set and a Tukey Honesty Test was used to determine significant differences in triglycerides between diet/exercise conditions within genotypes in Figures (E) and (F). Error bars represent SE±. Asterisk denotes ** $p<0.01$.

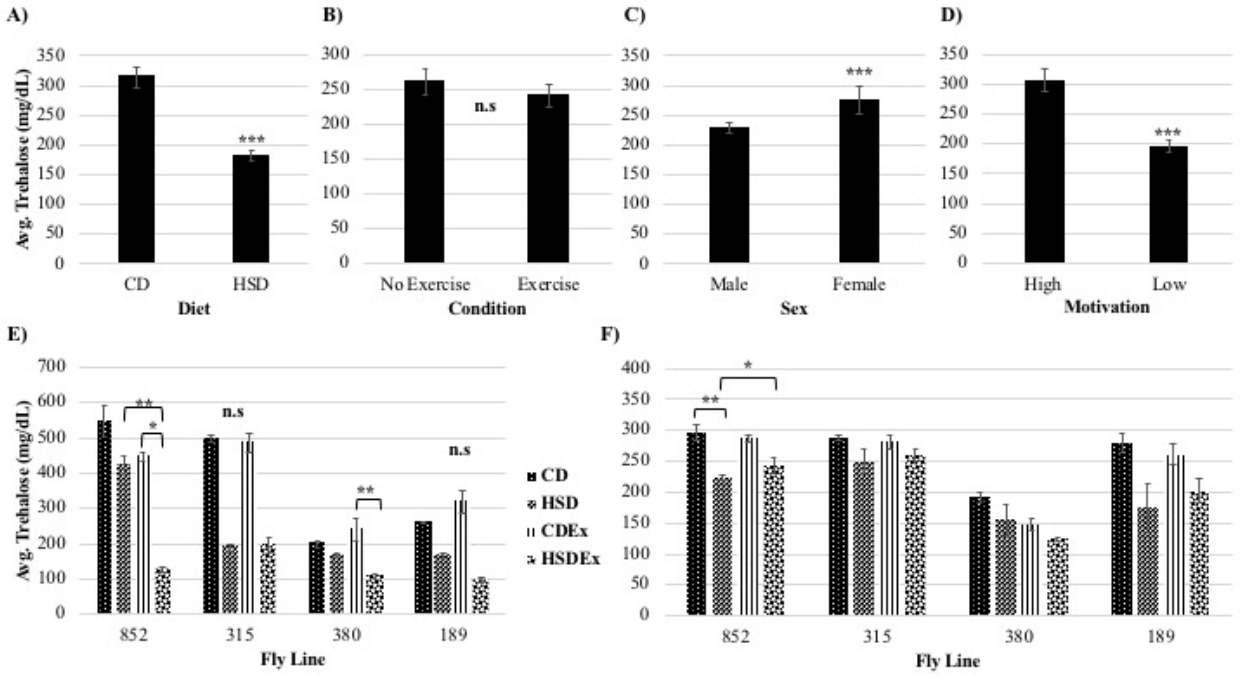

Figure 14. Trehalose content represented across different groups. Trehalose averaged across all genotypes, both sexes, and separated by (A) CD and HSD conditions, inclusive of exercise conditions, (B) no exercise versus exercise, inclusive of diet conditions, (C) sex, (D) high or low motivation. (E) Trehalose from female genotypes. Comparisons are between conditions within a genotype. (F) Trehalose from male genotypes. Comparisons are between conditions within a genotype. A Student's t-test was used to determine significant differences in trehalose between diet and diet with exercise conditions, as well as sex and motivational groups in Figures (A)-(D). An ANCOVA test was used to determine significant differences within the data set and a Tukey Honesty Test was used to determine significant differences in trehalose between diet/exercise conditions within genotypes in Figures (E) and (F). Error bars represent SE±. Asterisks denote the following: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

As with weight, I also analyzed restricted datasets separating the flies according to just diet or exercise condition (Figure 15; Figure 16; Figure 17; Figure 18). By limiting our analysis to flies on one exercise condition, I was able to resolve a small, but significant, effect of diet on glucose, glycogen, and trehalose concentrations but not triglycerides, despite finding a decrease in food consumption on the HSD (Figure 15A,C; Figure 16A,C; Figure 17A,C; Figure 18A,C). In contrast, limiting our analysis to flies on one diet, showed no significant impact of exercise on glucose, glycogen, triglycerides, or trehalose concentrations (Figure 15B,D; Figure 16B,D; Figure 17B,D; Figure 18B,D).

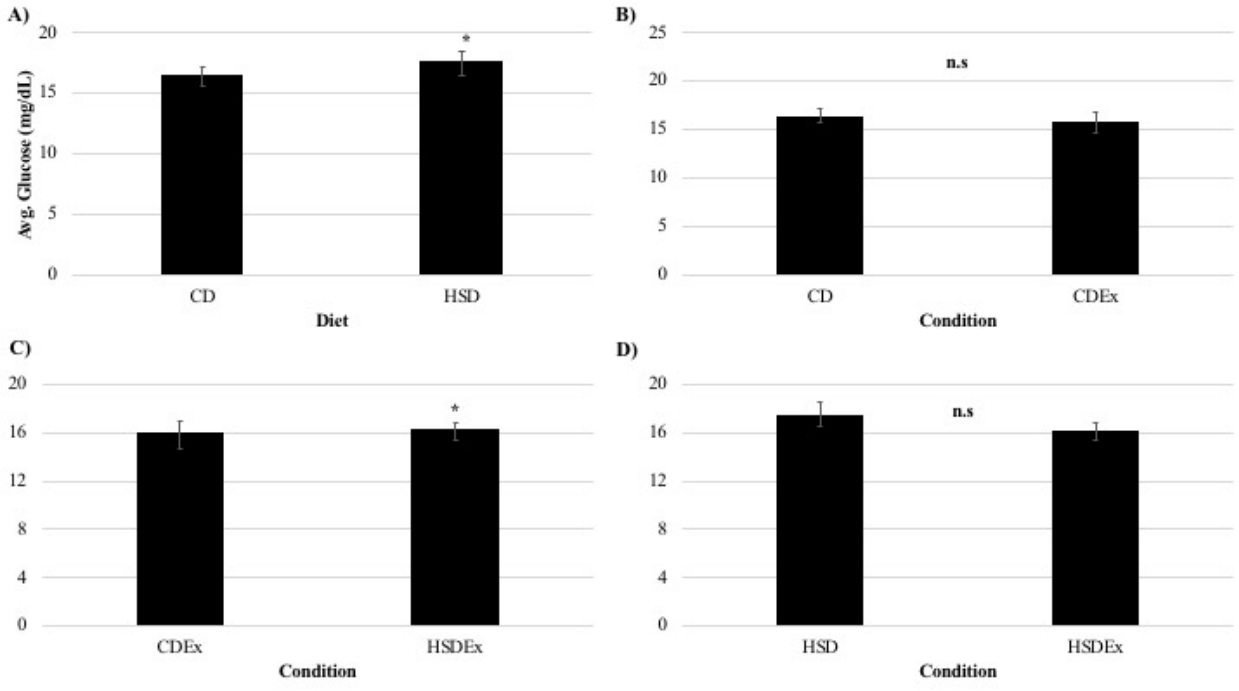

Figure 15. Effect of diet and exercise on glucose content. Glucose averaged across all genotypes, both sexes, and separated by (A) CD and HSD conditions, exclusive of exercise conditions, (B)

CD and CDEx conditions, exclusive of diet conditions, (C) CDEx and HSDEx conditions, (D) HSD and HSDEx conditions. A Student's t-test was used to determine significant differences in glucose between diet and diet with exercise conditions in Figures (A)-(D). Asterisks denote * $p<0.05$.

