University of Nebraska - Lincoln
DigitalCommons@University of Nebraska - Lincoln

Dissertations and Theses in Biological Sciences | Biological Sciences, School of

12-6-2007

Drosophila melanogaster Starvation Resistance: A Quantitative Genetic and Physiological Investigation in Relationship to Life History Traits

Mei-Hui Wang
University of Nebraska - Lincoln, mhwang@bigred.unl.edu

Follow this and additional works at: http://digitalcommons.unl.edu/bioscidiss

Part of the Life Sciences Commons

Wang, Mei-Hui, "*Drosophila melanogaster* Starvation Resistance: A Quantitative Genetic and Physiological Investigation in Relationship to Life History Traits" (2007). *Dissertations and Theses in Biological Sciences*. 3.
http://digitalcommons.unl.edu/bioscidiss/3

This Article is brought to you for free and open access by the Biological Sciences, School of at DigitalCommons@University of Nebraska - Lincoln. It has been accepted for inclusion in Dissertations and Theses in Biological Sciences by an authorized administrator of DigitalCommons@University of Nebraska - Lincoln.

www.manaraa.com

DROSOPHILA MELANOGASTER STARVATION RESISTANCE: A QUANTITATIVE GENETIC AND PHYSIOLOGICAL INVESTIGATION IN RELATIONSHIP TO LIFE HISTORY TRAITS

By

Mei-Hui Wang

A DISSERTATION

Present to the Faculty of

The Graduate College at the University of Nebraska

In Partial Fulfillment of Requirements

For the Degree of Doctor of Philosophy

Major: Biological Sciences

Under the Supervision of Professor Lawrence G. Harshman

Lincoln, Nebraska

November, 2007

المنارة للاستشارات

www.manaraa.com

DROSOPHILA MELANOGASTER STARVATION RESISTANCE: A QUANTITATIVE GENETIC AND PHYSIOLOGICAL INVESTIGATION IN RELATIONSHIP TO LIFE HISTORY TRAITS

Mei-Hui Wang, Ph.D.

University of Nebraska, 2007

Advisor: Lawrence G. Harshman

The starvation resistance is an important trait for insect survival. The studies presented in this dissertation investigated starvation resistance using *Drosophila melanogaster* in terms of genetic correlations, physiological mechanisms and evolutionary trajectory.

The increase of starvation can also extend lifespan is a controversial issue in *D. melanogaster*. Quantitative trait locus (QTL) mapping can provide a good evidence of this controversial problem. A QTL analysis was conducted to test the correlation between starvation resistance and longevity using a set of recombination inbred lines (RILs) from a natural population of *D. melanogaster*. We found the QTL effects were consistent for sexes and environments. Importantly, one QTL region was associated with increased starvation resistance and increased longevity. This QTL region provides support for the hypothesis that starvation resistance and longevity may have a common genetic basis as a result of coupled evolution of the two traits.

The same RILs derived from a natural population were used to investigate natural

المنارة للاستشارات

www.manaraa.com

genetic variation for lipid abundance and starvation resistance. QTLs for lipid proportion (lipid abundance normalized by weight or protein abundance) were present on all chromosomes; a lipid proportion QTL on the third chromosome correlated with a QTL for starvation resistance observed in a previous study using the same set of RILs suggesting it might underlie both traits

The laboratory selection lines are usually beneficial for us to compare the physiological changes under starvation. We have been doing laboratory selection on starvation resistance for over 20 generations. Selected flies in our laboratory had similar traits that other papers had: lower early fecundity, higher lipid contents and heavier, comparing to control populations. Besides, we found our starvation selected lines had longer lifespan which not all laboratory starvation selection discovered. The idea of if starvation resistant flies can use energy efficiently under stressful situation was also tested. The results showed that starvation resistant flies can maintain stable energy under stressful situation through physiological adjustment.

Differential lipid (triglycerides and phospholipids) abundances were measured in starvation selected lines and control lines to examine the Y model which indicating differential allocation of limited internal resources. We identified the physiological and genetically causes of life-history variation and trade-off in starvation resistance and cost of reproduction in *D. melanogaster* by differential lipid allocation.

المنارة للاستشارات
www.manaraa.com

ACKNOWLEDGEMENTS

I would like thank my advisor Dr. Harshman and my committees, Drs. Zera, Bachman, Kachman and Logan. During the last four years, I had the pleasure of having you all mentor my research. You gave me plenty of motivation and inspiration to finish my Ph.D. Special thank to Thomas M. Plucinak for revising my dissertation.

To my family: Grandma, Dad, Mom, my sisters, my husband and my daughter. You are the most important people to me no matter what. Thank you for your love, understanding and support.

Thank you all friends in Dr. Harshman's laboratory and all the faculty and staff in Manter Hall. Without your kindness and help, I can't have great time living in Nebraska. You opened my eyes and my mind.

المنارة للاستشارات

www.manaraa.com

TABLE OF CONTENTS

CONTENTS **Page**

ACKNOWLEDGEMENTS………………………………………………………..... i

TABLE OF CONTENTS……………………………………………………..... ii

CHAPTER I: INTRODUCTION……………………………………………..... 1

CHAPTER II: Environment-dependent survival of *Drosophila melanogaster*

: a quantitative genetic analysis

Abstract………………………………………………………………………. 11

Introduction………………………………………………………………….. 12

Materials and Methods………………………………………………………. 15

Results……………………………………………………………………….. 20

Discussion……………………………………………………………………. 22

Acknowledgements………………………………………………………….. 26

Tables………………………………………………………………………… 27

Figures……………………………………………………………………….. 33

References……………………………………………………………………. 35

CHAPTER III: Quantitative trait loci for lipid content in *Drosophila melanogaster*

Abstract………………………………………………………………………. 40

Introduction & Results....…………………………………………………….. 39

Materials and Methods………………………………………………………. 44

Acknowledgements………………………………………………………….. 46

Tables………………………………………………………………………… 47

المنارة للاستشارات

www.manaraa.com

Figures……………………………………………………………………….. 49

References……………………………………………………………………… 55

CHAPTER IV: Laboratory Selection of Starvation Resistance Using *Drosophila melanogaster*: Reproduction and Lipids

Introduction……………………………………………………………………. 58

Materials and Methods…………………………………………………………. 61

Results………………………………………………………………………….. 66

Discussion……………………………………………………………………… 70

Tables…………….…………………………………………………………….. 75

Figures………………………………………………………………………….. 77

References……………………………………………………………………… 88

CHAPTER V: Investigation of Different Lipid Abundance in Starvation Resistance and Reproduction

Introduction……………………………………………………………………. 93

Materials and Methods…………………………………………………………. 96

Results………………………………………………………………………….. 100

Discussion……………………………………………………………………… 103

Tables…………….…………………………………………………………….. 107

Figures………………………………………………………………………….. 108

References……………………………………………………………………… 116

المنارة للاستشارات

www.manaraa.com

Chapter I:

Introduction

المنارة للاستشارات
www.manaraa.com

A. Starvation resistance and Longevity

The definition of stress is exposure to any environmental factor that acts to reduce the fitness of an organism (Koehn and Bayne, 1989). Environmental stress might be one of the most important sources of natural selection, as indicated by many specific adaptations that have evolved to resist stress (Hoffman & Parsons, 1991; Randall et al., 1997). One important cause of stress is shortage of nutrition and certainly most species experience periodical starvation. Since most animals face periods of food shortage they are thus expected to have evolved adaptations resulting in starvation resistance.

The evolutionary relationship between starvation and longevity has been confirmed in some selection experiments. In flies, there is evidence for a relationship between starvation resistance and longevity. The relevant *Drosophila* data are based almost exclusively on studies using *D. melanogaster*. Laboratory lines selected for enhanced longevity are also relatively more resistant to starvation stress (Rose & Archer, 1996), moreover selection for stress resistance can increase longevity (Rose et al., 1992; Hoffmann & Parsons, 1993). In addition, long- lived *D. melanogaster* mutants are usually stress resistant (Lin & Benzer, 1998). The *chico* gene in *D. melanogaster* encodes an insulin receptor substrate that functions in an insulin/insulin-like growth factor (IGF) signaling pathway. Mutation of *chico* extends life-span by up to 48% and also increases starvation resistance in females (Clancy et al., 2001). However, these results are not always consistent since there are exceptions.

Hoffman and Harshman (1999) summarized the relationship between starvation resistance and longevity. In one experiment, populations selected for starvation resistance showed no correlated change in longevity (Harshman et al., 1999). Moreover, in another study, lines selected for greater longevity did not exhibit starvation resistance as a correlated response to selection (Force et al., 1995). In another study, selection for starvation resistance was initially accompanied by an increase in longevity, but in subsequent generations, as starvation increased, a decline in longevity was observed (Archer et al., 2003). Also, in another set of lines there was a genetically correlated change in increased stress resistance under selection for reduced longevity (Zwaan et al., 1995) and the other lines where increased virgin lifespan was selected for showed that

المنارة للاستشارات
www.manaraa.com

there was a genetic correlation for reduced starvation resistance (Vermeulen et al., 2006).

There is also evidence of processes which affecting starvation resistance and longevity independently. For example, the Target of Rapamycin (TOR) signaling pathway is thought to regulate lifespan (Tatar et al., 2003). Several genes involved in this pathway can be manipulated to increase life span with no associated change in starvation resistance (Kapahi et al., 2004). On the other hand, Adipokinetic hormones (AKHs, metabolic neuropeptides) which mediate mobilization of energy substrates can also regulate feeding behavior under starvation. Flies devoid of AKH neurons lacked hyperactivity under starvation and also display strong resistance to starvation compared to starved wild-type flies, but they didn't show longer life span (Lee & Park, 2004).

There is a genetic basis for increased lipid abundance which is associated with starvation resistance and longevity in *D. melanogaster*. These starvation resistant flies have greater lipid reserves that are genetically determined (Chippindale et al., 1996). Quantitative trait loci (QTL) mapping points to several genes that might influence starvation resistance by being involved in lipid accumulation (Harbison et al., 2004). QTL studies of starvation resistance and longevity are important because the literature suggests that the traits are genetically associated. Moreover, physiological studies of mutants indicate that increased starvation resistance can be associated with metabolic changes leading to the accumulation of lipids (Hader et al., 2003).

The generally positive correlation between life span and stress resistance is a controversial issue in *D. melanogaster.* There are also complex mechanisms underlying starvation resistance responses and longevity and it appears that alterations in lipid metabolism may be important.

B. *Quantitative trait loci (QTL) studies in starvation resistance*

Drosophila has been used to identify many quantitative trait loci (QTLs) that account for correlations observed in complex traits. Some of those QTLs are pleiotropic and interact epistatically. The list of quantitative trait phenotypes that can be investigated using *Drosophila* include sensory bristles number, a broad range of behaviors, stress resistance, drug tolerance, enzymatic activity, metabolic traits, fertility and longevity

المنارة للاستشارات
www.manaraa.com

(Mackay, 2004). Mapping of QTLs responsible for natural variation is facilitated by rearing large numbers of flies, and constructing recombinant (near-isoallelic) inbred lines. Mackay (2004) reviewed recent progress that had recently been made towards elucidating the complex genetic architecture of quantitative traits in *Drosophila*. Techniques allowing this progress include complementation of deficiency stocks enabling rapid high resolution QTL mapping, as well as complementation of mutations allowing the identification of positional candidate QTLs. Linkage disequilibrium decays rapidly in *Drosophila* regions of normal recombination (Long et al., 1998), enabling identification of specific molecular polymorphisms that correspond to QTLs. Finally, the genome sequence (Adams et al., 2000) and multiple platforms for whole genome transcriptional profiling facilitate genomic approaches for identifying genes affecting quantitative traits, and variation in quantitative traits.

Starvation resistance is a typical quantitative trait that displays considerable genetic variation in Drosophila (Service and Rose 1985; Da Lage et al. 1990; Hutchinson and Rose 1991; Hutchinson et al. 1991; Toda and Kimura 1997; van Herrewege and David 1997; Karan and Parkash 1998; Karan et al. 1998; Hoffmann and Harshman, 1999; Harbison et al. 2004) and in response to artificial selection (Harshman and Schmid 1998; Harshman et al. 1999). The form of stress resistance that indirectly responds to selection for longevity is inconsistent (Tower, 1996; Harshman & Hoffmann, 2000) because selection experiments are notorious for heterogeneous outcomes, which could be due to a range of factors including the intensity of selection (Harshman & Hoffmann, 2000). Consequently, a better test of the genetic relationship between these traits is whether or not they colocalize in QTLs that have a positive effect on starvation survival and longevity.

Recombinant inbred lines (RILs) can be used to identify QTLs as regions of the genome that account for a significant component of the segregating genetic variation and also to characterize genetic correlations in starvation resistance. Harbison et al. (2004) identified 383 *P*-element insertions affecting starvation resistance and found 13 loci (of those six with sex-specific effects) contributing to a difference in starvation resistance between two inbred laboratory strains. The sex-specific QTL effect implies that genes affecting development in both sexes may also exhibit sex-specific pleiotropy for adult

المنارة للاستشارات
www.manaraa.com

traits.

The characterization of natural genetic variation for the control of obesity in humans is an important goal in human health research. However, it is difficult to acquire this information because there are limited methods available for the study of the genetic architecture of complex traits in humans. Conversely, quantitative trait loci studies of lipid abundance in model organisms such as *Drosophila* are especially suited for this purpose. Because of its short life span, well-characterized biology, low cost, and excellent genetic and genomic resources, this organism has been useful in the genetic studies of several biological aspects of obesity and lipid biochemistry. The study of obesity in *Drosophila* is relevant to humans because pathways of lipid metabolism are generally conserved between vertebrates and invertebrates (Canavoso et al., 2001). Insights derived from the *Drosophila* model may help to elucidate the genetic architecture of obesity variation in humans.

Wang et al. (2005) found that a lipid proportion QTL on the third chromosome correlated with a QTL for starvation resistance. As lipid content is associated with starvation resistance, it is tempting to compare mapping results with whole genome microarray data on gene expression during starvation and sugar feeding (Zinke et al., 2002). In particular, the overlap of QTL and microarray data sets has been used to produce a list of candidate genes (Wayne and McIntyre, 2002). For example, the *Lsd2* gene (at cytological position 13A) belongs to the PAT family of proteins. *Lsd2* mutants have reduced levels of lipid indicating that the gene is involved in lipid storage (Teixeira et al., 2003). Another example is the *pumpless* gene located at 78C (Zinke et al., 1999) that is involved in amino acid-dependent signaling arising from fat bodies that induce cessation of feeding in larvae. The most interesting gene that has been found is a microRNA, miR14, located at 45F (Xu et al., 2003). miR14 suppresses cell death and is required for normal fat metabolism. miR14 appears to synchronize multiple physiological responses related to stress, as supported by the fact that miR14 loss-of-function flies display increased sensitivity to a variety of stress conditions.

Among the molecular family of lipids, triglycerides are the most abundant in insects. Triglyceride storage is a quantitative trait, and segregating variation has been shown to exist in natural populations of *D. melanogaster* (Clark and Keith, 1988; Clark, 1989). De

المنارة للاستشارات
www.manaraa.com

Luca et al., (2005) identified QTLs that contribute to variation in triacylglycerol (TAG) storage in *D. melanogaster*. QTLs have been found that contain the natural and variable genetic information that directly accounts for variation in TAG storage, and fine-scale mapping of these QTLs can identify candidate genes. The female-specific QTL for TAG storage includes acyl-coenzyme A oxidase (CoA) at 57D proximal (Acox57D-p) and acyl-CoA oxidase at 57D distal (Acox57D-d) that both encode a product with palmitoyl-CoA oxidase activity putatively involved in fatty acid-oxidation in peroxisomes. Genes encoding transforming growth factor β (TGF-β) receptor signaling pathway members have also been found to affect TAG storage in *D. melanogaster*. Increased TGF-β1 expression has been associated with Body Mass Index (BMI) and increase in abdominal adipose tissue in human morbid obesity (Petruschke et al., 1994), and variation in TGF-β1 has been associated with obesity phenotypes.

