UNIVERSITY OF CALIFORNIA, SAN DIEGO

Drosophila melanogaster as a Model to Dissect Obesity-Associated Mechanisms

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy

in

Biomedical Sciences

by

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The Dissertation of Erilynn Heinrichsen is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Chair

University of California, San Diego

2013

DEDICATION

I am so proud to be able to dedicate this dissertation to my grandmothers, Priscilla Ann Berge and Mary Jo Coony, two phenomenal women and life-long educators who have inspired me in so many ways.

TABLE OF CONTENTS

Signature Page……... iii

Dedication……... iv

Table of Contents……... v

List of Abbreviations…….. viii

List of Figures…… ix

Acknowledgements…… xi

Vita…… xiv

Abstract of the Dissertation…….. xv

Chapter 1: Role of High-Fat Diet in Stress Response of *Drosophila*……..……..…... 1

Abstract…… 1

Introduction……... 1

Methods……... 3

Results……... 7

Phenotypic Profile of Flies on High-fat Diet..……... 7

Effects of Intermittent or Constant Hypoxia on Flies on a High-fat Diet…….. 11

Discussion……... 19

Acknowledgements…… 22

Appendix 1.1. Additional phenotypic assessment of flies on high-fat diet (HFD): Protein levels and body weight……. 23

Appendix 1.2. Additional phenotypic assessment of flies on high-fat diet (HFD): Geotaxis measurement……... 24

Appendix 1.3. Additional phenotypic assessment of flies on high-fat diet (HFD): Activity and sleep measurements…… 25

Appendix 1.4. Additional phenotypic assessment of flies on high-fat diet (HFD): Response of male flies to HFD and intermittent hypoxia (IH)…… 26

Chapter 2: High-Fat Diet and Intermittent Hypoxia Lead to Transcriptional Changes in *Drosophila*…… 27

Abstract…… 27

Introduction……... 27

Methods……... 29

Results……... 31

Changes in gene expression due to high-fat diet in normoxia…….. 32

Screening genes involved in high-fat diet phenotype in normoxia..…….. 33

Changes in gene expression due to intermittent hypoxia……..……..…… 37

Screening genes involved in intermittent hypoxia phenotype in flies on high-fat diet……..……..……..……..……..…….. 40

CG9510 as candidate gene for further study……..…… 44

Discussion…….…….. 46

Acknowledgements……..……... 47

Appendix 2.1. Significantly affected KEGG pathways……..... 48

Appendix 2.2. List of RNAi stocks used in screening candidate genes from high-fat diet microarray…….…..... 49

Appendix 2.3. List of RNAi stocks used in screening candidate genes from intermittent hypoxia microarray…….…… 50

Appendix 2.4. Differentially Expressed Genes…….. 52

Chapter 3: Metabolic and Transcriptional Response to a High-Fat Diet in *Drosophila melanogaster*…….…… 56

Abstract…… 56

Introduction……..…… 56

Methods…….……... 58

Results…… 63

Metabolomic analysis of flies on HFD..…..……... 63

Role of *CG9510* in response to HFD……..... 65

CG9510 in *Drosophila* metabolism..……..…….. 74

Discussion…….... 76

Acknowledgements……..….. 81

Appendix 3.1. Lifespan of flies with down-regulation of *CG9510* in normoxia……... 82

Appendix 3.2. Verification of *CG9510* expression in high-fat diet and transgenic fly lines……...…..…….…….…… 83

Appendix 3.3. Supplemental Methods…..…….…….…….…. 84

References……..… 85

LIST OF ABBREVIATIONS

AcCoA	Acetyl-coenzyme A
Adp^{60}	$Adipose^{60}$
Arm-Gal4	Armadillo-Gal4
ASL	Argininosuccinate lyase
BCAA	Branch chain amino acid
CAFE	Capillary feeder
CF	Coconut food (same as HFD, high-fat diet)
CH	Constant hypoxia
CS	Canton-S
Da-Gal4	Daughterless-Gal4
EASE	Expression Analysis Systematic Explorer (DAVID analysis)
FAME	Fatty acid methyl ester
Fdr	False discovery rate
GC/MS	Gas chromatography/mass spectrometry
HFD	High-fat diet (same as CF, coconut food)
IH	Intermittent hypoxia
LD	Light:Dark cycle
MeOH	Methanol
N	Normoxia
Ore-R	Oregon-R
OSA	Obstructive Sleep Apnea
PCR	Polymerase chain reaction
PF	Palm food
RD	Regular diet (same as RF, regular food)
RF	Regular food (same as RD, regular diet)
RT-PCR	Reverse-transcriptase PCR
TBDMS	Tert-butyldimethylsilyl
TCA	Tricarboxylic acid
TG	Triglyceride
TMS	Trimethylsilyl
Tobi	Target of brain insulin
VDRC	Vienna Drosophila RNAi Center
WHO	World Health Organization
w^{1118}	white-1118

LIST OF FIGURES

Figure 1.1.	Changes in metabolic profile of flies on high-fat diet compared with regular diet……	9
Figure 1.2.	Altered lifespan due to high-fat diet……..	10
Figure 1.3.	Decreased tolerance to acute stress in flies on high-fat diet……	11
Figure 1.4.	Changes in metabolic profile due to high-fat diet and hypoxia……	13
Figure 1.5.	Altered lifespan due to high-fat diet and hypoxia……..	14
Figure 1.6.	Anoxia tolerance unaltered by hypoxia exposure……	15
Figure 1.7.	Cold survival altered by intermittent hypoxia, but not constant hypoxia..	16
Figure 1.8.	Changes due to intermittent hypoxia also seen in genetic mutant model of obesity……	17
Figure 1.9.	Similar food consumption observed in normoxia and intermittent hypoxia……..	18
Figure S1.1.	Altered starvation resistance due to high-fat diet……..	19
Figure 2.1.	Microarray results evaluating change in gene expression in *Drosophila* on a high-fat diet (HFD) compared to a regular diet (RD)……	33
Figure 2.2.	Paradigm for screening candidate genes from microarray analysis of high-fat diet compared to regular diet in normoxia……	34
Figure 2.3.	*CG9510* and *CG1934* show significant phenotypes from screen of candidate genes……..	36
Figure 2.4.	Microarray results evaluating change in gene expression in *Drosophila* in intermittent hypoxia (IH) compared to normoxia (N) while on a regular diet (RD) or a high-fat diet (HFD)……..	39
Figure 2.5.	Paradigm for screening candidate genes from microarray analysis of intermittent hypoxia (IH) compared to normoxia (N) in flies on a high-fat diet (HFD)……	40
Figure 2.6.	Screen of candidate genes for role in intermittent hypoxia (IH) phenotype……..	43
Figure 2.7.	Overlapping genes altered by HFD and IH with differential expression……..	44
Figure 2.8.	Down-regulation of *CG9510* leads to increased triglyceride levels in intermittent hypoxia…….....	45
Figure 3.1.	Increased fatty acid accumulation and altered carbohydrate and amino acid metabolism in *Drosophila* on a high-fat diet (HFD)……	64
Figure 3.2.	Increased oxygen consumption in *Drosophila* on a high-fat diet (HFD)..	65
Figure 3.3.	Down-regulating *CG9510* in various tissues simulates high-fat diet phenotype…….....	66

Figure 3.4.	Similar food consumption observed in control flies and flies with down-regulation of *CG9510*……..	67
Figure 3.5.	Total glucose levels unaffected in flies with down-regulation of *CG9510*	68
Figure 3.6.	Altered lifespan and heart function with ubiquitous down-regulation of *CG9510*……...…….....……...…….....……....	70
Figure 3.7.	Down-regulating *CG1315* simulates high-fat diet phenotype……...	72
Figure 3.8.	Up-regulation of *CG9510* decreases stored triglyceride levels, increases survival…….....	73
Figure 3.9.	Increased fatty acid accumulation and altered urea and TCA metabolite abundance in *Drosophila* with down-regulation of *CG9510*...	75
Figure 3.10.	Model of *Drosophila* response to a high-fat diet……..	78

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ABSTRACT OF THE DISSERTATION

Drosophila melanogaster as a Model to Dissect Obesity-Associated Mechanisms

by

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Doctor of Philosophy in Biomedical Sciences

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Obesity affects a tremendous number of adults and children in the United States and worldwide, enough to be classified as a global epidemic by the World Health Organization (WHO). Given the serious complications associated with obesity, including heart disease, diabetes and cancer, it is critical to improve our understanding of the mechanisms underlying the response to obesity. In order to explore the pathogenesis of diet-induced obesity, we use *Drosophila melanogaster* as a model to evaluate the phenotypic, metabolic and transcriptional changes occurring in response to a high-fat diet.

Similar to obesity in humans, *Drosophila* fed a high-fat diet (HFD) have increased triglyceride and glucose levels and decreased lifespan. Their tolerances to anoxic and cold stresses are diminished to only two-thirds and one-quarter, respectively, that of flies on regular diets (RD). In light of the multiple obesity-associated complications that involve hypoxia, we examined the effect of hypoxia on flies and found that intermittent hypoxia (IH), in particular, causes dramatic changes in phenotype. When flies spend a week in IH while on either RD or

HFD, their triglyceride levels decrease and cold stress tolerance increases to nearly 100% survival. Meanwhile, IH seems to exacerbate the glucose levels in flies on both diets, and even more so in flies on HFD.

We hypothesized that the phenotypic changes resulting from HFD or IH were likely accompanied by transcriptional changes in metabolic pathways. Indeed, microarray analysis reveals changes in amino acid metabolic pathways in flies on HFD and a candidate gene involved in arginine metabolism, *CG9510*, is able to simulate the HFD phenotype even when on regular diet (RD). Meanwhile, carbohydrate metabolic pathways are affected by IH in flies both on RD and HFD. The expression of several candidate genes, including *CG9510*, are differentially regulated by HFD and IH, suggesting that perhaps the two stresses are causing vastly different phenotypes by altering gene expression in opposite directions.

Analysis of metabolite abundance using mass spectrometry of normoxic flies on HFD compared to RD reveals changes in similar pathways as those showing transcriptional changes. Flies on a HFD have altered abundances of metabolites associated with fatty acid, amino acid, and carbohydrate metabolism. We confirmed that the candidate gene identified from the HFD microarray has a similar function as its human homologue, argininosuccinate lyase (ASL), as it alters levels of urea and TCA cycle intermediates and thus lies at a key junction between carbon and nitrogen metabolism. The importance of this gene is further verified when up-regulation of *CG9510* reverses aspects of the HFD phenotype.

Reminiscent of the metabolic consequences that accompany diet-induced obesity in humans, *Drosophila* on a high-fat diet demonstrate a severely worsened phenotype and disrupted metabolic homeostasis. As a part of the response to HFD, flies on HFD alter *CG9510* as a key enzyme at the junction of amino acid and carbohydrate metabolism. Importantly, manipulation of this single gene directly affects triglyceride storage and cold tolerance. As a result of these studies, we have identified a novel link between amino acid metabolism and obesity.

CHAPTER 1:

Role of High-fat Diet in Stress Response of *Drosophila*

Abstract

Obesity is associated with many diseases, one of the most common being obstructive sleep apnea (OSA), which in turn leads to blood gas disturbances, including intermittent hypoxia (IH). Obesity, OSA and IH are associated with metabolic changes, and while much mammalian work has been done, mechanisms underlying the response to IH, the role of obesity and the interaction of obesity and hypoxia remain unknown. As a model organism, *Drosophila* offers tremendous power to study a specific phenotype and, at a subsequent stage, to uncover and study fundamental mechanisms, given the conservation of molecular pathways. Herein, we characterize the phenotype of *Drosophila* on a high-fat diet in normoxia, IH and constant hypoxia (CH) using triglyceride and glucose levels, response to stress and lifespan. We found that female flies on a high-fat diet show increased triglyceride levels ($p<0.001$) and a shortened lifespan in normoxia, IH and CH. Furthermore, flies on a high-fat diet in normoxia and CH show diminished tolerance to stress, with decreased survival after exposure to extreme cold or anoxia ($p<0.001$). Of interest, IH seems to rescue this decreased cold tolerance, as flies on a high-fat diet almost completely recovered from cold stress following IH. We conclude that the cross talk between hypoxia and a high-fat diet can be either deleterious or compensatory, depending on the nature of the hypoxic treatment.

Introduction

Over 60% of the population in the United States is estimated to be obese or overweight, a number that has dramatically increased in recent decades and continues to climb [1]. A multitude of social and economic factors have contributed to the rise in obesity, not the least of which is an abundance of processed foods high in saturated fat and simple carbohydrates. With

obesity comes many disease complications, including sleep apnea, hypoxia, atherosclerosis, cardiovascular diseases and stroke [2].

In obstructive sleep apnea (OSA), one of the most common associated diseases, the upper airway collapses repeatedly during sleep, causing chronic intermittent hypoxia [3]. OSA patients therefore are often challenged with both obesity and intermittent hypoxia conditions. In order to investigate the potential interaction of obesity and hypoxia, we needed a model that would allow us to study metabolic changes and be able to survive hypoxic challenges. Furthermore, such a model would need to lend itself to molecular analysis to appreciate the basis for such phenotypic changes.

The fruit fly, *Drosophila melanogaster*, has served as a useful genetic model system in many situations, including development and human disease states. We undertook this investigation to determine whether we can develop a model in the fly that would be helpful for understanding the effects of hypoxia and obesity and their potential interactions. We believe that the fly could be an important model for various reasons, including the facts that a) many of the metabolic and signaling pathways involved in fat metabolism and insulin signaling in flies are conserved in humans [4] and b) *Drosophila* have many organ systems analogous to humans that control uptake, storage and metabolism; one of these is the fat body, which functions like human liver and white adipose tissue, metabolizing nutrients and storing reserves of glycogen and lipids [4]. The adult fat body is subject to diet-induced lipid overload, making *Drosophila* even more appealing as a genetic model in which to study obesity [5-7]. Furthermore, many human disease genes (>70%) have been found to exist in flies [8].

Since the early 1990s, our laboratory has used *Drosophila melanogaster* to investigate the effects of hypoxia on metabolism, gene regulation, and the role of gene regulation in resistance or susceptibility to injury. More recently, we have studied the effects of both intermittent (IH) and constant hypoxia (CH) [9-11], since each of these conditions occurs in many different diseases and causes significant stress on the organism. Additionally, we have shown

that the two separate paradigms of IH and CH result in a differential change in gene expression [12], making it all the more important to individually evaluate and compare the two stresses.

With the ability to put flies on a diet high in saturated fat while simultaneously exposing them to either normoxic or hypoxic conditions, we were able to investigate three different aims. These involved assessing the effects of a high-fat diet, the effects of intermittent hypoxia, and their potential interaction on the phenotype of *Drosophila*. We hypothesized that intermittent hypoxia alters lipid metabolism, leading to changes in stress tolerance in *Drosophila*, and the results show clear evidence of this interaction.

Materials and Methods

Fly Rearing and Collection.

All stocks were maintained on standard cornmeal *Drosophila* medium in an incubator at 25°C and 30-50% humidity. Adult flies were collected at 0-3 days and transferred to a separate vial of the standard cornmeal medium in room air. After aging for 3 more days, male and female flies were separated and only the females were transferred to the experimental diet and oxygen condition. In all experiments, unless otherwise noted, w^{1118} flies were used in order to facilitate future work with genetically mutated lines, since this is a common background of flies with P-element and UAS insertions. When $adipose^{60}$ mutants were studied, the wildtype strain used was *Oregon-R* in order to control for genetic background. The stocks of *Oregon-R, Canton-S* and w^{1118} were obtained from Bloomington Stock Center, while $adipose^{60}$ was a gift from Dr. Tania Reis.

Experimental Diets: Regular food and Coconut food.

Jazz Mix *Drosophila* food from New Horizon Foods was prepared as directed and placed in plastic vials as the Regular food (RF). The Coconut food (CF) diet was based on a recipe developed by Dr. Sean Oldham at the Sanford/Burnham Institute, adding coconut oil to the regular food as a source for increased saturated fat in the diet [7]. The recipe has been

specialized for the current model, with the coconut food diet consisting of 5, 10 or 20% weight per volume of food-grade coconut oil, with the last two higher than the maximum 7% saturated fat recommended by the American Heart Association. The 5% diet did not have an obvious enough effect, and no significant difference was found between the 10 and 20% supplemented food, so only results from the 20% diets are reported. For verification of results, we also tested a diet supplemented with palm oil in the same manner as described above for coconut oil.

Experimental Oxygen Conditions: Normoxia, Intermittent and Constant Hypoxia.

At age 3-6 days, female flies were sorted onto the diets and immediately placed in normoxia, intermittent hypoxia or constant hypoxia, with approximately 25 flies per vial. All three conditions were at room temperature (22-24°C) and in a similar environment.

In normoxic conditions, flies were kept in room air (21% O_2). For intermittent hypoxia (IH) and constant hypoxia (CH), flies were placed in specially designed chambers where the oxygen levels are carefully controlled using a combination of oxygen and nitrogen with the Oxycycler hydraulic system (Model A44x0, BioSpherix, Redfield, NY) and ANA-Win2 Software (Version 2.4.17, Watlow Anafaze, CA). In the chamber for IH, flies were exposed to O_2 levels alternating between 4 minutes at 1% and 4 minutes at 21% O_2. The total time for one IH cycle was 20 minutes, with a ramp time of 1 minute for 1%–21% O_2 and around 10 minutes for 21%– 1% O_2. In the CH chamber, flies were exposed to a constant oxygen level of 5% O_2. Oxygen levels were chosen based on previous observations in our laboratory in order to allow flies to be mobile and still consume their diets.

Flies remained in their oxygen condition for one week if they were to be assayed or as long as needed to examine lifespan. To maintain consistent food conditions, flies were transferred to fresh food of their respective diet every 3-4 days.

Metabolic Profile: Triglyceride, Glucose and Protein Measurement.

After one week in a specific oxygen condition, flies were collected in groups of five female flies and placed in 1.5 ml microcentrifuge tubes. Their live weight was determined and the flies were frozen on dry ice. They were then homogenized using the Precelly's 24 homogenizer and prepared as described in Grönke 2003 [13] to measure absolute triglyceride levels using the Thermo Infinity Triglyceride kit and protein levels using Pierce BCA protein assay (protein levels not shown, as normalizing triglycerides to protein was comparable with normalizing to bodyweight). Differing levels of triglycerides reported in the current literature can easily be accounted for by multiple factors, including the sex and age of the flies, and the method of extraction.

To determine whole-body glucose, groups of five female flies were homogenized in 1 ml deionized water. The homogenates were centrifuged and the supernatants transferred to a 96-well plate in triplicate. Glucose levels were quantified using the Glucose GO assay kit (Sigma–Aldrich, Saint Louis, MO) according to the manufacturer's instructions. In brief, glucose is oxidized to gluconic acid and hydrogen peroxide by glucose oxidase. The hydrogen peroxide then reacts with o-dianisidine in the presence of peroxide to form a colored product, and the oxidized o-dianisidine reacts with sulfuric acid to form a more stable colored product. The intensity of the color is proportional to the original glucose concentration. The whole-body free glucose was measured, although it is important to note that the major carbohydrate in *Drosophila* is trehalose. Trehalose is the product of two glycolytic intermediates, with the condensation of glucose-6-phosphate and the glucose moiety of uridine diphosphoglucose (UDP-glucose). Trehalose is then hydrolyzed into glucose by the enzyme trehalase. This study measures the glucose already available in the organism.

Capillary Feeder (CAFE) Assay.

Flies were placed on regular food (RF) and in normoxia or IH for 5 days. Following that time, groups of 5 flies were placed in a plastic vial with only a piece of filter paper containing

500µl water. Through the top plug, a capillary tube was inserted containing 5µl liquid food (5% yeast, 5% sucrose) as described in Ja et al [14]. The capillary tube was removed every 24 hours and replaced with a new capillary tube containing 5µl of food. The flies were allowed to adjust to the new setup for the initial 24 hours, after which measurements were taken each time the capillary was removed, measuring the difference in level of food (in mm). Knowing the initial height (in mm) of the 5µl, the calculated µl/mm could be multiplied by the change in food level to determine how much food (in µl) was consumed.

Stress Tolerance: Cold, Anoxia and Starvation.

All assays were performed in normoxia, immediately following the week of exposure to experimental diet and oxygen conditions. Flies exposed to the different diets were assayed simultaneously when possible (cold and starvation) or consecutively (anoxia).

Cold Stress: A -5°C bath was made using water, ice and salt. For each group, sets of 15-20 female flies were placed in empty plastic vials and into the water bath. Flies fell unconscious almost immediately and vials were checked to make sure all flies were at the bottom and thus submerged in the cold bath. They remained as such for 2 hours, with the temperature being checked regularly throughout. At the end of the 2 hours, vials were removed from the water bath, and flies transferred to regular food and left to recover at room temperature. After 24 hours, survival was recorded as the number of flies that had regained consciousness.

Anoxic Stress: A specially designed chamber was used to study flies under controlled O_2 levels [9]. Sets of 30 flies from a particular group were placed in the chamber and exposed to anoxic conditions (O_2 concentration= 0% with administration of 100% N_2) for 2 hours. They were then returned to a vial with regular food in room air, and the number of flies that regained consciousness after 24 hours of recovery was counted as the survival.

Starvation: For each group, sets of 10-15 female flies were placed in a plastic vial with no food. A small circular filter paper was placed in the bottom of the vial with 75ul of water to prevent

dehydration and this was replenished with water every 16 hours or as needed. Survival was recorded every 4-8 hours as the number of flies alive in each vial.

Lifespan.

Flies were placed on experimental diets and in oxygen conditions as described for previous assays. The lifespan of the flies was observed by recording the number of flies alive each day in that oxygen condition. Flies were transferred to fresh food every 3-4 days. The experiment was concluded when there were no flies remaining in a group or when one group was below 50% survival and there was a clear difference between groups.

Statistical Analysis.

Graphpad Prism was used for statistical analysis. A t-test was used to determine significance between RF and CF results in the metabolic and stress assays. To determine interaction between diets and oxygen a 2-way ANOVA was performed. In lifespan and starvation assays, the significance was determined by comparing the survival curves with a log-rank (Mantel-Cox) test.

Results

Phenotypic Profile of Flies on High-fat Diet

To evaluate the effect of a diet high in fat on *Drosophila*, we placed female w^{1118} flies on either a regular diet or food supplemented with coconut oil (rich in saturated fats) as 20% of the diet (see methods). After a week on these diets in normoxia, the flies on coconut oil supplemented food (CF) showed a large increase in whole-body triglyceride levels ($p<0.0001$) compared to those fed regular food (RF) (Figure 1.1 A). To evaluate whether this increase seen with the CF diet was specific to the strain of fly or type of food, we also assayed the triglyceride levels of *Canton-S* and *Oregon-R* flies on RF and CF diets, as well as tested the effect of palm oil in the diet (PF) rather than coconut oil. Both fly strains responded in the same way as w^{1118} flies,

with increased triglyceride levels when on the CF diet, and a high-fat PF diet resulted in similar increases as CF (Figures 1.1 B, C). Additionally we measured the whole-body free glucose levels in the w^{1118} flies, as it is well known that obesity also affects glucose metabolism [15,16]. Flies on the CF diet had significantly increased glucose levels ($p<0.001$) compared to those on the RF diet (Figure 1.1 D).

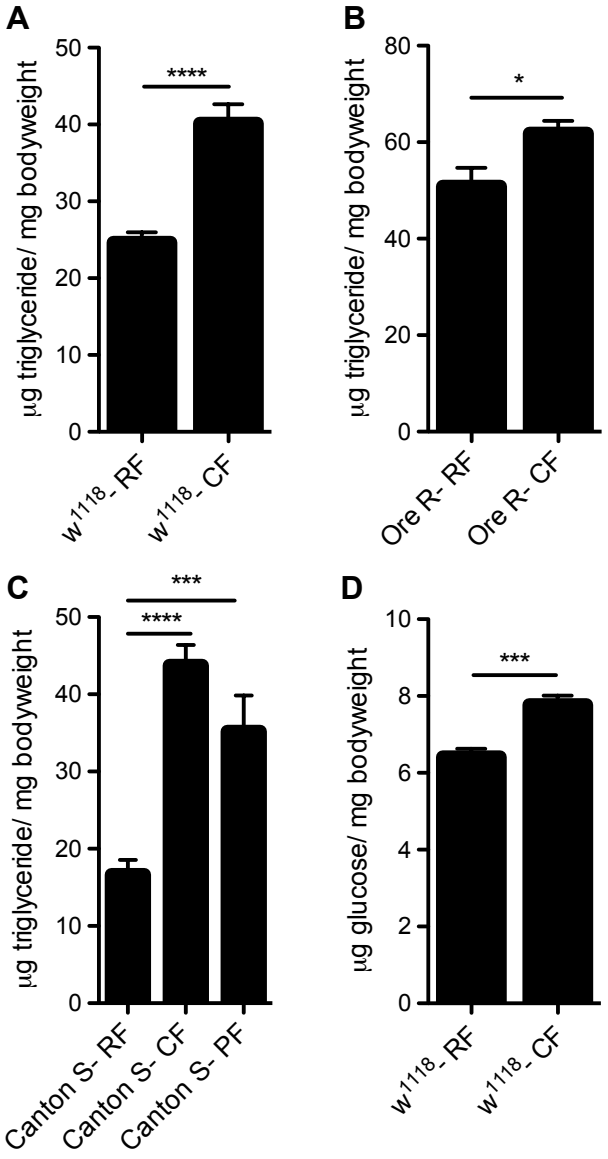


Figure 1.1. Changes in metabolic profile of flies on high-fat diet compared with regular diet. A-C) Triglyceride levels (per mg live weight) in whole-body homogenate of 10-12 day old adult female (A) *w*^{1118}, (B) *Oregon-R* and (C) *Canton-S* flies after one week on diets in room air. Flies were on regular food (RF), high-fat coconut food (supplemented with 20% coconut oil, CF) or high-fat palm food (supplemented with 20% palm oil, PF). D) Glucose levels (per mg live weight) in whole-body homogenate of 10-12 day old adult female *w*^{1118} flies, after one week on RF and CF diets in room air. Flies were homogenized in groups of five females: (A) n= 31 groups (155 flies), (B) n= 17 groups (85 flies), (C) RF n= 11 groups (55 flies), (D) n= 24 groups (120 flies). Error bars show SEM; *= p<0.05, ***= p<0.001, ****= p<0.0001.