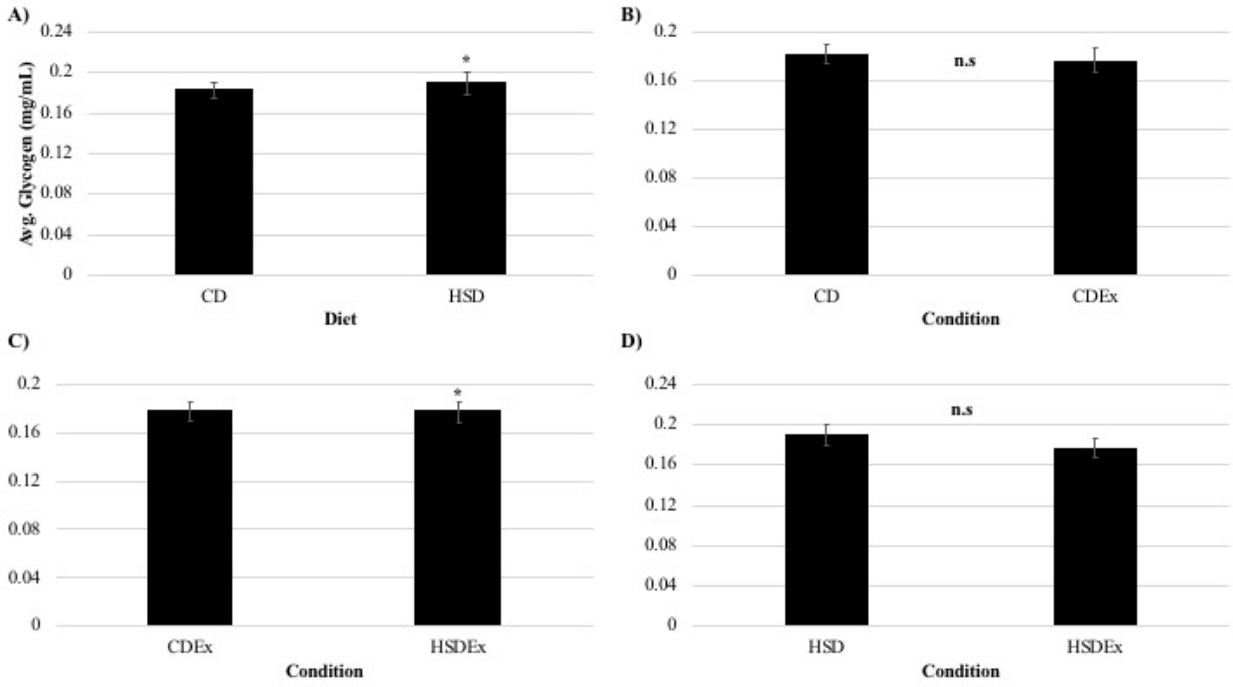

Figure 16. Effect of diet and exercise on glycogen content. Effect of diet and exercise on glycogen content. Glycogen averaged across all genotypes, both sexes, and separated by (A) CD and HSD conditions, exclusive of exercise conditions, (B) CD and CDEx conditions, exclusive of diet conditions, (C) CDEx and HSDEx conditions, (D) HSD and HSDEx conditions. A Student's t-test was used to determine significant differences in glycogen between diet and diet with exercise conditions in Figures (A)-(D). Asterisks denote * $p<0.05$.

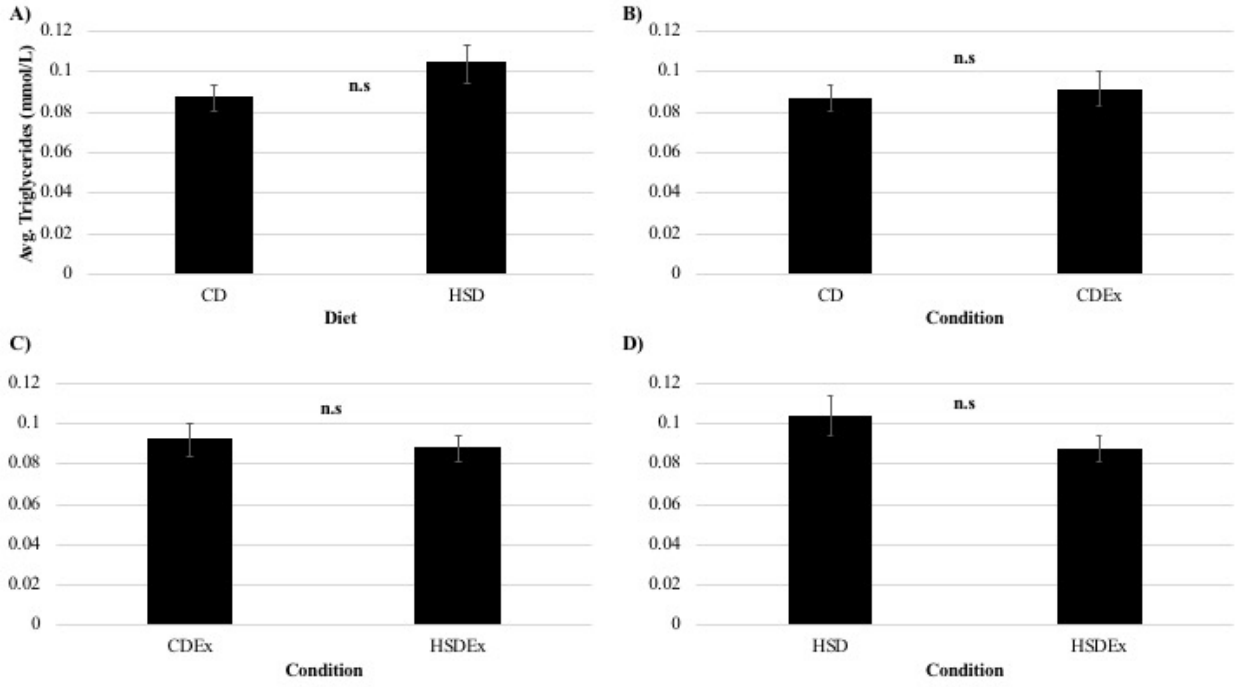

Figure 17. Effect of diet and exercise on triglyceride content. Triglycerides averaged across all genotypes, both sexes, and separated by (A) CD and HSD conditions, exclusive of exercise conditions, (B) CD and CDEx conditions, exclusive of diet conditions, (C) CDEx and HSDEx conditions, (D) HSD and HSDEx conditions. A Student's t-test was used to determine significant differences in triglycerides between diet and diet with exercise conditions in Figures (A)-(D).

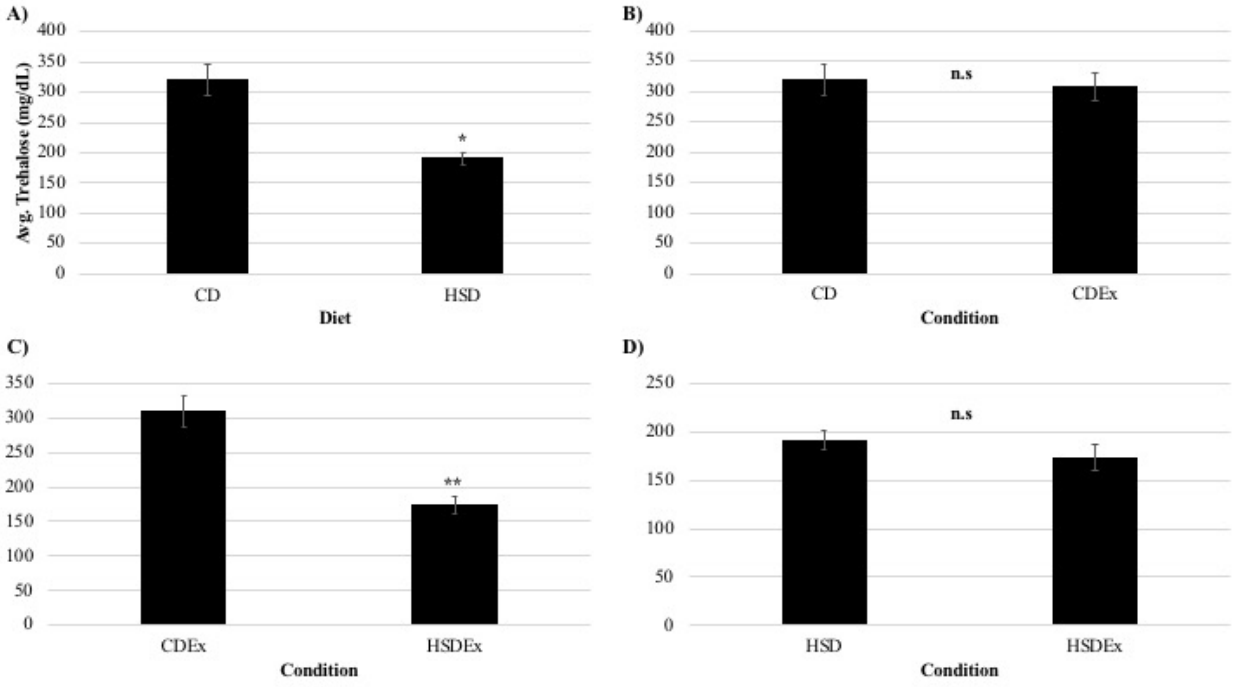

Figure 18. Effect of diet and exercise on trehalose content. Trehalose averaged across all genotypes, both sexes, and separated by (A) CD and HSD conditions, exclusive of exercise conditions, (B) CD and CDEx conditions, exclusive of diet conditions, (C) CDEx and HSDEx conditions, (D) HSD and HSDEx conditions. A Student's t-test was used to determine significant differences in trehalose between diet and diet with exercise conditions in Figures (A)-(D). Asterisks denote the following: * $p<0.05$, ** $p<0.01$.

2.3.5) Insulin Pathway Genes

Unlike metabolite concentration, expression of the IIS pathway genes were sensitive to exercise and sex, but not diet. Similar to the metabolite results, the overall lack of a response to diet could be explained by the variation in responses influenced by genetic background.

