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Selection for Increased Starvation Resistance Using Drosophila melanogaster: Investigating Physiological and Life History Trait Responses to Starvation and Dietary Supplementation in the Context of an Obese Phenotype

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Selection for Increased Starvation Resistance Using *Drosophila melanogaster*: Investigating Physiological and Life History Trait Responses to Starvation and Dietary Supplementation in the Context of an Obese Phenotype

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Selection for Increased Starvation Resistance Using *Drosophila melanogaster*: Investigating Physiological and Life History Trait Responses to Starvation and Dietary Supplementation in the Context of an Obese Phenotype

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Artificial selection for starvation resistance provided insight into the relationships between evolved physiological and life history trait responses following exposure to biologically induced stress. Investigations of alterations to body composition, metabolic rate, movement, and life history traits including development time, female egg production, and longevity in response to brief periods of starvation were conducted on genetically based starvation-resistant and control lines of *Drosophila melanogaster*. Analysis of the starvation-resistant lines indicated increased energy storage with increased triglyceride deposition and conversion of carbohydrates to lipid, as identified by respiratory quotient values. Correlations between reductions in metabolic rates and movement in the starvation-resistant lines, suggested the presence of an evolved physiological response resulting in energy conservation. Investigations of life history traits in the starvation-resistant lines indicated no significant differences in development time or reproduction between the selected and control lines. Measurements of longevity,

however, indicated a significant reduction in starvation-resistant *D. melanogaster* lifespan. These results suggested that elevated lipid concentrations, similar to that observed with obesity, were correlated with premature mortality. Exposure of the starvation-resistant and control lines to diets supplemented with glucose, palmitic acid, and a 2:1 mixture of casein to albumin were used to investigate alterations in body composition, movement, and life history traits. Results obtained from this study indicated that increased sugar in the diet led to increased carbohydrate, glycogen, total sugar, trehalose, and triglyceride concentrations, while increased fat and protein in the diet resulted in increased soluble protein, carbohydrate, glycogen, total sugar, and trehalose concentrations. Examination of life history trait responses indicated reduced fecundity in females exposed to increased glucose concentrations. Increased supplementations of palmitic acid was consistently correlated with an overall reduction in lifespan in both the starvation-resistant and control *Drosophila* lines, while measurements of movement indicated increased female activity levels in flies exposed to diets supplemented with fat and protein. Analyses of the physiological and life history trait responses to starvation and dietary supplementation on *Drosophila melanogaster* used in the present study has implications for investigating the mechanisms underlying the development and persistence of human obesity and associated metabolic disorders.

Dedication

I dedicate my dissertation to my husband Brandon, my parents Richard and Diane, and my sister Melinda. It was through your love, encouragement, and support that I was able to complete my doctoral degree and continue on to attend medical school. It was your belief in me that motivated me to succeed and never settle for anything other than my absolute best. I am forever grateful to each of you.

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I am eternally grateful to all my family, friends, and colleagues that have provided support and guidance throughout my academic career. It was your continual encouragement that allowed me to complete my doctoral degree and continue to pursue my academic goals in medicine and medical research.

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you. You stood by me through the long late nights, dealt with all of my tears and doubts in myself, and have been my sounding board when I felt no one else would listen. Thank you for always helping with my project. If you had not come to help me with my research, I would never have been able to leave Manter Hall. Thank you for always believing in me and encouraging me. I could not have come this far without you.

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To the members of the Harshman lab both past and present, thank you for all of your guidance, support, and friendship. You have made this place feel like home. I especially want to thank all of the technicians and undergraduates who have worked with me over the years. It is your hard work and dedication that have made this project a success. Thank you for your commitment and enthusiasm and for making those long hours of counting flies and collecting eggs an enjoyable experience. I will never forget the conversations and the fun we had. I also want to thank Dr. Adrianne Prokupek-Pickett for all of her insight and making time spent in the office something to look forward to. If it were not for your help I am not sure I would ever completely understand RNAi or know

how to condense 300 pages into a 45 minute presentation. I have enjoyed our conversations, and I am grateful for your friendship. It is my hope that we can always stay in touch and continue to share the closeness we have established over the past few years.

To my friends, thank you for sticking with me throughout this whole experience. As I look back on our friendships, I am grateful for the impact each of you has had on my life. You have truly helped me become a better person. Thank you for filling my life with surprises, laughter, and love.

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Table of Contents

CHAPTER 4. The effects of dietary alterations on body composition, life history traits, and movement using genetically altered starvation-resistant and control lines of *Drosophila melanogaster*

List of Multimedia Objects

Chapter 1. Introduction

Chapter 2. Investigations of physiological, metabolic, and life history trait responses to starvation in genetic-based starvation-resistant and control lines of *Drosophila melanogaster*

Chapter 3. Investigating respiration and movement in genetically based starvation-resistant and control lines of *Drosophila melanogaster*

Chapter 4. The effects of dietary alterations on body composition, life history traits, and movement using genetically altered starvation-resistant and control lines of *Drosophila melanogaster*

List of Abbreviations

ml	milliliters
mg	milligram
µg	microgram
mm	millimeter
nm	nanometers
µl	microliters
mM	milimolar
µg/ml	microgram per milliliter
rpm	revolutions per minute
°C	degrees Celsius
M	molar
N	normal
HPLC	high performance liquid chromatography grade water
HCL	hydrochloric acid
KCL	potassium chloride
NaOH	sodium hydroxide
BSA	bovine serum albumin
BCA	bicinchoninic acid
CoA	coenzyme A
ATP	adenosine triphosphate
rRNA	ribosomal ribonucleic acid

DNA	deoxyribonucleic acid
RNA	ribonucleic acid
D. melanogaster	Drosophila melanogaster
DILP	*Drosophila* insulin-like peptides
InR	insulin receptor
Carbs	carbohydrates
AKH	adipokinetic hormone (insect glucagon)
AKHR	adipokinetic hormone receptor
TAG	triglycerides
TOR	target of rapamycin
PI3K	phosphoinositide-3-kinase
AKT	protein kinase B in *Drosophila*
dFOXO	*Drosophila* forkhead transcription factor
S6K	S6 kinase
eIF4G	eukaryotic initiation factor 4 G
4E-BP	eukaryotic translation initiation factor 4E binding protein
ifg-1	initiation factor G 1
rsks-1	ribosomal protein S6 kinase
Lsd1	lipid storage droplet 1
Lsd2	lipid storage droplet 2
PAT	perilipin, adipophilin, and tail-interacting protein

UDP-glucose	uridine diphosphate glucose
cAMP	cyclic adenosine monophosphate
O2	oxygen
CO2	carbon dioxide
VO_2	volume of oxygen
RQ	respiratory quotient
FiO_2	fractional volume of oxygen entering the chamber
FeO_2	fractional volume of oxygen leaving the chamber
FR	flow rate
VCO_2	volume of carbon dioxide
STP	standard temperature and pressure of ambient air
$FiCO_2$	fractional volume of carbon dioxide entering the chamber
$FeCO_2$	fractional volume of carbon dioxide leaving the chamber
25R2	two generations of relaxed selection following 25 generations of selection for starvation resistance
26R2	two generations of relaxed selection following 26 generations of selection for starvation resistance
27R2	two generations of relaxed selection following 27 generations of selection for starvation resistance

Chapter 1.

Introduction

Obesity prevalence and associated risk factors

Over the past three decades, the world has witnessed a dramatic increase in the prevalence of obesity within the human population (Wang & Beydoun, 2007). Current estimates indicate that approximately 400 million people worldwide are identified as clinically obese, with an additional 155 million children recognized as either overweight or obese (Seidell, 2000; Hossain et al., 2007; Hofker & Wijmenga, 2009). As such, obesity has reached epidemic proportions and is believed to continue to increase in prevalence due to the 1.1 billion individuals worldwide that are currently identified as overweight (Hossain et al., 2007). Obesity is the leading risk factor for the development of insulin resistance resulting in type II diabetes, hypertension, hyperlipidemia, cardiovascular disease including atherosclerosis, and certain forms of cancer (Baker & Thummel, 2007; Bharucha, 2009; Hong & Park, 2010). Statistical correlations between obesity and mortality rates indicate obesity as a leading risk factor for premature death, with some experts suggesting it to be the second leading cause of preventable death (Wang & Beydoun, 2007). Currently, obesity is not recognized as a cause of death in the International Classification of Diseases established by the World Health Organization, nor can physicians determine cause of death to be directly associated with obesity, so implications of this disease as a leading cause of preventable deaths is based solely on statistical correlations.

Genetic and environmental interactions are the main contributors to the development and persistence of obesity (Hong & Park, 2010). Studies conducted in mice and other model organisms have indicated that genetic alterations can have a dramatic

effect on the amount of food consumed, energy expenditure and utilization of calories, and the amount of lipid storage within adipose tissue (Hong & Park, 2010). Genetic based predispositions to increased caloric intake and lipid storage are correlated with an increased prevalence of obesity. Additionally, reductions in energy expenditure can result in increased fat deposition within the individual leading to the development and persistence of this disease. Reductions in exercise coupled with a positive energy balance due to greater food intake are correlated with increased risk for the development of obesity and associated metabolic diseases (Hong & Park, 2010).

Drosophila melanogaster as a model for studying metabolism

The increased prevalence of obesity within the human population has led to renewed interest in understanding the molecular mechanisms underlying this disease. Researchers are currently utilizing model organisms to conduct phenotypic perturbations to gain insight into correlations between genetic and environmental alterations and the resulting physiological responses. One model that has become important for use in the investigation of metabolism is the fruit fly, *Drosophila melanogaster* (Baker & Thummel, 2007). This organism is ideal for metabolic studies in that the central pathways of intermediary metabolism, including the metabolic and signaling pathways associated with fat metabolism, adipocyte development, and insulin signaling have been conserved throughout evolution (Ruden at al., 2005; Baker & Thummel, 2007). In addition to the conservation in metabolic pathways and signaling cascades, a strong genetic association

has been established where approximately 61 percent of human genes implicated in disease development have homologs or orthologs identified in *Drosophila melanogaster*.

The conservation in metabolic pathways and molecular function of specific components is illustrated in similarities between humans and *Drosophila* in nutrient uptake, transport, and storage (Ruden et al., 2005; Baker & Thummel, 2007). Digestion and absorption of dietary derived nutrients occurs in the midgut of *Drosophila*. This organ is the functional equivalent to the stomach and small intestine in mammals in that both are involved in the catabolism of food and absorption of nutrients (Baker & Thummel, 2007). Following absorption, nutrients are transported through the haemolymph to the fat body in lipophorin particles, which are similar in structure and function to the low and high density lipoproteins in human circulation (Baker & Thummel, 2007). Within the fat body, lipids and carbohydrates are metabolized and stored in the form of triglycerides and glycogen, respectively (Ruden et al., 2005; Baker & Thummel, 2007).

The main objective of this research is to investigate physiological and life history trait responses to starvation and dietary supplementation in the context of an obese phenotype. The following sections provide an overview of metabolism in *Drosophila melanogaster* focusing on metabolic organ structure and function, metabolic pathways of lipid, carbohydrate, and protein synthesis and degradation, and a description of energetic homeostasis and responses to starvation. Each section will additionally discuss similarities between *Drosophila* and humans to illustrate the importance of this organism in studying human metabolic diseases. Investigations of correlations between phenotypic

perturbations including starvation and dietary supplementation with body composition, life history traits, and movement will provide insight into the molecular mechanisms underlying the development and persistence of obesity.

The *Drosophila* fat body

Prior to metamorphosis in *Drosophila*, the larva possess a fat body that performs similar metabolic functions as those occurring in the adult fly (figure1.1). During metamorphosis, the organism undergoes a series of dramatic changes that ultimately result in alterations to tissues and organ structures. One example of this change is the autophagy of the larval fat body and the formation of the adult fat body de novo (Bharucha, 2009). Contrary to the discrete location of the fat body within larvae, the adult fat body is more dispersed throughout the organism (Bharucha, 2009). Sheets of adipose tissue composed of adipocytes are distributed underneath the integument and surrounding the gut and reproductive tract within each individual fly (Arrese & Soulages, 2010; Hong & Park, 2010). The thin lobes of the fat body tissue are maintained in close proximity to the haemolymph, allowing for an effective exchange of nutrients between the fat body and the insect blood (Arrese & Soulages, 2010). Within the fat body, the capacity for lipogenesis is higher than glycogen synthesis with lipid droplets occupying the majority of the intercellular space. The remaining space within this organ consists of a small concentration of glycogen and protein granules (Arrese & Soulages, 2010).

Intermediary metabolism is modulated in the fat body through the regulation of carbohydrate metabolism, protein synthesis, and amino acid and nitrogen catabolism

(Arrese & Soulages, 2010). In order to efficiently regulate metabolic activities, the fat body must possess nutrient sensors to determine the nutritional status of the organism. Amino acid transporters located within the membrane of the fat body act as nutrient sensors stimulating the fat body to store nutrients when food is plentiful, and release energy during times of nutrient deprivation (Arrese & Soulages, 2010). Regulation of carbohydrate metabolism within the organism occurs in the fat body where signals are generated to stimulate insulin producing cells in the brain to secrete insulin-like peptides, thus promoting carbohydrate uptake and storage (Leopold & Perrimon, 2007; Bharucha, 2009; Hong & Park, 2010). Beyond the regulatory properties of the fat body, this organ also serves an endocrine function through the production of antimicrobial peptides. These particular proteins are involved in the detoxification of nitrogen metabolism resulting from intercellular protein degradation (Arrese & Soulages, 2010). Based on similarities in metabolic functions, the *Drosophila* fat body has been equated functionally to the human liver.

Lipid metabolism

One of the main functions of the fat body in *Drosophila* is to store lipids in the form of triglycerides (Hong & Park, 2010). As such, this organ shares analogous functions with mammalian white adipose tissue. Approximately 6.5 percent of adult *Drosophila melanogaster* body weight is composed of lipid (Baker & Thummel, 2007). The proportion of dry mass accounted for by lipid is approximately 50 percent with 90 percent of all stored lipids within in the organism identified as triglyceride molecules

(Arrese & Soulages, 2010). Stored triglycerides within the individual are derived form dietary carbohydrates, fatty acids, or protein (figure 1.2) through various metabolic conversions (Arrese & Soulages, 2010).

Triglycerides are stored within the *Drosophila* fat body in intercellular lipid droplets (Arrese & Soulages, 2010). Similarly in humans, triglycerides accumulate in storage vesicles surrounded by a monolayer of phospholipids. However, the composition of the lipid droplets in *Drosophila* are more structurally similar to human lipoproteins, rather than lipid storage vesicles, in that they consists of a core of neutral lipids, triglycerides and cholesterol esters, surrounded by a monolayer of phospholipids and embedded proteins (Arrese & Soulages, 2010). Proteins embedded in the surface of the *Drosophila* lipid droplet consist of Lsd1 and Lsd2 which are identified as PAT family proteins (Bharucha, 2009). Lsd2 is a homolog to mammalian perilipin and is thought to promote lipid accumulation within the intercellular lipid droplets (Arrese & Soulages, 2010). Lsd1 serves an opposing function in that it promotes lypolysis and the release of stored triglycerides to be used for energy production (Bharucha, 2009). Liberation of free fatty acids is additionally achieved during prolonged periods of starvation through the actions of Brummer lipase residing on the outer surface of the lipids droplet (Baker & Thummel, 2007). Lipids derived from the fat body are released into the haemolymph in the form of diacylglycerols that can later be cleaved by oenocytes to release free fatty acids for conversion into acetyl-CoA used in ATP synthesis (Arrese & Soulages, 2010).

Carbohydrate metabolism

Carbohydrate levels within *Drosophila melanogaster* are maintained by neurosecretory cells, specifically located in the ring gland complex of the brain, that receive input of nutritional status from the fat body (Leopold & Perrimon, 2007). Excess carbohydrates within the organism are stored in the form of glycogen that is deposited primarily in the muscle and fat body. Glycogen is synthesized from UDP-glucose that is derived from dietary carbohydrates or amino acids (Arrese & Soulages, 2010). During times of nutrient deprivation (figure 1.3), dietary derived carbohydrates can be converted into trehalose; however, this process is energy dependent and is only used upon depletion of stored glycogen and triglycerides (Arrese & Soulages, 2010).

In response to depleted nutrients, especially carbohydrates, adipokinetic hormone (AKH) is released from the corpora cardiaca within the ring gland complex, located in the *Drosophila* brain (Baker & Thummel, 2007). The release of AKH is due to an inverse alteration in intracellular calcium stores mediated by ATP-sensitive potassium channels (Baker & Thummel, 2007). The resulting rapid influx of calcium increases intercellular cAMP levels, thus activating protein kinase A (Arrese & Soulages, 2010). Phosphorylation of a glycogen phosphorylase by protein kinase A stimulates the catabolism of stored glycogen resulting in increased trehalose concentrations in the haemolymph (Leopold & Perrimon, 2007). When the energetic needs of the organism are met, AKH binds to its receptor located on the outer surface of the fat body stimulating a signaling cascade that prevents further AKH release and catabolism of glycogen reserves (Bharucha, 2009). The strong functional similarities between AKH regulation in

Drosophila and glucagon signaling in humans illustrate the high degree of metabolic signaling conservation throughout evolution (Baker & Thummel, 2007).

Protein metabolism

A number of gene products have been identified to function as nutrient sensors within *Drosophila melanogaster*. One example is a TOR-dependent molecular sensor for amino acids that is involved in the regulation of feeding behavior (Leopold & Perrimon, 2007). TOR sensors located on the surface of the fat body detect variations in the levels of circulating amino acids and generate responses that stimulate neuroendocrine cells located within the *Drosophila* brain to increase feeding when circulating protein levels are significantly reduced. In the presence of sufficient quantities of circulating amino acids, TOR kinase becomes activated and in turn phosphorylates S6 kinase. This reaction increases protein synthesis and organismal growth during times of adequate nutrition (Baker & Thummel, 2007). An additional nutrient sensor on the surface of the fat body, known as slimfast (figure 1.2), signals through TOR to reduce growth and metabolism during periods of inadequate nutrition. During food deprivation, TOR signaling declines resulting in a subsequent reduction in insulin signaling to prevent the continued storage of carbohydrates. This leads to the catabolism of stored glycogen and triglycerides, thus allowing for energy liberation within the organism until food consumption is restored (Baker & Thummel, 2007).

Energy homeostasis and the response to starvation

Drosophila melanogaster adapt to reductions in available nutrients through the regulation of metabolism (Leopold & Perrimon, 2007). Tissues that regulate metabolic homeostasis within the organism include the fat body, oenocytes, the gastrointestinal tract, Malphigian tubes, and the brain (Bharucha, 2009). During starvation (figure 1.3), diacylglycerols are released from the fat body into the haemolymph to be taken up by specialized cells, known as oenocytes (Leopold & Perrimon, 2007). These cells are derived from the ectoderm and distributed in discrete paired clusters along the body wall allowing for direct contact with the haemolymph (figure 1.1) (Bharucha, 2009; Arrese & Soulages, 2010). Oenocytes are functionally similar to mammalian hepatocytes in that they perform lipid metabolism to generate energy necessary for survival during periods of starvation (Leopold & Perrimon, 2007).

During the early responses to starvation, AKH activates cAMP-dependent protein kinase, which in turn stimulates Lsd1 and Brummer lipase at the surface of the intercellular lipid droplets to release diacylglycerols for transport through the haemolymph by lipophorins (Leopold & Perrimon, 2007). Upon reaching the oenocytes, the liberated diacylglycerols are converted to free fatty acids through the actions of lipases. A series of enzymatic reactions convert the fatty acids to acetyl-CoA, which can then be used in the Krebs cycle for ATP synthesis (Hong & Park, 2010). During periods of prolonged starvation, the liberated free fatty acids are converted into ketone bodies to be used in the *Drosophila* brain for energy (Baker & Thummel, 2007; Hong & Park, 2010).

Specific aims

The objective of this research was to investigate physiological and life history trait responses to starvation and dietary supplementation in the context of an obese phenotype using genetically based starvation-resistant and control lines of *Drosophila melanogaster*. The purpose of the initial study was to investigate correlations between the direct and a series of indirect responses to the presence or absence of a brief period of starvation within starvation-resistant and control *Drosophila melanogaster* lines. In this study an artificial selection experiment was conducted to measure indirect responses to selection including alterations to body composition specifically quantifying triglycerides, carbohydrates, and soluble protein concentrations. The resulting data were combined with measurements of movement and life history traits, including development time, longevity, and fecundity, to identify correlations between the response to selection and observed alterations in body composition and activity levels. Results obtained from this study were used to gain insight into alterations to nutrient storage in obese individuals and the resulting correlations between development, lifespan, and reproduction.

The second study investigated metabolic and physiological responses of genetically based starvation-resistant and control lines of *Drosophila melanogaster* to the presence or absence of starvation. The main objective of this research was to analyze oxygen consumption and carbon dioxide production of the starvation-resistant and control lines that are either starved or unstarved to determine metabolic rate. In addition, respiratory quotient values were used to determine the primary metabolic fuel utilized in the starved and unstarved states. Measurements of the number of movements of each

individual were coupled with the respiration data to identify correlations between metabolism and movement. This analysis provided insight into metabolism and behavior in lines that possess an evolved resistance to starvation. In addition, information obtained from this study will be used to gain insight into metabolic rate and associations with activity levels in obese individuals.

The final study investigated correlations among four separate dietary alterations and body composition, life history traits, and movement within the starvation-resistant and control lines of *Drosophila melanogaster*. In this study, *Drosophila* diets were supplemented with one of the following three constituents: glucose, palmitic acid, or a 2:1 mixture of casein and albumin. Investigations of the effects of dietary supplementation were conducted to identify correlations between increased dietary components and alterations to body composition and life history traits. Comparisons of the results obtained from each of the diets were made with the base diet for identification of specific alterations associated with each form of supplementation. Results obtained from this study provided insight into correlations between nutrient storage and potential long-term effects associated with dietary supplementation.

This research represents a comprehensive approach to investigating metabolic and physiological responses to starvation and dietary alterations in the context of an obese phenotype. Investigations of correlations between the responses to starvation and dietary supplementation with alterations in body composition, life history traits, movement, and metabolic rate provided insight into the molecular mechanisms underlying the development of an obese phenotype. The author of this research recognizes that results

obtained in model organisms do not necessarily directly correlate with responses in humans; however, data obtained from this study has implications for further investigation of obesity and associated metabolic disorders within the human population.

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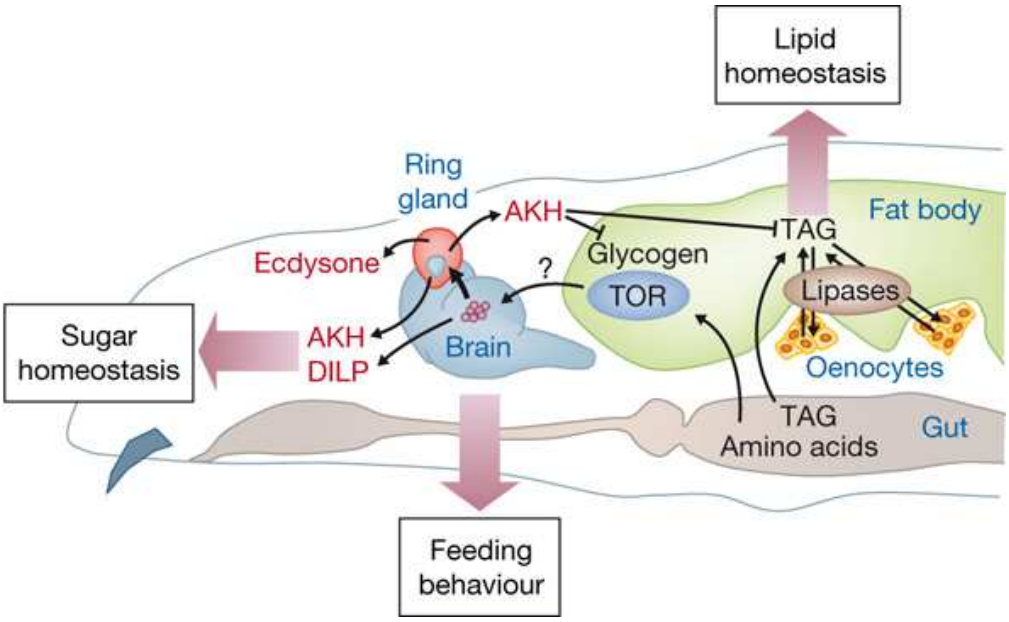

Figure 1.1 Metabolic signaling pathways in *Drosophila melanogaster*. Hormones are featured in red, while tissue names are shown in blue. This figure was obtained from Leopold, 2007.

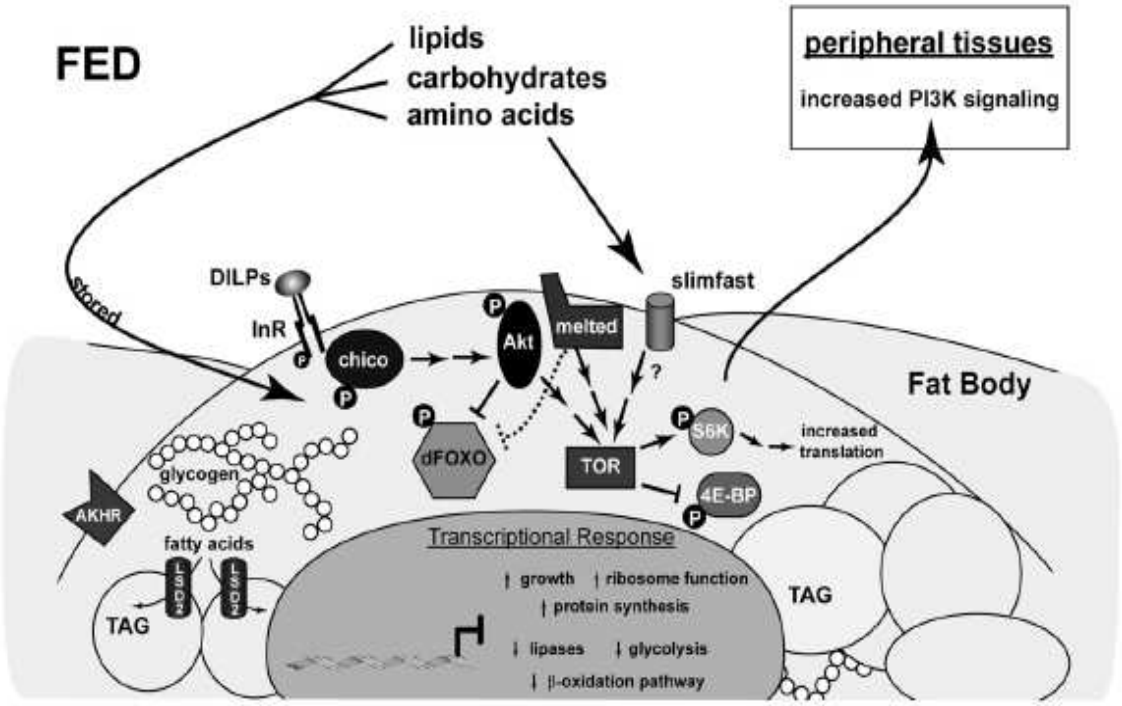

Figure 1.2 Metabolic signaling in *Drosophila melanogaster* during the fed state. This figure was obtained from Baker, 2007.

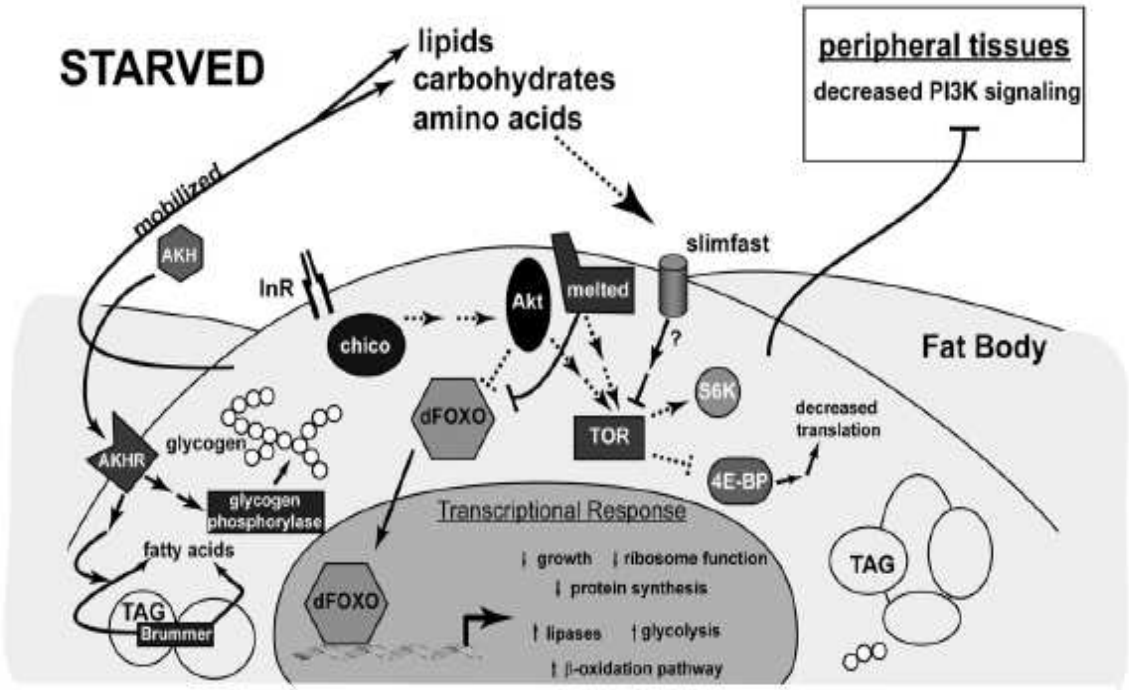

Figure 1.3 Metabolic signaling in *Drosophila melanogaster* during the starved state. This figure was obtained from Baker, 2007.

Chapter 2.

Investigations of physiological, metabolic, and life history trait responses to starvation in genetic-based starvation-resistant and control lines of *Drosophila melanogaster*

Abstract

Correlated responses obtained from artificial selection experiments for starvation resistance provide insight into the evolved metabolic and physiological alterations associated with increased survival following exposure to biologically induced stress. Investigations of genetically based starvation-resistant and control *Drosophila melanogaster* used in this study were conducted under starved and fully-fed conditions. Measurements of body composition within the selected and control lines of *D. melanogaster* exposed to the presence or absence of a brief period of starvation revealed an increase in metabolic fuel storage, specifically triglycerides and carbohydrates, within the starvation-resistant lines. Additional comparisons of body composition indicated increased concentrations of proteins following starvation, suggesting that protein production is correlated with the response to starvation. A reduction in movement recorded in the selected lines, suggested the presence of a coordinated physiological response that resulted in the conservation of energy reserves during periods of starvation. Measurements of life history traits within the selected and control lines indicated no statistically significant differences in development time or fecundity, while longevity was decreased in the starvation-resistant lines as compared to the control lines. Similarities in metabolic and physiological responses to starvation between insects and higher order vertebrates, including humans, provide a logical parallel to studying metabolic disorders including an obese phenotype. As such, results obtained from this study have implications for research focused on gaining insight into human metabolic diseases.

Introduction

Natural populations of most species have been exposed to periods of unsuitable or insufficient food resources (White, 1993). Malnutrition and starvation resulting from limited availability of nutrients has led to the evolution of adaptive mechanisms allowing for efficient energy utilization and survival while exposed to biologically induced stress (Rion & Kawecki, 2007). The evolved resistance to periods of starvation within organisms likely result in a series of physiological responses that alter the phenotype to withstand changes in environmental conditions (Hoffmann & Parsons, 1991). Limits to the degree of physiological adaptations in response to environmental variation play an important role in identifying constraints of evolution with regards to individuals and the population as a whole (Somero & Hochachka, 1971). Investigations of biological responses to starvation within natural populations and in a laboratory setting are essential for gaining insight into the evolution of traits correlated to starvation resistance at the cellular level, and with regards to life history traits (Rion & Kawecki, 2007).

The majority of knowledge regarding the genetic and physiological basis for organismal adaptations to starvation resistance were derived from laboratory studies conducted on *Drosophila melanogaster* (Rion & Kawecki, 2007). Artificial selection experiments utilizing *Drosophila* have identified patterns of variation within and among populations, evolutionary correlations among traits, and trade-offs between life history traits in response to selection (Hoffmann & Harshman, 1999; Zera & Harshman, 2001). Previous experiments selecting for starvation resistance within *D. melanogaster* resulted in significant alterations to body composition coupled with alterations to lifespan and

fecundity (Djawdan et al., 1998; Harshman et al., 1999a; Baldal et al., 2006). The purpose of this study is to investigate correlations between the direct and a range of indirect responses to selection for starvation resistance.

Three physiological mechanisms have been proposed to account for the observed response to selection leading to enhanced starvation resistance (Ballard et al., 2008). The first mechanism addressed the increase in stored energy reserves, especially lipids, while the second and third mechanisms addressed a reduction in the rate of energy utilization and decreases in the minimal resources necessary for somatic maintenance (Hoffman et al., 2005; Rion & Kawecki, 2007). In support of the first mechanism of increased starvation resistance, previous studies of laboratory based selection for starvation resistance in *Drosophila melanogaster* indicated an increased abundance of lipids and carbohydrates (Djawdan et al., 1998; Harshman et al., 1999a), which are stored within the fat body. Additional responses correlated with artificial selection including life history traits, body composition, metabolism, and movement are of interest in relationship to the second and third hypothesized mechanisms of increased starvation resistance.

There are a number of experiments using *Drosophila* that address correlations between body composition, life history traits, and starvation resistance. Artificial selection studies quantifying alterations in body composition in *Drosophila melanogaster* indicated increased carbohydrate and lipids stores, while total body protein concentrations remain unaltered (Djawdan et al., 1998; Harshman et al., 1999b; Baldal et al., 2006). Several experiments conducted in *Drosophila* have shown a relationship between starvation survival and life history traits, but the results are variable. In some

instances, selection for starvation resistance was positively correlated with increased survival, but in other experiments no correlations were observed between starvation and lifespan. Selection experiments for increased longevity exhibit similar trends with a few studies resulting in enhanced survival, while others exhibit no relationship between longevity and starvation resistance (Harshman, 2003). A study conducted by Rose (1984) indicated that increased resistance to starvation was correlated with an extension of lifespan, while studies conducted by Luckinbill (1984), Zwann (1995), Harshman (1999b) and Stearns (2000) indicated no statistically significant differences in longevity between starvation-resistant and control lines. An opposing trend was observed in experiments using isofemale lines of *Drosophila simulans*, indicative of a negative relationship between lifespan and starvation survival (Ballard et al., 2008). Studies conducted on increased accumulation of lipids associated with selection for starvation resistance in *Drosophila melanogaster* have indicated an increase in the amount of time required for egg-to-adult development (Chippindale et al.,1998; Harshman et al., 1999a). It has been suggested that the slower developmental time could allow for the accumulation of energy stores, specifically lipids, prior to adult emergence (Chippindale et al., 1998). Studies correlating selection responses and female fecundity indicated a negative correlation between the two traits representative of a possible trade-off between starvation resistance and reproduction (Harbison et al., 2004; Baldal et al., 2006).

The relationship between life history traits and survival under biologically induced stress is relevant to a number of investigations in biological research. One related aspect is the conserved evolutionary response of organisms to dietary restriction. In most

organisms, reduced nutrient intake to the minimum level required for somatic maintenance led to an extension of lifespan (Sinclair, 2005). It has been suggested that organisms have evolved biochemical and physiological responses to periods of starvation that allow individuals to breed when food is more abundant (Partridge et al., 2005; Sinclair, 2005). An associated topic regarding life history traits and the stress response focused on evolution of life history traits and the influence of metabolism. The Y-model of life history trait evolution argued that a negative correlation between life history traits was expected if response traits were competing for a common pool of energy storage molecules (van Noordwijkand & de Jong,1986; Zera & Harshman, 2001).

In the present study, a selection experiment was conducted to measure indirect responses to selection including body composition. This data was coupled with movement data to gain insight into utilization and storage of energy reserves following exposure to the presence or absence of brief periods of starvation. Life history traits including developmental time, female fecundity, and longevity were measured to identify correlations with the response to selection and observed alterations in body composition and movement.

Materials and Methods

Artificial selection for starvation resistance

The base population of *Drosophila melanogaster* utilized in artificial selection for starvation resistance was derived from inbred lines collected in a manner designed to preserve natural genetic variation. Initially, a large number of inseminated females were collected from the Wolfskill Experimental Orchard located in Yolo County in Northern California. Following collection, the females were brought into a laboratory setting and the resulting progeny were used for 20 generations of inbreeding. From this population, flies were randomized into a series of ten individual lines that were intercrossed using all possible pair-wise crosses between the lines, with all reciprocal crosses conducted. From the approximately 100 crosses generated, 150 progeny were randomly selected from each cross and placed within a large population cage (12 inches high, 36 inches wide, and 24 inches deep) to ensure a balanced representation of genetic variation. The initial population density of approximately 15,000 adults was maintained through an overlapping generation population regime where 4 of the 20 open bottle food sources within the population cage were replaced weekly to ensure an adequate food supply. This population regime contributed to the maintenance of lifespan and stress resistance, which are normally lost when flies are maintained in a laboratory culture with one generation placed in each bottle (Hoffmann et al., 2001; Linnen et al., 2001).

The base population used for selection was divided into eight subpopulations, which were used to produce the four replicate matched selected and control lines. Adult

flies utilized for selection were obtained by collecting a large number of eggs from 10 cut glass bottles randomly placed throughout the initial population. The bottles contained a food source consisting of yeast extract, water, dextrose, agar, and a mixture of acids that prevent mold and fungal growth on the surface of the media. Each day approximately 100 eggs were collected from the surface of the media and were placed within vials of food that were stored at a temperature of 25°C with 12 hours of light and 12 hours of darkness. Following collection, the surface of the media was removed and the bottles were replaced within the cage overnight to obtain additional eggs used to produce the replicate selected and control line populations. This process was repeated until each of the eight subpopulations consisted of approximately 4,000 individuals. Adults within each subpopulation were allowed to randomly mate prior to egg collection for individuals in the next generation. This procedure was repeated for four generations with the population size for each of the replicate selected and control lines consistently maintained at a density of 2,000 males and 2,000 females. Artificial selection for starvation resistance on the four subpopulations designated as the selected lines commenced in the following generation.

Selection for starvation resistance was conducted on adult mated females and males that were approximately five days old upon initiation of selection. Separation and scoring of the sexes was performed following two days post eclosure, by briefly exposing the flies to a small amount of ethyl ether to anesthetize the flies for separation. The 2,000 males and 2,000 females obtained from each of the individual matched selected and control lines were placed in sixteen population cages with dimensions of 12 inches wide

by 18 inches long by 12 inches high. One circular opening in each cage allowed for placement and removal of flies while conducting selection. This opening was sealed to prevent the escape of test subjects using a sleeve of finely woven cloth that was tied in a knot. Males and females within the replicate control lines were provided with 6 petri-plates (9mm in diameter) containing food, while flies in the replicate selected lines received 6 plates (9mm in diameter) containing solidified agar as a water source. Plates were changed every other day during the morning time-point to provide flies with adequate food or water sources, in addition to preventing the growth and development of mold and microbes on the surface of the media. Moreover, the plates within the cages were replaced to prevent the introduction of progeny into experimentation. Relative humidity within the experimental environment was maintained at a moderate level by placing the cages within a clear plastic bag containing a damp paper towel. The moistened paper towel was replaced in conjunction with removal and replacement of food and agar plates.