C. Physiological studies of starvation resistance with respect to lipid metabolism

Drosophila experiences periods of food shortage in nature, yet the suite of mechanisms or genes affecting their physiological and behavioral responses to starvation remain unclear. Physiological studies of starvation resistance are used to determine the specific physiological mechanisms used by organisms to resist famine since these can have substantial adaptive significance. For starvation, there is good evidence that an increase in the lipid content of adults underlies increased resistance to starvation (Chippindale et al., 1996; Harshman & Schmid, 1998; Harshman et al., 1999; Hoffmann et al., 2005). *D. melanogaster* demonstrates a strong response to selection for starvation resistance through a genetically determined increase in lipids but not carbohydrates or glycogen (Djawdan et al., 1998). Carbohydrate and glycogen reserve increases are significantly correlated with desiccation resistance (Djawdan et al., 1998; Graves et al., 1992). This suggests that different forms of stress are tolerated by distinct physiological mechanisms and that the evolutionary responses of flies to stress selection are specific to the stress imposed.

Some data suggest that lipid abundance accounts for much of all the variation in

المنارة للاستشارات
www.manaraa.com

starvation resistance. For instance, Chippindale et al. (1996) scored lipid and starvation levels in different sets of lines selected for starvation or changes in life history traits. It was found that there was a close correlation between starvation and lipid levels when all lines were considered. The association between lipid level and starvation resistance has also been documented from a set of starvation-selection lines derived from a different base population (Harshman et al., 1999), in comparisons of allozyme genotypes (Oudman et al., 1994), and the way in which lipid levels and starvation change with age (Service, 1987). Similar experiments have not been conducted in other species of *Drosophila*, although at the species level there is a correlation between starvation resistance and lipid level, as already noted (Van Herrewege & David, 1997).

The enzymes indirectly associated with lipid biogenesis also increased in starvation selected populations (Harshman et al., 1999). Important for lipid biogenesis, glucose-6-phosphate dehydrogenase (G6PD, a pentose shunt regulatory enzyme) exhibited higher activity in starvation selected lines but 6-phosphogluconate dehydrogenase (PGD, another pentose shunt regulatory enzyme) and glycogen phosphorylase (GP, relative to glycogen use) activities decreased in selected lines in response to starvation (Harshman et al., 1999).

Triglycerides are preferentially allocated to somatic tissue and phospholipids are important in egg development (Zhao and Zera, 2002). Flight-capable Morph Crickets contain a substantially greater amount of total lipid and triglyceride, the main flight fuel of this species (Zera et al, 1999), than the flightless morph, whereas the reproductive morph contains a greater amount of phospholipids (Grapes et al, 1989; Zhao and Zera, 2002). In *D. melanogaster*, higher starvation resistance is correlated to early fecundity in that female flies that are able to tolerate starvation well usually have lower early fecundity. If there is existing trade-off between starvation resistance and early fecundity, it is possible to detect the trade-off between triglycerides and phospholipids in starvation resistant and non-starvation resistant flies.

D. Significant Studies Included

In this dissertation, I used quantitative genetic and physiological methods to

المنارة للاستشارات
www.manaraa.com

investigate starvation resistance and lipid abundance. The overall goal of these present studies is to demonstrate viable mechanisms which explain starvation resistance in fruit flies. The following questions and topics were addressed:

1. Are there any significant QTLs for life span survival when food is present and starvation survival when food is absent? Do longevity and starvation survival coincide in one or more QTLs? The method used in this section is QTL mapping. We found that one QTL region was associated with increased starvation resistance and increased longevity. This QTL region provides support for the hypothesis that starvation resistance and longevity may have a common genetic basis as a result of coupled evolution of the two traits.

2. Lipid levels are found correlated to starvation survival. Is there any genetic correlation between lipid abundance and starvation resistance of *D. melanogaster*? The method used in this section is QTL mapping. QTLs for lipid proportion (lipid abundance normalized by weight or protein abundance) were present on all chromosomes; a lipid proportion QTL on the third chromosome correlated with a QTL for starvation resistance observed in a previous study using the same set of recombinant inbred lines suggesting it might underlie both traits.

3. Here the life history traits of laboratory starvation selected lines and control lines are compared. In addition, lipid abundance at different starvation time points and the lipid level in eggs are measured to determine whether or not if starvation resistant flies have more lipid reserves. The results of this study show that starvation resistant flies have greater weight, longer life span, more lipids and lower fecundity. The starvation selected females also maintain stable triglycerides after 36 hours of starvation. Starvation resistant females have more lipids in their stage-14 oocytes but lose some proportion of their lipid reserves after 36 hours of starvation stress.

4. Is there any trade-off between starvation resistance and fecundity? At the base of the ‘Y’ model, energy resources are allocated to reproduction versus the functions of the

المنارة للاستشارات
www.manaraa.com

somatic tissues. The method used in this study was to measure the differential lipid (triglycerides and phospholipids) distribution. In soma, higher triglycerides are found in starvation resistant females and more phospholipids are found in control females. After 36 hours of starvation, resistant flies still have similar amount of triglycerides and greater enzyme activity.

المنارة للاستشارات
www.manaraa.com

Chapter II

Environment-dependent survival of *Drosophila melanogaster*: a quantitative genetic analysis

[Published by Mei-Hui Wang, Oleg Lazebny, Lawrence G. Harshman and Sergey V. Nuzhdin (2004) In Aging Cell 3: 133-140]

المنارة للاستشارات

www.manaraa.com

Abstract

Survival under starvation conditions was investigated in relationship to survival when food was present because these traits could be linked by evolutionary history. Recombinant inbred lines derived from natural populations of *Drosophila melanogaster* were used to test genetic correlations and architecture of these survival traits. Sexes were genetically correlated within traits and there was significant correlation between survival traits. A number of quantitative trait loci (QTLs) were present for starvation survival and/or survival on food. In general, the QTL effects were consistent for sexes and environments. QTL effects were found on each major chromosome, but the major effects were largely localized on the second chromosome. Importantly, the 'four-allele' progenitor of the recombinant inbred lines used in the present study allowed the sign and magnitude of effects to be assigned to linkage groups. One such linkage group on the second chromosome conferred starvation resistance and longevity, supporting the hypothesis of an association between starvation resistance and lifespan.

www.manaraa.com

Introduction

Survival under starvation conditions might be a driving factor in the evolution of longevity. Omnipresent environmental variation could produce a universal adaptation that promotes survival during times of insufficient food and allows for subsequent reproduction when resources are more plentiful. A gene with pleiotropic effects on both starvation survival and survival when food is present, or closely linked genes affecting both traits, could evolve when starvation is the agent of selection. Therefore, we expected to find colocalization of genetic variation for starvation resistance and longevity in the genome of *Drosophila melanogaster*. Of the model species used for genetic studies of longevity, *D. melanogaster* is particularly useful for ecologically relevant research. Importantly, genetic methods and other technologies have been developed for *D. melanogaster* that are potentially useful for genetic identification of complex trait candidates, including genome-wide deletion mapping and microarrays.

Quantitative trait loci (QTL) studies of longevity and stress resistance are important because the literature suggests that the traits are genetically associated. Artificial laboratory selection experiments on longevity in *D. melanogaster* have documented genetic correlations between longevity and stress resistance (Luckinbill et al., 1984; Rose, 1984; Service et al., 1985; Service, 1987; Dudas & Arking, 1995; Force et al., 1995; Arking et al., 2000a,b). However, the form of stress resistance that indirectly responds to selection for longevity is inconsistent (Tower, 1996; Harshman & Hoffmann, 2000). For example, one set of lines responded to longevity selection by evolving resistance to starvation and desiccation (Rose, 1984; Service et al., 1985; Service, 1987), but another set of longevity-selected lines evolved resistance to oxidative stress and not to starvation or desiccation (Luckinbill et al., 1984; Dudas & Arking, 1995; Force et al., 1995; Arking et al., 2000a,b). Similarly, artificial selection for starvation resistance increased lifespan in one set of selected lines (Rose et al., 1992), but not in another set of lines (Harshman et al., 1999). Selection experiments are notorious for heterogeneous outcomes, which could be due to a range of factors including the intensity of selection (Harshman & Hoffmann, 2000). Consequently, a better test of the genetic relationship between these traits is

المنارة للاستشارات
www.manaraa.com

whether or not they colocalize in QTLs that have a positive effect on starvation survival and longevity.

Recombinant inbred lines (RILs) can be used to identify QTLs as regions of the genome that account for a significant component of the segregating genetic variation and characterize genetic correlations between traits. One set of RILs derived from laboratory stocks has been used to map quantitative trait loci for longevity and stress-related traits in female and male *D. melanogaster* (Nuzhdin et al., 1997). Using a dense molecular map, a genome-wide screen was conducted for QTLs that affect the lifespan of individually held virgin males and females. Five QTLs were identified that had a significant effect on longevity, but they affected lifespan in only one sex or the other. Vieira et al. (2000) extended this study by measuring the lifespan of virgin females or males under high and low temperature conditions and after heat shock. In addition, survival was determined under starvation conditions. Seventeen QTLs were identified that affected lifespan under different conditions as well as starvation survival. The QTLs typically were sex- and condition-dependent and there was no statistically significant positive covariation between starvation survival and longevity QTLs. Longevity QTL studies based on mixed sex cohorts have produced different results in that no sex-specific QTLs were observed in the same panel of RILs (Reiwitch & Nuzhdin, 2002) or in a different set of RILs (Curtsinger & Khazaeli, 2002). Only in one study has positive QTL covariation between longevity and stress resistance (oxidative stress resistance) been reported (Curtsinger & Khazaeli, 2002). In this case, the RILs were derived from a laboratory selection experiment for extended longevity. Given that the lines had been maintained for many generations in the laboratory before inbreeding to generate the RILs, it was not clear that the QTL represented naturally occurring positive genetic covariation between longevity and stress resistance.

The purpose of the present study was to investigate the genetic relationship between longevity and starvation resistance in *D. melanogaster* using recombinant inbred lines that were recently derived from the field. The RILs employed were derived from heterozygous individuals taken from a natural population (Kopp et al., 2003). The

www.manaraa.com

following questions were of particular interest given that our RIL lines are relatively representative of natural genetic variation:

1. Is survival with food present genetically correlated with survival under starvation conditions?
2. Are there significant QTLs for survival when food is present and when it is absent?
3. Do longevity and starvation resistance coincide in one or more QTL?

Of particular note, a major QTL for extended longevity and starvation resistance was identified.

المنارة للاستشارات
www.manaraa.com

Materials and Methods

Recombinant inbred lines (RILs) and genotypes

A panel of 144 RILs was generated by crossing a single virgin female from the F1 progeny of a fertilized female caught in the wild (Winters, CA, USA) to a single male from the F1 progeny of a different fertilized female caught at the same location. Chromosomes of the parental flies were allowed to recombine for one generation, and recombinant F2 genomes were isogenized by 25 generations of full-sib inbreeding. Because the parental individuals were heterozygous, up to four different alleles may be segregating at each locus. The genetic crosses employed, and genotyping of the derivative lines, is described elsewhere in more detail (Kopp et al., 2003).

As markers, we have used positions of transposable elements that are highly heterogeneous in natural populations (see Charlesworth & Langley, 1989, for a review). Positions of the roo elements were determined by in situ hybridization to polytene salivary gland chromosomes, using a biotinylated DNA probe (Shrimpton et al., 1986). For use as probe, a plasmid carrying a full-length roo element was labelled with bio-7-dATP (BRL) by nick translation. Hybridization was detected using the Elite Vectastain ABC kit (Vector Laboratories) and visualized with diaminobenzidine. Element locations were determined from the images at the level of cytological bands on the standard Bridges' map of *D. melanogaster* polytene chromosomes (Lefevre, 1976). We genotyped five individuals per line. The marker was recorded as present if detected in all larvae, as absent if not detected in any larva and as segregating otherwise. On the four homologous chromosomes per autosome, and similarly on the X chromosome, initial linkage of markers was inferred from residual linkage disequilibrium in the population of RILs (Proc CORR, SAS Institute, 1988). In some cases, roo elements either were uninformative (present in more than one complementation group) or gave a hybridization signal that was too weak to be scored reliably. Such elements were dropped from further analysis, leaving 152 markers that segregated between parental chromosomes (see figure 2 in Kopp et al., 2003). Given that the genome size of *D. melanogaster* is approximately 170 Mb, the average density of markers is approximately 0.9 per Mb, but as indicated in Kopp et al. (2003), the markers are not evenly distributed. All chromosomes were

المنارة للاستشارات
www.manaraa.com

homosequential, with the exception of an inversion on 3R that contains 7% of the physical genome (approximately 89EF−96A). This inversion was not associated with either starvation or lifespan in either males or females (P = 0.69, 0.30, 0.78, 0.70), and is not be discussed further.

Phenotypic measurements

Lifespan

For each measurement of lifespan, all RILs were expanded for one generation at a density of ten pairs of flies per vial and six vials per line to obtain a sufficient number of individuals for testing. In the following generation, ten pairs of flies were placed in each of ten vials per line for 3 days. Virgin males and females were collected at 8-h intervals over a 24-h period, 9 days after the parents were discarded. The following day, 25 males and females per RIL were placed in population cages made from 500-mL plastic Dixie Cups with attached glass vials containing 8 mL of cornmeal–agar–molasses medium plus yeast. Dead flies were removed by aspiration and deaths recorded at 2-day intervals. The food was changed every fourth day.

Starvation resistance

For each measurement of survival under starvation conditions (no food present), all RILs were expanded in one generation using a low density of females and males in each vial. Adults emerging from these vials were used for egg production; approximately 75 eggs were transferred to each of three vials per RIL. A vial contained 8–10 mL of cornmeal–agar–molasses medium plus yeast. For the starvation assay, males and females emerging in the egg transfer vials were collected within a 24-h period for most lines, but some lines required another day to accumulate the target number of flies. Fifteen males and females were held together in vials for 3 days and then transferred to fresh food and this procedure was repeated a total of three times. Thereafter, females and males were separated into single sex vials for 2 days based on the expectation that this was enough time for the flies to have completely recovered from the brief exposure to ether used to separate them. First, this pre-assay fly housing regime allowed us to emulate the conditions of the mixed sex cohort used to assess longevity. It also allowed us to record

المنارة للاستشارات

www.manaraa.com

the time interval of death of males and females in separate vials, which would have been difficult if the sexes had not been previously sorted into separate vials for starvation. Ten females or the same number of males were transferred to empty vials with a water-saturated fibre plug. Replicate vials were assayed for each sex for each line. Every 8 h, mortality was recorded in each vial until all flies died. Recording mortality every 8 h has been shown to be sufficient to differentiate populations that exhibit relatively small differences in starvation survival time (Harshman & Schmid, 1998). Both experiments were fully replicated twice.