Across the entire data set, there was no significant effect of diet or motivation on gene expression of any of the four genes I examined, *Dilp2*, *InR*, *Chico*, or *Akt* (Figures 19A,D; 20A,D; 21A,D; 22A,D), while exercise resulted in significantly higher expression of *Dilp2* and *InR,* but not *Chico* or *Akt* (Figures 19B; 20B; 21B; 22B). Similar to our metabolite results, there were significant differences in IIS pathway gene expression between males and females (Figures 19C; 20C; 21C; 22C); female flies had significantly lower levels of expression of the *Dilp2*, *InR*, and *Chico* genes and there was reduced in *Akt* gene expression than male flies. Also similar to the metabolite data, there were substantial differences in the patterns of gene expression between the lines (Figures 19E,F; 20E,F; 21E,F; 22E,F). Interestingly, although there were substantial differences in metabolite concentrations between high and low motivation flies, there were no significant differences in gene expression of IIS pathway genes (Figure 19D; Figure 20D; Figure 21D; Figure 22D). The variation in IIS pathway gene expression is consistent with the variation in metabolite data; fly response to diet and exercise is highly influenced by genotype and sex. The lack of significance overall most likely reflects the variability in responses to diet and exercise and supports that fly response is highly genotype and sex specific.

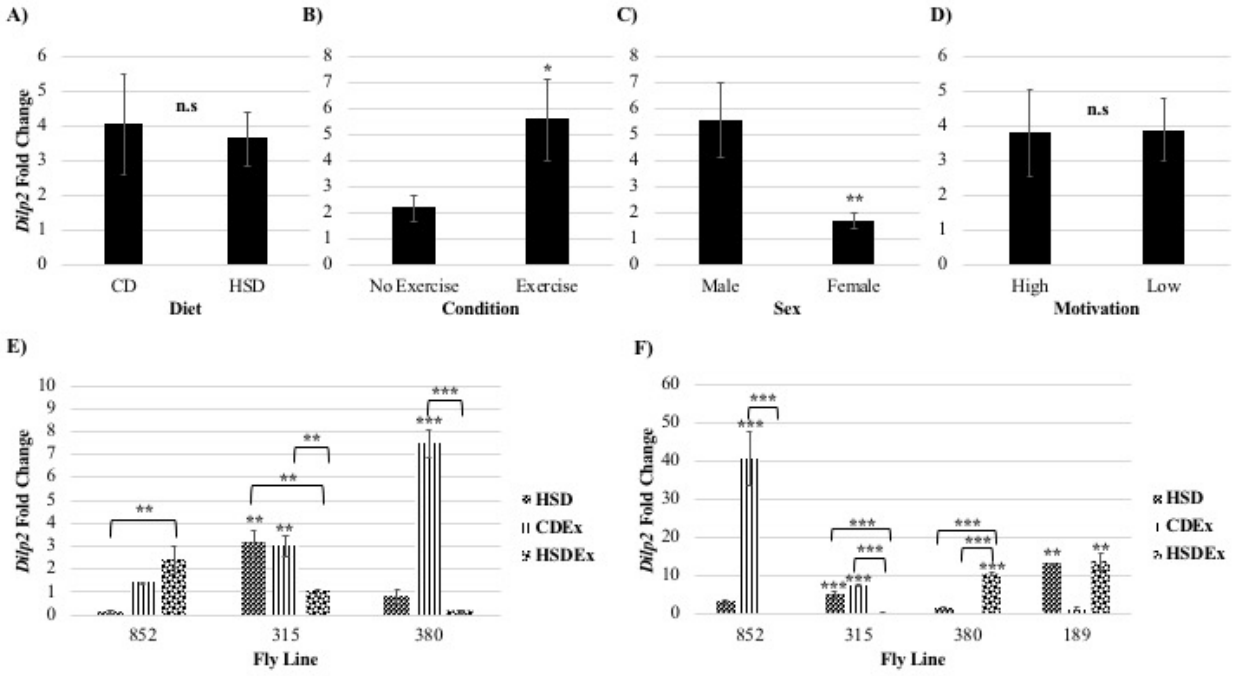

Figure 19. Influence of diet and exercise on *Dilp2* expression. *Dilp2* gene activity averaged across all genotypes, both sexes, and separated by (A) CD and HSD conditions, inclusive of exercise conditions, (B) no exercise versus exercise, inclusive of diet conditions, (C) sex, (D) high or low motivation. (E) *Dilp2* gene activity from female genotypes. Comparisons are between conditions within a genotype. (F) *Dilp2* gene activity from male genotypes. Comparisons are between conditions within a genotype. Asterisks labelled with no bar indication are comparisons to their respective control. Asterisks denote the following: * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Expression was normalized to *RpL39*. Relative expression was calculated using the ΔΔCT method (Livak and Schmittgen 2001). A Student's t-test was used to determine significant differences in *Dilp2* expression between diet, exercise, sex, and motivation groupings in Figures (A)-(D). An ANCOVA test was used to determine significant differences within the data set and a Tukey

Honesty Test was used to determine significant differences in *Dilp2* expression between diet/exercise conditions within genotypes in Figures (E) and (F). Error bars represent SE±.

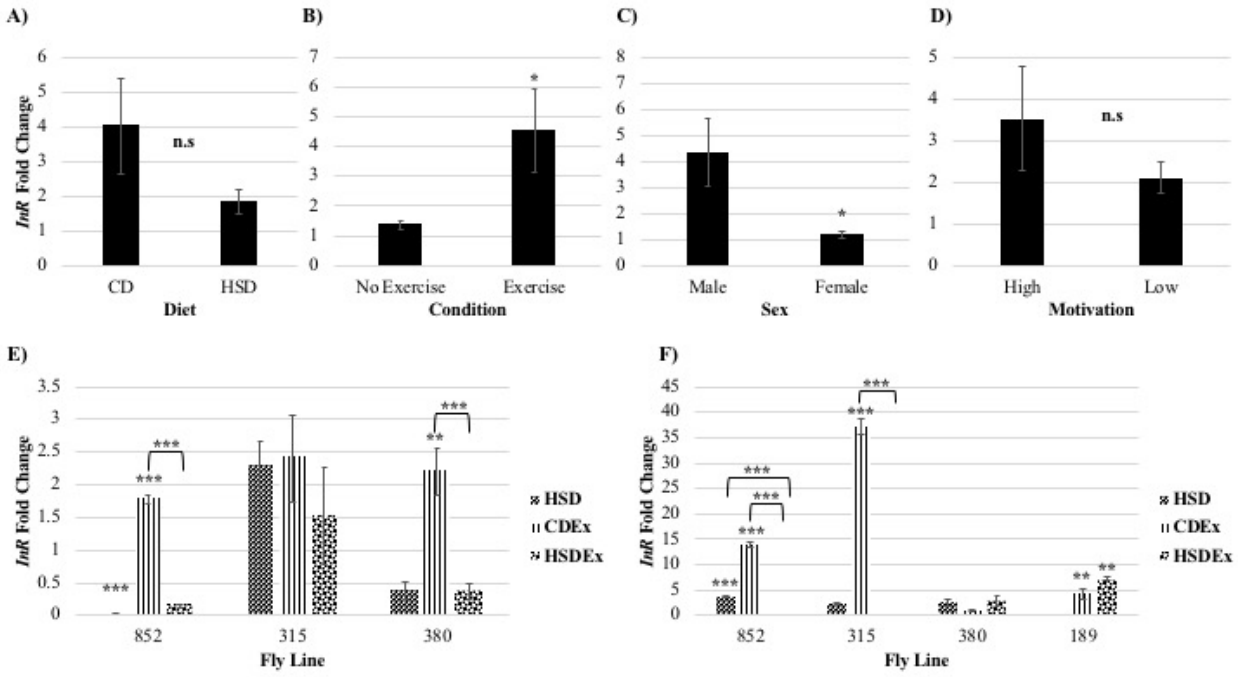

Figure 20. Influence of diet and exercise on *InR* expression. *InR* gene activity averaged across all genotypes, both sexes, and separated by (A) CD and HSD conditions, inclusive of exercise conditions, (B) no exercise versus exercise, inclusive of diet conditions, (C) sex, (D) high or low motivation. (E) *InR* gene activity from female genotypes. Comparisons are between conditions within a genotype. (F) *InR* gene activity from male genotypes. Comparisons are between conditions within a genotype. Asterisks labelled with no bar indication are comparisons to their respective control. Asterisks denote the following: * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Expression was normalized to *RpL39*. Relative expression was calculated using the ΔΔCT method (Livak and

Schmittgen 2001). A Student's t-test was used to determine significant differences in *InR* expression between diet, exercise, sex, and motivation groupings in Figures (A)-(D). An ANCOVA test was used to determine significant differences within the data set and a Tukey Honesty Test was used to determine significant differences in *InR* expression between diet/exercise conditions within genotypes in Figures (E) and (F). Error bars represent SE±.