During the process of selection for starvation resistance, the response to selection was assessed in each of the selected cages by tabulating mortality levels at 12 hour intervals and removing dead flies by aspiration. To ensure consistency in experimental procedures and to determine natural mortality rates at 12 hour intervals, each of the control cages was assessed and dead flies were removed. Upon each selected line reaching approximately fifty percent mortality levels, flies from the matched selected and control lines were removed from the cages by aspiration following brief exposure to carbon dioxide to anesthetize the flies within the cage. The surviving flies from each line

were placed at moderate densities in plastic bottles containing 50 ml of food to allow for refeeding following selection. Once all of the four selected lines had reached fifty percent mortality levels, the flies were allowed a two-day recovery period prior to breeding for the next generation used in selection for starvation resistance.

To generate the next generation used in selection, a standard number of 75 males and 75 females from each of the replicate selected and control lines were allowed to mate in 6 cut glass bottles, with egg collection beginning after a one-day adaptation period within the bottles. Approximately 100 eggs were collected per vial to produce a series of 200 vials for each replicate matched selected and control line. From the collected eggs, a population of 2,000 males and 2,000 females for each individual replicate selected or control line was obtained for each generation of selection.

Cohort mortality assays were conducted every fifth generation on flies that were two generations removed from selection to assess the response to selection. In these total mortality studies, flies from each replicate selected and control line were provided with only agar as a water source to measure percent survival under starvation. Mortality levels were tabulated at 12 hour intervals until all flies in each replicate selected and control line reached complete mortality. Each assay for the response to selection was conducted at 25°C with 12 hours of light and 12 hours of dark with moderate relative humidity maintained as previously described.

Following generation 15 of selection for starvation resistance, flies from each selected line underwent one to six generations of relaxed selection, which is defined as the absence of selection. Starting at the third generation of relaxation from selection, a

series of experiments were conducted to determine the response of the control and selected lines to 32 hours of starvation compared to lines that were maintained on food. The rational for selecting 32 hours of starvation was to induce a stress response within the individuals while preventing mortality of subjects used for experimentation. Following exposure to the presence or absence of starvation, flies used in life history trait measurements were returned to food. Development time, female fecundity, and longevity were measured on flies that were five generations removed from starvation selection to identify correlations between selection, reproduction, and lifespan. In addition, a large number of flies that were subjected to either starvation or maintained on food were flash frozen in liquid nitrogen to be used in determining the concentrations of soluble proteins, total carbohydrates, total sugars, glycogen, trehalose, triglycerides, and glycerol. All replicates used in each of the body composition, life history trait, and movement assays were based on separate sets of flies.

Soluble protein concentrations

Total soluble protein quantification was conducted using the Pierce BCA Protein Assay Kit. The methodology employed with this kit coupled the reduction of copper in a basic solution with colorimetric detection of the cuprous cation by reaction with a bicinchoninic acid (BCA) reagent. A series of standard solutions were created by combining known amounts of a 2 μg/ml solution of albumin with known quantities of HPLC water to create a dilution series that ranged in concentration from 0 to 2,000 μg/ml of protein.

Determination of total soluble protein concentrations within each of the replicate selected and control lines were conducted by homogenizing ten males or ten females in 500 µl of HPLC water. Each sample was homogenized using a 5/32 inch stainless steel grinding ball from OPS Diagnostics in a Talboys High Throughput Homogenizer (OPS Diagnostics) at 1,120 rpm for 3 minutes. Twenty-five microliters of each standard solution or unknown sample were added to individual wells within a 96 well microplate. Additionally, two hundred microliters of the combined kit reagents were added to each well prior to thorough mixing on an orbital shaker for 30 seconds. Following mixing, the plate was covered and incubated at 37°C for 30 minutes prior to cooling to room temperature. Optical density readings of total soluble proteins within the samples were determined using a Versa Max microplate reader (Molecular Devices) set at a wavelength of 562 nm. A standard curve was generated by plotting the absorbance versus the concentration of the standard solutions using a quadratic best-fit curve. Total protein concentrations within each of the three replicates of the matched selected and control lines in either the starved or unstarved states were calculated utilizing the linear portion of the standard curve.

Total carbohydrate concentrations

Total carbohydrate concentrations were determined utilizing methods described by Van Handel (1985). A glucose calibration curve was formed by the addition of increasing amounts (25, 50, 100, 150, and 200 µg) of a 1 mg to 1 ml glucose in 25 percent ethanol solution to anthrone reagent consisting of a mixture of distilled water,

concentrated sulfuric acid, and dissolved anthrone to generate a total reaction volume of 5 milliliters. Each solution was heated at 90°C for 17 minutes prior to cooling to room temperature. Optical densities of each standard were determined using the microplate reader at wavelengths of 625 and 555 nm. Readings were recorded at two wavelengths to generate increased linearity of the standard curve. The microgram concentrations of glucose were plotted with the optical density readings to produce the standard curve.

To determine total carbohydrate concentrations in the starved and unstarved selected and control samples, ten males or ten females were homogenized in 1 ml of anthrone reagent using the high throughput homogenization procedure previously described. Following homogenization, additional anthrone was added to generate a total reaction volume of 5 ml. Optical densities of the solutions were measured at 625 and 555 nm following 17 minutes of heating at 90°C prior to cooling to room temperature. Carbohydrate concentrations for each of the three replicate starved and unstarved selected and control lines were determined based on extrapolation from the glucose standard curve.

Glycogen and total sugar concentrations

Quantification of glycogen and total sugar concentrations was preformed using methods described by Van Handel (1985). Ten male or ten female starved and unstarved selected and control *D. melanogaster* samples were homogenized in 200 µl of a two percent sodium sulfate solution in water using the high throughput homogenization methodology previously described. One milliliter of methanol was added to the

homogenate prior to a 1 minute centrifugation at 2,000 rpm. Following centrifugation, the supernatant containing the sugars was decanted and evaporated to a volume of 100 to 200 µl, while the glycogen within the sample remained in the precipitated sodium sulfate and fly tissue. Anthrone reagent was added to both the sugar and the glycogen solutions to generate a reaction volume of 5 ml. Each solution was thoroughly mixed, heated to 90 °C, and cooled to room temperature prior to determining optical densities at wavelengths of 625 and 555 nm. Glycogen and total sugar concentrations for each of the three replicate starved and unstarved selected and control lines were determined using the glucose standard curve.

Trehalose concentrations

Trehalose concentrations for the starved and unstarved selected and control lines were determined using methodology described by Van Handel (1985), which separated trehalose from the additional sugars present in the total sugars fraction. A trehalose standard curve was created through the addition of increasing concentrations (25, 50, 100, 150, and 200 µg) of a 1 mg to 1 ml of trehalose in 25 percent ethanol solution to 50 µl of 1N hydrochloric acid. The resulting solution was heated at 90°C for 7 minutes to hydrolyze sucrose to glucose and fructose leaving trehalose intact. Addition of 150 µl of 1N sodium hydroxide heated at 90°C for 7 minutes led to the preclusion of anthrone reactivity to glucose and fructose, allowing for the quantification of trehalose. Anthrone reagent was added to each standard solution, producing a total reaction volume of 5 milliliters. Each solution was heated at 90°C for 17 minutes. Following cooling to room

temperature, the optical densities were measured in a Versa Max microplate reader at 625 and 555 nm. Plotting the trehalose concentrations versus the optical density readings produced the trehalose standard curve.

Trehalose concentrations in the starved and unstarved selected and control lines were determined by homogenizing ten males or ten females in a two percent sodium sulfate solution in water utilizing the high throughput homogenization methods previously described. One milliliter of methanol was added to the homogenates prior to a 1 minute centrifugation at 2,000 rpm. The solution containing the sugar fraction was decanted, while the pellet was resuspended in 300 µl of water. One milliliter of methanol was added to the resuspension prior to an additional 1 minute centrifugation at 2,000 rpm. The resulting supernatant was combined with the previously decanted solution prior to concentrating the sugar fraction to a volume of 500 µl.

One hundred microliters of the concentrated sugar fraction was added to 50 µl of 1N hydrochloric acid prior to heating at 90°C for 7 minutes. Following heating, 150 µl of sodium hydroxide was added to prior to an additional 7 minutes of heating at 90°C. A total reaction volume of 5 ml was generated through the addition of anthrone reagent prior to heating the solution for 17 minutes at 90°C. Optical densities were determined at wavelengths of 555 and 625 nm using the microplate reader for each of the three replicate staved and unstarved selected and control lines following cooling. Trehalose concentrations for each sample were calculated using the trehalose standard curve.

Triglyceride and glycerol concentrations

Triglyceride and glycerol concentrations for the starved and unstarved selected and control *D. melanogaster* lines were obtained using the BioVision Triglyceride Quantification Kit. A standard curve was generated through the addition of increasing amounts (0, 2, 4, 6, 8, 10 μl) of a 1 mM triglyceride standard solution to the provided buffer resulting in a total reaction volume of 50 μl. Prior to constructing each standard curve, triglycerides in the standard solution were dissolved into the aqueous phase to ensure accurate concentration readings. Solubilization of the standard was achieved by heating the solution to 80°C in a water bath for 1 minute prior to vortexing the solution for 30 seconds. This step was repeated to ensure complete and accurate concentration readings ranging from 0 to 10 nanomoles.

Whole body samples of five males or five females from each of the matched selected and control lines in either the starved or unstarved states were homogenized in 500 μl of a five percent Titon-X-100 in water solution using the previously described homogenization procedure. The samples were then heated at 80°C for 5 minutes. The homogenates were cooled to room temperature and reheated to ensure complete solubilization of triglycerides and glycerol in solution. A 1 minute centrifugation at 2,000 rpm removed insoluble materials prior to conducting a tenfold dilution of the samples in HPLC water. This dilution ensured that the triglyceride and glycerol concentrations of each sample fell within range of the triglyceride standard curve.

Two microliters of lipase were added to the standard solutions and the samples used for triglyceride quantification to allow for cleavage of the triglyceride molecules

into free fatty acids and glycerol. Samples that were used to measure free glycerol concentrations did not receive the addition of lipase to ensure that the measurements were quantifying only free glycerol. A combined reaction mixture consisting of 46 µl of triglyceride assay buffer, 2 µl of the probe, and 2 µl of an enzymatic mixture were added to each sample and standard solution prior to a 30 minute incubation at room temperature in the absence of light. During the incubation, glycerol within each sample was oxidized to generate a product that reacted with the provided probe to produce a colorimetric response. This response was measured as an optical density reading in the Versa Max microplate reader set to a wavelength of 570 nm. Triglyceride and free glycerol concentrations were determined for each of the three replicates per selected and control line in the starved and unstarved states through extrapolation from the triglyceride standard curve.

Weight measurements and data normalization

Body composition concentrations were normailized using the dry weight, the weight in the absence of water, and the lean mass, the weight in the absence of lipid, of the individual sexes. Flies from each replicate selected and control line subjected to the presence or absence of starvation were flash frozen with liquid nitrogen and stored in a -80°C freezer prior to obtaining individual weight measurements. Dry weights were acquired by placing five replicates of ten males or ten females from each of the selected and control lines that were either starved or unstarved in opened microfuge tubes positioned in a 65°C drying oven for 24 hours. Each fly was weighed individually and the

average dry weight was obtained for each line in the starved or unstarved states. Lean masses were determined in a similar manner as dry weights with a standard Bligh and Dyer lipid extraction (chloroform and methanol) conducted prior to drying the samples in the oven for 24 hours. Additionally, each measurement obtained for body composition was recorded per fly to use in comparisons on the level of a single biological unit.

Development time

Adult emergence times were determined using *D. melanogaster* five generations removed from fifteen generations of selection for starvation resistance from each of the replicate selected and control lines. Parental flies were subjected to either 32 hours of starvation, with only agar as a water source, or were placed in bottles containing food at seven days post eclosion. Following 32 hours of either the presence or absence of starvation, the mated adults were returned to bottles with food allowing for egg collection. Approximately 100 eggs were collected and placed in individual vials with a total of ten vials collected per replicate selected or control line for each starvation treatment. The vials were maintained at 25°C with 12 hours of light exposure and 12 hours of darkness. Emerging adult flies were collected and scored based on sex from each of the vials at 8 hour time intervals until all flies had emerged.

Female fecundity

Female egg production was quantified using flies six generations removed from fifteen generations of selection for starvation resistance. Adult *Drosophila melanogaster* selected and control lines were subjected to either 32 hours of starvation or 32 hours in the presence of food at one day post eclosion. Following the treatments, flies were returned to food prior to conducting matings within each line. Fifteen replicates of single-pair matings between one male and one female obtained from each of the replicate selected and control starved and unstarved lines were used to obtain an average number of eggs produced by females per 24 hour time period. The number of eggs produced by each individually mated female was scored at the same time in the afternoon each day for 40 days.

Longevity

Life span determination for each of the replicate selected and control flies were conducted during the fifth generation removed from fifteen generations of starvation selection. Flies one day post eclosion were subjected to either a 32 hour period of starvation or 32 hours on food. Following this time period, 3 replicates of 30 males and 30 females from each line exposed to each treatment were placed in cages supplied with a food source kept at 25°C with 12 hours of light and 12 hours of darkness. The food was replaced every 48 hours and dead flies within the cages were removed by aspiration and scored based on sex. This study was conducted until total mortality was reached in all lines.

Movement

The number of movements of individual *Drosophila melanogaster* from the selected and control lines following either 32 hours of starvation or 32 hours in the presence of food were determined using individuals that were six generations removed from fifteen generations of artificial selection for starvation resistance. Mated flies selected for movement analysis were exposed to the presence or absence of starvation at 7 to 8 days post eclosion prior to placement within glass capillary tubes (5 millimeter diameter and 65 millimeter length). Once inside the tubes, the individuals were allowed to recover from ethyl ether exposure used to separate the sexes prior to experimentation. Food sources were provided to the individual *D. melanogaster* at both ends of the capillary tube, with one end only partially covered to allow airflow.

The placement of each individual within the 64 total spaces between the two *Drosophila* activity monitors (TriKinetics) was determined using a statistical randomization scheme generated by SAS 9.2 (SAS, 2009). Implementation of statistical randomization of sample placement within each of the monitors was used to reduce or eliminate positional effects in the acquisition of data. Each capillary tube was centered with respect to the monitor and secured in place with a rubber band.

Detection of the number of movements of individual flies was accomplished using an infrared beam that bisected the glass capillary tube located within each position of the monitors. Each time an individual fly crossed the beam, the computer recorded the movement. The number of movements were quantified for each of the six total replicates obtained from each selected and control line exposed to the presence or absence of

starvation over a ten minute time interval for a duration of 48 hours. This allowed for the identification of variation within and among lines and treatments with respect to alterations in light cycle. Environmental conditions were held constant during experimentation at 25°C with a 12 hour light/dark cycle. Relative humidity was maintained using moistened cotton balls placed within clear plastic bags surrounding each monitor. The cotton balls were moistened with distilled water daily to prevent desiccation of the experimental subjects.

Statistical analysis

A mixed model analysis was conducted using SAS 9.2 software (SAS, 2009) to analyze the effect of selection and starvation, and to determine the presence or absence of significant interactions between selection and starvation. The model included fixed effects for replication, selection, starvation, and the interaction between selection and starvation with a random effect of selected lines. Statistical significance within the data was defined as possessing a p-value equal to or less than 0.05. Least squares means estimates of statistically significant data were compared to determine the direction of change in concentrations within and among the lines exposed to each treatment.

Results

Artificial selection for starvation resistance

A direct response to artificial selection for starvation resistance was observed in the selected lines. Comparisons of percent survival under starvation at generations one and fifteen indicated a two-fold increase in survival at the fifty percent mortality level in both sexes (figure 2.1). Survival rates under starvation measured in the control lines compared at generations one and fifteen indicated no significant difference in male and female survival over the generations (figure 2.2). Average survival rates under starvation were compared within the starvation-resistant and control lines following fifteen generation of selection and six generations of relaxation from selection (figure 2.3). A slight reduction in the response to selection was observed in the starvation–resistant lines following six generations of relaxed selection; however, no statistically significant differences in mortality levels were observed in the comparison between generations one and six. This indicated that the selected lines maintained levels of resistance to starvation during the relaxation period.

Soluble protein concentrations

Comparisons of soluble protein concentrations obtained from male and female *Drosophila melanogaster* exposed to each treatment indicated a statistically significant effect of starvation in both sexes (tables 2.9 and 2.10), while the effect of selection reached statistical significance only in males (table 2.8). Whole body soluble protein

concentrations (table 2.1) measured per fly indicated significantly lower concentrations of proteins in the starved state as compared to the unstarved state in both sexes (p-values <0.0001 females, 0.0391 males). Measurements normalized by dry weight and lean mass, however, resulted in the opposing response in males and females. Flies exposed to starvation possessed significantly greater protein concentrations as compared to flies that remained in the presence of food (p-values <0.0001). Female concentrations accounting for lean mass did not reach statistical significance, but showed similar trends in protein concentrations. These results when coupled to the per fly measurements indicated an effect of weight normalization that made the proportion of protein appear to be increased in the presence of starvation. Male concentrations normalized by dry weight indicated a statistically significant effect of selection (table 2.8), where selected lines possessed significantly reduced soluble protein concentrations as compared to control lines (p-values 0.0406). Concentrations in males reported per fly resulted in selected males with significantly greater protein concentrations than control males (p-value, 0.0222). This suggests that the alterations in concentrations reported per fly and with regards to mass may be due to alterations in the accumulation of stored energy between the selected and control lines.

Analysis of the interaction between starvation and selection indicated reduced soluble protein concentrations in starved selected male (table 2.11) measurements normalized by dry weight and lean mass, with no statistically significant differences recorded between starvation-resistant and control lines in the fed state. Unstarved males in each measurement possessed greater quantities of total protein than males in the

starved state regardless of the presence or absence of selection for starvation resistance. Concentrations normalized by lean mass and dry weight indicated that starved selected males possessed reduced protein concentrations as compared to starved control males (p-values 0.0151, and 0.0178 respectively). This result may perhaps be attributed to alterations in energy storage between the selected and control lines.

Carbohydrate concentrations

Starvation (tables 2.9 and 2.10) and selection (table 2.8) had a statistically significant effect on carbohydrate concentrations. Measurements of carbohydrates within starvation-resistant and control *Drosophila melanogaster* (table 2.2) indicated a statistically significant effect of starvation, with starved flies possessing reduced concentrations of carbohydrates as compared to flies remaining in the fed state in both sexes (p-values <0.0001). Selection had a statistically significant effect in female measurements reported per fly, and normalized with respect to dry weight and lean mass (table 2.8) with selected females possessing increased carbohydrate concentrations as compared to control females (p-values <0.0001 and 0.0035 respectively). A similar result was observed in male measurements recorded per fly where the selected lines acquired increased carbohydrate concentrations as compared to the control lines (p-value 0.0227), which is indicative of increased energy storage in lines with an evolved resistance to starvation.

Interactions between starvation and selection suggested that starvation resulted in reduced quantities of carbohydrates, with increased concentrations detected in selected

lines as compared to controls (table 2.11). Females carbohydrate measurements recorded per fly indicated a statistically significant interaction between selection and starvation with the starved females possessing significantly reduced carbohydrate concentrations as compared to unstarved females regardless of the presence or absence of selection. Moreover, female measurements recorded per fly indicated an increased concentration of carbohydrates in the selected lines as compared to the control lines regardless of starvation conditions (p-value 0.0308). Male carbohydrate concentrations normalized by dry weight indicated that males in the starved state possessed reduced concentrations of total carbohydrates as compared to males that remained fed regardless of selective pressures. Additionally, starved selected males were found to possess greater carbohydrate concentrations than starved control males (p-values 0.0191). No statistically significant differences were detected between selected and control lines with regards to carbohydrate concentrations when flies remained in the presence of food, suggesting that brief exposure to starvation has a more significant effect on carbohydrate concentrations than the evolved resistance to starvation.

Measurements of stored carbohydrates, in the form of glycogen, indicated a statistically significant effect of starvation in all recorded measurements (tables 2.9 and 2.10), while no significant interactions between starvation and selection were observed in either sex (tables 2.3 and 2.11). Exposure to a brief period of starvation resulted in a reduction in glycogen concentrations in both males and females (p-values <0.0001). The observed reduction in glycogen in response to starvation suggested the mobilization of energy to withstand periods of food deprivation.

Comparisons of total sugar concentrations indicated a significant effect of starvation in both sexes (tables 2.9 and 2.10), while an interaction between starvation and selection was detected only in males (table 2.11). Total sugar concentrations obtained from whole body samples of selected and control lines in the starved or unstarved states (table 2.4) indicated a statistically significant effect of starvation resulting in reduced total sugar concentrations in the starved state as compared to the unstarved state in all recorded measurements (p-values <0.0001). Statistically significant interactions between selection and starvation were detected in all measurements recorded in males. Concentrations that were normalized per fly and by lean mass exhibited significantly reduced sugar concentrations in the starved state as compared to the unstarved state regardless of the presence or absence of selection. No statistically significant differences were detected between selected and control males in the unstarved state; however, starved selected males were found to possess significantly greater concentrations of sugars than starved control males (p-values 0.0092 and 0.0001 respectively). In male concentrations normalized with respect to dry weight, the selected lines acquired increased amounts of total sugars as compared to the control lines regardless of the presence or absence of starvation (p-value <0.0001). This interaction suggested an underlying pattern that resulted in a reduction of total sugar concentrations in response to starvation with the selected lines possessing increased quantities of sugar as compared to the matched control lines.

Measurements of trehalose concentrations indicated a significant effect of starvation in both sexes (tables 2.9 and 2.10), while a significant effect of selection was

observed only in males (table 2.8). Comparisons of total body trehalose indicated a statistically significant effect of starvation in both sexes in all recorded measurements (table 2.5), with decreased trehalose concentrations detected in the starved state as compared to the unstarved state (p-values <0.0001). Additionally, a statistically significant effect of selection was observed in male concentrations normalized per fly with increased trehalose concentrations in the selected lines as compared to the control lines (p-value 0.0468). Significant interactions between selection and starvation were recorded in male measurements normalized with respect to dry weight and lean mass, with selected males possessing greater trehalose concentrations than control males regardless of the presence or absence of starvation (p-values 0.0125 and 0.0284 respectively). Moreover, starved selected males were found to possess greater whole body trehalose concentrations than starved control males. Interactions between starvation and selection indicated that the selected lines possessed increased trehalose concentrations with the starved state resulting in an overall reduction of whole body trehalose.

Triglyceride and glycerol concentrations

Quantification of triglyceride concentrations indicated significant effects of starvation (tables 2.9 an 2.10) and selection (table 2.8) in both sexes, while a significant interaction between selection and treatment occurred only in female measurements recorded per fly (table 2.11). Triglyceride concentrations obtained from whole body samples of starvation-resistant and control *Drosophila melanogaster* males and females

indicated a statistically significant effect of starvation among all measurements (table 2.6) with the starved lines possessing significantly reduced triglyceride concentrations as compared to lines remaining in the presence of food (p-values <0.0001). Measurements of triglycerides recorded per fly in females indicated that selected lines possessed significantly greater concentrations of triglycerides as compared to control lines (p-value 0.0400). A similar observation occurred in male measurements recoded per fly and normalized with respect to dry weight and lean mass (p-values 0.0135, 0.0163, and 0.0153 respectively). The only statistically significant interaction between selection and starvation occurred in female measurements recorded per fly. No significant differences were observed between selected and control females in the starved state; however, selected females possessed significantly greater concentrations of triglycerides as compared to control females in the unstarved state (p-value 0.0508). This interaction indicates an overall reduction in triglyceride concentrations in response to starvation with the selected lines possessing increased quantities of stored lipids.

Measurements of free glycerol concentrations indicated a significant effect of starvation in females (table 2.9), while selection had a significant effect on glycerol concentrations in males (table 2.8). No significant interactions were detected between selection and starvation in comparisons of glycerol concentrations among the lines. Comparisons of whole body glycerol concentrations (table 2.7) indicated a statistically significant effect of starvation in females when measurements were recorded per fly and normalized with respect to lean mass. In this comparison, the starved lines possessed significantly reduced free glycerol concentrations as compared to the unstarved lines.

Selection had a statistically significant effect on male measurements recorded per fly resulting in selected lines with greater free glycerol concentrations than control lines. The altered effects of starvation and selection on glycerol concentrations obtained from females and males respectively indicated that perhaps females respond to starvation directly, while males have a greater response to an evolved resistance to starvation.

Life History Traits

Analysis of the effects of starvation and selection on life history traits indicated no significant differences in egg-to-adult developmental time or female fecundity, while measurements of longevity indicated a significant reduction in lifespan for the starvation-resistant lines. Measurements of egg-to-adult development time in starvation-resistant and control males and females indicated no significant difference between lines in either the starved or unstarved states (figures 2.4 and 2.5). Comparisons between male and female development time indicated that females emerge slightly before males; however, the difference in development was not statistically significant. Quantification of egg production by mated selected and control females that were either starved or unstarved indicated no statistically significant difference in the number of eggs produced (figure 2.6). Slight fluctuations in egg production occurred during the first 5 days past initial mating and spanning days 15 to 19 with the number of eggs produced by females declining past day fifteen. The initial alterations in egg production may be due to adaptation to the experimental environment, while the alterations spanning days 15 to 19 could perhaps be due to increased egg deposition prior to the decline in eggs production

observed in females at 15 days post eclosion. Longevity studies conducted on starvation-resistant and control *D. melanogaster* lines indicated a significant reduction in lifespan in selected males and females as compared to the control lines (figures 2.7 and 2.8). Furthermore, starvation had an effect on control female longevity with a reduction in lifespan recorded in the starved control lines (figure 2.7). Additional comparisons following 32 hours of starvation indicated no significant differences between selected and control lines in either sex; however, trends in the data indicated a reduction in lifespan in the selected lines as compared to the control lines.

Movement

Light had a significant effect on the number of movements recorded from males and females resulting in increased movement during the 12 hours of darkness as compared to the12 hours of light (p-values <0.0001). An interaction between starvation and light resulted in increased activity in both sexes during the darkness in lines exposed to either the starved or unstarved states (p-values 0.0109, <0.0001 females, <0.0001 males respectively). A significant interaction between selection and light in females resulted in control lines moving substantially more during the dark phase of the light cycle, with no effect of light detected in the selected lines (p-values <0.0001, 0.0241 respectively). The interaction between selection and light in males resulted in selected lines with reduced movements as compared to control lines (p-values <0.001) in either light cycle, with a greater number of movements recorded during the darkness regardless of selective pressures (p-values <0.0001, 0.0243 respectively). For males, selection had a

significant effect on movement resulting in selected lines moving less than the control lines (p-value 0.0006). The interaction between selection and starvation in males resulted in increased movement in the control lines regardless of the presence or absence of starvation. (p-values starved 0.0076, unstarved 0.0077).

Discussion

Starvation is a significant form of biological stress encountered by most organisms residing within natural populations (White, 1993). In response to periods of starvation, organisms from diverse taxa ranging from insects to vertebrates have evolved physiological responses that result in more efficient energy utilization during periods of starvation (Baker & Thummel, 2007). Three mechanisms have been proposed to explain the physiological adaptations associated with an evolved resistance to starvation (Ballard et al., 2008). The first mechanism addressed increased storage of energy molecules, while the second and third mechanisms focused on a reduction in the rate of energy utilization and an overall reduction in the minimal resources necessary for survival (Ballard et al., 2008). The purpose of the present study is not to prove or disprove these theories of increased starvation resistance; however, results obtained from this study investigating correlations between the direct and a series of indirect responses to selection were supportive of the first two proposed theories.

Artificial selection for starvation resistance on the population of *Drosophila melanogaster* utilized in this study resulted in a measurable direct response to selection. Selected males and females exhibited an approximately two-fold increase in survival under starvation following fifteen generations of selection for starvation resistance. Analysis of body composition of the starvation-resistant and control lines indicated a significant increase in the amount of triglycerides, glycerol, and total carbohydrates within the selected lines, which is consistent with previous studies measuring starvation resistance in *D. melanogaster* (Service, 1987; Zwann et al., 1991; Chippindale et al.,

1996; Harshman & Schmid, 1998; Djawdan et al., 1998; Baldal et al., 2006; Ballard et al., 2008). Triglyceride concentrations in both sexes were approximately two fold greater in the selected lines, while free glycerol concentrations were approximately three fold greater in selected males. Perhaps the increased glycerol concentrations in selected males can be attributed to increased liberation of free fatty acids to use in generating energy for survival. Measurements of total carbohydrates indicated that selected females possessed significantly greater concentrations as compared to control females. This result supports previous findings that starvation-resistant lines have adapted mechanisms to increase energetic storage in response to starvation (Chippindale et al., 1996; Harshman & Schmid, 1998; Djawdan et al., 1998). Measurements of whole body trehalose in selected males indicated an increased amount of circulating sugars. Perhaps, the increased concentrations can be explained by increased transport of sugars to the fat body to be used in synthesizing glycogen or triglycerides for storage.

Reduced concentrations of soluble proteins obtained from the selected males in the present study countered previous results that indicated no significant differences in protein concentrations between the starvation-resistant and control lines (Baldal et al., 2006). One possible explanation is that the selected lines are storing increased concentrations of high-energy molecules resulting in reduced availability for protein deposition. Another possible reason for the reduction in protein concentrations in the selected lines could be attributed to a reduction in translation. This phenomenon has been observed in caloric restricted *Drosophila melanogaster* lines that receive food with reduced nutrient content, producing a similar phenotype to flies that have been starved for

a brief period of time (Bjedov et al., 2010). The transition from a fed to starved state may require extensive changes in genes transcribed and expressed, thus accounting for reductions in RNA and protein concentrations. Overall, body composition data obtained from the selected and control lines indicated an increase in energy storage associated with increased resistance to starvation.

Analysis of the effects of starvation on both the starvation-resistant and control lines indicated the utilization of energy resources by both of the lines when exposed to starvation. Triglyceride and carbohydrate reserves, including glycogen, total sugars, and circulating trehalose concentrations, were reduced during starvation, perhaps for conversion into energy necessary for survival. Additionally, glycerol concentrations were reduced during starvation, suggesting that free glycerol was possibly converted into glucose for use in ATP production (Sugden et al., 1989). These results indicated the presence of a coordinated metabolic response that mobilized necessary energy by converting stored triglycerides and carbohydrates to energetic compounds for use in withstanding periods of starvation. Measurements of protein concentrations in the starved and fed lines indicated increased concentrations following exposure to starvation. One potential explanation for this occurrence is that starvation may result in an overall remodeling of metabolism that alters RNA expression and protein production within the organism. Perhaps, this adaptive response to biological stress may lead to a dramatic series of changes that alter metabolic function to be more efficient in processing stored energy compounds. As such, the organism may need to synthesize new RNA and proteins to be able to survive during periods of starvation.

In addition to measuring body composition in the starvation-resistant and control lines exposed to the presence or absence of a brief period of starvation, the numbers of movements were recorded to investigate correlations between metabolism and energy expenditure. Analysis of movement indicated that selected and control males and females exhibited greater activity during the 12 hours dark phase of the light cycle, with selected males exhibiting reduced movement as compared to control males. This observation of increased sedentary behavior in the selected lines holds true when comparisons are made regarding starvation, implying that selected lines conserve more energy by reducing the number of movements in both the presence and absence of starvation. Similar results were observed in a previous study measuring locomotion in starvation-resistant *Drosophila melanogaster*, where the selected lines exhibited more sedentary behavior (Hoffmann & Parsons, 1993; Williams et al., 2004). An additional study correlating circulating trehalose concentrations with movement in *D. melanogaster* indicated that a reduction in trehalose concentrations in males resulted in a correlated reduction in movement (Belgacem & Martin, 2006), which is supportive of results obtained in the present study.

Analysis of life history traits within the starvation-resistant and control lines resulted in no significant difference in egg-to-adult development time between the lines. This is contrary to previous studies in that the starvation-resistant lines possessed an increased egg-to-adult development time (Chippindale et al., 1996, Harshman et al., 1999a). One possible explanation for this discrepancy is that the flies used in this study had not been exposed to the high levels of mortality used for selection in previous

studies. Additionally, flies used in this study underwent only fifteen generations of artificial selection for starvation resistance, while other studies have used flies that have been selected for thirty or more generations. Measurements of female fecundity within the lines indicated no significant difference in the number of eggs produced by females in the selected or control lines exposed to the presence or absence of starvation, while previous studies indicated a negative relationship between fecundity and selection for starvation resistance (Harbison et al., 2004; Baldal et al., 2006). These studies conducted in *D. melanogaster* imply a cost of reproduction that was not observed within the lines used in the present study.

Measurements of lifespan indicated that selected males and females have reduced longevity as compared to the control lines. This result correlates with results obtained from studies using isofemale lines of *Drosophila simulans* that indicated a negative relationship between starvation-resistance and longevity (Ballard et al., 2008). Both the present study and the study conducted by Ballard support the Y model of life history evolution stating that a negative correlation exists between life history traits if the traits are competing for a common pool of reserved energy (vanNoordwijk & de Jong, 1986; Zera & Harshman, 2001). Additional studies analyzing the relationship between starvation-resistance and longevity have resulted in no association between the two traits (Luckinbill et al., 1984; Zwaan et al., 1995; Harshman et al., 1999b; Stearns et al., 2000), while other studies have demonstrated a positive correlation between starvation-resistance and longevity (Rose, 1984). These results suggest that longevity is a highly complex phenotype controlled by numerous genes that could respond differentially in the

various selection experiments analyzed (Harshman & Hoffmann, 2000; Ackermann et al., 2001).

The results obtained from this study measuring both the metabolic and physiological responses to starvation and genetic based starvation-resistance represent the most comprehensive investigation of the response to starvation and selection in *Drosophila melanogaster*. As such, these results have implications for organisms ranging from insects to vertebrates that reside in natural populations. One application of this study is investigating parallels between starvation-resistance and caloric restriction. It has been observed that limitations to caloric intake result in an extension of lifespan in insects as well as higher order mammals (Sinclair, 2005). The mechanisms underlying enhanced survival are suggested to be similar to starvation due to a highly conserved stress response that evolved in early life forms to increase survival of organisms exposed to periods of adverse environmental conditions (Sinclair, 2005). An additional application of results obtained from this study is the investigation of the development and persistence of metabolic diseases including obesity. This particular disease has reached epidemic proportions within the human population and has been statistically correlated with increased premature mortality (Hossain et al., 2007). By analyzing modifications to body composition and the resulting alterations in physiological responses in model organisms, one can begin to investigate the molecular basis resulting in an obese phenotype. Studies such as the one conducted in this paper can be utilized to further investigate metabolism and physiological responses associated with an obese phenotype in human populations.

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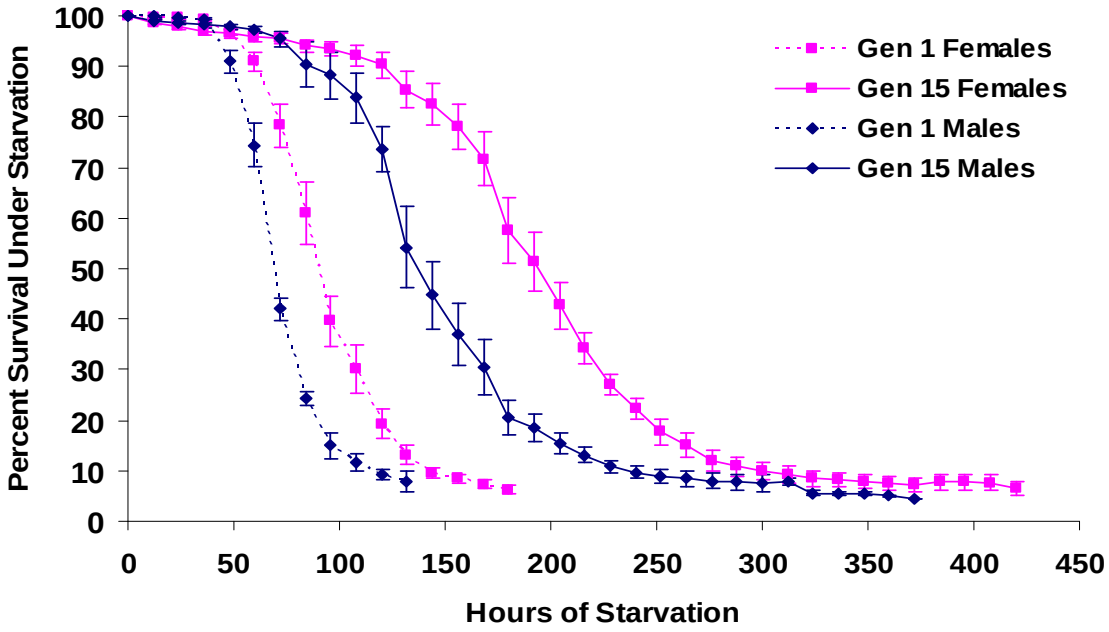

Figure 2.1 The response to selection of *Drosophila melanogaster* selected for starvation resistance. Percent survival under starvation (and standard error) was assayed after one generation of selection and following fifteen generations of selection for starvation resistance.

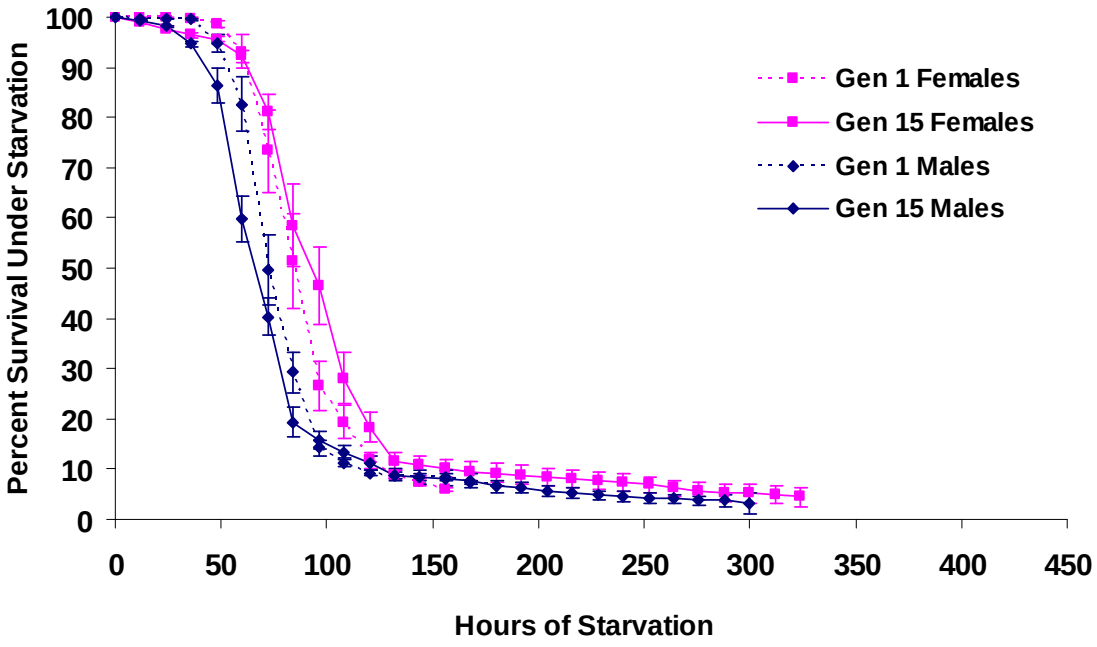

Figure 2.2 The response to starvation in control lines of Drosophila melanogaster. Percent survival under starvation (and standard error) was assayed following one and 15 generations of selection conducted on the starvation resistant lines.