The elevated triglyceride levels seen in obesity are often correlated with a decreased lifespan, both in humans [17] and *Drosophila* [18,19]. We found that the high-fat diet had a detrimental effect on lifespan, as the lifespan of w^{1118} flies on a CF diet was significantly shortened compared to RF flies ($p<0.0001$) when given full access to food (Figure 1.2). This significant decrease was also seen in the *Oregon-R* flies on CF diets ($p<0.0001$) and *Canton-S* flies on CF ($p<0.001$) or PF ($p<0.0001$) diets when compared to RF flies (Figure 1.2). Not surprisingly, under starvation conditions the w^{1118} CF flies were more resistant to the stress of starvation than flies on RF diet and survived longer ($p<0.05$) (Figure S1.1 A).

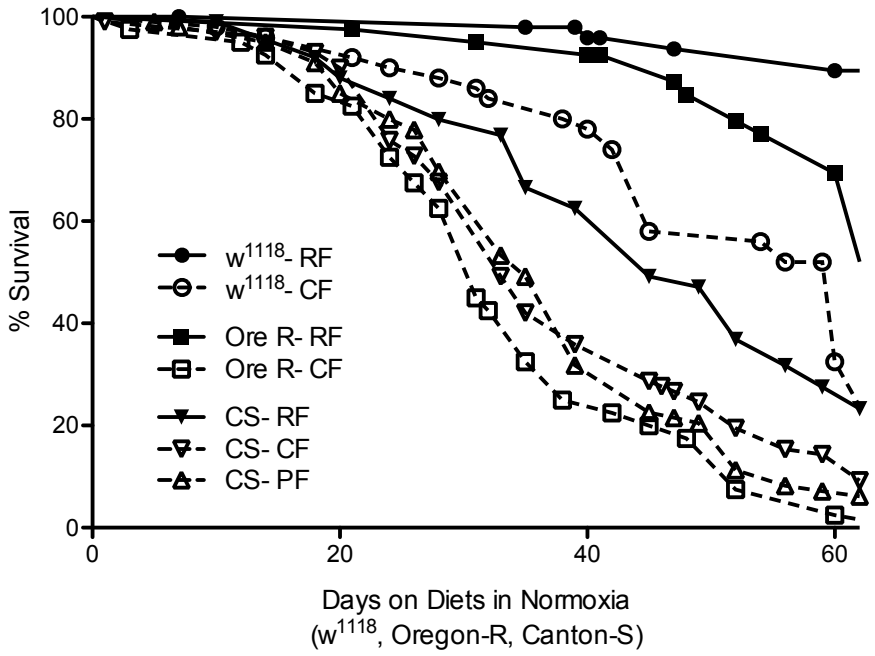


Figure 1.2. Altered lifespan due to high-fat diet. Adult female w^{1118}, *Ore-R,* and *Canton-S* flies (d3-5, n= 50 per diet) were placed on regular (RF) and high-fat (CF) diets in room air. Additionally one group of *Canton-S* flies was placed on a high-fat diet using palm oil (PF). Flies were counted daily, with the number alive recorded. There was a significant difference between the regular and high-fat diet curves in each comparison; w^{1118} RF vs. CF $p< 0.0001$, *Ore-R* RF vs. CF $p<0.0001$, *Canton-S* RF vs. CF $p<0.001$, RF vs. PF $p<0.0001$ (Log-rank test).

Since lifespan can be an indication of the ability of an organism to tolerate stress [20], we also tested stress resistance in these flies to cold and to anoxia (Figure 1.3 A). The flies on the CF diet showed a marked decrease in tolerance to these two acute stresses. After 2 hours in anoxia (0% oxygen), nearly 90% of flies on the RF diet were able to recover during the

subsequent 24 hours, compared with less than 60% of flies on the CF diet (Figure 1.3 B). After exposure to 2 hours in -5°C, flies on a CF diet survived significantly less than their RF counterparts, with just a quarter of the RF survival rate (Figure 1.3 C).

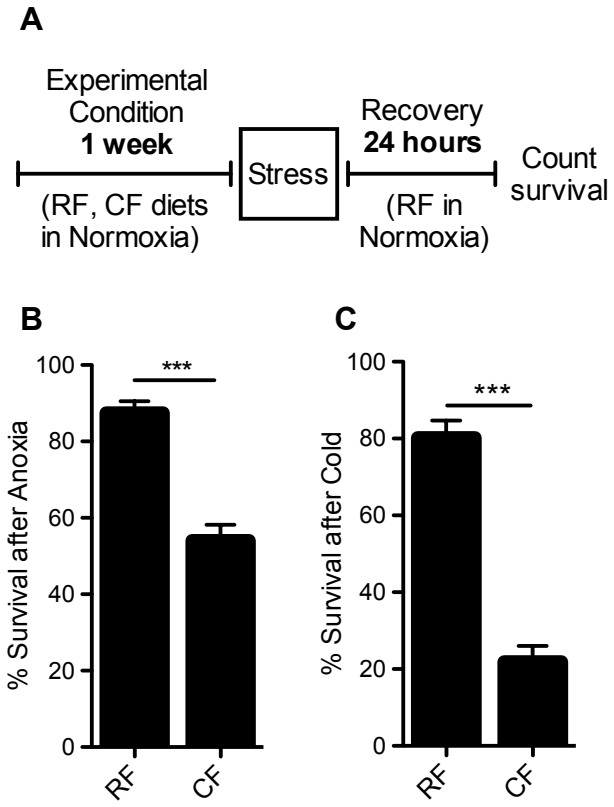


Figure 1.3. Decreased tolerance to acute stress in flies on high-fat diet. A) Diagram of experimental paradigm for stress assays. Adult female w^{1118} flies were placed on regular (RF) or high-fat (CF) diets in room air for one week, after which they were stressed for 2 hours in B) anoxia (0% oxygen) or C) extreme cold (-5°C water bath). After recovering for 24 hours on regular food in room air, survival was counted as number of flies alive. Flies were tested in groups of 15 females per vial: (B) n= 18 groups (270 flies), (C) n= 16 groups (240 flies). Error bars show SEM; ***= $p<0.001$.

Effects of Intermittent or Constant Hypoxia on Flies on a High-fat Diet

Similar to the evaluation of flies on the RF and CF diets in normoxia, flies were placed on the diets and immediately put in a hypoxic chamber in either intermittent (IH) or constant hypoxia (CH) for 7 days. IH and CH have been shown to have differential effects [21-23] and alter expression of different genes [12], so it was important to separately evaluate the response of the flies on the diets in both hypoxia conditions.

The alterations in the metabolic profile seen in normoxia were also present in both IH and CH, with increased TG levels in flies on the coconut-supplemented food ($p<0.001$) (Figure 1.4 A, C). Although the difference in TG levels due to diet was persistent, there was also a difference in TG levels between flies on the same diet, depending on the oxygen condition. When triglyceride levels were compared between hypoxic and normoxic conditions in flies on the same diet, IH was associated with lower TG levels in the flies on both the RF and the CF diet (Figure 1.4 A). There was no significant interaction between diet and oxygen level, so IH was not causing the flies to respond differently to the diet in terms of TG, but rather just causing an overall decrease regardless of diet. On the other hand, CH provided a very different response. While the TG levels in flies on the RF diet significantly decreased in CH compared to normoxia, the TG levels in flies on the CF diet increased (Figure 1.4 C). The interaction between oxygen and diet was significant ($p<0.01$), indicating that being in constant low oxygen (i.e. in CH) affected flies on the CF diet differently than the RF diet.

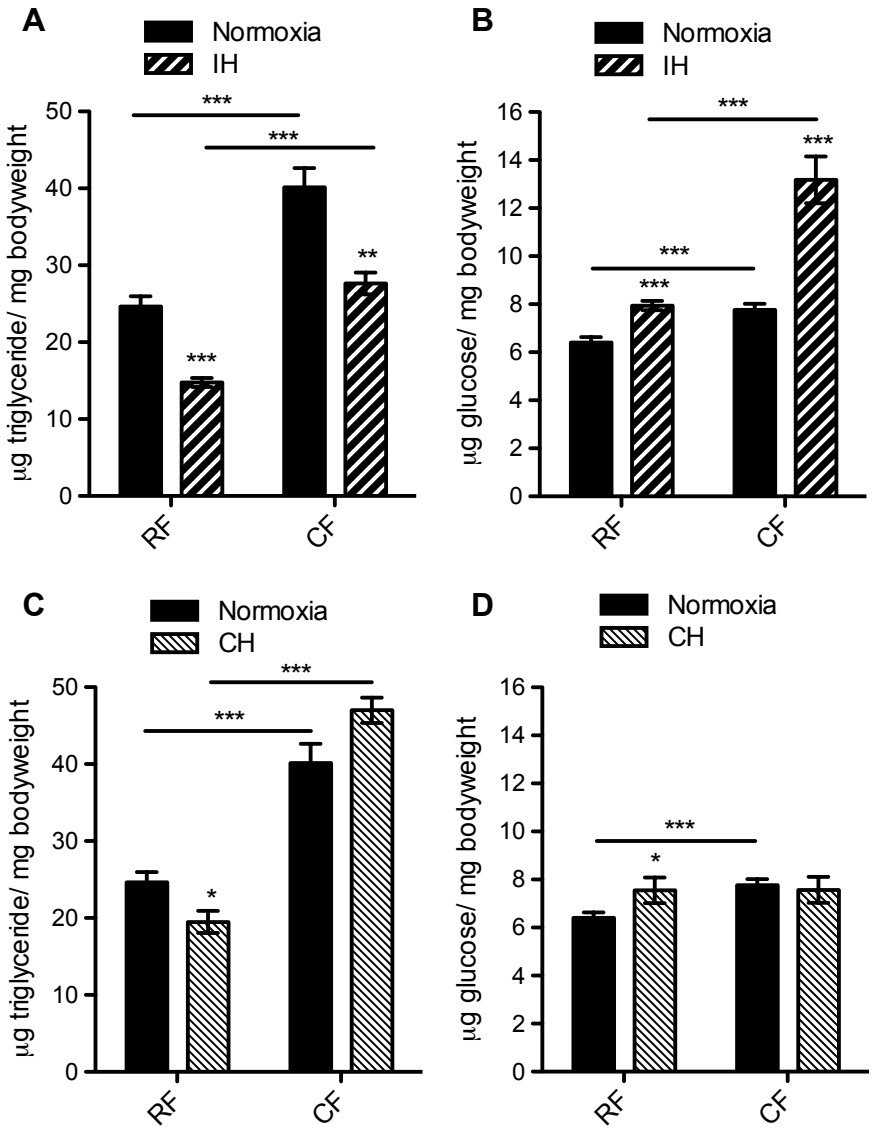


Figure 1.4. Changes in metabolic profile due to high-fat diet and hypoxia. A, C) Triglyceride levels (per mg live weight) and B, D) glucose levels (per mg live weight) were assayed in whole-body homogenate of 10-12 day old adult female w^{1118} flies. Measurements were taken after one week on diets in normoxia (solid bars, A-D), intermittent hypoxia (IH, pattern bars, A and B) or constant hypoxia (CH, pattern bars, C and D). During that week, flies were on diets of either regular (RF) or high-fat diet (CF). Flies were homogenized in groups of five females: normoxia triglyceride (TG) n= 31 (155 flies), glucose n= 24 groups (120 flies); IH TG n= 17 (85 flies), glucose n= 18 groups (90 flies); CH TG n= 20 (100 flies), glucose n= 12 groups (60 flies). Error bars show SEM; *= $p<0.05$, **= $p<0.01$, ***= $p<0.001$.

As in normoxia, glucose levels were significantly increased in flies on the CF diet compared to the RF diet when exposed to IH (Figure 1.4 B). However, the flies in IH showed a much larger increase than those in normoxia, indicating that there was a significant interaction

between diet and IH when the regulation of glucose levels was considered. Flies exposed to CH showed no significant difference in glucose levels between diets, although there was a slight increase in glucose levels between normoxia and CH in flies on the RF diet (Figure 1.4 D). Similar to results in normoxia, flies on the CF diet in IH were more resistant to starvation than those on RF ($p<0.0001$) (Figure S1.1 B) and had a significantly shortened lifespan ($p<0.0001$) (Figure 1.5). A comparable lifespan difference was seen in flies in constant hypoxia.

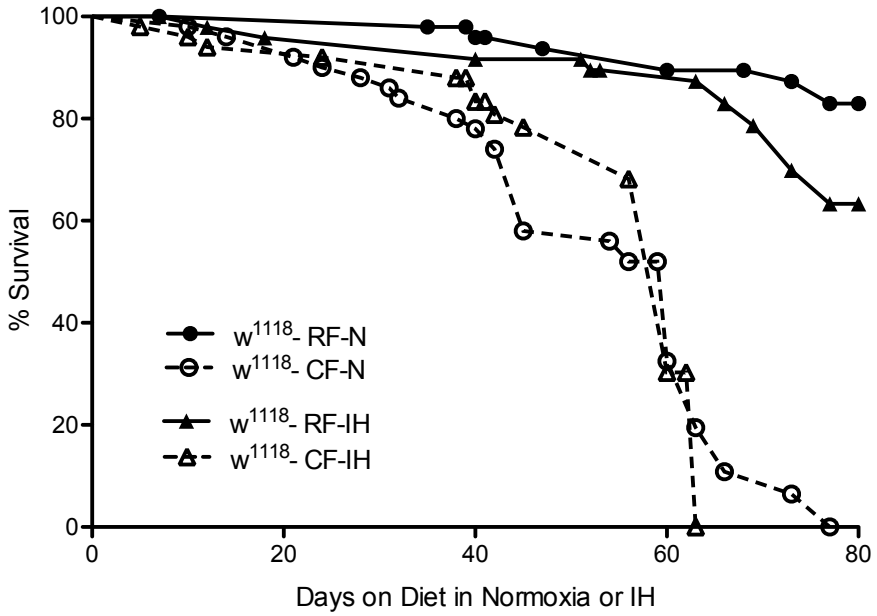


Figure 1.5. Altered lifespan due to high-fat diet and hypoxia. Adult female *w*^{1118} flies (d3-5) were placed on regular (RF) and high-fat (CF) diets in normoxia (also shown in Figure 1.2) or intermittent hypoxia (IH) (n= 50 flies per diet). Flies were counted daily, with the number alive recorded. There was a significant difference between the regular and high-fat diet curves in each comparison, as well as between normoxia and IH when flies were on a regular diet; *w*^{1118} RF-Normoxia vs. CF-Normoxia $p< 0.0001$, *w*^{1118} RF-IH vs. CF-IH $p< 0.0001$, *w*^{1118} RF-N vs. RF-IH $p< 0.05$ (Log-rank test).

The response to stress also varied with the two hypoxic treatments. Flies on a CF diet in IH and CH had decreased survival after anoxic stress when compared to RF, similar to the response seen in normoxia (Figure 1.6). In contrast, survival after cold stress was very much affected by the hypoxia paradigm. There was a marked increase in survival after cold stress even in flies on the RF diet in IH compared to normoxia. Flies on the CF diet in IH saw a remarkable change in response, with nearly full survival in IH compared to just over 20% survival in normoxia (Figure 1.7 A, B). There was no difference in survival between diets in the IH flies, but there was

a highly significant interaction ($p<0.0001$) in how the oxygen condition affected the flies on the two diets. This difference is not seen in CH, as the flies responded similarly as in normoxia, with a decreased survival in the CF flies after cold stress (Figure 1.7 C).

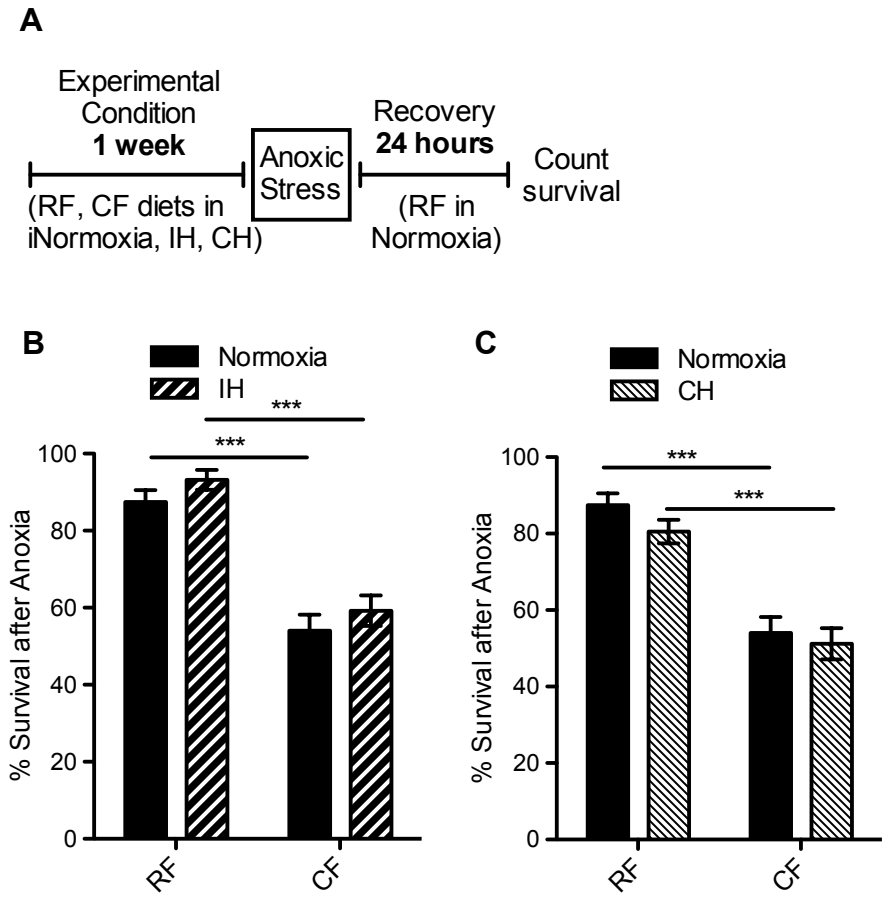


Figure 1.6. Anoxia tolerance unaltered by hypoxia exposure. Effect of prior exposure to intermittent hypoxia (IH) or constant hypoxia (CH) on survival of flies after 2 hours in anoxia (0% oxygen) was measured. A) Diagram of experimental paradigm for anoxia assay following hypoxia exposure. Adult female w^{1118} flies (10-12 days old) were placed on regular (RF) or high-fat (CF) diets in normoxia (B, C solid bars), IH (B, pattern bars), or CH (C, pattern bars) for one week prior to anoxia assay. Flies were removed from hypoxia and immediately assayed for tolerance to 2 hours of acute anoxia. After recovering for 24 hours on RF in room air, survival was measured as number of flies alive. Flies recovered in groups of 15 flies per vial: normoxia n= 18 groups (270 flies); IH n= 13 groups (195 flies); CH n= 17 groups (255 flies). Error bars show SEM; ***= $p<0.001$.

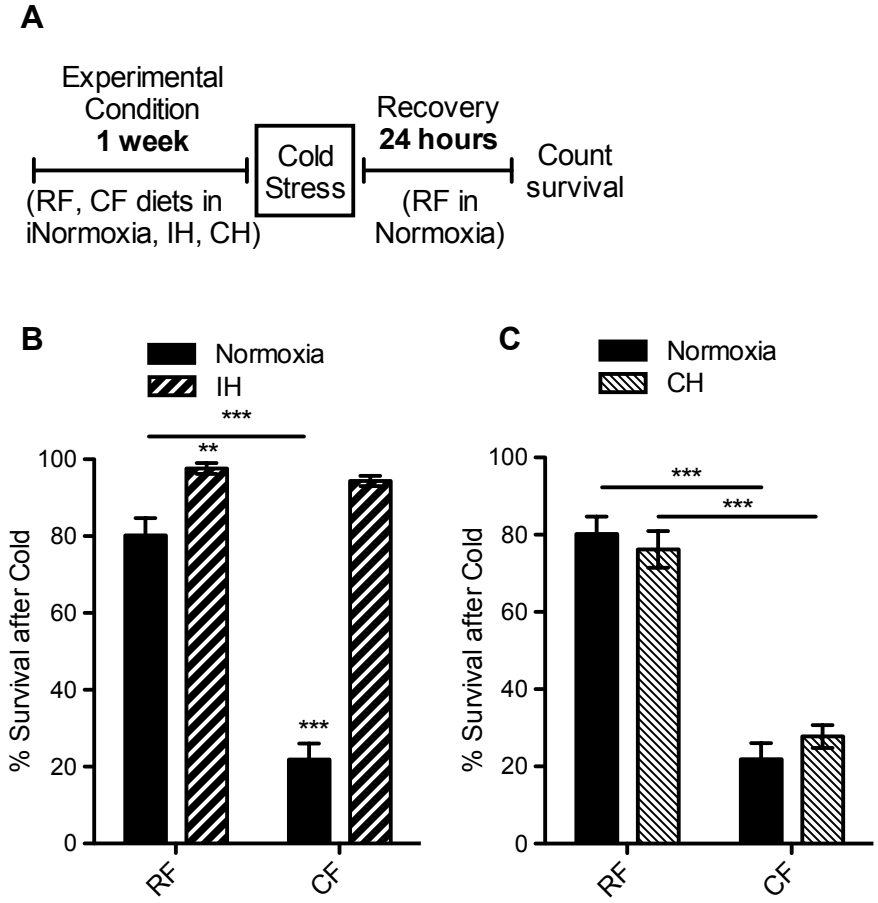


Figure 1.7. Cold survival altered by intermittent hypoxia, but not constant hypoxia. Effect of prior exposure to intermittent hypoxia (IH) or constant hypoxia (CH) on survival of flies after 2 hours in extreme cold (-5°C water bath) was measured. A) Diagram of experimental paradigm for cold stress assay following hypoxia exposure. Adult female w^{1118} flies (10-12 days old) were placed on regular (RF) or high-fat (CF) diets in normoxia (B, C solid bars), IH (B, pattern bars), or CH (C, pattern bars) for one week prior to cold stress assay. Flies were removed from hypoxia and immediately assayed for tolerance to acute cold stress. After recovering for 24 hours on RF in room air, survival was measured as number of flies alive. Flies were tested in groups of 15 flies per vial: normoxia n= 17 groups (255 flies); IH n= 14 groups (210 flies); CH n= 22 groups (330 flies). Error bars show SEM; **= $p<0.01$, ***= $p<0.001$.

To further describe the effect of IH on cold stress recovery, it was important to test an additional paradigm causing increased fat storage. In order to do this, we utilized the well-characterized genetic mutant *adipose*^{60} (*adp*^{60}). On a regular diet, these mutants have significantly higher triglyceride levels than wildtype flies, and even surpass the triglyceride level of wildtype flies on a high-fat diet. These triglyceride levels drop slightly after IH, as they do in the

wildtype flies on both diets (Figure 1.8 A). Importantly, IH almost fully restores cold stress survival in the *adp*^{60} mutant flies, as was seen with wildtype flies on both diets (Figure 1.8 B).

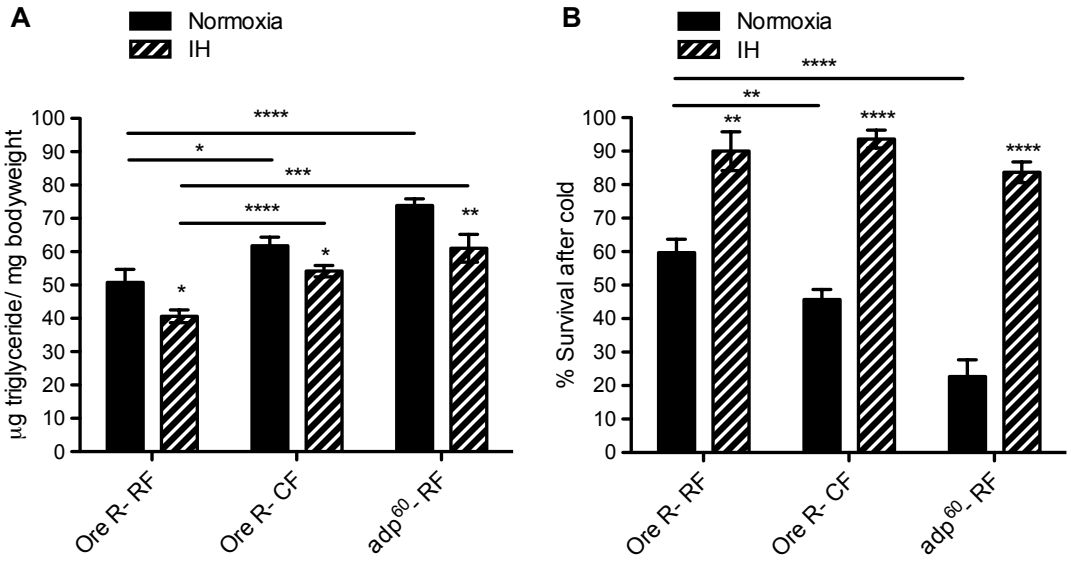


Figure 1.8. Changes due to intermittent hypoxia also seen in genetic mutant model of obesity. A genetic mutant model of obesity, *adipose*^{60} *(adp*^{60}*)*, was assayed in normoxia and intermittent hypoxia (IH) with the *Oregon-R (Ore-R)* strain as control. Adult female *adp*^{60} flies (10-12 days old) were placed on the regular (RF) diet and adult *Ore-R* females were placed on RF and high-fat (CF) diets. The flies were kept in normoxia (solid bars) or IH (pattern bars) for one week. Following that week, the flies were assayed for A) triglyceride levels (per mg live weight) in whole-body homogenate and B) survival of flies after 2 hours in extreme cold (-5°C water bath), measured 24 hours after return to room air. For triglyceride determination, flies were homogenized in groups of five females, normoxia: *Ore-R* n= 17 groups (85 flies), *adp*^{60} n=20 groups (100 flies); IH: *Ore-R* n= 13 groups (65 flies), *adp*^{60} n=15 groups (75 flies). In the cold assay, flies were tested in groups of 15 flies per vial: normoxia *Ore-R* n= 13 groups (65 flies), *adp*^{60} n=12 groups (60 flies); IH *Ore-R* n= 8 groups (40 flies), *adp*^{60} n=11 groups (55 flies). Error bars show SEM; *= p<0.05, **= p<0.01, ***= p<0.001, ****= p<0.0001.

Given the consistent decrease in triglyceride levels in flies on both diets in IH, we felt it was important to determine whether this change was due to an altered level of food consumption. We adapted the CAFE set-up described in Ja et al [14] for normoxic and IH conditions, using flies previously on RF in normoxia or IH. After an initial decrease in food consumption in IH flies on the first day of CAFE measurement, there appears to be no significant difference between the slopes of the cumulative consumption, as determined by a Deming (Model II) Linear Regression (Figure 1.9 A) and no significant differences on the 2nd and 3rd day in the daily food consumption between flies in normoxia as compared to IH (Figure 1.9 B).