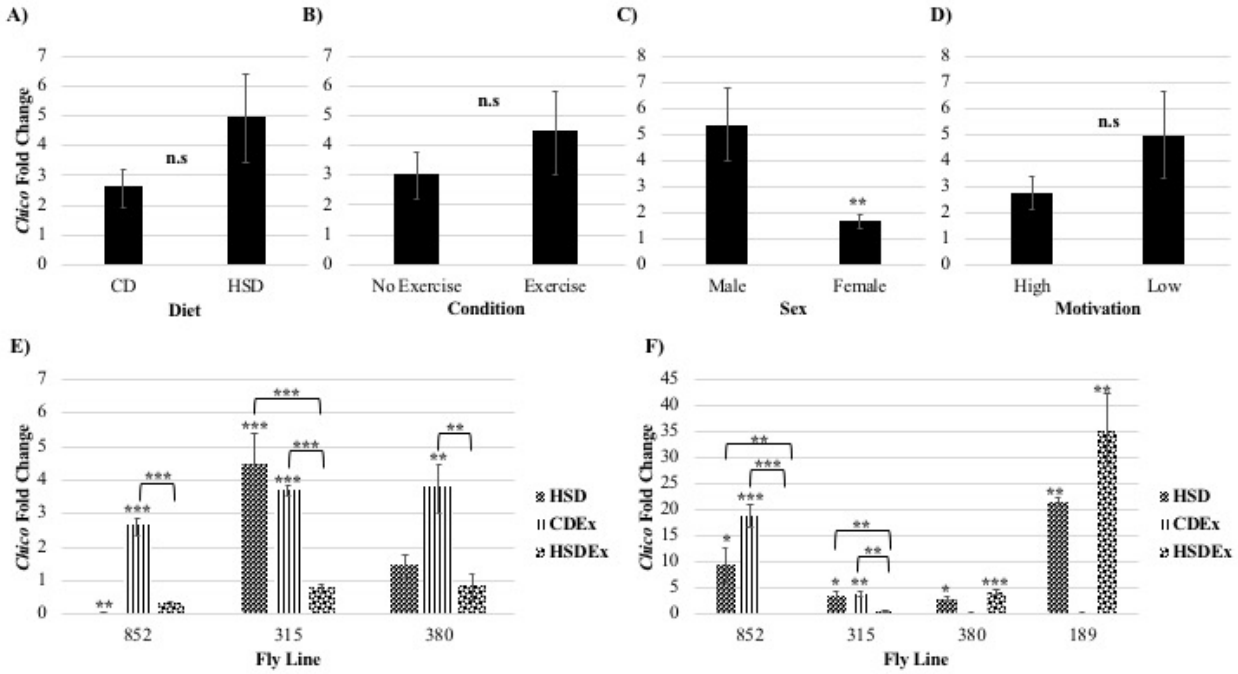

Figure 21. Influence of diet and exercise on *Chico* expression. *Chico* gene activity averaged across all genotypes, both sexes, and separated by (A) CD and HSD conditions, inclusive of exercise conditions, (B) no exercise versus exercise, inclusive of diet conditions, (C) sex, (D) high or low motivation. (E) *Chico* gene activity from female genotypes. Comparisons are between conditions within a genotype. (F) *Chico* gene activity from male genotypes. Comparisons are

between conditions within a genotype. Asterisks labelled with no bar indication are comparisons to their respective control. Asterisks denote the following: * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Expression was normalized to *RpL39*. Relative expression was calculated using the ΔΔCT method (Livak and Schmittgen 2001). A Student's t-test was used to determine significant differences in *Chico* expression between diet, exercise, sex, and motivation groupings in Figures (A)-(D). An ANCOVA test was used to determine significant differences within the data set and a Tukey Honesty Test was used to determine significant differences in *Chico* expression between diet/exercise conditions within genotypes in Figures (E) and (F). Error bars represent SE±.

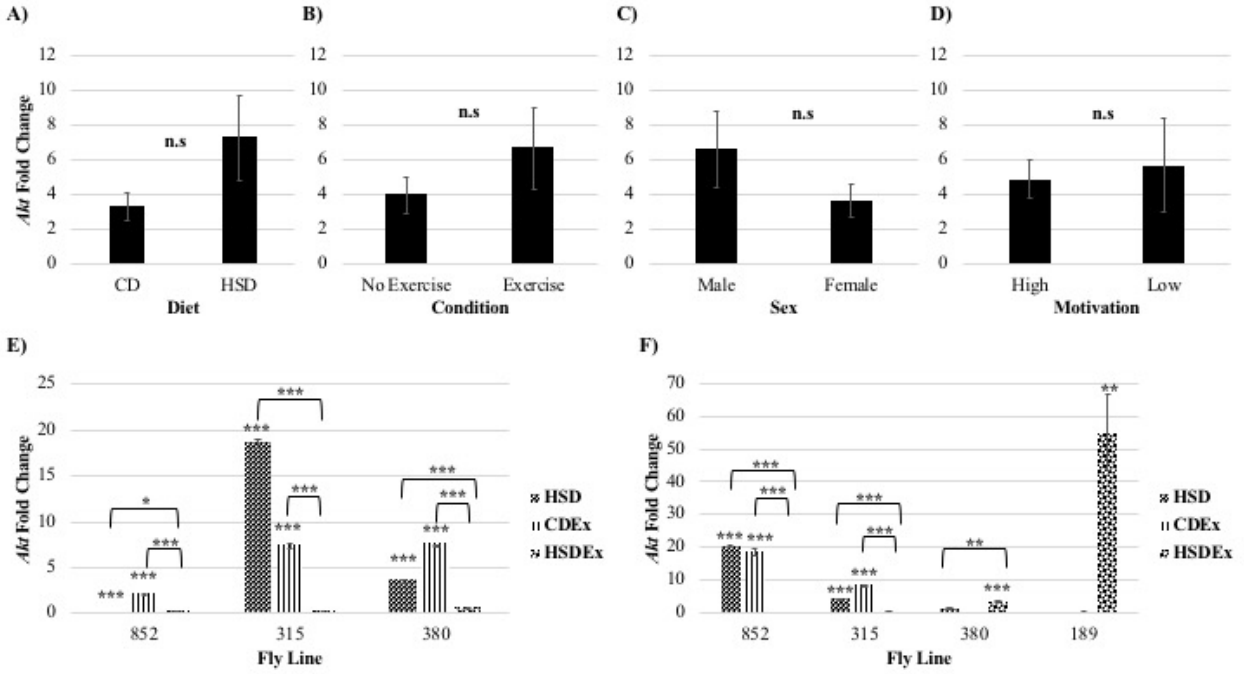

Figure 22. Influence of diet and exercise on *Akt* expression. *Akt* gene activity averaged across all genotypes, both sexes, and separated by (A) CD and HSD conditions, inclusive of exercise

conditions, (B) no exercise versus exercise, inclusive of diet conditions, (C) sex, (D) high or low motivation. (E) *Akt* gene activity from female genotypes. Comparisons are between conditions within a genotype. (F) *Akt* gene activity from male genotypes. Comparisons are between conditions within a genotype. Asterisks labelled with no bar indication are comparisons to their respective control. Asterisks denote the following: * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Expression was normalized to *RpL39*. Relative expression was calculated using the ΔΔCT method (Livak and Schmittgen 2001). A Student's t-test was used to determine significant differences in *Akt* expression between diet, exercise, sex, and motivation groupings in Figures (A)-(D). An ANCOVA test was used to determine significant differences within the data set and a Tukey Honesty Test was used to determine significant differences in *Akt* expression between diet/exercise conditions within genotypes in Figures (E) and (F). Error bars represent SE±.

As with weight and metabolites, to determine small effects of diet and exercise, I separately analyzed flies that differed only in diet or exercise (Supplemental Figure 1; Supplemental Figure 2; Supplemental Figure 3; Supplemental Figure 4). By limiting our analysis to flies on one exercise condition, there was a significant effect of diet on *Dilp2*, *InR*, *Chico*, and *Akt* gene expression (Supplemental Figure 1A,C; Supplemental Figure 2A,C; Supplemental Figure 3A,C; Supplemental Figure 4A,C). Similarly, when I discriminated our analysis to flies on one diet, there was a significant of exercise on *Dilp2*, *InR*, *Chico*, and *Akt* gene expression (Supplemental Figure 1B,D; Supplemental Figure 2B,D; Supplemental Figure 3B,D; Supplemental Figure 4B,D).

All four genes have been previously shown to be involved in insulin response and resistance, but the *Dilp* genes are known to behave independently from one another, and the DILPs are released from various tissues under different conditions (Nässel et al. 2013; Semaniuk et al. 2018). Therefore, I pooled all data from *InR*, *Chico*, *Akt* and examined expression of the group for both diets, both exercise and control, all four genotypes, both sexes. In this pooled expression data, exercise, but not diet, had a significant effect on gene expression (Figure 23A,B). As with the other phenotypes, there were the expected significant differences in IIS pathway regulation between sexes (Figure 23C). Interestingly, however, although motivation seemed to have a strong impact on metabolite concentrations, there was no significant effect of motivation class on the IIS pathway gene regulation (Figure 23D).