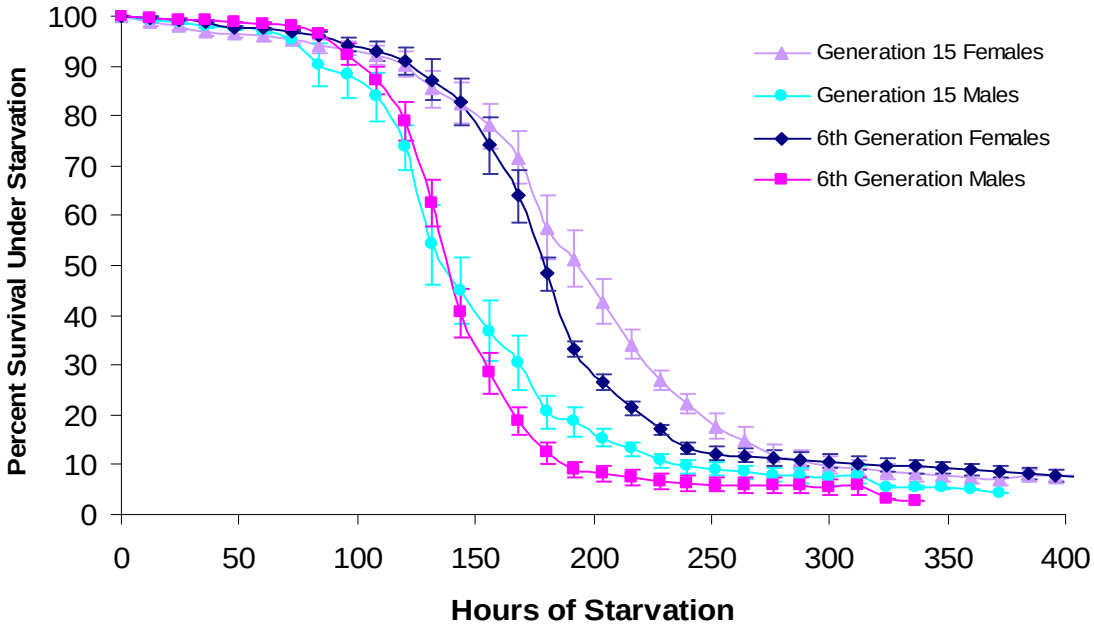

Figure 2.3 Comparisons of the direct response to selection in the starvation-resistant lines immediately following fifteen generations of selection and after six generations of relaxed selection. Percent survival under starvation (and standard error) of the starvation-resistant lines.

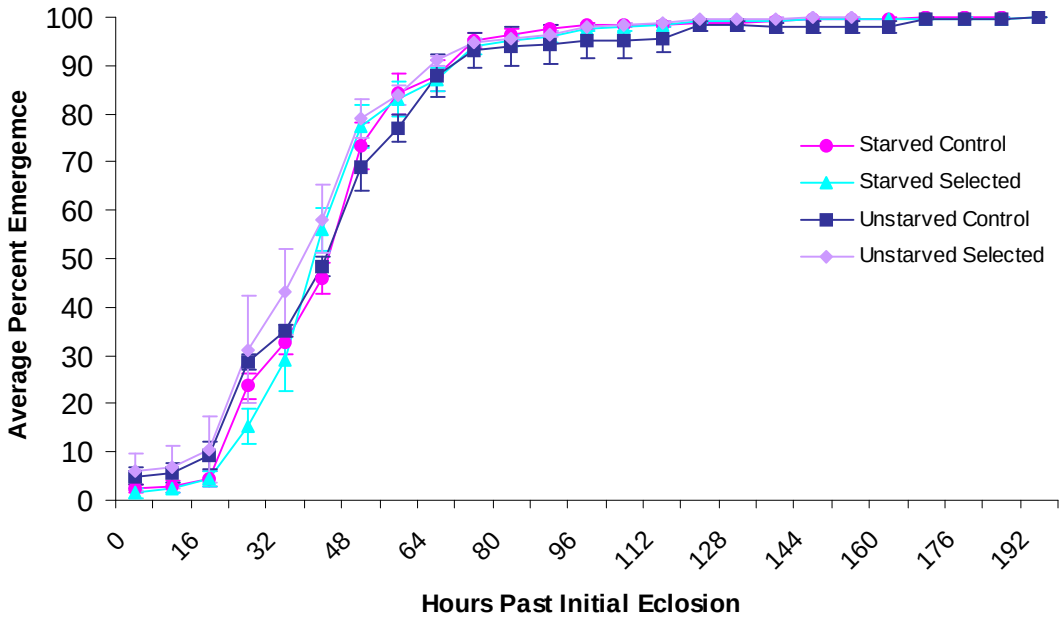

Figure 2.4 Development times (and standard error) of the selected and control lines of female *Drosophila melanogaster* following 15 generations of selection for starvation resistance.

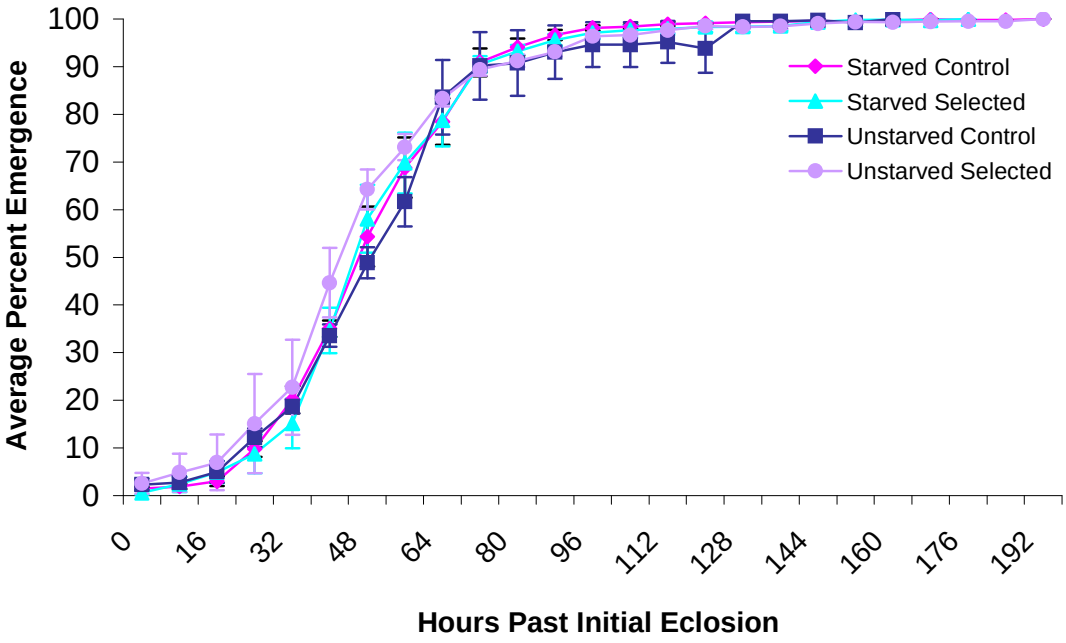

Figure 2.5 Development times (and standard error) of the selected and control lines of male *Drosophila melanogaster* following 15 generations of selection for starvation resistance.

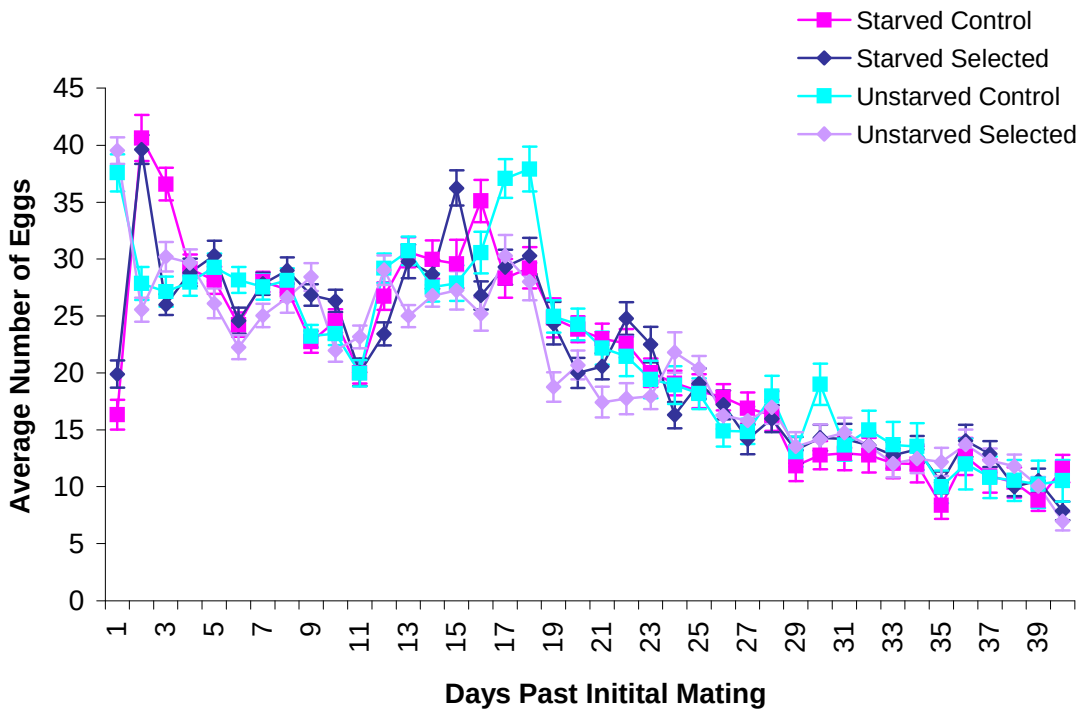

Figure 2.6 The average number of eggs produced by the selected and control lines (and standard error) following 15 generations of selection for starvation resistance

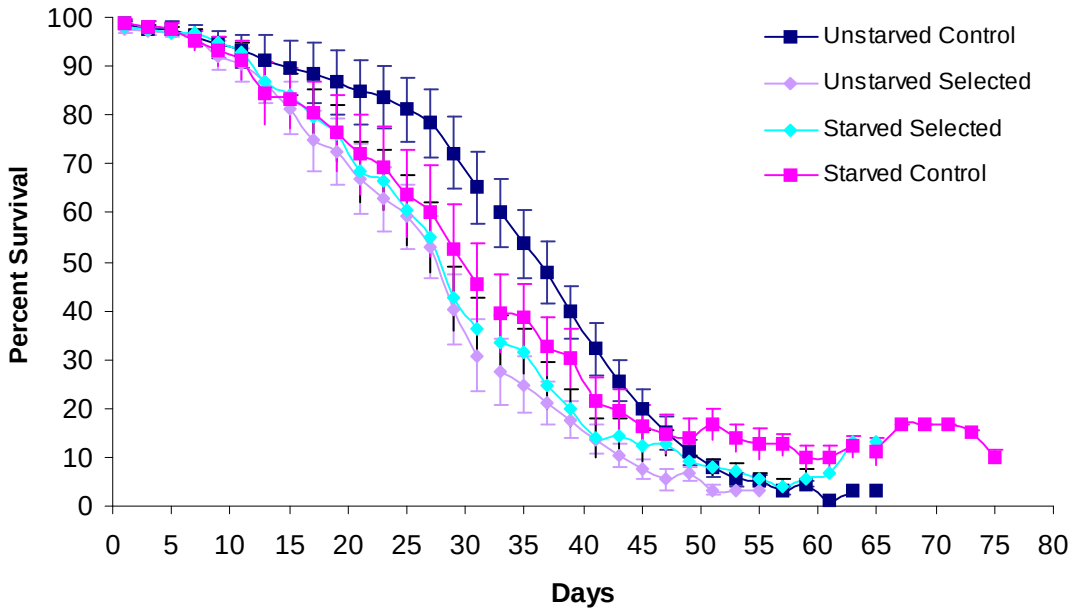

Figure 2.7 Average longevity (and standard error) of selected and control lines of female *Drosophila melanogaster* following 15 generations of selection for starvation resistance.

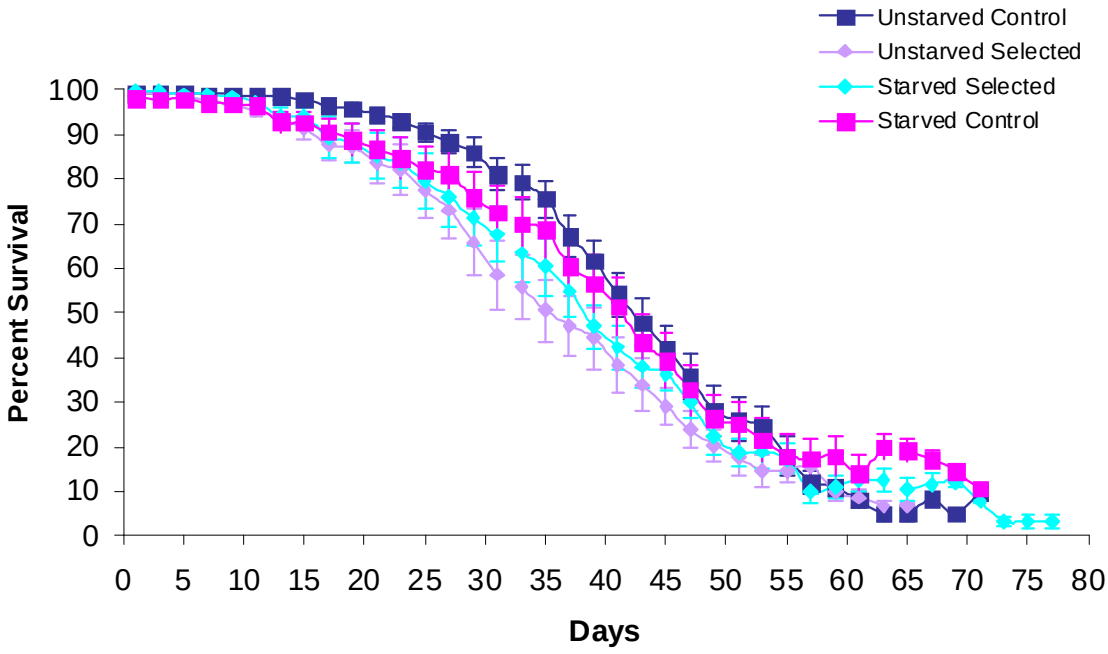

Figure 2.8 Average longevity (and standard error) of selected and control lines of male *Drosophila melanogaster* following 15 generations of selection for starvation resistance.

Table 2.1 Soluble protein concentrations within starvation resistant and control lines reported per fly and normalized with respect to lean mass and dry weight. Mean protein concentration (standard error) following 15 generations of selection for starvation resistance.

Line	Sex	Treatment	Protein concentration per fly	Protein concentration per lean mass	Protein concentration per dry weight
Selected	Male	Starved	0.2161 (0.0036)	1.1074 (0.0205)	0.8694 (0.0198)
Control	Male	Starved	0.2074 (0.0034)	1.1999 (0.0173)	0.9842 (0.0132)
Selected	Female	Starved	0.2391 (0.0057)	0.7243 (0.0096)	0.5812 (0.0096)
Control	Female	Starved	0.2343 (0.0076)	0.7877 (0.0320)	0.6546 (0.0260)
Selected	Male	Unstarved	0.2354 (0.0074)	1.0212 (0.0386)	0.6998 (0.0223)
Control	Male	Unstarved	0.2110 (0.0056)	0.9883 (0.0285)	0.7214 (0.0215)
Selected	Female	Unstarved	0.2632 (0.0063)	0.7648 (0.0192)	0.4930 (0.0094)
Control	Female	Unstarved	0.2547 (0.0093)	0.7951 (0.0324)	0.5522 (0.0202)

Table 2.2 Carbohydrate concentrations within starvation resistant and control lines reported per fly and normalized with respect to lean mass and dry weight. Mean carbohydrate concentration (standard error) following 25 generations of selection for starvation resistance.

Line	Sex	Treatment	Carbohydrate concentration per fly	Carbohydrate concentration per lean mass	Carbohydrate concentration per dry weight
Selected	Male	Starved	0.0050 (0.0002)	0.0256 (0.0009)	0.0201 (0.0008)
Control	Male	Starved	0.0027 (0.0002)	0.0154 (0.0014)	0.0126 (0.0011)
Selected	Female	Starved	0.0059 (0.0001)	0.0179 (0.0003)	0.0143 (0.0002)
Control	Female	Starved	0.0036 (0.0000)	0.0120 (0.0004)	0.0099 (0.0003)
Selected	Male	Unstarved	0.0149 (0.0005)	0.0644 (0.0015)	0.0442 (0.0012)
Control	Male	Unstarved	0.0127 (0.0006)	0.0594 (0.0027)	0.0433 (0.0020)
Selected	Female	Unstarved	0.0168 (0.0001)	0.0487 (0.0003)	0.0314 (0.0004)
Control	Female	Unstarved	0.0131 (0.0001)	0.0409 (0.0005)	0.0284 (0.0005)

Table 2.3 Glycogen concentrations within starvation resistant and control lines reported per fly and normalized with respect to lean mass and dry weight. Mean glycogen concentration (standard error) following 15 generations of selection for starvation resistance.

Line	Sex	Treatment	Glycogen concentration per fly	Glycogen concentration per lean mass	Glycogen concentration per dry weight
Selected	Male	Starved	0.0015 (0.0001)	0.0075 (0.0003)	0.0058 (0.0002)
Control	Male	Starved	0.0004 (0.0001)	0.0022 (0.0005)	0.0018 (0.0004)
Selected	Female	Starved	0.0022 (0.0002)	0.0065 (0.0007)	0.0052 (0.0006)
Control	Female	Starved	0.0010 (0.0001)	0.0035 (0.0003)	0.0029 (0.0003)
Selected	Male	Unstarved	0.0080 (0.0011)	0.0344 (0.0044)	0.0236 (0.0030)
Control	Male	Unstarved	0.0061 (0.0007)	0.0286 (0.0032)	0.0208 (0.0026)
Selected	Female	Unstarved	0.0144 (0.0010)	0.0417 (0.0032)	0.0269 (0.0017)
Control	Female	Unstarved	0.0123 (0.0003)	0.0384 (0.0011)	0.0266 (0.0009)

Table 2.4 Total sugar concentrations within starvation resistant and control lines reported per fly and normalized with respect to lean mass and dry weight. Mean sugar concentration (standard error) following 15 generations of selection for starvation resistance.

Line	Sex	Treatment	Total sugar concentration per fly	Total sugar concentration per lean mass	Total sugar concentration per dry weight
Selected	Male	Starved	0.0036 (0.0001)	0.0186 (0.0005)	0.0145 (0.0003)
Control	Male	Starved	0.0015 (0.0001)	0.0084 (0.0008)	0.0069 (0.0007)
Selected	Female	Starved	0.0048 (0.0003)	0.0144 (0.0009)	0.0115 (0.0007)
Control	Female	Starved	0.0028 (0.0001)	0.0092 (0.0005)	0.0076 (0.0004)
Selected	Male	Unstarved	0.0066 (0.0006)	0.0284 (0.0025)	0.0195 (0.0017)
Control	Male	Unstarved	0.0058 (0.0001)	0.0270 (0.0001)	0.0197 (0.0001)
Selected	Female	Unstarved	0.0100 (0.0008)	0.0291 (0.0022)	0.0187 (0.0015)
Control	Female	Unstarved	0.0068 (0.0006)	0.0213 (0.0020)	0.0147 (0.0012)

Table 2.5 Trehalose concentrations within starvation resistant and control lines reported per fly and normalized with respect to lean mass and dry weight. Mean trehalose concentration (standard error) following 15 generations of selection for starvation resistance.

Line	Sex	Treatment	Trehalose concentration per fly	Trehalose concentration per lean mass	Trehalose concentration per dry weight
Selected	Male	Starved	0.0005 (0.0001)	0.0028 (0.0006)	0.0021 (0.0004)
Control	Male	Starved	0.0001 (0.0001)	0.0003 (0.0007)	0.0002 (0.0005)
Selected	Female	Starved	0.0019 (0.0001)	0.0049 (0.0002)	0.0050 (0.0002)
Control	Female	Starved	0.0010 (0.0001)	0.0040 (0.0005)	0.0029 (0.0003)
Selected	Male	Unstarved	0.0018 (0.0001)	0.0076 (0.0003)	0.0052 (0.0002)
Control	Male	Unstarved	0.0014 (0.0001)	0.0064 (0.0003)	0.0046 (0.0002)
Selected	Female	Unstarved	0.0041 (0.0004)	0.0070 (0.0012)	0.0095 (0.0007)
Control	Female	Unstarved	0.0033 (0.0002)	0.0071 (0.0006)	0.0085 (0.0005)

Table 2.6 Triglyceride concentrations within starvation resistant and control lines reported per fly and normalized with respect to lean mass and dry weight reported per fly and normalized with respect to lean mass and dry weight. Mean triglyceride concentration (standard error) following 15 generations of selection for starvation resistance.

Line	Sex	Treatment	Triglyceride concentration per fly	Triglyceride concentration per lean mass	Triglyceride concentration per dry weight
Selected	Male	Starved	0.4382 (0.0741)	2.0871 (0.3939)	1.6231 (0.2921)
Control	Male	Starved	0.9073 (0.1945)	0.7334 (0.1391)	0.6012 (0.1139)
Selected	Female	Starved	0.1281 (0.0252)	2.8174 (0.6204)	2.2206 (0.4831)
Control	Female	Starved	0.4070 (0.0772)	1.4666 (0.2451)	1.2270 (0.2107)
Selected	Male	Unstarved	3.1036 (0.3807)	7.5241 (1.6782)	5.1395 (1.1366)
Control	Male	Unstarved	5.5440 (0.8830)	3.2521 (0.2876)	2.3765 (0.2119)
Selected	Female	Unstarved	0.6934 (0.0605)	15.818 (2.4761)	10.424 (1.6644)
Control	Female	Unstarved	1.7333 (0.3859)	9.6617 (1.1754)	6.7428 (0.8533)

Table 2.7 Free glycerol concentrations within starvation resistant and control lines reported per fly and normalized with respect to lean mass and dry weight. Mean free glycerol concentration (standard error) following 15 generations of selection for starvation resistance.

Line	Sex	Treatment	Glycerol concentration Per fly	Glycerol concentration per lean mass	Glycerol concentration per dry weight
Selected	Male	Starved	0.0683 (0.0216)	0.4201 (0.1920)	0.3195 (0.1439)
Control	Male	Starved	0.0559 (0.0175)	0.2405 (0.0873)	0.1971 (0.0713)
Selected	Female	Starved	0.0410 (0.0146)	0.1717 (0.0552)	0.1353 (0.0432)
Control	Female	Starved	0.0816 (0.0375)	0.2346 (0.0760)	0.1956 (0.0632)
Selected	Male	Unstarved	0.1003 (0.0175)	0.6543 (0.1304)	0.4470 (0.0880)
Control	Male	Unstarved	0.1098 (0.0160)	0.2709 (0.0676)	0.1971 (0.0494)
Selected	Female	Unstarved	0.0580 (0.0145)	0.3142 (0.0447)	0.2062 (0.0306)
Control	Female	Unstarved	0.1501 (0.0294)	0.3150 (0.0561)	0.2173 (0.0382)

Table 2.8 Statistically significant least squares means values for the effects of selection on body composition measurements obtained from the selected and control lines exposed to the presence or absence of starvation. Least squares means (standard error) following 15 generations of selection for starvation resistance.

Effect	Sex	Dependent Variable	p-value	Selected	Control
Selection	Male	Protein per fly	0.0222	0.2257 (0.0038)	0.2092 (0.0038)
Selection	Male	Protein per dry weight	0.0406	0.7846 (0.0185)	0.8528 (0.0185)
Selection	Female	Total carbs per dry weight	0.0035	0.0228 (0.0005)	0.0192 (0.0005)
Selection	Female	Total carbs per lean mass	0.0004	0.0331 (0.0006)	0.0264 (0.0006)
Selection	Female	Total carbs per fly	<0.0001	0.0112 (0.0002)	0.0083 (0.0002)
Selection	Male	Total carbs per fly	0.0227	0.0099 (0.0005)	0.0076 (0.0005)
Selection	Male	Trehalose per fly	0.0468	0.0012 (0.0001)	0.0007 (0.0001)
Selection	Female	Triglyceride per fly	0.0400	3.2256 (0.3937)	1.7709 (0.3937)
Selection	Male	Triglyceride per fly	0.0135	1.0702 (0.1349)	0.4107 (0.1349)
Selection	Male	Triglyceride per dry weight	0.0163	3.3813 (0.4049)	1.4888 (0.4049)
Selection	Male	Triglyceride per lean mass	0.0153	4.8056 (0.5924)	1.9927 (0.5924)
Selection	Male	Glycerol per fly	0.0447	0.1159 (0.0185)	0.0495 (0.0185)

Table 2.9 Statistically significant least squares means values for the effects of starvation on female body composition measurements obtained from the selected and control lines exposed to the presence or absence of starvation. Least squares means (standard error) following 15 generations of selection for starvation resistance.

Effect	Sex	Dependent Variable	p-value	Starved	Unstarved
Starvation	Female	Protein per fly	<0.0001	0.2367 (0.0081)	0.2589 (0.0081)
Starvation	Female	Protein per dry weight	<0.0001	0.6179 (0.0194)	0.5226 (0.0194)
Starvation	Female	Glycogen per dry weight	<0.0001	0.0040 (0.0011)	0.0267 (0.0011)
Starvation	Female	Glycogen per lean mass	<0.0001	0.0050 (0.0018)	0.0400 (0.0018)
Starvation	Female	Glycogen per fly	<0.0001	0.0016 (0.0005)	0.0132 (0.0005)
Starvation	Female	Total carbs per dry weight	<0.0001	0.0123 (0.0005)	0.0297 (0.0005)
Starvation	Female	Total carbs per lean mass	<0.0001	0.0151 (0.0006)	0.0445 (0.0006)
Starvation	Female	Total carbs per fly	<0.0001	0.0047 (0.0002)	0.0148 (0.0002)
Starvation	Female	Total sugars per fly	<0.0001	0.0037 (0.0005)	0.0084 (0.0005)
Starvation	Female	Total sugars per dry weight	<0.0001	0.0096 (0.0011)	0.0168 (0.0011)
Starvation	Female	Total sugars per lean mass	<0.0001	0.0118 (0.0017)	0.0251 (0.0017)
Starvation	Female	Trehalose per dry weight	<0.0001	0.0036 (0.0006)	0.0074 (0.0006)
Starvation	Female	Trehalose per lean mass	<0.0001	0.0044 (0.0008)	0.0111 (0.0008)
Starvation	Female	Trehalose per fly	<0.0001	0.0014 (0.0002)	0.0037 (0.0002)
Starvation	Female	Triglyceride per fly	<0.0001	0.6728 (0.3701)	4.3238 (0.3701)
Starvation	Female	Triglyceride per dry weight	<0.0001	1.7238 (0.7495)	8.5838 (0.7495)
Starvation	Female	Triglyceride per lean mass	<0.0001	2.1420 (1.0487)	12.740 (1.0487)
Starvation	Female	Glycerol per lean mass	0.0327	0.2032 (0.0435)	0.3146 (0.0435)
Starvation	Female	Glycerol per fly	0.0095	0.0620 (0.0130)	0.1051 (0.0130)

Table 2.10 Statistically significant least squares means values for the effects of starvation on male body composition measurements obtained from the selected and control lines exposed to the presence or absence of starvation. Least squares means (standard error) following 15 generations of selection for starvation resistance.

Effect	Sex	Dependent Variable	p-value	Starved	Unstarved
Starvation	Male	Protein per fly	0.0391	0.2117 (0.0038)	0.2232 (0.0038)
Starvation	Male	Protein per lean mass	<0.0001	1.1537 (0.0250)	1.0048 (0.0250)
Starvation	Male	Protein per dry weight	<0.0001	0.9268 (0.0161)	0.7106 (0.0161)
Starvation	Male	Glycogen per dry weight	<0.0001	0.0038 (0.0019)	0.0222 (0.0019)
Starvation	Male	Glycogen per lean mass	<0.0001	0.0048 (0.0026)	0.0313 (0.0026)
Starvation	Male	Glycogen per fly	<0.0001	0.0009 (0.0006)	0.0070 (0.0006)
Starvation	Male	Total carbs per dry weight	<0.0001	0.0163 (0.0014)	0.0437 (0.0014)
Starvation	Male	Total carbs per lean mass	<0.0001	0.0205 (0.0017)	0.0618 (0.0017)
Starvation	Male	Total carbs per fly	<0.0001	0.0038 (0.0004)	0.0137 (0.0004)
Starvation	Male	Total sugars per fly	<0.0001	0.0025 (0.0003)	0.0061 (0.0003)
Starvation	Male	Total sugars per dry weight	<0.0001	0.0107 (0.0010)	0.0195 (0.0010)
Starvation	Male	Total sugars per lean mass	<0.0001	0.0134 (0.0014)	0.0276 (0.0014)
Starvation	Male	Trehalose per dry weight	<0.0001	0.0016 (0.00004)	0.0049 (0.00004)
Starvation	Male	Trehalose per lean mass	<0.0001	0.0022 (0.0006)	0.0069 (0.0006)
Starvation	Male	Trehalose per fly	0.0468	0.0004 (0.0001)	0.0015 (0.0001)
Starvation	Male	Triglyceride per fly	<0.0001	0.2676 (0.1349)	1.2133 (0.1349)
Starvation	Male	Triglyceride per dry weight	<0.0001	1.1121 (0.4049)	3.7580 (0.4049)
Starvation	Male	Triglyceride per lean mass	<0.0001	1.4102 (0.5924)	5.3881 (0.5924)

Table 2.11 Statistically significant least squares means values for the effects of the interaction between selection and starvation on body composition measurements obtained from the selected and control lines exposed to the presence or absence of starvation. Least squares means (standard error) following 15 generations of selection for starvation resistance.

Sex	Dependent Variable	p-value	Control Starved	Control Unstarved	Selected Starved	Selected Unstarved
Male	Protein per lean mass	0.0151	1.1999 (0.0354)	0.9883 (0.0354)	1.1074 (0.0354)	1.0212 (0.0354)
Male	Protein per dry weight	0.0178	0.9842 (0.0228)	0.7214 (0.0228)	0.8695 (0.0228)	0.6998 (0.0228)
Male	Total carbs per dry weight	0.0191	0.0127 (0.0020)	0.0433 (0.0020)	0.0200 (0.0020)	0.0442 (0.0020)
Female	Total carbs per fly	0.0308	0.0036 (0.0002)	0.0130 (0.0002)	0.0059 (0.0002)	0.0166 (0.0002)
Male	Total sugars per fly	0.0092	0.0014 (0.0004)	0.0057 (0.0004)	0.0036 (0.0004)	0.0065 (0.0004)
Male	Total sugars per dry weight	<0.0001	0.0068 (0.0014)	0.0197 (0.0014)	0.0145 (0.0014)	0.0194 (0.0014)
Male	Total sugars per lean mass	0.0001	0.0083 (0.0020)	0.0270 (0.0020)	0.0185 (0.0020)	0.0282 (0.0020)
Male	Trehalose per dry weight	0.0125	0.0002 (0.0006)	0.0046 (0.0006)	0.0031 (0.0006)	0.0052 (0.0006)
Male	Trehalose per lean mass	0.0284	0.0002 (0.0009)	0.0063 (0.0009)	0.0041 (0.0009)	0.0076 (0.0009)
Male	Trehalose per fly	<0.0001	5.1×10^{-5} (0.0001)	0.0013 (0.0001)	0.0008 (0.0001)	0.0017 (0.0001)
Female	Triglyceride per fly	0.0508	0.4382 (0.5234)	3.1036 (0.5234)	0.9073 (0.5234)	5.5440 (0.5234)

Chapter 3.

Investigating respiration and movement in genetically based starvation-resistant and control lines of *Drosophila melanogaster*

Abstract

Resistance to biological stress, including starvation, is an adaptive mechanism that alters the phenotype to withstand deleterious environments. One theory of increased resistance to starvation suggests the presence of a coordinated physiological response resulting in a reduction of metabolic rate coupled to a reduction in energy expenditure. The purpose of this study is to investigate respiration and movement in genetically based starvation-resistant and control lines of *Drosophila melanogaster* exposed to the presence or absence of a brief period of starvation. An overall reduction in metabolic rate was observed in the starvation-resistant lines as compared to the control lines based on measurements of carbon dioxide production and oxygen consumption. Additionally, measurements of carbon dioxide production following 32 hours of starvation indicated a reduction in metabolic rate within the starvation-resistant lines. Respiratory quotient values reported per fly suggested increased conversions of carbohydrates to lipids in the starvation-resistant lines, while analysis of the control lines indicated carbohydrate oxidation as the primary source of metabolic fuel. The number of movements recorded using individual starvation-resistant and control flies previously used for respiration indicated an overall reduction in movement in the selected lines. The results obtained from this study indicated a positive correlation between respiration and movement supportive of the theory that an evolved resistance to starvation is correlated with decreased metabolic rate and a reduction in energy expenditure to promote survival under adverse conditions.

Introduction

Biological stress has been previously defined as an alteration in the environment that results in a reduction of organismal fitness (Koehn & Bayne, 1989; Sibly & Calow, 1989; Hoffman & Parsons, 1991). Inadequate or inedible food resources leading to acute starvation conditions represent a significant form of biological stress, presumably encountered by most organisms residing in natural populations (White, 1993; Harshman & Schmid, 1998; Harshman et al., 1999; Rion & Kawecki, 2007). In response to alterations in the environment, including periods of starvation, organisms have evolved adaptive physiological mechanisms that alter the phenotype to promote enhanced survival (Djawdan et al., 1998; Harshman & Schmid, 1998; Rion & Kawecki, 2007). An evolved resistance to starvation could be achieved through a coordinated physiological response that couples a reduction in metabolism with an overall conservation of energy reserves (Harshman & Schmid, 1998). This is accomplished, in part, through the storage of nutrients during times when food is plentiful to allow for increased energetic reserves when food resources are later depleted (Chippindale et al., 1996; Hoffman et al., 2005; Rion & Kawecki, 2007). Additionally, a reduction in metabolic rate and movement serve as a means to conserve energy during periods when food is scarce (Harshman & Schmid, 1998; Knoppien et al., 2000; Williams et al., 2004). The purpose of this study is to investigate correlations between respiration and movement in genetically based starvation-resistant and control lines of *Drosophila melanogaster* to identify physiological relationships between metabolism and energy expenditure in the presence or absence of starvation.

An overall reduction in metabolic rate has been proposed as a key mechanism utilized in an evolved resistance to starvation (Hoffmann & Parsons, 1991). In *Drosophila*, the mitochondria produce carbon dioxide as a metabolic byproduct during oxidative phosphorylation (Williams et al., 1997; Harshman & Schmid, 1998). The carbon dioxide released during ATP production is then transported through a highly branched system of cuticle-lined tubes located throughout the organism to the trachea, where it is released to the external environment (Hetz & Bradley, 2005). Diffusion of carbon dioxide out of the body during gas exchange occurs at valve-like structures known as spiracles that are located on the surface of the abdomen and thorax (Hetz & Bradley, 2005; Lehmann & Schutzner, 2009). Since *D. melanogaster* only respire aerobically, measuring the concentration of expelled carbon dioxide using respirometry provided a reasonable estimation of metabolic rate (Djawdan et al., 1997; Harshman & Schmid, 1998).

Artificial selection for starvation resistance in *Drosophila melanogaster* has been frequently used to investigate phenotypic alterations associated with increased tolerance to starvation (Djawdan et al., 1998; Harshman et al., 1999; Baldal et al., 2006; Rion & Kawecki, 2007). Previous studies in *Drosophila* have indicated a significant relationship between stress responses, metabolism, and the maintenance of energetic homeostasis within the organism (Hoffman & Parsons, 1991). Resistance to biological stress, including starvation, can be attained through alterations in the storage and metabolism of high-energy molecules resulting in the conservation of resources until nutrient intake can be resumed (Harshman et al., 1999). Experiments utilizing laboratory-based artificial

selection for starvation resistance in *Drosophila melanogaster* have indicated an increased abundance of triglycerides and glycogen in response to selection (Chippendale et al., 1998; Djawdan et al., 1998; Harshman et al., 1999; Marron et al., 2003). Additional studies utilizing respiration to measure metabolism in starvation-resistant lines indicated a reduction in metabolic rate in response to selection, with reduced carbon dioxide production in lines exposed to brief periods of starvation (Djawdan et al., 1997). Similar results were observed in a related study with reduced specific metabolic rates obtained from starvation-resistant females as compared to control females (Harshman & Schmid, 1998). Further research of metabolic rates normalized with respect to body mass in *D. melanogaster* has indicated no significant differences between starvation-resistant and control lines (Djawdan et al., 1998; Harshman & Schmid, 1998). This observation could be attributed to the increased weight of metabolically inert lipid and carbohydrate stores present within the selected lines (Harshman et al., 1999). When carbon dioxide production was normalized with respect to weight, the metabolic rates appeared lower in flies with increased body mass (Harshman et al., 1999).

A reduction in energetic expenditures of the organism in response to starvation has been suggested to be an added mechanism to allow for the preservation of metabolic fuels (Harshman et al., 1999). Previous analysis of movement patterns in *Drosophila* revealed that activity occurred in discrete bouts that were separated by periods of inactivity, with males exhibiting a more continuous pattern of walking than females (Martin et al., 1999). Measurements of activity levels within lines selected for starvation resistance indicated a reduction in the number of movements obtained from the selected

lines (Williams et al., 2004). When exposed to starvation, the selected lines exhibited decreased activity levels as compared to the controls (Knoppien et al., 2000). Behavioral studies conducted on *Drosophila melanogaster* indicated that selected lines exposed to starvation exhibited a reduced search response to obtain food as compared to the control lines that have not previously evolved mechanisms to withstand starvation (Bell & Tortorici, 1987; Rion & Kawecki, 2007).

The main objectives of this study were to analyze oxygen consumption and carbon dioxide production in starvation-resistant and control *Drosophila melanogaster* lines exposed to the presence or absence of a brief period of starvation to investigate the associated physiological responses. Calculations of respiratory quotients obtained from each individual provided information on metabolic fuels utilized in both the starvation-resistant and control lines in the presence or absence of 32 hours of starvation. Additionally, measurements of the number of movements recorded from individuals previously used for analysis of metabolic rates were acquired to identify correlations between respiration and movement. Results obtained from this study provided insight into energy utilization in response to starvation in lines with an evolved resistance to starvation and those reared in the absence of selective pressures.