Analysis of variance

For each trait, we first log transformed the data to normalize them (tested with the UNIVARIATE procedure, SAS Institute, 1989; option normal). Two-way anovas were then performed with sex as the fixed effect, and line and line by sex as random effects. Significance of the component of variance was assayed with the GLM procedure. The genetic component of variance was estimated with the VARCOMP procedure. Genetic correlations between traits and sexes were estimated with the CORR procedure, after averaging measurements between replicates.

Single marker analysis (SMA)

For each of the marker loci, the significance of the difference in the mean trait values was tested between two groups of lines, one with the marker allele containing the roo insert and the other with the marker allele having no roo insert (GLM procedure). An empirical distribution of the test statistics under the null hypothesis of no association between any of the markers and trait values was obtained by randomly permuting the trait data 1000 times and calculating the most significant probability for marker–trait associations across all markers for each permutation. Statistics from the original data that were exceeded fewer than 50 times by those from the permutation are significant at $P = 0.05$ (Doerge & Churchill, 1996). The contribution of the QTL in the overall genetic variance was estimated with the VARCOMP procedure with the marker effect as random.

Composite interval mapping (CIM)

المنارة للاستشارات
www.manaraa.com

The standard QTL mapping software was not designed for the type of RILs used in the present study. These software packages fit a single difference in effects of alleles fixed between two isogenic parental stocks, which segregate among RILs. However, the standard mapping software has been adapted to the RILs used in the present study. For that, we test whether an allele from one homologous chromosome codes a trait value significantly different from the average trait due to combined effects of other alleles: two for the X or three for the second chromosomes. Except the chromosome tips, two of the parental third chromosomes appear identical (Kopp et al., 2003), and thus we modelled only three parental third chromosomes. For each of the parental chromosomes, we generate a separate likelihood profile: three for the X, four for the second and three for the third chromosome. Note that the number of different allelic effects we can independently estimate is equal to the number of segregating chromosomes minus 1. With the above logic, likelihood profiles were generated with the CIM procedure in QTL Cartographer (Basten et al., 1994, 1999). QTL effects (Table 3) were expressed as additive effect (a) on log transformed data, as estimated by QTL Cartographer software. Recombination distances between markers were reconstructed as described in detail elsewhere (Mezey, unpubl. data). Note that recombination distances reflect history of recombination events during derivation of RILs, and thus for some chromosomes they are contracted and for others expanded in comparison with the standard *D. melanogaster* recombination map (Fig. 1). The options were two (number of background markers), 30 (window size in cM) and two (Kosambi mapping function). Every parental chromosome has its own set of positions of roo markers. Only those were followed in the CIM analyses for a given chromosome; the other markers were disregarded. The significance of a QTL was tested with 1000 permutations for each trait and chromosome. Significance thresholds – likelihood ratios corresponding to 5% of the highest values obtained on permuted data sets – substantially varied (from 6.49 to 27.23), perhaps due to uneven sample sizes for different marker alleles. For convenience of comprehension we scaled experimental likelihoods by permutation thresholds. These likelihood ratios show significant QTL when in excess of unity. Note that we performed ten tests for four traits, or 40 analyses in total. By chance alone, we expect to detect two QTLs, whereas in fact we detected 11 (Fig. 1). We might have further adjusted significance thresholds for

المنارة للاستشارات
www.manaraa.com

multiple tests to verify significance of every individual QTL. This, however, would substantially increase the type 2 error. As our composite interval mapping is exploratory, and intended to generate a list of candidate regions for the follow-up genetic analyses, we chose to make no such adjustment.

المنارة للاستشارات

www.manaraa.com

Results

Analysis of variance

The mean longevity (± standard deviation) for females was 39.03 (11.405) days and for males 44.25 (12.770) days. The mean survival under starvation conditions for females was 32.58 (9.070) h and males 33.47 (9.021) h. Using anova, there was a significant genetic component of variance for starvation and lifespan among RILs for both sexes ($P < 0.0001$). For starvation survival, anovas indicated a line by sex interaction ($P < 0.0001$). Starved females lay few eggs in the empty vials in comparison with egg laying in the presence of food. This makes it unlikely that the significant line by sex interaction is explained by variation in egg laying rate during starvation. There was a significant line effect ($P < 0.0001$) when tested over the line by sex term plus error. For survival on food, anovas indicated significant line and sex effects ($P < 0.0001$), but no line by sex interaction ($P = 0.9581$). There was a significant correlation between sexes for the same trait, and the lifespan and starvation survival traits were correlated. As indicated by the statistical analysis of genetic correlation, the correlation between male and female mean longevity was 0.83 ($P < 0.0001$) and between male and female starvation survival the correlation was 0.59 ($P < 0.0001$). The correlation between longevity and starvation survival was positive and significant within sexes, but only marginally significant across sexes (Table 1).

QTL analyses

Single marker analysis

Table 2 presents probabilities and genetic variance components accounted for by the strongly supported markers that are bolded when they exceed the whole genome permutation threshold. Across the centromere of chromosome 2 (approximately corresponding to cytological positions 38A−41F as described, Kopp et al., 2003), there are markers that are associated with longevity and starvation survival (Table 2, 38A and 41CE). A nearby region of the genome (Table 2, 42D), has a strongly supported QTL longevity as well as starvation survival. Other markers that exceed permutation threshold in relationship to male longevity and female survival under starvation conditions are

المنارة للاستشارات
www.manaraa.com

listed in Table 2. Other regions (48A and 48D) are associated with male and female longevity, but not starvation resistance of either sex. Importantly, alleles of the most significant QTLs account for approximately half genetic variation in both traits.

Composite interval mapping

Figure 1 represents the outcome of composite interval mapping (starvation resistance of males in cyan, females in pink, lifespan of males in blue, females in red), with the data for three X chromosomes in the panels A–C, four second chromosomes D–G, three third chromosomes H–J. Out of five significant QTLs identified by single marker analysis, three hold unchanged, and one is nearby a composite interval QTL (42D) illustrating the robustness of inferences. As expected from the higher power of composite interval analysis it identifies five additional QTLs listed in Table 3. The major QTL effects are associated with the second chromosome. On this chromosome, the QTL 2-1/48D (see Figure 1 legend for explanations on designations) and the three third chromosome QTLs affect lifespan of males and females but not starvation survival. The second homolog of the second chromosome QTLs affects starvation survival but not survival in the presence of food (longevity). The rest of the QTLs have significant and largely concordant effects on both starvation survival and survival on food, accounting for much of genetic correlation between these traits. One of these QTLs is associated with longevity and starvation resistance, whereas the other QTL decreases survival when food is absent or present.

Because we compare the effect of a QTL allele with the mean effect of many alleles, we can record whether this allele decreases lifespan and starvation survival (presumably deleterious mutations) or improves performance for these traits. Interestingly, one of the QTL alleles (41CE−42B, Table 3) appears to belong to the latter category.

المنارة للاستشارات
www.manaraa.com

Discussion

The present study has identified QTLs that affect survival. QTLs were detected that affected survival only under starvation conditions or that affected survival only when food was present. However, there were QTLs that affected survival under both environmental conditions (food present or absent). Importantly, one QTL region was associated with increased starvation resistance and increased longevity. This QTL region provides support for the hypothesis that starvation resistance and longevity may have a common genetic basis as a result of coupled evolution of the two traits.

Natural selection for the almost invariable association of the two traits might be expected in variable food resource environments (potentially most environments), where individuals must survive starvation in a sufficiently youthful condition to be able to reproduce. If this prediction describes some aspects of evolutionary reality, then a common genetic basis for longevity and starvation resistance might be expected. A partial test of the hypothesis is to determine if the two traits have a common genetic basis and the present study takes one step toward that goal. It provides evidence that one, or more, allele segregating in a natural population has a positive effect on both traits and that the effect can be localized to a genomic region that encompasses hundreds of genes. It does not yet identify a common genetic basis for starvation resistance and longevity, but makes that goal realizable.

Lifespan QTLs have previously been mapped in *D. melanogaster*. To form 98 RILs, Nuzhdin et al. (1997) crossed two homozygous laboratory strains with one allele at a variable site contributed by each of the parental strains. Using these RILs, five quantitative trait loci for longevity were identified for virgin flies. The genetic markers that comprise the QTLs account for approximately 50% of the genetic variation among the lines. The QTLs were sex-specific, in accord with the low level of genetic correlation between female and male longevity among the lines. However, the effects of two of the QTLs were in the same direction, positive or negative, in males and females. In this regard, Reiwitch & Nuzhdin (2002) and Curtsinger (2002) have pointed out that some of the sexual dimorphism attributed to QTLs might be due to limited statistical power. In

المنارة للاستشارات
www.manaraa.com

Nuzhdin et al. (1997), one QTL was associated with a significant positive effect on males and a non-significant positive effect on females. This QTL was located in map position interval 38A to 44C, a region that encompasses the QTL for female and male longevity in the present study (38CD−41F).

QTLs for longevity and starvation survival have been identified using the set of 98 RILs (Vieira et al., 2000). In this study, starvation survival was investigated as well as longevity at low and high temperature, longevity after heat shock and longevity at the control environment. The genetic correlation between the starvation environment and other environments was not significantly different from zero, but it typically was negative. For example, and of most relevance to the present study, the genetic correlation between starvation survival and longevity in the control environment was 0.021. There were multiple significant QTLs for starvation survival that were not significantly different from zero in terms of correlations with QTLs for longevity in the control environment. It might be noteworthy to mention that for one QTL (map region 38A−48D), starvation survival was positively associated with female longevity in the control environment, but negatively associated with male longevity. This cytogenetic region includes significant QTL regions in the present study corresponding to starvation survival and survival with food present. Deficiency mapping in the 32F−44E map region of chromosome 2 delineated a relatively fine-scale longevity QTL for males in the 38–39 map region with evidence for line effects on male longevity and line-by-genotype interaction for both sexes (Pasyukova et al., 2000).

Results from the aforementioned studies (Nuzhdin et al., 1997; Vieira et al., 2000) overlap to variable degrees with subsequent QTL studies on *D. melanogaster* lifespan. Substantial differences are associated with the mating status of flies used for assays and the origin of the RILs employed. Using the panel of 98 RILs, longevity was assayed by holding males and females together in cages (Reiwitch & Nuzhdin, 2002). Lifespan declined in both sexes presumably due to courtship, mating and increased gamete production. Generally, the location of longevity QTLs did not change, with the noteworthy exception of the loss of the lifespan QTL in the region 38E−43A. However, the sexual dimorphism characteristic of earlier studies was not observed in Reiwitch & Nuzhdin (2002). Similarly, results from a set of 120 RILs, derived from a longevity

المنارة للاستشارات
www.manaraa.com

selection experiment, indicated an absence of sexual dimorphism associated with lifespan QTLs (Curtsinger & Khazaeli, 2002). In this study lifespan was tested with females and males housed together, and most of the genetic variation for longevity was found on the third chromosome. In contrast to the negative association between longevity and other traits reported in Vieira et al. (2000), the study by Curtsinger & Khazaeli (2002) observed a positive association between QTLs for longevity, mid-life fertility and resistance to oxidative stress. A study of age-specific mortality in the RILs used in the present study, based on large sample size and mixed sex cohorts, has been conducted and the data analysed (Nuzhdin et al., unpubl. data). There is substantial overlap with lifespan QTLs in the present study, regardless of the fact that the diet and other environmental conditions were different in the two studies.

To date, RILs have been derived from three sources: established laboratory stocks (Nuzhdin et al., 1997), lines selected for extended longevity in the laboratory (Curtsinger & Khazaeli, 2002) and lines inbred immediately after collection from natural populations (Kopp et al., 2003). Laboratory populations evolve shortened lifespan (Promislow & Tatar, 1998; Hoffmann et al., 2001) and thus inbred lines from laboratory-adapted populations could represent a pattern of genetic variation for life history and related traits that deviates considerably from natural populations. Lines from natural populations could include the effect of ecological conditions that maintain genetic variation with pleiotropic effects on lifespan. However realistic in concept, lines recently derived from natural populations have only been assessed in laboratory environments and it remains to be determined if the results extend to natural environments. The assay conditions may also affect the results of lifespan QTL studies. Temperature, larval density and genetic background can affect the location of QTLs that affect lifespan of virgin males and females (Vieira et al., 2000; Leips & Mackay, 2000). Mating status changed whether sexual dimorphism was observed for lifespan QTLs (Nuzhdin et al., 1997; Reiwitch & Nuzhdin, 2002). Studies on virgin flies indicate that QTLs have antagonistic pleiotropic effects on lifespan in different environments (Vieira et al., 2000), whereas QTLs defined using mated flies exhibit positive genetic covariation between longevity and stress resistance or fecundity traits as shown in Curtsinger & Khazaeli (2002) and the present study.

المنارة للاستشارات
www.manaraa.com

Mutation analysis of longevity using model species for genetic studies indicates a general relationship between lifespan and stress resistance. Yeast (Saccharomyces cerevisiae) mutations that extend longevity can confer resistance to various environmental stresses (Jazwinski, 1996), including starvation resistance (Kennedy et al., 1995). Worm (Caenorhabditis elegans) mutations that extend longevity tend also to confer resistance to various stresses (Guarente & Kenyon, 2000). Mutations in the insulin signalling pathway can considerably extend the lifespan of C. elegans (Johnson & Wood 1982; Kenyon et al., 1993). Mutations in daf-2, and other genes in the insulin pathway, accumulate lipid and glycogen in adults (Kimura et al., 1997). Similarly, mutation in the insulin signalling pathway extend the lifespan of *D. melanogaster* (Clancy et al., 2001; Tatar et al., 2001). In addition to extended lifespan, mutation in an insulin receptor substrate gene (chico) results in adult lipid accumulation (Bohni et al., 1999) and starvation resistance (Clancy et al., 2001). Thus, a single insulin signalling gene could underlie longevity and starvation resistance. Research on insulin signalling mutations in C. elegans has led to the hypothesis that food scarcity might modulate lifespan in diverse species (Kenyon, 2001) and similar research on *D. melanogaster* has led to the related hypothesis of an evolutionarily conserved mechanism based on a process that matches reproduction to food supply (Partridge & Gems, 2002). The latter hypothesis is not yet specified in detail, but presumably the idea is that joint regulation of reproduction and metabolism maximizes the former and serves to maintain endogenous resources at an evolutionarily 'optimal' level, including the ability to withstand starvation.

Multiple stress resistance is an issue of considerable general interest in relationship to longevity. One long-lived mutant of *D. melanogaster* has been shown to be resistant to multiple forms of environmental stress (Lin et al., 1998), whereas a different long-lived mutation in an insulin signalling pathway gene was resistant to starvation but not to oxidative stress (Clancy et al., 2001). An extended question for the set of RILs used in the present study is whether there are QTLs that confer resistance to more than one environmental stress and whether such regions of the genome are associated with longevity in the absence of environmental stress. Starvation resistance might be especially salient because it presumably evolved from food scarcity, which might have

المنارة للاستشارات
www.manaraa.com

been the selective agent producing a conserved mechanism that mediates aging and lifespan. In any case, QTL studies ultimately have the goal of identifying genes that are polymorphic for alleles that control genetic variation in populations (DeLuca et al., 2003) and this is also our goal with respect to the QTLs for extended starvation resistance and longevity.

ACKNOWLEDGEMENTS

This study was supported by a U.S. Army grant to L.G.H. and S.V.N. (DAAD), 19-03-1-0152, and a National Institute of Health grant to S.V.N., NIH1 RO1 G61773-01.