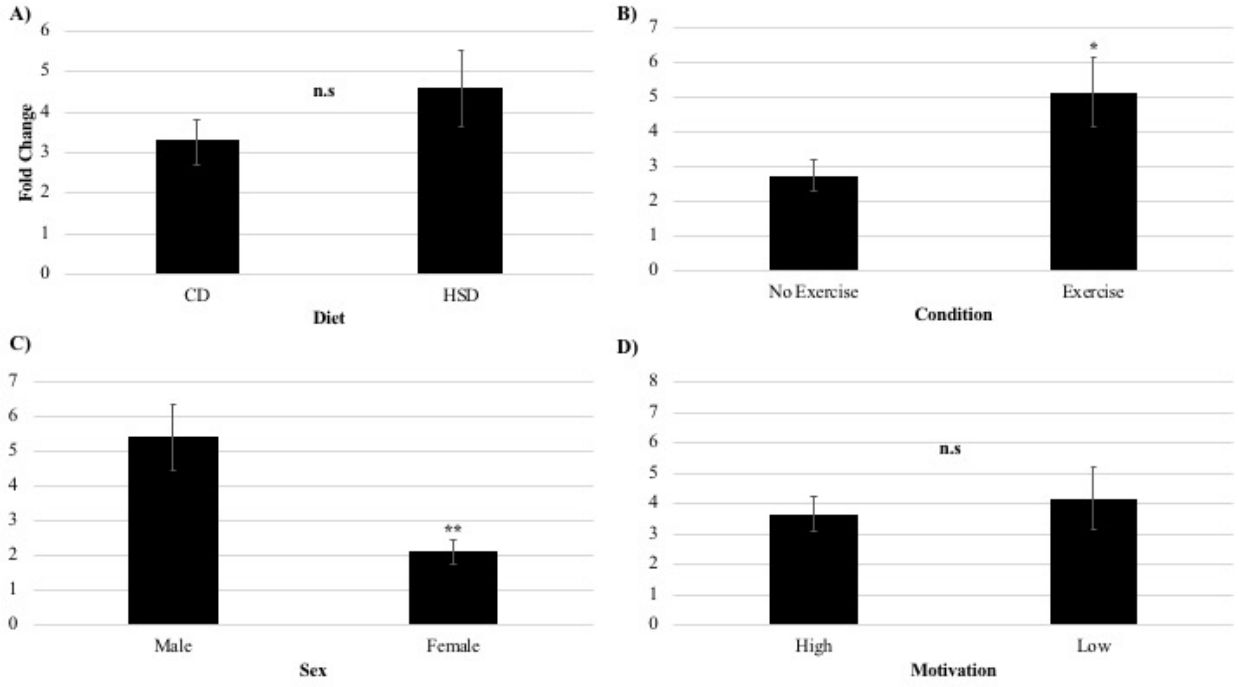

Figure 23. Influence of diet and exercise on the expression of non-*Dilp* insulin pathway genes. Gene activity averaged across all non-*dilp* genes, genotypes, both sexes, and separated by (A) CD and HSD conditions, inclusive of exercise conditions, (B) no exercise versus exercise, inclusive of diet conditions, (C) sex, (D) high or low motivation. Expression was normalized to *RpL39*. Relative expression was calculated using the ΔΔCT method (Livak and Schmittgen 2001). A Student's t-test was used to determine significant differences in insulin pathway gene expression between diet, exercise, sex, and motivation groupings. A MANOVA test was used to determine significant differences within the data set. Error bars represent SE±. Asterisks denote the following: * $p<0.05$, ** $p<0.01$.

In addition to hyperglycemia, glucose intolerance, and an increase in glucose, glycogen, and triglyceride concentrations found in the DGRP 852 fly line, the patterns of IIS gene expression in DGRP 852 were consistent with an insulin resistant phenotype. Transcriptional data gave insight to what I believe, are two sex dependent responses in the regulation of *Dilp2* gene expression, as well as IIS pathway activity. Male DGRP 852 flies displayed hyperinsulinemia, an increase in insulin production, as well as upregulation in *InR*, *Chico*, and *Akt* transcripts. In female DGRP 852 flies, a strong downregulation in *Dilp2* expression was observed, which may suggest female flies have further progressed to complete insulin resistance. The suggestion of female DGRP 852 flies displaying complete insulin resistance is further supported by a strong downregulation in *InR*, *Chico*, and *Akt* transcripts, showing overall reduction in IIS pathway activity.

2.4 Discussion

I examined four lines, including both sexes, to explore how exercise and diet could interact to impact the progression of insulin resistance, and interestingly, found that fly response to diet and exercise is highly genotype and sex-dependent. Strikingly, a HSD that is known to induce insulin resistance in *Drosophila*, specifically the BAE 318 line, only did so in one of the four genetic backgrounds I assayed, line DGRP 852. All fly lines responded substantially differently to diet and exercise, across all the phenotypes I examined, including fasting glucose and oral glucose tolerance tests, weight and metabolite concentrations, metabolomic, and transcriptomic phenotypes. Only the DGRP 852 line consistently presented symptoms of hyperglycemia, glucose intolerance, and/or hyperinsulinemia, symptoms of classical insulin resistance. Moving forward, DGRP 852 can be used as a potential candidate for future insulin resistance studies. In addition, this study demonstrates the complex nature of insulin resistance, that is not only found in humans, but other model species as well. Most importantly, all genotypes and both sexes responded in distinct ways in this study emphasizing the importance of studying disease related states using multiple genetic backgrounds.

Flies Subjected to HSD Display Hyperglycemia but not Glucose Intolerance

Flies who consumed the HSD as well as flies that were exercised versus non-exercised flies, displayed elevated glucose levels, commonly known as hyperglycemia, but ultimately responded with typical glucose clearing kinetics following a fasting period (12-16hrs), refeeding period (1 hour), and recovery time periods (every 15 minutes, up to 45 minutes). Phenotypes associated with

human insulin resistance correlate with the phenotypes found in *Drosophila* which include hyperglycemia, glucose intolerance, and/or hyperinsulinemia (Haselton *et al*. 2010; Musselman *et al*. 2011; Morris *et al*. 2012). Previous work has demonstrated that a diet rich in sucrose can lead to insulin resistant phenotypes in *Drosophila*, at least in the genotypes tested (Musselman *et al*. 2011; Morris *et al*. 2012) Although, overall, flies in this experiment displayed hyperglycemia, an insulin resistant characteristic, fly response to the oral glucose tolerance test was varied and the lack of an overall pattern across all genotypes and both sexes, suggests that the glucose response is sensitive to both. Of the lines I examined, only DGRP 852, displayed classical hyperglycemia and glucose intolerance, and, in fact, across all the lines there was an overall pattern of glucose tolerance (see Figure 5).

Flies Consume Less Food on HSD

Flies that were subjected to the HSD consumed significantly less food than the control food. The reduction in food consumption on the HSD may reflect caloric intake, ie. flies have to eat less food to maintain the same number of calories. Caloric breakdown of each diet shows the HSD is roughly 2 × that of the control diet (Supplemental Table 1-2). The reduced consumption, then, may reflect internal regulation by the flies to maintain consistent caloric intake (Lee *et al*. 2008). Despite the differences observed in food consumption when looking at the overall pattern by diet, flies on the HSD remained hyperglycemic. The differences in food consumption that I demonstrate suggest the importance of quantifying food intake in diet studies.

Fly exercise on the 'Flygometer 2.0' induces metabolomic changes

Flies that were exercised for two hours a day for five days on the 'Flygometer 2.0' experienced physiological changes consistent with an exercise response. An untargeted mass spectrometry-based metabolomic approach detected numerous metabolites in male and female flies (23,604 metabolites in males, 28,068 metabolites in females). Differences between exercise versus no exercise conditions in males and females could be resolved (Figure 7), showing the clear impact exercise has on the metabolome of each sex, and how different each sex responds to exercise. Using the detected metabolites, I unveiled that roughly 11-13% of the metabolome changed in response to exercise in both male and female flies (see Table 2). Looking at the lines individually, roughly 20-29% of the metabolome changed in response to exercise, suggesting response to exercise is influenced by genotype (Table 3; Figure 8). Changes in metabolites in response to gentle fly exercise have been previously demonstrated by Mendez *et al*. 2016, and I found that same effect on a broad metabolomic scale. The changes detected in the metabolomes of each sex as well as the individual lines, demonstrates the effectiveness of our exercise regime. This study further supports *Drosophila melanogaster* as an exercise model, and validates the use of our unique fly treadmill, the 'Flygometer 2.0', to induce gentle fly exercise.

Genotype, Sex, and Motivation Influence Metabolic Phenotypes

I monitored weight and the concentrations of various metabolites known to respond to diet and exercise in a wide suite of species including *Drosophila melanogaster* (Vieira *et al*. 2009; Gordon

et al. 2014; Hansen *et al*. 2015; Mendez *et al*. 2016). Broadly, I found that the biggest impact on weight and metabolites was, strikingly, not diet and exercise, but sex of the fly and motivational state. The differences in response to diet and exercise between fly lines made it difficult to draw broad conclusions, although the large differences likely indicate that the responses across these phenotypes are driven by genetic background and sex. These large line-specific differences mean that, overall, there isn't a single, general, pattern or statistically significant response. A major finding of this study, is, then, that fly response to diet and exercise is genotype and sex specific. Genotype and sex specific responses to diet have been previously found in the mouse, rat, and human (Parks *et al*. 2015; Nadal-Casellas *et al*. 2012; Geer and Shen 2009; respectively).