Materials and Methods

Drosophila melanogaster stocks

The *Drosophila melanogaster* base population used in artificial selection for starvation resistance was initially derived from a series of inbred lines collected in a manner designed to preserve natural genetic variation. Experimentation began by obtaining a large population of inseminated females from the Woflskill Experimental Orchard in Yolo County, located in Northern California. The collected females were placed in a laboratory setting with the resulting progeny used in 20 generations of inbreeding to produce a series of lines. Ten of the established lines were intercrossed using one virgin female and one male to generate all possible combinations of pair-wise matings with all reciprocal crosses conducted between the lines. From the approximately 100 crosses performed, 150 progeny per cross were selected for placement within a large population cage (12 inches high, 36 inches wide, and 24 inches deep. The food source within the population cage consisted of a series of twenty open food bottles distributed throughout the cage. Four of the twenty total food sources consisting of yeast extract, molasses, cornmeal, glucose, water, agar, and a mixture of acids to prevent mold growth were replaced weekly, thus ensuring that the oldest food was removed and continually replaced with a fresh food source. This overlapping population regime contributed to the preservation of lifespan duration and stress resistance responses, which can be lost when flies are maintained in a laboratory culture (Hoffmann et al., 2001, Linnen, 2001). The resulting base population of *D. melanogaster* was maintained for one year prior to

establishing a derived population used for conducting artificial selection for starvation resistance.

The population of *D. melanogaste*r used in experimentation was divided into eight subpopulations, which were randomly separated into the four starvation–resistant and four control lines. Flies used in the artificial selection experiment were obtained by placing ten cut glass bottles containing food in randomized locations throughout the population cage. Bottles were removed each day to collect eggs residing on the surface of the media. Approximately 100 eggs were collected and placed in individual vials containing food to generate a series of vials for each line. Vials containing the collected eggs were stored in a controlled environment maintained at a temperature of 25 degrees Celsius with a 12 hour light/dark cycle. Prior to replacing the bottles in the population cage for resuming egg collection on the following day, the surface of the media was removed to eliminate any residual eggs or larvae. This process was repeated until each subpopulation consisted of approximately 4,000 individuals. Adults emerging from the collected eggs were allowed to randomly mate within each subpopulation prior to collecting eggs for the next generation. A series of four generations of matings were conducted prior to initiating selection for starvation resistance on the following generation of adult flies. Population sizes for each of the replicate selected and control lines were consistently maintained at a density of 2,000 males and 2,000 females.

Artificial selection for starvation resistance was performed using adult mated flies that were collected five days post eclosion. Males and females from each line were separated and placed in cages that either contained food for the control lines or agar for

the selected lines. Prevention of desiccation within the selected lines was achieved by supplying individuals with solidified agar as a water source. Petri-plates containing either food or agar were routinely removed from the cages every 48 hours to supply the flies with a fresh food or water source and prevent the introduction of progeny. The temperature, light exposure, and relative humidity were held constant during experimentation with subjects being kept at 25 degrees Celsius with a 12 hour light/dark cycle. Relative humidity was maintained by placing a moist paper towel in a clear plastic bag surrounding each cage. The paper towel was changed in conjunction with the plates to ensure adequate moisture and prevent desiccation of experimental subjects.

Direct responses to selection were measured within each individual replicate cage of selected males or females by calculating the mortality levels at 12 hour intervals. *D. melanogaster* that had died during that time frame were aspirated out of the experimental cages and were disposed of following the determination of mortality rates. In order to maintain consistency in experimentation, the control cages were additionally observed every 12 hours to determine mortality levels in the presence of food. Flies that had died during this time frame were removed and counted in the manner previously described. Upon reaching 50 percent mortality levels in one of the selected male or female cages, individuals within the matched selected and control lines were removed from the cages following brief exposure to carbon dioxide, which acted as an anesthetic. Individuals removed from the cages were placed in plastic bottles containing food that were maintained at 25 degrees Celsius with a12 hour light/dark cycle. Flies were allowed a 2 day recovery period prior to being mated to produce progeny for the next generation of

selection for starvation resistance. A population size of approximately 450 males and 450 females was used for mating each of the four selected and control populations prior to collecting eggs for the next generation. A total of 25 to 27 generations of selection for starvation resistance were conducted prior to collecting individuals from each of the three generations to obtain measurements of respiration and movement.

Respiration and movement values were obtained using flies collected from generations 25, 26, and 27 of selection for starvation resistance. Two generations of relaxation from selection were conducted following each of the three generations used for experimentation to prevent parental effects. Relaxation of selection was defined as the absence of artificial selection on the starvation-resistant lines. Individuals obtained from generations 25 and 26 were used to measure the volume of oxygen consumption, carbon dioxide production, and movement of individuals obtained from each line. Additionally, a pool of five flies was used to determine oxygen consumption and carbon dioxide production within larger samples. *D. melanogaster* obtained following generation 27 of artificial selection for starvation resistance were subjected to either 32 hours of starvation, with exposure to only agar as a water source, or 32 hours remaining on food. Following exposure to the presence or absence of starvation, selected and control males and females from each of the four replicate lines were used for measurements of respiration and movement, as previously described.

Respiration

Respiration was conducted on twelve replicate individuals and pools of five selected and control *Drosophila melanogaster* following generations 25 and 26 of selection for starvation resistance. Additionally, seven replicates per selected and control line were obtained following generation 27 to evaluate the effects of 32 hours of starvation or 32 hours in the presence of food with regards to oxygen consumption and carbon dioxide production. Mated flies randomly selected to undergo experimentation were collected five to seven days post eclosion. Following brief exposure to ether, males and females from generations 25 and 26 were separated and allowed a one day acclimation period in the presence of food prior to measuring respiration. Flies from generation 27 were additionally exposed to the presence or absence of 32 hours of starvation prior to measuring the amount of oxygen consumed and carbon dioxide produced by each individual and pool of five flies. All measurements were recorded during the 12 hour light phase of the light cycle to eliminate potential alterations in respiration that can be attributed to changes in behavior associated with changes in light. Following respiratory measurements, each replicate individual and pool of five flies were aspirated out of the syringes and placed in the presence of food for a one day recovery period prior to quantifying the number of movements of the individual flies used in respiration.

Two types of respiratory designs have been previously used to determine metabolic rate and calculate respiratory quotients in *Drosophila melanogaster*. Stop-flow respiration consists of gasses held at a constant volume, while flow through respiration

allows gasses to flow continuously through the recording chambers. Stop flow respiration was selected for this experiment because it provided an accurate method of high throughput analysis of respiratory gas exchange. Due to the increased number of samples used for analysis, this method was essential for obtaining precise readings in a reasonable amount of time.

To obtain accurate readings of oxygen consumption and carbon dioxide production from flies selected for experimentation, the air used within the respiratory apparatus, otherwise known as scrubbed air, was filtered to remove impurities that can alter the data (figure 3.1). Air within the system was initially acquired from the surrounding environment by a Tygon tube placed within a Nalgene carboy, which was used to reduce air turbulence (20 liter capacity). Following collection, water vapor and carbon dioxide were removed using a column of drierite and sodalime, respectively. Airflow within the system was consistently regulated between 50 to 80 milliliters per minute using the SS3 subsampler (Sable Systems). This particular airflow rate used in experimentation was selected to optimize data normalization during analysis.

Following the 24 hour acclimation period in the presence of food, individuals or pools of five *D. melanogaster* were aspirated into separate five milliliter plastic syringes. Aspiration of the flies was used to prevent additional effects of ether on the experimental subjects that could potentially alter measurements of respiration. Ambient air within the syringes was flushed and replaced with 3 milliliters of scrubbed air at the flush junction of the respiratory apparatus (figure 3.1). A total of 12 syringes per individual time point possessing either one individual or a pool of five flies obtained from generations 25 or 26

were placed within the PELT-5 environmental chamber maintained at 25 degrees Celsius with a 12 hour light cycle until respiration measurements were obtained. A series of seven syringes were placed within the chamber during a single time point for flies collected from generation 27. The time points used for generations 25 and 26 consisted of three separate three hour windows that spanned from 8:00 am to l1:00 am, noon to 3:00 pm, and 5:00 pm to 8:00 pm. These three time points were selected to identify potential alterations in respiration measurements due time of day. A singular time point was used for respiration in individuals selected from generation 27 that spanned from 3:00 pm to 7:00 pm.

At approximately seven to twenty minutes following the flushing of residual air from the syringes, the respired air from the individual or pool of five flies was injected into the respiration system at the injection port (figure 3.1). Respired air was passed through a scrubber containing magnesium perchlorate to remove water from the air collected from each syringe. Following water removal, the respired air was passed to the CA-10 carbon dioxide analyzer (Sable Systems), where carbon dioxide production values were recorded. The respired air was then passed through an ascarite scrubber to remove any residual carbon dioxide prior to entering the FC-10 oxygen analyzer (Sable Systems). Upon obtaining a reading for the amount of oxygen consumed by the individual or pool of five flies within each syringe, the respired air was returned to the surrounding environment.

Carbon dioxide production and oxygen consumption data were collected by the UI2 apparatus (Sable Systems) prior to analysis. Baseline readings for carbon dioxide and

oxygen concentrations were calculated using Expedata software (Sable Systems). Prior to experimentation, the carbon dioxide analyzer was set to zero, while values for oxygen were corrected by 20.95 during data analysis to account for the percentage of oxygen within the surrounding environmental air. To adjust for fluctuations in the carbon dioxide and oxygen readings due to the rate of injecting the respired air into the system, a blank syringe possessing only scrubbed air was injected following every five readings. The values obtained from the blank syringes were subtracted from the initial experimental readings, thus normalizing the data.

Carbon dioxide and oxygen concentrations within the respired air were obtained by aligning the oxygen and carbon dioxide peaks collected from each syringe. The area under each of the peaks was calculated and transformed into values of oxygen consumption (equation 3.1) and carbon dioxide production (equation 3.2). The values for oxygen and carbon dioxide were converted into a rate by dividing the milliliter volume of each gas by the time in minutes that the individuals or pools of five flies were respiring within the syringe. Additionally, respiratory quotients were calculated (equation 3.3) to be used to determine the primary metabolic fuel utilized by starvation-resistant and control flies subjected to the presence or absence of a brief period of starvation.

Movement

The number of movements of each individual *Drosophila melanogaster* obtained from each of the three generations subjected to respiratory studies was measured to identifypossible correlations between respiration and movement in the selected and

control lines exposed to the presence or absence of starvation. Following experimentation to determine the amount of oxygen consumption and carbon dioxide production, individuals were allowed one day of recovery on food prior to being aspirated into separate glass capillary tubes (5 millimeters in diameter and 65 millimeters in length). Food sources were provided within the tubes at either end, with only one end partially covered by food to allow for airflow during experimentation. Positioning within the 64 total spaces in the two *Drosophila* activity monitors (TriKinetics) was completed using a statistical randomization scheme obtained from SAS 9.2 (SAS, 2009). This method of randomization was used to reduce or potentially eliminate a positional effect in data acquisition. Once each tube containing an individual fly was correctly positioned, the tubes were centered with respect to the monitor and secured in place using a rubber band.

Activity levels for each of the twelve replicates per selected and control line collected from generations 25 and 26, or seven replicates per selected and control line in the starved and unstarved states in generation 27 were recorded every ten minutes for a total of 24 hours. The numbers of movements within this time frame were detected when an individual *Drosophila melanogaster* crossed an infrared beam that bisects the individual capillary tubes positioned within each of the monitors. Measuring the number of movements over a 24 hour time period allowed for the detection of variations in movement patterns within the lines spanning the entire light cycle. During experimentation, the environmental conditions were held constant at a temperature of 25 degrees Celsius with 12 hours of light and 12 hours of dark. The relative humidity was also maintained by placing a moistened cotton ball within a clear plastic bag surrounding

each monitor to prevent desiccation of the individual *Drosophila melanogaster* used in experimentation.

Weight measurements and data normalization

Dry weight measurements for both the individual flies that were used in respiration and movement studies, and in the pools of five flies used to measure respiration were obtained by flash freezing the individual flies or pools of five flies in liquid nitrogen following the conclusion of activity monitoring. The frozen flies were stored at -80 degrees Celsius until dry weight measurements were obtained. In order to ensure the complete removal of water from the tissues prior to obtaining a weight, the flies were placed in a 65 degree Celsius oven for 24 hours. Following this time period, flies were individually weighed to obtain dry weights for each individual fly and an average weight for the pool of five flies from each of the selected and control lines in the presence or absence of starvation. Data obtained from the respiratory studies was recorded per fly and was additionally normalized with respect to dry weight.

Statistical analysis

The respiration and movement data obtained from the starvation-resistant and control lines following generations 25, 26, and 27 were analyzed using SAS 9.2 software (SAS, 2009) to conduct a linear mixed model analysis for identifying variations in oxygen consumption, carbon dioxide production, respiratory quotient values, and the number of movements. The model used in analysis included fixed effects factors for

selection, starvation, generation of selection, and generation removed. When only one level of a factor was present, that factor was dropped from the analysis. Additionally, the model included random effects for the selection line nested within selection criteria and each replicate. When examining the association of movement and respiration, movement was added as a covariate to the respiration models. Statistically significant results were identified as those possessing a p-value equal to or less than 0.05. Once identified as statistically significant, the least squares means values were compared to determine the direction and magnitude of the change in the starvation-resistant lines as compared to the controls with respect to respiration and movement.

Results

Respiration

Respiration data was considered separately for each generation of selection due to differences in experimental design. In generation 27, flies were exposed to the presence or absence of starvation, while generation 25 and 26 flies remained in the presence of food. Comparisons between generations 25 and 26 indicated a significant difference between the generations with generation 25 values of carbon dioxide production and oxygen consumption consistently greater than generation 26 values. Measurements of carbon dioxide production obtained from pools of five males or five females indicated increased values collected from generation 25 as compared to generation 26 (p-values 0.0103 and 0.0080 respectively). Oxygen consumption measurements recorded per fly in both sexes (p-values <0.0001 females and 0.0104 males) and with respect to dry weight in females (p-value 0.0162) indicated increased concentrations obtained from generation 25 flies as compared to generation 26 flies. Due to the significant differences between generations 25 and 26, the main focus of the analysis will be on the generations separately.

Measurements obtained from pools of five flies collected two generations removed from generation 25 indicated a significant effect of selection (table 3.16) on oxygen consumption and respiratory quotient values (tables3.5 and 3.7), while no significant effect of selection was observed in comparisons of carbon dioxide production. Oxygen consumption values normalized with respect to dry weight indicated a reduction

in oxygen concentrations in selected females as compared to control females (p-value 0.0385). Additionally, respiratory quotient values recorded per fly in males and females indicated increased values obtained from the selected lines as compared to the controls (p-values 0.0004 and 0.0028 respectively). Measurements of respiratory quotients normalized with respect to dry weight, however, indicated an opposing effect where the selected females possessed decreased respiratory quotient values as compared to control females (p-value 0.0494). Average respiratory quotient values recorded from generation 25 ranged from 1.0 to 1.3 indicating carbohydrate oxidation at respiratory quotient values of 1.0 and increased conversion of carbohydrates to lipids at respiratory quotients values greater than 1.0 within the control and selected lines, respectively.

Analysis of generation 26 flies indicated a significant effect of selection (table 3.16) on carbon dioxide production, oxygen consumption, and respiratory quotients in measurements obtained from individuals and pools of five flies (tables 3.2, 3.4, 3.6, and 3.8). Measurements of carbon dioxide production normalized with respect to dry weight in pools of five flies of each sex indicated a reduction in carbon dioxide values obtained from the selected lines as compared to the controls (p-value 0.0282 females and 0.0364 males). Similar effect were observed in oxygen consumption values normalized with respect to dry weight obtained from pools of five males or five females indicating a reduction in oxygen consumption by the selected lines (p-values 0.0088 and 0.0053 respectively). Comparisons of respiratory quotients obtained from generation 26 flies indicated a significant effect of selection in female measurements normalized with respect to dry weight (p-value 0.0507) resulting in selected females with a reduced

respiratory quotient as compared to control females. Average respiratory quotient values obtained from generation 26 flies ranged from 0.7 to 1.4 indicating increased fat oxidation in lines where the values were below 1.0 and increased carbohydrate to lipid conversions in lines where the respiratory quotient exceeded a value of 1.0.

Starvation had a statistically significant effect (table 3.18) on carbon dioxide production values obtained from generation 27 flies (tables 3.9 and 3.10). Analysis of carbon dioxide production indicated a reduction in carbon dioxide values obtained from the starved lines as compared to flies in the fed state in measurements recorded per fly and with respect to dry weight (p-values 0.0423 and 0.0215 respectively). No significant differences in oxygen consumption or respiratory quotients were detected between the selected and control lines in either the starved or fed states. Respiratory quotient values obtained from generation 27 flies were all close to 1.0 indicating carbohydrate oxidation as the primary metabolic fuel.

Single fly measurements of carbon dioxide production in generations 26 and 27 females indicated a significant effect of selection in generation 26 (table 3.16) and starvation in generation 27 (table 3.18) in measurements analyzed using movement as a covariate. Carbon dioxide production measured per fly in generation 26 females indicated increased concentrations obtained from the selected lines as compared to the controls (p-value 0.0475). Analysis of the effects of starvation in generation 27 females indicated reduced carbon dioxide production in starved females as compared to fed females when measurements were recorded per fly and with respect to dry weight (p-values 0.0330 and 0.0237 respectively).

Movement

Selection had a significant effect on the number of movements (tables 3.16 and 3.17) recorded in flies obtained from generations 26 and 27 (tables 3.2, 3.13, 3.14, and 3.15). Movement values obtained from generation 26 males during the 12 hours of darkness indicated a reduction in the number of movements recorded in the selected lines as compared to the controls (p-value 0.0122). Additionally, a significant effect of selection was detected in the number of movements recorded during time point three in generation 26 males with the selected lines moving significantly less than the controls (p-value 0.0123). Movement values obtained from generation 27 males indicated a significant effect of selection in both the dark and light phases of the 12 hour light cycle. During the 12 hours of darkness, selected males moved significantly less than control males (p-value 0.0014). A similar effect was observed during the 12 hour light phase with selected males moving significantly less than control males (p-value 0.0055). Comparisons of the number of movements between generations 25 and 26 indicated increased movement in generations 25 females during the dark phase (p-value 0.0295).

Discussion

Acute exposures to periods of starvation are an ecologically significant form of biological stress encountered by most organisms residing in natural populations (White, 1993). In response to increased environmental stress, organisms have evolved adaptive physiological responses that allowed for enhanced survival during periods of inadequate or unsuitable food resources (Harshman & Schmid, 1998; Harshman et al., 1999; Rion and Kawecki, 2007). Overall reductions in metabolic rate and conservation of energy reserves have been suggested to be the primary mechanisms used for survival (Knoppien et al., 2000; Williams et al., 2004). Previous studies of stress resistance in *Drosophila melanogaster* have indicated a strong positive association between increased stress resistance and reductions in metabolic rate (Hoffman & Parsons, 1991). This suggested that reductions in metabolism to the level of somatic maintenance were a key component in the plastic response associated with resistance to starvation employed by organisms in both natural and laboratory based populations (Djawdan et al., 1997; Marron et al., 2003).

Reductions in energy expenditure have been frequently correlated with reduced metabolic rates in organisms with an evolved resistance to biological stress (Williams et al., 2004). In *Drosophila*, movement and flight are energetically costly with the metabolic rate increased approximately fifty to one hundred fold during flight (Arrese & Soulages, 2010). To conserve energy, starvation-resistant organisms have implemented behavioral modifications that result in increased sedentary behavior with an overall reduction in flight and movement until food resources were restored (Hoffmann & Parsons, 1993; Williams et al., 2004; Rion & Kawecki, 2007; Ballard et al., 2008). This combination of

reduced metabolism coupled with a conservation of energy allowed organisms to survive during adverse environmental conditions. In the present study, quantification of metabolic rate was achieved through measurements of respiration in starvation-resistant and control lines of *Drosophila melanogaster* that were either briefly exposed to starvation or remained continuously fed. The numbers of movements in individuals previously measured for respiration were correlated with respiratory values to identify significant associations between metabolism and energy expenditure in starvation-resistant and control lines exposed to the presence or absence of a brief period of starvation.

A statistically significant difference between comparisons of selected and control lines collected from generations 25 and 26 was observed in carbon dioxide production, oxygen consumption, and the number of movements. Within these measurements, generation 25 values were consistently greater than generation 26. Due to the observed differences between the generations, values from each generation were considered separately. Reductions in carbon dioxide production in generation 26 flies supported the theory that increased selection for starvation resistance resulted in reduced metabolic rates (Djawdan et al., 1997; Marron et al., 2003). The magnitude of the observed change was interesting in that one would expect the lines to approach a steady state with only slight alterations observed in responses correlated with starvation resistance from generations 25 to 26. This suggested that perhaps an extraneous environmental variable could be altering the data between the generations, thus producing the observed differences.

Carbon dioxide concentrations normalized with respect to dry weight obtained from generations 25, 26, and 27 indicated that the selected lines produced significantly less carbon dioxide than the control lines. This result was in contrast with previous studies in starvation-resistant *Drosophila* lines that indicated an increase in carbon dioxide production by the starvation-resistant lines (Djawdan et al., 1997). Potential explanations for the observed differences between the present and past studies were differences in respiratory measurements, stop-flow respiration used in this study versus flow through respiration in previous studies, and alterations in design and degree of selection. Measurements of carbon dioxide production with regards to the presence or absence of starvation obtained from flies collected from generation 27 indicated a reduction in carbon dioxide production in lines exposed to a brief period of starvation as compared to the unstarved lines recorded per fly and with respect to dry weight. This result was consistent with previous findings in *D. melanogaster* that indicated a reduction in carbon dioxide production in control lines exposed to starvation as compared to lines remaining in the presence of food (Djawdan et al., 1997; Marron et al., 2003). Previous studies using starvation in *Drosophila melanogaster* indicated an overall reduction in metabolic rate in response to starvation (Djawdan et al., 1997; Marron et al., 2003), which supports the findings obtained from the current study.

A correlation between carbon dioxide production and movement was detected in flies obtained from generations 26 and 27. Reductions in the number of movements were correlated with reduced carbon dioxide production in selected lines as compared to control lines obtained from generation 26. Additionally, a reduction in movement was

correlated with a reduction in carbon dioxide production in the starved lines as compared to the fed lines in measurements obtained from generation 27. The correlation between movement and carbon dioxide production was supportive of the theory of increased energy conservation within starvation-resistant flies and flies exposed to brief periods of starvation.

Starvation-resistant females obtained from generations 25 and 26 consumed less oxygen than control females. Similar observations of oxygen consumption occurred in generation 27 flies; however, the results did not reach statistical significance. Reduced oxygen consumption in the selected lines indicated a reduction in metabolic rate within selected females as compared to control females, which was supported by previous studies of respiration in *Drosophila melanogaster* (Harshman et al., 1999). Reductions in oxygen consumption within the selected lines have been suggested as a possible mechanism for enhanced resistance to starvation. Increased oxygen levels within the organism can be toxic due to increased concentrations of oxygen free radicals. These particular molecules in high concentrations can result in increased tissue damage, which can lead to premature mortality (Hetz & Bradley, 2005). The results obtained from this study suggest that the reduction in oxygen concentrations observed in the starvation-resistant lines may perhaps be an adaptive mechanism for enhanced survival through reductions in free radical formation.

Calculations of the respiratory quotient obtained from generation 25 and 26 indicated that selected females possessed reduced metabolic quotients as compared to control females when measurements were normalized with respect to dry weight. This

was indicative of a reduction in metabolic rate in the selected lines; however, this result must be considered with caution because normalizing by dry weight accounts for a large volume of metabolically inert lipids stored within the starvation-resistant lines (Harshman et al., 1999). Respiratory quotient values reported per fly in generation 25 indicated increased values obtained from the selected lines as compared to the controls. Similar results were observed in generations 26 and 27; however, the respiratory quotient values did not reach statistical significance. Respiratory quotient values close to 1.0 were indicative of carbohydrate oxidation, while values close to 0 indicated fat oxidation (Chadwick, 1947; Lighton, 2008). Values exceeding 1.0 for the respiratory quotient were indicative of the conversion of carbohydrate into fat, resulting in increased lipid storage within the organism (Chadwick, 1947; Lighton, 2008). Respiratory quotient values that exceeded 1.0 have increasingly greater error associated with the measurement, so values that were significantly greater than 1.0 were considered with caution. In general, the respiratory quotient values measured per fly obtained from the selected lines slightly exceeded 1.0 indicating increased carbohydrate to fat conversions. This result was consistent with previous findings that increased selection for starvation resistance resulted in increased lipid deposition within the selected lines (Chippendale et al., 1998; Djawdan et al., 1998; Harshman et al., 1999; Marron et al., 2003).

The number of movements recorded in individual flies previously measured for respiration indicated a reduction in movement in the starvation-resistant lines as compared to the control lines. Measurements recorded in both the 12 hours of darkness and 12 hours of light indicated a reduction in male movement across all three

generations, which was consistent with previous findings of reduced movement in the starvation-resistant lines (Hoffman& Parsons, 1993; Williams et al., 2004; Rion & Kawecki, 2007; Ballard et al., 2008). The reduction in movement observed in the selected lines used the present study supported the theory that starvation-resistant lines were more sedentary, and were thus capable of conserving greater amounts of energy when food resources were depleted (Hoffman & Parsons, 1993; Williams et al., 2004; Ballard et al., 2008). Analysis of movement in the control lines indicated increased movement following exposure to starvation. These values were not statistically significant within this study; however, it illustrated the increased search behavior displayed by organisms that have not evolved a resistance to starvation (Knoppien et al., 2000). Previous studies have suggested that males exhibited more continuous patterns of walking, while females have a more periodic pattern of movement with bouts of inactivity (Martin et al., 1999). The sex related differences in activity levels could potentially explain the lack of statistical significance in variations of female movement patterns observed within the context of this study.

Comparisons of respiration values and activity levels obtained from this study indicated a positive correlation between metabolism and movement. Analysis of carbon dioxide production and oxygen consumption in the selected lines using movement as a covariate indicated that a reduction in movement was correlated with reduced metabolism. Moreover, exposure to a brief period of starvation resulted in reduced carbon dioxide production that when coupled with the movement data supported an overall reduction in metabolic rate with conservation of energy reserves. The results

obtained from this study indicated a significant positive association between metabolism and movement in starvation-resistant lines of *Drosophila melanogaster*. An overall reduction in metabolic rate coupled to a reduction in movement recorded in the selected lines supported the theory that an evolved resistance to starvation resulted in a reduction of metabolic rate coupled to an increased conservation of energy.

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Equation 3.1

$$\mathbf{VO_2 = (FiO_2\text{-}FeO_2) * FR / (1\text{-}FeO_2)}$$

The volume of oxygen consumed (VO_2) was calculated by taking the difference of the fractional concentration of oxygen entering the chamber (FiO_2), and the oxygen concentration leaving the chamber (FeO_2), multiplied by the flow rate (FR). This value is then divided by the difference of one minus the concentration of oxygen leaving the chamber (FeO_2) to obtain the volume of oxygen consumed by the organism. FiO_2 is equivalent to 0.2095 and is used to account for atmospheric oxygen content. The volume of oxygen consumed is measured in milliliters per minute.

Equation 3.2

$$\mathbf{VCO_2 = STP * FR * (FeCO_2 - FiC0_2) – (FeCO_2 * V0_2) / (1\text{-}FeCO_2)}$$

The volume of carbon dioxide expelled (VCO_2) was calculated by taking the standard temperature and pressure of the ambient air (STP) and multiplying it by both the flow rate (FR) and the difference between the concentration of carbon dioxide leaving the chamber ($FeCO_2$) and the fractional concentration of carbon dioxide entering the chamber ($FiCO_2$). This value is then subtracted from the product of the carbon dioxide concentration leaving the chamber ($FeCO_2$) and the volume of oxygen from equation 1 (VO_2). The resulting value is divided by the difference between one minus the carbon dioxide concentration leaving the chamber ($FeCO_2$). $FiCO_2$ is equivalent to zero based on experimental setup. The volume of carbon dioxide production was measured in milliliters per minute.

Equation 3.3

$$\mathbf{RQ = VCO_2 / VO_2}$$

The respiratory quotient (RQ) is calculated by taking the volume of carbon dioxide expelled (VCO_2) by the fly or series of flies and dividing it by the volume of oxygen consumed (VO_2) by the individual or series of five flies.

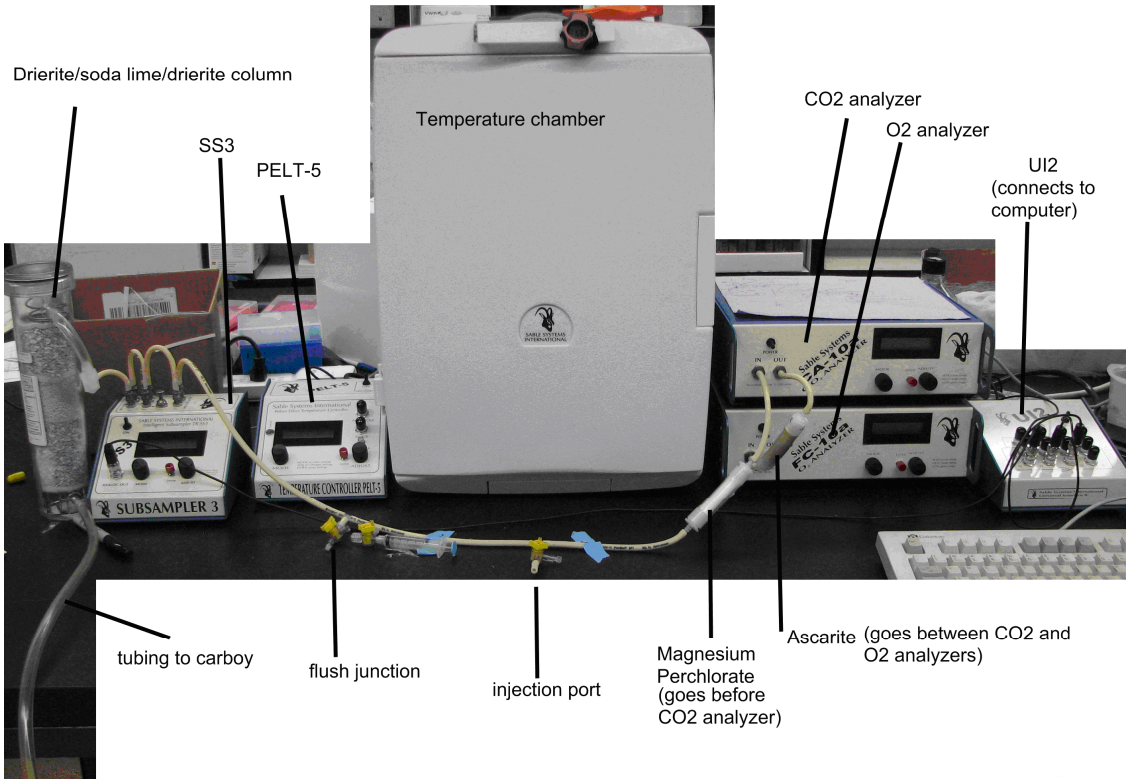

Figure 3.1 Diagram of the equipment setup for conducting respiration.

Table 3.1 Generation 25 respiratory and movement measurements for individual starvation resistant and control flies reported per fly. Mean respiratory or movement measurement (standard error) after 2 generations of relaxed selection following 25 generations of selection for starvation resistance.

Generation of Selection	Generations Removed	Replicate	Selection	Sex	O2_RATE	CO2_RATE	RQ	Movement
25	2	1	Control	Female	0.00010 ($1.3x10^{-5}$)	0.00012 ($1.4x10^{-5}$)	1.38914 (0.1301)	3.09825 (0.9589)
25	2	2	Control	Female	0.00008 ($1.0x10^{-5}$)	0.00010 ($1.1x10^{-5}$)	1.41709 (0.2302)	3.06140 (0.9928)
25	2	3	Control	Female	0.00013 ($1.9x10^{-5}$)	0.00014 ($2.5x10^{-5}$)	1.05875 (0.7616)	4.50000 (0.6305)
25	2	1	Selected	Female	0.00012 ($1.7x10^{-5}$)	0.00014 ($1.4x10^{-5}$)	1.29167 (0.1072)	2.40789 (0.6398)
25	2	2	Selected	Female	0.00008 ($1.0x10^{-5}$)	0.00010 ($1.0x10^{-5}$)	1.37943 (0.1068)	2.31955 (0.7274)
25	2	3	Selected	Female	0.00011 ($1.6x10^{-5}$)	0.00013 ($2.2x10^{-5}$)	1.24213 (0.1319)	4.68045 (0.8488)
25	2	1	Control	Male	0.00009 ($2.3x10^{-5}$)	0.00009 ($2.1x10^{-5}$)	1.35581 (0.1824)	7.45263 (2.1667)
25	2	2	Control	Male	0.00006 ($7.0x10^{-6}$)	0.00008 ($1.3x10^{-5}$)	1.32476 (0.1407)	3.30175 (1.3804)
25	2	3	Control	Male	0.00010 ($1.3x10^{-5}$)	0.00010 ($1.6x10^{-5}$)	1.05615 (0.1180)	7.80567 (1.3585)
25	2	1	Selected	Male	0.00006 ($1.0x10^{-5}$)	0.00009 ($9.0x10^{-6}$)	1.25702 (0.2299)	5.01404 (1.4438)
25	2	2	Selected	Male	0.00008 ($1.9x10^{-5}$)	0.00009 ($2.0x10^{-5}$)	1.53902 (0.3915)	6.00810 (2.4750)
25	2	3	Selected	Male	0.00008 ($1.1x10^{-5}$)	0.00009 ($1.2x10^{-5}$)	1.19312 (0.1365)	4.53947 (1.1053)

Table 3.2 Generation 26 respiratory and movement measurements for individual starvation resistant and control flies reported per fly. Mean respiratory or movement measurement (standard error) after 2 generations of relaxed selection following 26 generations of selection for starvation resistance.

Generation of Selection	Generations Removed	Replicate	Selection	Sex	O2_RATE	CO2_RATE	RQ	Movement
26	2	1	Control	Female	0.00008 ($1.2x10^{-5}$)	0.00010 ($1.3x10^{-5}$)	1.64337 (0.3141)	2.33772 (0.7860)
26	2	1	Control	Male	0.00007 ($2.7x10^{-5}$)	0.00009 ($2.9x10^{-5}$)	1.62703 (0.5285)	7.42807 (1.3108)
26	2	2	Control	Female	0.00007 ($1.5x10^{-5}$)	0.00009 ($9.0x10^{-6}$)	0.43424 (0.6593)	2.79649 (0.5944)
26	2	2	Control	Male	0.00007 ($1.1x10^{-5}$)	0.00008 ($1.5x10^{-5}$)	1.51607 (0.3594)	3.34035 (1.0335)
26	2	3	Control	Female	0.00010 ($2.3x10^{-5}$)	0.00009 ($2.3x10^{-5}$)	0.12789 (0.7753)	4.32794 (0.7640)
26	2	3	Control	Male	0.00006 ($6.0x10^{-6}$)	0.00006 ($5.7x10^{-5}$)	1.11131 (0.1466)	8.23509 (1.8451)
26	2	1	Selected	Female	0.00009 ($1.4x10^{-5}$)	0.00013 ($9.0x10^{-6}$)	3.60113 (1.4877)	4.94737 (1.0801)
26	2	1	Selected	Male	0.00006 ($7.0x10^{-6}$)	0.00007 ($6.0x10^{-6}$)	1.31846 (0.1574)	4.26316 (1.0656)
26	2	2	Selected	Female	0.00009 ($1.1x10^{-5}$)	0.00011 ($1.6x10^{-5}$)	1.33739 (0.2706)	2.83806 (0.9651)
26	2	2	Selected	Male	0.00013 ($6.8x10^{-5}$)	0.00014 ($5.9x10^{-5}$)	1.65698 (0.4280)	2.79352 (0.6240)
26	2	3	Selected	Female	0.00010 ($3.3x10^{-5}$)	0.00010 ($3.0x10^{-5}$)	7.92602 (4.2269)	4.26692 (0.6594)
26	2	3	Selected	Male	0.00005 ($1.3x10^{-5}$)	0.00006 ($1.0x10^{-5}$)	3.03091 (2.0106)	6.03158 (1.1038)

Table 3.3 Generation 25 respiratory and movement measurements for individual starvation resistant and control flies reported per dry weight. Mean respiratory or movement measurement (standard error) after 2 generations of relaxed selection following 25 generations of selection for starvation resistance.

Generation of Selection	Generations Removed	Replicate	Selection	Sex	O2_RATE	CO2_RATE	RQ	Movement
25	2	1	Control	Female	0.00024 ($3.7x10^{-5}$)	0.00028 ($4.7x10^{-5}$)	3.25000 (0.3939)	3.09825 (0.9925)
25	2	1	Control	Male	0.00030 ($8.3x10^{-5}$)	0.00029 ($5.1x10^{-5}$)	5.22532 (0.9207)	7.45263 (2.1667)
25	2	2	Control	Female	0.00020 ($2.3x10^{-5}$)	0.00022 ($2.3x10^{-5}$)	2.7382 (0.2708)	3.06140 (0.9928)
25	2	2	Control	Male	0.00019 ($2.5x10^{-5}$)	0.00023 ($3.0x10^{-5}$)	4.66596 (0.7677)	3.30175 (1.3804)
25	2	3	Control	Female	0.00028 ($5.9x10^{-5}$)	0.00029 ($7.4x10^{-5}$)	2.17284 (0.1883)	4.50000 (0.6305)
25	2	3	Control	Male	0.00037 ($5.5x10^{-5}$)	0.00039 ($7.1x10^{-5}$)	4.23509 (0.6934)	7.80567 (1.3585)
25	2	1	Selected	Female	0.00020 ($1.8x10^{-5}$)	0.00025 ($1.9x10^{-5}$)	2.61909 (0.2319)	2.40789 (0.6398)
25	2	1	Selected	Male	0.00022 ($3.8x10^{-5}$)	0.00031 ($3.6x10^{-5}$)	4.16946 (0.8534)	5.01404 (1.4438)
25	2	2	Selected	Female	0.00017 ($2.3x10^{-5}$)	0.00022 ($3.0x10^{-5}$)	2.64190 (0.3353)	2.31955 (0.7274)
25	2	2	Selected	Male	0.00033 ($9.3x10^{-5}$)	0.00036 ($9.4x10^{-5}$)	6.47304 (2.3534)	6.00810 (2.4750)
25	2	3	Selected	Female	0.00026 ($4.9x10^{-5}$)	0.00032 ($6.6x10^{-5}$)	2.77834 (0.3272)	4.68045 (0.8488)
25	2	3	Selected	Male	0.00028 ($5.5x10^{-5}$)	0.00027 ($4.1x10^{-5}$)	3.78553 (0.4259)	4.53947 (1.1053)

Table 3.4 Generation 26 respiratory and movement measurements for individual starvation resistant and control flies reported per dry weight. Mean respiratory or movement measurement (standard error) after 2 generations of relaxed selection following 26 generations of selection for starvation resistance.