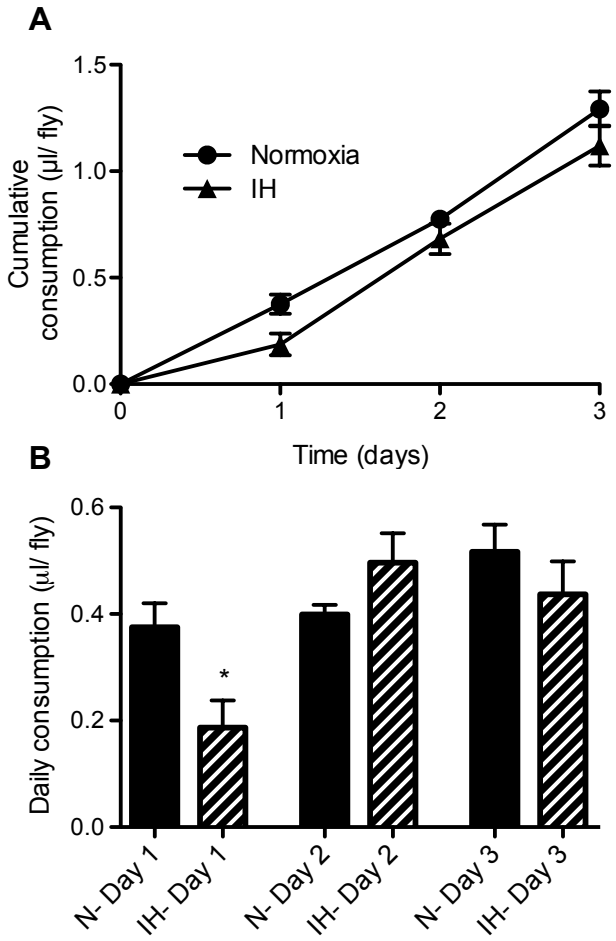
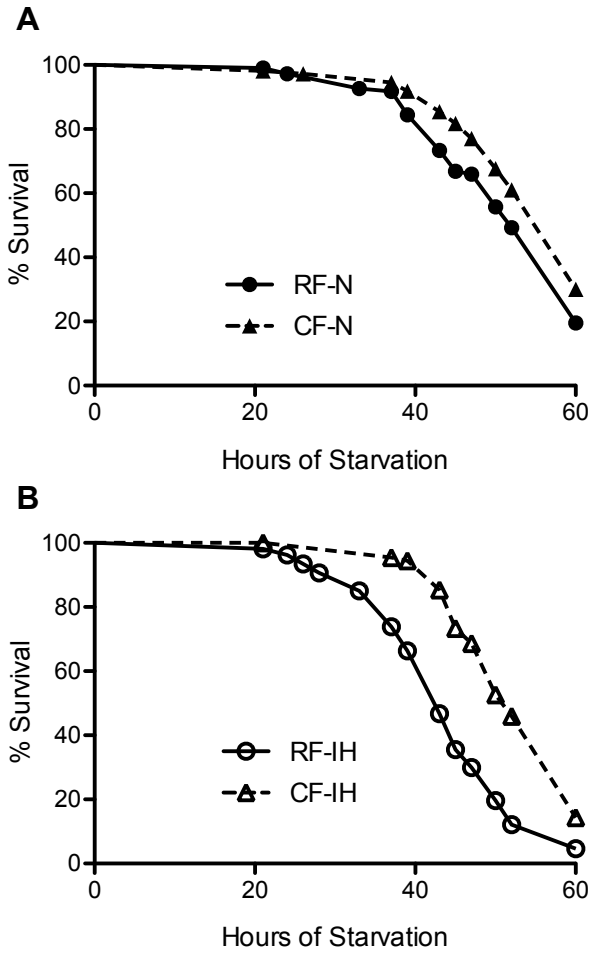


Figure 1.9. Similar food consumption observed in normoxia and intermittent hypoxia. Adult female w^{1118} flies (3-5 days old) were placed on regular (RF) for 5 days in normoxia or intermittent hypoxia (IH). Following that time, flies were transferred to the CAFE vials and returned to normoxia or IH. In the CAFE setup, flies had access to water but had to obtain their food out of a capillary tube through the top of the vial. Measurements of the change in liquid food level in the capillary tube allowed for determination of the total food consumed A) cumulatively over 3 days and B) on a daily basis. Flies were tested in groups of 5, n=14 groups (70 flies). Error bars show SEM; *= $p<0.05$.



Supporting Figure S1.1. Altered starvation resistance due to high-fat diet. Adult female w^{1118} flies (3-5 days old) were placed on regular (RF) or high-fat (CF) diets in A) normoxia (N) or B) intermittent hypoxia (IH) for one week (n=110 flies per group). Following that week, flies were transferred to plastic vials without food, but with access to water. Flies were kept in room air and counted every 4-6 hours, with the number alive recorded. There was a significant difference between the survival curves; A) p= 0.02, B) p<0.0001 (Log-rank test).

Discussion

We have generated a model showing the detrimental effects of a high-fat diet on lifespan and stress tolerance in *Drosophila*. Flies on a diet supplemented with coconut oil (or palm oil) significantly increased their triglyceride and glucose levels and shortened their lifespan. This diet, high in saturated fat, not only hindered flies from living as long as others on the regular diet, but it also greatly diminished their tolerance to acute stresses. When challenged with either anoxia or

severe cold, few flies on the high-fat diet survived, while most of those on the regular diet did. Conversely, under starvation conditions the flies on the high-fat diet showed increased resistance, due to their increased triglyceride and energy stores.

The link between hypoxia and metabolism has been demonstrated in models from flies and mice to humans [24-28]. Intermittent hypoxia in particular has been strongly implicated as having a role in altering glucose and lipid metabolism. In rats, IH has been shown to lead to oxidative stress, lipid peroxidation, neuronal apoptosis and up-regulation of stress responsive proteins [29,30]. Chronic IH has been shown to cause insulin resistance and glucose intolerance in obese leptin-deficient mice [25,31] as well as inducing hyperlipidemia in lean mice [25]. Although studies in humans are more limited, experimental evidence supports a detrimental effect of hypoxia on metabolism, with diminished insulin sensitivity in subjects after just 30 minutes of hypoxia exposure [24]. Our model presents an essential first step in understanding the *Drosophila* response to a high-fat diet and modulation of this response by IH.

When investigating the phenotype of flies on a high-fat diet, both independently and in conjunction with hypoxia, we made two important observations and pose here two interesting questions that arise from these results. First, since the coconut oil diet led to a decreased survival following cold stress, our question is: how does this high-fat diet cause decreased survival in this stressful condition? Second, and of interest, IH led to rescuing flies from decreased survival after a severe cold stress; how does IH allow flies to fully recover from this stress?

Normally, cells convert excess non-esterified free fatty acids into triglycerides, which is why the coconut oil diet led to increased levels of TG in our model. With this excess storage of triglycerides, we showed that flies had decreased tolerance to stresses such as anoxia and cold. Keeping in mind the diminished recovery from cold stress in flies fed coconut oil, it is likely that the high-fat diet alters energy and metabolic pathways in such a way that flies are unable to activate some of the key mechanisms needed to recover from the cold stress. This assumes that genes are activated not only during the cold exposure itself but also during recovery from cold. Indeed, Clark and Worland have demonstrated just that [32]. For example, it is known that heat

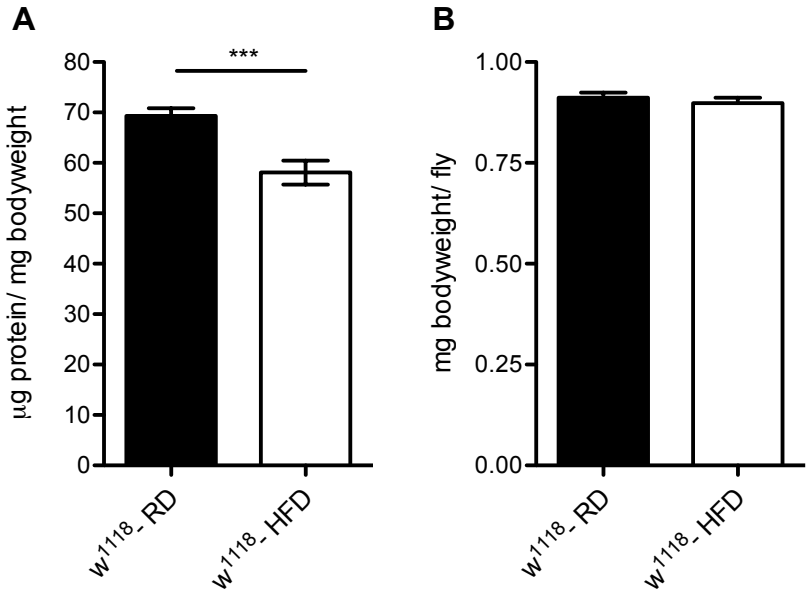
shock proteins, which are well characterized in their response to stress, are important in recovery. Newly discovered genes in this area, such as *starvin* and *frost* [33-36], have also been implicated. While its exact function in recovery from cold stress is not known, starvin is up-regulated in the recovery phase following cold stress and is believed to be a co-chaperone regulating the Hsp70 complex during recovery from cold. A mucin-like protein, frost is thought to play a role in protecting against oxidative stress and maintaining membrane integrity, thus aiding in the ability to recover from cold stress. We therefore hypothesize that flies on the CF diet were unable to activate mechanisms such as these, jeopardizing their recovery and survival from cold.

The inability of flies on the high-fat diet to recover from the stress of cold was completely rescued when these flies had been in intermittent hypoxia prior to the cold. Since this rescue of survival did not occur in flies in CH, these data would indicate that IH specifically, and not just hypoxia in general, is necessary for this rescue phenotype. It appears that intermittent hypoxia alters processes in such a way to override the negative effect of increased triglycerides, whether from a high-fat diet or as a result of a genetic mutation (adp^{60}). One possible explanation could involve the increase in glucose levels seen in flies on both RF and CF diets following IH. Sugars such as glucose and trehalose are considered cryoprotective molecules and have been implicated in maintenance of cell function at low temperatures [37,38]. With this accumulation of free glucose during IH, the fly may be better prepared to survive the cold stress. Another potential mechanism may be related to other alterations in gene expression. For example, we have previously shown that IH induces expression of genes important in transport and defense, including the high affinity inorganic phosphate: sodium symporter, l(2)08717 [12]. Ion transport appears to play an important role in survival to extreme cold, as lower temperatures can lead to decreased ion pump activity, decreased membrane fluidity and inhibited ion channel gating [32,39]. If exposure to IH up-regulated expression of genes is important in ion transport, we speculate here that this IH is tantamount to a pre-conditioning of the flies to stress, allowing them to resist the effects of the high-fat diet in the survival from cold stress.

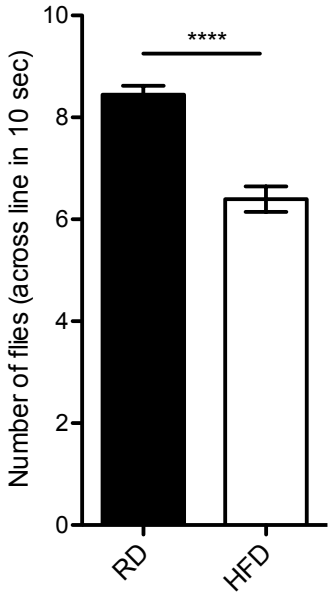
In summary, we found that flies on a high-fat diet have a drastically worsened phenotype, with decreased resilience to stress and decreased survival compared to those on a regular diet, and this phenotype is altered by exposure to hypoxia. With significantly increased triglyceride and glucose levels in normoxic conditions, flies on a high-fat diet have a shortened lifespan and decreased tolerance to stress. While the triglyceride and glucose levels are altered with hypoxia, the most interesting change was seen when exposure to IH appeared to rescue survival after cold stress. The detrimental effects of a high-fat diet are clear in this *Drosophila* model, and there appears to be both deleterious and compensatory cross talk occurring between hypoxia and the high-fat diet. With the climbing global obesity levels and lack of understanding of the interaction between obesity and hypoxia, developing an animal model that lends itself to an investigation of this interaction becomes vital.

Acknowledgements

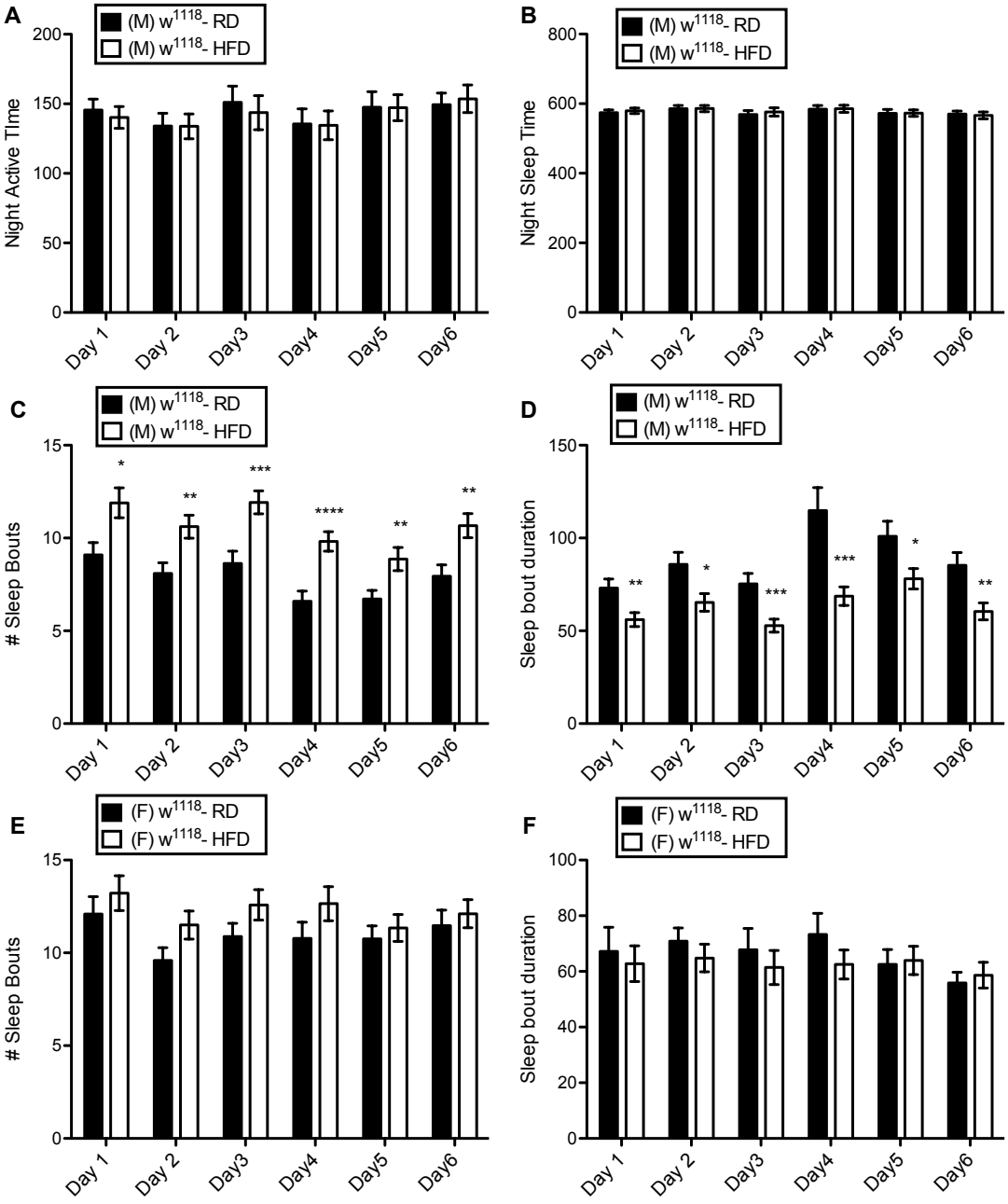
Chapter 1, in full, is a reprint of the material as it appears in PLoS ONE. **Heinrichsen ET**, Haddad GG. Role of High-Fat Diet in Stress Response of *Drosophila. PLoS ONE* 2012; 7(8): e42587. Additional phenotypic assessments included in the appendices are unpublished. In Appendix 1.2, geotaxis measurements were done by groups of Advanced Biology students at Mira Mesa High School (San Diego, CA) as a part of the NSF GK-12 Socrates Fellowship, under the supervision of Lynda Spendlove and the dissertation author. In Appendix 1.3, activity measurements were done by the dissertation author in the laboratory of William Joiner, and MATLAB analysis done by James Robinson. The dissertation author was the primary investigator and author of this material. Gabriel Haddad supervised the project and provided editing and direction.



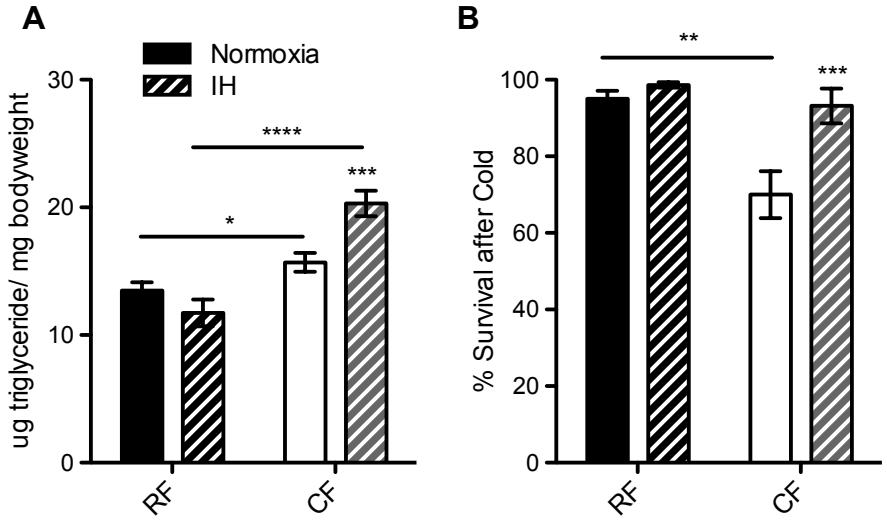
Appendix 1.1. **Additional phenotypic assessment of flies on high-fat diet (HFD): Protein levels and body weight.** A) Whole-body protein levels were decreased in HFD flies (HFD was referred to as coconut food or CF in Chapter 1). B) There was no change in body weight between RD and HFD. Protein levels and weights were determined from the same samples as in Figure 1.1, with methods described in Chapter 1. Significance was measured with a t-test, ***=p<0.001.



Appendix 1.2. Additional phenotypic assessment of flies on high-fat diet (HFD): Geotaxis measurement. Flies on HFD have decreased geotaxic ability than flies on a regular diet (RD) (HFD was referred to as coconut food or CF in Chapter 1). Methods: Ability and speed of flies to climb against gravity was measured by knocking the flies down to the base of an empty vial and measuring how many flies were able to cross a line 8 cm above before 10 seconds had passed. Each measurement (n) used was the average of 10 trials done with 2 minutes recovery between trials, with 10 flies per vial and RD and HFD flies being counted simultaneously in separate vials; n=36 per group. Measurements were done by groups of Advanced Biology students at Mira Mesa High School (San Diego, CA) as a part of the NSF GK-12 Socrates Fellowship. Significance was measured with a t-test, ****=$p<0.0001$.



Appendix 1.3. Additional phenotypic assessment of flies on high-fat diet (HFD): Activity and sleep measurements. Activity measurement in adult male and female *w*^{1118} flies, on regular diet (RD) or HFD (coconut food/CF in Chapter 1). No change in overall activity or sleep time is seen, however there are significant changes in the number of sleep bouts and sleep bout duration in males on HFD compared to RD. A similar pattern is seen in females, although the data is not significant. Methods: Adult flies were collected at Day 1 and kept in a 12 hr:12 hr light:dark (LD) cycle for two days. To assay for sleep, single flies were transferred to glass tubes containing either RD or HFD in the end of the tube. Males and females were monitored separately using *Drosophila* Activity Monitoring System (Trikinetics) in a 25º incubator, n=32 per group. As previously described [40], activity counts were collected in 1-minute bins in LD for 6 days, and sleep was considered periods of inactivity lasting at least 5 minutes [40,41]. Results were analyzed with MATLAB-based software. Measurements were done in the laboratory of William Joiner and analysis done by James Robinson.



Appendix 1.4. Additional phenotypic assessment of flies on high-fat diet (HFD): Response of male flies to HFD and intermittent hypoxia (IH). Assays to measure A) triglyceride levels and B) survival after cold stress were performed identically in males as in females, as described in Chapter 1. A similar response to HFD was seen in males as females, although in most situations the male response showed less of a difference between the diets. The main difference between males and females was found in the response to IH. While IH decreased triglyceride levels in females on the RF and CF diets, males on a CF diet actually had increased triglyceride levels after IH (A). Interestingly, even with these increased triglyceride levels, IH was still able to rescue the survival of male flies from cold stress (B). Flies were assayed in groups of 8 males for triglyceride levels, n= 22-28 (176-224 flies per group) and 10 males per vial for cold tolerance, n= 15-18 (150-180 flies per group).

CHAPTER 2:

High-Fat Diet and Intermittent Hypoxia Lead to Transcriptional Changes in *Drosophila*

Abstract

We have previously developed a *Drosophila melanogaster* model of response to a high-fat diet (HFD) and observed significant phenotype changes when these high-fat flies live in intermittent hypoxia (IH). Given the conservation of several mechanisms that regulate metabolism and transcription in hypoxia, *Drosophila* may provide important insights to the interaction of obesity and hypoxia at the transcriptional level. In this work, we performed microarray analyses to elucidate transcriptional changes involved in the responses to HFD and IH. Based on affected KEGG pathways and screening of candidate genes, we show that metabolic pathways are greatly affected both by the HFD and by IH. Flies on HFD seem to alter amino acid metabolism, and a candidate gene involved in arginine metabolism, *CG9510*, is able to simulate the HFD phenotype even when on regular diet (RD). Meanwhile, carbohydrate metabolic pathways are affected by IH in flies both on RD and HFD. Several candidate genes in carbohydrate metabolism also have a significant change in cold stress tolerance. Two of these genes, *target of brain insulin* (*tobi*) and *maltase A7*, as well as *CG9510* from the HFD screen, are differentially regulated by HFD and IH, further strengthening the importance of transcriptional changes in carbohydrate and amino acid metabolism in the phenotype changes.

Introduction

The increased prevalence of obesity has created a huge burden on our healthcare system and our economy, largely due to its many associated complications and diseases [2,3,42,43]. One of the most common of these are sleep disorders such as obstructive sleep apnea (OSA) [3]. OSA in turn is strongly associated with type 2 diabetes and many cardiovascular diseases [44,45]. OSA is also associated with several risk factors for

atherosclerosis, including inflammation, dyslipidemia, and lipid peroxidation [45]. OSA involves repeated collapsing of the upper airway during sleep, which causes chronic intermittent hypoxia (IH). IH is able to directly induce atherosclerosis in mice [45,46], as well as leading to hypercholesterolemia, increasing lipid peroxidation in myocardial tissue of rats, and activating inflammatory pathways in vitro [46-49]. Obesity is also associated with cardiovascular diseases, as well as biochemical changes such as low high-density lipoprotein cholesterol, high serum triglycerides, glucose intolerance and hyperinsulinemia [3]. While obesity, OSA and hypoxia are all associated with both cardiovascular and metabolic disorders, the underlying mechanisms and interplay between pathways are unclear.

While mammalian models have greatly increased our understanding of obesity and OSA, only a small portion of genes influencing obesity have been uncovered or examined in depth [50,51]. Although the exact mechanism are still being sorted out, there is an important link between IH and fat metabolism [51,52]. With immense power of genetic analysis, non-mammalian genetic models such as Drosophila are becoming important paradigms for elucidating the fundamental basis of mammalian obesity.

The fruit fly, *Drosophila melanogaster*, serves as a potentially useful genetic tool to uncover these pathways. It is a well-studied organism with a small number of chromosomes, short generation time and many stocked mutants and P-element lines that encompass the majority of its genome. *Drosophila* is a powerful genetic model due to the abundance of molecular tools available to probe into the importance of specific genes, including P-element lines that down-regulate certain genes and the UAS-Gal4 system to up-regulate genes in specific tissues or even silence a gene with an RNAi cross. Additionally, there is high conservation of biological and genetic pathways from Drosophila to man. For example, many of the proteins that control cardiac development, physiology and disease are conserved, making it feasible to study the effect of stress on the cardiovascular system in Drosophila [53]. Of importance for this study, Drosophila has several genes for regulation of metabolism, signaling and transcription during hypoxia that are similar in both sequence and function to human genes [8,54-58].

Over the past decades, our lab has explored in depth the molecular responses and genetic alterations when Drosophila are exposed to varying intensities and duration of hypoxia [10,12,21,23,59-62]. We have also described the importance of metabolism in response to hypoxia [27,28,63].

As described in Chapter 1, we recently developed a high-fat diet *Drosophila* model and observed the impact of both constant and intermittent hypoxia on the phenotype [64]. In this model, we observed that flies on a high-fat diet (HFD) had increased triglyceride storage, shortened lifespan and decreased stress tolerance. Interestingly, exposure to intermittent hypoxia (IH) causes triglyceride levels to decrease and survival from cold stress to increase in flies on both a regular diet (RD) and HFD. These drastically altered phenotypes lead us to ask: 1. What genes are playing a role in the worsened phenotype seen in HFD flies and 2. What genes or pathways are important in the change in phenotype seen after IH exposure? In this chapter, we examine the transcriptional changes underlying these phenotypes.

Methods

Fly Rearing and Collection.

All stocks were maintained on standard cornmeal *Drosophila* medium in an incubator at 25°C and 30-50% humidity. Adult flies were collected at 0-3 days and transferred to a separate vial of the standard cornmeal medium. After aging for 3 more days, male and female flies were separated and only the females were transferred to the regular (RD) or high-fat diet (HFD) and kept in room air.

Drosophila stocks.

All UAS-RNAi transgenic lines, as well as their controls w^{1118} and *y,w[1118];P{attP,y[+],w[3`]}*, were obtained from the VDRC [65]. The Gal4 driver stocks and P-element (w^{1118}; *PBac{PB}CG9510*^{c05706} *CG9515*^{c05706}) were obtained from Bloomington *Drosophila* Stock Center (Bloomington, IN, USA).

Experimental Oxygen Conditions: Normoxia and Intermittent Hypoxia.

At age 3-6 days, female flies were sorted onto the diets and immediately placed in normoxia or intermittent hypoxia, with approximately 20 flies per vial. All three conditions were at room temperature (22-24°C) and in a similar environment. Normoxia and intermittent hypoxia conditions were as described in Chapter 1.