Although I report small, but significant, changes in metabolites (ie. glucose, glycogen, trehalose) when I separated the flies according to diet or exercise condition, I was able to verify the exercise regime by detecting changes in the metabolome of exercised flies versus non-exercised flies in both sexes and all lines. Flies differing in motivation class have been previously categorized by Mendez *et al*. 2016 and have been shown to differ in phenotypes such as weight and glucose, consistent with results found in our study (weight, glucose, glycogen, and trehalose).

Surprisingly, a diet rich in excess carbohydrates that other groups have shown to increase fly weight (Morris *et al*. 2012) did not produce overweight flies in individual fly lines or overall in our larger analysis. As our HSD did not produce heavier flies, this may suggest that some or all of the lines may be metabolically obese but normal weight (MONW), due to additional phenotypes found in this study such as insulin resistance observed in the line DGRP 852. This phenomenon,

MONW, has been observed in other species, such as human, rat, and mouse models (Conus *et al*. 2007; Kim *et al*. 2007; Cifre *et al*. 2018).

Insulin Pathway Genes are Sensitive to Diet and Exercise

I monitored the expression of four genes from the IIS pathway, *Dilp2*, *InR*, *Chico*, *Akt*, to analyze the impact of diet and exercise on IIS pathway activity. Although metabolite concentrations appeared to be impacted less by diet and exercise, IIS pathway genes were found to be sensitive to exercise but not diet (Figure 19-22). Like weight and metabolite concentrations, gene expression was sensitive to the sex of the fly (Figure 19-21), as expected from previous literature (Graze *et al*. 2018). Similar to our metabolite results, the absence of a response to diet could be explained by the different responses influenced by genetic background and sex. A strong downregulation in *Dilp2* expression on a HSD has been associated with insulin resistance in *Drosophila* (Morris *et al*. 2012), but due to large differences in responses to diet, I was unable to resolve those changes with a larger analysis. Exposure to a HSD has been found to diminish insulin response in flies, eventually becoming completely resistant at advanced ages (Morris *et al*. 2012; Álvarez-Rendón *et al*. 2018), a result in our study that is shown to be genotype and sex specific. The change in gene expression as a consequence of exercise has been previously shown in Sujkowski *et al*. 2015 and Mendez *et al.* 2016, specifically in mitochondrial related genes. Changes in both IIS pathway and mitochondrial related genes can be expected from exercise, given that both play a role in maintaining cellular energy homeostasis (Babbar and Sheikh 2013). A grouped IIS pathway gene analysis revealed exercise, but not diet, had a significant effect on gene expression. The overall

lack of response to diet stems from the influential role genotype and sex have on diet response, thus not showing signs of insulin resistance. An increase in IIS pathway expression was expected since exercise is known to stimulate rapid glucose uptake in various species (Richter *et al*. 2001; Bradley *et al*. 2008; Medeiros *et al*. 2011). Despite flies lacking an overall pattern of insulin resistance transcriptionally, I did happen to find, one line, DGRP 852, that responded to diet in an insulin resistant fashion. Male DGRP 852 flies showed a hyperinsulinemic response and increased insulin pathway activity, while female DGRP 852 displayed a strong downregulation in insulin pathway activity, indicative of complete insulin resistance (see Figures 19-22E,F). Transcriptional data for both male and female DGRP 852 along with other characterized insulin resistant phenotypes in our study, suggests DGRP 852 is a potential candidate for insulin resistant studies.

Although one of four fly lines displayed phenotypes of insulin resistance, these results suggest that genotype and sex play a role in the development and progression of insulin resistance in flies. Across all experiments, all genotypes and both sexes responded to diet and exercise substantially different, a result that correlates with previous work found by Reed *et al.* 2014, which suggests genotype and genotype-by-diet interactions are a major component of transcriptional and metabolomic variation. The variation observed in flies is also observed in humans, where not all individuals will develop T2D, but some individuals are genetically predisposed for the metabolic disease (ie. *TCF7L2* and *HHEX*), and when combined with certain environmental factors, develop T2D (Ali 2013). Much like humans, when studying insulin resistance using flies, it is crucial that we study both sexes and multiple genotypes to further understand how genotype and sex-dependent mechanisms play a role in the diversity of insulin resistance. The current issue in the *Drosophila* insulin resistance field, is that all studies primarily consist of one fly line and one sex

across all experiments. Moving forward, to understand the diverse nature of insulin resistance across different genetic backgrounds and both sexes, it is imperative not to make false assumptions that all flies respond uniformly.

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Chapter 3

3 Discussion & Future Directions

3.1 Quantification of Glucose and Trehalose

In this study, I found both diet, and diet and exercise, conditions influenced both glucose and trehalose levels, when I pooled all lines and both sexes. I found significant differences in glucose levels under both diet and diet and exercise conditions, when I pooled both sexes and all the lines. For trehalose, I found significant differences in only diet and exercise conditions when all lines and both sexes were grouped. Interestingly, when I pooled all the conditions and lines and examined the sexes independently, I found a significant increase in glucose and trehalose levels in female flies. Further, when I grouped the flies by lines identified as high, or low, motivation, I found a significant increase in glucose and trehalose levels in high motivation flies. When I separated the data by genotype and condition, I observed each genotype and sex behaved distinctly for both glucose and trehalose.

In my thesis, I explored possible symptoms of insulin resistance in flies and quantified how diet and exercise impacted fly physiology. I used whole animal homogenates to estimate circulating glucose and trehalose levels, but this approach cannot provide a completely accurate depiction of circulating glucose and trehalose levels. The bidirectionality of glucose transporters allow free glucose to travel within intracellular and extracellular compartments, resulting in most free glucose being found in the circulating hemolymph rather than within cells (Tennessen *et al*. 2014). Using samples of hemolymph rather than whole animal homogenate would give a much more precise representation of circulating metabolite levels, which is important in insulin

resistant studies (Tennessen *et al*. 2014). Measurement of circulating sugar levels in the hemolymph of the fly gives an accurate representation of whether the fly displays hyperglycemia, a symptom of insulin resistance.

Future research could explore the fly's true circulating glucose and trehalose levels using hemolymph samples to determine an accurate representation of the fly's glycemic state. Future research could also incorporate injections of exogenous insulin to determine glucose tolerance or intolerance. Injecting the fly with exogenous insulin tests the ability of the cells to respond to circulating glucose after being fed a HSD. Hemolymph samples would then be collected to analyze glucose tolerance/intolerance in the fly (Haselton and Fridell 2011). This combination of approaches could give more insight into whether the fly displays symptoms of insulin resistance. These approaches are, however, time and labour intensive and require unique injection equipment.

3.2 Isocaloric Diets

In this study, overall, I found that flies that were fed the HSD consumed less food when compared to the CD, possibly because of the HSD containing almost double the number of calories. This difference in the amount of food consumed suggests that the flies may not have to eat as much food to maintain approximately the same number of calories. This difference in food consumption leads me to question if the result I found is in fact due to caloric content. Future research could address this question by placing the flies on an isocaloric diet, diets with the same caloric density, to analyze whether the HSD food consumption pattern is associated with calories or not. Isocaloric diets have been implemented in multiple model species such as fruit fly, mice, rat, and pig (Lee

2015; Donovan *et al*. 2007; Axen *et al*. 2017; Liu *et al*. 2017). If food consumption is in fact regulated by calorie content, then future sucrose experiments will need to incorporate isocaloric diets to standardize the amount of food being eaten. Additionally, the reduction in food consumption on the HSD leads to a reduction in protein intake. Moving forward, diet studies should be performed by manipulating the content of protein while keeping a constant sucrose concentration, and analyzing the relationship between sucrose:protein and food consumption, and further the effect on the longevity of the fly.

3.3 RT-qPCR of *Dilps*

Insulin and IIS pathway genes were regulated in a manner that was significantly influenced by sex and genotype. In this thesis, I quantified gene expression of *Dilp2* and other IIS pathway genes (*InR*, *Chico*, *Akt*) to analyze the presence or absence of an insulin resistant state within the flies. I found that a particular line, DGRP 852, behaved with symptoms of insulin resistance. Both male and female DGRP 852 displayed independent insulin resistant-like responses that lead me to believe both sexes of DGRP 852 present different insulin resistant progressions. Unfortunately, due to the COVID-19 virus, I did not have time to quantify *Dilp3* and *Dilp5*, additional *Dilps* produced and secreted from the IPC's of the *Drosophila* brain. Although I did not quantify these *Dilps*, the lack of information did not play a crucial role in analyzing the activity of the IIS pathway. It is however, important to study these *Dilps* together since they are produced from the same location, as well as all *Dilps* are regulated independently of one another and have diverse roles (Nässel *et al*. 2013; Semaniuk *et al*. 2018). It has been shown that when adult flies are placed

on a HSD, *Dilp3*, and *Dilp5* are downregulated in response to excess carbohydrates, and on a high protein diet they are slightly upregulated in response to excess protein (Morris *et al*. 2012). The modification of diets (i.e. high/low protein, high/low sucrose) have a direct effect on *Dilp* regulation, and therefore I recommend quantifying *Dilp2* and *Dilp3* in specific, but further studies should not be limited to these particular *Dilps*. Future research analyzing all *Dilps* will give more insight into how exercise and diet impact *Dilp* regulation and provide more information on how the *Dilps* are independently regulated.