Generation of Selection	Generations Removed	Replicate	Selection	Sex	O2_RATE	CO2_RATE	RQ	Movement
26	2	1	Control	Female	0.00018 ($3.0x10^{-5}$)	0.00023 ($3.0x10^{-5}$)	4.63451 (1.3317)	2.49393 (0.7860)
26	2	1	Control	Male	0.00036 ($1.6x10^{-4}$)	0.00042 ($1.7x10^{-4}$)	3.87787 (0.9450)	7.42807 (1.3108)
26	2	2	Control	Female	0.00013 ($3.0x10^{-5}$)	0.00022 ($2.0x10^{-5}$)	0.37058 (0.2966)	2.79649 (0.5944)
26	2	2	Control	Male	0.00028 ($7.0x10^{-5}$)	0.00033 ($9.0x10^{-5}$)	6.59354 (2.2416)	3.34035 (1.0335)
26	2	3	Control	Female	0.00032 ($1.2x10^{-4}$)	0.00030 ($1.3x10^{-4}$)	0.31106 (0.2619)	4.32794 (0.7640)
26	2	3	Control	Male	0.00022 ($4.0x10^{-5}$)	0.00020 ($3.0x10^{-5}$)	3.45011 (0.5580)	8.23509 (1.8451)
26	2	1	Selected	Female	0.00016 ($4.0x10^{-5}$)	0.00025 ($2.0x10^{-5}$)	7.45689 (5.7721)	4.94737 (1.0801)
26	2	1	Selected	Male	0.00018 ($3.0x10^{-5}$)	0.00022 ($3.0x10^{-5}$)	5.02142 (0.8278)	4.26316 (1.0656)
26	2	2	Selected	Female	0.00018 ($3.0x10^{-5}$)	0.00023 ($5.0x10^{-5}$)	3.26564 (1.0701)	2.83806 (0.9651)
26	2	2	Selected	Male	0.00090 ($7.4x10^{-4}$)	0.00081 ($6.4x10^{-4}$)	3.12237 (0.4402)	2.79352 (0.6240)
26	2	3	Selected	Female	0.00022 ($8.0x10^{-5}$)	0.00024 ($8.0x10^{-5}$)	20.1320 (19.479)	4.26692 (0.6354)
26	2	3	Selected	Male	0.00016 ($5.0x10^{-5}$)	0.00022 ($3.0x10^{-5}$)	10.5529 (7.5382)	6.03158 (1.1038)

Table 3.5 Generation 25 respiratory measurements for pools of five starvation resistant and control flies reported per fly. Mean respiratory measurement (standard error) after 2 generations of relaxed selection following 25 generations of selection for starvation resistance.

Generation of Selection	Generations Removed	Replicate	Selection	Sex	O2_RATE	CO2_RATE	RQ
25	2	1	Control	Female	0.00008 ($6.0x10^{-6}$)	0.00009 ($7.0x10^{-6}$)	1.17266 (0.0511)
25	2	2	Control	Female	0.00008 ($2.0x10^{-6}$)	0.00009 ($4.0x10^{-6}$)	1.12925 (0.0284)
25	2	3	Control	Female	0.00009 ($5.0x10^{-6}$)	0.00009 ($4.2x10^{-6}$)	1.09077 (0.0455)
25	2	1	Control	Male	0.00005 ($3.0x10^{-6}$)	0.00006 ($4.0x10^{-6}$)	1.07947 (0.0517)
25	2	2	Control	Male	0.00006 ($4.0x10^{-6}$)	0.00007 ($5.0x10^{-6}$)	1.10393 (0.0454)
25	2	3	Control	Male	0.00006 ($6.0x10^{-6}$)	0.00007 ($8.0x10^{-6}$)	1.11618 (0.0404)
25	2	1	Selected	Female	0.00008 ($3.0x10^{-6}$)	0.00010 ($4.0x10^{-6}$)	1.25097 (0.0333)
25	2	2	Selected	Female	0.00008 ($5.0x10^{-6}$)	0.00010 ($5.0x10^{-6)}$	1.25549 (0.0429)
25	2	3	Selected	Female	0.00008 ($7.0x10^{-6}$)	0.00009 ($2.9x10^{-5}$)	1.17191 (0.0320)
25	2	1	Selected	Male	0.00005 ($5.0x10^{-6}$)	0.00006 ($5.0x10^{-6}$)	1.23190 (0.0443)
25	2	2	Selected	Male	0.00006 ($1.0x10^{-5}$)	0.00008 ($1.3x10^{-5}$)	1.35710 (0.1170)
25	2	3	Selected	Male	0.00005 ($4.0x10^{-6}$)	0.00007 ($4.0x10^{-6}$)	1.27929 (0.0531)

Table 3.6 Generation 26 respiratory measurements for pools of five starvation resistant and control flies reported per fly. Mean respiratory measurement (standard error) after 2 generations of relaxed selection following 26 generations of selection for starvation resistance.

Generation of Selection	Generations Removed	Replicate	Selection	Sex	O2_RATE	CO2_RATE	RQ
26	2	1	Control	Female	0.00007 ($5.0X10^{-6}$)	0.00007 ($4.0x10^{-6}$)	1.38395 (0.2593)
26	2	1	Control	Male	0.00005 ($2.0x10^{-6}$)	0.00005 ($3.0x10^{-6}$)	1.11262 (0.0612)
26	2	2	Control	Female	0.00007 ($6.0x10^{-6}$)	0.00008 ($8.0x10^{-6}$)	1.19220 (0.0515)
26	2	2	Control	Male	0.00005 ($3.0x10^{-6}$)	0.00006 ($4.0x10^{-6}$)	1.13101 (0.0530)
26	2	3	Control	Female	0.00006 ($2.0x10^{-6}$)	0.00006 ($7.0x10^{-6}$)	1.00591 (0.1028)
26	2	3	Control	Male	0.00005 ($5.0x10^{-6}$)	0.00004 ($5.0x10^{-6}$)	0.99807 (0.1027)
26	2	1	Selected	Female	0.00007 ($3.0x10^{-6}$)	0.00009 ($4.0x10^{-6}$)	1.31239 (0.0610)
26	2	1	Selected	Male	0.00005 ($3.0x10^{-6}$)	0.00006 ($3.0x10^{-6}$)	1.16860 (0.0416)
26	2	2	Selected	Female	0.00007 ($6.0x10^{-6}$)	0.00008 ($8.0x10^{-6}$)	1.17684 (0.0366)
26	2	2	Selected	Male	0.00005 ($4.0x10^{-6}$)	0.00006 ($4.0x10^{-6}$)	1.21147 (0.0889)
26	2	3	Selected	Female	0.00007 ($5.0x10^{-6}$)	0.00006 ($8.0x10^{-6}$)	0.99112 (0.1079)
26	2	3	Selected	Male	0.00005 ($4.0x10^{-6}$)	0.00005 ($5.0x10^{-6}$)	1.17420 (0.1061)

Table 3.7 Generation 25 respiratory measurements for pools of five starvation resistant and control flies reported per dry weight. Mean respiratory measurement (standard error) after 2 generations of relaxed selection following 25 generations of selection for starvation resistance.

Generation of Selection	Generations Removed	Replicate	Selection	Sex	O2_RATE	CO2_RATE	RQ
25	2	1	Control	Female	0.00017 ($1.0x10^{-5}$)	0.00019 ($1.5x10^{-5}$)	2.41706 (0.0856)
25	2	1	Control	Male	0.00025 ($2.4x10^{-5}$)	0.00026 ($2.4x10^{-5}$)	4.21288 (0.2228)
25	2	2	Control	Female	0.00023 ($4.0x10^{-5}$)	0.00025 ($4.2x10^{-5}$)	4.73501 (0.4903)
25	2	2	Control	Male	0.00015 ($1.2x10^{-5}$)	0.00018 ($1.5x10^{-5}$)	2.46965 (0.1668)
25	2	3	Control	Female	0.00019 ($1.4x10^{-5}$)	0.00021 ($1.4x10^{-5}$)	4.00544 (0.2263)
25	2	3	Control	Male	0.00028 ($4.6x10^{-5}$)	0.00030 ($4.5x10^{-5}$)	3.87327 (0.1387)
25	2	1	Selected	Female	0.00016 ($1.1x10^{-5}$)	0.00018 ($1.4x10^{-5}$)	2.36012 (0.1373)
25	2	1	Selected	Male	0.00020 ($1.2x10^{-5}$)	0.00022 ($1.3x10^{-5}$)	4.12936 (0.2742)
25	2	2	Selected	Female	0.00020 ($8.0x10^{-6}$)	0.00022 ($1.2x10^{-5}$)	4.20584 (0.2603)
25	2	2	Selected	Male	0.00014 ($8.0x10^{-6}$)	0.00017 ($8.0x10^{-6}$)	2.18139 (0.0844)
25	2	3	Selected	Female	0.00017 ($1.8x10^{-5}$)	0.00012 ($1.8x10^{-5}$)	3.96923 (0.1614)
25	2	3	Selected	Male	0.00017 ($1.3x10^{-5}$)	0.00021 ($1.2x10^{-5}$)	4.00696 (0.2030)

Table 3.8 Generation 26 respiratory measurements for pools of five starvation resistant and control flies reported per dry weight. Mean respiratory measurement (standard error) after 2 generations of relaxed selection following 26 generations of selection for starvation resistance.

Generation of Selection	Generations Removed	Replicate	Selection	Sex	O2_RATE	CO2_RATE	RQ
26	2	1	Control	Female	0.00015 ($9.0x10^{-6}$)	0.00016 ($1.2x10^{-5}$)	2.50463 (0.1714)
26	2	1	Control	Male	0.00021 ($2.6x10^{-5}$)	0.00023 ($3.5x10^{-5}$)	4.95335 (0.5986)
26	2	2	Control	Female	0.00015 ($1.9x10^{-5}$)	0.00018 ($2.2x10^{-5}$)	2.61781 (0.1378)
26	2	2	Control	Male	0.00021 ($1.9x10^{-5}$)	0.00025 ($2.7x10^{-5}$)	4.90286 (0.4363)
26	2	3	Control	Female	0.00014 ($6.0x10^{-6}$)	0.00014 ($1.8x10^{-5}$)	2.13459 (0.2944)
26	2	3	Control	Male	0.00017 ($1.4x10^{-5}$)	0.00017 ($1.8x10^{-5}$)	4.00324 (0.3501)
26	2	1	Selected	Female	0.00012 ($6.0x10^{-6}$)	0.00015 ($8.0x10^{-6}$)	2.14918 (0.1167)
26	2	1	Selected	Male	0.00016 ($1.1x10^{-5}$)	0.00019 ($1.1x10^{-5}$)	3.89384 (0.1610)
26	2	2	Selected	Female	0.00012 ($1.0x10^{-5}$)	0.00014 ($1.1x10^{-5}$)	2.26653 (0.2639)
26	2	2	Selected	Male	0.00017 ($1.4x10^{-5}$)	0.00020 ($1.5x10^{-5}$)	4.09366 (0.3856)
26	2	3	Selected	Female	0.00012 ($1.5x10^{-5}$)	0.00012 ($1.3x10^{-5}$)	1.79474 (0.1525)
26	2	3	Selected	Male	0.00014 ($1.0x10^{-5}$)	0.00016 ($1.4x10^{-5}$)	3.98914 (0.2688)

Table 3.9 Generation 27 respiratory and movement measurements for individual starvation resistant and control flies reported per fly. Mean respiratory or movement measurement (standard error) after 2 generations of relaxed selection following 27 generations of selection for starvation resistance.

Generation of Selection	Generations Removed	Selection	Sex	Starvation	O2_RATE	CO2_RATE	RQ	Movement
27	2	Control	Female	Starved	0.00005 ($1.0x10^{-5}$)	0.00006 ($7.0x10^{-6}$)	1.00812 (0.4048)	2.93584 (0.8339)
27	2	Control	Male	Starved	0.00006 ($1.1x10^{-5}$)	0.00008 ($9.0x10^{-6}$)	1.90455 (0.4025)	5.69609 (0.8396)
27	2	Control	Female	Unstarved	0.00009 ($1.6x10^{-5}$)	0.00012 ($2.1x10^{-5}$)	1.28961 (0.0889)	2.63187 (0.3771)
27	2	Control	Male	Unstarved	0.00007 ($1.2x10^{-5}$)	0.00008 ($1.2x10^{-5}$)	1.20819 (0.0719)	4.42687 (0.8785)
27	2	Selected	Female	Starved	0.00007 ($1.5x10^{-5}$)	0.00006 ($1.3x10^{-5}$)	1.01208 (0.0662)	3.20339 (1.4281)
27	2	Selected	Male	Starved	0.00015 ($8.5x10^{-5}$)	0.00015 ($7.9x10^{-5}$)	1.14403 (0.0862)	2.70373 (0.4106)
27	2	Selected	Female	Unstarved	0.00018 ($9.6x10^{-5}$)	0.00019 ($8.7x10^{-5}$)	1.26401 (0.0765)	3.93601 (0.8538)
27	2	Selected	Male	Unstarved	0.00008 ($1.2x10^{-5}$)	0.00010 ($1.4x10^{-5}$)	1.21914 (0.0756)	3.76239 (0.6695)

Table 3.10 Generation 27 respiratory and movement measurements for individual starvation resistant and control flies reported per dry weight. Mean respiratory or movement measurement (standard error) after 2 generations of relaxed selection following 27 generations of selection for starvation resistance.

Generation of Selection	Generations Removed	Selection	Sex	Starvation	O2_RATE	CO2_RATE	RQ	Movement
27	2	Control	Female	Starved	0.00012 ($3.0x10^{-5}$)	0.00013 ($2.1x10^{-5}$)	2.15352 (1.2500)	2.93584 (0.8339)
27	2	Control	Male	Starved	0.00023 ($4.8x10^{-5}$)	0.00029 ($4.1x10^{-5}$)	7.39583 (2.7446)	5.69609 (0.9129)
27	2	Control	Female	Unstarved	0.00022 ($5.1x10^{-5}$)	0.00028 ($6.5x10^{-5}$)	3.03515 (0.1780)	2.63187 (0.3771)
27	2	Control	Male	Unstarved	0.00028 ($6.2x10^{-5}$)	0.00032 ($6.2x10^{-5}$)	4.70258 (0.2588)	4.42687 (0.8785)
27	2	Selected	Female	Starved	0.00015 ($4.6x10^{-5}$)	0.00015 ($3.7x10^{-5}$)	2.31496 (0.2475)	3.20339 (1.4281)
27	2	Selected	Male	Starved	0.00045 ($2.7x10^{-4}$)	0.00046 ($2.5x10^{-4}$)	3.89903 (0.4119)	2.70373 (0.4106)
27	2	Selected	Female	Unstarved	0.00046 ($2.7x10^{-4}$)	0.00049 ($2.4x10^{-4}$)	2.70861 (0.3269)	3.93601 (0.8538)
27	2	Selected	Male	Unstarved	0.00031 ($5.7x10^{-5}$)	0.00035 ($7.0x10^{-5}$)	4.14767 (0.3054)	3.76239 (0.6695)

Table 3.11 Generation 27 respiratory measurements for pools of five starvation resistant and control flies reported per fly. Mean respiratory measurement (standard error) after 2 generations of relaxed selection following 27 generations of selection for starvation resistance.

Generation of Selection	Generations Removed	Selection	Sex	O2_RATE	CO2_RATE	RQ
27	2	Control	Female	0.000072 ($5.0x10^{-6}$)	0.00007 ($5.0x10^{-6}$)	1.02800 (0.0239)
27	2	Control	Male	0.000049 ($3.0x10^{-6}$)	0.00005 ($3.0x10^{-6}$)	1.02088 (0.0229)
27	2	Selected	Female	0.000059 ($5.0x10^{-6}$)	0.00006 ($6.0x10^{-6}$)	1.05760 (0.0304)
27	2	Selected	Male	0.000049 ($5.0x10^{-6}$)	0.00005 ($5.0x10^{-6}$)	1.06383 (0.0281)

Table 3.12 Generation 27 respiratory measurements for pools of five starvation resistant and control flies reported per dry weight. Mean respiratory measurement (standard error) after 2 generations of relaxed selection following 27 generations of selection for starvation resistance.

Generation of Selection	Generations Removed	Selection	Sex	O2_RATE	CO2_RATE	RQ
27	2	Control	Female	0.00015 ($1.3x10^{-5}$)	0.00016 ($1.4x10^{-5}$)	2.17563 (0.0922)
27	2	Control	Male	0.00017 ($1.2x10^{-5}$)	0.00017 ($1.3x10^{-5}$)	3.48164 (0.1578)
27	2	Selected	Female	0.00010 ($8.0x10^{-6}$)	0.00011 ($1.0x10^{-5}$)	1.87513 (0.0703)
27	2	Selected	Male	0.00015 ($1.9x10^{-5}$)	0.00016 ($1.9x10^{-5}$)	3.31692 (0.1105)

Table 3.13 Generation 25 movement measurements for individual starvation resistant and control flies reported per fly. Mean number of movements (standard error) after 2 generations of relaxed selection following 25 generations of selection for starvation resistance.

Generation of Selection	Generations Removed	Replicate	Selection	Sex	Number of movements in the light	Number of movements in the dark
25	2	1	Control	Female	3.03221 (0.4896)	4.06103 (0.6316)
25	2	2	Control	Female	3.23695 (0.7589)	4.70423 (0.9358)
25	2	3	Control	Female	3.66733 (0.5165)	6.00822 (1.5053)
25	2	1	Control	Male	5.26404 (1.2769)	8.80282 (2.0057)
25	2	2	Control	Male	4.17291 (1.2410)	7.01878 (1.6190)
25	2	3	Control	Male	3.67830 (0.3462)	6.18202 (1.0506)
25	2	1	Selected	Female	3.75174 (0.6465)	4.37764 (0.6039)
25	2	2	Selected	Female	2.74386 (0.4343)	5.62777 (0.9955)
25	2	3	Selected	Female	3.97498 (0.6085)	4.02012 (0.6446)
25	2	1	Selected	Male	3.54098 (0.7181)	6.47606 (1.0084)
25	2	2	Selected	Male	6.57200 (1.4834)	6.93608 (0.9615)
25	2	3	Selected	Male	3.03452 (0.5327)	3.15728 (0.7002)

Table 3.14 Generation 26 movement measurements for individual starvation resistant and control flies reported per fly. Mean number of movements (standard error) after 2 generations of relaxed selection following 26 generations of selection for starvation resistance.

Generation of Selection	Generations Removed	Replicate	Selection	Sex	Number of movements in the light	Number of movements in the dark
26	2	1	Control	Female	1.96733 (0.5552)	2.94691 (0.7135)
26	2	1	Control	Male	4.23370 (0.6079)	5.43099 (0.6444)
26	2	2	Control	Female	3.81911 (0.8015)	4.20563 (0.6096)
26	2	2	Control	Male	3.63342 (0.4618)	7.64601 (1.1954)
26	2	3	Control	Female	3.26205 (0.5702)	3.42470 (0.6014)
26	2	3	Control	Male	4.95069 (1.1711)	6.52488 (1.8042)
26	2	1	Selected	Female	3.80943 (0.4790)	4.69131 (0.5436)
26	2	1	Selected	Male	3.19099 (0.8324)	4.49899 (0.8814)
26	2	2	Selected	Female	2.35467 (0.6928)	3.27302 (0.9161)
26	2	2	Selected	Male	3.67605 (0.5432)	4.67931 (0.9521)
26	2	3	Selected	Female	2.98048 (0.3537)	3.91851 (0.6183)
26	2	3	Selected	Male	4.54965 (0.7393)	3.67042 (0.6164)

Table 3.15 Generation 27 movement measurements for individual starvation resistant and control flies reported per fly. Mean number of movements (standard error) after 2 generations of relaxed selection following 27 generations of selection for starvation resistance.

Generation of Selection	Generations Removed	Replicate	Selection	Sex	Starvation	Number of movements in the light	Number of movements in the dark
27	2	1	Control	Female	Starved	2.93584 (0.8339)	4.34507 (1.0404)
27	2	1	Control	Male	Starved	5.69609 (0.9129)	7.86620 (0.9854)
27	2	1	Control	Female	Unstarved	2.63187 (0.3771)	3.72535 (0.5594)
27	2	1	Control	Male	Unstarved	4.42687 (0.8785)	6.61268 (1.4446)
27	2	1	Selected	Female	Starved	3.20339 (1.4281)	3.79930 (1.0225)
27	2	1	Selected	Male	Starved	2.70373 (0.4106)	4.72300 (0.7113)
27	2	1	Selected	Female	Unstarved	3.93601 (0.8538)	4.50960 (0.9314)
27	2	1	Selected	Male	Unstarved	3.76239 (0.6695)	3.75352 (0.7526)

Table 3.16 Statistically significant least squares means values for the effects of selection in the selected and control lines. Least squares means (standard error) following generations 25, 26, and 27 of selection for starvation resistance.

Generation	Removal	Effect	Sex	Number of flies	Dependent variable	Covariate	p-value	Control	Selected
25	2	Selection	Female	5	O2 per dry weight	none	0.0427	$1.6x10^{-4}$ ($1.4x10^{-6}$)	$1.4x10^{-4}$ ($1.8x10^{-6}$)
25	2	Selection	Female	5	RQ per dry weight	none	0.0494	2.3619 (0.0916)	2.1656 (0.0933)
25	2	Selection	Female	5	RQ per fly	none	0.0028	1.1302 (0.0256)	1.2278 (0.0265)
25	2	Selection	Male	5	RQ per fly	none	0.0004	1.0998 (0.0361)	1.2883 (0.0369)
26	2	Selection	Female	1	CO2 per fly	movement	0.0475	$9.0x10^{-5}$ ($1.2x10^{-5}$)	$1.2x10^{-4}$ ($1.0x10^{-5}$)
26	2	Selection	Female	5	CO2 per dry weight	none	0.0282	$1.6x10^{-4}$ ($1.0x10^{-6}$)	$1.4x10^{-4}$ ($1.2x10^{-6}$)
26	2	Selection	Female	5	O2 per dry weight	none	0.0088	$1.5x10^{-4}$ ($1.3x10^{-5}$)	$1.2x10^{-4}$ ($1.0x10^{-5}$)
26	2	Selection	Female	5	RQ per dry weight	none	0.0507	2.4495 (0.1658)	2.0791 (0.1633)
26	2	Selection	Male	1	Movement in the dark	none	0.0122	6.5319 (0.6526)	4.2347 (0.6810)
26	2	Selection	Male	1	Movement time point 3	none	0.0123	8.3064 (0.6549)	5.8793 (0.6861)
26	2	Selection	Male	5	CO2 per dry weight	none	0.0364	$2.2x10^{-4}$ ($1.5x10^{-5}$)	$1.8x10^{-4}$ ($1.0x10^{-5}$)
26	2	Selection	Male	5	O2 per dry weight	none	0.0053	$2.0x10^{-4}$ ($1.2x10^{-5}$)	$1.6x10^{-4}$ ($1.7x10^{-5}$)
27	2	Selection	Male	1	Movement in the dark	none	0.0014	7.5798 (0.6954)	4.2382 (0.6794)
27	2	Selection	Male	1	Movement in the light	none	0.0055	5.2931 (0.5026)	3.2330 (0.4910)
25 and 26	2	Selection	Female	1	CO2 per fly	movement	0.0385	$1.0x10^{-4}$ ($1.3x10^{-6}$)	$1.2x10^{-4}$ ($1.1x10^{-6}$)
25 and 26	2	Selection	Female	5	CO2 per dry weight	none	0.0037	$1.7x10^{-4}$ ($1.4x10^{-6}$)	$1.5x10^{-4}$ ($1.0x10^{-6}$)
25 and 26	2	Selection	Female	5	O2 per dry weight	none	0.0121	$1.6x10^{-4}$ ($1.0x10^{-5}$)	$1.3x10^{-4}$ ($1.3x10^{-5}$)
25 and 26	2	Selection	Female	5	RQ per dry weight	none	0.0012	2.4045 (0.0909)	2.1252 (0.0907)
25 and 26	2	Selection	Male	1	Movement in the dark	none	0.0049	6.8763 (0.5110)	4.9228 (0.5141)
25 and 26	2	Selection	Male	5	O2 per dry weight	none	0.0013	$2.0x10^{-4}$ ($1.3x10^{-5}$)	$1.7x10^{-4}$ ($1.1x10^{-5}$)
25 and 26	2	Selection	Male	5	RQ per fly	none	0.0104	1.0904 (0.0314)	1.2352 (0.0319)

Table 3.17 Statistically significant least squares means values for the effects of generation in the selected and control lines. Least squares means (standard error) following generations 25 and 26of selection for starvation resistance.

Generations Compared	Effect	Sex	Number of flies	Dependent variable	Covariate	p-value	Generation 25 R2	Generation 26 R2
25 and 26	Generation	Females	1	Movement in the dark	none	0.0295	4.7598 (0.3222)	3.7500 (0.3277)
25 and 26	Generation	Females	5	CO2 per fly	none	0.008	$9.0x10^{-5}$ ($1.3x10^{-7}$)	$8.0x10^{-5}$ ($1.8x10^{-7}$)
25 and 26	Generation	Females	5	O2 per dry weight	none	0.0162	$1.5x10^{-4}$ ($1.0x10^{-5}$)	$1.3x10^{-4}$ ($1.2x10^{-5}$)
25 and 26	Generation	Females	5	O2 per fly	none	<0.0001	$8.0x10^{-5}$ ($1.1x10^{-7}$)	$7.0x10^{-5}$ ($1.6x10^{-7}$)
25 and 26	Generation	Male	5	CO2 per fly	none	0.0103	$7.0x10^{-5}$ ($1.0x10^{-7}$)	$5.0x10^{-5}$ ($1.4x10^{-7}$)
25 and 26	Generation	Male	5	O2 per fly	none	0.0104	$6.0x10^{-5}$ ($1.2x10^{-7}$)	$5.0x10^{-5}$ ($1.0x10^{-7}$)

Table 3.18 Statistically significant least squares means values for the effects of starvation in the selected and control lines. Least squares means (standard error) following generation 27 of selection for starvation resistance.

Generation Compared	Effect	Sex	Number of flies	Dependent variable	Covariate	p-value	Starved	Unstarved
27	Starvation	Female	1	CO2 per dry weight	none	0.0215	$1.3x10^{-4}$ ($8.0x10^{-5}$)	$4.0x10^{-4}$ ($8.3x10^{-5}$)
27	Starvation	Female	1	CO2 per dry weight	movement	0.0237	$1.3x10^{-4}$ ($8.6x10^{-5}$)	$4.0x10^{-4}$ ($8.1x10^{-5}$)
27	Starvation	Female	1	CO2 per fly	none	0.0423	$6.0x10^{-5}$ ($3.0x10^{-5}$)	$1.5x10^{-4}$ ($3.2x10^{-5}$)
27	Starvation	Female	1	CO2 per fly	movement	0.033	$6.0x10^{-5}$ ($3.7x10^{-5}$)	$1.6x10^{-4}$ ($3.7x10^{-5}$)

Chapter 4.

The effects of dietary alterations on body composition, life history traits, and movement using genetically altered starvation-resistant and control lines of *Drosophila melanogaster*

Abstract

Dietary alterations can have a dramatic effect on body composition, life history traits, and behavior. In this study, genetically based starvation-resistant and control *Drosophila melanogaster* lines were exposed to diets supplemented with glucose, palmitic acid, and a 2:1 mixture of casein and albumin to identify correlations between dietary alterations and body composition, life history traits, and movement in the context of an obese phenotype. Results obtained from this study indicated that increased levels of dietary protein and fat led to increased soluble protein concentrations in selected and control males and females. Increased sugar, fat, and protein in the diet resulted in increased concentrations of total carbohydrates, glycogen, total sugars, and trehalose in both sexes. Furthermore, increased levels of dietary sugar promoted triglyceride accumulation in all lines. Analysis of life history traits indicated a negative relationship between high sugar consumption and fecundity in both the selected and control lines. Supplementation with either a high fat or high protein diet resulted in a significant reduction in lifespan in both sexes of the selected and control lines, with the most dramatic effects observed in control females. Comparisons of movement indicated that all of the supplemented diets resulted in increased movement in males, while supplementation with only the high fat and high protein diets producing increased movement in females. Results obtained from this study indicated that alterations to nutrient composition within the diet can have dramatic effects on body composition and life history traits. Information obtained from this study has implications for the analysis of dietary alterations in humans in regards to management and treatment of obesity.

Introduction

Alterations to nutrient composition in the diet frequently occur in natural populations due to changes in environmental conditions (Somero & Hochachka, 1971; White, 1993). For example, variations in soil nutrients alter the quantity and type of metabolites present in plants, which in turn alters the composition of food consumed by herbivores and omnivores. Analyses of phenotypic perturbations associated with alterations to the diet provide insight into correlations between consumption of specific nutrients and alterations in body composition and life history traits (Skorupa et al., 2008; Katewa & Kapahi, 2010). This area of research is important for the investigation of associated human health issues including correlations between nutrient consumption and the development and persistence of metabolic diseases, including obesity. Gaining insight into the responses to altered diets can lead to improved recommendations for dietary consumption resulting in enhanced performance and an improved quality of life (Miller et al., 2009).

Nutrient intake and absorption are key components in the maintenance of energy homeostasis within organisms (Buch et al., 2008). The need for a constant supply of energy coupled with variability in food resources has resulted in the evolution of mechanisms to detect nutritional status. Analysis of metabolic reserves stored within the organism and available environmental nutrients allows for a coordinated response between metabolic regulation and physiological adaptation resulting in alterations to organismal development, growth, and reproductive status based on the amount of available energy (Baker & Thummel, 2007). During periods of excess intake, most

organisms absorb the additional nutrients and store the unused energy in the form of triglycerides and glycogen. However, some organisms, including mice, increase energy expenditure through mitochondrial respiration, thus limiting energy storage following excess consumption (Obici et al, 2002). When energy or food resources are depleted, biochemical mechanisms within the organism trigger the release of free fatty acids and glucose for conversion into energy through ATP synthesis (Gronke et al., 2005). Alterations to energy homeostasis resulting in a chronic imbalance of excess nutrient intake and reduced energy expenditure have been linked to the development and persistence of metabolic diseases including obesity, diabetes mellitus, cardiovascular disease, and certain forms of cancer (Jiang et al., 2005; Baker & Thummel., 2007; Gronke et al., 2007). Attaining a proper balance between the quantity and type of nutrients is therefore essential for maintaining energy homeostasis within the organism and preventing the development of disease. The present study used genetically based starvation-resistant and control lines of *Drosophila melanogaster* to investigate correlations among four separate diets with body composition, life history traits, and movement in the context of an obese phenotype.

The use of the model organism *Drosophila melanogaster* in metabolic studies is warranted due to the high degree of evolutionary conservation between insects and vertebrates in metabolic responses including the mechanisms of nutrient absorption, transport, storage, and mobilization (De Luca et al., 2005; Ruden et al., 2005; Wang & Beydoun, 2005; Baker & Thummel., 2007). Absorption of dietary nutrients occurs in the midgut within *Drosophila*, which is analogous in function to the stomach and small

intestine in vertebrates (Bharucha, 2009). Following absorption, nutrients are transported through the haemolymph, similar to mammalian blood, to the fat body, which performs metabolic functions comparable to those observed in the mammalian liver and white adipose tissue. Within the fat body, lipids and carbohydrates are metabolized and stored in the form of triglycerides and glycogen (Ruden et al., 2005; Beller et al., 2006; Baker & Thummel, 2007). During nutrient deprivation within the organism, these high-energy molecules are mobilized for energy utilization to withstand periods of starvation (Baker & Thummel, 2007; Gutierrez et al., 2007).

Previous dietary studies conducted on *Drosophila melanogaster* have provided insight into correlations between individual diets and life history traits (Mair et al.; 2005; Piper & Partridge, 2007; Skorupa et al., 2008). Manipulations of the basal diet resulting in reduced nutrient availability without malnutrition, commonly referred to as dietary restriction, prolongs life in a diverse subset of species including *Drosophila*, mice, primates, and perhaps humans (Carvalho et al., 2005). Additionally, caloric restriction has been identified to delay or arrest the onset of age-related deteriorations and the development of diseases such as cancer and cardiovascular disease in a diverse set of species studied (Carvalho et al., 2005; Skorupa et al., 2008; Lefevre et al., 2009; Witte et al., 2009). The observed extension in lifespan was initially attributed to an overall reduction in caloric intake; however, more recent studies have indicated that reductions in specific dietary components including sugar and protein have a more robust effect on lifespan extension in *Drosophila melanogaster* than caloric content alone (Mair et al., 2005; Piper et al., 2007; Lee et al., 2008).

Correlations between life history traits and dietary supplementation in *Drosophila* have indicated that diets consisting of relatively low protein and moderate carbohydrate concentrations resulted in increased longevity and fecundity (Skorupa et al, 2008; Ja et al, 2009). Increases in lifespan observed in dietary alteration studies have been attributed to the combination of a relatively low protein to moderate carbohydrate diet resulting in a ratio of protein to carbohydrates at 1:16 (Lee et al., 2008; Skorupa et al., 2008; Ja et al., 2009; Katewa & Kapahi, 2009). Reductions in dietary protein intake in *D. melanogaster* have been indicated to enhance mitochondrial function resulting in increased longevity, while the converse of high protein supplementation in the diet resulted in increased protein intake and reduced adiposity, which was correlated with a reduction in lifespan (Skorupa et al., 2008; Katewa & Kapahi, 2009; Zid et al., 2009). Additionally, excess protein has been suggested to reduce lifespan through increased oxygen free radical production by the mitochondria, changes in insulin and amino acid signaling pathways, and increased organ damage due to high concentrations of nitrogen degradation products (Lee et al., 2008). Consumption of a high carbohydrate to moderate protein diet resulted in an obese phenotype with increased triglyceride stores and a suppression of protein storage, that in combination were associated with an overall reduction in lifespan (Skorupa et al., 2008; Katewa & Kapahi, 2009). Measurements of the effects of dietary alterations on fecundity in *Drosophila* have indicated a reduction in the number of offspring produced by females exposed to high carbohydrate diets (Skorupa et al., 2008). Conversely, diets possessing a protein to carbohydrate ratio of 1:2 resulted in maximal

egg laying rates with a dietary ratio of 1:4 producing maximal lifetime egg production (Lee et al., 2008).

Alterations to dietary composition have become common for promoting weight loss in human populations. Some of the more prevalent diets that are implemented for weight loss include the Atkins, South Beach, Zone, and Ornish diets. Similarities in recommended dietary consumption are present in the Atkins and South Beach diets, which promote a reduction in carbohydrate intake with increased fat consumption (Gardner et al, 2007). The Ornish diet, however, focuses on the combination of a high carbohydrate intake with relatively low amounts of fat consumed (Miller et al., 2009). Dietary recommendations for the Zone diet present a more balanced dietary approach where the nutrient composition is 40 percent carbohydrates, 30 percent fat, and 30 percent protein (Gardner et al., 2007). Due to the increased popularity of these diets, it is important to establish not only the effects of dietary alterations on body composition, but also the long-term effects on life history traits, including survival.

In the present study, starvation-resistant selected and control *Drosophila melanogaster* were supplied with increased concentrations of glucose, palmitic acid, and a 2:1 mixture of casein and albumin to determine the effects of dietary alterations on body composition and life history traits. Comparisons of results obtained from each of the diets were made with the basal diet to identify alterations associated with each form of dietary supplementation. Measurements of body composition including the abundance of soluble proteins, carbohydrates, glycogen, total sugars, trehalose, and triglycerides were correlated with movement data to gain insight into associations between activity levels

and body composition following exposure to each of the diets. Life history traits including female egg production and longevity in both sexes were measured to identify the effects of dietary supplementation on survival and reproduction.

Materials and Methods

Drosophila melanogaster stocks

The initial base population of *Drosophila melanogaster* used in artificial selection for starvation resistance was derived from a series of inbred lines collected in a manner designed to preserve natural genetic variation. Initially, a large population of inseminated females was collected from the Wolfskill Experimental Orchard in Winters, California. Once moved into a laboratory setting, 20 generations of inbreeding was conducted using progeny obtained from the female lines. From this series of inbred lines, ten were randomly selected to be intercrossed by conducting all possible pair-wise crosses between lines with all possible reciprocal crosses conducted. From the series of approximately 100 crosses, 150 progeny per cross were randomly selected and placed in a large population cage (12 inches high, 36 inches wide, and 24 inches deep). Selection of an equal number of progeny from each of the crosses ensured a balanced representation of naturally occurring genetic variation within the base population of *Drosophila melanogaster*. The population density of 15,000 individuals was sustained through an overlapping generation population regime. Initially, twenty bottles of food consisting of yeast extract, cornmeal, molasses, glucose, agar, and acids to prevent mold growth were placed in random locations throughout the cage. Each week, four of the oldest bottles were removed and replaced with a fresh food source. This form of population management contributed to the maintenance of lifespan and stress resistance, which are

normally lost when individuals are maintained in laboratory culture (Hoffmann et al., 2001, Linnen et al., 2001).

The base population used for experimentation was divided into eight discrete subpopulations, of which four were randomly designated as the selected lines while the remaining four were identified as the control lines. Ten cut glass bottles containing a food source were placed at random locations throughout the cage to collect flies for each subpopulation. All bottles used for progeny collection were removed daily from the population cage to obtain eggs from the surface of the media. Approximately 100 eggs were collected per vial with the resulting series of vials placed in a controlled environment maintained at 25°C with a 12 hour light/dark cycle. Following the first egg collection, the surface of the media was removed in order to eliminate any residual eggs and larvae from the previous egg laying period. Bottles were then replaced within the population cage and egg collection resumed on the following day. This process was continued until each subpopulation consisted of approximately 4,000 individuals. Adult *D. melanogaster* that emerged from the vials were allowed to randomly mate prior to egg collection for the next generation. This process was repeated for four generations with the sample size of the subpopulations consistently maintained at 2,000 females, and 2,000 males within each of the eight lines. Artificial selection for resistance to starvation commenced on the selected lines in the following generation.

Individuals selected for starvation resistance were collected at 5 days post eclosion. Two thousand males and two thousand females from each line were separated following brief exposure to ethyl ether, which acted as an anesthetic, prior to placement

in population cages that were supplied with either a food source for the control lines or agar for the selected lines. Solidified agar was placed within the selection cages to ensure an adequate supply of water and prevent desiccation of the flies exposed to starvation. The petri-plates (9mm in diameter) containing either food or agar were replaced every 48 hours to provide fresh media and prevent the introduction of progeny. Environmental conditions were held constant during selection with a temperature of 25°C, and 12:12 light/dark cycle. The relative humidity was maintained during experimentation by placing moist paper towels within a clear plastic bag surrounding the cage. The paper towels were replaced in conjunction with the media to sustain the relative humidity levels.

During each generation of artificial selection for starvation resistance, mortality levels within each selected line were monitored at 12 hour intervals. Individuals that had died during this time period were removed by aspiration and counted to determine mortality rates under starvation, prior to being discarded. The control populations were additionally assayed at 12 hours intervals in order to maintain consistency in experimental conditions and to determine mortality rates in the presence of food. When one of the selected lines reached approximately fifty percent mortality, the surviving *D. melanogaster* from the matched selected and control lines were removed from the cages following brief exposure to carbon dioxide, which was used to anesthetize the individuals within in the cage. Flies removed from each of the cages were then placed at a moderate density in plastic bottles containing food. Bottles containing the flies from each individual line were kept at 25°C with 12 hours of light and dark until all lines had reached optimal mortality levels. A two day recovery period was extended to all of the

lines prior to mating individuals to produce the next generation used in selection. A population size of approximately 450 males and 450 females were used to propagate each line for each of the generations of artificial selection for starvation resistance. Twenty generations of selection were conducted prior to obtaining a subpopulation of *Drosophila melanogaster* to be used in the analysis of effects of dietary supplementation on body composition, life history traits, and movement.