المنارة للاستشارات
www.manaraa.com

Table 1

Pearson correlation coefficients (r, below the diagonal) and probability (P, above the diagonal) that the absolute value of r is greater than zero for female and male survival with (lifespan = ‘Life’) and without (starvation condition = ‘Starve’) food present

المنارة للاستشارات

www.manaraa.com

	Life male	Life female	Starve male	Starve female
Life male	1.0000	*P* < 0.0001	*P* = 0.0332	*P* = 0.1072
Life female	0.8264	1.0000	*P* = 0.0764	*P* = 0.0505
Starve male	0.2121	0.1772	1.0000	*P* = 0.0001
Starve female	0.16374	0.1981	0.5852	1.0000

المنارة للاستشارات

www.manaraa.com

Table 2

Single marker analysis for female and male survival traits. The proportion of variance accounted by marker segregation is shown for each sex and condition corresponding to the cytogenetic region of the marker (for example, 38C). The probability (P) reported is that marker segregation is not associated with trait segregation. The values in bold type indicate marker effects whose probability is less than the permutation threshold (life male = 0.000461, life female = 0.000489, starve male = 0.000523, starve female = 0.000468). 'Life' refers to survival when food is present and 'Starve' refers to survival when no food is present

المنارة للاستشارات

www.manaraa.com

Marker region	Life male	Life female	Starve male	Starve female
38C	**0.34** **($P < 0.000001$)**	0.20 ($P = 0.00086$)	**0.30** **($P = 0.00001$)**	0.13 ($P = 0.00254$)
41F	**0.40** **($P < 0.000001$)**	**0.23** **($P = 0.00026$)**	**0.20** **($P = 0.00023$)**	0.11 ($P = 0.00448$)
42B	**0.39** **($P < 0.000001$)**	**0.27** **($P = 0.00006$)**	**0.19** **($P = 0.00034$)**	0.12 ($P = 0.00235$)
44C	0.00 ($P = 0.444370$)	0.01 ($P = 0.26769$)	0.20 ($P = 0.00062$)	**0.25** **($P = 0.00008$)**
48D	**0.29** **($P = 0.00004$)**	0.09 ($P = 0.02237$)	0.18 ($P = 0.00075$)	0.25 ($P = 0.05210$)
49B	**0.25** **($P = 0.00003$)**	0.06 ($P = 0.04033$)	0.02 ($P = 0.17224$)	0.00 ($P = 0.75553$)

المنارة للاستشارات
www.manaraa.com

Table 3

QTL effects on starvation survival and lifespan. QTL effects are indicated by positive or negative magnitude of table values and statistically significant effects are given in bold type. In the table, chromosome is followed by complementation group (chromosome-complementation), which is one of the four linkage groups in the starting population used to produce the RILs. 'Life' refers to mean longevity and 'Starve' refers to mean survival time when no food is present

www.manaraa.com

ChromosomeTable 2 QTL*	QTL position (interval)	Life males	Life females	Starve male	Starve female
2–1/48D	33F (22C−48D)	**0.18**	0.11	0.03	−0.01
2–2	37C (36F−49F)	−0.00	0.01	0.09	**0.10**
2–2	54C (54A−55C)	−0.03	−0.01	**0.11**	0.07
2–3/41F	36AB−41CE (35CD−41F)	−0.26	**−0.20**	**−0.19**	**−0.15**
2–3/42B	41F (41CE−42B)	0.26	0.20	**0.20**	**0.16**
2–4/44C, 49B	49B (44C−50C)	**0.16**	**0.18**	0.06	0.02
3–1	66B (66A−67D)	**0.09**	0.06	0.04	0.02
3–4	62E (61A−64B)	**−0.24**	**−0.17**	−0.10	0.00
3–4	65E (65C−73D)	**−0.23**	**−0.23**	−0.10	−0.00

*QTL intervals corresponding to single marker analyses in Table 2 are shown after the back-slash. Note that the marker 38C does not correspond to a QTL identified by composite interval mapping.

www.manaraa.com

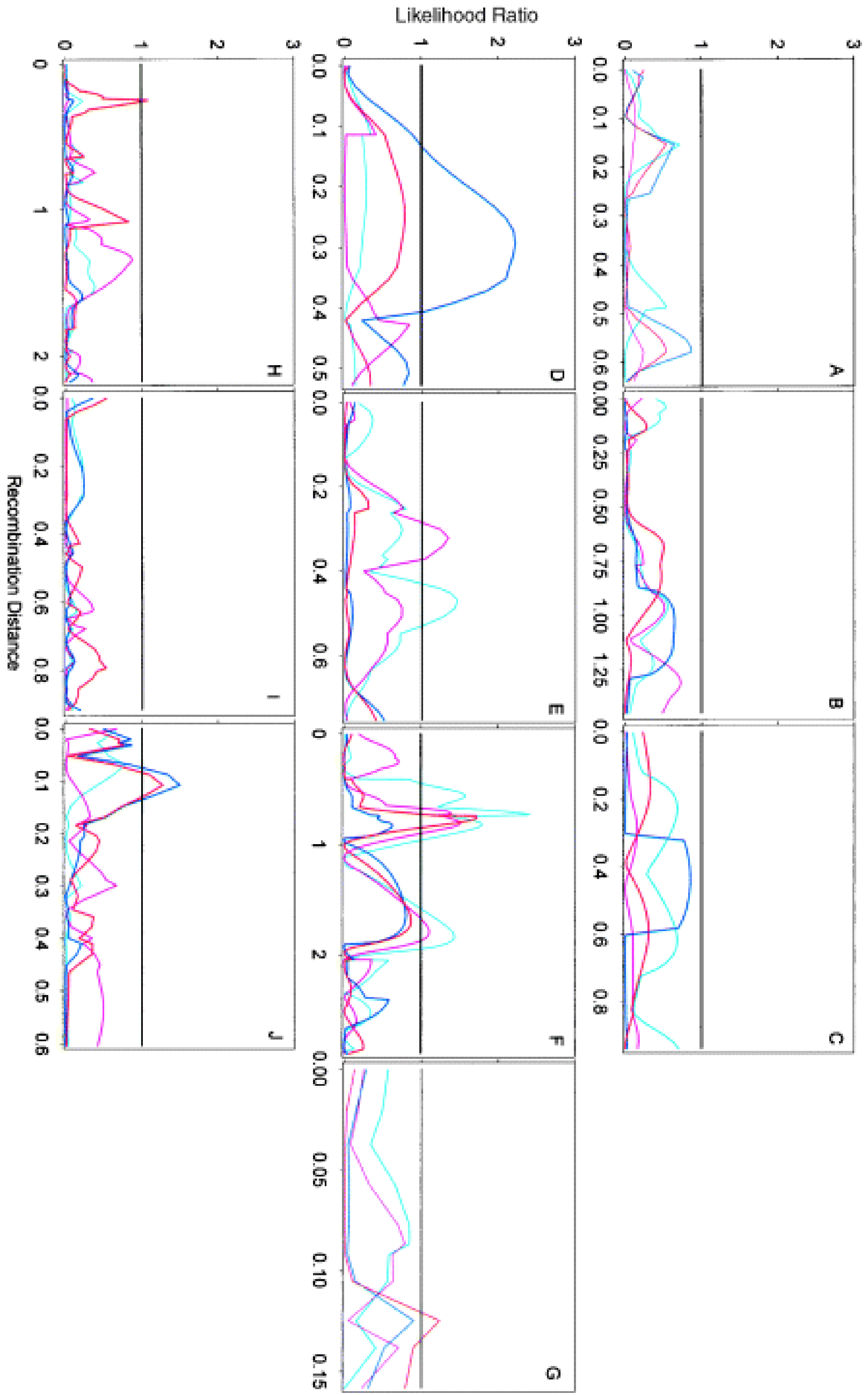
Figure 1

The QTL likelihoods for starvation of males (cyan) and females (pink), and the lifespan of males (blue) and females (red) on X chromosome panels (A–C), second chromosome panels (D–G) and third chromosome panels (H–J). The ordinate of each panel shows the strength of support for QTL regions and the line at 1 indicates the permutation threshold for significant QTLs above this level. The abscissa is scaled to recombination distance across the genome.

(Imagine was made by Sergey V. Nuzhdin)

المنارة للاستشارات

www.manaraa.com



www.manaraa.com
المنارة للاستشارات

References

Arking R, Burde V, Graves K, Haris R, Feldman E, Zeevi A, Soliman S, Saraiya A, Buck S, Vettraino J, Sathrasala K, Wehr N, Levine RL (2000a)Forward and reverse selection for longevity in *Drosophila* is characterized by alteration of antioxidant gene expression and oxidative damage patterns. Exp. Gerontol. 35, 167–185.

Arking R, Burde V, Graves K, Haris R, Feldman E, Zeevi A, Soliman S, Saraiya A, Buck S, Vettraino J, Sathrasala K (2000b) Identical longevity phenotypes are characterized by different patterns of gene expression and oxidative damage. Exp. Gerontol. 35, 353–373.

Basten CJ, Weir BS, Zeng Z-B (1994) Zmap-a QTL cartographer. In Proceedings of the 5th World Congress on Genetics Applied to Livestock Production: Computing Strategies and Software (eds Smith C, Gavora JS, Benkel B, Chesnais J, Fairfull W, Gibson JP, Kennedy BW). Guelph, Ontario: E. B. Burnside, pp. 65–66.

Basten CJ, Weir BS, Zeng Z-B (1999) QTL Cartographer, Version 1.13. Raleigh, NC: Department of Statistics, North Carolina State University. Bohni R, Riesgo-Escovar J, Oldham S, Brogiolo W, Stocker H, Andruss BF, Beckingham K, Hafen E (1999) Autonomous control of cell and organ size by CHICO, a *Drosophila* homolog of vertebrate IRS1-4. Cell 97, 865–875.

Charlesworth B, Langley CH (1989) The population genetics of *Drosophila* transposable elements. Ann. Rev. Genetics 23, 251–287.

Clancy DJ, Gems D, Harshman LG, Oldham S, Stocker H, Hafen E, Leevers SJ, Pattridge L (2001) Extension of life–span by loss of CHICO, a *Drosophila* insulin receptor substrate protein. Science 292, 104–106.

Curtsinger JW (2002) Sex specificity, life-span QTLs, and statistical power. J. Gerontol. A Biol. Sci. Med. Sci. 57, B409–B414.

Curtsinger JW, Khazaeli AA (2002) Life span, QTLs, age-specificity and pleiotropy in *Drosophila*. Mechanisms Aging Dev. 123, 81–93.

DeLuca M, Roshina NV, Geiger-Thornsberry GI, Lyman RF, Pasyukova EG, Mackay T (2003) Dopa decarboxylase (Ddc) affects variation in *Drosophila* longevity. Nature Genet. 34, 429–433.

Doerge RW, Churchill GA (1996) Permutation tests for multiple loci affecting a

المنارة للاستشارات

www.manaraa.com

quantitative character. Genetics 142, 285–294.

Dudas SP, Arking RA (1995) A coordinate upregulation of antioxidant gene activities is associated with the delayed onset of senescence in a long-lived strain of *Drosophila*. J. Gerontol. Biol. Sci. 50A, B117–B127.

Force AG, Staples T, Soliman T, Arking R (1995) A comparative biochemical and stress analysis of genetically selected *Drosophila* strains with different longevities. Dev. Genet. 17, 340–351.

Guarente L, Kenyon C (2000) Genetic pathways that regulate ageing in model organisms. Nature 408, 255–262.

Harshman LG, Hoffmann AA (2000) Laboratory selection experiments using *Drosophila*: what do they really tell us? Trends Ecol. Evol. 15, 32–36.

Harshman LG, Moore KM, Sty MA, Magwire MM (1999) Stress resistance and longevity in selected lines of *Drosophila melanogaster*. Neurobiol. Aging 20, 521–529.

Harshman LG, Schmid J (1998) Evolution of starvation resistance in *Drosophila melanogaster*: aspects of metabolism and counter-impact selection. Evolution 52, 1679–1685.

Hoffmann AA, Hallas R, Sinclair C, Partridge L (2001) Rapid loss of stress resistance in *Drosophila melanogaster* under adaptation to laboratory culture. Evolution 52, 436–438.

Jazwinski M (1996) Longevity, genes and aging. Science 273, 54–59.

Johnson TE, Wood WB (1982) Genetic analysis of life-span in Caenorhabditis clegans. PNAS 79, 6603–6607.

Kennedy BK, Austriaco NR, Zhang J, Guarente L (1995) Mutation in the silencing gene SIR4 can delay aging in S. cerevisiae. Cell 80, 485–496.

Kenyon C (2001) A conserved regulatory system for aging. Cell 105, 165–168.

Kenyon C, Chang J, Gensch E, Rudner A, Tablang R (1993) A C. elegans mutant that lives twice as long as wild type. Nature 366, 461–464.

Kimura KD, Tissenbaum HA, Liu Y, Ruvkum G (1997) daf-2, an insulin receptor-like gene that regulates longevity and diapause in Caenorhabditis elegans. Science 277, 942–946.

Kopp A, Graze RM, Xu S, Carroll SB, Nuzhdin SV (2003) Quantitative trait loci

المنارة للاستشارات
www.manaraa.com

responsible for variation in sexually dimorphic traits in *Drosophila* melangaster. Genetics 163, 771–787.

Lefevre GJ (1976) A photographic representation and interpretation of the polytene chromosomes of *D. melanogaster* salivary glands. The Genetics and Biology of *Drosophila*, Vol. 1a (eds Ashburner M., Novitski E.) pp. 31–66. Academic Press, London.

Leips J, Mackay TF (2000) Quantitative trait loci for life span in *Drosophila melanogaster*: interactions with genetic background and larval density. Genetics 155, 1773–1778.

Lin YJ, Seroude L, Benzer S (1998) Extended life-span and stress resistance in the *Drosophila* mutant _Methuselah. Science 282, 943–946.

Luckinbill LS, Arking R, Clare MJ, Cirocco WC, Buck SA (1984) Selection for delayed senescence in *Drosophila melanogaster*. Evolution 38, 996–1004.

Nuzhdin SV, Pasyukova EG, Dilda CL, Zeng ZB, Mackay TF (1997) Sex-specific quantitative trait loci affecting longevity. Proc. Natl Acad. Sci. USA 94, 9734–9739.

Partridge L, Gems D (2002) Mechanisms of ageing: public or private? Nat. Rev. Genet. 3, 165–175.

Pasyukova EG, Vieira C, Mackay TFC (2000) Deficiency mapping of quantitative trait loci affecting longevity in *Drosophila melanogaster*. Genetics 156, 1129–1146.

Promislow DE, Tatar M (1998) Mutation and senescence: where genetics and demography meet. Genetica 102/103, 299–314.

Reiwitch SG, Nuzhdin SV (2002) Quantative trait loci of life span of mated *Drosophila melanogaster* affects both sexes. Genet. Res. 80, 1–6.

Rose MR (1984) Laboratory evolution of postponed senescence in *Drosophila melanogaster*. Evolution 38, 1004–1010.

Rose MR, Vu LN, Park SU, Grave JL Jr. (1992) Selection on stress resistance increases longevity in *Drosophila melanogaster*. Exp. Gerontol. 27, 241–250.

SAS Institute Inc (1988) SAS/STAT User's Guide. Release 6.03 Edition. Cary NC: SAS Institute Inc.

Service PM, Hutchinson EW, MacKinley MD, Rose MR (1985) Resistance to environment stress in *Drosophila melanogaster* selected for postponed senescence.

المنارة للاستشارات
www.manaraa.com

Evolution 42, 708–716.