Experimental Diets: Regular food and Coconut food.

Regular (RD) and high-fat diet (HFD) were prepared as described in Chapter 1, using Jazz Mix *Drosophila* food from New Horizon Foods as the base food and 20% weight per volume coconut oil as a source for increased saturated fat in the diet [64].

Microarrays and Data Analysis.

GeneChipH Drosophila Genome 2.0 Arrays (Affymetrix, Santa Clara, CA) were used in this study, with three arrays per group (w^{1118} on RD-N, R-IH, HFD-N and HFD-IH). Following one week on RD or HFD, flies were homogenized and total RNA was extracted from each sample using Trizol (Invitrogen, Carlsbad, CA) followed by purification with RNeasy Kit (Qiagen, Carlsbad, CA) as per manufacturer's instructions. Hybridization of the RNA to the Affymetrix GeneChipsH, washing and scanning were done according to protocols recommended by Affymetrix using an Affymetrix GeneChip fluidic station and scanner (Affymetrix, Santa Clara, CA). The raw data were normalized using Bioconductor Affy software (www.bioconductor.org/packages/2.0/bioc/html/affy.html), followed by analysis of the normalized data for each array spot with VAMPIRE microarray suite [66]. A spot was considered differentially expressed between two samples when the threshold of false discovery rate (fdr) was smaller than 0.05. Significance limits were set at changes of 1.5-fold (for up-regulated genes) and 0.67-fold (for down-regulated genes). Further analysis was done with genes at or above +/-2.0-fold change. DAVID Bioinformatics software was used to identify genes involved in significantly affected KEGG

pathways and functional clusters, as determined by the EASE (Expression Analysis Systematic Explorer) analysis implemented in DAVID functional annotation tool (http://david.niaid.nih.gov/david/ease.htm) [67,68]. The microarray analysis data can be retrieved using access number GSE46358 in the Gene Expression Ominibus database at http://www.ncbi.nlm.nih.gov/geo.

Candidate Gene Screening: Triglyceride Measurement and Cold Stress.

Triglyceride levels and tolerance to cold stress were assayed as described in Chapter 1.

Statistical Analysis.

Graphpad Prism was used for statistical analysis. A t-test was used to determine significance between RD and HFD results in most assays. In lifespan measurement, the significance was determined by comparing the survival curves with a log-rank (Mantel-Cox) test.

Results

We previously showed that adult female flies on a high-fat diet have a worsened phenotype compared to their counterparts on a regular diet, with increased triglyceride levels and decreased stress tolerance [64]. Meanwhile, exposure to intermittent hypoxia during the week on either RD or HFD caused a change in the phenotype, with decreased TG levels and increased cold tolerance in flies on both diets. We used microarray analysis to evaluate the transcriptional changes underlying each of these observations—the worsened phenotype of HFD flies in normoxia, the slight change in phenotype in flies on RD in IH and the drastic change in flies on HFD in IH. The four groups of flies used for the microarray included flies on RD in normoxia (RD-N), RD in IH (RD-IH), HFD in normoxia (HFD-N) and HFD in IH (HFD-IH). We compared the groups to identify the changes in gene expression for each of the two main phenotypes: 1. the effect of HFD compared to RD in normoxia (HFD-N vs. RD-N) and 2. the effect of IH both on RD and HFD flies compared to their normoxic counterparts (RD-IH vs. RD-N and HFD-IH vs. HFD-N).

Based on these comparisons, we developed criteria to select genes and study their role in cold tolerance and triglyceride levels.

Changes in gene expression due to high-fat diet in normoxia

In examining the effect of HFD compared to RD in normoxia, we found that HFD altered the expression of 1327 genes (1030 up, 297 down), with 381 genes (273 up, 108 down) changing ≥ two-fold (Figure 2.1 A). Using specific criteria intended to sort out genes with a potentially higher likelihood of impacting the phenotype, the initial list of genes was narrowed down to 33 candidate genes. These criteria selected genes a) within the group of 381 genes with a ≥ two-fold change in gene expression and b) that are part of a statistically significant pathway or functional cluster as determined with DAVID bioinformatics software [67,68]. The functional clusters with the highest biological significance ranking contained gene-annotation groups that included extracellular region or secretion, immune response, carbohydrate binding, and ion transport (data not shown). Significantly affected pathways included several pathways important in transport within the cell, the metabolism of amino acids and breakdown of sugars and waste products (Figure 2.1 B).

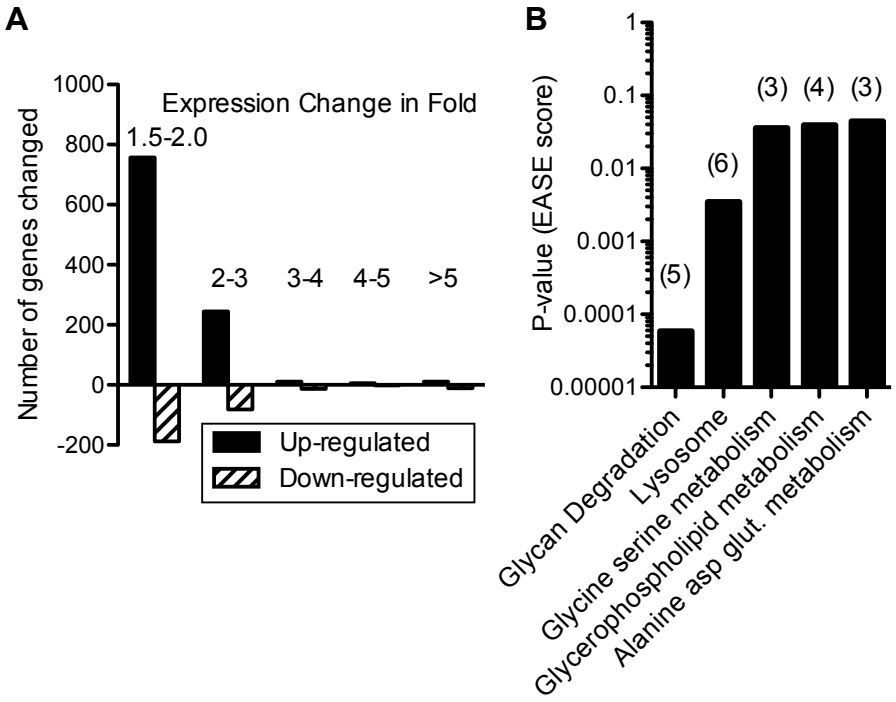


Figure 2.1. Microarray results evaluating change in gene expression in *Drosophila* on a high-fat diet (HFD) compared to a regular diet (RD). Adult female w^{1118} flies (d3-5) were placed on RD or HFD for one week prior to microarray analysis. A) Distribution of fold-changes of gene expression in *Drosophila* on a HFD (vs. RD). B) Analysis of genes with at least 2-fold change in expression showed significant enrichment in several KEGG pathways when flies were on a HFD. P-values are shown on y-axis on a log scale, and the number of genes significantly changed within the pathway is listed in parenthesis at the top of each bar. Pathways and p-values are from DAVID, listed in Appendix 2.1.

Screening genes involved in high-fat diet phenotype in normoxia

To further understand the role of these candidate genes, we evaluated the phenotype of flies in which the gene of interest was down-regulated by ubiquitous expression of an RNAi to the specific gene using the UAS/Gal4 system [69]. For this, we used the ubiquitously expressed daughterless (da)-Gal4 or armadillo (arm)-Gal4 drivers (Appendix 2.2). The rationale behind down-regulating genes of interest was to simulate the effect of HFD (if the gene was down-regulated in the microarrays) or to counteract the effect of HFD on transcription and phenotype (if the gene was up-regulated in the microarrays) (Figure 2.2).

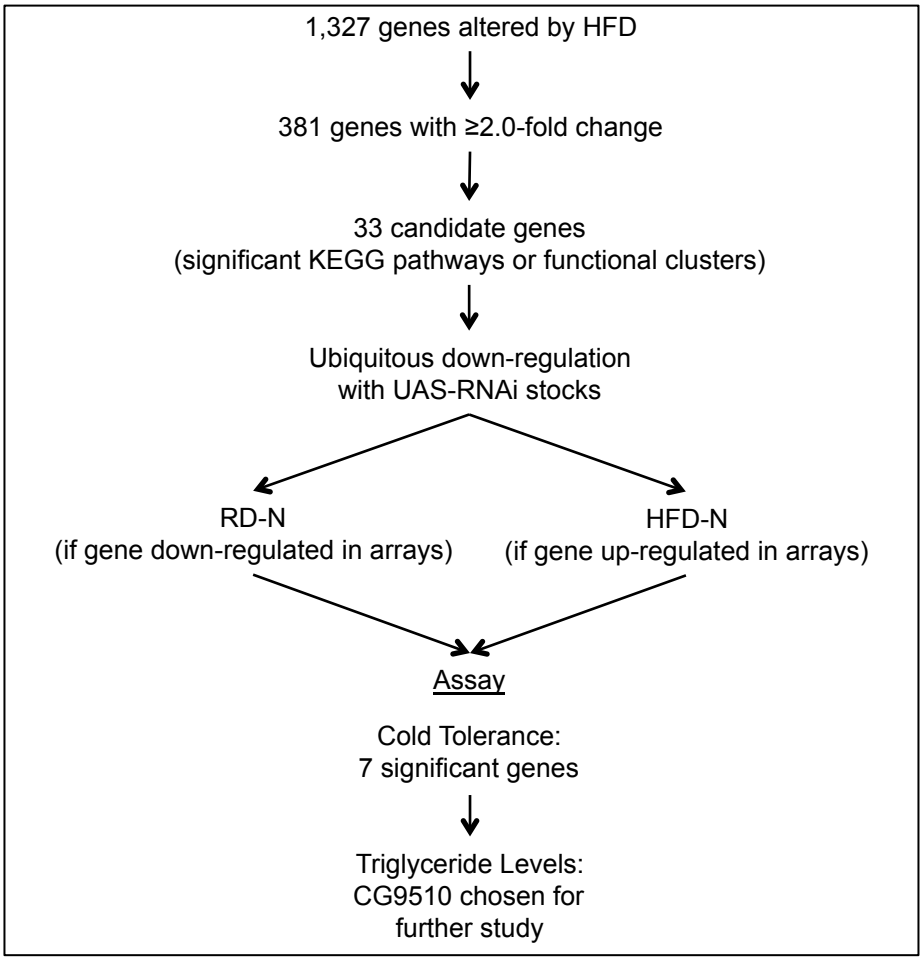


Figure 2.2. Paradigm for screening candidate genes from microarray analysis of high-fat diet compared to regular diet in normoxia. Candidate genes with available UAS-RNAi lines were crossed with daughterless (da)- or armadillo (arm)-Gal4 to ubiquitously down-regulate the gene. Adult female progeny (d3-5) of these crosses were placed on a regular diet (RD) or high-fat diet (HFD) depending on the direction of change in the microarray. After one week on the diets, progeny were compared with control flies on similar diets and assayed to see if down-regulation of the gene caused a change either simulating the HFD phenotype (for those on RD) or diminishing the severity of the HFD phenotype (for those on HFD).

After the transgenic lines had been on their respective diets for one week, they were stressed with severe cold and their survival compared to control flies on the same diet (Figure 2.3 A, B). Most control flies on RD were able to survive the cold stress (71%), while only 32% of those on HFD survived. Later measurement of controls specific for the Gal4 drivers (w^{1118} x da-Gal4 and w^{1118} x arm-Gal4) showed similar results. This phenotype implies that changes have occurred in flies on HFD that prevent them from activating mechanisms necessary for recovery from a severe stress. Four RNAi lines and a P-element insertion line (three different genes) on

RD showed significantly decreased survival than their control counterparts, indicating that down-regulating those candidate genes gave a similar cold stress response as flies on a HFD (Figure 2.3 A). Meanwhile, four different RNAi lines on HFD showed significantly increased survival compared to control on HFD, which implied that down-regulating these genes rendered the corresponding flies more resistant to cold stress than control flies, on HFD (Figure 2.3 B). Based on the cold tolerance results, seven candidate genes stood out as potentially having a role in the HFD phenotype, including *CG9510, CG5322, CG6806, CG18108, CG32356, CG5873*, and *CG1934*.

Triglyceride (TG) levels are another important part of the HFD phenotype, with control flies on HFD storing between 66-81 μg/mg bodyweight compared to 39-65μg/mg bodyweight in the flies on RD (range due to different genetic backgrounds). To further examine the role of the previously mentioned seven candidate genes that stood out as significant after the cold screen, TG levels in these transgenic lines fed RD or HFD were quantified (Figure 2.3 C, D). These lines were carefully compared to background-specific controls for the Gal4 line (Gal4 line crossed with the control for the RNAi background). In case of significance, we tested an additional control for the RNAi line (RNAi line crossed with the control for the Gal4 background) (data not shown). Of the seven genes, two showed a significant difference in triglyceride levels from the Gal4 controls. *CG1934*, or ecdysone-inducible gene E2, is associated with imaginal disc morphogenesis and was up-regulated by 4.4 fold in the microarray, while *CG9510* is involved with the arginine biosynthesis process and was down-regulated by -2.3 fold. Importantly, ubiquitous knockdown with two RNAi lines (on different chromosomes) and a P-element insertion line, all down-regulating *CG9510* (Appendix 3.2), showed significant changes in both the cold tolerance and TG content.

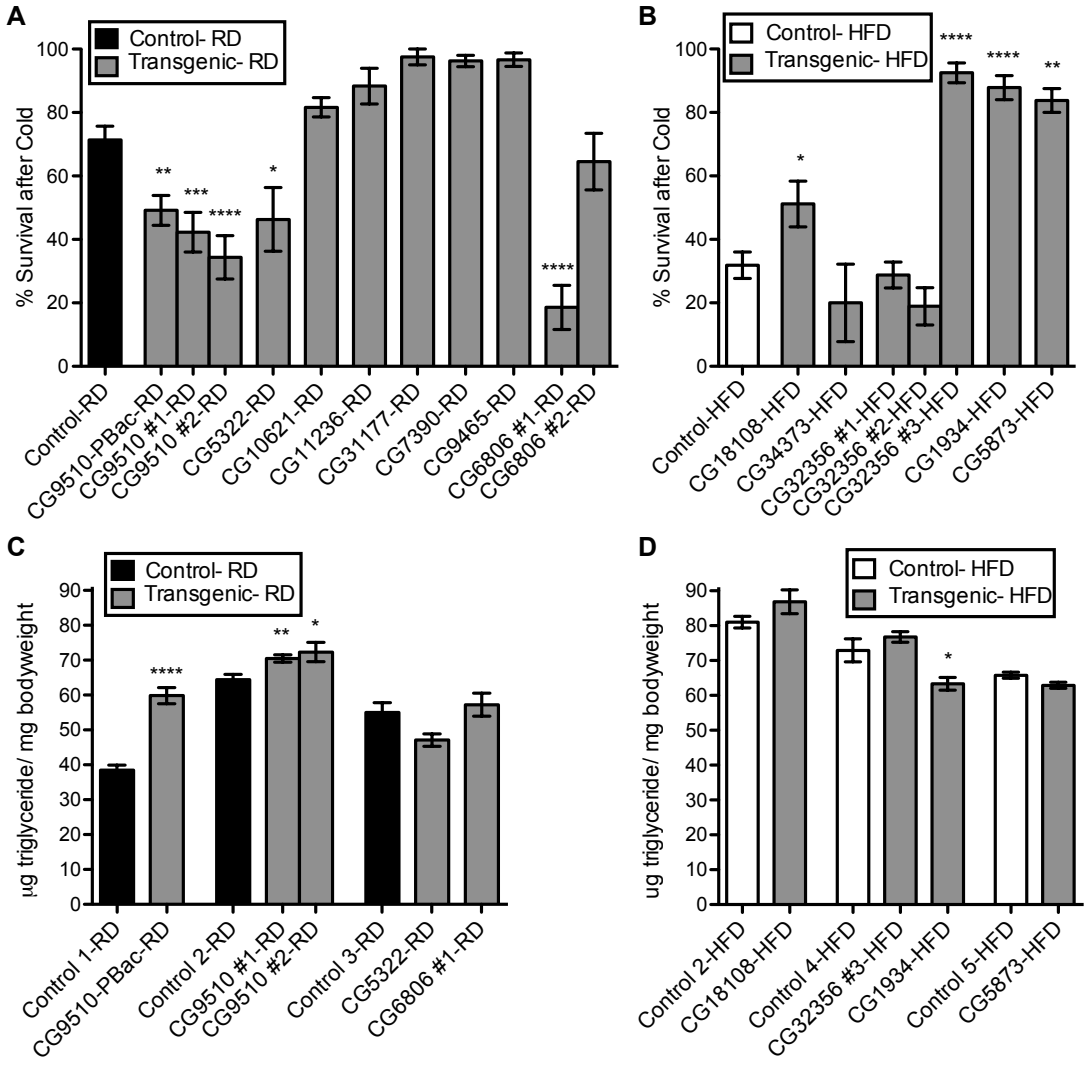


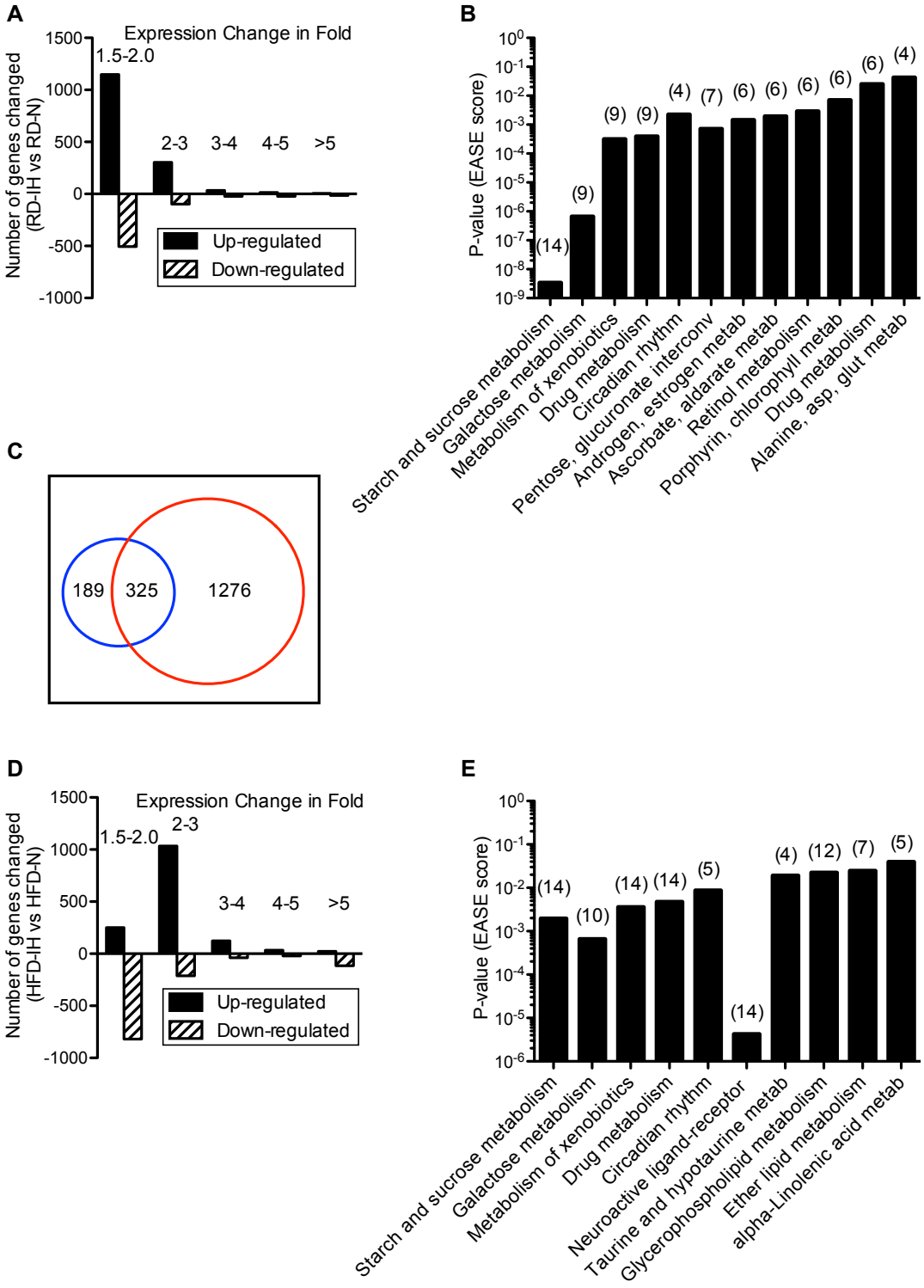
Figure 2.3. *CG9510* and *CG1934* show significant phenotypes from screen of candidate genes. Using the paradigm described in Figure 2.2, following one week on a regular diet (RD) or high-fat diet (HFD), transgenic flies with down-regulated expression of candidate genes were assayed for cold tolerance and triglyceride levels. A, B) Percent survival after cold stress of transgenic flies (progeny of UAS-RNAi x daughterless (da)-Gal4 or UAS-RNAi x armadillo (arm)-Gal4, labeled with gene name) on RD (A) or HFD (B) compared to control (w^{1118}) flies on the same diet. Multiple UAS-RNAi lines were evaluated for *CG9510*, *CG6806*, and *CG32356* and are marked with a #1-3 following the gene name. C, D) Triglyceride levels from whole-body homogenate of transgenic flies (P-element line "CG9510-PBac" and progeny from UAS-RNAi x da-Gal4 or UAS-RNAi x arm-Gal4, labeled with gene name) on RD (C) or HFD (D), compared to specific controls for background of flies and diets (Control-1: w^{1118}; Control 2: w^{1118} x da-Gal4 ; Control 3: *yw* x da-Gal4; Control 4: *yw* x arm-Gal4; Control 5: w^{1118} x arm-Gal4). Specific RNAi stocks and Gal4 drivers used are listed in Appendix 2.2. Flies were tested in sets of 10 females per vial for cold tolerance, 5 females for TG levels: (A, B) n= between 6-15 sets (60-150 flies per experimental group), (C, D) n= 5-14 sets (25-70 flies per experimental group). Significance between values for each experimental group and its respective control was determined with a t-test, *= p<0.05 **=p<0.01 ***=p<0.001 ****=p<0.0001.

Changes in gene expression due to intermittent hypoxia

While flies on a HFD showed a phenotype of decreased cold tolerance and increased TG, IH seemed to counteract this phenotype in both tests, with an increase in cold survival and decrease in TG levels. This was seen in flies both on RD and HFD in IH compared to their normoxic counterparts, although the HFD flies showed a more drastic change in phenotype. This accentuated change in phenotype is reflected in the microarray results of flies on the two diets in IH compared to normoxia (RD-IH vs. RD-N and HFD-IH vs. HFD-N). Although flies on RD-IH had 2168 genes altered, less than a quarter of those (514 genes) had over 2-fold expression change (Figure 2.4 A), while those on HFD-IH had 2671 genes changed compared to HFD-N, 1600 of which showed over 2-fold change (Figure 2.4 D).

A large number of the genes altered ≥2-fold by IH in flies on RD also were changed by IH in flies on HFD, with nearly two-thirds of those genes in the RD-IH array also significantly changed in HFD-IH (Figure 2.4 C). The KEGG pathways with significantly altered genes showed some differences and some overlap between the two groups (Figure 2.4 B, E). KEGG pathways involved in carbohydrate metabolism, drug metabolism and circadian rhythm all were greatly affected by IH in flies on both diets, suggesting that these pathways may be important in the change in phenotype due to IH independent of the specific diet.

Figure 2.4. Microarray results evaluating change in gene expression in *Drosophila* in intermittent hypoxia (IH) compared to normoxia (N) while on a regular diet (RD) or a high-fat diet (HFD). Adult female w^{1118} flies (d3-5) were placed in IH or N conditions and on RD or HFD for one week prior to microarray analysis. A) Distribution of fold-changes of gene expression in *Drosophila* on RD-IH (vs. RD-N). B) Analysis of genes with at least 2-fold change in expression showed significant enrichment in several KEGG pathways when flies were in IH compared to N while on RD. P-values from DAVID analysis are shown on y-axis on a log scale, and the number of genes significantly changed within the pathway is listed in parenthesis at the top of each bar. Pathways are listed in Appendix 2.1. C) Overall effect of IH on gene expression, shown by the overlap between genes changed due to IH in flies on RD and HFD. Shown is the number of genes with over 2-fold expression change in flies exposed to IH compared to N in flies on RD (blue circle) and HFD (red circle). The number in the overlapping area indicates genes that existed in both groups. D) Distribution of fold-changes of gene expression in *Drosophila* on HFD-IH (vs. HFD-N). E) Analysis of genes with at least 2-fold change in expression showed significant enrichment in several KEGG pathways when flies were in IH while on HFD. P-values are shown on y-axis on a log scale, and the number of genes significantly changed within the pathway is listed in parenthesis at the top of each bar. Pathways are listed in Appendix 2.1.


A
Expression Change in Fold
1.5-2.0
2-3
3-4
4-5
>5
Number of genes changed (RD-IH vs RD-N)
1500
1000
500
0
-500
-1000
Up-regulated
Down-regulated
B
P-value (EASE score)
(14)
(9)
(9)
(9)
(4)
(7)
(6)
(6)
(6)
(6)
(6)
(4)
Starch and sucrose metabolism
Galactose metabolism
Metabolism of xenobiotics
Drug metabolism
Circadian rhythm
Pentose, glucuronate interconv
Androgen, estrogen metab
Ascorbate, aldarate metab
Retinol metabolism
Porphyrin, chlorophyll metab
Drug metabolism
Alanine, asp, glut metab
C
189
325
1276
D
Expression Change in Fold
1.5-2.0
2-3
3-4
4-5
>5
Number of genes changed (HFD-IH vs HFD-N)
1500
1000
500
0
-500
-1000
Up-regulated
Down-regulated
E
P-value (EASE score)
(14)
(10)
(14)
(14)
(5)
(14)
(4)
(12)
(7)
(5)
Starch and sucrose metabolism
Galactose metabolism
Metabolism of xenobiotics
Drug metabolism
Circadian rhythm
Neuroactive ligand-receptor
Taurine and hypotaurine metab
Glycerophospholipid metabolism
Ether lipid metabolism
alpha-Linolenic acid metab

Screening genes involved in intermittent hypoxia phenotype in flies on high-fat diet

Given that the HFD-IH vs. HFD-N comparison had the most dramatic change in phenotype and a large number of genes changed, we used this microarray analysis to select candidate genes. Using the same criteria as described for the HFD-N vs. RD-N comparison, we selected 95 candidate genes for further study. Using UAS-RNAi stocks for these genes in the UAS/Gal4 system, we examined each of these genes independently down-regulated in the whole fly. To drive the RNAi expression in the whole fly, we used the ubiquitously expressed daughterless (da)-Gal4 or armadillo (arm)-Gal4 drivers. Arm-Gal4 was used in cases when the UAS-RNAi x da-Gal4 progeny was too weak to maintain on diets for a week (Appendix 2.3). As with the previous candidate gene screen, the rationale behind down-regulating genes of interest was to simulate the effect of IH on HFD flies even when flies were in normoxia (if the gene was down-regulated in the HFD-IH vs. HFD-N microarrays) or to counteract the effect of IH on transcription and phenotype of HFD flies while in IH (if the gene was up-regulated in the microarrays) (Figure 2.5).