Diets

Upon concluding 20 generations of artificial selection for starvation resistance, flies from each replicate selected line were subjected to one to six generations of relaxed selection. To maintain consistency in experimentation, the control lines were maintained in the same manner as the selected lines. Relaxation of selection within this experiment refers to the absence of selection on the starvation-resistant lines for a series of discrete generations. Measurements of body composition, life history traits, and movement were conducted on flies that were two to six generations removed from selection to identify the effects of increased supplementation of sugar, fat, and protein to the standard diet consisting of a mixture of yeast extract, glucose, water, agar, and a combination of phosphoric and propionic acids to prevent mold growth. *Drosophila melanogaster* placed on the high sugar diet were provided with 1200 grams of glucose, which is twice the normal amount in the base diet. Individuals on the high fat diet were provided with an additional 3 grams of palmitic acid, while individuals on the high protein diet received an additional 60 grams of protein consisting of a 2 to 1 mixture of casein to albumin.

Supplementation of the diet with casein alone does not provide an adequate balance of amino acids due to deficiencies in methionine and cystine. To correct for the absence of these amino acids, albumin was added resulting in the 3:1 casein to albumin mixture used for protein supplementation (Dussutour & Simpson, 2008; Simpson & Abisgold, 1985 after Dadd, 1961). To investigate the effects of each dietary alteration, an additional sub-population of flies were maintained on the base diet to be used for comparison.

Individuals used for the analysis of body composition were six generations removed from selection and were maintained in the presence of each diet for seven days prior to being flash frozen with liquid nitrogen. Female fecundity and longevity in both sexes were measured on flies that were two and six generations removed from selection, respectively. These individuals were continually exposed to the diets following one day post eclosion to obtain the full effect of dietary modification on life history traits. *Drosophila melanogaster* used to quantify alterations in movement were exposed to each of the altered dietary conditions for seven days prior to being placed within the activity monitors to determine the number of movements in a 48 hour time period. Flies used for measuring the number of movements of each individual exposed to each of the four diets were six generations removed from selection for starvation resistance.

Soluble protein concentrations

The Pierce BCA Protein Assay Kit was used to determine soluble protein concentrations within whole body homogenizations of selected and control *Drosophila melanogaster* lines placed on each of the four diets. Ten males or ten females from each of the replicate lines on each diet were homogenized in 500 µl of HPLC water for 3 minutes at 1,120 rpm using a Talboys High Throughput Homogenizer (OPS Diagnostics) with a 5/32 inch stainless steel grinding ball (OPS Diagnostics). Twenty-five microliters of each homogenate was added to 200 µl of the combined kit reagents within individual wells of a 96 well microplate. Each sample was thoroughly mixed for 30 seconds using an orbital shaker prior to a 30 minute incubation at 37°C in the absence of light. Following cooling of the samples to room temperature, the optical density reading of each homogenized sample was obtained using a Versa Max microplate reader (Molecular devices) set to a wavelength of 562 nm.

Quantification of soluble protein concentrations was obtained by creating a series of standard solutions for use in generating a standard curve. Standards consisted of known amounts of a 2 mg/ml solution of albumin with known quantities of HPLC water. The dilution series resulted in protein concentrations ranging from 0 to 2,000 µg/ml. Twenty-five microliters of each standard was combined with 200 µl of reagents from the kit within individual wells of a 96 well plate. Each standard solution was thoroughly mixed on an orbital shaker for 30 seconds prior to being incubated at 37°C for 30 minutes in complete darkness. Following incubation, the solutions were cooled to room temperature and the optical density of each standard was read at 562 nm (Versa Max

microplate reader from Molecular Devices). The resulting standard curve comparing optical density readings and known protein concentrations was used to determine the amount of soluble protein in each of the three replicates for each selected and control line on each of the four diets.

Total carbohydrate concentrations

Quantification of total carbohydrate concentrations in both the selected and control lines on each of the four diets were achieved using methodology described by Van Handel (1985). Homogenization of ten males or ten females from each line on each of the diets was conducted in 1 ml of an anthrone reagent composed of distilled water, concentrated sulfuric acid, and dissolved anthrone using the homogenization procedure previously described. The resulting homogenate was decanted and an additional volume of anthrone was added to produce a total reaction volume of 5 ml. Activation of the anthrone used to detect the concentration of carbohydrates was achieved by heating the samples at 90°C for 17 minutes. Upon cooling the solutions to room temperature, optical densities of each sample were measured at both 625 and 555 nanometers in a Versa Max microplate reader (Molecular Devices). Measurements were recorded at each of the two wavelengths to extend the linear portion of the standard curve.

Carbohydrate concentrations for each of the three replicates of each selected and control line placed on one of the four diets were determined through extrapolation from a glucose calibration curve. Standard solutions of known glucose concentrations were generated by the addition of increasing amounts of a 1 mg to 1 ml glucose in 25 percent

ethanol solution (25, 50, 100, 150, 200 μg) to known volumes of anthrone reagent producing a total reaction volume of 5 ml. The solutions were heated for 17 minutes at 90°C to activate the anthrone reagent. Optical densities for each of the standards were measured at wavelengths of 625 and 555 nm in a Versa Max microplate reader (Molecular Devices) following cooling of the samples to room temperature. Measurement of two wavelengths allowed the linear portion of the standard curve to be extended as advocated by Van Handel (1985). Total carbohydrate concentrations for each replicate selected and control line exposed to each of the four diets were obtained by comparison with the standard curve consisting of the microgram concentrations of glucose plotted against the optical density readings.

Glycogen and total sugars concentrations

Concentrations of glycogen and total sugars within the selected and control flies on each of the four diets were determined using methods described by Van Handel (1985). Homogenization of ten males or ten females from each of the replicate selected and control lines on each diet was completed in 200 μl of a 2 percent sodium sulfate solution in water using the procedures previously described. Following homogenization, 1 ml of methanol was added to each sample prior to a 1 minute centrifugation at 2,000 rpm. The supernatant containing the sugars was decanted and evaporated to a volume of 100 to 200 μl, while the glycogen remained in the pellet with the precipitated sodium sulfate and homogenized tissue. A total reaction volume of 5 ml was generated by the addition of anthrone reagent to each tube containing either the sugars or the glycogen

extracted from each of the three replicate samples for each line exposed to each diet. Solutions containing the mixture of extracts and anthrone were heated at 90°C for 17 minutes prior to cooling the samples to room temperature. Optical density readings were obtained for each sample using the Versa Max microplate reader (Molecular Devices) reading at 625 and 555 nm. Both the glycogen and total sugar concentrations for each replicate of the selected and control lines on one of the four diets were determined by comparison to the glucose standard curve.

Trehalose concentrations

Determinations of trehalose concentrations were obtained using methodology adapted from Van Handel (1985). Ten males or ten females from each of the replicate selected and control lines on each of the four diets were homogenized in a 2 percent sodium sulfate solution in water using the high throughput homogenization procedure described previously. One milliliter of methanol was combined with the homogenates prior to a 1 minute centrifugation at 2,000 rpm. The resulting solution containing the sugar fraction was decanted, while the pellet of homogenized tissue and precipitated sodium sulfate was resuspended in 300 μl of HPLC water. An additional 1 ml of methanol was added to the resuspended solution to ensure complete recovery of sugars from each of the samples prior to a second 1 minute centrifugation at 2,000 rpm. The supernatant obtained following centrifugation was combined with the initial solution containing the sugar fraction prior to concentrating the fraction by reducing the volume of the solution to 500 μl.

Fifty microliters of 1N hydrochloric acid was added to 100 µl of the concentrated sugar fraction. The combined solution was heated at 90°C for 7 minutes to ensure hydrolyzation of sucrose in the samples to glucose and fructose. The reactivity of glucose and fructose to the anthrone reagent was inhibited by the addition of 150 µl of 1N sodium hydroxide heated at 90°C for 7 minutes. Following the additions of HCL and NaOH, the reactivity of sucrose, glucose, and fructose to anthrone was inhibited leaving only trehalose to be quantified. Anthrone reagent was added to each sample resulting in a total reaction volume of 5 ml prior to heating the combined mixture at 90°C for 17 minutes. Each of the three replicate samples for each selected and control line on the four diets were cooled to room temperature prior to obtaining an optical density reading at wavelengths of 625 and 555 nm using the Versa Max microplate reader (Molecular Devices).

The amount of trehalose within each of the replicate selected and control lines on each of the diets were determined through extrapolation of values from a trehalose standard curve. Increasing amounts of known volumes of a 1 mg to 1 ml of trehalose in 25 percent ethanol solution (25, 50, 100, 150, and 200 µg) were added to 50 µl of 1N hydrochloric acid. Each standard trehalose solution was heated for 7 minutes at 90°C prior to the addition of 150 µl of 1N sodium hydroxide. Following the addition of sodium hydroxide, the solutions were again heated at 90°C for 7 minutes. Anthrone reagent was added to each of the standard solutions resulting in total reaction volume of 5 ml prior heating the solutions at 90°C for 17 minutes. The optical densities for each of the standards were determined using a microplate reader set 625 and 555 nm following

cooling of the solutions to room temperature. Concentrations of trehalose present in each of the three replicate samples of selected and control lines on each of the diets were determined using the standard curve plotting the optical density readings of the standards against the known concentrations of trehalose.

Triglyceride concentrations

The BioVision Triglyceride Quantification Kit was used to determine the concentration of triglycerides in the selected and control *Drosophila melanogaster* lines exposed to each of the four diets. Homogenization of five males or five females from each of the lines on each diet was preformed using the procedures previously described. Flies were homogenized for 3 minutes at 1,120 rpm in 500 µl of a 5 percent Triton-X-100 in water solution. The resulting homogenized solution was decanted and heated at 80°C for 5 minutes. Samples were cooled to room temperature prior to repeating the heating step to ensure complete solubilzation of triglycerides in solution. Insoluble materials were removed by conducting a 1 minute centrifugation at 2,000 rpm. To ensure that the concentrations of triglycerides fell within the range of a series of known triglyceride standards, each sample was diluted tenfold with HPLC water prior to placing 50 µl of the diluted solution within a well of a 96 well plate.

To determine the concentration of triglycerides within each of the three replicate samples from each line on each diet, a standard triglyceride concentration curve was generated. Increasing amounts of a 1mM standard triglyceride solution (0, 2, 4, 6, 8, 10 µl) were added to a known volume of buffer resulting in total reaction volume of 50 µl. In

order to ensure accurate concentrations of the triglycerides, the standard solution was heated in an 80°C water bath for 1 minute prior to vortexing the solution for 30 seconds. The heating and mixing steps were repeated to ensure the complete solubilzation of triglycerides within the standard solution to obtain accurate concentration readings that ranged from 0 to 10 nanomoles.

Two microliters of lipase was added to all standard solutions and samples used to quantify triglyceride concentrations. The resulting enzymatic reaction cleaved the triglyceride molecules to release glycerol and free fatty acids into solution. Each well containing either the series of standard solutions or the samples for quantification received 50 µl of a combined reaction mixture consisting of 46 µl of buffer, 2 µl of the probe, and 2 µl of an enzyme mixture. The samples were thoroughly mixed on an orbital shaker prior to a 30 minute incubation at room temperature in the absence of light. This resulted in the oxidation of glycerol to generate a product that reacted with the provided probe to produce a colorimetric response used for determining triglyceride concentrations. The optical density readings for the standards and the samples were obtained using a Versa Max microplate reader (Molecular Devices) reading at a wavelength of 570 nm. Triglyceride concentrations for each of the three replicates of the selected and control lines that were placed on one of the four diets were determined using the triglyceride standard curve that compared optical density readings with known triglyceride concentrations.

Dry weight measurements

Each of the body composition measurements were normalized by dry weight, which is a measure of the mass of an individual fly excluding weight attributed to water. Five replicates of *D. melanogaster* from each replicate selected and control line placed on one of the four diets were flash frozen following a seven day exposure to the high sugar, high protein, high fat, or base diets. Ten males or ten females from each line on each of the four diets were placed in an open microfuge tube kept in a 65°C drying oven for 24 hours. All of the flies used for dry weight determination were individually weighed and an average was obtained for each of the selected and control lines on each of the diets. In addition to the dry weight normalizations of the body composition data, each measurement was recorded per fly. In order to accurately account for the differences in body mass between the selected and control lines of each sex, only the dry weight measurements were considered further for comparisons of body composition.

Female fecundity

The number of eggs produced by females was quantified for each of the replicate selected and control lines on each of the four diets every day for a duration of 30 days post eclosion starting on day one. Egg counts were obtained during a consistent one to two hour time interval to ensure the accuracy of the data and prevent alterations to counts based on time of day. Ten single-pair matings between one randomly selected adult female and male in each of the replicate selected and control lines were generated to obtain an average number of eggs produced by mated females within each line on each

diet. Each of the vials containing the individually mated flies was kept at 25 °C with a 12 hour light/dark cycle for the duration of experimentation.

Longevity

The duration of lifespan was measured in three replicates of each of the selected and control lines on each of the four diets. Thirty males and thirty females from each of the lines were collected one day post eclosion to be placed in cages containing one of the four diets. Apart from dietary manipulations, environmental conditions were held constant for experimentation with flies kept at 25°C with a 12 hour light/dark cycle. The population cages used in longevity measurements were monitored every 48 hours to replace the food source and remove dead individuals. Flies that had died within this time frame were removed by aspiration with each sex counted to determine mortality levels. Measurements of lifespan duration were continued until all experimental subjects had reached total mortality.

Movement

The number of movements of individual *Drosophila melanogaster* from the selected and control lines on each of the four diets were determined using flies that were six generations removed from selection for starvation resistance. Individuals selected for experimentation were placed in vials containing one of the four diets for seven days following one day post eclosion. Following exposure to the diets, flies were anesthetized with a small amount of ethyl ether prior to allow placement within glass capillary tubes

(5 mm in diameter and 65 mm in length) used for monitoring activity levels. Within the glass tubes, individuals were supplied with a food source on either end, with one end only partially covered by food to allow airflow during experimentation.

Flies from the selected and control lines placed on each of the four diets were randomized with respect to position in each of the 64 total spaces within the two *Drosophila* activity monitors (TriKinetics) to prevent positional effects in the data. Each tube was centered with respect to the monitor and secured using a rubber band to ensure that the position of the tube remained constant throughout experimentation. Readings of the number of movements of each individual were detected by an infra-red beam that bisected the tube within each position of the monitor. Each time the individual crossed the beam, a reading was recorded in the computer. Activity levels for each of the six total replicates in the selected and control lines on one of the four diets were recorded at 10 minute time intervals for a 48 hour time period to identify variations in movement due to dietary alterations. During experimentation, environmental conditions were held at a constant temperature of 25°C with a 12 hour light/dark cycle. Relative humidity was maintained by placing moistened cotton balls within clear plastic bags surrounding the individual activity monitors. Each of the cotton balls was moistened once daily to prevent drying of the food sources and desiccation of the flies.

Statistical analysis

The body composition data obtained from the selected and control lines on each of the four diets were analyzed using a linear mixed model generated by SAS 9.2 (SAS, 2009) to identify variations in concentrations of proteins, lipids, and carbohydrates. The model included fixed effects for replication, selection, dietary supplementation, and the interaction between selection and each of the diets, along with a random effect of selected line. Statistically significant results were identified as those that possessed a p-value equal to or less than 0.05. Following identification as statistically significant, the least squares means values obtained from the analysis were compared to determine the direction and magnitude of dietary associated changes in the selected lines as compared to the controls.

Results

Artificial selection for starvation resistance

The direct response to selection was quantified for the starvation-resistant lines following twenty generations of artificial selection. Comparisons between selected *D. melanogaster* following one and twenty generations of selection for starvation resistance indicated a two-fold increase in survival under starvation in males, and a 2.5 fold increase in survival under starvation in females at the 50 percent mortality level (figure 4.1). Survival rates measured in the control lines indicated no significant alterations from generations one to twenty, with mortality levels remaining relatively consistent throughout the generations of artificial selection on the starvation-resistant lines (figure 4.2).

A previous study following fifteen generations of selection for starvation resistance indicated no significant reduction in the response to selection following six generations of relaxation from artificial selection. Comparisons of survival rates under starvation between generations fifteen and twenty resulted in a slight increase in survival rates of females with no significant difference in male survival under starvation (figure 4.3). Based on the results obtained from the earlier study conducted at generation fifteen, it was reasonable to assume no significant alterations in the response to selection following six generations of relaxed selection for starvation resistance starting at generation twenty.

Soluble protein concentrations

Soluble protein concentrations obtained from whole body homogenizations of the selected and control lines placed on each of the four diets revealed a significant effect of selection on females (table 4.8) and dietary treatment (table 4.9) on both sexes, with a significant interaction (table 4.10) between selection and dietary treatment observed in males (table 4.1). Measurements normalized with respect to dry weight indicated that selected females possessed reduced concentrations of soluble proteins as compared to control females (p-values 0.0011). Additionally, a significant effect of dietary treatment (p-value <0.0001) on female soluble protein concentrations indicated that females placed on the base and high protein diets possessed significantly greater concentrations of soluble proteins than females on either the high fat or high sugar diets with no statistically significant differences detected between females placed on the base and high protein diets. Comparisons between the high fat and high sugar diets indicated significantly greater soluble protein concentrations in females while exposed to the high fat diet. Soluble protein concentrations obtained from selected and control females on each of the four diets indicated that the selected lines possessed reduced soluble protein concentrations as compared to control lines with the high protein and base diets resulting in increased protein concentrations as compared to the high fat and high sugar diets.

In males, dietary treatment had a statistically significant effect on measurements of soluble protein concentrations normalized per dry weight (p-value <0.0001). Exposure of males to the high fat or high protein diets resulted in increased soluble protein concentrations as compared to either the base diet or the high sugar diet, with no

statistically significant differences observed between high fat and high protein diets. Measurements of soluble proteins in males placed on either the base or high sugar diets indicated a statistically significant increase in protein concentrations in males on the base diet. Soluble protein concentrations recorded in males indicated increased concentrations of protein while placed on the high fat and high protein diets, followed sequentially by the base and high sugar diets.

A statistically significant interaction between selection and treatment (table 4.10) was detected in male protein concentrations normalized by dry weight (p-value 0.0026). Exposure of males to either the base or the high sugar diets resulted in no statistically significant differences between the selected and control lines. Following placement of males on the high protein or high fat diets, a significant decrease in soluble protein concentrations were detected in the selected males as compared to the control males. Measurements of the interaction between selection and the four dietary treatments indicated that the high protein and high fat diets resulted in decreased soluble protein concentrations in the selected males as compared to the control males, with no detectable differences between the lines when males were exposed to the high sugar or base diets.

Soluble protein concentrations recorded in control and selected males indicated similar results between the lines with the high protein diet producing increased soluble protein concentrations as compared to the high sugar diet. Measurements obtained from control males indicated a significantly increased concentration of soluble proteins in males placed on the high protein or high fat diets as compared to either the base or high sugar diets, with no statistically significant difference between males on the base and the

high sugar diets. Additionally, no statistically significant differences were detected between males exposed to the high protein and high fat diets. Soluble protein concentrations measured in selected males indicated no statistically significant differences in concentrations among all dietary alterations, except for the comparison between males on the high protein and high sugar diets. Within this comparison, males on the high protein diet possessed increased soluble protein concentrations as compared to males exposed to the high sugar diet. Measurements recorded in selected and control males indicated increased protein concentrations in control males on the high fat and high protein diets as compared to the base and high sugar diets, while measurements recorded in selected males indicated increased protein concentrations in males on the high protein diet as compared to the high sugar diet.

Total carbohydrate concentrations

Measurements of total carbohydrate concentrations normalized with respect to dry weight in the selected and control lines on each of the four diets resulted in a statistically significant effect of dietary treatment (tables 4.2 and 4.9) in both sexes (p-values <0.0001 females and 0.0004 males). Females exposed to the high sugar diet possessed increased concentrations of carbohydrates as compared to the other three dietary alterations, with no significant differences in carbohydrate concentrations detected between females exposed to the high protein, high fat, or base diets. Males exposed to the high fat diet possessed increased carbohydrates as compared to males placed on the other three dietary alterations. Additionally, no significant differences in carbohydrate concentrations were

detected between males exposed to the high protein, high sugar, or base diets. Measurements of carbohydrate concentrations in both sexes indicated increased carbohydrate concentrations in females placed on the high sugar diet and males exposed to the high fat diet.

A significant interaction between selection and the dietary treatments (table 4.10) was identified in female carbohydrate concentrations normalized by dry weight (p-value 0.0079). Selected females placed on the high sugar or base diets possessed reduced carbohydrate concentrations as compared to control females, while exposure of females to either the high fat or high protein diets resulted in no statistically significant differences between the selected and control lines. Comparisons of the effects of dietary supplementation in control females indicated increased carbohydrate concentrations in females exposed to the high sugar diet as compared to the other three diets, with no statistically significant differences in carbohydrate concentrations detected in females on the high fat, high protein, or base diets. Dietary comparisons in selected females indicated that all three of the dietary alterations resulted in increased carbohydrate concentrations as compared to the base diet, with no statistically significant difference in carbohydrate concentrations observed in females placed on the high sugar, high fat, or high protein diets. Measurements of the interaction between selection and the dietary treatments indicated a reduction in carbohydrate concentrations in females exposed to the high sugar and base diets, with the high sugar diet resulting in increased concentrations in control females as compared to the other three dietary alterations. Measurements in selected

females indicated that all three of the dietary alterations resulted in increased carbohydrate concentrations as compared to females exposed to the base diet.

Glycogen concentrations

Analysis of the selected and control lines placed on each of the four diets indicated a significant effect of dietary treatment (tables 4.3 and 4.9) on glycogen concentrations in both sexes (p-values <0.0001 females and 0.00126 males). Comparisons of glycogen concentrations obtained from females exposed to the high sugar diet resulted in increased glycogen as compared to females placed on the high fat, high protein, or base diets. Females on either the high fat or base diets possessed increased concentrations of glycogen as compared to females on the high protein diet, with no statistically significant differences detected between females exposed to high fat or base diets. Measurements of glycogen concentrations normalized by dry weight in males resulted in no statistically significant differences between the diets, except for the comparison between males on the high fat and base diets. Within this comparison, males exposed to the high fat diet possessed increased concentrations of glycogen as compared to males on the base diet. Measurements of glycogen concentrations in females exposed to each of the diets resulted in increased concentrations of glycogen obtained from females placed on the high sugar diet as compared to the high fat and base diets, with females exposed to the high protein diet possessing the least amount of glycogen. Measurements obtained from males indicated that males placed on the high fat diet resulted in increased glycogen as compared to males on the base diet.

Glycogen concentrations recorded in males indicated a statistically significant interaction between selection and the dietary treatments (table 4.10), with no significant differences in glycogen concentrations detected between selected and control males placed on each of four the diets (p-value 0.0210). Measurements of glycogen concentrations in control males indicated that each of the dietary alterations resulted in increased glycogen content as compared to concentrations obtained from males on the base diet. Moreover, no statistically significant differences in concentrations were observed in control males exposed to the high fat, high sugar, or high protein diets. Glycogen concentrations obtained from selected males indicated that males exposed to the high fat diet possessed increased glycogen concentrations as compared to males on the high sugar diet, with no statistically significant differences detected between comparisons of males on the additional diets. Measurements of the interaction between selection and the dietary treatments indicated no significant differences in glycogen concentrations obtained from selected and control males, with the three dietary alterations in control males resulting in increased glycogen concentrations as compared to the base diet. Glycogen concentrations obtained from selected males indicated increased concentrations in males placed on the high fat diet as compared to the high sugar diet.

Total sugar concentrations

Total sugar concentrations obtained from selected and control lines exposed to each of the four diets indicated a statistically significant effect of dietary treatments (tables 4.4 and 4.9) in both sexes (p-values <0.0001). Comparisons of total sugar concentrations in females indicated that exposure to the high protein diet resulted in greater sugar concentrations than the other three diets, with no statistically significant differences in sugar concentrations detected between females on the high sugar, high fat, or base diets. Comparisons of males placed on each of the four dietary alterations indicated that males on the high protein diet possessed increased concentrations of sugars as compared to males exposed to the high fat, high sugar, or base diets. Moreover, measurements of sugars in males on the high fat and high sugar diets resulted in increased sugar concentrations as compared to males exposed to the base diet. Comparisons of total sugar concentration in males and females placed on each of the four diets indicated an increase in sugar concentrations in individuals exposed to the high protein diet as compared to the additional three diets.

A statistically significant interaction was detected between selection and dietary treatment (table 4.10) in female sugar concentrations normalized with respect to dry weight (p-value 0.0172). Comparisons of selected and control females on each of the four diets indicated no statistically significant difference between the lines on all of the diets, except for the high sugar diet. Selected females placed on the high sugar diet possessed reduced concentrations of sugars as compared to control females exposed to the same diet. Measurements of total sugar concentrations in selected and control females indicated

that females on the high protein diet possessed increased concentrations of sugars as compared to the other three diets, with no significant differences in concentrations obtained from females on the high fat, high protein, or base diets. The interaction between selection and the dietary treatments indicated that selected females possessed reduced concentrations of sugars as compared to the control lines, with females placed on the high sugar diet resulting in increased total sugar concentrations as compared to females on the additional three diets.

Trehalose concentrations

Dietary treatment had a statistically significant effect on trehalose concentrations (tables 4.5 and 4.9) in both sexes of the selected and control lines (p-values <0.0001). Measurements of trehalose concentrations in females exposed to each of the four diets indicated a significantly increased concentration of trehalose in females placed on the high fat diet as compared to the other three diets. Trehalose concentrations were increased in females exposed to the base diet as compared to females on the high protein and high sugar diets, with no significant differences detected between females exposed to the high protein or high sugar diets. Males exposed to either the base or high fat diets possessed increased concentrations of trehalose as compared to males placed on either the high protein or high sugar diets, with no statistically significant differences observed between males exposed to the high protein or high sugar diets. Additionally, no significant differences in trehalose concentrations were observed in males placed on either the high fat or base diets. Measurements of trehalose concentrations obtained from

selected and control females and males indicated that the high fat diet resulted in increased quantities of trehalose as compared to the other three diets.

In females, a statistically significant interaction between selection and treatment (table 4.10) was observed in measurements normalized with respect to dry weight (p-value 0.0266). Comparisons of selected and control females on each of the diets indicated no statistically significant differences between the lines on all of the diets, except for the high protein diet. When exposed to the high protein diet, selected females possessed significantly increased concentrations of trehalose as compared to control females. Measurements of trehalose concentrations in control females indicated a progression of concentrations among the diets with females placed on the high fat diet possessing the greatest quantity of trehalose followed by females on the base diet. Females placed on the high sugar diet possessed reduced trehalose concentrations compared to females on the base diet, with females on the high protein diet possessing the least amount of trehalose. Similar results were observed in selected females, except for the comparison between the high sugar and high protein diets. In this comparison, no statistically significant differences were detected between females exposed to the high sugar or high protein diets. The interaction between selection and the dietary alterations indicated increased trehalose concentrations in selected females as compared to control females while on the high protein diet, while comparisons within the selected and control lines indicated that females exposed to the high fat diet resulted in increased trehalose concentrations as compared to the other three diets.

Triglyceride concentrations

Measurements of triglyceride concentrations normalized by dry weight in selected and control lines indicated a significant effect of dietary treatment (table 4.9) in both sexes (p-values <0.0001), with a significant effect of selection identified in males (tables 4.6 and 4.8). Measurements of triglyceride concentrations indicated that females on the high sugar diet possessed increased triglyceride concentrations as compared to females placed on either the high fat or high protein diets, with no statistically significant difference in concentrations detected between females exposed to the high fat or high protein diets. Triglyceride concentrations obtained from females on each of the three dietary alterations resulted in increased triglycerides as compared to females on the base diet. A statistically significant effect of selection in males resulted in selected lines possessing increased concentrations of triglycerides as compared to control lines (p-value 0.0018). Comparisons of dietary treatments indicated that males placed on the high sugar diet possessed increased triglyceride concentrations as compared to males on the base diet. Measurement of triglyceride concentrations in males on the high fat, high protein, or base diets resulted in the opposing tend observed in females, where males on the high fat or high protein diets possessed reduced concentrations of triglycerides as compared to males on the base diet. Comparisons of triglyceride concentrations in males and females indicated that selected males possessed increased triglyceride concentrations as compared to control males with exposure to the high sugar diet resulting in increased triglyceride concentrations in both sexes.

A statistically significant interaction between selection and the dietary treatments (table 4.10) was observed in females (p-value <0.0001). Measurements of triglyceride concentrations indicated that selected females possessed significantly increased concentrations of triglycerides as compared to control females placed on the high sugar, high protein, or base diets, while selected and control females placed on the high fat diet did not possess significantly different triglyceride concentrations. Control females exposed to the high sugar diet resulted in the greatest concentrations of triglycerides followed by control females placed on the high fat diet. Females on the high protein and base diets possessed reduced concentration of triglycerides as compared to the high fat diet, with no significant differences observed between the high protein and base diets. Measurements of triglyceride concentrations in selected females on the high sugar and high protein diets indicated increased triglycerides as compared to females on either the high fat or base diets. No significant differences in concentrations were observed between females on the high sugar or high protein diets, or in females on the high fat or base diets. Comparisons of the interaction between selection and the dietary treatments in females indicated that the selected females possessed increased triglycerides as compared to control females on all diets, except the high fat diet where no differences between the lines were observed. Exposure to the high sugar diet in each of the female lines resulted in increased triglyceride concentrations as compared to the other three diets.

In males, a statistically significant interaction between selection and treatment (table 4.10) was detected in concentrations normalized by dry weight (p-value 0.0022). Comparisons made with regards to selection resulted in selected males possessing

increased quantities of triglycerides as compared to control males when exposed to each of the dietary alterations. Measurements in the control males indicated that the high sugar diet resulted in increased quantities of triglycerides as compared to all other diets, with no statistically significant differences in concentrations observed between males exposed to the high protein, high fat, and base diets. Similar results were observed in selected males, with males on the high sugar diet possessing increased triglyceride concentrations as compared to males on the base diet. Selected males placed on the high fat or high protein diets resulted in reduced triglyceride concentrations as compared to males on the base diet, with no statistically significant difference in concentrations detected between the high fat and high protein diets. Measurements of triglyceride concentrations in males indicated that the selected lines possessed increased triglyceride concentrations as compared to control lines, with males on the high sugar diet resulting in increased triglycerides as compared to the other three dietary alterations.

Dry weight measurements

Selection for starvation resistance and dietary supplementation were shown to have a statistically significant effect on body mass in *Drosophila melanogaster* (tables 4.7, 4.8, and 4.9). Comparisons of dry weight values obtained from selected and control males and females indicated that selection for starvation resistance resulted in increased dry weight of selected males and females as compared to control lines (p-values 0.0092 males, 0.0003 females). Additionally, dry weight measurements of selected and control males and females indicated a significant effect of dietary treatment in both sexes (p-

values <0.0001). In females, exposure to the high sugar diet resulted in the greatest accumulation of dry weight, which was followed by females placed on the high fat diet. Females on the high protein diet possessed reduced weight as compared to females on the high fat diet, with the least amount of dry mass observed in females placed on the base diet. Comparisons in males indicated increased dry weight measurements obtained from males exposed to the high sugar diet followed by males placed on the high fat diet. Males placed on either the high protein or base diets resulted in reduced dry mass as compared to males exposed to the high fat diet, with no statistically significant differences in mass detected between males on either the high protein or base diets. Measurements of dry weight obtained from the selected and control males and females indicated the greatest increase of dry mass in flies exposed to the high sugar diet as compared to the other three diets.

Female egg production

Analysis of fecundity in selected and control females indicated no statistically significant difference in egg production between the selected and control lines when fed the same diet for each of the four diets; however, alterations to the diet resulted in significant changes in female egg production. Comparisons of fecundity obtained from females placed on the high sugar diet resulted in decreased egg production as compared to the base diet (figure 4.4), while no differences in egg production were detected between females exposed to the base diet and either the high fat or high protein diets (figures 4.5 and 4.6). Females placed on the high protein diet produced a significantly

increased number of eggs as compared to females on the high sugar diet (figure 4.7). Evaluation of egg quantity obtained from females on the high fat diet compared to the high protein diet indicated an increased amount of eggs produced from females on the high protein diet within the first 15 days of experimentation (figure 4.8). This situation was reversed in comparisons of the high fat and high sugar diets with increased egg production observed from females on the high fat diet in the last 15 days of experimentation (figure 4.9).

Longevity

The duration of lifespan was recorded for female and male selected and control lines exposed to each of the four diets. Comparisons of female longevity indicated that the selected lines lived significantly shorter than control lines (figures 4.10, 4.12, 4.14). Female responses to each of the supplemented diets resulted in decreased longevity as compared to females exposed to the base diet. Lifespan measurements of females on the high protein diet resulted in increased longevity as compared to either the high sugar or high fat diets (figures 4.16, 4.18) with females placed on the high sugar diet living appreciably more than those on the high fat diet (figure 4.20). In males, no significant differences were detected between the diets, except for the comparison made between the base and the high fat diets (figures 4.11, 4.15, 4.17, 4.19, 4.21). Within this comparison, males placed on the high fat diet lived significantly less than males exposed to the base diet (figure 4.13).

Movement

There were no main effects of selection or environmental variation on the number of movements, except that light resulted in a reduction in activity levels recorded in males and females from the selected and control lines. The number of movements obtained from selected and control females exposed to each of the four diets indicated a statistically significant interaction between selection and light (p-value 0.0104). Within this interaction, no significant differences in movement were detected between the selected and control lines exposed to 12 hours of darkness. During the light phase, the selected females exhibited a reduction in the number of movements as compared to control females. Additionally, increased movements were detected within the light phase in the control lines as compared to movements in the dark (p-value 0.0198). No statistically significant differences in movements were detected for selected lines during either the light or dark phases.

Interactions between the dietary alterations and the light cycle were recorded during the 12 hours of darkness (p-value 0.0365). Within this comparison, females on the high fat and high protein diets exhibited increased movements as compared to females on the high sugar diet. All other dietary comparisons using females resulted in no statistically significant differences in the number of movements recorded. In males, a statistically significant effect of light was identified with increased movements recorded during the 12 hours of darkness as compared to the 12 hours of light (p-value 0.0119). An interaction between the diets and the light cycle indicated a statistically significant

increase in movements during the dark in males exposed to the high fat, high protein, and high sugar diets (p-values 0.0133, 0.0059, and 0.0470 respectively).

Discussion

Alterations to dietary nutrient composition frequently occur within natural populations due to changing environmental conditions, such as alterations in temperature and available nutrients (Somero & Hochachka, 1971). Due to the variability of available resources, organisms must adapt to the changing environment to ensure survival. One example of evolved adaptations to changes in nutrient consumption in natural populations is the metabolic and physiological modifications associated with evolved resistance to starvation. In response to reductions in available food resources, most organisms have adapted mechanisms that sense nutrient availability allowing for increased storage of triglycerides and carbohydrates when food is plentiful (Djawden et al., 1998; Harshman et al, 1999). During periods of food deprivation, organisms ranging from insects to vertebrates have evolved a coordinated physiological response that allows for efficient utilization of energy coupled to a conservation of metabolic fuels through reduced movement. These mechanisms of starvation resistance have been shown to enhance survival while exposed to starvation (Sugden et al., 1989).

The present study utilized genetically based starvation-resistant and control lines of *Drosophila melanogaster* to gain insight into correlations between dietary supplementation and associated alterations in body composition, life history traits, and movement. Artificial selection for starvation resistance resulted in a two fold increase in survival under starvation in selected males and a 2.5 fold increase in female survival under starvation at the fifty percent mortality level following 20 generations of selection for starvation resistance. Measurements of survival within the control lines, used as the

basis for comparison, resulted in no statistically significant alterations to survival under starvation following 20 generations in culture.

Analysis of soluble proteins indicated a significant increase in protein concentrations when males and females were exposed to the high protein diet. A significant reduction in soluble protein concentrations were detected in selected lines as compared to control lines on the high fat and high protein diets, with no detectable difference between the lines on the high sugar and base diets. A related study conducted in *Caenorhabditis elegans* analyzing translation initiation factors *ifg-1*, the worm homologue of eIF4G, and *rsks-1*, the worm homologue of S6 kinase, in starvation resistant lines (Pan et al., 2007), indicated a reduction in protein synthesis during starvation due to inhibition of these two initiation factors. Inhibition of translation in starvation-resistant lines has been suggested as a mechanism to preserve somatic maintenance and decrease organismal growth during periods of starvation (Pan et al., 2007). This decrease in translation could perhaps be one potential explanation for the reduction in soluble protein concentrations observed in the starvation-resistant lines used in the present study. Protein concentrations were increased in individuals exposed to the high protein diet. This observation was consistent with previous findings that *D. melanogaster* exposed to a high protein diet, consisting of increased yeast concentrations, resulted in increase protein deposition within the organism (Skorupa et al., 2008). Protein measurements recorded in the males with regards to dietary treatments indicated increased protein concentrations measured in individuals exposed to the high fat diet. Analysis of metabolic pathways (figure 4.22) indicated that when individuals are exposed

to increased fat concentrations in the diet some of the fatty acids can be converted into acetyl-CoA through the citric acid cycle, which can then be converted into protein (Randle, 1995; Stipanuk, 2006). Comparisons of protein concentrations in males and females exposed to each of the diets indicated that the high protein diet resulted in increased protein concentrations in females, while the high fat and high protein diets resulted in increased concentrations of soluble protein in males, supporting the theory that increased protein consumption can lead to increased protein storage (Skorupa et al., 2008).

Body composition analysis of total carbohydrate and glycogen concentrations in males and females indicated increased concentrations associated with placement on the high sugar diet in females, while males on the high fat diet possessed increased amounts of carbohydrates and glycogen. Perhaps the sex differences in carbohydrate storage on the altered diets were associated with the cost of reproduction where females convert dietary sugar to stored carbohydrates for nutrition for the developing eggs, while males convert excess fat to sugar to be used for energy in movement or flight. Comparisons of the selected and control females indicated that within the control lines, females exposed to the high sugar diet possessed the largest amount of total carbohydrates. This result was supported by observations that exposure to increased levels of sugar in the diet results in enhanced sugar concentrations (Stipanuk, 2006). Comparisons within selected females indicated that each of the dietary alterations resulted in increased carbohydrates as compared to the base diet. Analyses of metabolic pathways (figure 4.23) illustrated the potential conversions of increased sugar, protein, and fat obtained from the diet into

increased carbohydrates, especially glucose. Selected lines possesses significantly reduced concentrations of carbohydrates as compared to the control lines when placed on the high sugar diet, while no differences were detected between selected and control line glycogen concentrations. Thus, selected lines may be converting the additional sugar into other high-energy molecules, such as triglycerides, while the control lines utilize the additional sugar for carbohydrate storage.