Service PM (1987) Physiological mechanisms of increased stress resistance in *Drosophila melanogaster* selected for postponed senescence. Physiol. Zool. 60, 321–326.

Shrimpton AE, Montgomery EA, Langley CH (1986) Om mutations in *Drosophila* ananassae are linked to insertions of a transposable element. Genetics 114, 125–135.

Tatar M, Kopelman A, Epstein D, Tu MP, Yin CM, Garofalo RS (2001) A mutant *Drosophila* insulin receptor homolog the extends life-span and impairs neuroendocrine function. Science 292, 107–110.

Tower J (1996) Aging mechanisms in fruit flies. Bioessay 18, 799–807.

Vieira C, Pasyukova EG, Zeng ZB, Hackett JB, Lyman RF, Mackay TF (2000) Genotype–environment interaction for quantitative trait loci affecting life in *Drosophila melanogaster*. Genetics 154, 213–227.

المنارة للاستشارات
www.manaraa.com

Chapter III:
Quantitative trait loci for lipid content in *Drosophila melanogaster*.

[Published by Mei-Hui Wang, Lawrence G. Harshman and Sergey V. Nuzhdin (2005) In Obesity Research 13: 1891-1897]

المنارة للاستشارات

www.manaraa.com

Abstract

Recombinant inbred lines derived from a natural population were used to investigate natural genetic variation for lipid abundance, protein abundance and weight of *D. melanogaster*. Females were heavier and contained more lipid and soluble protein than males. Lipid and protein abundance were genetically correlated with female weight, but male weight was not correlated with lipid or protein. Lipid and protein abundance were genetically correlated in males, but not in females. Quantitative trait loci (QTLs) for weight and protein abundance were predominantly on the X chromosome whereas QTLs for lipid abundance were found on the second and third chromosome. QTLs for lipid proportion (lipid abundance normalized by weight or protein abundance) were present on all chromosomes; a lipid proportion QTL on the third chromosome correlated with a QTL for starvation resistance observed in a previous study using the same set of recombinant inbred lines suggesting it might underlie both traits. Candidate genes are discussed in relationship to lipid abundance, lipid proportion and starvation resistance.

المنارة للاستشارات

www.manaraa.com

The characterization of natural genetic variation for the control of obesity in humans is an important goal in human health research. However, it is difficult to acquire this information because there are limited methods available for the study the genetic architecture of complex traits in humans. Conversely, quantitative trait loci studies of lipid abundance in model organisms like *Drosophila* are especially suited for this purpose. The study of obesity in *Drosophila* is relevant to humans because pathways of lipid metabolism are generally conserved between vertebrates and invertebrates (1). Insights from the *Drosophila* model may help to elucidate genetic architecture of obesity variation in humans. We have previously constructed a set of 144 recombinant inbred lines (RILs) that segregate for up to four natural alleles per locus (2). Here, we weigh males and females from these lines and assay for lipid and protein abundance, and for lipid proportion which is analogous to obesity. The goal was to map quantitative trait loci (QTLs) for genetic variation in these characters.

Protein and lipid content, and body weight were all significantly higher in females (0.793 mg/ml, 0.552 ug/ml, 0.315 mg respectively) than males (0.424 mg/ml, 0.369 ug/ml, 0.217 mg). The genetics of body size differences in females and males is not known, but it may be relevant to note that body size is controlled by a nutrient sensor mechanism during *Drosophila* growth (3). Scaled by body weight, lipid content was no longer different between sexes (1.78 ug/ml versus 1.80 ug/ml in males and females respectively) but protein content remains sexually differentiated (1.99 ug/ml versus 2.55 ug/ml). In two way ANOVAs, effects of sex, line, and sex by line interactions were significant at $P<0.0001$ level for every trait (data not shown). Male female line means were correlated within traits (Table 1). In males, neither protein nor lipid abundance was correlates with body weight, but in females, these correlations were positive and highly significant. Protein and lipid abundance were significantly correlated in males but not in females. Genetic correlations between some of these characters have been previously studied by selection in the laboratory, but the results were not consistent between experiments (4). Partially due to this reason, selection experiment results are marginally suitable for comparison to the present study.

The QTL likelihood profiles for three X chromosome haplotypes (linkage groups), four second chromosome haplotypes, and three third chromosome haplotypes are

المنارة للاستشارات
www.manaraa.com

represented on the left panels of the Figures 1, 2, and 3. Two haplotypes of the third chromosome (2 and 3) are indistinguishable in this set of RILs have been merged together into a "2-3" haplotype. Altogether, we have detected 12 QTLs significant at P=0.05 level while 3 are expected by chance alone. The false discovery rate might then be approximated as 0.25. The QTL effects are summarized in the Table 2. A majority of the protein abundance and weight QTLs were located on the X chromosome. QTLs affecting lipid abundance were observed on the second and third chromosomes. Contiguous haplotype QTL might in fact represent a single large effect QTL, for for instance protein abundance QTLs 5C-12C on the X chromosome. As another consideration, estimates of QTL effects on different haplotypes of the same chromosome might not be independent. For instance, protein content QTLs at 5C on haplotype 1 and 5D on the haplotype 3 of the X chromosome (italicized in the Table 2), and body weight QTLs at 22A on the haplotype 2 and 22B on the haplotype 3 of the second chromosome (underlined in the Table 2) might in fact represent a the effect of a single allele (see research methods and procedures for more explanations). In spite of these caveats, the effect of QTLs was similar to the pattern of line means.

As we were primarily interested in obesity QTLs we standardized lipid abundance by protein content and body weight which also has the effect of improving the power of detection by reducing unaccounted variation. Lipid proportion QTLs scaled by body weight and by protein content are presented in the right panels of Figures 1, 2, and 3. We detected an additional lipid proportion QTL at cytological position 11CD on the X chromosome (haplotype 3) and two additional lipid proportion QTLs at cytological position 51A on the second chromosome (haplotype 2). Notably, a third chromosome (cytological position 73C) female lipid proportion QTL (scaled by body weight) was also significant for lipid proportion in males indicating a region of the genome responsible for a general impact on lipid proportion irrespective of sex. We conclude that the genetic architecture of lipid and protein abundance, and lipid proportion, variation in flies is likely to be multi-factorial comprised of major and smaller effects to varying degree depending on the character.

As lipid content is associated with starvation resistance, it is tempting to compare our mapping results with whole genome microarray data on gene expression during

المنارة للاستشارات
www.manaraa.com

starvation and sugar feeding (5). In particular, the overlap of QTL and microarray data sets has been used to produce a list of candidate genes (6). However, it is not clear that phenotypic manipulations are expected to produce a similar pattern of gene expression to that observed for related evolved responses (7) including extant genetic variation maintained by selection.

Genetic variation in lipid composition might be due to allelic variation at the loci previously implicated in control of this trait. One can speculate about candidate genes corresponding to QTLs, as numerous genes are positioned in the QTL proximity. For instance Lsd2 gene (at cytological position 13A) belongs to the PAT family of proteins. Lsd2 mutants have reduced level of neutral lipid indicating that the gene is responsible for normal lipid storage (8). Another example is *pumpless* located at 78C (9) is a gene involved in amino acid-dependent signaling arising from fat body that induces cessation of feeding in the larva. Perhaps the most interesting example of a gene co-located with one of our QTLs is that of micro RNA miR14 located at 45F (10). miR14 suppresses cell death and is required for normal fat metabolism. Micro RNAs appear to be involved in systemic regulation of multiple genes including that respond to stress conditions (11). *C. elegans* miR234 is probably induced as a consequence of nutrient stress. *D. melanogaster* miR14 appears to synchronize multiple physiological responses related to stress, as supported by the fact that miR14 loss-of-function flies display increased sensitivity to a variety of stress conditions. Interestingly, lipid content was frequently increased in the lines selected for increased stress resistance (12). Intriguingly, QTL mapping in the same set of RILs as used in the present study identified a chromosomal region in proximity of 45F which was associated with *D. melanogaster* survival under starvation conditions (13). We therefore hypothesize that miR14 is a candidate locus potentially accounting for differential lipid abundance and starvation resistance. We will employ quantitative complementation tests (6) to test this prediction.

www.manaraa.com

RESEARCH METHODS AND PROCEDURES

Recombinant Inbred lines (RILs)

A panel of 144 RILs was generated by crossing a single virgin female from the F_1 progeny of a fertilized female caught in the wild (Winters, CA) to a single male from the F_1 progeny of a different fertilized female caught at the same location. The genetic crosses employed and methods for genotyping the lines are described in detail in (2). Briefly, recombinant F_2 genomes were isogenized by 25 generations of full-sib inbreeding. We assayed positions of *roo* transposable elements by *in situ* hybridization to polytene salivary gland chromosomes with a biotinylated DNA probe (14). Locations were determined at the level of cytological bands on the standard Bridges' map in five individuals per line. A marker was recorded as present if detected in all larvae, absent if not detected in any larva, and otherwise segregating. 123 markers were retained for the analysis, and recombination distances between them were calculated as in (15). Two of the founding third chromosomes appeared to be identical except for chromosome tips (2), thus we assumed three parental haplotypes for the third chromosome (see 16 for the list of marker positions).

Fly Rearing

Flies were reared at 22°C in vials with 7 ml of *Drosophila* medium. This medium was made as follows. To 1.5 ml of water, 11 gm of agar, 90 ml of molasses, 150 g of cornmeal, 125g of Torula yeast was added and the mixture cooked and mixed followed by addition of 30 ml ethanol, 3 ml of propionic acid and 3 g of Tegosept as anti-fungal agents. All flies from all lines were reared from vials that were seeded with approximately 75 eggs to control rearing density. Adult flies were transferred to fresh vials (15 males and 15 females) when they were 2-6 days old. Subsequently, flies were transferred to fresh food every three days for three successive transfers. At the end of the third transfer the flies were frozen at –80°C for the assays described below.

Lipid Assay

Total lipid was measured using a method described by van Handel (17) adopted by Zera et al. (18) and slightly modified for the present study. Three females or three

المنارة للاستشارات
www.manaraa.com

males were homogenized in 100 µl of chloroform:methanol (2:1) using plastic pestles and 1.5ml Eppendorf tubes. After low speed centrifugation (5,000 rpm) the solvent was transferred to a glass test tube. Triolein (0.08, 0.1, 0.3, 0.5, 0.7, 0.9, 1.0 and 1.2 mg/ml) in 2:1 chloroform:methanol was used for standards. Glass tubes with samples and standards were incubated in a 85-90ºC water bath for 10 minutes to evaporate the solvent before addition of sulfuric acid and additional incubation in the 85-90ºC water bath for 10 minutes. The tubes were removed and allowed to cool to room temperature before addition of vanillin-phosphoric acid reagent followed by gentle vortexing. 250 µl of each sample or standard was transferred to a well in a 96-well plate and read in a spectrophotometer at 525 nm.

Protein Assay

Soluble protein was measured using the BCA Protein Assay Kit (Pierce Company, Rockford Illinois). Five males or females from each line were homogenized as described in the lipid assay section, but in 250 µl of Tris-HCL pH 8.0. The homogenate was duplicated 1:3 in homogenizing buffer and 10 µl was added to each well (96 well plate) for The Microplate Assay Procedure. The following standard concentrations of protein were employed: 2 mg/ml, 1 mg/ml, 0.5 mg/ml, 0.25 mg/ml, and 0.125 mg/ml. The BCA reagents were added and after a 30 minute incubation at 37°C the plate was read at 562 nm.

Weight

Total body weight was determined after previously frozen flies were placed in a vacuum drier for at least 24 hours. For each recombinant inbred line, the weight of five females and five males was determined using a Satorius M2P Microbalance (1µg to 1g). Samples were removed
from the vacuum drier such that flies were weighed within 2 hr after removal.

Statistical Analysis

For the analysis of variance, we first transformed the data to improve normality of residuals. Box-Cox transformation was found that maximized Shapiro-Wilkinson test

المنارة للاستشارات
www.manaraa.com

statistics (19, SAS MACRO using UNIVARIATE procedure, option "normal", is available from SVN). Two way ANOVAs were then performed with effects due to sex (fixed), line (random), and line * sex interaction (random). Statistical significance of components of variance was assayed with SAS procedure GLM. Genetic correlations between traits and sexes were approximated as correlations between line means, and estimated with the SAS procedure CORR.

Our study makes use of wild, outbred founder flies. We adapted standard QTL mapping software that was designed for homozygous founders to analyze our data (see 13 for a detailed explanation). Briefly, we test whether an allele from one haplotype encodes a trait value significantly different from the average trait due to the combined effects of other haplotypes. The number of different allelic effects that we can independently estimate is equal to the number of segregating haplotypes minus one. Using haplotype specific marker alleles, we generated a separate likelihood profile with the CIM procedure in QTL Cartographer (20). Options in the CIM module were set to two background parameters, window size of 30 cM, and Kosambi mapping function. Significance thresholds were determined by 1000 permutations for each trait and chromosome.

ACKNOWLEDGEMENTS

This study was supported by an ARO grant to L. Harshman and S. Nuzhdin (DAAD 19-03-1-0152.

المنارة للاستشارات

www.manaraa.com

Table 1

Correlation matrix of line means between lipid and protein abundance, and body weight of males and females. Correlation coefficients above the diagonal (italicized if significant and bold faced if P = 0.001 or less) with the level of statistical significance indicated below the diagonal.

Trait		Protein abundance		Lipid abundance		Body weight	
	sex	male	female	male	female	male	female
Protein content	male	-	**.376**	*.215*	.091	.034	.168
	female	<.0001	-	.138	.053	*.233*	**.285**
Lipid content	male	.013	ns	-	**.640**	-.114	.152
	female	ns	ns	<.0001	-	.037	**.307**
Body weight	male	ns	.011	ns	ns	-	**.613**
	female	ns	.002	ns	.001	<.0001	-

المنارة للاستشارات
www.manaraa.com

Table 2

Quantitative trait loci affecting lipid and protein abundance, and body weight. Significant effects are bold-faced.