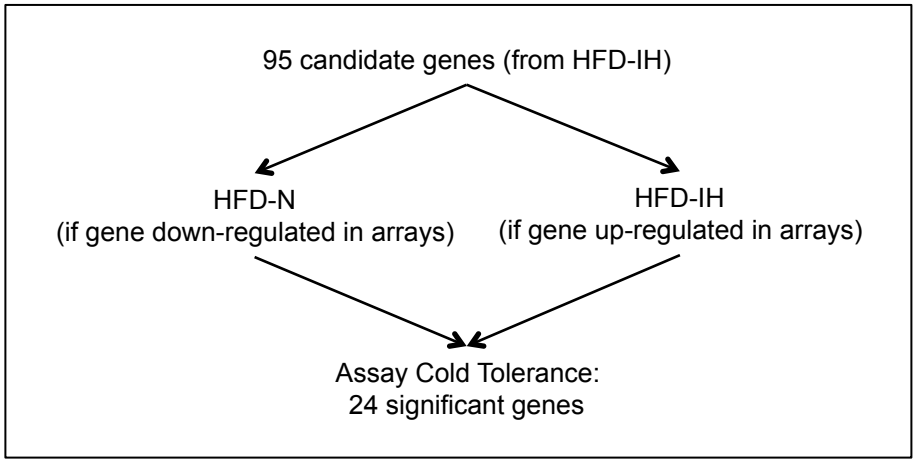
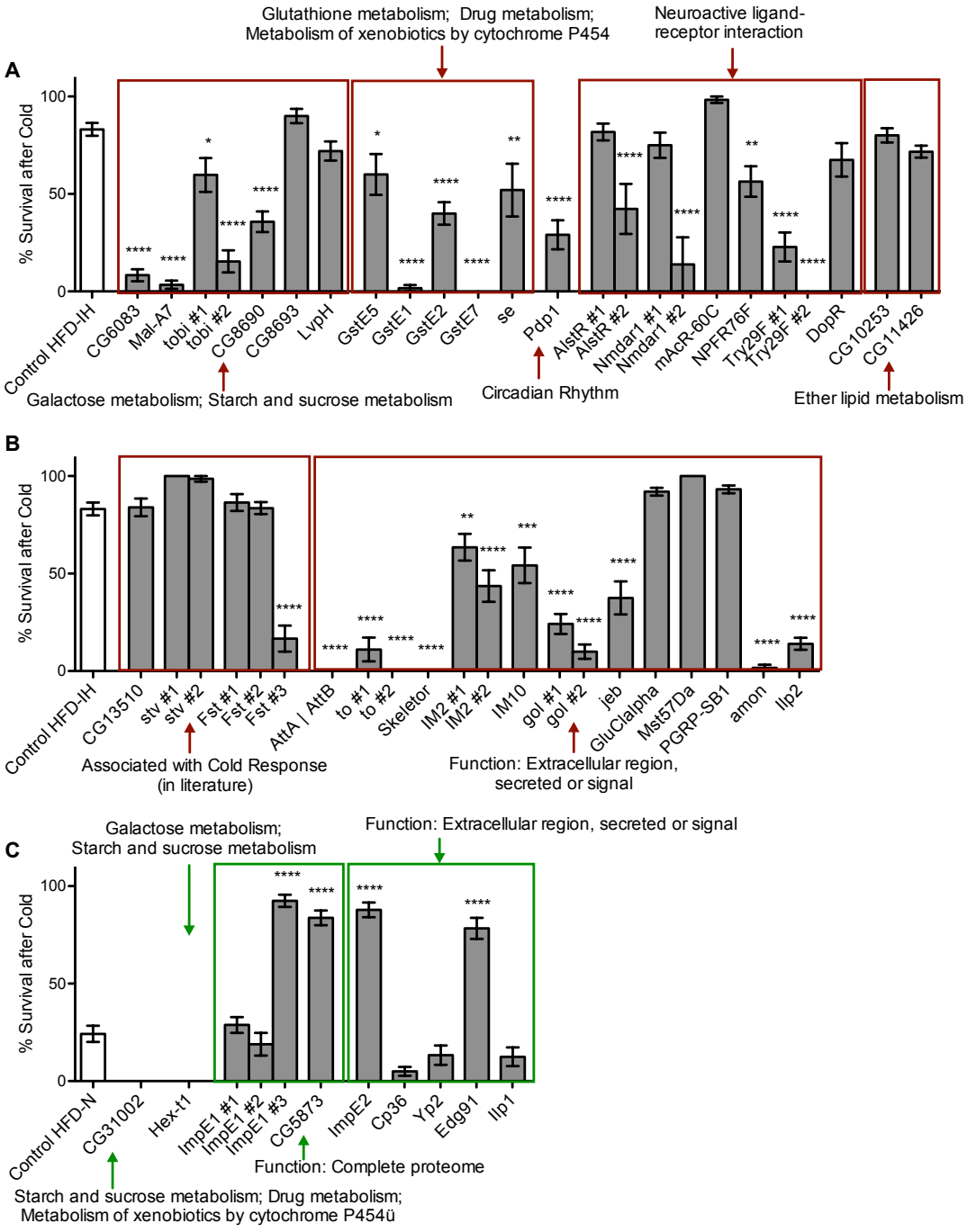


Figure 2.5. Paradigm for screening candidate genes from microarray analysis of intermittent hypoxia (IH) compared to normoxia (N) in flies on a high-fat diet (HFD). Candidate genes with available UAS-RNAi lines were crossed with daughterless (da)- or armadillo (arm)-Gal4 to ubiquitously down-regulate the gene. Adult female progeny (d3-5) of these crosses were placed on a HFD and in IH or N conditions depending on the direction of change in the microarray. After one week on the HFD and in their respective oxygen condition, progeny were compared with control flies on HFD and in a similar oxygen condition and assayed to see if down-regulation of the gene caused a change either simulating the HFD-IH phenotype (for those on HFD-N) or diminishing the effect of IH on the phenotype (for those on HFD-IH).

After the transgenic lines had been on HFD and in normoxia or IH for one week, they were stressed with severe cold and their survival compared to HFD control flies in the same oxygen condition (Figure 2.6). Most control flies on HFD in IH were able to survive the cold stress (83%) (Figure 2.6 A, B), while only 24% of those on HFD in normoxia survived (Figure 2.6 C). Given this large disparity in survival, changes must have occurred during IH to allow flies on HFD to be able more easily access the necessary stress recovery mechanisms. Independently altering expression of 21 different genes in IH and HFD caused flies to have significantly decreased survival than their control HFD-IH counterparts, indicating that by preventing up-regulation of those genes in IH, flies were unable to activate the mechanisms normally turned on by IH (Figure 2.6 A, B). Meanwhile, three transgenic lines on HFD in normoxia showed significantly increased survival compared to control on HFD-N, suggesting that by down-regulating those genes even in normoxia, flies were able to mimic the phenotype seen in IH (Figure 2.6 C). In four additional genes, a significant change was observed in one transgenic line, but no significance in another line for the same gene, so these were not included in the significant genes. Within the candidate genes that stood out in the screen for cold tolerance, several pathways were strongly represented. These include pathways involved in circadian rhythm, drug metabolism, carbohydrate metabolism, amino acid metabolism, neuroactive ligand-receptor interaction, and genes involved in secretion or in the extracellular region.

Figure 2.6. Screen of candidate genes for role in intermittent hypoxia (IH) phenotype. Using the paradigm described in Figure 2.5, following one week on a high-fat diet (HFD) in either IH or normoxia (N), cold tolerance was measured in transgenic flies with down-regulated expression of candidate genes. Percent survival after stress of da-gal4 and UAS-RNAi progeny (labeled with gene name) compared to control (w^{1118}) flies. Transgenic and control flies were on HFD and in IH (A, B) or N (C). In some cases, multiple UAS-RNAi lines were evaluated for the same gene. Specific RNAi stocks and Gal4 drivers used are listed in Appendix 2.3. Transgenic lines are grouped by the pathway or functional cluster associated with the candidate gene, as defined by DAVID analysis. Flies were tested in sets of 10 females per vial: (A, B) n= between 5-14 sets (50-140 flies per experimental group), (C) n= 5-17 sets (50-170 flies per group). Significance between each experimental group and its control was determined with a t-test, **=p<0.01 ***=p<0.001 ****=p<0.0001.


A
Glutathione metabolism; Drug metabolism;
Metabolism of xenobiotics by cytochrome P454
Neuroactive ligand-
receptor interaction
% Survival after Cold
100
50
0
Control HFD-IH
CG6083
Mal-A7
tobi #1
tobi #2
CG8690
CG8693
LvpH
GstE5
GstE1
GstE2
GstE7
se
Pdp1
AlstR #1
AlstR #2
Nmdar1 #1
Nmdar1 #2
mAcR-60C
NPFR76F
Try29F #1
Try29F #2
DopR
CG10253
CG11426
Galactose metabolism; Starch and sucrose metabolism
Circadian Rhythm
Ether lipid metabolism
B
% Survival after Cold
100
50
0
Control HFD-IH
CG13510
stv #1
stv #2
Fst #1
Fst #2
Fst #3
AttA | AttB
to #1
to #2
Skeletor
IM2 #1
IM2 #2
IM10
gol #1
gol #2
jeb
GluClalpha
Mst57Da
PGRP-SB1
amon
Ilp2
Associated with Cold Response
(in literature)
Function: Extracellular region,
secreted or signal
C
Galactose metabolism;
Starch and sucrose metabolism
Function: Extracellular region, secreted or signal
% Survival after Cold
100
50
0
Control HFD-N
CG31002
Hex-t1
ImpE1 #1
ImpE1 #2
ImpE1 #3
CG5873
ImpE2
Cp36
Yp2
Edg91
Ilp1
Function: Complete proteome
Starch and sucrose metabolism; Drug metabolism;
Metabolism of xenobiotics by cytochrome P454ü

CG9510 as candidate gene for further study

In Chapter 1, we observed that both IH and HFD in Normoxia affect TG and cold tolerance levels, although in opposing ways, with HFD increasing TG and lowering cold tolerance, and IH decreasing TG and increasing cold tolerance. This suggests that perhaps similar genes may be altered by both conditions, but in opposite directions. Of the genes altered by HFD (HFD-N vs. RD-N) and by IH (HFD-IH vs. HFD-N), we observed 100 genes with changes in both groups, and differentially expressed between the two groups (Figure 2.7). Forty-five of the genes up-regulated by HFD-N are also changed by IH, but are instead down-regulated, and 24 of these have over 2-fold change in both diet and IH. Meanwhile, of the genes down-regulated by HFD-N, 55 are up-regulated by IH, and 18 of these have over 2-fold change in both arrays (Appendix 2.4). Interestingly, these differentially expressed genes included several candidate genes that stood out in the gene screens, including *CG9510* in HFD, and tobi and Maltase A7 in IH.

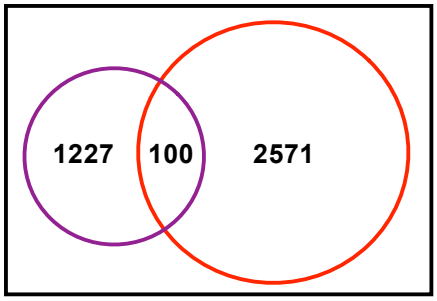


Figure 2.7. Overlapping genes altered by HFD and IH with differential expression. Shown is the total number of genes from the microarray analysis of HFD-N vs. RD-N (purple circle) and HFD-IH vs. HFD-N (red circle). The number in the overlapping area indicates the genes that exist in both arrays and are differentially expressed between the arrays.

Given the potential for *CG9510* as having a role in HFD phenotype, we tested its impact on the IH phenotype. *CG9510* is down-regulated by -2.3-fold change HFD, and in IH on HFD it is up-regulated by 2.4-fold. Interestingly it is also up-regulated by RD-IH by 1.6-fold. Two transgenic RNAi lines for *CG9510* (crossed with da-Gal4 for ubiquitous expression) had increased TG levels following a week in IH and on HFD, suggesting that knocking-down the gene seemed to diminish the ability of IH to decrease TG levels (Figure 2.8). These results further demonstrate the need for further study of the role of *CG9510* in *Drosophila*.

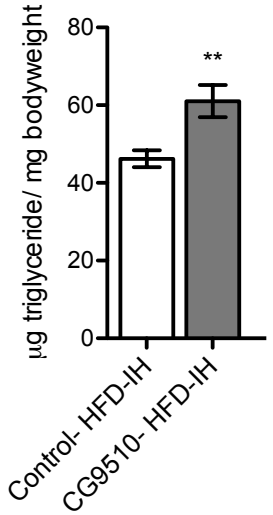


Figure 2.8. Down-regulation of *CG9510* leads to increased triglyceride levels in intermittent hypoxia. Adult female progeny (d3-5) from crosses between a UAS-RNAi line (CG9510 #1) and daughterless-Gal4 driver, as well as control progeny from a cross between w^{1118} and the UAS-RNAi line (CG9510 #1) were put on a high-fat diet (HFD) and in intermittent hypoxia (IH) for one week prior to the assay. Triglyceride levels (per mg of bodyweight) were determined from whole-body homogenate. Flies were tested in sets of 5 females for TG levels, n=8 sets (40 flies per group). Significance between values of the experimental group and its control was determined with a t-test, **=p<0.01.

In summary, we showed through microarray analysis that both HFD and IH alter the expression of a large number of genes. In response to HFD alone, flies have several metabolic pathways altered and the primary candidate gene that stood out after screening for both cold tolerance and TG levels, *CG9510*, is also involved in amino acid metabolism. In response to IH, flies have a wide variety of pathways altered. The consistent theme between the pathways modulated by IH in flies on both RD and HFD seemed to be carbohydrate and drug metabolism. This also was true in the screen of candidate genes affected by IH (on HFD), along with genes with functions in extracellular region, secreting or signaling. There were some genes that showed up in both the HFD and IH arrays, with their expression altered in different directions by the two conditions. Interestingly, this included the HFD candidate gene *CG9510*, suggesting it to be a gene to further study.

Discussion

In this chapter, we uncovered key transcriptional changes that seem to be involved in the response of Drosophila to a high-fat diet and intermittent hypoxia. It seemed that amino acid and carbohydrate metabolism plays a role in the HFD phenotype, both based on the significantly affected KEGG pathways and candidate gene screen. In response to a HFD, there was modulated expression of genes involved in several metabolic pathways, including amino acid metabolism and glycan degradation. In particular, one of the genes with altered transcription in flies on HFD, *CG9510*, also impacted the same phenotypic characteristics as HFD, suggesting that the gene and its function may be important in the response of the fly to HFD. Transgenic flies with decreased expression of *CG9510* had significant changes in triglyceride (TG) levels and cold tolerance even on RD, similar to control flies on a HFD.

Carbohydrate metabolism seems to potentially be playing a role in the change in phenotype seen in flies exposed to IH. Starch and sucrose metabolism and galactose metabolism pathways are significantly altered in both of the two IH comparisons (RD-IH vs. RD-N and HFD-IH vs. HFD-N) (Figure 2.4 B, E) and several of the genes in these pathways have a significant phenotype when down-regulated in flies (Figure 2.6 A). This suggests that IH may be affecting metabolism in such a way to speed up fat oxidation in the flies and decrease TG storage. Interestingly, flies in IH have altered glucose levels (Chapter 1) and this is reflected in the finding that several genes involved in starch and sucrose metabolism seem to play a significant role in the phenotype.

In examining the genes differentially expressed between the HFD and IH arrays, it is important to point out that among these were several of the strong candidate genes. *Tobi* and *maltase A7* were both significantly down-regulated by HFD alone (-2.0 and -2.4-fold, respectively) yet flies in IH up-regulated these genes by 9.5 and 4.8-fold, respectively. The knockdown transgenic flies for each of these genes had significantly reduced survival after cold stress even after IH exposure, indicating that by preventing IH from up-regulating these genes, the flies were unable to activate the pathways necessary to survive cold stress. The fact that these genes are

down-regulated by HFD further suggests that the down-regulation could be important in the decreased survival to cold stress and links carbohydrate metabolism to this phenotype.

CG9510 was also differentially regulated in the two arrays, with a down-regulation of -2.3-fold in HFD and up-regulation by 2.4-fold in IH. Importantly, transgenic flies with decreased expression of *CG9510* not only mimic the HFD phenotype when on RD, but they also seem to diminish the effects of IH on the phenotype. Typically IH would decrease TG levels in HFD flies, but when the knockdown transgenic flies are unable to up-regulate *CG9510* in IH, they show increased TG levels compared to control HFD flies in IH. This indicates a strong role for *CG9510* in the TG and cold tolerance phenotype of *Drosophila*; a role that will be further examined in Chapter 3.

Acknowledgements

Chapter 2, in part, has been submitted for publication. The dissertation author was the primary investigator and author of this material. Gabriel Haddad supervised the project and provided editing and direction.

Appendix 2.1. Significantly affected KEGG pathways. (From Figures 2.1 and 2.4)

HFD-N vs. RD-N (Figure 2.1 B)

Term	Count (significantly affected genes)	P-value
dme00511: Other glycan degradation	5	5.86E-05
dme04142: Lysosome	6	3.47E-03
dme00260: Glycine, serine and threonine metabolism	3	3.60E-02
dme00564: Glycerophospholipid metabolism	4	3.91E-02
dme00250: Alanine, aspartate and glutamate metabolism	3	4.43E-02

RD-IH vs. RD-N (Figure 2.4 B)

Term	Count (significantly affected genes)	P-value
dme00500: Starch and sucrose metabolism	14	3.42E-09
dme00052: Galactose metabolism	9	6.69E-07
dme00980: Metabolism of xenobiotics by cytochrome P450	9	3.18E-04
dme00982: Drug metabolism	9	3.95E-04
dme00040: Pentose and glucuronate interconversions	7	7.11E-04
dme00150: Androgen and estrogen metabolism	6	1.46E-03
dme00053: Ascorbate and aldarate metabolism	6	1.95E-03
dme04711: Circadian rhythm	4	2.23E-03
dme00830: Retinol metabolism	6	2.91E-03
dme00860: Porphyrin and chlorophyll metabolism	6	7.05E-03
dme00983: Drug metabolism	6	2.51E-02
dme00250: Alanine, aspartate and glutamate metabolism	4	4.24E-02

HFD-IH vs. HFD-N (Figure 2.4 E)

Term	Count (significantly affected genes)	P-value
dme04080: Neuroactive ligand-receptor interaction	14	4.26E-06
dme00052: Galactose metabolism	10	6.56E-04
dme00500: Starch and sucrose metabolism	14	1.96E-03
dme00980: Metabolism of xenobiotics by cytochrome P450	14	3.60E-03
dme00982: Drug metabolism	14	4.76E-03
dme04711: Circadian rhythm	5	8.67E-03
dme00430: Taurine and hypotaurine metabolism	4	1.92E-02
dme00564: Glycerophospholipid metabolism	12	2.23E-02
dme00565: Ether lipid metabolism	7	2.46E-02
dme00592: alpha-Linolenic acid metabolism	5	3.94E-02

Appendix 2.2. List of RNAi stocks used in screening candidate genes from high-fat diet microarray. RNAi stocks from Vienna Drosophila RNAi Center (VDRC) were used to screen candidate genes from microarray analysis of flies on high-fat diet (HFD) in normoxia (N) compared to regular diet (RD) in normoxia. Listed is the candidate gene as shown in Figure 2.3, the Transformant ID for the RNAi stock, the Gal4 driver used to create transgenic progeny (daughterless (da)-Gal4 or armadillo (arm)-Gal4), and conditions to test the transgenic flies (HFD-N or RD-N).

Candidate Gene in Figure 2.3	Transformant ID (VDRC)	Gal4 driver	Condition for testing transgenic flies
CG9510 #1	44682	da	RD-N
CG9510 #2	44683	da	RD-N
CG5322	106609	da	RD-N
CG10621	31291	da	RD-N
CG11236	38460	da	RD-N
CG31177	30962	da	RD-N
CG7390	35230	da	RD-N
CG9465	52270	da	RD-N
CG6806 #1	14069	da	RD-N
CG6806 #2	50715	da	RD-N
CG18108	8812	da	HFD-N
CG34373	105369	da	HFD-N
CG32356 #1	12854	da	HFD-N
CG32356 #2	12855	da	HFD-N
CG32356 #3	104613	arm	HFD-N
CG1934	100148	arm	HFD-N
CG5873	14374	arm	HFD-N

Appendix 2.3. List of RNAi stocks used in screening candidate genes from intermittent hypoxia microarray. RNAi stocks from Vienna Drosophila RNAi Center (VDRC) were used to screen candidate genes from microarray analysis of flies on high-fat diet (HFD) in intermittent hypoxia (IH) compared to HFD in normoxia (N). Listed is the candidate gene as shown in Figure 2.6, the Transformant ID for the RNAi stock, the Gal4 driver used to create transgenic progeny (daughterless (da)-Gal4 or armadillo (arm)-Gal4), and conditions to test the transgenic flies (HFD-IH or HFD-N).

Candidate Gene in Figure 2.6	Transformant ID (VDRC)	Gal4 driver	Condition for testing transgenic flies
CG6083	27549	da	HFD-IH
Mal-A7	15795	da	HFD-IH
tobi #1	14734	da	HFD-IH
tobi #2	103544	da	HFD-IH
CG8690	15800	da	HFD-IH
CG8693	105321	da	HFD-IH
LvpH	106220	da	HFD-IH
GstE5	25270	da	HFD-IH
GstE1	110529	da	HFD-IH
GstE2	32945	da	HFD-IH
GstE7	47251	da	HFD-IH
se	34227	da	HFD-IH
Pdp1	110551	da	HFD-IH
AlstR #1	48495	da	HFD-IH
AlstR #2	101395	da	HFD-IH
Nmdar1 #1	37333	da	HFD-IH
Nmdar1 #2	104773	da	HFD-IH
mAcR-60C	101407	da	HFD-IH
NPFR76F	9379	da	HFD-IH
Try29F #1	45717	da	HFD-IH
Try29F #2	48863	da	HFD-IH
DopR	107058	da	HFD-IH
CG10253	3321	da	HFD-IH
CG11426	42599	da	HFD-IH
CG13510	28434	da	HFD-IH
stv #1	34408	da	HFD-IH
stv #2	34409	arm	HFD-IH
Fst #1	16604	da	HFD-IH
Fst #2	17258	da	HFD-IH
Fst #3	102049	da	HFD-IH
AttA | AttB	9287	da	HFD-IH
to #1	51463	da	HFD-IH
to #2	100079	da	HFD-IH

Appendix 2.3. List of RNAi stocks used in screening candidate genes from intermittent hypoxia microarray, continued. RNAi stocks from Vienna Drosophila RNAi Center (VDRC) were used to screen candidate genes from microarray analysis of flies on high-fat diet (HFD) in intermittent hypoxia (IH) compared to HFD in normoxia (N). Listed is the candidate gene as shown in Figure 2.6, the Transformant ID for the RNAi stock, the Gal4 driver used to create transgenic progeny (daughterless (da)-Gal4 or armadillo (arm)-Gal4), and conditions to test the transgenic flies (HFD-IH or HFD-N).

Candidate Gene in Figure 2.6	Transformant ID (VDRC)	Gal4 driver	Condition for testing transgenic flies
Skeletor	49663	da	HFD-IH
IM2 #1	10586	da	HFD-IH
IM2 #2	43372	da	HFD-IH
IM10	12843	da	HFD-IH
gol #1	37435	da	HFD-IH
gol #2	107822	arm	HFD-IH
jeb	30799	da	HFD-IH
GluClalpha	105754	da	HFD-IH
Mst57Da	40694	da	HFD-IH
PGRP-SB1	101298	da	HFD-IH
amon	37023	da	HFD-IH
Ilp2	102158	arm	HFD-IH
CG31002	48226	da	HFD-N
Hex-t1	46574	da	HFD-N
ImpE1 #1	12854	da	HFD-N
ImpE1 #2	12855	da	HFD-N
ImpE1 #3	104613	da	HFD-N
CG5873	14374	da	HFD-N
ImpE2	100148	arm	HFD-N
Cp36	14824	da	HFD-N
Yp2	30859	da	HFD-N
Edg91	39554	da	HFD-N
Ilp1	5198	da	HFD-N

Appendix 2.4. Differentially Expressed Genes. Genes in common but differentially expressed in high-fat diet (HFD) array (HFD-normoxia vs. regular diet-normoxia) and intermittent hypoxia (IH) array (HFD-IH vs. HFD-normoxia).