Comparisons of total sugar and trehalose concentrations within the selected and control lines indicated that increased dietary protein resulted in increased total sugars, while increased dietary fat led to increased trehalose concentrations. In females, total sugar concentrations were reduced in the selected lines as compared to the controls, while the situation was reversed in male measurements with the selected lines possessing increased amounts of total sugars. This result indicated that the sexes may respond differently to selection with females utilizing sugars and trehalose for fuel, while selected males stored more sugars to be utilized for energy demanding processes including movement and flight. Trehalose concentrations were increased in the selected lines as compared to the controls. Additionally, increased sugars were detected in flies exposed to the high protein diet. This observation was supported by previous research indicating that high levels of protein can be toxic, and as such are frequently converted into glucose to be stored or utilized in energy production (Stipanuk, 2006). In the present study, a high fat diet resulted in increased whole body trehalose. This observation may be explained in that high levels of fat within the organism can be converted into glucose (Randle, 1995)

through the conversion of free fatty acids into acetyl-CoA that can then be converted into glucose through gluconeogenesis (figure 4.23).

Increased concentrations of triglycerides were observed in the selected lines, with females on the high sugar diet possessing the greatest concentrations of stored lipids. In males, the selected lines possessed significantly greater concentrations of triglycerides than control lines on all of the diets, while measurements in females indicate that selected lines possess greater triglycerides on all diets, except for the high fat diet where no differences were detected between the lines. All comparisons of triglyceride concentrations indicated that the high sugar diet resulted in increased quantities of triglycerides. This observation was supported by previous studies in starvation-resistant lines of *D. melanogaster* with increased carbohydrates in the diet promoting triglyceride storage (Skorupa et al., 2008).

Selected lines possessed increased dry mass as compared to control lines in both sexes. Males and females placed on the high sugar diet resulted in increased dry weight as compared to exposure of each of the sexes to the additional three diets. Additionally, results obtained from the analysis of body composition indicated an increase in total carbohydrates, glycogen, and triglycerides in flies exposed to the high sugar diet. Presumably, the increased concentrations of these energetic molecules were contributing to the overall increase in body mass observed in individuals placed on the high sugar diet.

Analysis of fecundity indicated a negative relationship between increased sugar in the diet and egg production, with no correlation observed between fecundity and selection for starvation resistance. Fecundity measurements indicated no statistically

significant differences between the selected and control lines on any of the altered diets indicating that selection pressures were not correlated with the number of eggs produced by females in the present study. Increased sugar concentrations were, however, negatively correlated with reproduction. This observation was supported by previous research indicating that increased sugar concentrations in the diet suppressed egg deposition rates (Lee et al., 2008; Skorupa et al., 2008). Measurements of egg production on the high protein diet revealed increased egg deposition, which was supported by previous research indicating a positive correlation between increased dietary protein supplementation, in the form of yeast, and fecundity (Skorupa et al., 2008; Katewa & Kapahi, 2010).

An overall negative correlation between dietary alterations and longevity was observed, with the most dramatic effects recorded in control females. Analysis of longevity between the selected and control lines indicated a reduced lifespan in the selected females as compared to control females. Comparisons of dietary effects on longevity indicated that all of the supplemented diets were negatively associated with lifespan in control females, with the high fat diet resulting in reduced lifespan in both sexes of the selected and control lines. This observation was supported by previous studies where increased protein, in the form of yeast, and increased carbohydrates, in the form of glucose, resulted in reduced longevity (Mair et al., 2005; Sanchez-Blanco et al., 2006; Lee et al., 2008; Skorupa et al., 2008; Ja et al., 2009). Additional research into alterations in food composition indicated that changes in nutrients could have negative

effects through potential food toxicity, which can dramatically reduce the lifespan of individuals exposed to the altered food source (Piper et al., 2007).

Analysis of movement patterns in the selected and control lines placed on one of the four diets indicated a significant effect of light on the number of movements recorded. The only significant alteration in the number of movements correlated with the diets occurred in females, with the high fat and high protein diets resulting in increased movements as compared to the high sugar diet. This results was probably not solely limited to nutrient intake, but rather could be attributed to increased amounts of energy obtained from metabolism of fats as compared to the energy obtained from the metabolism of glucose, thus allowing for increased movement of females on the high fat diet. In the dark phase, no statistically significant differences were detected between the selected and control lines in the number of movements. Comparisons of control females indicated increased movements in the light phase as compared to the dark, while no significant differences were detected in the number of movements in comparisons between the light and dark cycles in the selected lines.

Comparisons of movement and body composition data indicated that exposure to the high protein or high fat diets resulted in increased movements as well as increased concentrations of proteins, sugar, and trehalose. Perhaps the increased amounts of protein are deposited in muscles allowing for increased stamina, and thus an increase in the number of movements. Additionally, the increased amount of sugars present in females exposed to the high protein or high fat diets may be attributed to the increased amount of energy necessary to promote increased movement. Comparisons between movement and

life history traits indicated that females placed on the high protein diet have a significantly greater number of movements in addition to an increase in egg production. Perhaps females with increased protein have an increased number of movements to allow for greater distribution of eggs as a function of increased fecundity. Analysis of movement and longevity within the lines indicated that lines on the high fat and high protein diets have reduced lifespan, while movements are increased in lines exposed to these diets. Increased activity levels were negatively correlated with longevity in that energy is expended through increased movements, thus reducing the available energy to promote survival during times when energy resources are limited. Alternatively, the stress response may elicit a reduction in longevity that is independent of the increased number of movements.

The results of this study represent one of the most comprehensive studies in correlating dietary supplementation with alterations in body composition, life history traits, and movement. The outcome of this study suggests that alterations to dietary composition can lead to dramatic changes in body composition, life history traits, and activity levels. Further analysis of alterations to body composition within the selected and control lines needs to include an analysis of caloric content of each of the diets to identify whether the observed changes were due to alterations in macronutrient composition or changes in caloric content. Additionally, the amount of food consumed by each of the experimental subjects placed on each of the supplemented diets would provide insight into potential correlations between increases of individual dietary components with increased consumption. Correcting for these values would provide a more accurate

analysis of the individual alterations associated with increased supplementation of glucose, palmitic acid, and a 2:1 mixture of casein and albumin. Alterations to nutrient intake can have dramatic effects on the phenotype of an individual through the concerted actions of interlinked metabolic pathways. As such, alterations to specific nutrients including fat, carbohydrates, and proteins can result in changes that can enhance or reduce performance and quality of life. Studies examining an overall reduction in caloric intake, known as dietary restriction, have shown that intake of relatively low protein and moderate carbohydrate concentrations can result in increased longevity in a number of diverse organisms ranging from insects to humans (Mair et al., 2005; Lee et al., 2008; Ja et al., 2009; Katewa & Kapahi, 2010;).

In the present study, dietary supplementation had a significant effect on body composition with a consistently negative effect on survival. Results obtained from the current study have implications in the context of human health. One example is the use of dietary alterations in the promotion of weight loss within obese individuals. By restricting certain dietary components for a period of time, increased weight loss can be promoted resulting in improvements to overall health (Siedell, 2000; Hossain et al., 2007). Studies analyzing individuals on the Atkins diet indicate increased weight loss as compared to either the Zone or Ornish diets; however, both the South Beach and Ornish diets were better in weight management once the initial weight was lost (Gardner et al., 2007; Miller et al., 2009). These results must be considered with caution in that long-term effects have not been quantified. Results obtained from this study and others reviewing correlations with dietary alterations, body composition, and life history traits suggest that alterations

to the diet can promote an overall change in metabolism that can potentially have dramatic effects on body mass, reproduction, and longevity.

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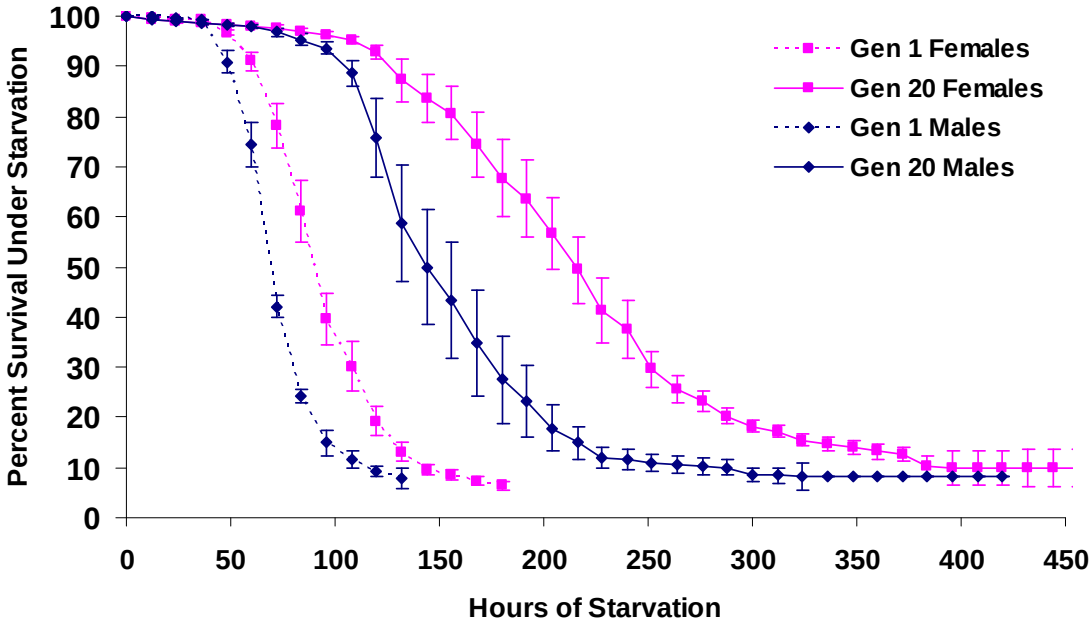

Figure 4.1 The response to selection of *Drosophila melanogaster* selected for starvation resistance. Percent survival (and standard error) of the starvation-resistant lines was assayed after one generation of selection and following twenty generations of selection for starvation resistance.

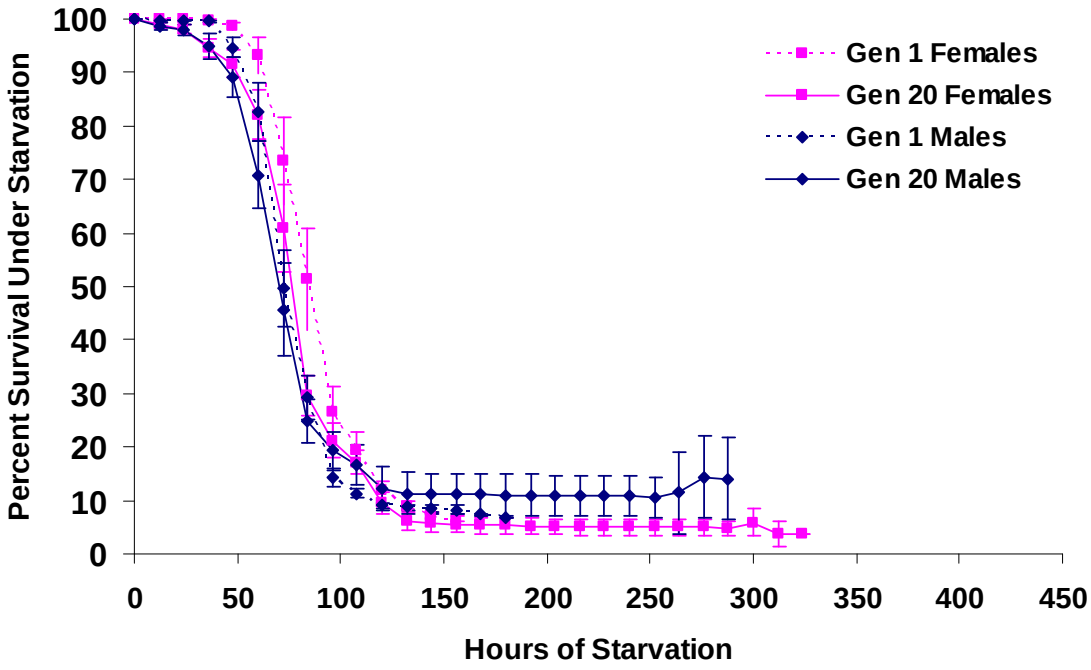

Figure 4.2 The response to starvation in control lines of Drosophila melanogaster. Survival under starvation (and standard error) was assayed following one and twenty generations of selection conducted on the starvation resistant lines.

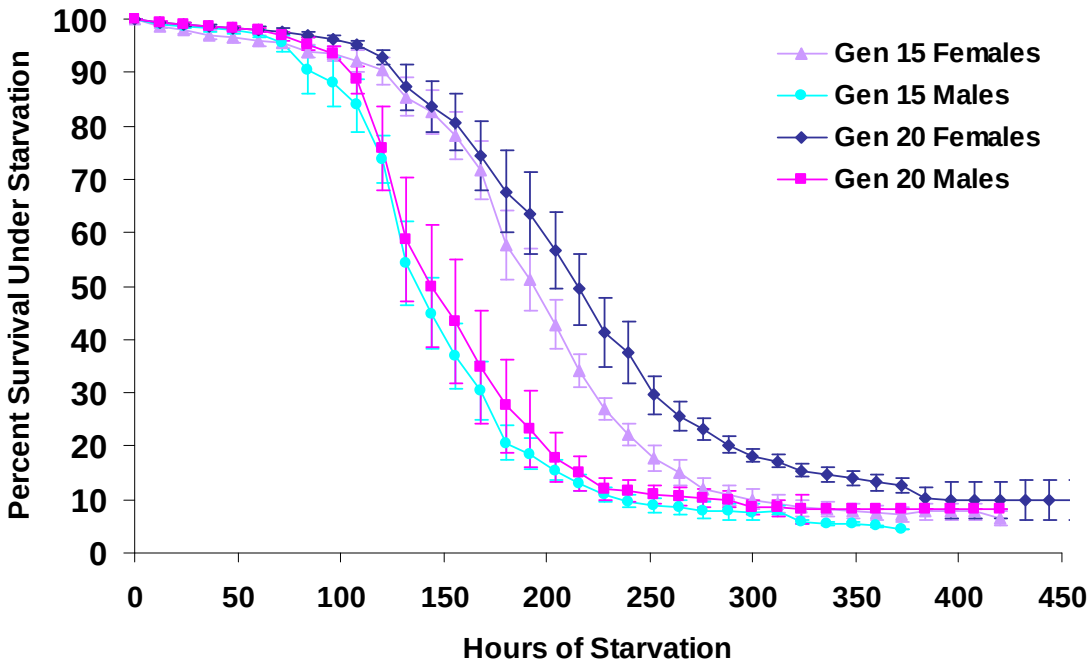

Figure 4.3 Comparisons of the direct response to selection in the starvation-resistant lines following fifteen and twenty generations of selection for starvation resistance. Percent survival (and standard error) of the starvation-resistant lines.

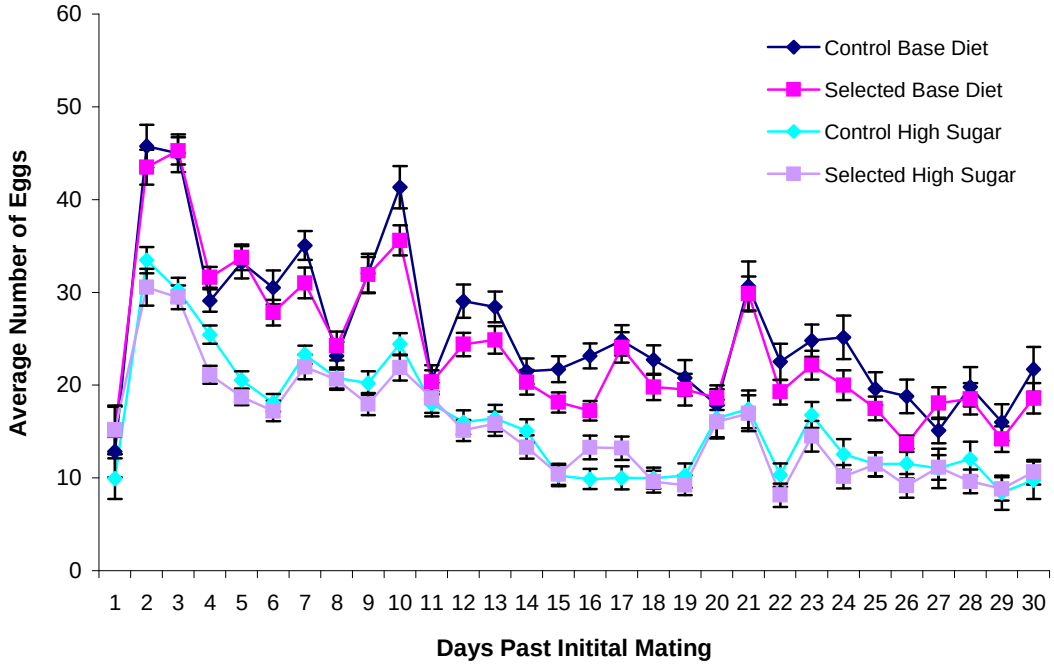

Figure 4.4 The average number of eggs (and standard error) produced by the selected and control lines on the base diet and high sugar diet following 20 generations of selection for starvation resistance.

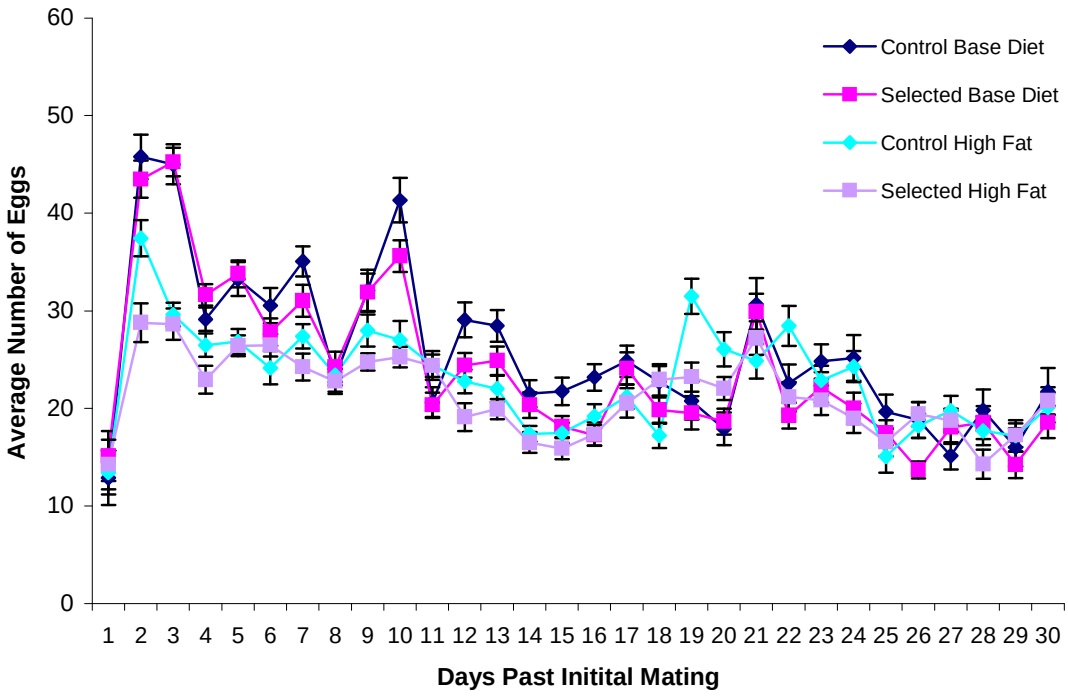

Figure 4.5 The average number of eggs (and standard error) produced by the selected and control lines on the base diet and high fat diet following 20 generations of selection for starvation resistance.

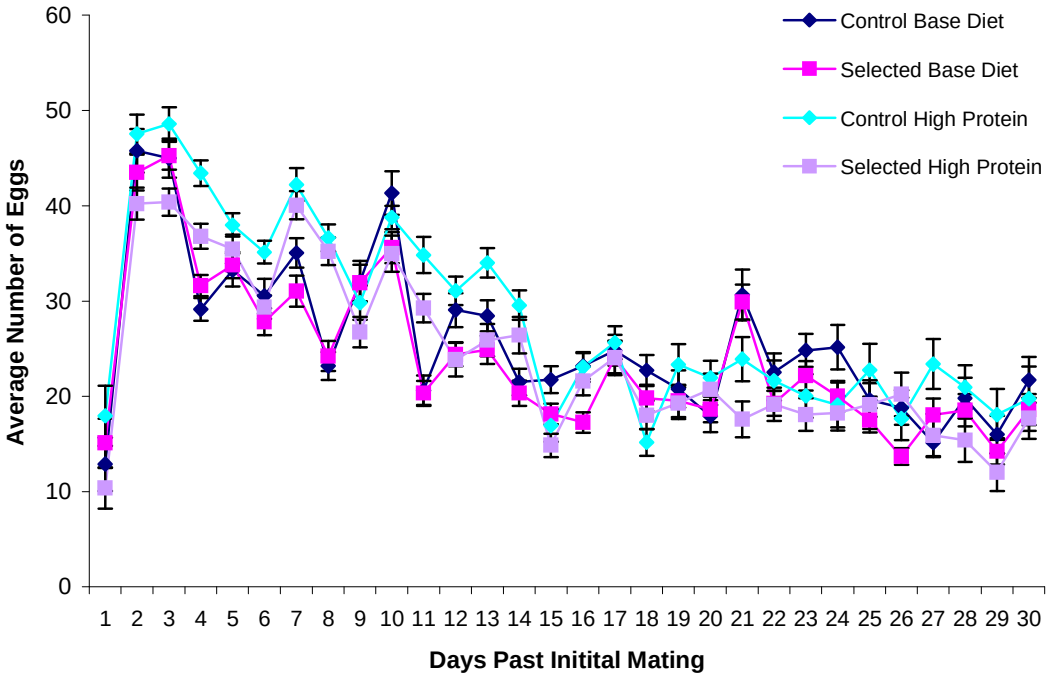

Figure 4.6 The average number of eggs (and standard error) produced by the selected and control lines on the base diet and high protein diet following 20 generations of selection for starvation resistance.

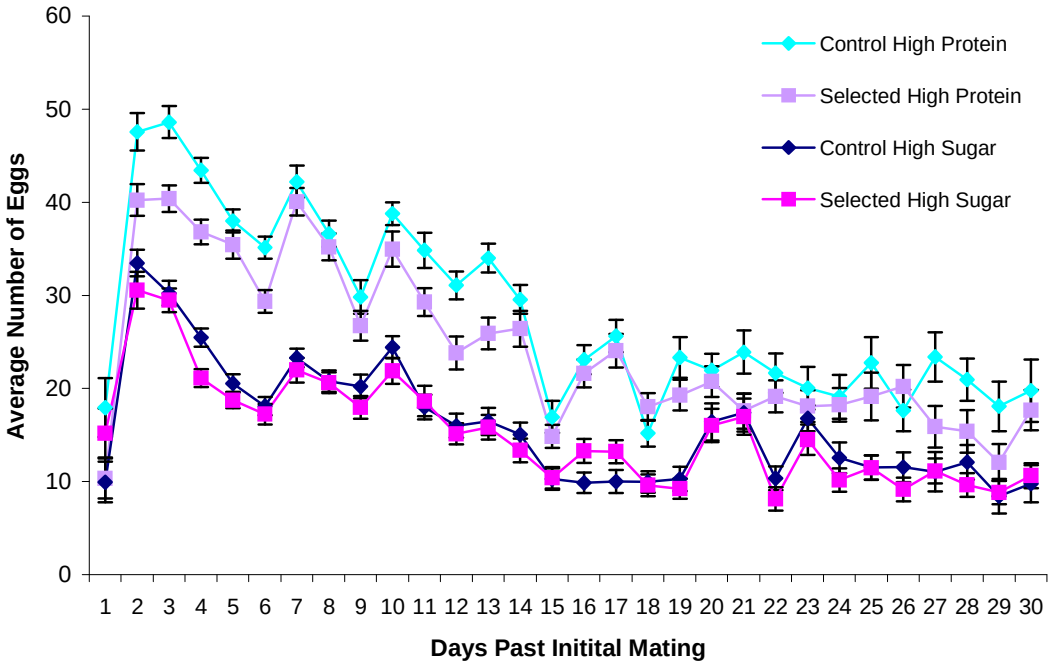

Figure 4.7 The average number of eggs (and standard error) produced by the selected and control lines on the high protein diet and sugar protein diet following 20 generations of selection for starvation resistance.

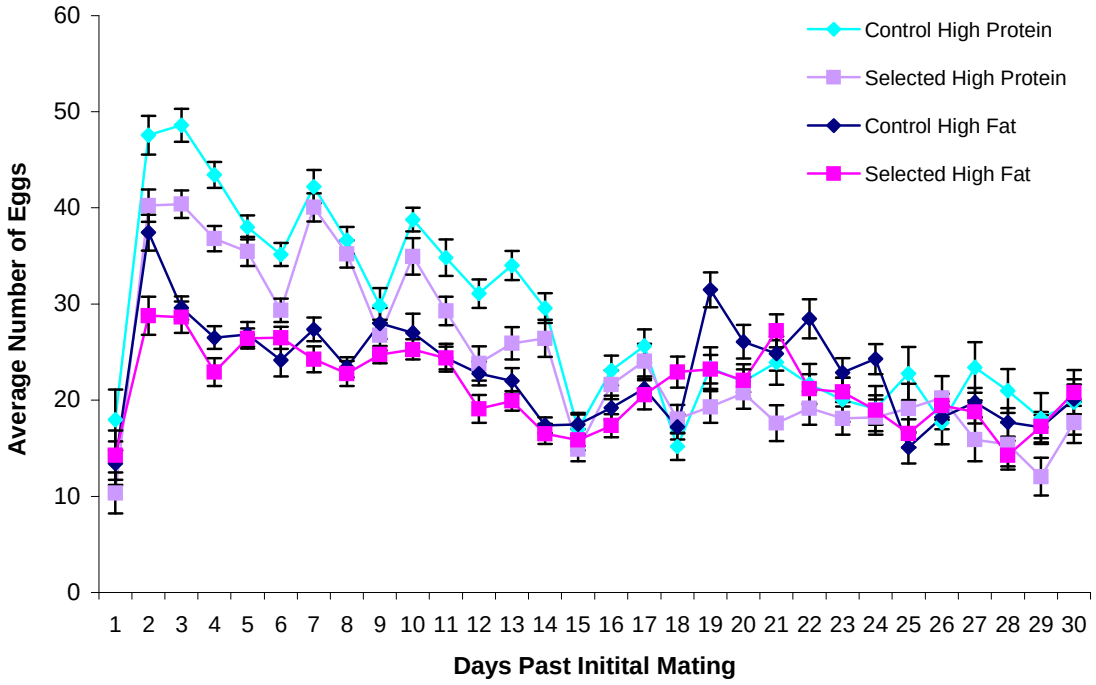

Figure 4.8 The average number of eggs (and standard error) produced by the selected and control lines on the high protein diet and high fat diet following 20 generations of selection for starvation resistance.

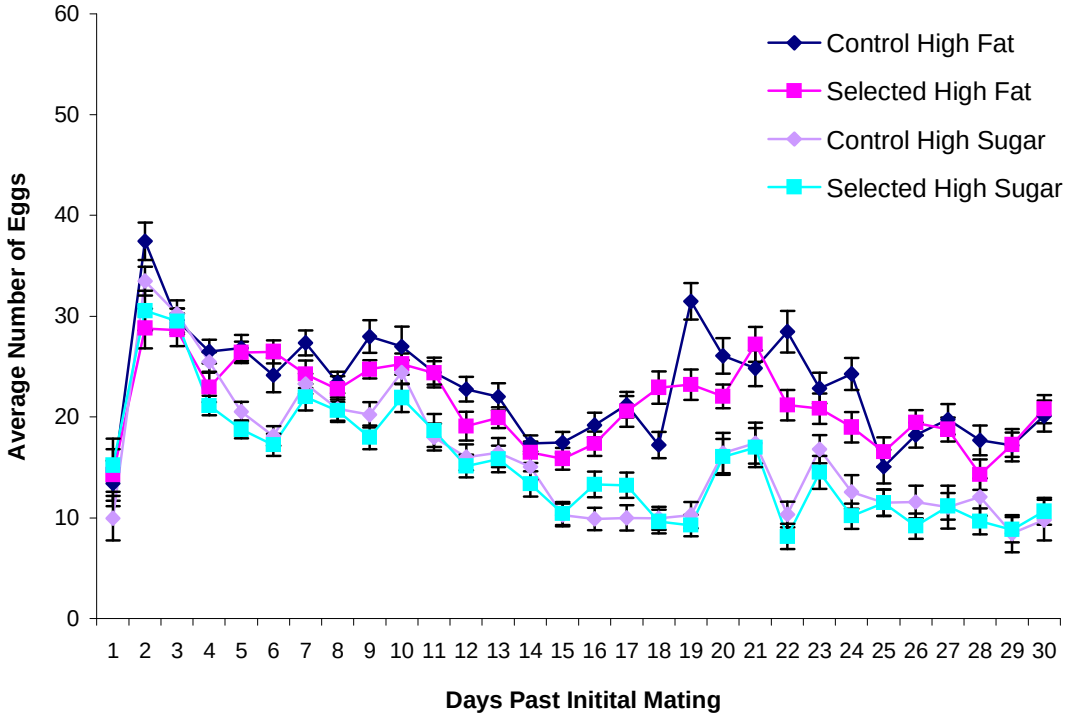

Figure 4.9 The average number of eggs (and standard error) produced by the selected and control lines on the high fat diet and high sugar diet following 20 generations of selection for starvation resistance.

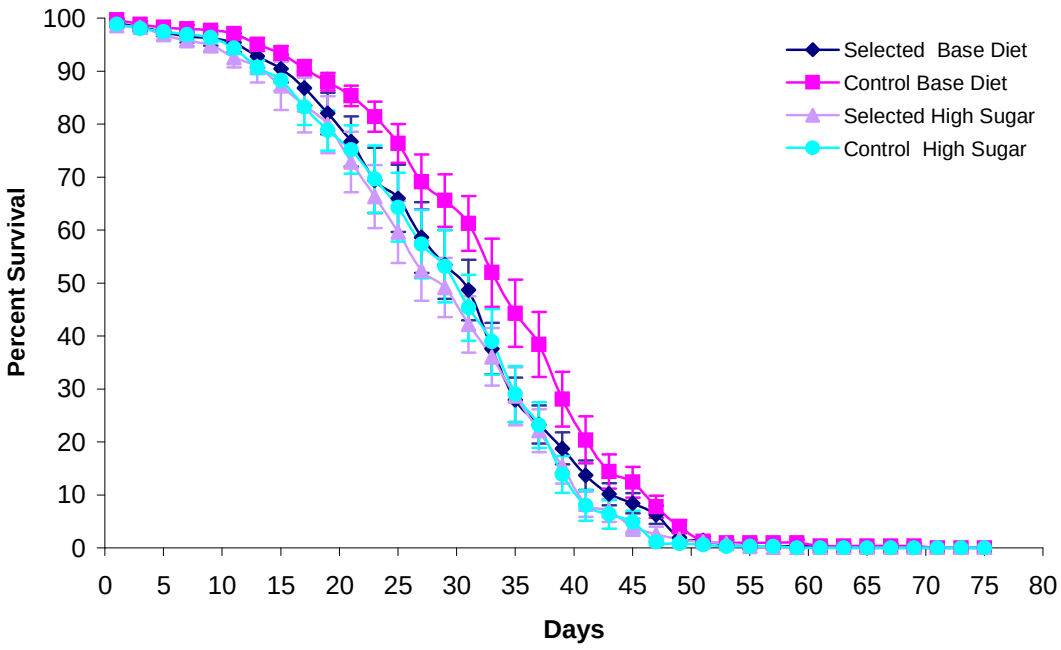

Figure 4.10 Average longevity (and standard error) of selected and control lines of female *Drosophila melanogaster* placed on the base and high sugar diets following 20 generations of selection for starvation resistance.

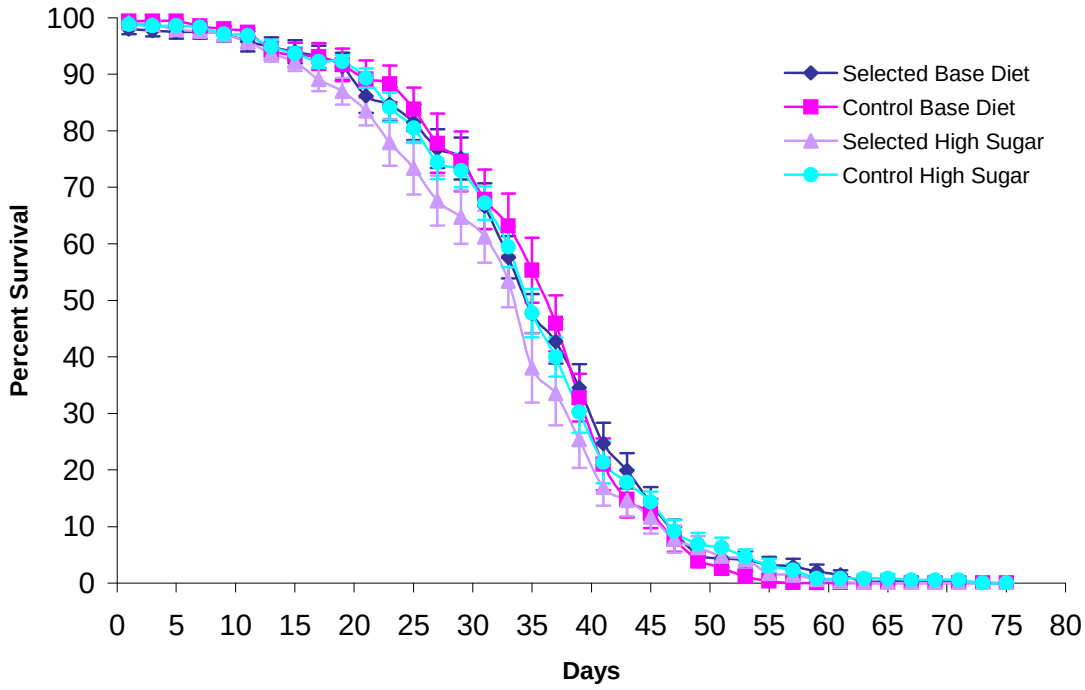

Figure 4.11 Average longevity (and standard error) of selected and control lines of male *Drosophila melanogaster* placed on the base and high sugar diets following 20 generations of selection for starvation resistance.

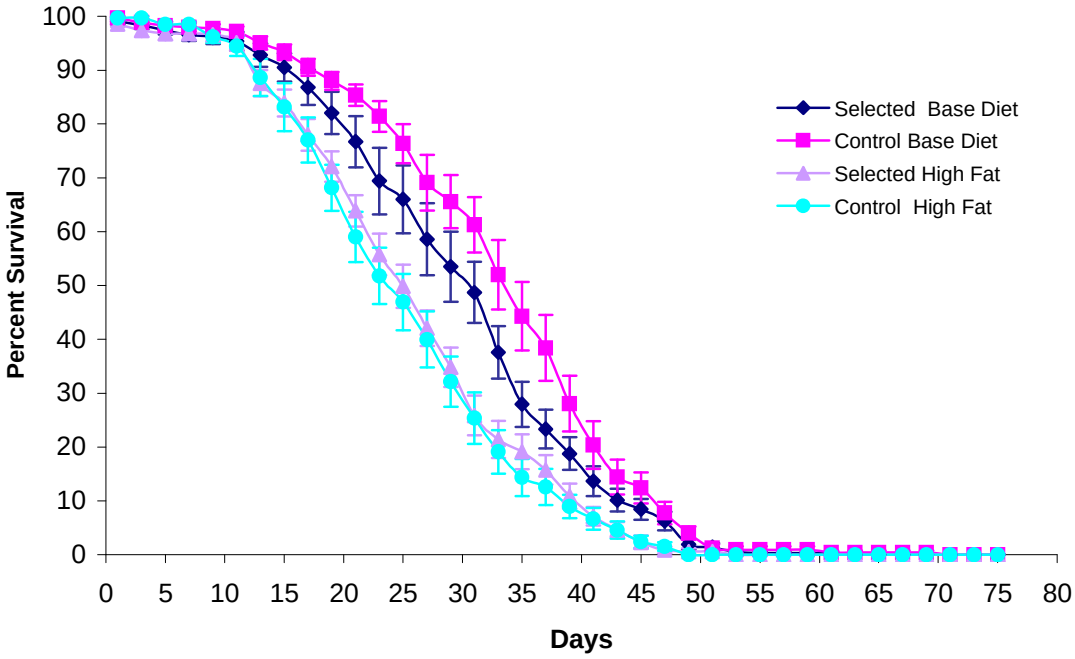

Figure 4.12 Average longevity (and standard error) of selected and control lines of female *Drosophila melanogaster* placed on the base and high fat diets following 20 generations of selection for starvation resistance.

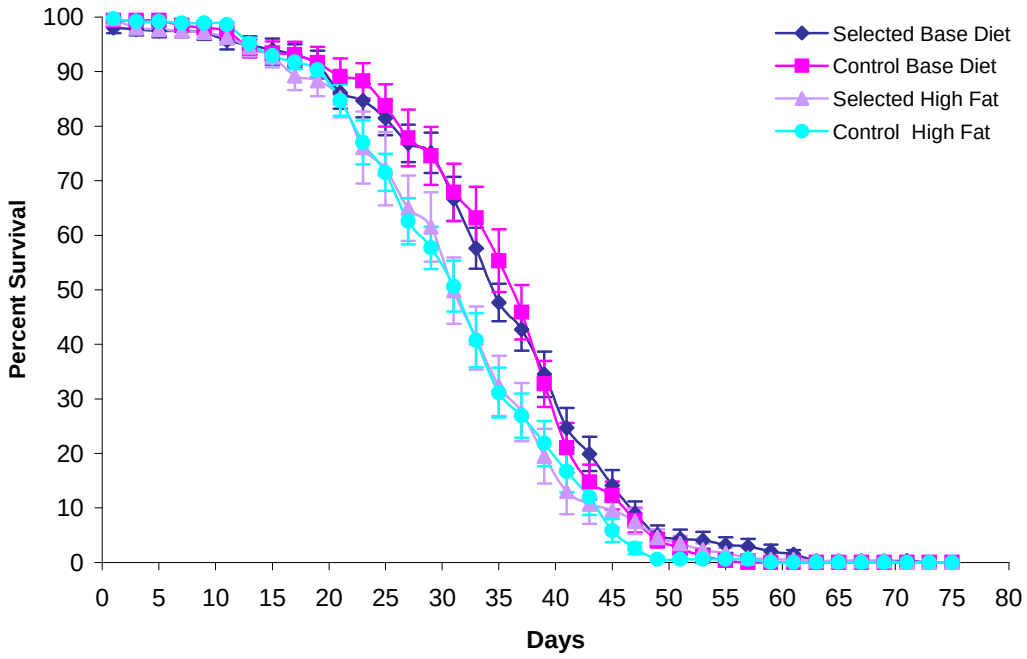

Figure 4.13 Average longevity (and standard error) of selected and control lines of male *Drosophila melanogaster* placed on the base and high fat diets following 20 generations of selection for starvation resistance.