Chromosome	Marker	Protein	content	Lipid	content	Body	weight
		male	female	male	female	male	female
X-1	4C	.012	-.014	-.002	.001	**.01**	-.002
X-1	*5C*	**.037**	.018	.007	.027	.014	.009
X-1	9C	**.038**	.027	.006	.016	.007	.013
X-1	12C	**.039**	.033	-.007	.018	.007	**.018**
X-3	*5D*	-.016	-.005	.007	-.000	**-.016**	-.013
2-2	22A	**.045**	.015	.024	-.004	-.000	.005
2-3	22B	**-.026**	-.025	-.015	.002	.002	.004
2-3	47E	.005	.024	**.037**	.008	-.001	.010
3-23	63E	.008	.005	.009	.008	**.011**	.008
3-23	73C	-.001	.004	**-.041**	-.026	.003	-.001
3-23	82C	.009	.023	**-.043**	-.009	.002	.005

المنارة للاستشارات
www.manaraa.com

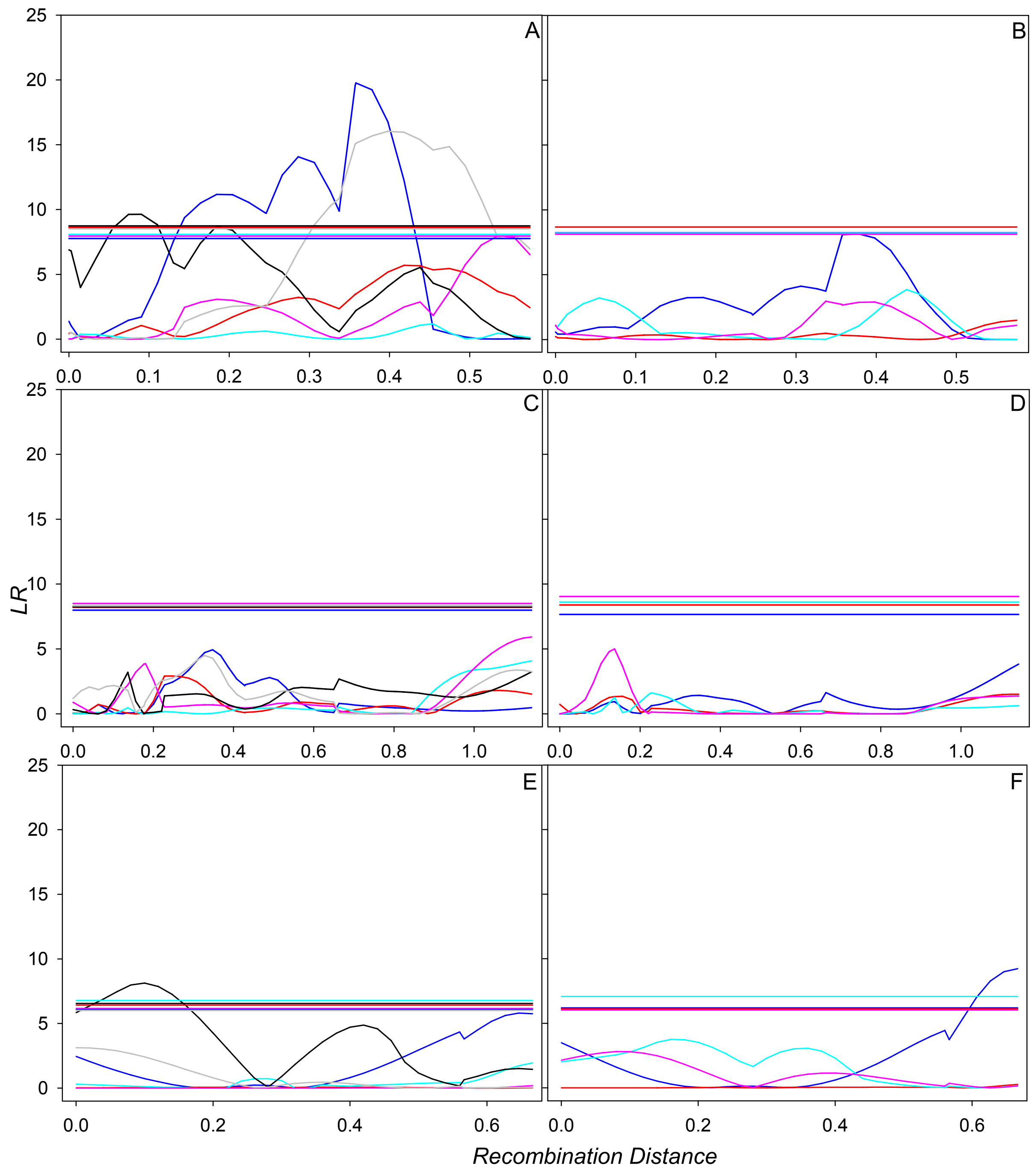
Figure 1

Left panels (A, C, and E) represent quantitative trait loci affecting male (blue) and female (red) protein content, lipid content (cyan and pink) and body weight (black and gray) on haplotypes 1, 2, and 3 of the X chromosome. QTLs for lipid content weighed by protein content (blue and red), and for lipid content weighed by body weight (cyan and pink) are in the three right panels. Horizontal lines correspond to significance thresholds for same colored traits.

(Imagine was made by Sergey V. Nuzhdin)

للاستشارات المنارة

www.manaraa.com



المنارة للاستشارات
www.manaraa.com

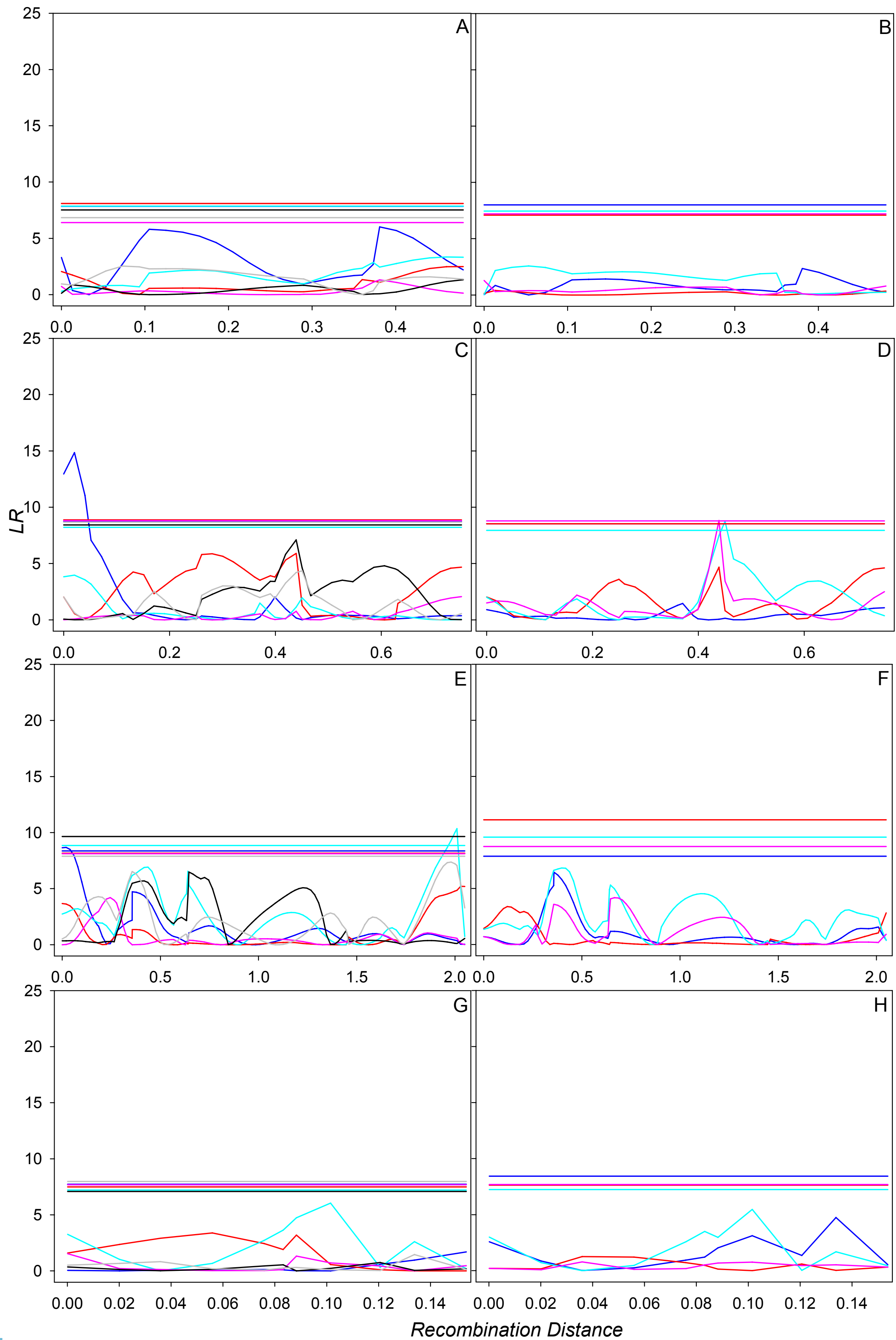
Figure 2

QTLs for lipid and protein abundance, and body weight on haplotypes 1, 2, 3, and 4 of the second chromosome.

(Imagine was made by Sergey V. Nuzhdin)

المنارة للاستشارات

www.manaraa.com



www.manaraa.com

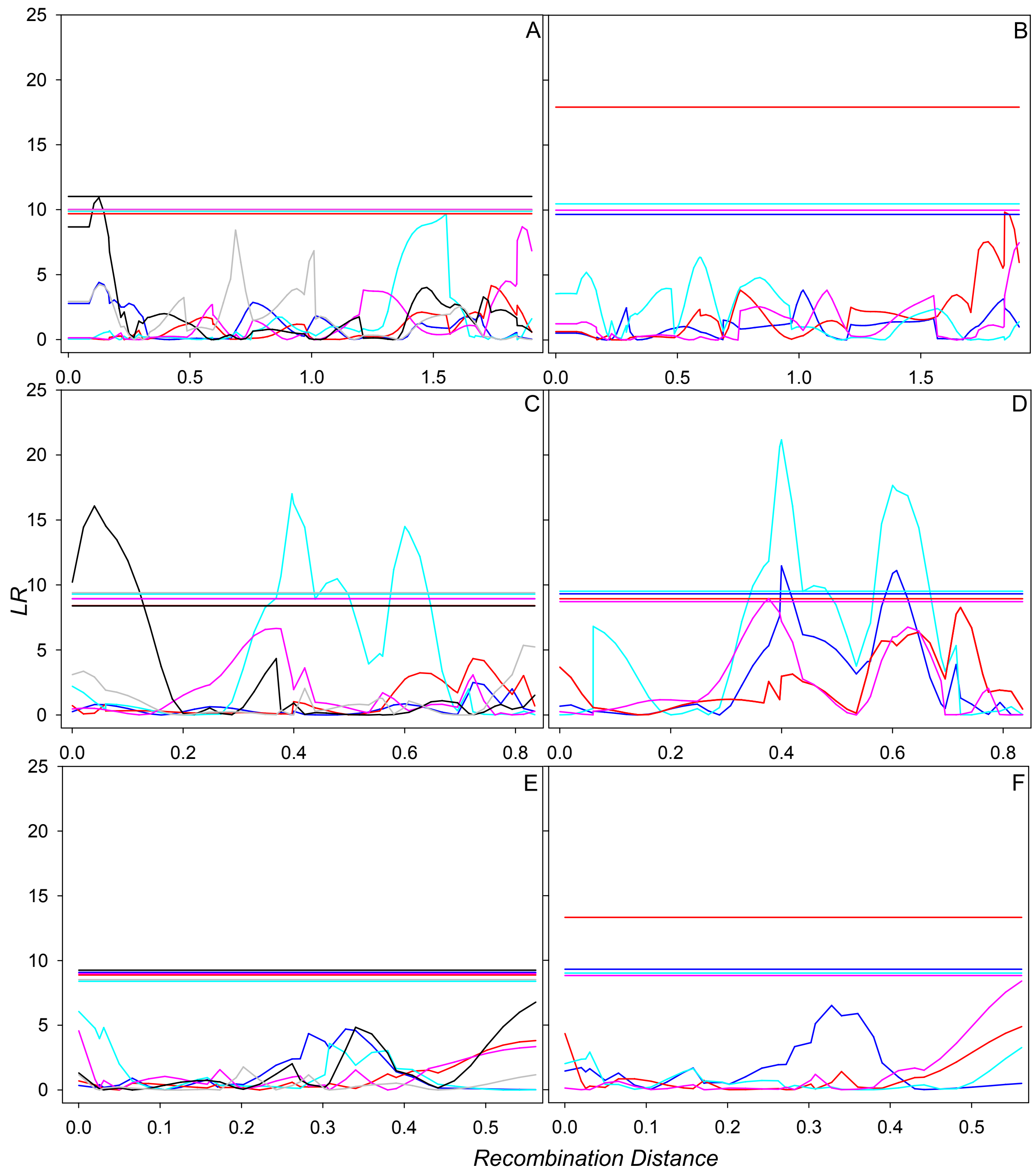
Figure 3

QTLs for lipid and protein abundance, and body weight on haplotypes 1, 2-3, and 4 of the third chromosome.

(Imagine was made by Sergey V. Nuzhdin)

المنارة للاستشارات

www.manaraa.com



المنارة للاستشارات
www.manaraa.com

References

1. Canavoso LE, Jouni ZE, Karnas KJ, Pennington JE, Wells MA. Fat metabolism in insects. *Annu. Rev. Nutr.* 2001;21:23-46.

2. Kopp A, Graze RM, Xu S, Carroll SB, Nuzhdin SV. Quantitative trait loci responsible for variation in sexually dimorphic traits in *Drosophila melanogaster*. *Genetics* 2003;163: 771-787.

3. Colombani J, Raisin S, Pantalacci S, Radimerski T, Montagne J, Leopold P. A nutrient sensor mechanism controls *Drosophila* growth. *Cell* 2003;114:739-749.

4. Harshman LG, Hoffman AA. Laboratory selection experiments using *Drosophila*: what do they really tell us? *TREE* 2000;15:32-36.

5. Zinke I, Schutz CS, Katzenberger JD, Bauer M, Pankratz MJ. Nutrient control of gene expression in *Drosophila*: microarray analysis of starvation and sugar-dependent response. *EMBO J*. 2002;21:6162-6173.

6. Wayne ML, McIntyre LM. Combining mapping and arraying: An approach to candidate gene identification. *Proc Natl. Acad. Sci.* USA 2002;23: 14903-14906.

7. Harshman LG, Schmid JL. Evolution of starvation resistance in *Drosophila melanogaster*: aspects of metabolism and counter-impact selection. *Evolution* 1998;52:1679-1685.

8. Teixeira L, Rabouille C, Rorth P, Ephrussi A, Vanzo NF. *Drosophila* Perilipin/ADRP homologue Lsd2 regulates lipid metabolism. *Mech. Develop.* 2003;120:1071-1081.

9. Zinke I, Kirchner C, Chao LC, Tetzlaff MT, Pankratz MJ. Suppression of food intake and growth by amino acids in *Drosophila*: the role of *pumpless*, a fat body expressed gene with homology to vertebrate glycine cleavage system. *Development* 1999;126:5275-5284.

10. Xu P, Vernooy SY, Guo M, Hay BA. The *Drosophila* micro RNA Mir-14 supresses cell death and is required for normal fat metabolism. *Current Biology* 2003;13:790-795.

11. Llave C. MicroRNAs: more than a role in plant development? *Mol. Plant Pathology* 2004;5:361-366.

12. Chippindale AK, Chu TJF, Rose MR. Complex trade-offs and the evolution of starvation resistance in *Drosophila melanogaster. Evolution* 1996;50:753-766.

المنارة للاستشارات
www.manaraa.com

13. Wang MH, Lazebny O, Harshman LG, Nuzhdin SV. Environment-dependent survival of *Drosophila melanogaster*: a quantitative genetic analysis. *Aging Cell* 2004;3:133-140.
14. Shrimpton AE, Montgomery EA, Langley CH. Orc mutations in *Drosophila ananassae* are linked to insertions of a transposable element. *Genetics* 1986;114:125–135.
15. Mezey JG, Houle D, Nuzhdin SV. Quantiative trait loci affecting wing shape in *Drosophila melanogaster. Genetics* (in press).
16. Nuzhdin SV, Khazaeli AA, Curtsinger JW. Survival analysis of life span QTL in *Drosophila melanogaster. Genetics* (accepted pending revision).
17.van Handel E. Rapid determination of total lipids in mosquitoes. *J. Am. Mosq. Control Assoc.* 1985;1:302-304.
18. Zera AJ, Sall J, Otto K. Biochemical aspects of flight and flightlessness in Gryllus: flight, fuels, enzyme activities and electrophoretic profiles of flight muscles from flight-capable and flightless morphs. *J. Insect Physiol*. 1999;45:275-285.
19. SAS Institute, 1989 SAS/STAT User's Guide, Release 6.03. SAS Institute, Cary, NC.
20. Basten CJ, Weir BS, Zeng ZB. 1999 QTL Cartographer: Version 1.13. Department of Statistics, North Carolina State University, Raleigh, NC.