Affy ID	Fly Base ID	Gene Name	HFD fold change (HFD-N vs. RD-N)	IH fold change (HFD-IH vs. HFD-N)	Pathway/ Information
1636458_at	FBgn0037992	CG4702	6.56	-500.00	
1625382_at	FBgn0027527	Osi6	4.76	-90.91	plasma membrane
1623372_at	FBgn0038511	CG5873	4.68	-4.18	ion binding
1634928_at	FBgn0001254	ImpE2	4.37	-71.43	imaginal disc; extracellular
1624990_at	FBgn0039851	CG12063	3.96	-6.37	
1635686_at	FBgn0036352	CG14110	3.51	-16.39	
1624245_at	FBgn0029681	CG15239	3.49	-24.39	
1626327_s_at			3.26	-2.11	
1639376_at	FBgn0035621	CG10591	3.19	-11.24	
1627954_a_at	FBgn0035208	CG9184	2.91	-166.67	
1635556_at			2.78	-2.67	
1640837_a_at	FBgn0001256	ImpL1	2.77	-4.67	extracellular
1626893_at	FBgn0026077	Gasp	2.73	-1.93	chitin binding
1639474_at	FBgn0033721	CG13159	2.71	-52.63	
1628376_x_at	FBgn0031277	CG13947	2.62	-3.94	
1634259_at	FBgn0035077	CG9083	2.60	-10.99	
1625625_at	FBgn0001253	ImpE1	2.51	-2.07	imaginal disc; extrinsic to membrane
1636782_at	FBgn0038028	CG10035	2.51	-7.25	
1639190_at	FBgn0011653	mas	2.49	-4.18	serine endopepsidase activity
1633077_at	FBgn0037430	Osi20	2.41	-9.01	plasma membrane
1636184_at	FBgn0051813	CG31813	2.39	-166.67	
1628720_at	FBgn0040984	CG4440	2.25	-31.25	
1629764_at			2.20	-2.30	
1632625_a_at	FBgn0035315	CG8960	2.14	-3.13	
1635380_at	FBgn0050413	CG30413	2.07	-2.80	
1634409_at	FBgn0028537	CG31775	1.99	-41.67	
1628084_at	FBgn0040487	Brother of Bearded A	1.97	-20.41	
1627159_at	FBgn0034514	CG13427	1.95	-29.41	gastrulation
1636449_at	FBgn0052984	CG32984	1.94	-1.75	
1633404_at	FBgn0035381	CG9965	1.90	-2.22	
1630716_at			1.89	-2.07	
1638610_at	FBgn0039704	CG7802	1.77	-3.92	embryo shape

Appendix 2.4. Differentially Expressed Genes, Continued. Genes in common but differentially expressed in high-fat diet (HFD) array (HFD-normoxia vs. regular diet-normoxia) and intermittent hypoxia (IH) array (HFD-IH vs. HFD-normoxia).

Affy ID	Fly Base ID	Gene Name	HFD fold change (HFD-N vs. RD-N)	IH fold change (HFD-IH vs. HFD-N)	Pathway/ Information
1637939_at	FBgn0024294	Spn43Aa	1.76	-7.69	serine protease inhibitor
1626373_at	FBgn0052833	CG32833	1.76	-2.23	serine endopepsidase activity
1630715_at	FBgn0037037	CG10588	1.76	-1.81	
1640255_at			1.72	-1.76	
1641679_at	FBgn0036900	CG8765	1.68	-2.11	
1623950_s_at	FBgn0000071	Ama	1.68	-8.20	antigen binding
1631624_at	FBgn0040360	CG14626	1.67	-1.92	
1635598_at	FBgn0031724	CG18266	1.64	-1.96	
1629313_at	FBgn0030837	CG8661	1.60	-2.35	
1631714_a_at	FBgn0029128	SP71	1.59	-2.37	serine endopepsidase activity
1639641_at	FBgn0035781	CG8560	1.58	-3.85	zinc ion binding
1627986_s_at	FBgn0043576	PGRP-SC1a	1.54	-1.91	
1623821_at	FBgn0035257	CG12011	1.51	-18.52	
1631806_at	FBgn0033294	CG8693	-1.51	2.16	starch and sucrose metab, etc.
1638038_at	FBgn0038795	CG4335	-1.53	2.45	oxidation-reduction
1639934_at	FBgn0051272	CG31272	-1.54	2.88	transporter activity, lipase activity
1631801_at	FBgn0030928	CG15044	-1.54	4.30	
1635862_at	FBgn0053120	CG33120	-1.54	2.03	
1628657_at	FBgn0063491	GstE9	-1.55	2.18	glutathione transferase; response to toxin
1636682_at	FBgn0051778	CG31778	-1.55	2.22	serine endopeptidase inhibitor activity
1624945_a_at	FBgn0034289	CG10910	-1.56	2.29	
1634786_at	FBgn0002940	CG4550	-1.57	2.92	
1632087_at	FBgn0032472	CG9928	-1.58	2.18	
1636119_at	FBgn0030157	CG1468	-1.58	2.23	
1627463_at	FBgn0033659	Damm	-1.60	3.24	
1637129_at	FBgn0063497	GstE3	-1.61	3.49	glutathione transferase
1631873_at	FBgn0040342	CG3706	-1.62	1.87	

Appendix 2.4. Differentially Expressed Genes, Continued. Genes in common but differentially expressed in high-fat diet (HFD) array (HFD-normoxia vs. regular diet-normoxia) and intermittent hypoxia (IH) array (HFD-IH vs. HFD-normoxia).

Affy ID	Fly Base ID	Gene Name	HFD fold change (HFD-N vs. RD-N)	IH fold change (HFD-IH vs. HFD-N)	Pathway/ Information
1634960_at	FBgn0038147	CG14375	-1.63	3.15	neuropeptide hormone activity
1636470_at	FBgn0054026	CG34026	-1.64	2.71	
1632735_at	FBgn0033659	CG18188	-1.66	2.18	
1630740_at	FBgn0034663	CG4363	-1.66	4.36	
1623601_at	FBgn0020506	Amyrel	-1.67	2.66	starch and sucrose metab, etc.
1634815_at	FBgn0051104	CG31104	-1.69	4.06	transferase activity
1639268_at	FBgn0033789	CG13324	-1.69	4.59	
1633251_at	FBgn0035743	CG15829	-1.70	1.89	cellular acyl-CoA homeostasis; enzyme inhibitor
1641627_at	FBgn0037714	CG9396	-1.71	2.09	
1634697_at	FBgn0052667	CG32667	-1.72	2.57	extracellular
1633214_at	FBgn0035607	CG4835	-1.73	2.05	chitin binding
1628258_at	FBgn0027578	CG14526	-1.77	2.47	metallo-endopeptidase activity
1639424_at	FBgn0036262	CG6910	-1.77	4.11	Ascorbate and aldarate metabolism, Inositol phosphate metabolism
1631220_at	FBgn0034903	CG9850	-1.78	2.18	metallo-endopeptidase activity
1637567_at	FBgn0050360	CG30360	-1.78	5.18	cation binding
1640755_at	FBgn0013772	Cyp6a8	-1.78	1.87	oxidation-reduction
1638974_at	FBgn0035409	CG14963	-1.79	3.92	
1624590_at	FBgn0032304	CG17134	-1.81	2.82	proteolysis
1634257_at	FBgn0030828	CG5162	-1.82	1.85	lipid metabolic process
1632984_s_at	FBgn0040606	CG6503	-1.83	2.08	
1634143_at	FBgn0033065	Cyp6w1	-1.84	2.77	oxidation-reduction; membrane
1624695_at	FBgn0037801	CG3999	-1.91	2.51	oxidoreductase
1628405_at	FBgn0036948	CG7298	-1.95	4.19	extracellular region, chitin binding
1629853_at	FBgn0040349	CG3699	-1.97	2.02	oxidoreductase

Appendix 2.4. Differentially Expressed Genes, Continued. Genes in common but differentially expressed in high-fat diet (HFD) array (HFD-normoxia vs. regular diet-normoxia) and intermittent hypoxia (IH) array (HFD-IH vs. HFD-normoxia).

Affy ID	Fly Base ID	Gene Name	HFD fold change (HFD-N vs. RD-N)	IH fold change (HFD-IH vs. HFD-N)	Pathway/ Information
1639262_at	FBgn0033296	CG11669	-2.00	4.82	starch and sucrose metab, etc.
1631523_at	FBgn0038865	CG10824	-2.00	2.26	
1639323_at	FBgn0044812	TotC	-2.02	1.99	response to stress; response to bacteria
1635549_at	FBgn0028396	TotA	-2.02	2.25	
1633147_at	FBgn0032839	CG10659	-2.03	2.36	acetyl-transferase
1625583_at	FBgn0040732	CG16926	-2.21	3.29	
1630800_s_at	FBgn0054040	CG34040	-2.29	21.21	
1640488_a_at	FBgn0032076	CG9510	-2.30	2.41	argininosuccinate lyase
1626086_at	FBgn0032726	CG10621	-2.32	2.21	cysteine and methionine metab; methyltransferase activity
1623521_at	FBgn0039330	CG11909	-2.36	9.52	starch and sucrose metab, galactose metab; glocosidase
1626613_at	FBgn0031860	CG11236	-2.36	2.18	amino acid metab; oxidoreductase
1634477_at	FBgn0259237	CG31177	-2.44	2.56	ion binding
1625202_at			-2.77	5.72	
1625235_at	FBgn0033792	CG13325	-3.00	4.23	transferase activity
1636973_at	FBgn0038257	smp-30	-3.07	3.08	associated with cold response (literature)
1635450_a_at	FBgn0038257	smp-30	-3.53	3.76	associated with cold response (literature)
1641634_at	FBgn0002565	Lsp2	-66.67	2.81	pepsidase

CHAPTER 3:

Metabolic and Transcriptional Response to a High-Fat Diet in *Drosophila melanogaster*

Abstract

Considering the importance of overall metabolic homeostasis in the obesity response, it is important to consider the interaction of multiple metabolic pathways within an organism. In order to examine these fundamental changes, we previously developed a *Drosophila* model of diet-induced obesity using a high-fat diet (HFD), as described in Chapter 1 [64]. With this model, we performed mass spectrometry-based metabolomics to elucidate metabolic changes involved in the response to a HFD. We found that flies on a HFD have altered abundances of metabolites associated with fatty acid, amino acid, and carbohydrate metabolism. In previous microarray analysis and candidate gene screening (Chapter 2), we identified the homologue of human argininosuccinate lyase (ASL), *CG9510*, as having an important role in the HFD phenotype. When *CG9510* expression is knocked down in a wide variety of tissues in flies on a regular diet (RD), they show a phenotype similar to control flies on a HFD, with significantly increased triglyceride (TG) levels and decreased cold tolerance. Overexpression of *CG9510*, on the other hand, ameliorates some of the negative consequences of a HFD. Metabolomic analysis of flies with altered *CG9510* confirmed that this gene has a similar metabolic role as human ASL, regulating the balance of carbon and nitrogen metabolism. We have demonstrated that flies on a HFD down-regulate *CG9510* and manipulation of this gene in transgenic flies directly affects TG storage, stress tolerance and lifespan. This draws a clear and important link between regulation of amino acid metabolism and the response to diet-induced obesity.

Introduction

The negative impact of a high-fat diet on the human population is becoming increasingly evident, with over 60% of the US population obese or overweight [1] including a growing

percentage of children and adolescents [70]. The strain that obesity creates within the body contributes to a multitude of diseases, including type 2 diabetes, stroke, sleep apnea, and cardiovascular diseases [2]. With a severely increased risk of morbidity and mortality in obese adults, as well as a predisposition for adult-onset disease in obese children and adolescents [17,71-73], it is vital to better understand the underlying mechanistic changes that occur with the onset of obesity.

It has been hypothesized that metabolic homeostasis is deranged in disorders associated with obesity, such as insulin resistance and diabetes. It has been recognized that while many of the major metabolic regulatory pathways that are affected in these disorders, including AMPK, mTOR, and insulin, each have their specific function, they are all interconnected in a larger network to regulate organismal and cellular response [74]. In order to elucidate the mechanisms underlying the response to dietary changes, it is necessary to look beyond a single metabolic pathway and instead at the interplay between fat, sugar and amino acid metabolism. A clear example of this is seen in research into the mechanisms of obesity-related insulin resistance and type 2 diabetes, where the majority of studies have focused on glucose metabolism. Although this is an important pathway to examine given the nature of the complications, there is increasing evidence that there are other metabolic disturbance at play, including changes in fat and amino acid metabolism [75,76], which could contribute to the disorders.

Given the complexity of the whole-body response to dietary changes, model organisms can serve as useful tools to examine the interplay between genes, signaling pathways, and metabolism [6,77-79]. For example, these systems have taught us that regulatory pathways such as insulin/IGF signaling and TOR are highly conserved and exert significant control over metabolism, including lipid and glucose homeostasis [6,7,55,80-83]. These phenotypic changes resemble those seen in human obesity [17,70,84,85], strengthening the use of *Drosophila* as a model for studying obesity.

In Chapter 1, we developed a model to study the effects of a high-fat diet (HFD) using *Drosophila melanogaster*. We found that a HFD had a clearly deleterious phenotype in the fly,

characterized by increased triglyceride and glucose levels, decreased stress tolerance and decreased lifespan [64]. We then evaluated gene expression in our HFD *Drosophila* model using microarray analysis, and identified transcriptional changes in metabolic pathways and key candidate genes that influenced the HFD phenotype (Chapter 2).

In this chapter, we further examine the metabolic response to HFD in our Drosophila model, given that a) many of the metabolic and signaling pathways involved in fat metabolism and insulin signaling in humans are conserved in flies and b) *Drosophila* have many organ systems analogous to humans that control uptake, storage and metabolism [4]. To better understand the functional changes associated with the HFD, we performed mass spectrometry-based metabolomics. We then took advantage of the genetic tools available in fruit flies to further evaluate the role of *CG9510*, a candidate gene identified in Chapter 2, in the response to HFD. In this chapter, our aims were to: 1. Define the metabolic changes occurring when flies are on a high-fat diet; and 2. Identify and probe the role of the candidate gene *CG9510* in the phenotype. As a result of these aims, we present important metabolic pathways altered by HFD and hypothesize that key transcriptional changes are occurring in response to the disrupted metabolic homeostasis.

Methods

Fly Rearing and Collection.

All stocks were maintained on standard cornmeal *Drosophila* medium in an incubator at 25°C and 30-50% humidity. Adult flies were collected at 0-3 days and transferred to a separate vial of the standard cornmeal medium. After aging for 3 more days, male and female flies were separated and only the females were transferred to the regular (RD) or high-fat diet (HFD) and kept in room air.

Drosophila stocks.

All UAS-RNAi transgenic lines, as well as their controls w^{1118} and *y,w[1118];P{attP,y[+],w[3`]}*, were obtained from the VDRC [65]. The Gal4 driver stocks and P-element (w^{1118}*; PBac{PB}CG9510*^{c05706} *CG9515*^{c05706}) were obtained from Bloomington *Drosophila* Stock Center (Bloomington, IN, USA). The transgenic *P{CaryP}attP2-UAS9510* (UAS-*CG9510*) line was created in our lab. To create this line, *CG9510* cDNA was subcloned into the pUAST-attB vector from GM20637 (DGRC) using EcoRI and XbaI sites appended to the PCR product using the following primers: 5'-ATATAGAATTCATGAGCAACTTTTGTTTTAAAGATAAC-3' and 5'-ATATATCTAGACTACGACTGTTTGGCCAAATC-3'. Transgenic flies were generated by injecting embryos carrying P{CaryP}attP2 at 68A4 (Rainbow Transgenic Flies, Inc).

Experimental Diets: Regular food and Coconut food.

Regular (RD) and high-fat diet (HFD) were prepared as described in Chapter 1, using Jazz Mix *Drosophila* food from New Horizon Foods as the base food and 20% weight per volume coconut oil as a source for increased saturated fat in the diet [64].

Metabolite extraction and GC/MS analysis.

Metabolite extraction, derivatization and analysis are described in detail in Appendix 3.3. Briefly, female flies were kept on RD or HFD for one week, groups of 5 flies were placed in 2ml ceramic bead tubes (1.4mm, MoBio 13113-50) and weighed. Immediately -80ºC 9:1 methanol water mixture (MeOH, Cat# 34860, Sigma-Aldrich) was added and the tube was placed in a cooling bath of dry ice and isopropanol (approximately -78ºC) [86]. Flies were homogenized, transferred to an eppendorf tube and mixed with ice cold water. To separate polar and non-polar phases, ice-cold chloroform was added and the tube was vortexed and centrifuged at room temperature. For analysis of general free polar metabolites and uric acid, two aliquots of the aqueous phase were transferred to the gas chromatography/mass spectrometry (GC/MS) sampler tube for evaporation in a refrigerated vacuum centrifuge. For analysis of total fatty acids, the aliquot of the organic phase containing the non-polar fraction was collected in the eppendorf

tube and evaporated under airflow at room temperature. For derivatization of polar metabolites, dried polar metabolite aliquots were dissolved in 2% methoxyamine hydrochloride in pyridine and incubated at 37ºC. Subsequent conversion to their tert-butyldimethylsilyl (TBDMS) and trimethylsilyl (TMS) derivatives was accomplished by adding *N*-methyl-*N*-(tert-butyldimethylsilyl) trifluoroacetamide + 1% tert-butyldimethylchlorosilane or *N*-Methyl-*N*-(trimethylsilyl) trifluoroacetamide and incubating at 37ºC. Fatty acid methyl esters were generated by dissolving dried fatty acid aliquots in 2% (v/v) methanolic sulfuric acid and subsequently extracted in hexane with saturated NaCl.

Gas chromatography/mass spectrometry (GC/MS) analysis was performed using an Agilent 7890A with a 30m DB-35MS capillary column (Agilent Technologies) connected to an Agilent 5975C MS. For quantification of metabolites, selected ion fragments were integrated using a MATLAB-based in-house algorithm [87-89]. Additional ions are listed in Appendix 3.3. The relative quantification of total fly metabolites was determined by normalizing to the intensity of the added internal standards during extraction and measured fly body weights.

Quantitative Real-time PCR analysis.

Following one week on RD or HFD, flies were homogenized and total RNA was extracted using Trizol (Invitrogen, Carlsbad, CA), followed by purification with the RNeasy kit (Qiagen, Carlsbad, CA) as per manufacturer's instructions. cDNA was produced from total RNA through RT-PCR using Superscript III First-Strand Synthesis system (Invitrogen, Carlsbad, CA). Real-time PCR was performed using a GeneAmp 7500 sequence detection system using POWER SYBR Green chemistry (Applied Biosystems, Foster City, CA). Two primers used were used for *CG9510*: CG9510-A (fwd: GAAGATTCTACCCGGTGACG; rev: GACCACCTGGTCATTTCTGC); and CG9510-B (fwd: ATCCATCATGACCCAGATCC; rev: TAGAGGCGAGAATCGTAGGG) [90]. The expression level of actin was used as an internal control to normalize the results (fwd: CTAACCTCGCCCTCTCCTCT; rev: GCAGCCAAGTGTGAGTGTGT). The fold change was calculated relative to the expression level of the respective control (w^{1118} or the progeny of w^{1118}

crossed with the UAS-RNAi or UAS-*CG9510*) on RD.

Triglyceride and Glucose Measurements.

Triglyceride levels were determined as described in Chapter 1. Whole-body total glucose was determined as described in Birse 2010 [7]. Groups of five female flies were homogenized in 500µl 100mM PIPES buffer (Sigma P6757) with 1.25µl porcine kidney trehalase (Sigma T8778), allowing for measurement of total available glucose by hydrolyzing trehalose into glucose. The homogenates were incubated at 37ºC for 1 hour and the supernatants transferred to a 96-well plate in triplicate. Glucose levels were quantified using the Glucose GO assay kit (Sigma–Aldrich, Saint Louis, MO) according to the manufacturer's instructions. In brief, glucose is oxidized to gluconic acid and hydrogen peroxide by glucose oxidase. The hydrogen peroxide then reacts with o-dianisidine in the presence of peroxide to form a colored product, and the oxidized o-dianisidine reacts with sulfuric acid to form a more stable colored product. The intensity of the color is proportional to the original glucose concentration and was measured at 520nm using a BioTek Synergy HT microplate reader.

Capillary Feeding (CAFE) Assay.

Food consumption was measured in normoxia using adult female w^{1118} and *CG9510*-PBac flies, as described in Chapter 1.

Cold Stress Tolerance.

The cold tolerance assay was performed immediately following a week on RD or HFD, as described in Chapter 1.

Lifespan.

Female adult flies were placed on RD or HFD, with 10 flies per vial of food. Lifespan was recorded as described in Chapter 1.

Semi-intact *Drosophila* heart preparation and digital high-speed movie analysis.

All the dissection steps were done in artificial hemolymph containing 108 mM NaCl2, 5 mM KCl, 2 mM CaCl2, 8 mM MgCl2, 1 mM NaH2PO4, 4mM NaHCO3, 15 mM HEPES, 10 mM Sucrose, 5 mM trehalose, at pH 7.1 [91]. The flies were anesthetized with fly-nap, followed by transfer to a Petri dish coated with Vaseline for dissection [92]. After the dissection, the submerged hearts were oxygenated for 15 minutes at room temperature for equilibration. Next, the dish chamber is installed on a Leica DM-LFSA microscope with a 10x water immersion lens. The digital high-speed movies of the heartbeats are acquired using a Hamamatsu EM-CCD digital high-speed camera and HCI image capture software (Compix Imaging System). Movie analysis of the heart activity is then carried out according to previous reports [91,93].

Oxygen Consumption.

Oxygen consumption rates in normoxia were measured as previously described [94], with some modifications. Adult female flies were placed on either RD or HFD on Day 1 after eclosion and remained on their diet for 7 days. On the final day, flies were transferred to a 5ml vial in groups of 150 flies per oxygen consumption measurement. After 20 minutes of acclimation, the testing vial was sealed and oxygen levels were monitored over 35-60 minutes. Following measurements, flies were frozen and weighed.

Statistical Analysis.

Graphpad Prism was used for statistical analysis. A t-test was used to determine significance between RD and HFD results in most assays. In lifespan measurement, the significance was determined by comparing the survival curves with a log-rank (Mantel-Cox) test. A chi (χ) square test was used in analyzing heart dysfunction.

Results

Metabolomic analysis of flies on HFD

In Chapter 1, we demonstrated that adult female flies on a high-fat diet (HFD) have a deteriorated phenotype than their counterparts on a regular diet (RD), with increased triglyceride levels and decreased stress tolerance [64]. To further understand the changes in metabolism occurring in these flies, we used mass spectrometry to analyze the relative abundance of metabolites in wildtype flies on HFD compared to flies on RD. We found that the HFD significantly increases (1.2-2.7-fold of normal) total fatty acid abundance, including myristate, palmitate, oleate and stearate (Figure 3.1 A). Flies on a HFD had altered carbohydrate metabolism, as evidenced by dramatically increased lactate (20-fold of normal) and pyruvate (2.8-fold of normal) levels. Amino acid metabolism was also affected by HFD, with an increase in both urea and uric acid abundance and an overall decrease in many amino acids (Figure 3.1 B, C). Additionally, we also observed slight decreases in several tricarboxylic acid (TCA) metabolites, including fumarate, alpha-ketoglutarate, and malate, though these changes were not statistically significant. Little or no change was seen in citrate or succinate abundances (Figure 3.1 B). As seen in other HFD models [95-97], the overall metabolic rate of flies on HFD was significantly increased, with a 1.4-fold increase in oxygen consumption in flies on a HFD compared to RD (Figure 3.2).

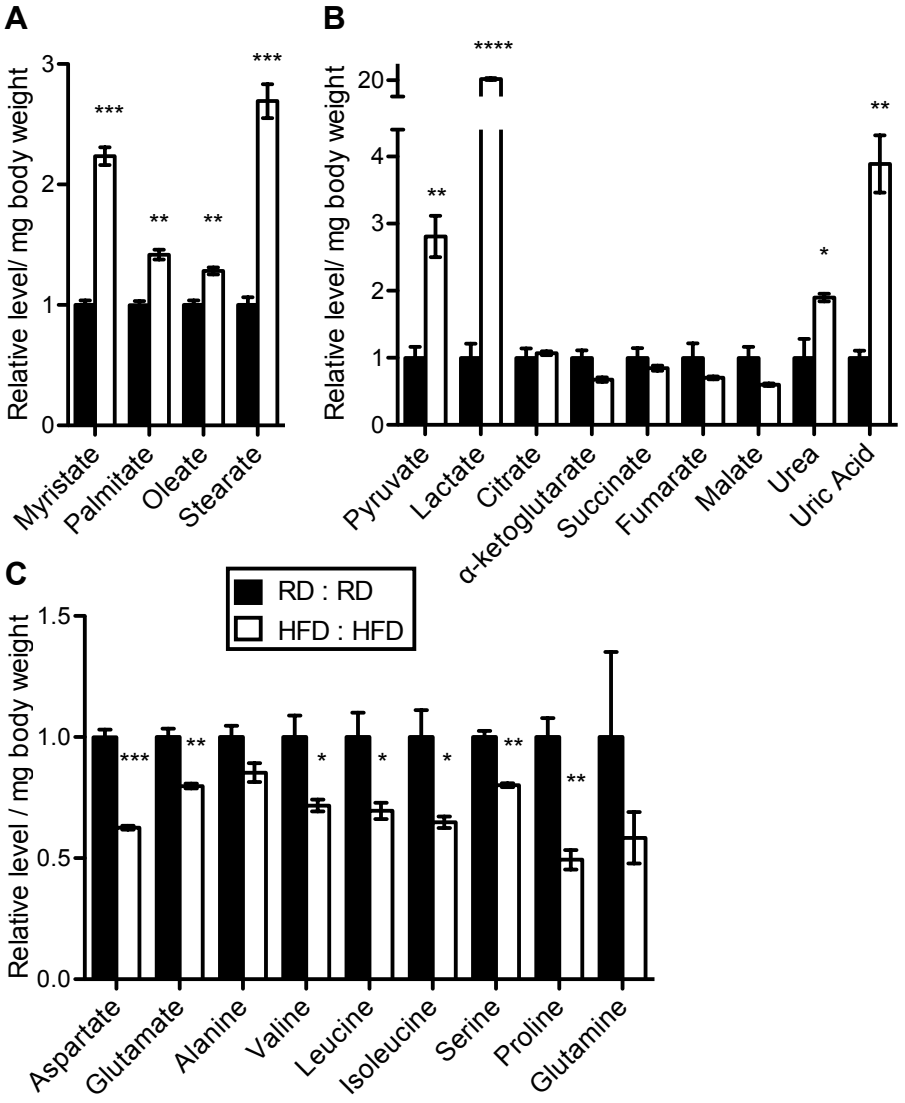


Figure 3.1. Increased fatty acid accumulation and altered carbohydrate and amino acid metabolism in *Drosophila* on a high-fat diet (HFD). Adult female w^{1118} flies were put on a regular diet (RD) or HFD for one week prior to metabolite extraction with whole-body homogenate and GC/MS analysis. Shown is the relative abundance of A) fatty acids, B) organic acids and C) amino acids in flies on RD and HFD, normalized to body weight and divided by the average metabolite abundance in the RD flies. For metabolites with multiple fragments available, consistent changes were observed in each and one fragment was selected to represent the metabolite. Three sets of five female flies were analyzed for each diet condition. Significance between relative abundance values of the HFD and RD groups for each metabolite was determined with a t-test, *= $p<0.05$ **=$p<0.01$ ***=$p<0.001$ ****=$p<0.0001$.