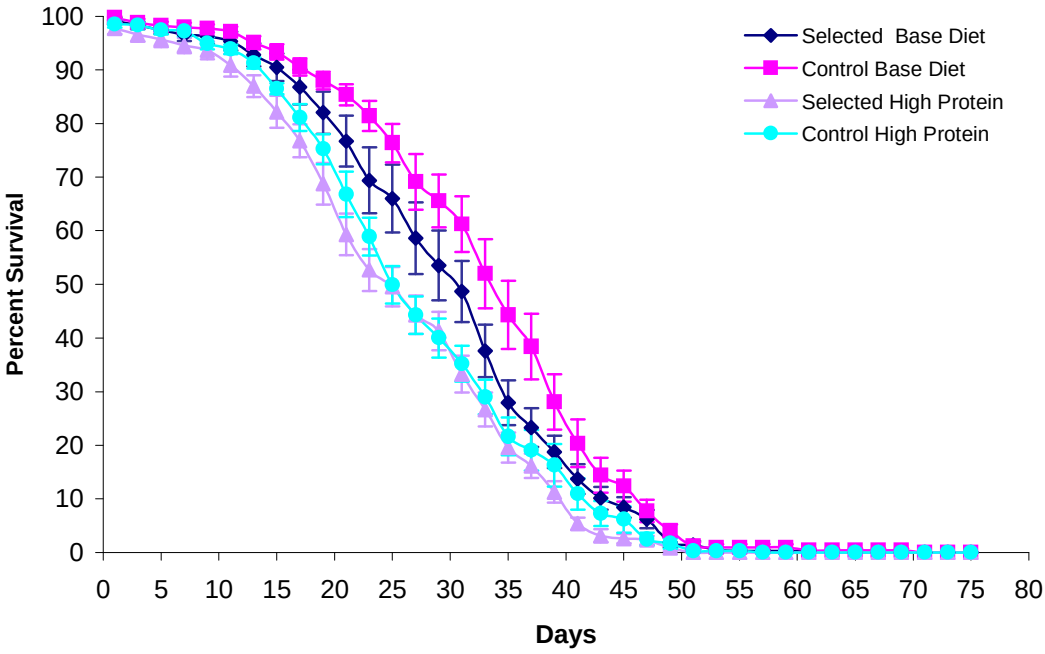

Figure 4.14 Average longevity (and standard error) of selected and control lines of female *Drosophila melanogaster* placed on the base and high protein diets following 20 generations of selection for starvation resistance.

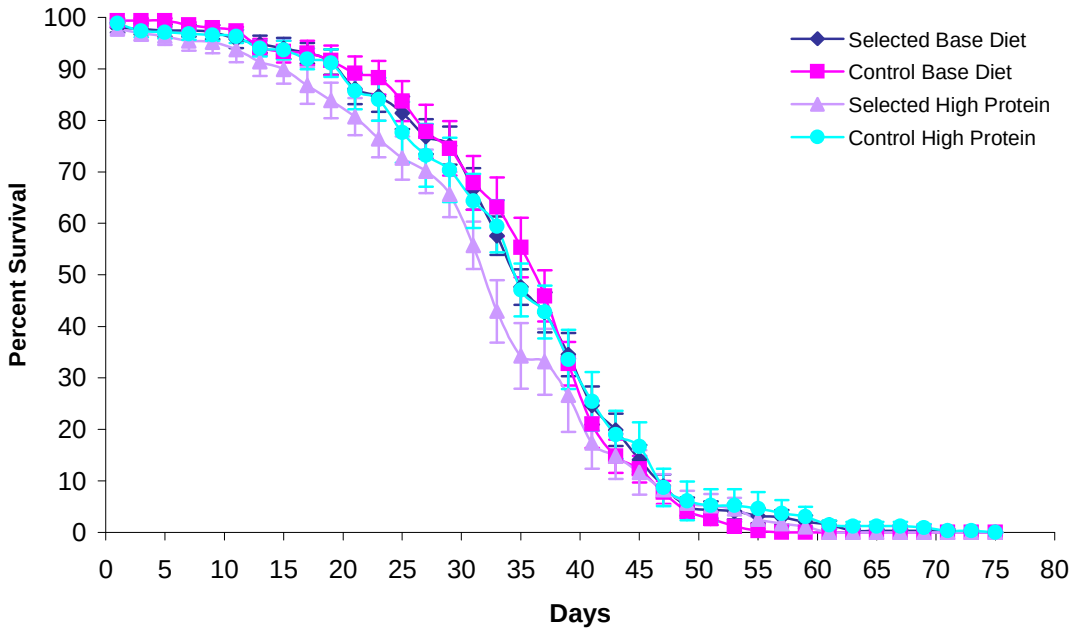

Figure 4.15 Average longevity (and standard error) of selected and control lines of male *Drosophila melanogaster* placed on the base and high protein diets following 20 generations of selection for starvation resistance.

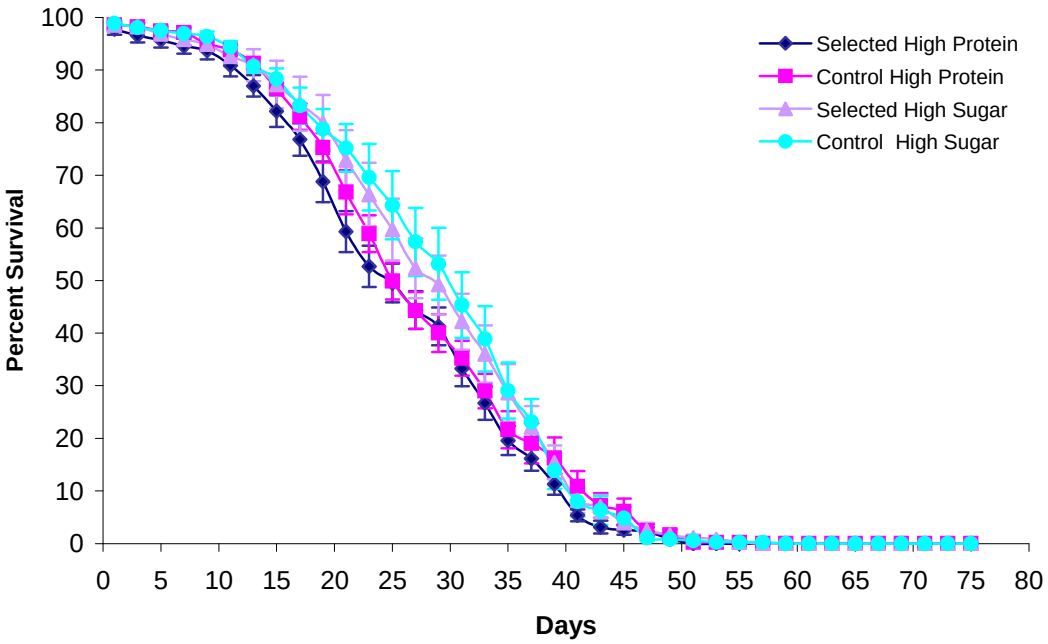

Figure 4.16 Average longevity (and standard error) of selected and control lines of female *Drosophila melanogaster* placed on the high protein and high sugar diets following 20 generations of selection for starvation resistance.

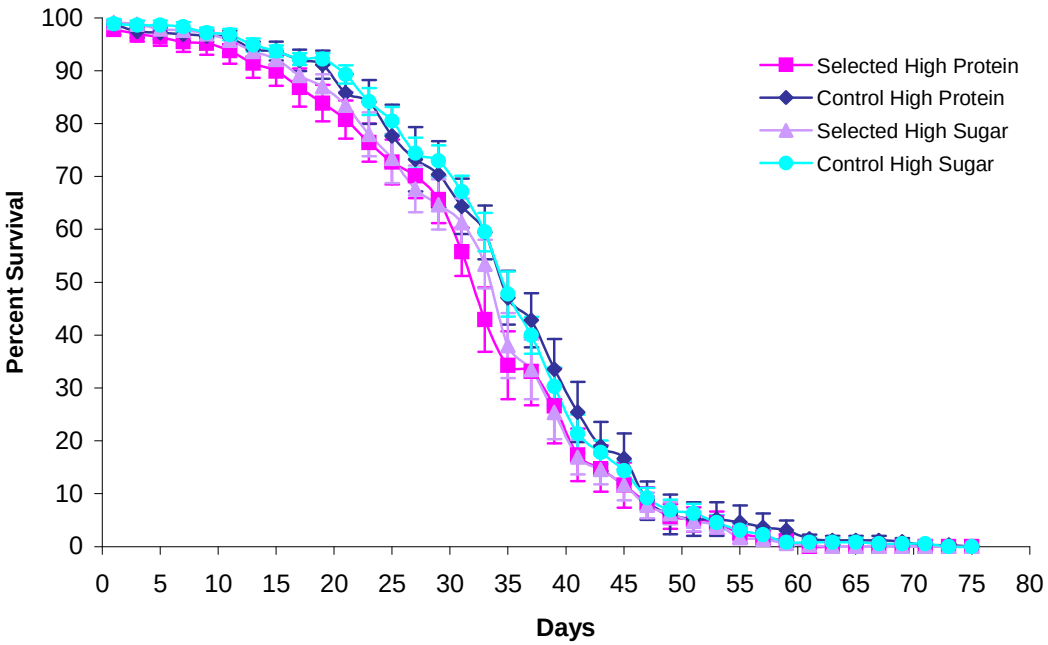

Figure 4.17 Average longevity (and standard error) of selected and control lines of male *Drosophila melanogaster* placed on the high protein and high sugar diets following 20 generations of selection for starvation resistance.

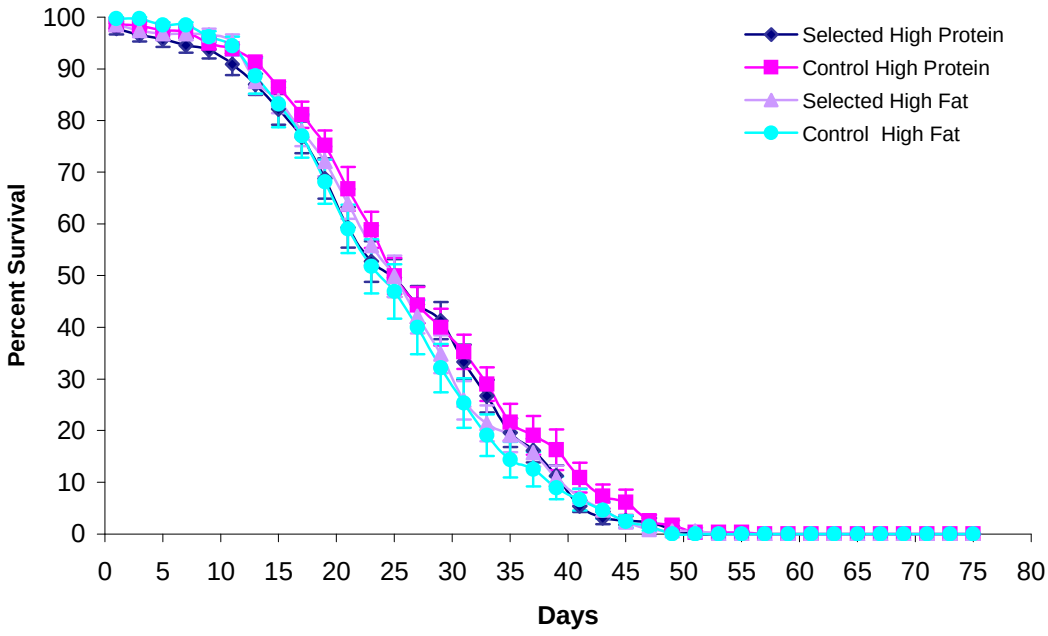

Figure 4.18 Average longevity (and standard error) of selected and control lines of female *Drosophila melanogaster* placed on the high protein and high fat diets following 20 generations of selection for starvation resistance.

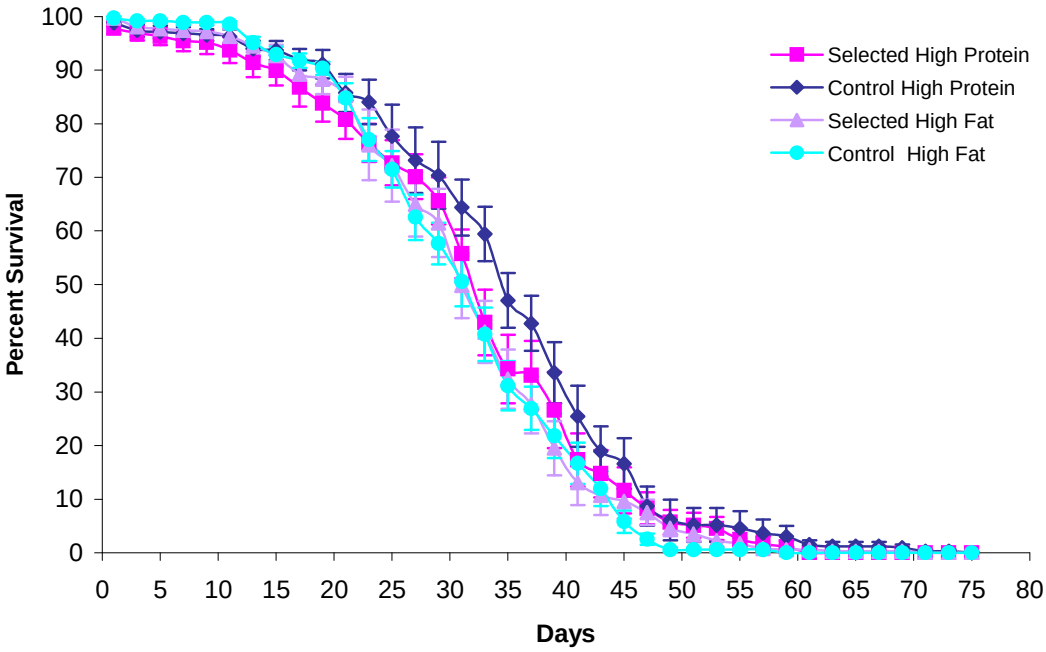

Figure 4.19 Average longevity (and standard error) of selected and control lines of male *Drosophila melanogaster* placed on the high protein and high fat diets following 20 generations of selection for starvation resistance.

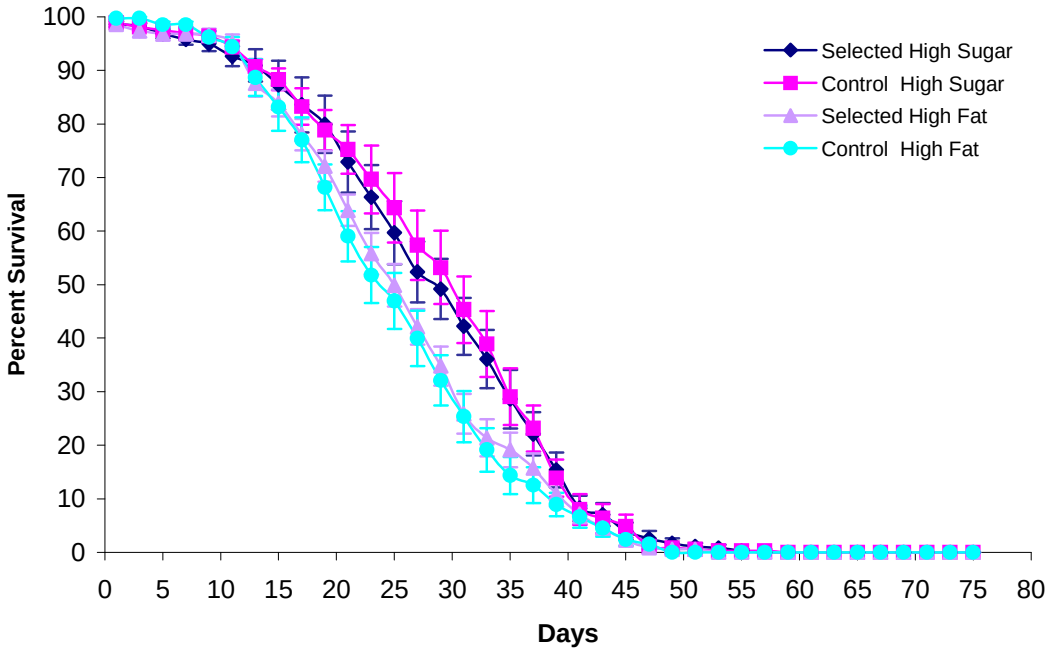

Figure 4.20 Average longevity (and standard error) of selected and control lines of female *Drosophila melanogaster* placed on the high sugar and high fat diets following 20 generations of selection for starvation resistance.

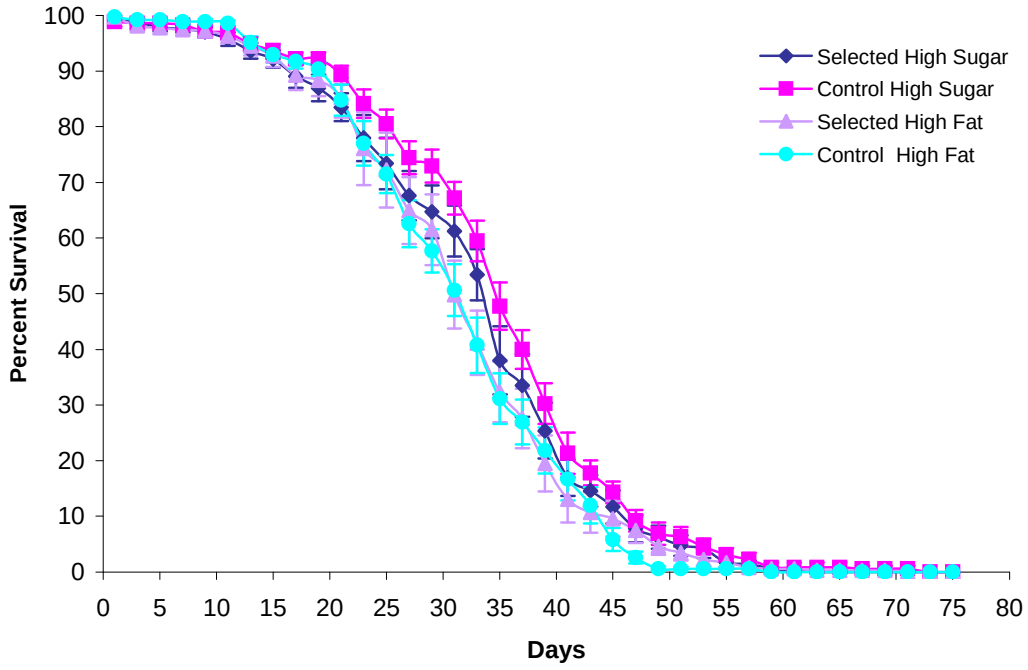

Figure 4.21 Average longevity (and standard error) of selected and control lines of male *Drosophila melanogaster* placed on the high sugar and high fat diets following 20 generations of selection for starvation resistance.

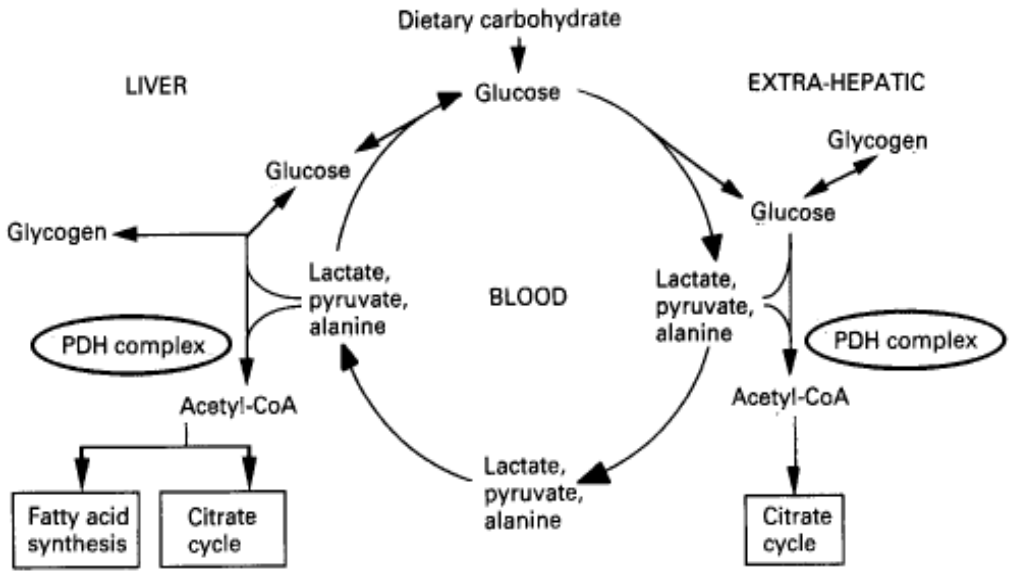

Figure 4.22 The Cori Cycle and partitioning of glucose metabolism in mammals. This figure was obtained from Randle, 1995.

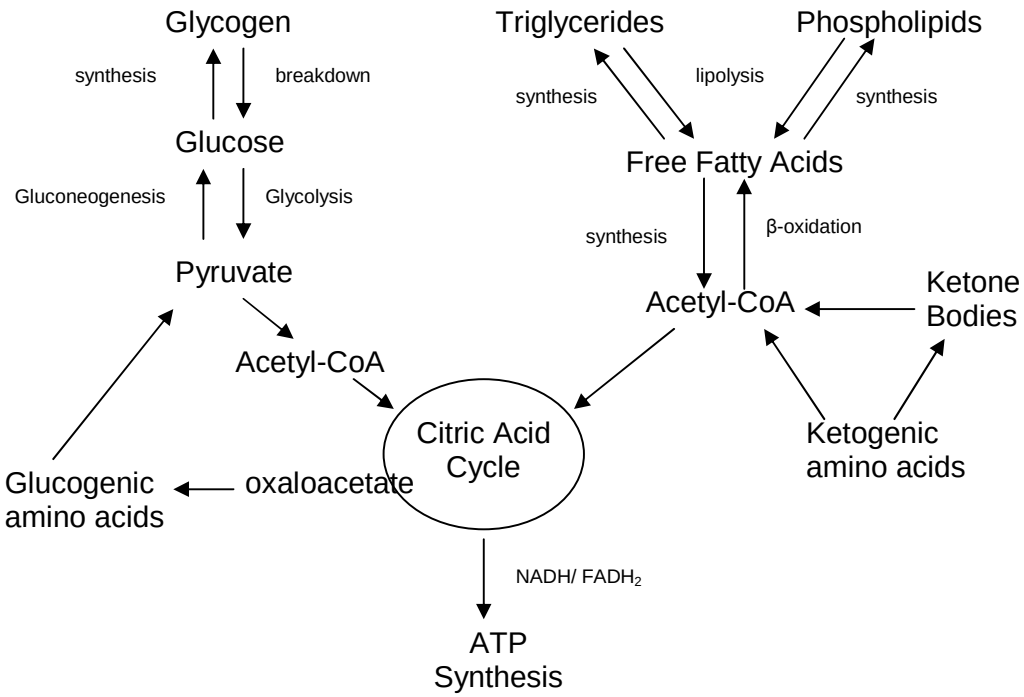

Figure 4.23 Metabolic pathways for the conversion of dietary nutrients into energy. The figure was adapted from Voet, 2006.

Table 4.1 Soluble protein concentrations within starvation resistant and control lines exposed to each of the four diets. Mean protein concentration (standard error) following 20 generations of selection for starvation resistance.

Line	Sex	Treatment	Protein concentration per fly	Protein concentration per dry weight
Selected	Male	Sugar	0.2183 (0.0039)	0.6333 (0.0212)
Control	Male	Sugar	0.2024 (0.0034)	0.6502 (0.0212)
Selected	Female	Sugar	0.2886 (0.0029)	0.6803 (0.0414)
Control	Female	Sugar	0.2730 (0.0024)	0.4900 (0.0666)
Selected	Male	Fat	0.2209 (0.0032)	0.6863 (0.0116)
Control	Male	Fat	0.2127 (0.0025)	0.7781 (0.0144)
Selected	Female	Fat	0.2901 (0.0032)	0.5311 (0.0141)
Control	Female	Fat	0.2797 (0.0038)	0.6176 (0.0101)
Selected	Male	Protein	0.2145 (0.0029)	0.6939 (0.0105)
Control	Male	Protein	0.2120 (0.0052)	0.8192 (0.0180)
Selected	Female	Protein	0.2983 (0.0018)	0.5690 (0.0445)
Control	Female	Protein	0.2794 (0.0035)	0.6522 (0.0799)
Selected	Male	Base	0.2032 (0.0048)	0.6822 (0.0188)
Control	Male	Base	0.2074 (0.0048)	0.6803 (0.0378)
Selected	Female	Base	0.2867 (0.0031)	0.5840 (0.0119)
Control	Female	Base	0.2754 (0.0036)	0.6588 (0.0960)

Table 4.2 Carbohydrate concentrations within starvation resistant and control lines exposed to each of the four diets. Mean carbohydrate concentration (standard error) following 20 generations of selection for starvation resistance.

Line	Sex	Treatment	Carbohydrate concentration per fly	Carbohydrate concentration per dry weight
Selected	Male	Sugar	0.0060 (0.0004)	0.0174 (0.0013)
Control	Male	Sugar	0.0057 (0.0003)	0.0182 (0.0026)
Selected	Female	Sugar	0.0065 (0.0005)	0.0126 (0.0020)
Control	Female	Sugar	0.0055 (0.0003)	0.0111 (0.0018)
Selected	Male	Fat	0.0053 (0.0002)	0.0165 (0.0030)
Control	Male	Fat	0.0046 (0.0001)	0.0169 (0.0014)
Selected	Female	Fat	0.0063 (0.0006)	0.0116 (0.0010)
Control	Female	Fat	0.0053 (0.0005)	0.0117 (0.0015)
Selected	Male	Protein	0.0064 (0.0000)	0.0206 (0.0019)
Control	Male	Protein	0.0055 (0.0003)	0.0213 (0.0015)
Selected	Female	Protein	0.0080 (0.0005)	0.0152 (0.0014)
Control	Female	Protein	0.0082 (0.0002)	0.0190 (0.0017)
Selected	Male	Base	0.0047 (0.0002)	0.0159 (0.0027)
Control	Male	Base	0.0038 (0.0001)	0.0126 (0.0033)
Selected	Female	Base	0.0061 (0.0005)	0.0124 (0.0017)
Control	Female	Base	0.0052 (0.0004)	0.0124 (0.0023)

Table 4.3 Glycogen concentrations within starvation resistant and control lines exposed to each of the four diets. Mean glycogen concentration (standard error) following 20 generations of selection for starvation resistance.

Line	Sex	Treatment	Glycogen concentration per fly	Glycogen concentration per dry weight
Selected	Male	Sugar	0.0085 (0.0007)	0.0245 (0.0018)
Control	Male	Sugar	0.0093 (0.0009)	0.0297 (0.0028)
Selected	Female	Sugar	0.0176 (0.0011)	0.0299 (0.0021)
Control	Female	Sugar	0.0169 (0.0002)	0.0353 (0.0014)
Selected	Male	Fat	0.0103 (0.0008)	0.0321 (0.0020)
Control	Male	Fat	0.0080 (0.0007)	0.0294 (0.0019)
Selected	Female	Fat	0.0150 (0.0013)	0.0275 (0.0022)
Control	Female	Fat	0.0127 (0.0015)	0.0281 (0.0033)
Selected	Male	Protein	0.0080 (0.0005)	0.0259 (0.0017)
Control	Male	Protein	0.0074 (0.0006)	0.0287 (0.0020)
Selected	Female	Protein	0.0096 (0.0007)	0.0182 (0.0015)
Control	Female	Protein	0.0083 (0.0002)	0.0193 (0.0009)
Selected	Male	Base	0.0081 (0.0008)	0.0271 (0.0028)
Control	Male	Base	0.0064 (0.0005)	0.0211 (0.0021)
Selected	Female	Base	0.0121 (0.0008)	0.0246 (0.0016)
Control	Female	Base	0.0132 (0.0014)	0.0317 (0.0026)

Table 4.4 Total sugar concentrations within starvation resistant and control lines exposed to each of the four diets. Mean sugar concentration (standard error) following 20 generations of selection for starvation resistance.

Line	Sex	Treatment	Total sugar concentration Per fly	Total sugar concentration per dry weight
Selected	Male	Sugar	0.0060 (0.0004)	0.0174 (0.0013)
Control	Male	Sugar	0.0057 (0.0003)	0.0182 (0.0014)
Selected	Female	Sugar	0.0065 (0.0005)	0.0126 (0.0008)
Control	Female	Sugar	0.0055 (0.0003)	0.0111 (0.0006)
Selected	Male	Fat	0.0053 (0.0002)	0.0165 (0.0009)
Control	Male	Fat	0.0046 (0.0001)	0.0169 (0.0007)
Selected	Female	Fat	0.0063 (0.0006)	0.0116 (0.0012)
Control	Female	Fat	0.0053 (0.0005)	0.0117 (0.0009)
Selected	Male	Protein	0.0064 (0.0000)	0.0206 (0.0006)
Control	Male	Protein	0.0055 (0.0003)	0.0213 (0.0012)
Selected	Female	Protein	0.0080 (0.0005)	0.0152 (0.0010)
Control	Female	Protein	0.0082 (0.0002)	0.0190 (0.0007)
Selected	Male	Base	0.0047 (0.0002)	0.0159 (0.0009)
Control	Male	Base	0.0038 (0.0001)	0.0126 (0.0007)
Selected	Female	Base	0.0061 (0.0005)	0.0124 (0.0010)
Control	Female	Base	0.0052 (0.0004)	0.0124 (0.0009)

Table 4.5 Trehalose concentrations within starvation resistant and control lines exposed to each of the four diets. Mean trehalose concentration (standard error) following 20 generations of selection for starvation resistance.

Line	Sex	Treatment	Trehalose concentration per fly	Trehalose concentration per dry weight
Selected	Male	Sugar	0.0019 (0.0001)	0.0055 (0.0003)
Control	Male	Sugar	0.0017 (0.0001)	0.0055 (0.0002)
Selected	Female	Sugar	0.0007 (0.0001)	0.0078 (0.0002)
Control	Female	Sugar	0.0007 (0.0001)	0.0012 (0.0003)
Selected	Male	Fat	0.0027 (0.0002)	0.0083 (0.0010)
Control	Male	Fat	0.0028 (0.0000)	0.0102 (0.0003)
Selected	Female	Fat	0.0031 (0.0002)	0.0057 (0.0003)
Control	Female	Fat	0.0026 (0.0001)	0.0058 (0.0003)
Selected	Male	Protein	0.0018 (0.0001)	0.0059 (0.0005)
Control	Male	Protein	0.0016 (0.0001)	0.0063 (0.0005)
Selected	Female	Protein	0.0010 (0.0002)	0.0020 (0.0005)
Control	Female	Protein	0.0002 (0.0001)	0.0006 (0.0003)
Selected	Male	Base	0.0023 (0.0001)	0.0077 (0.0003)
Control	Male	Base	0.0024 (0.0002)	0.0078 (0.0009)
Selected	Female	Base	0.0017 (0.0001)	0.0035 (0.0003)
Control	Female	Base	0.0015 (0.0001)	0.0036 (0.0004)

Table 4.6 Triglyceride concentrations within starvation resistant and control lines exposed to each of the four diets. Mean triglyceride concentration (standard error) following 20 generations of selection for starvation resistance.

Line	Sex	Treatment	Triglyceride concentration per fly	Triglyceride concentration per dry weight
Selected	Male	Sugar	9.6444 (0.7418)	27.7878 (1.8289)
Control	Male	Sugar	4.1182 (0.3233)	13.2972 (1.0716)
Selected	Female	Sugar	18.5696 (1.4681)	31.4500 (2.3968)
Control	Female	Sugar	12.6872 (0.6219)	26.5198 (1.4315)
Selected	Male	Fat	4.4538 (0.7687)	13.9729 (2.4484)
Control	Male	Fat	1.8375 (0.4995)	6.7409 (1.8656)
Selected	Female	Fat	11.2370 (1.4420)	20.4925 (2.5633)
Control	Female	Fat	8.9113 (1.3302)	19.9027 (3.1115)
Selected	Male	Protein	4.8512 (0.4654)	15.6733 (1.4653)
Control	Male	Protein	2.3552 (0.1883)	9.0940 (0.6970)
Selected	Female	Protein	15.3850 (1.2864)	29.2548 (2.3142)
Control	Female	Protein	5.4821 (0.6611)	12.7648 (1.5414)
Selected	Male	Base	6.3951 (0.5908)	21.4595 (1.9510)
Control	Male	Base	1.8058 (0.1863)	6.1859 (0.7404)
Selected	Female	Base	9.3303 (1.7877)	19.0076 (0.7404)
Control	Female	Base	4.9698 (0.8593)	11.7758 (1.9431)

Table 4.7 Average dry weight of starvation resistant and control lines exposed to each of the four diets. Mean dry weight following 20 generations of selection for starvation resistance.

Line	Sex	Treatment	Average Dry Weight (mg)	Standard Error
Selected	Male	Sugar	0.3447	0.0034
Control	Male	Sugar	0.3112	0.0052
Selected	Female	Sugar	0.5890	0.0062
Control	Female	Sugar	0.4800	0.0060
Selected	Male	Fat	0.3219	0.0034
Control	Male	Fat	0.2734	0.0023
Selected	Female	Fat	0.5462	0.0058
Control	Female	Fat	0.4528	0.0046
Selected	Male	Protein	0.3092	0.0043
Control	Male	Protein	0.2587	0.0028
Selected	Female	Protein	0.5243	0.0058
Control	Female	Protein	0.4283	0.0049
Selected	Male	Base	0.2979	0.0027
Control	Male	Base	0.3049	0.0022
Selected	Female	Base	0.4909	0.0063
Control	Female	Base	0.4180	0.0054

Table 4.8 Statistically significant least squares means values for the effects of selection in the selected and control lines. Least squares means (standard error) following generation 20 of starvation selection.

Effect	Sex	Dependent Variable	p-value	Selected	Control
Selection	Female	Protein per dry weight	0.0011	5.4471 (0.0964)	6.2517 (0.0964)
Selection	Male	Triglyceride per dry weight	0.0018	19.723 (1.4553)	8.8295 (1.4553)
Selection	Female	Dry weight	0.0003	0.5376 (0.0088)	0.4448 (0.0088)
Selection	Male	Dry weight	0.0092	0.3184 (0.0076)	0.2776 (0.0076)

Table 4.9 Statistically significant least squares means values for the effects of dietary treatment in the selected and control lines. Least squares means (standard error) following generation 20 of starvation selection.

Effect	Sex	Dependent Variable	p-value	Protein	Fat	Sugar	Base
Diet	Female	Glycogen per dry weight	<0.0001	0.0188 (0.0018)	0.0278 (0.0018)	0.0326 (0.0018)	0.0279 (0.0018)
Diet	Male	Glycogen per dry weight	0.0126	0.0272 (0.0020)	0.0304 (0.0020)	0.0269 (0.0020)	0.0243 (0.0020)
Diet	Female	Protein per dry weight	<0.0001	6.1119 (0.0814)	5.7628 (0.0814)	5.2968 (0.0814)	6.2262 (0.0814)
Diet	Male	Protein per dry weight	<0.0001	7.5780 (0.1960)	7.3389 (0.1960)	6.4612 (0.1960)	6.9305 (0.1960)
Diet	Female	Triglyceride per dry weight	<0.0001	21.009 (2.0280)	20.197 (2.0280)	28.984 (2.0280)	15.391 (2.0280)
Diet	Male	Triglyceride per dry weight	<0.0001	12.383 (1.3471)	10.356 (1.3471)	20.542 (1.3471)	13.822 (1.3471)
Diet	Female	Total carbs per dry weight	<0.0001	0.0382 (0.0014)	0.0365 (0.0014)	0.0434 (0.0014)	0.0346 (0.0014)
Diet	Male	Total carbs per dry weight	0.0004	0.0388 (0.0023)	0.0448 (0.0023)	0.0400 (0.0023)	0.0369 (0.0023)
Diet	Female	Trehalose per dry weight	<0.0001	0.0012 (0.0002)	0.0057 (0.0002)	0.0013 (0.0002)	0.0035 (0.0002)
Diet	Male	Trehalose per dry weight	<0.0001	0.0060 (0.0004)	0.0089 (0.0004)	0.0055 (0.0004)	0.0083 (0.0004)
Diet	Female	Total sugars per dry weight	<0.001	0.0171 (0.0008)	0.0116 (0.0008)	0.0113 (0.0008)	0.0123 (0.0008)
Diet	Male	Total sugars per dry weight	<0.0001	0.0210 (0.0007)	0.0168 (0.0007)	0.0178 (0.0007)	0.0143 (0.0007)
Diet	Female	Dry weight	<0.0001	0.4763 (0.0076)	0.4995 (0.0076)	0.5345 (0.0076)	0.4545 (0.0076)
Diet	Male	Dry weight	<0.0001	0.2839 (0.0065)	0.2976 (0.0065)	0.3278 (0.0065)	0.2826 (0.0065)

Table 4.10 Statistically significant least squares means values for the interaction of selection and dietary treatment in the selected and control lines. Least squares means (standard error) following generation 20 of starvation selection.

Sex	Dependent Variable	p-value	Control Protein	Control Fat	Control Sugar	Control Base	Selected Protein	Selected Fat	Selected Sugar	Selected Base
Male	Glycogen per dry weight	0.0210	0.0283 (0.0029)	0.0291 (0.0029)	0.0295 (0.0029)	0.0217 (0.0029)	0.0260 (0.0029)	0.0318 (0.0029)	0.0242 (0.0029)	0.0269 (0.0029)
Male	Protein per dry weight	0.0026	8.2059 (0.2771)	7.8008 (0.2771)	6.5496 (0.2771)	7.0289 (0.2771)	6.9501 (0.2771)	6.8771 (0.2771)	6.3728 (0.2771)	6.8321 (0.2771)
Female	Triglyceride per dry weight	<0.0001	12.764 (2.8681)	19.902 (2.8681)	26.519 (2.8681)	11.775 (2.8681)	29.254 (2.8681)	20.492 (2.8681)	31.450 (2.8681)	19.007 (2.8681)
Male	Triglyceride per dry weight	0.0022	9.0940 (1.9050)	6.7409 (1.9050)	13.297 (1.9050)	6.1859 (1.9050)	15.673 (1.9050)	13.972 (1.9050)	27.787 (1.9050)	21.459 (1.9050)
Female	Total carbs per dry weight	0.0079	0.0378 (0.0020)	0.0368 (0.0020)	0.0459 (0.0020)	0.0394 (0.0020)	0.0386 (0.0020)	0.0361 (0.0020)	0.0409 (0.0020)	0.0299 (0.0020)
Female	Trehalose per dry weight	0.0266	0.0005 (0.0004)	0.0057 (0.0004)	0.0014 (0.0004)	0.0035 (0.0004)	0.0019 (0.0004)	0.0057 (0.0004)	0.0012 (0.0004)	0.0035 (0.0004)
Female	Total sugars per dry weight	0.0172	0.0191 (0.0012)	0.0116 (0.0012)	0.0116 (0.0012)	0.0123 (0.0012)	0.0151 (0.0012)	0.0116 (0.0012)	0.0110 (0.0012)	0.0124 (0.0012)