3.4 Insulin Pathway Analysis by Western Blotting

Future research could examine post-translational modification of IIS pathway proteins to confirm insulin resistance in flies. Future work could quantify the phosphorylation of Ser505-AKT and Thr398-S6K, two proteins found in the insulin signaling pathway. I had intended to monitor the phosphorylation status of these two proteins, but due to lack of replication, no conclusions could be drawn. Future work could optimize the Western Blot analysis of both Ser505-AKT and Thr398-S6K so that replicative results are achieved. Generally, studies utilize these proteins to monitor insulin pathway activity, specifically PI3K and TOR pathways respectively (Bjedov *et al*. 2010; Lindquist *et al*. 2011; Kim *et al*. 2018). In this study, I aimed to monitor the phosphorylation status of these proteins in whole fly extracts, to analyze whether the HSD was reducing IIS pathway activity, overall giving an insulin resistant phenotype. An insulin resistant state would result in lower IIS pathway activity, and in turn reduce kinase activity (Bjedov *et al*. 2010). The optimization of each antibody concentration was completed. Unfortunately, the replication

between western blots was not consistent and therefore no conclusions were drawn. Alternatively, the lack of consistent replicates may be due to a tissue specific response. Moving forward, this experiment should incorporate samples of fat body tissue, as IIS signaling activity has been shown to decrease in fat body tissue upon overfeeding states (Morris *et al*. 2012; Álvarez-Rendón *et al*. 2018). The fat body of the fly is functionally equivalent to adipose tissue and liver found in humans (Zhang and Xi 2015).

3.5 Conclusion

The progression of human T2D is still poorly understood, and the cases not only affect individuals with advanced age, but now young adolescents. This project, using *Drosophila melanogaster*, is the starting foundation for insulin resistant studies in the Merritt lab. My research has identified clear distinctions in metabolite levels between high and low motivational groups. My work has demonstrated that motivation plays a key role in influencing physiological responses to both diet and exercise. Along with motivation playing a role, my research has shown that both sex and genotype heavily influence the outcome of an insulin resistant phenotype. In addition, my work has given insight to a particular fly line, DGRP 852, that displays insulin resistant-like symptoms such as hyperglycemia, glucose intolerance, and hyperinsulinemia in males. Despite not finding hyperinsulinemia in females, IIS signaling activity was reduced which, interestingly enough, may suggest the finding of different insulin resistant progressions in both sexes. While the other fly lines do not display signs of insulin resistance, it is clear here that genetic background and sex play a role. Future work will include further confirming insulin resistance in the DGRP 852 fly line by

exploring circulating glucose levels by hemolymph samples, *Dilp* regulation by RT-qPCR, as well as quantifying the phosphorylation of both Ser505-AKT and Thr398-S6K by Western Blot analysis. Once confirmed, a transcriptomic approach can be used to try and identify genes that genetically predispose for insulin resistance. An additional approach could be to perform untargeted metabolomics, where identified metabolites would undergo pathway analysis to shed light on the progression of insulin resistance. This work will give a better understanding of the complex disease and can be potentially translated to Diabetes research in humans.

3.6 References

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Appendices

Appendix A: Supplemental Tables & Figures

1. Caloric Breakdown of Diets

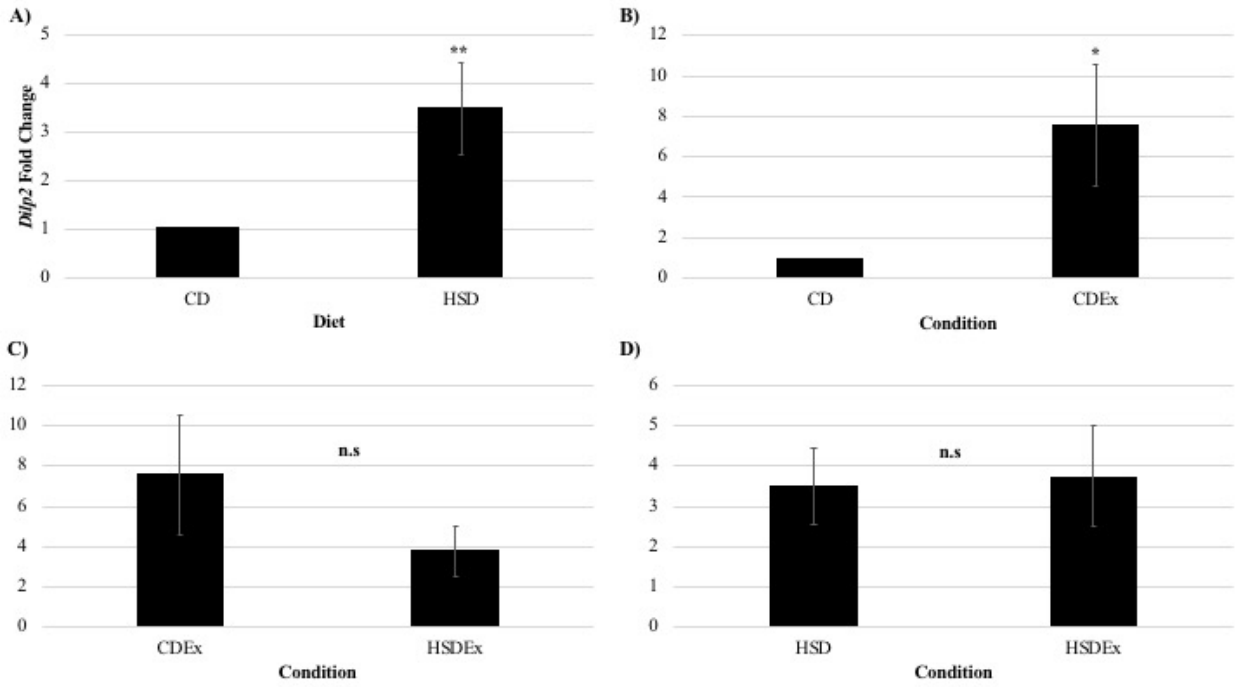
Reagent	Amount (g/L) Food	Carbs (g)	Protein (g)	Fat (g)	Total kCal
Agar	10	8.9	0	0.1	
Brewer's Yeast	80	32	36	0.8	
Yeast Extract	20	3.3	10.8	0	
Peptone	20	0.1	14.6	0	
Sucrose	100	100	0	0	
Propionic Acid		6	0	0	
Methyl Paraben		11	0	0	
Total Grams		144.3	61.4	0.9	
Total kCal		577.2	245.7	7.7	830.6

Supplemental Table 1. Nutrient content of CD food. Diets were modified from Bloomington's semi-defined food to contain only sucrose. Sucrose content was adjusted from larval (Musselman *et al*. 2011) to adult fly concentrations (Morris *et al*. 2012). Calculations were based on manufacturer's datasheets.

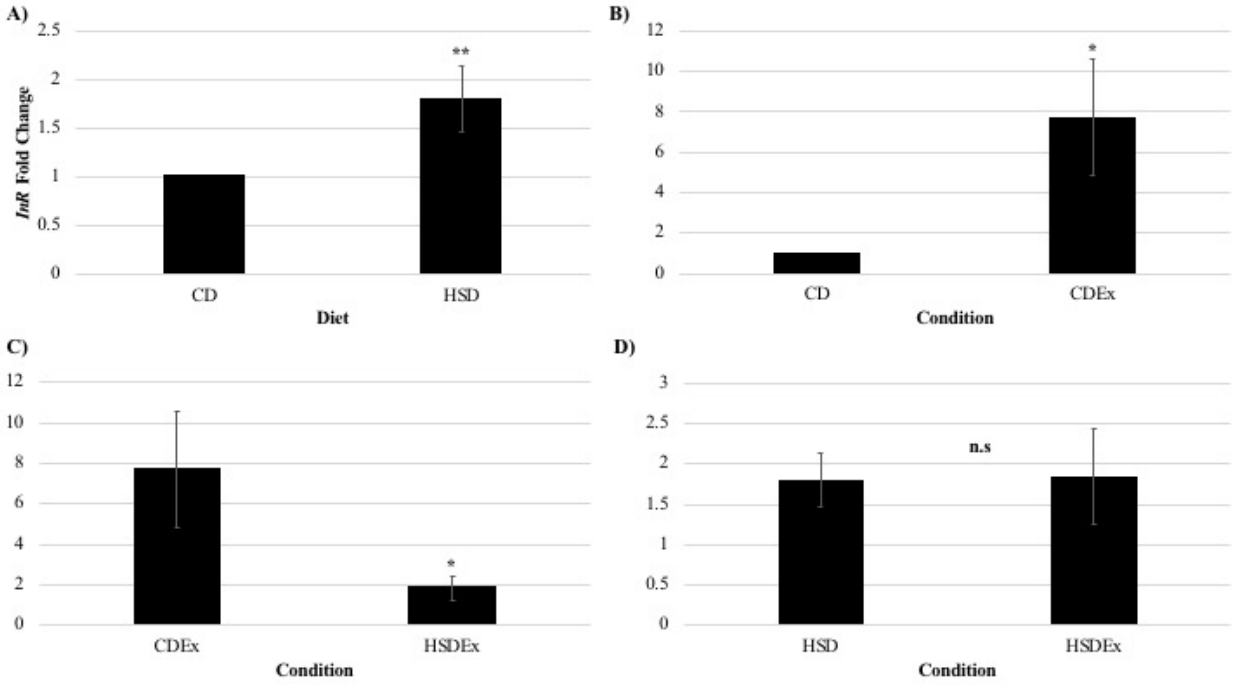
Reagent	Amount (g/L) Food	Carbs (g)	Protein (g)	Fat (g)	Total kCal
Agar	10	8.9	0	0.1	
Brewer's Yeast	80	32	36	0.8	
Yeast Extract	20	3.3	10.8	0	
Peptone	20	0.1	14.6	0	
Sucrose	300	300	0	0	
Propionic Acid		6	0	0	
Methyl Paraben		11	0	0	
Total Grams		344.3	61.4	0.9	
Total kCal		1377.2	245.7	7.7	1630.6