المنارة للاستشارات
www.manaraa.com

Chapter IV:
Laboratory Selection of Starvation Resistance Using *Drosophila melanogaster*: Reproduction and Lipids

للاستشارات المنارة
www.manaraa.com

Introduction

For most of species, essential nutrition may sometimes be limited during their life cycles. For *Drosophila,* food abundance changes depending on season or location (Hoffmann and Parson, 1991; Gibbs, 1999) and the ability to survive starvation is presumably an important fitness trait. Survival strategies may include genetic, physiological or behavioral changes (Hoffmann and Parsons 1991; Harshman, Hoffmann and Clark 1999; Zou et al, 2000; Lee and Park, 2004; Harbison et al 2005). Starvation resistance is important in evolutionary physiology (Hoffmann and Parsons 1991; Zera and Harshman, 2001), impinging on a wide variety of traits, from fitness, to longevity, to fecundity. Starvation resistance is correlated to lifespan and reproduction, such that there sometimes is a positive genetic correlation between starvation and longevity, and a negative genetic correlation between resistance and early fecundity (Leroi et al. 1994a.b; Prasad and Joshi 2003). The abundance of lipids, the most important energy storage molecules, is commonly correlated with starvation resistance as well (Service, 1987; Chippindale et al, 1996; Harshman 2003; Wang et al. 2005). Further evidence supporting the notion that lipids are be the primary resources involved is that they are differentially allocated in survival (longevity) and reproduction.

Many questions remain regarding the physiological mechanism of starvation resistance in *D. melanogaster*, especially the dynamics of lipid usage and the physiological trade-off between starvation and reproduction. The physiological traits correlated to starvation response and life history tradeoffs, especially reproduction, have been examined by comparing laboratory populations that have undergone selection. By utilizing laboratory selection to explore the physiological and genetic changes associated with the starvation selection response, insights can be gained into the physiology basis of the phenomenon. Selection studies can aid in elucidating the genetic basis of physiological changes, while comparing physiology between selected populations and control populations may explain common resistance mechanisms and the interaction between life history traits (Gibbs, 1999; Zera and Harshman, 2001; Telonis-Scott et al., 2006).

For starvation resistant females, it has been previously shown that a tradeoff exists

المنارة للاستشارات
www.manaraa.com

which points to a decrease in fecundity and an increase in lifespan or stress resistance (Williams 1966; Roff 1992). Decreased reproduction costs ascertained by phenotypic measurements confirmed the tradeoff between somatic and germ line success. Although lower fecundity is related to increased stress resistance, there are no studies that have been done to examine the differences in nutritional distribution of the eggs between non-selected and selected females. Nor are there data showing any difference in oocyte maturation between them. Is it possible that starvation resistant flies have a lower number of more nutritious eggs? Do starvation resistant flies have more or less oocytes in their ovaries? Can fruit flies scavenge energy from their ovaries under stressful environmental situations? In *Eretmocerus eremicus*, a short-lived whitefly parasitoid that produces anhydropic oocytes, the existence of oosorption by yolk degradation has been verified (Asplen and Byrne, 2006). Messina and Fox (2001) think chorion nutrient recycling is important in females producing large egg numbers at the cost of lowered yolk investment per egg.

The ovary and the fat body of *D. melanogaster* both synthesize vitellogenins *in vivo*. The ovary contributes nearly as much vitellogenin to the yolk of an oocyte as does the fat body (Isaac and Bownes, 1982). There have not been any studies done that specifically quantified and compared the lipid content of oocytes of starvation sensitive and resistance flies. Most research has focused on differential allocation of internal resources to either combat starvation or reduce reproduction but the specific mechanisms underlying tradeoffs at the physiological level remain poorly understood (Rose and Brandley, 1998; Zera and Harshman, 2001; Harshman and Zera, 2007).

We confirmed basic life history traits with existing methods proposed in early papers by using our starvation resistant flies. Our results demonstrated that our selected flies had similar traits to those described in other references e.g. lower early fecundity, higher lipid contents and larger mass, compared to control populations. We also found that our starvation selected flies had longer lifespans which was not a universal discovery of all previous starvation selected lines. Some hypotheses have been provided in the attempt to explain how starvation resistant flies can live longer, but there are still some unknown physiological mechanisms relating to starvation resistance and longevity. In this study, we asked the following unexplored questions which can provide a brand new insight for

المنارة للاستشارات
www.manaraa.com

studying starvation resistance.

1. If selected females had more fecundity, did they have more oocytes in their ovaries?
2. What are the lipid dynamics in flies at different starvation time points?
3. Did resistant females have more or less lipids in their eggs?

المنارة للاستشارات

www.manaraa.com

Materials and Methods

Population Source and Procedure of Starvation Selection

A base population from 20 inbred lines of *D. melanogaster* was initiated and propagated in the lab for approximately 2 years before selection. These inbred lines were derived from inseminated females collected in the field (Wolfskill Orchards, maintained by the University of California at Davis, Yolo County, CA, USA). To establish the base population, each of the inbred lines was reciprocally crossed to a subset of other inbred lines in a balanced design based on the goal of equal representation of all lines. A standard number of progeny was harvested from each cross.

Thousands of these heterozygous progeny were released at the same time into a 91 X 60 X 31 cm population cage.. The next generation, the population of adult flies in the cage, consisted of over 10,000 individuals. Twenty bottles containing a standard Drosophila food (yeast, cornmeal, molasses and agar) were kept in the cage with the adult flies at any one time, each one added a week apart. Every week five fresh bottles of food were added to the cage replacing the five oldest spent food bottles. This overlapping generation regime was maintained for 26 months before the start of laboratory selection.

For laboratory selection we established three selected and three control populations. All experimental lines (three independent control lines and three independent selection lines) were derived from this mass culture simultaneously. About 500 individuals (250 males and 250 females) were initiated for each control line or selection lines and adult flies were randomly sampled from the wild population cage. The control lines are designated SC (starvation control) and the selection lines are designated ST (starvation selection). Flies were reared at 25 °C under constant illumination. All flies were only cultured on a standard cornmeal, yeast, sugar, agar *Drosophila* medium in this study. This study was conducted from generations 1 to 21 of laboratory selection for starvation resistance in both sexes.

The regime of selection was based on differential survival of adult males and females in the absence of food. About 500 flies (males or females) were placed in to two

المنارة للاستشارات
www.manaraa.com

empty bottles per selection line. The cotton plug for each bottle was saturated with water and thus flies were held in a high-humidity environment in constant proximity to moisture. The plugs were inspected every 12 hours to ensure that they remained wet. After approximately 50% mortality, the survivors, including females and males both, were transferred to laboratory medium to produce the next generation.

After one round of starvation selection, selected flies or control flies were transferred to fresh food in bottles, where they laid eggs overnight. We used the same number of flies for ST1 and SC1, ST2 and SC2 and ST3 and SC3. For example, if there were 1000 ST1 females that survived selection during the first round, exactly the same number of flies from SC1 would be transferred to bottles. Approximately 100 eggs were collected into each vial. Overall, 10 vials seeded with eggs were collected from each selected and control line. These vials were placed at 25 °C in a 12h:12h light and darkness incubator. Within 2-3 days after eclosion, approximately 15 females and 15 males were collected into vials with fresh food for 2 days, providing sufficient time for sexual maturation, and then transferred to another vial for another 2 days to prevent the increasing adhesiveness of the food from restricting or trapping the adult flies.

Flies were separated by sex under ether anesthesia and held for at least 24 hours on medium before flies were used in the following experiments. Selected and control line flies were reared for at least one generation without selection and appropriate egg densities were collected and placed on laboratory *Drosophila* medium prior to any experiments. Flies used in the assays were 6-7 days old and were raised and tested at room temperature (25 °C).

Starvation Resistance & Longevity Assays

We compared starvation resistance between control and selected lines every a couple of generations in order to monitor their response to starvation selection. For the starvation survival assay, flies (females and males) from all lines (control lines 1-3 and starvation selected lines 1-3) were tested for survival in the absence of food at room temperature. Ten females or 10 males were placed in an empty plastic vial capped with a

المنارة للاستشارات
www.manaraa.com

water-saturated cotton plug. The number of dead flies was determined every 12 hours until all flies had died. Five replicate vials for each sex and six total populations, as stated above, were used to test for starvation resistance.

To obtain lifespan measurements, control lines and selected lines were selection relaxed at least one generation at a density of 90-100 eggs per vial, with five vials per line, to obtain a sufficient number of individuals for testing. In the following generation, fifteen pairs of flies per line were placed in each of three vials for 3 days. For 6-7 days after eclosion, 15 males and 15 females were placed in population cages made from 500-mL plastic Dixie Cups with attached plastic vials containing 8 mL of cornmeal–agar–molasses medium plus yeast, standard food. For each line, there were three replicates and experiments were done at two different generations. Dead flies were removed by aspiration and mortality recorded at 3-day intervals. Food was changed every three days.

Dry Weight and Protein Assays

To conduct these assays, it was decided that we would measure dry flies instead of wet flies because this way any effects caused by humidity could be eliminated. We reasoned that dry weight would be most accurate at estimating the exact energy stored in the bodies of the flies. For each line and sex, 20 single flies were weighted. All flies were 6-7 days old and were not virgins. Body weight was determined after previously frozen flies were lyophilized for at least 24 hours. The masses of the flies were determined using a Satorius M2P Microbalance (1 µg to 1 gram). Samples were weighed within 2 hours after removal from the vacuum drier.

Soluble protein was measured using the BCA Protein Assay Kit (Pierce Company, Rockford Illinois). Five males or females from each line were homogenized in 250 µl of Tris-HCL pH 8.0. The homogenate was duplicated 1:3 in homogenizing buffer and 10 µl was added per well in a 96-well plate for the Microplate Assay Procedure. The following standard concentrations of protein were employed: 2 mg/ml, 1 mg/ml, 0.5 mg/ml, 0.25 mg/ml, and 0.125 mg/ml. The BCA reagents were added and after a 30 minute incubation at 37° C, the plate was read in a spectrophotometer at 562 nm.

المنارة للاستشارات
www.manaraa.com

Fecundity

Virgin females and males were collected within 8 hours after eclosion, and one female and male from each line were put into a vial, containing standard fly food, for mating. Flies were transferred to new vials every day and eggs were counted daily. For each control or selection line, there were ten replicates. If a male happened to die during the experiment, a male of the same age would be replace it.

Determination of oocyte stages and stage-14 oocyte collection.

Ovaries were dissected in Ringer's solution (130 mM NaCl, 5 mM KCl, 2 mM CaCl2, 50 mM Na_2HPO_4, pH 7.0). Ovaries were further dissected into single ovarioles and the oocyte stages counted according to the procedure of King (1970). Stage-14 oocytes are easy to detect due to the obvious presence of two breathing tubes, making them easily distinguishable from oocytes in other stages. From each female, we collected 20 stage-14 oocytes into 1.5 ml plastic tubes containing 50µl Ringer's solution. We then added 100µl 2:1 chloroform: methanol immediately after collection into the Ringer's solution. The lipid content of 20 stage-14 eggs was determined by the assay described below.

Total Lipid Assays

Total lipid was measured using a method described by van Handel (1985), adopted by Zhao and Zera (2002) and slightly modified for the present study. We placed all flies in a -20° C freezer in preparation for the lipid assay. Three female flies or 20 stage-14 eggs were homogenized in 1.5ml Eppendorf tubes containing 100 µl of a Chloroform/Methanol (2:1) solution using plastic pestles and then centrifuged for 3 minutes. Triolein (0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 2.0 and 3.0 mg/ml) solutions in 2:1 chloroform: methanol were used for standards. The sample supernatants and standards were transferred into glass tubes and put into an 85-90°C water bath for 5 minutes. This step was conducted to evaporate the solvent before addition of sulfuric acid, which was followed by an additional incubation in an 85-90ºC water bath for 10 minutes. The tubes were removed and allowed to cool to room temperature before addition of 1 ml of vanillin-phosphoric acid reagent, which was followed by a gentle vortex. Two hundred

المنارة للاستشارات
www.manaraa.com

and fifty µl of each sample or standard was transferred to a well in a 96-well plate and read in a spectrophotometer at 525 nm.

Triglyceride Assays

Three flies for each treatment or line were homogenized in 1 ml Chloroform/Methanol (2:1) solution. Next, 250µl of a 0.88% KCl solution was added, to extract lipids from the solvent, and then the mixture was centrifuged for 5 minutes at 7000 rpm. The supernatant was removed into columns suspended in glass tubes and 2 ml of a Hexane/Ethyl Ether (80% / 20%) solution was added to the columns to extract triglycerides in the flow-through liquid. Triolein (0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 2.0 and 3.0 mg/ml) solutions in 2:1 chloroform:methanol were used as standards. The following steps of this assay from here on are the same as those of total lipid assay. Briefly, vanillin-phosphoric acid reagent was added to all samples and standards and concentration data was read by spectrophotometer set to read at 525nm.

Statistics

All data were analyzed using SAS statistical software (SAS Institute Inc. 2003). Differences among the lines were tested using a regression model on the selection response with line and sex as independent factors. Data for dry weight, soluble protein abundance, fecundity, total lipids and triglycerides and oocyte distribution were analyzed using two-way ANOVA or PROC GLM unless indicated otherwise.

المنارة للاستشارات

www.manaraa.com

Results

Starvation Response and Longevity after selection

Selection for starvation resistance (ST) had an increased resistance response in both sexes (Fig. 1a). After 17 generations of selection, ST lines survived starvation approximately 106.8 ±8.4 hours and 124.4 ± 11.7 hours in males and females respectively which was significantly longer than the survival window of SC lines ($F_{3, 55}$= 53.46, $P<0.0001$). The average length of starvation survival in SC males and females were 73.4 ± 9.1 hours and 88.0 ± 15.6 hours respectively. By generation 20, flies subjected to starvation selection could survive up to 127.5 ± 10.3 hours and 158.4 ± 9.3 hours in ST males and females respectively.

The starvation resistance response in selected females is stronger than in males. Starvation resistance of ST males increased 70% while ST females' ability to resist starvation increased 80%. However, the longevity results weren't consistent (Figure 1b). SC female were shorter lived than SC males or ST males and females ($F_{3, 30}$= 3.56, P= 0.0257). This may indicate that reproduction squanders energy and reduces the longevity of these SC females. Also, both SC and ST males lived longer than females, which further suggests that reproduction is one of the main factors in the reduction of longevity. Although SC and ST females had shorter life spans than males, most likely due to reproduction costs, as expected ST females had greater longevity than SC females ($F_{1, 15}$= 6.61, P= 0.0213) This seems to signify that increased starvation resistance can also increase lifespan in females. There was no significant difference between ST and SC males with respect to lifespan ($F_{1, 15}$= 0.02, P= 0.9033).