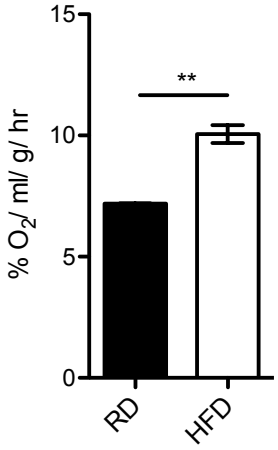


Figure 3.2. Increased oxygen consumption in *Drosophila* on a high-fat diet (HFD). Adult female w^{1118} flies were put on a regular diet (RD) or HFD for one week prior to assay. Measurements of 150 flies at a time were made for each group to measure the difference in oxygen levels in a sealed 5 ml vial. Three sets of flies for each group were measured. Oxygen consumption was determined by the total change in oxygen level was divided by the size of the vial, the weight of the 150 flies and the time length of the experiment. Significance between the two groups was determined with a t-test, **=p<0.01.

Role of *CG9510* in response to HFD

Given the results of the metabolomic analysis and the affected pathways uncovered by the microarrays in Chapter 2, we decided to pursue the relation between amino acid metabolism and HFD by further characterizing the function of *CG9510* in the HFD phenotype. *CG9510* was down-regulated by HFD in normoxia, and down-regulation of the gene in flies on RD led to a phenotype similar to that of wildtype flies on HFD (Chapter 2). We used real-time PCR to verify the down-regulation of *CG9510* by HFD seen in the microarray results as well as the decreased expression of *CG9510* in the P-element and RNAi lines used to evaluate the phenotype of *CG9510* (Appendix 3.2). Using the UAS-RNAi line for *CG9510*, we took advantage of tissue-specific Gal4 drivers to regulate expression in various parts of the *Drosophila* system, including several neuronal groups, glia, muscle, fat body, imaginal discs and malpighian tubules. In most tissues examined, down-regulating *CG9510* led to decreased cold tolerance and increased TG levels (7 of 9 drivers in cold assay, 8 of 9 in TG), with the strongest effect seen when down-regulated in dopaminergic and serotonergic neurons (Figure 3.3). This indicates that down-

regulation of this gene in many tissues seems to generate a likely systemic effect, which supports a critical role of *CG9510* in stress responses and metabolic homeostasis.

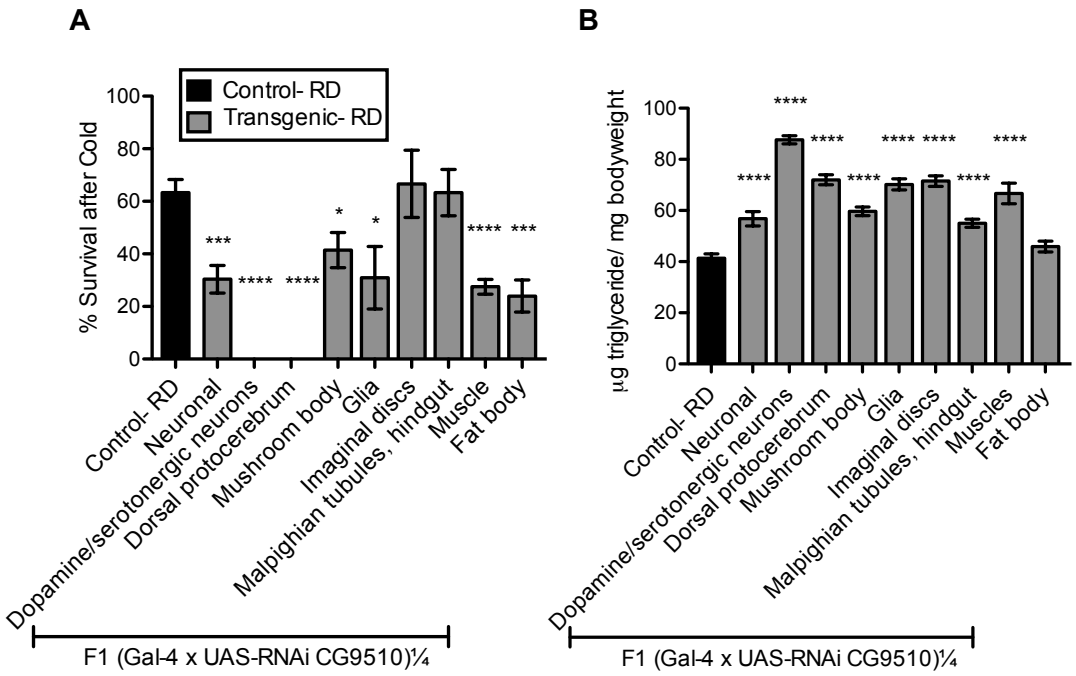


Figure 3.3. Down-regulating *CG9510* in various tissues simulates high-fat diet phenotype. Adult female progeny (d3-5) from crosses between the UAS-RNAi line (CG9510 #1) and various Gal4 drivers, as well as control progeny from a w^{1118} x *CG9510* UAS-RNAi cross were put on a regular diet (RD) for one week prior to the assay. A) Percent survival after cold stress (2 hours at -5º, with 24 hours recovery in room air) and B) triglyceride levels (per mg of bodyweight) from whole-body homogenate of flies with *CG9510* down-regulated in specific tissues were compared to control values. Tissues affected by Gal4 drivers included: neurons (elav-Gal4), dopaminergic and serotonergic neurons (Ddc-Gal4), neurons of the dorsal protocerebrum, mushroom body and fan-shaped body (c061-Gal4), mushroom body (Tab2-Gal4), glia (repo-Gal4), imaginal discs (c601-Gal4), malpighian tubules and hindgut (c591-Gal4), muscles (24B-Gal4) or fat body (lsp2-Gal4). Flies were tested in sets of 10 females per vial for cold tolerance, 5 females for TG levels: (A) n= 5-12 sets (50-120 flies per experimental group), (B) n= 6-10 sets (30-50 flies per experimental group). Significance between values for each experimental group and control was determined with a t-test, *= p<0.05 ***=p<0.001 ****=p<0.0001.

We verified that the increase in TG levels in flies with *CG9510* knockdown was unlikely due to a change in food consumption (Figure 3.4). Flies down-regulating *CG9510* deviate in some aspects from the HFD phenotype, as there is no change in total glucose levels in transgenic flies compared to wildtype on RD, unlike the increased glucose seen in wildtype on HFD (Figure 3.5).

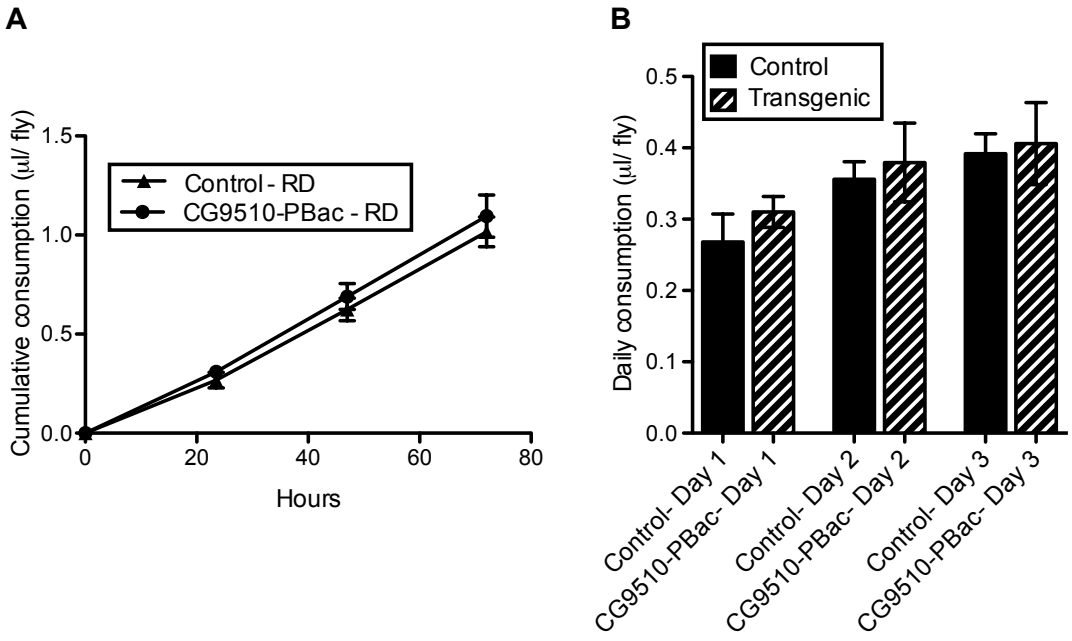


Figure 3.4. Similar food consumption observed in control flies and flies with down-regulation of *CG9510*. Adult female w^{1118} (Control) and *CG9510*-PBac flies (3–5 days old) were placed on a regular diet (RD) for 3 days. Following that time, flies were transferred to the CAFE vials. In the CAFE setup, flies had access to water but had to obtain their food out of a capillary tube through the top of the vial. They were allowed to adjust to the new environment for one day prior to recording data. Measurements of the change in liquid food level in the capillary tube allowed for determination of the total food consumed A) cumulatively over 3 days and B) on a daily basis. Flies were tested with 5 flies per vial, n = 8 vials (40 flies) per control or experimental group. Error bars show SEM.

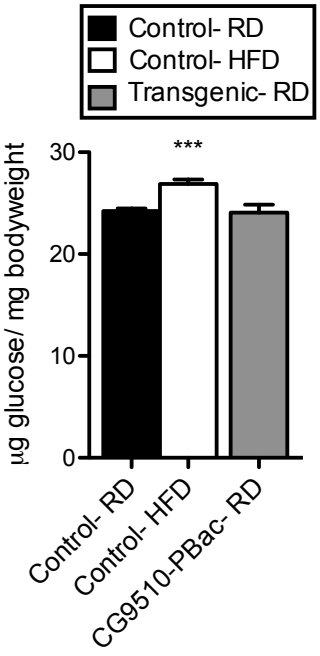


Figure 3.5. Total glucose levels unaffected in flies with down-regulation of *CG9510*. Adult female flies (d3-5) from a P-element line down-regulating *CG9510* (CG9510-PBac) were put on a regular diet (RD) and control female w^{1118} flies were put on RD or high-fat diet for one week prior measurement of trehalose and glucose levels in whole-body homogenate. Flies were tested in sets of 5 flies, n= 8 sets (40 flies per group). Significance between values for the experimental group and control was determined with a t-test, ***=p<0.001.

Having seen a shortened lifespan in flies on HFD compared to RD [64], we examined whether ubiquitous down-regulation of *CG9510* would also decrease lifespan compared to control flies on RD. Indeed, *CG9510* knockdown flies on RD, as well as wildtype flies on HFD, exhibited a shortened lifespan, indicating that reduced *CG9510* function possibly affects lifespan in a similar fashion as HFD, although not as severely (Figure 3.6 A). These results were corroborated with similar results using the second UAS-RNAi line for *CG9510* (Appendix 3.1).

HFD has been previously shown to lead to heart dysfunction in flies [7]. Therefore, we wondered whether down-regulation of *CG9510* was also associated with heart dysfunction, and the observed abnormalities were similar to those observed in wildtype flies exposed to the HFD regime employed in this study. One week of HFD feeding, as used in this paper (20% coconut oil-supplemented diet) of wildtype flies leads to somewhat constricted heart tube (Figure 3.6 B), which is similar but less pronounced than a previously study that employed a 30% coconut oil-

enriched protocol [7]. However, homozygous *CG9510-PBac* flies on RD also showed a more dramatically decreased diastolic diameter (Figure 3.6 B). Previously, it was shown that 30% HFD also induces in the fly numerous heart dysfunction phenotypes, including non-contractile heart regions, dysfunctional ostia, asynchronous beating pattern between anterior and posterior regions (akin to a 'two-to-one block' as in [7,98,99]). The 20% HFD regime used here also produces some of these heart phenotypes, but to a lesser extent (Figure 3.6 C). Interestingly, as for the diastolic diameter phenotype, *CG9510-PBac* homozygotes again show similar but more pronounced heart defects than flies on 20% HFD, again resembling heart dysfunction due to 30% HFD exposure [7]. These findings demonstrate that reduced function of *CG9510* causes systemic TG accumulation and associated heart dysfunction like wildtype flies on a HFD, thus implicating the *CG9510* gene in metabolic homeostasis and heart function.

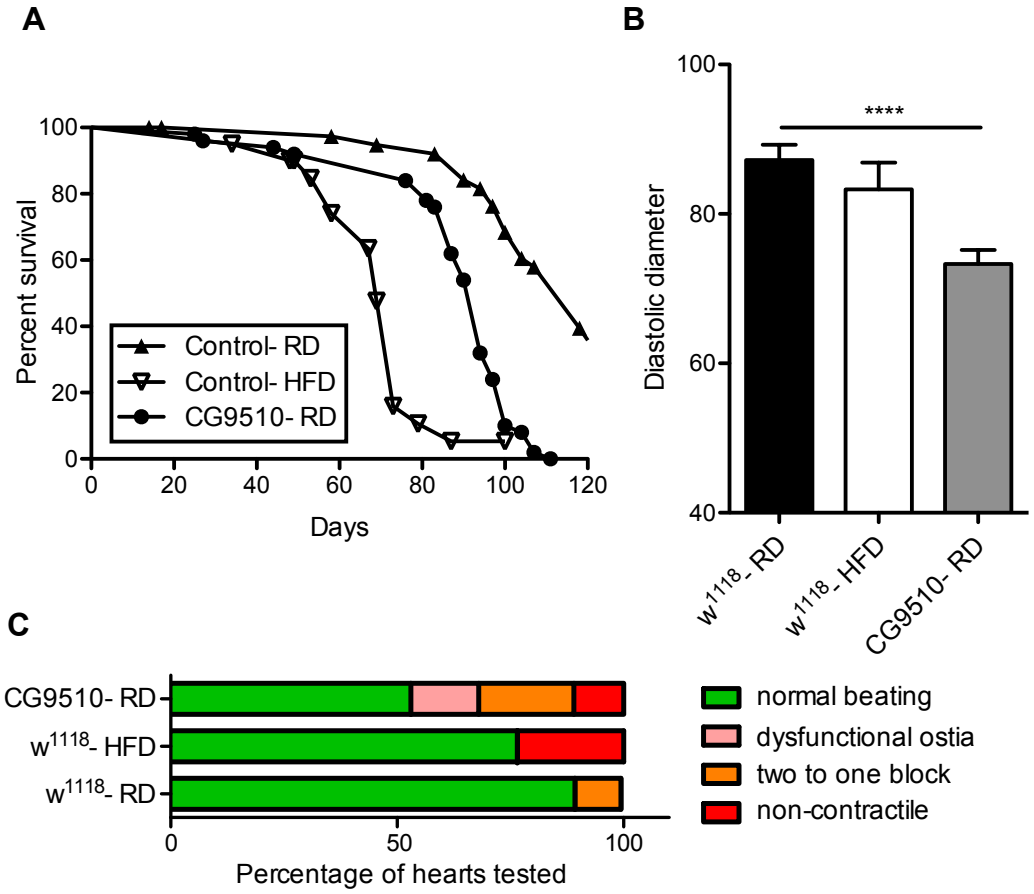


Figure 3.6. Altered lifespan and heart function with ubiquitous down-regulation of *CG9510*. A) Lifespan measurement of flies with down-regulated *CG9510* compared to controls. Adult female progeny (d3-5) with down-regulated *CG9510*, from the cross between UAS-RNAi line (CG9510 #1) and daughterless (da)-Gal4, were placed on a regular diet (RD). Control flies were adult female progeny (d3-5) of the cross between UAS-RNAi line (CG9510 #1) and w^{1118}, and were placed on either RD or high-fat diet (HFD). Flies with down-regulation of *CG9510* on a regular diet (RD) had a significantly shorter lifespan than control flies on RD ($p<0.0001$), and longer lifespan than control flies on HFD ($p<0.0001$) (n=40 per group). B) Diastolic heart diameter measurements when *CG9510* is down-regulated. Bar graph representation of changes in diastolic diameter of wildtype w^{1118} (w^{1118}-RD, n=33; w^{1118}-HFD, n=17) and homozygous *CG9510-PBac* (CG9510-RD, n=30) hearts. Note significant decrease in diastolic diameter of CG9510-RD hearts, compared to w^{1118}-RD (****=$p<0.0001$). C) Heart dysfunction analysis. Graph represents the percentage of hearts (n= same as Figure 3.6 B) displaying non-contractile regions, asynchronous beating pattern and dysfunctional ostia (inflow tracks). Note increased heart defects in w^{1118}-HFD and CG9510-RD compared to w^{1118}-RD. Statistical analysis was chi (χ) square test. w^{1118}-RD compared to CG9510 homozygote flies: $\chi^2 = 18.84$, $p<0.001$. w^{1118}-RD compared to w^{1118}-HFD: $\chi^2 = 1.42$, $p=0.1$. w^{1118}-HFD compared to CG9510-RD: $\chi^2 = 2.45$, $p=0.1$.

While relatively uncharacterized in *Drosophila*, *CG9510* has sequence homology with the human gene argininosuccinate lyase, or ASL, which is implicated in amino acid metabolism as part of the urea cycle. ASL lies at the junction of the urea and aspartate-argininosuccinate shunt

of the tricarboxylic acid (TCA) cycle, catalyzing the breakdown of argininosuccinate into arginine and fumarate. To verify the importance of this pathway in the HFD phenotype, we evaluated UAS-RNAi flies down-regulating *CG1315*, the homologue of human argininosuccinate synthase. Argininosuccinate synthase lies immediately upstream to ASL in the arginine biosynthesis pathways, and is responsible for catalyzing the synthesis of argininosuccinate from citrulline and aspartate (Figure 3.7 A). When *CG1315* was ubiquitously down-regulated, the flies had significantly decreased cold tolerance and increased TG levels compared to the control for the Gal4 (w^{1118} x da-Gal4) (Figure 3.7 B, C) and this was verified by comparing it to the control for the RNAi line (w^{1118} x *CG1315* UAS-RNAi) (data not shown). These results provide independent evidence that modulation of this pathway through a separate enzyme generates a similar phenotypic response/adaptation to HFD.

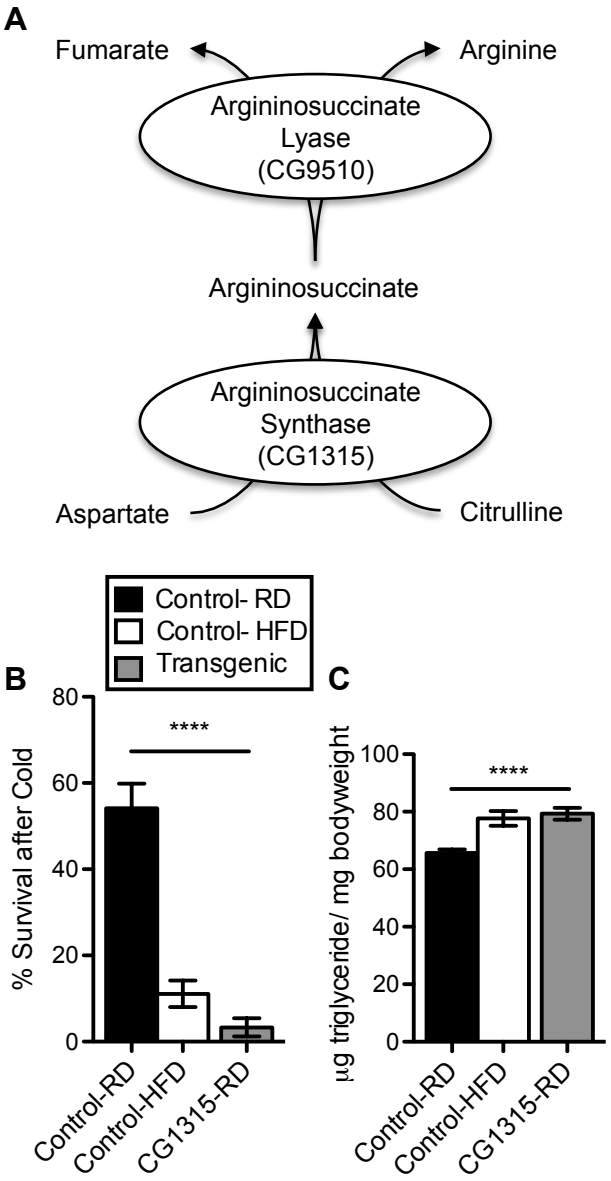


Figure 3.7. Down-regulating *CG1315* simulates high-fat diet phenotype. Evaluating the phenotype of *CG1315*, gene immediately upstream of *CG9510* in argininosuccinate metabolism as shown in (A). Adult female progeny (d3-5) from a *CG1315* UAS-RNAi x daughterless (da)-Gal4 cross were put on a regular diet (RD) and control progeny from a w^{1118} x da-Gal4 cross were put on RD and a high-fat diet (HFD) for one week prior to the assay. B) Percent survival after cold stress (2 hours at -5º, with 24 hours recovery in room air) and C) triglyceride levels (per mg of bodyweight) from whole-body homogenate of control flies and flies with *CG1315* down-regulated ubiquitously. Flies were tested in sets of 10 females per vial for cold tolerance, 5 females for TG levels: (B) n= 6-8 sets (60-80 flies per group), (C) n= 12-15 sets (60-75 flies per group). Significance between values for the experimental group and control was determined with a t-test, ****=p<0.0001.

Given that down-regulation of the ASL pathway via *CG9510* causes an increase in TG levels of RD flies, we asked whether up-regulation of *CG9510* could restore normal TG levels and

improve their stress tolerance. To address this question, we made a transgenic line with *CG9510* under UAS control. UAS-*CG9510*/da-Gal4 flies up-regulated *CG9510* expression nearly 10-fold compared to controls (Supporting Figure 2). Importantly, ubiquitous overexpression of UAS-*CG9510* under da-Gal4 control led to significantly increased cold tolerance (Figure 10A) and lower TG levels both on a RD and HFD compared to control RD flies (Figure 10B). These flies also had a longer lifespan when on RD than control flies (Figure 3.8 C).

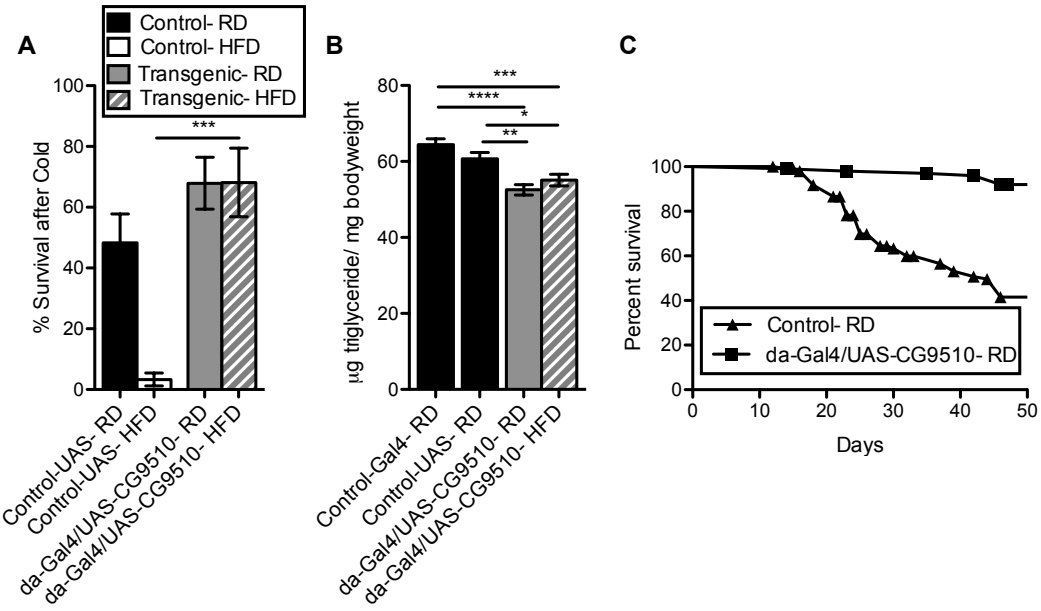


Figure 3.8. Up-regulation of *CG9510* decreases stored triglyceride levels, increases survival. A) Survival following cold stress was counted for flies with up-regulated *CG9510* (da-Gal4 x UAS-*CG9510*) and control flies (w^{1118} x UAS-*CG9510*) on regular (RD) and high-fat diet (HFD). Flies were tested in sets of 10 females, n= 6 sets (60 flies per group). B) Triglyceride (TG) levels (per mg of bodyweight) were determined in whole-body homogenate of flies with up-regulated *CG9510* expression compared to control. Adult female progeny (d3-5) from the Gal4-control cross (w^{1118} x da-Gal4) or UAS-control cross (w^{1118} x UAS-*CG9510*) were placed on RD and progeny from the experimental cross (UAS-*CG9510* x da-Gal4) were placed either RD or HFD for one week prior to the assay. Flies were tested in sets 5 females for TG levels, n= 15-20 sets (75-100 flies per group). C) Lifespan of control flies (w^{1118} x UAS-CG9510) and up-regulated *CG9510* (da-Gal4 x UAS-*CG9510*) on RD. 10 flies were kept in each vial, n= 10 vials (100 flies per group). Significance between values for the experimental group and control in A and B was determined with a t-test, *= p<0.05 **=p<0.01 ***=p<0.01****=p<0.0001.

CG9510 in *Drosophila* metabolism

In mammals, ASL functions at a key intersection between carbon and nitrogen metabolism. We used mass spectrometry to characterize the role of *CG9510* in *Drosophila* metabolism. Down-regulating *CG9510* led to an overall increase in fatty acid abundance even though the flies ate a RD (Figure 3.9 A), with no change in the glycolytic metabolites pyruvate and lactate (Figure 3.9 B). This supports the whole-body quantitative analysis of TG and glucose levels, which showed that down-regulating *CG9510* increases TG levels (Figure 3.3 B) but does not affect glucose levels (Figure 3.5). Flies with down-regulated *CG9510* showed a drastic decrease in the whole-body relative abundance of urea, alpha-ketoglutarate and citrate, while increasing aspartate (Figure 3.9 B, C) and leaving the pool of free amino acids unchanged (Figure 3.9 C). Importantly, uric acid levels were significantly increased, consistent with the observed deficiency in urea production and need for an alternate route for nitrogen disposal (Figure 3.9 B). Given the observed alterations in nitrogen metabolism and citric acid cycle intermediates, these data confirm that *CG9510* has a similar role to human ASL in its effect on the balance of carbon and nitrogen metabolism.