Supplemental Table 2. Nutrient content of HSD food. Diets were modified from Bloomington's semi-defined food to contain only sucrose. Sucrose content was adjusted from larval (Musselman *et al*. 2011) to adult fly concentrations (Morris *et al*. 2012). Calculations were based on manufacturer's datasheets.

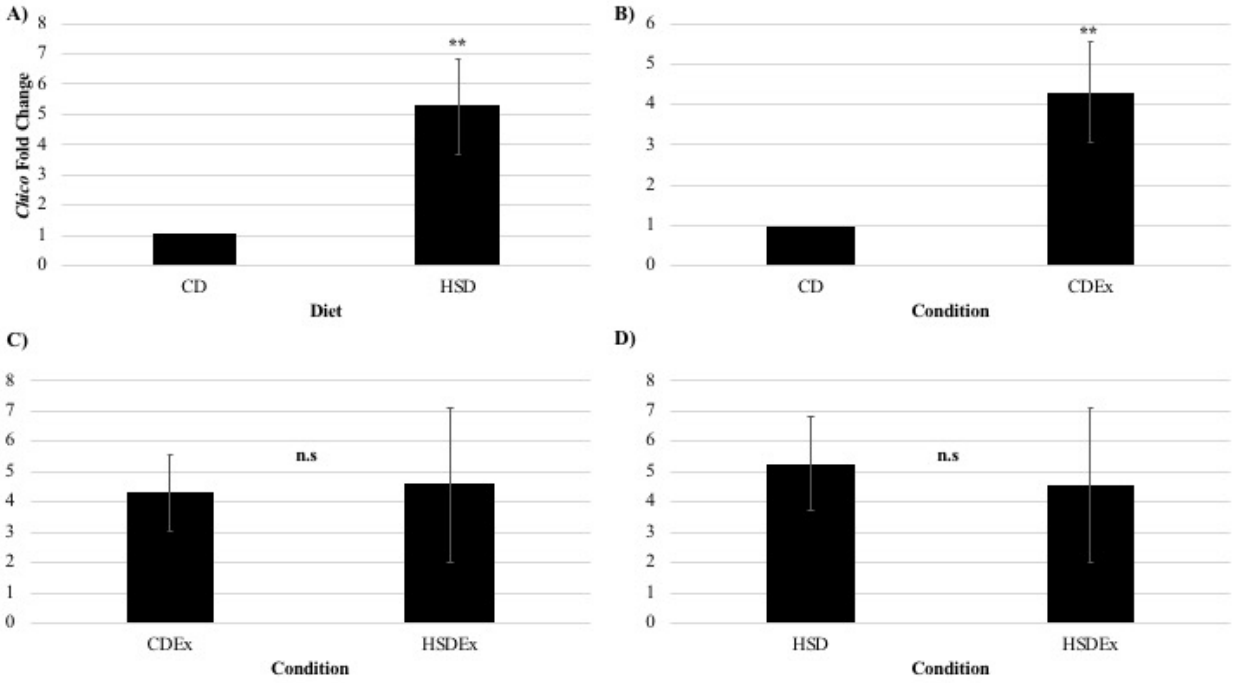
2. Insulin Pathway Genes



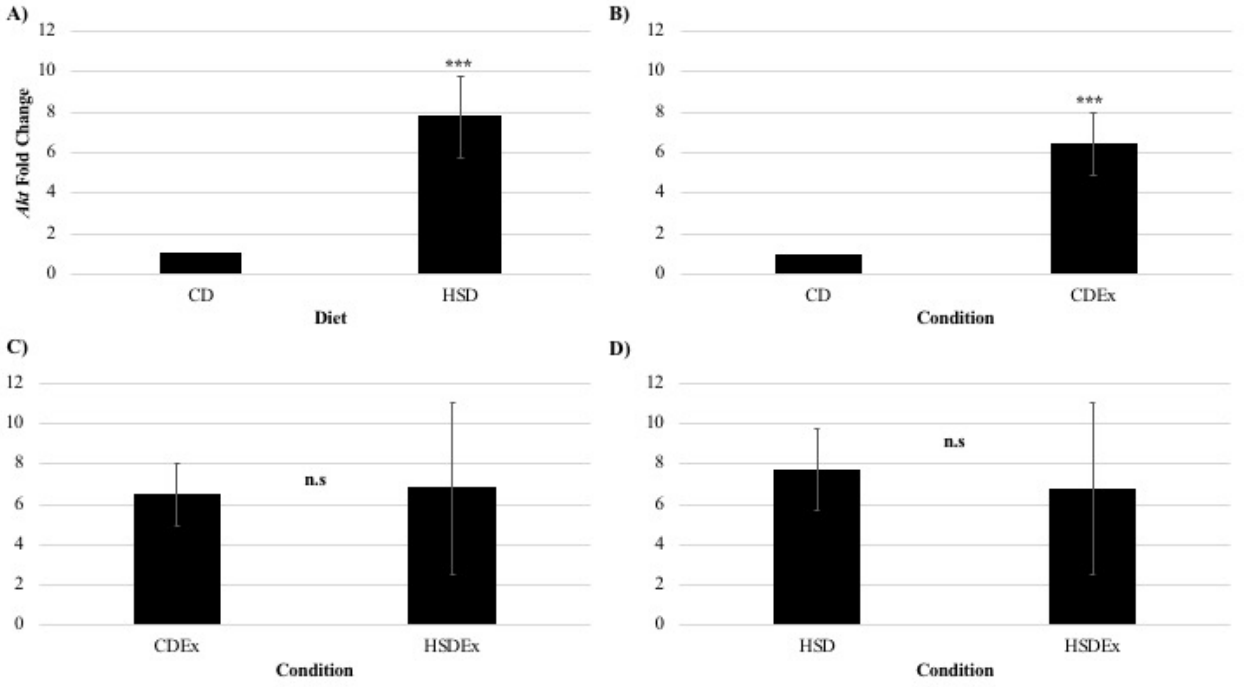
Supplemental Figure 1. Effect of diet and exercise on *Dilp2* expression. *Dilp2* gene expression averaged across all genotypes, both sexes, and separated by (A) CD and HSD conditions, exclusive of exercise conditions, (B) CD and CDEx conditions, exclusive of diet conditions, (C) CDEx and HSDEx conditions, (D) HSD and HSDEx conditions. A Student's t-test was used to determine significant differences in *Dilp2* expression between diet and diet with exercise conditions in Figures (A)-(D). Asterisks denote the following: * $p<0.05$, ** $p<0.01$.



Supplemental Figure 2. Effect of diet and exercise on *InR* expression. *InR* gene expression averaged across all genotypes, both sexes, and separated by (A) CD and HSD conditions, exclusive of exercise conditions, (B) CD and CDEx conditions, exclusive of diet conditions, (C) CDEx and HSDEx conditions, (D) HSD and HSDEx conditions. A Student's t-test was used to determine significant differences in *InR* expression between diet and diet with exercise conditions in Figures (A)-(D). Asterisks denote the following: * $p<0.05$, ** $p<0.01$.



Supplemental Figure 3. Effect of diet and exercise on *Chico* expression. *Chico* gene expression averaged across all genotypes, both sexes, and separated by (A) CD and HSD conditions, exclusive of exercise conditions, (B) CD and CDEx conditions, exclusive of diet conditions, (C) CDEx and HSDEx conditions, (D) HSD and HSDEx conditions. A Student's t-test was used to determine significant differences in *Chico* expression between diet and diet with exercise conditions in Figures (A)-(D). Asterisks denote ** $p<0.01$.



Supplemental Figure 4. Effect of diet and exercise on *Akt* expression. *Akt* gene expression averaged across all genotypes, both sexes, and separated by (A) CD and HSD conditions, exclusive of exercise conditions, (B) CD and CDEx conditions, exclusive of diet conditions, (C) CDEx and HSDEx conditions, (D) HSD and HSDEx conditions. A Student's t-test was used to determine significant differences in *Akt* expression between diet and diet with exercise conditions in Figures (A)-(D). Asterisks denote *** $p<0.001$.