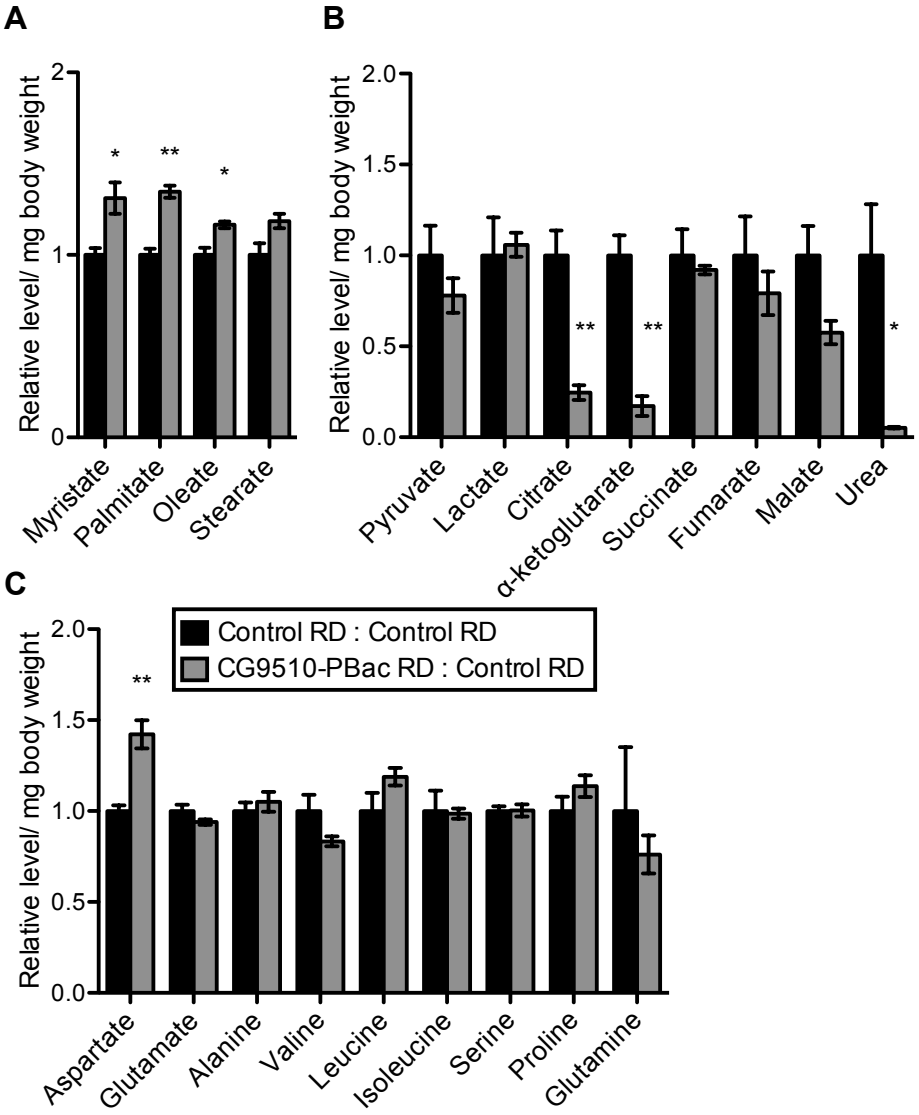


Figure 3.9. Increased fatty acid accumulation and altered urea and TCA metabolite abundance in *Drosophila* with down-regulation of *CG9510*. Adult female flies (d3-5) from a P-element line down-regulating *CG9510* (CG9510-PBac) and control female w^{1118} flies were put on a regular diet (RD) for one week prior to metabolite extraction with whole-body homogenate and GC/MS analysis. Shown is the relative abundance of A) fatty acids, B) organic acids and C) amino acids in CG9510-PBac and control flies on RD, normalized to body weight and divided by the average metabolite abundance in the control flies. For metabolites with multiple fragments available, consistent changes were observed in each and one fragment was selected to represent the metabolite. Three sets of five female flies were analyzed for each diet condition. Significance between relative abundance values of the CG9510-PBac and control group was determined with a t-test, *= $p<0.05$ **=$p<0.01$.

In summary, we found that the effects of a HFD on *Drosophila* extend beyond lipid metabolism, perturbing the balance of amino acid and carbohydrate metabolism. In metabolomic studies, flies on a HFD have increased fatty acid, glycolytic metabolites and urea abundance, and

decreased free amino acid levels. Our microarray analysis allowed us to identify a critical gene, *CG9510*, associated with amino acid metabolism that is involved in response to HFD. Down-regulating *CG9510* altered the phenotype in such a way to increase TG levels, decrease cold survival and lifespan, as well as induce heart dysfunction, even when on a RD diet. Further characterization of *CG9510* revealed that it affects carbon and nitrogen metabolism, similar to its human homologue ASL. These results demonstrate that flies on a HFD have an imbalance in amino acid and carbohydrate metabolism and a HFD causes transcriptional changes in a key enzyme at the junction of these two metabolic pathways.

Discussion

In this chapter, we have identified a strong association between amino acid metabolism and HFD by uncovering key metabolic changes in flies consuming a HFD and a transcriptional response to these changes. Flies on a HFD exhibited significant alterations in the abundance of compounds involved in fatty acid, carbohydrate and amino acid metabolism. We had previously shown that HFD modulates gene expression in several metabolic pathways, including the candidate gene *CG9510* (Chapter 2). Knockdown of one of the genes with reduced transcription in flies on HFD, *CG9510*, phenocopied the effects of HFD, indicating that this gene is a key regulator of HFD. Transgenic flies with decreased expression of *CG9510* had significant changes in triglyceride (TG) levels and cold tolerance even on RD, similar to control flies on a HFD. *CG9510* is a homologue to the human argininosuccinate lyase (ASL) and, like ASL, affects both carbon and nitrogen metabolism. The observations we made in this work lead us to ask two key questions: 1. What are the metabolic events that occur during HFD that cause such dramatic changes in amino acid and carbohydrate metabolite abundance; and 2. What effect does the modulation of *CG9510* have during HFD?

Metabolic imbalance in flies on HFD

The link between an imbalance in metabolism and HFD has been previously demonstrated in literature. HFD is known to influence glucose metabolism, including increased levels of lactate in obese human subjects [76,100], and increased pyruvate and lactate in mice on a HFD [101]. Mice on a HFD show elevated TCA cycle flux [102], as do cows with high liver fat content [103]. Recent studies have consistently revealed alteration of normal amino acid metabolism in obese, insulin resistant states and type 2 diabetes [75]. Additionally, a HFD has been shown to diminish skeletal muscle growth in mice [104], similar to decreased protein levels seen in flies on HFD in our studies (Appendix 1.1). Our work expands on this by demonstrating for the first time a link between HFD and metabolic imbalance in a *Drosophila* model. Further analysis allowed us to link an amino acid metabolic regulator to this imbalance due to HFD.

Our targeted metabolomic analysis of flies fed a control diet (RD) or a HFD identified changes in compounds involved in carbohydrate, fatty acid, and amino acid metabolism (indicated by green arrows in Figure 3.10). The increased pyruvate and lactate observed in flies consuming a HFD provide evidence that fatty acid oxidation may occur at the expense of pyruvate metabolism by the pyruvate dehydrogenase complex in mitochondria. Both processes serve as important sources of acetyl-coenzyme A (AcCoA) to fuel the TCA cycle, and the increased availability of triglycerides and fatty acids may displace the use of pyruvate as a substrate for oxidative mitochondrial metabolism. In turn, pyruvate accumulates and is subsequently diverted to generate large amounts of lactate (Figure 3.10, green arrows). Meanwhile, we observed significant decreases in the abundance of nearly all amino acids detected in our analysis. Along with the increases in urea and uric acid, this demonstrates a tangible change in nitrogen/amino acid metabolism. Such an effect can be explained by the need for anaplerotic substrates in the TCA cycle (indicated by purple arrows in Figure 3.10).

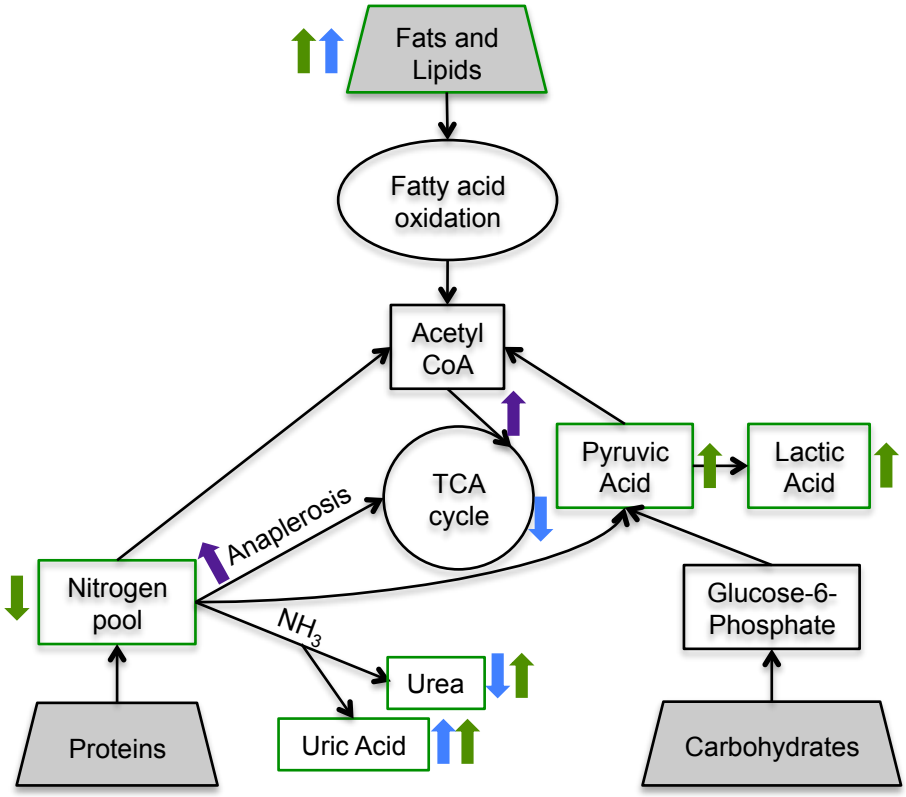


Figure 3.10. Model of *Drosophila* response to a high-fat diet. Green arrows indicate changes in metabolite abundance in flies on a HFD, purple arrows are hypothesized effects on a HFD, and blue arrows show metabolic effect of down-regulating a gene altered by HFD, *CG9510*. Flies show increased fatty acids, as well as a significant increase in pyruvate and lactate, decreased free amino acids, and increased urea levels on a HFD. This indicates that an increased abundance of acetyl co-a is entering the TCA cycle, increasing the need for anaplerosis in order to replenish TCA intermediates. By down-regulating expression of *CG9510*, flies still have increased fatty acids, but are also decreasing TCA intermediates and urea levels.

In order for AcCoA to enter the TCA cycle oxaloacetate is required to act as a co-substrate for citrate synthesis. Unlike glucose and amino acids, lipids cannot enter the TCA cycle via anaplerotic pathways and generate AcCoA via β-oxidation. In the context of HFD, glucose may be primarily metabolized to lactate. Therefore, increased amino acid catabolism can serve as a more significant source of anaplerotic substrates to sustain TCA cycling when HFD is provided. In turn, amino acids that are catabolized in the TCA cycle become more depleted under these conditions. Importantly, the observed increase in oxygen consumption indicates that oxidative phosphorylation and flux through the TCA cycle are maintained or elevated on a HFD, despite the fact that glycolytic metabolism increases. This provides independent support for the

notion that amino acid oxidation is increased in flies on a HFD to sustain anaplerosis in mitochondria.

Metabolomic profiling in human subjects has also showed a change in amino acid metabolism, specifically branch chain amino acid (BCAA) catabolism, in obese compared to lean subjects [76]. While we observed a decrease in BCAA abundance in the *Drosophila* model, human studies have shown an overall increase in plasma BCAA levels. This is likely due to the fact that human subjects are eating a typical American diet with high levels of both fat and protein. Dietary protein has >20% BCAA, and given that dietary BCAAs reach the blood at nearly the same levels as in the diet [105], this would lead to a large increase in circulating BCAA levels. In our study, on the other hand, *Drosophila* consume a HFD with an increase only in saturated fat levels. Furthermore, as amino acids are broken down, nitrogen is released which must be converted to urea or uric acid. Indeed, we observed significantly elevated levels of both in flies on HFD.

Decreased expression of *CG9510* in flies on HFD

The impact of HFD on metabolic pathways was seen at a transcriptional level with its decreased expression of *CG9510* and the ability to impact TG levels and cold tolerance with independent manipulation of this gene (Figure 3.3). *CG9510* has sequence homology to human ASL, an enzyme at the intersection between carbon and nitrogen metabolism, catalyzing the breakdown of argininosuccinate into arginine and fumarate. Interestingly, mammals on HFD or with high liver fat also present decreased levels of urea cycle enzymes [103], including decreased ASL [101] and we confirmed this in HFD mouse liver samples using real time PCR analysis for *CG9510* expression (unpublished preliminary data using samples from Dr. Jerrold Olefsky [106]).

Metabolomic analysis confirmed the function of *CG9510* in flies as similar to ASL. As would be expected given the complexity of an entire organism responding to dietary change, not all changes seen in the HFD were manifested in the *CG9510* transgenic flies. Transgenic flies with decreased expression of *CG9510* had increased fatty acid abundance, similar to those on

HFD, even though the transgenic flies were on RD. It is possible that the transgenic *CG9510* flies are unable to oxidize fatty acids as efficiently, which also would explain the increased TG levels seen in these flies. The fact that the relative abundance of aspartate is elevated, while urea and citrate are decreased, establishes that *CG9510*, similar to ASL, plays a role in maintaining the balance of carbon and nitrogen metabolism. In support of this role, the transgenic flies had increased uric acid levels, as the dramatic decrease in urea production would have required them to rely instead on the uric acid pathway for disposing of nitrogen. This demonstrates that by modulating *CG9510* during HFD, flies attempt to compensate for specific components of the metabolic imbalance caused by the diet (indicated by blue arrows in Figure 3.10). In doing so, however, the end result may be detrimental to the overall phenotype given the increased TG and decreased stress tolerance and lifespan that accompany down-regulation of *CG9510* in transgenic flies. Although more detailed studies are required to display changes in pathway fluxes proposed in this model, our metabolomic, transcriptional, and phenotypic results provide a compelling link between HFD and an imbalance in carbohydrate, lipid, and amino acid metabolism.

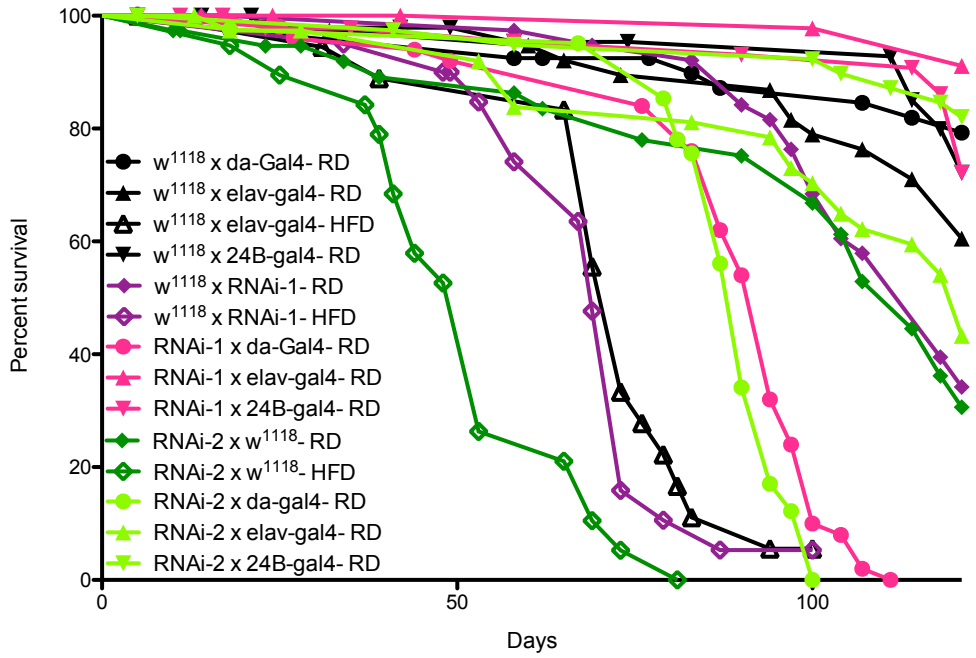
HFD dramatically alters the balance of metabolism in *Drosophila*. With an increased availability of fatty acids from the diet, flies seem to shift away from pyruvate as a source for AcCoA, leaving pyruvate to be converted into lactate. We see a decrease in amino acid abundance and an increase in urea levels in HFD flies, likely due to an increased demand for TCA cycle intermediates through anaplerosis in order for the increased supply of AcCoA to enter the cycle. The influence of HFD on nitrogen metabolism is seen through transcriptional changes as well. During HFD, flies alter expression of the homologue for human ASL, *CG9510*, potentially to compensate for the imbalance in nitrogen metabolism. The importance of regulating this gene is also seen in respect to TG storage, stress tolerance and lifespan, as down-regulating *CG9510* in transgenic flies phenocopies the effect of HFD, while overexpression of the gene ameliorates some of the negative consequences of HFD. The role of this gene in the balance of carbon and

nitrogen metabolism further strengthens the link between HFD and the regulation of metabolic balance.

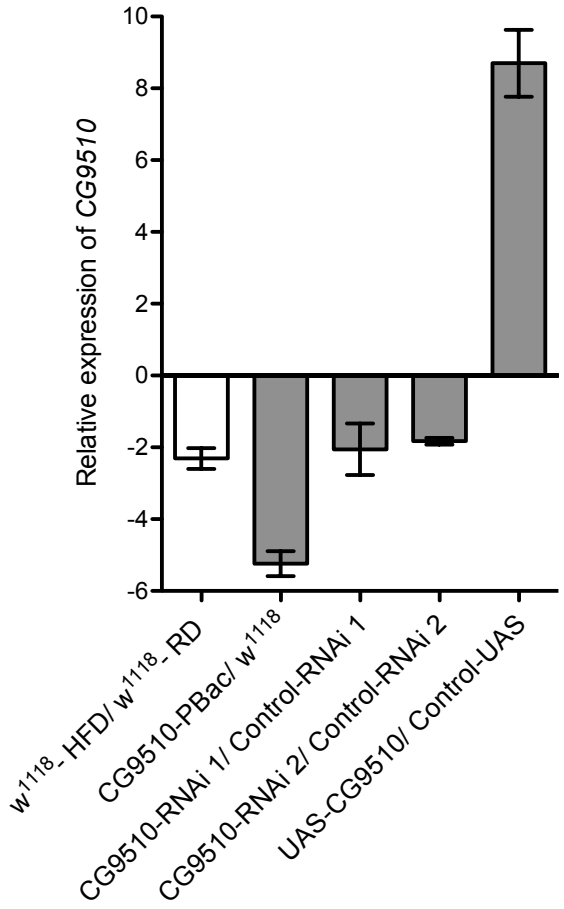
We have shown that *Drosophila* on HFD have a severely worsened phenotype (Chapter 1), altered transcription of genes involved in amino acid metabolism (Chapter 2), and disrupted metabolic homeostasis (Chapter 3). As a part of the response to HFD, flies on HFD alter *CG9510* as a key enzyme at the junction of amino acid and carbohydrate metabolism. Importantly, manipulation of this single gene directly affects triglyceride storage, cold tolerance and lifespan. As a result of these studies, we have identified a novel link between amino acid metabolism and obesity.

Acknowledgements

Chapter 3, in part, has been submitted for publication. The dissertation author was the primary investigator and author of this material. John Ngo assisted with triglyceride assays and real-time PCR experiments. Rolf Bodmer and Soda Diop performed heart function imaging and analysis. William Joiner and James Robinson assisted with the design and creation of the transgenic fly line. Christian Metallo and Hui Zhang provided advice and performed metabolic extraction and mass-spectrometry analysis. Gabriel Haddad supervised the project and provided editing and direction.



Appendix 3.1. **Lifespan of flies with down-regulation of *CG9510* in normoxia.** (This graph includes the data from Figure 3.6) Two RNAi stocks (RNAi-1 and RNAi-2) for *CG9510* down-regulation were tested with multiple gal4 drivers (da= ubiquitous, elav= neurons, 24B= skeletal muscle). Controls included w^{1118} crossed with each gal4 driver and each RNAi line. Adult female progeny (d3-5, n=40 per group) of the experimental groups were placed on a regular diet (RD). The progeny of the control crosses were either placed on RD or high-fat diet (HFD). Flies with down-regulation of *CG9510* on a regular diet (RD) had a significantly shorter lifespan than control flies on RD ($p<0.0001$), and longer lifespan than control flies on HFD ($p<0.0001$).



Appendix 3.2. Verification of *CG9510* expression in high-fat diet and transgenic lines. Expression of *CG9510* was measured using real-time PCR analysis. Adult female flies (d3-5) were placed on regular (RD) or high-fat diet (HFD) for one week prior to RNA extraction and cDNA synthesis. The real-time PCR values were normalized with actin, and fold-change was calculated in comparison to relative mRNA levels in control flies. Relative expression of *CG9510* in w^{1118} flies on HFD compared to w^{1118} on RD (-2.3-fold change) was evaluated to verify microarray results of *CG9510* down-regulation in HFD flies (w^{1118}-HFD/ w^{1118}-RD). Down-regulation of *CG9510* in the transgenic P-element and RNAi lines was verified by comparing relative expression of *CG9510* in the following three transgenic flies on RD: *CG9510-PBac* to w^{1118} (CG9510-PBac/ w^{1118}, -5.2-fold change); progeny of *CG9510*-RNAi #1 x daughterless (da)-Gal4 to progeny of *CG9510*-RNAi #1 x w^{1118} (CG9510-RNAi 1/ Control-RNAi 1, -2.1-fold change); progeny of *CG9510*-RNAi #2 x da-Gal4 to progeny of *CG9510*-RNAi #2 x w^{1118} (CG9510-RNAi 2/ Control-RNAi 2, -1.8-fold change). Up-regulation of *CG9510* in the injected transgenic UAS-*CG9510* fly line was verified by comparing relative expression of *CG9510* in the progeny of UAS-*CG9510* x da-Gal4 to progeny of UAS-*CG9510* x w^{1118} (UAS-CG9510/ Control-UAS, 8.7-fold change).

Appendix 3.3. Supplemental Methods.

Metabolite extraction and GC/MS analysis.

After female flies were kept on RD or HFD for one week, groups of 5 flies were placed in 2ml ceramic bead tubes (1.4mm, MoBio 13113-50) and weighed. Immediately 0.4 ml of -80ºC 9:1 methanol water mixture (MeOH, Cat# 34860, Sigma-Aldrich) was added and the tube was placed in a cooling bath of dry ice and isopropanol (approximately -78ºC) [86]. Flies were homogenized using the Precelly's 24 homogenizer and the homogenate removed to an eppendorf tube on ice. The addition of 0.1 ml 9:1 methanol water mixture was added to the ceramic bead tube to rinse any remaining homogenate off the beads and transferred to the eppendorf tube for a total volume of 0.5 ml (0.45 ml of MeOH and 0.05 ml of water). The addition of 0.25 ml ice-cold water was added to the eppendorf tube. To separate polar and non-polar phases, 0.45 ml of ice-cold chloroform (CHCl3, Cat# 366927, Sigma-Aldrich) was added and the eppendorf tube was vortexed at 4ºC for 30 minutes. The extract was further centrifuged at 14,000g at room temperature for 10 minutes. For analysis of general free polar metabolites and uric acid, two 0.1 ml aliquots of the aqueous phase were transferred to the GC/MS sampler tube for evaporation in a refrigerated vacuum centrifuge (Labconco CentriVap Concentrator). For analysis of total fatty acids, the 0.1 ml aliquot of the organic phase containing the non-polar fraction was collected in the eppendorf tube and evaporated under airflow at room temperature.

Derivatization of polar metabolites was performed using a Gerstel MultiPurpose Sampler (MPS 2XL). Dried polar metabolite aliquots were dissolved in 7.5 µl of 2% methoxyamine hydrochloride (MOX, Cat# 155405, MP Biomedicals) in pyridine (Cat# 270407, Sigma-Aldrich) and held at 37ºC for 30 minutes. Subsequent conversion to their tert-butyldimethylsilyl (TBDMS) and trimethylsilyl (TMS) derivatives was accomplished by adding 15 µl *N*-methyl-*N*-(tert-butyldimethylsilyl) trifluoroacetamide (MBTSTFA) + 1% tert-butyldimethylchlorosilane (TBDMCS; Cat# 270143, Regis Technologies) or 15 µl *N*-Methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA; Cat# 270590, Regis Technologies) and incubating at 37ºC for 60 minutes. Fatty acid methyl esters (FAMEs) were generated by dissolving dried fatty acid aliquots in 0.5 ml 2% (v/v) methanolic sulfuric acid (H_2SO_4, Cat# 320501, Sigma-Aldrich) and incubating at 50 ºC for 2 hours. FAMEs were subsequently extracted in 1 ml hexane with 0.1 ml saturated NaCl.

Gas chromatography/mass spectrometry (GC/MS) analysis was performed using an Agilent 7890A with a 30m DB-35MS capillary column (Agilent Technologies) connected to an Agilent 5975C MS. GC/MS was operated under electron impact (EI) ionization at 70 eV. One µl sample was injected in splitless mode at 270ºC, using helium as the carrier gas at a flow rate of 1 ml/min. For analysis of organic and amino acid derivatives, the GC oven temperature was held at 100ºC for 2 minutes, increased to 255ºC at 3.5ºC/min, then ramped to 320ºC at 15ºC/min for a total run time of approximately 50 minutes. For measurement of FAMEs, the GC oven temperature was held at 100ºC for 3 minutes, then to 205ºC at 25ºC/min, further increased to 230ºC at 5ºC/min and ramped up to 300ºC at 25ºC/min for a total run time of approximately 15 minutes. The MS source and quadrupole were held at 230ºC and 150ºC, respectively, and the detector was operated in scanning mode, recording ion abundance in the range of 100 – 650 m/z.

For quantification of metabolites, selected ion fragments were integrated using a MATLAB-based in-house algorithm [87-89]. Additional ions integrated include: urea, 231 m/z; uric acid, 441 m/z and 456 m/z; myristate, 242 m/z; palmitate, 270 m/z; oleate, 264 m/z and 296 m/z; and stearate, 298 m/z. The relative quantification of total fly metabolites was determined by normalizing to the intensity of the added internal standards during extraction and measured fly body weights. Norvaline 1µg (Cat# N7502, Sigma-Aldrich), ribitol 1µg (adonitol, Cat# A5502, Sigma-Aldrich) and glyceryl triheptadecanoate 1µg (Cat# T2151, Sigma-Aldrich) were added to each sample during extraction as standards for TBDMS derivatives, TMS derivatives and FAMEs, respectively.

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