Dry Weight and Protein Content

Weight is the simplest index with which to estimate energy storage, and an increase in weight may correlate with high starvation resistance. The average dry weight of three independent control male lines was 0.276±0.004 mg while female lines weighed 0.524±0.001 mg; selected male lines weighed 0.283±0.006 mg and selected female lines

المنارة للاستشارات
www.manaraa.com

weighed 0.571±0.004 mg (table 1a). There were statistical differences between lines and sexes (table 1b). Selection showed significant effects versus control female lines ($F_{1, 118}$= 44.36, P< 0.0001). Starvation selected males were not heavier than control males though ($F_{1, 117}$= 2.56, P= 0.1211), but they did survive starvation longer. In females, an increase in body weight was correlated with have high starvation resistance but this was not the case for males. Therefore, we speculated that weight is not the sole factor influencing starvation tolerance.

Proteins are the preliminary products of gene translation and a great deal of body tissues are composed largely of proteins. Therefore, protein content is presumably a fairly good representative measurement of body mass. Our results showed that soluble protein was slightly higher in starvation selected lines but there was no statistical significance to these data (table 1a). Although ST females were heavier than SC females, there was no difference at the protein level (table 1c).

Fecundity

Fecundity was measured by the sexual production of single pairs of flies upon 4 hours of enclosure and isolation. Figure 2, shows the daily pattern of accumulated fecundity or egg production (day 1 through day 14) for control and selected females. ST lines had lower fecundity than SC lines ($F_{1, 52}$= 6.02, P= 0.0176). Because ST females had longer life spans and higher famine tolerance, all available evidence showed that increased starvation resistance decreases early fecundity. It appears that there exists a life history trade-off between longevity/starvation resistance and reproduction cost.

Oocyte distribution (stage-10 to stage-14)

Many research examples have demonstrated that starvation resistant flies have lower fecundity, and due to this effect, another interesting subject to explore is the developmental distribution of oocytes. Our fecundity data (Fig. 3a) also agreed that starvation control female lines produced more eggs early (early fecundity) than selected female lines. In Figure 3b, we found that starvation selected females had more oocytes (stage-10 to stage-14) in their ovaries than control females before and after starvation ($F_{3,89}$=5.91 , P=0.001) as well as stage-14 only oocytes ($F_{3,89}$=48.54 , P<0.001). This

www.manaraa.com

result is contradictory to our fecundity results in which SC females laid more eggs.

SC and ST females both had increased numbers of stage-14 oocytes, matured from stages 10-13, after 36 hours of starvation. When flies were submitted to starvation, there was inadequate substrate or energy with which to lay eggs. They could only hold all the mature (stage-14) oocytes. The total number of egg chambers at stage-13 to stage-10 before starvation is closed to the increased number of stage 14 oocytes after starvation. Meanwhile, there were very few stage-10 to stage-13 egg chambers presented in the female ovaries after starvation.

Lipid Dynamic- Total lipid & Triglycerides

We wanted to know how starvation selected flies gain lipids after eclosion and how they consume lipids upon starvation. Total lipid content of female flies was measured at three different stages: within, 7-days after eclosion and after 36 hours of starvation. The dynamics of total lipid content is showed in Figure 4. Both ST and SC females had similar lipid increase and declining rates from eclosion to 7 days and after 36 hours of starvation ($F_{1,10}$=0.90, P=0.3655). After 1 hour past eclosion, selected females did not have significantly higher lipid content than control females (P=0.3571). This result may indicate that our selected flies didn't store much lipid during the larval stage but relied on food consumption at the adult stage to increase their lipid level. This result was contrary to Chippindale et al. (1996) found. They found that larval lipid acquisition played a major role in adult starvation resistance. If starvation resistant flies store more lipids at the larval stage, we should have seen more lipids immediately after eclosion.

Starvation selected females had more lipid content relative to control females at 7 days post eclosion and after 36 hours of starvation ($F_{3, 20}$= 6.02, P=0.0006). However, the declination of lipid content caused by food deprivation was similar (ST lipid levels lowered about 32% and SC lipid levels lowered about 38% after the starvation period). Overall, shortage of food may require that both ST and SC lines use similar amounts of lipid to survive.

Based on the utilization rate of total lipid content after 36 hours of starvation, we only know that lipid demand between SC and ST lines was similar, but lipids are diverse and we did not know if there was differential lipid utilization in these *Drosophila* lines

المنارة للاستشارات
www.manaraa.com

when exposed to famine. Therefore, we measured the triglyceride content of SC and ST females at different starvation time points to see how triglycerides were used in response to starvation. Starvation resistant females had stable triglyceride levels after having been starved for 0 hr, 8 hr, 16 hr, 24 hr, 32 hr and 36 hr($F_{5,27}$=0.46 , P=0.8055); conversely, control line females showed decreased triglyceride levels after 36 hours of starvation. Overall, selected females had higher and more stable levels of triglycerides during 36 hours of starvation compared to control female lines ($F_{11,54}$=4.28 , P=0.0001).

Lipid content of mature oocytes (stage-14)

While trade-off seems to exists between early fecundity and starvation resistance, we further tested how starvation selected females distributed their energy between the soma or reproductive tissues. If starvation resistance burns more resources from a limited energy pool, then energy available for reproductive expense should be less. Twenty stage-14 oocytes were collected from SC and ST females before or after 36 hours of starvation. Total lipid was measured and the result is shown in Figure 5. Starvation resistant females had higher lipid level in their eggs relative to control females before starvation ($F_{1,57}$=9.2, P=0.0036) but the lipid content of their eggs didn't show any difference after starvation ($F_{1,21}$=0.06, P=0.8022). This result suggested that starvation resistant females allocated more energy to their eggs but lost some proportion of these lipids to the soma during starvation.

www.manaraa.com

Discussion

Starvation resistance and life history traits

Selection for starvation resistance over 20 generations showed a significant response in the present study. Chippindale et al. (1996) found that increased starvation resistance can also have an indirect response on larval development time, and can cause higher adult lipid content and larger body size. In our selection lines, we also found that resistant female flies had a higher lipid content (7 days post eclosion), higher early fecundity, and higher dry weight, all of which are consistent with the findings of the aforementioned study. However, the selected male flies did not show significant differences in body size, protein content or even triglyceride level, but they did have higher starvation resistance. It will be an interesting topic for further research to explore why these males can survive under famine without extra energy stores. In our selection regime, we selected both males and females. Resistant females had more lipids and lowered their reproduction cost to extend their lifespan and survive during starvation, but none of these observations were made in males. What kind of physiological mechanism did resistant males use to survive starvation? From figure 1b, there was no difference between ST and SC males in their longevity. It could be that ST males might reduce their mating frequency or courtship costs to conserve energy and resist starvation. They may have behavioral adaptations or other modified physiological mechanisms that enable starvation resistance. To verify the behavioral adaptation hypothesis, mating frequencies could be monitored sometime in the future.

Increased body weight has been associated with starvation resistance in some studies (Chippindale et al, 1996; Hoffman and Harshman, 1999), and body weight may reflect the total reserves of energy storage compounds such as lipids, carried by organisms (Djawdan et al., 1998). Chippindale et al (1996) showed that the progeny of starvation resistant flies had higher lipid levels 3 hours after eclosion than controls but we did not observe the same thing when measurements were taken within one hour after eclosion. The different outcomes of our experiments might have arisen from discrepancies in lipid content measurements or selection regimes.

Approximately 25% of the transcriptome was affected in response to starvation

المنارة للاستشارات
www.manaraa.com

stress, including genes involved in growth, maintenance and protein biosynthesis (Harbison et al. 2005). They reasoned that increased protein biosynthesis and hydrolase activity could enhance nourishment as substantial protein and organelle degradation could be then conducted to feed starving cells. In our results, however, ST females had a slightly elevated protein level but it was not statically significant when compared to SC females. For males, ST individuals had similar amounts of soluble protein as compared to SC males. Again, males under our selection regime must have had a different mechanism with which to resist starvation.

Oocyte distribution between SC and ST females

During oogenesis in Drosophila, the egg chambers are matured along the ovariole and increase in size due to yolk proteins (YP) consumption by oocytes. YP synthesis (Vitellogenesis) and consumption by growing oocytes proceeds during stages 8–10 of oocyte development. During stages 1–7, the egg chambers contain only previtellogenic oocytes. In the last (14th) stage, egg chambers contain mature eggs (Bownes, 1986; Spradling, 1993). According to our fecundity results, starvation resistant females laid fewer eggs compared to the control females. This makes sense as it would be reasonable for resistant females to maintain and produce fewer oocytes within their ovaries. Unfortunately, this finding did not correlate with results derived from a closer look at oocyte development and quantity. We dissected females that had mated 7 days earlier from both populations and recorded their oocytes numbers, especially those that had reached stages 10-14. This inspection showed that ST females had higher numbers of oocytes for each developmental stage. This result is contradictory to our fecundity data, as mentioned above. The appropriate explanation for this phenomenon could be that ST females slow down their egg laying rate so they just accumulated more stage-10 to stage-14 occytes in ovaries.

After 36 hours of starvation treatment, both ST and SC females had stage-14 oocyte accumulation and most of oocytes at stage10 to stage 13 had disappeared. When we compared the sum of oocytes from stage-10 to stage-14 before starvation and after starvation, we found that the numbers didn’t show any difference between SC and ST females. This further shows that Drosophila females mature more eggs, especially from

المنارة للاستشارات
www.manaraa.com

stage 10, 11, 12 and 13 to stage 14, while they were exposed to a stressful environment: starvation. This is a beneficial physiological change in evolution because females can lay eggs quickly right after food becomes available again. Also important to note is that it didn't take much time or nutritional investment for oocytes to progress from stage-10 to stage-14.

Total lipid declination under starvation

SC and ST females didn't show much divergence in their lipid content soon after eclosion. Not until 7 days after eclosion on regular fly food did ST females have higher lipid content than SC females. This result revealed that resistant females gained more weight after eclosion. One possible explanation is that ST females had more efficient feeding strategies during their adult stage but this notion needs to be experimentally confirmed. Higher feeding rates would also be a correlated response to starvation resistance, but this hypothesis has not been tested (Rion and Kawecki, 2007). In relation to energy distribution between reproduction and survival, it is also possible that SC females allocate more lipids to reproduction. Overall, the resistant females in this study did have higher levels of lipids, a finding which is corroborated by other studies. Athough, these previous findings do not contain any lipid level data at eclosion. We think that resistant females don't have more lipids stored during larval stage (there was no lipid difference when SC and ST flies were eclosed) or they may use other forms of energy for storage, such as carbohydrates. ST females also had an altered biosynthesis or metabolism that increased body weight as well as lipid content after eclosion.

When we starved 7 day old ST or SC females for another 36 hours, we found that the lipid content of both lines declined. The rate of this decrease seemed similar between the ST and SC females, but ST females still had more lipids in their bodies. These results can only tell us that starvation resistance is highly associated with lipid content, which is not surprising. Meanwhile, the utilization rate of lipids under starvation in both populations also reflected that ST females didn't have a slower metabolism rate (Harshman and Schmid, 1998) because SC and SC females had a similar rate of decrease in lipid content.

المنارة للاستشارات
www.manaraa.com

Triglycerides don't decline during starvation

Although there was no difference in the total lipid decline, we used the same idea to test if there was a change in triglycerides at different starvation time points. We showed that SC females reduced their lipid contents but that ST females could maintain stable levels of triglycerides in their bodies after 36 hours of starvation. This outcome was contradictory to the total lipid result. How did resistant flies decrease their lipid but not their triglycerides levels? Theoretically, since triglycerides are important storage lipids in *Drosophila*, resistant flies would have to burn triglycerides as an energy source under starvation. Did resistant flies gain triglycerides or convert other lipids or resources into triglycerides from some place else in the body? Or is it possible that ST females had stable triglycerides not because they didn't use any of them but because they were able to replenish them?

In insects, the lipid trade-off between somatic tissues and reproductive tissues is also the trade-off between triglycerides and phospholipids. For example, long-wing crickets have more triglycerides in their bodies and lower phospholipids in their ovaries while short-wing crickets have less triglyceride in the body and more phospholipids in their ovaries (Zhao and Zera, 2002). We speculated that ST females might retrieve lipids from ovaries to maintain their level of triglycerides. To verify our hypothesis, the distribution of triglycerides and phospholipids in the soma and ovaries was measured next.

Replenishment of Triglycerides using ovary lipids

Resistant female flies can maintain stable triglyceride concentrations after 36 hours of starvation which means they may be replenishing as much as they use. Furthermore, the source of this replenishment must be from somewhere in their bodies. When we measured the lipid level of stage-14 oocytes in SC and ST females, we found that ST individuals had more lipids stored in their stage-14 oocytes. However, the lipid level of mature eggs in ST flies decreased after 36 hours of starvation. After starvation, SC and ST had no difference in their lipid content in their stage-14 oocytes. SC females displayed a quite consistent oocyte lipid level before or after starvation and they had a decrease of triglycerides or total lipids after starvation. On the other hand, ST females had a decrease

المنارة للاستشارات
www.manaraa.com

of lipids in stage-14 oocytes but not triglycerides after starvation, but they did decrease their overall lipid level. This evidence leads us to reason that ST females might preferentially scavenge energy from their ovaries for somatic use when exposed to starvation. Under normal environmental conditions, ecdysteroids and juvenile hormone (JH) induce yolk protein (YP) synthesis in the fat body and follicular cells immediately after the eclosion, maintain this synthesis at a specific level after fertilization, and regulate YP consumption by oocytes (Postlethwait and Shirk, 1981; Richard et al., 1998; Soller et al., 1999; Rauschenbach et al, 2004; Rauschenbach et al, 2007). Therefore, the hormone changes induced by starvation may contribute to the loss of lipid from stage-14 eggs in ST females.

In the future, our core interest is to figure out the physiological mechanism governing how starvation resistant females retrieve lipids from oocytes for somatic usage and how this is upregulated in flies selected for starvation resistance. If we could find the physiological mechanism of energy relocation after starvation, it may help to explain the evolution of increasing fitness for starvation resistance in females.

In this study, the evolution of starvation resistance by using laboratory selection has helped us explore physiological responses in flies. We have shown that in starvation resistance there may be more than one solution for *D. melanogaster* to choose from. These flies can either increase lipid content, reduce early fecundity, or maintain stable triglycerides, all of which are strategies that appeared helpful during life in this harsh environment. A novel insight provided by this study is that starvation resistant females may break down some of their ovary lipids for somatic use under starvation. The differential lipid allocation between the soma and the reproductive tissues can explain how starvation selected lines resist famine. Moreover, using energy more efficiently can also elevate competitive ability and fitness when food is scarce.

المنارة للاستشارات
www.manaraa.com

Table 1

(a) The average weight (±SE) and soluble protein (±SE) of starvation control and starvation selection lines in both sexes

(b)Analysis of variance for dry weight to SC and ST in both sexes.

(c) Analysis of variance for soluble protein to SC and ST in both sexes

SC: starvation control lines; ST: starvation selected lines

المنارة للاستشارات

www.manaraa.com

(a)

Line	Weight (mg)	Soluble Protein (mg/ml)
SC male	0.2765 ± 0.0044 (a)	0.74 ± 0.05 (a)
SC female	0.5236 ± 0.0009 (b)	1.54 ± 0.09 (b)
ST male	0.2816 ± 0.0061 (a)	0.69 ± 0.05 (a)
ST female	0.5706 ± 0.0044 (c)	1.83 ± 0.23 (b)

*Different letters shows $P < 0.05$

(b)

Source	DF	F Value	Pr > F
line	1	46.30	<.0001
Sex	1	4188.18	<.0001
line*Sex	1	19.95	<.0001

(c)

Source	DF	F Value	Pr > F
line	1	1.33	0.2821
Sex	1	86.19	<.0001
line*Sex	1	2.58	0.1467

www.manaraa.com

Figure 1

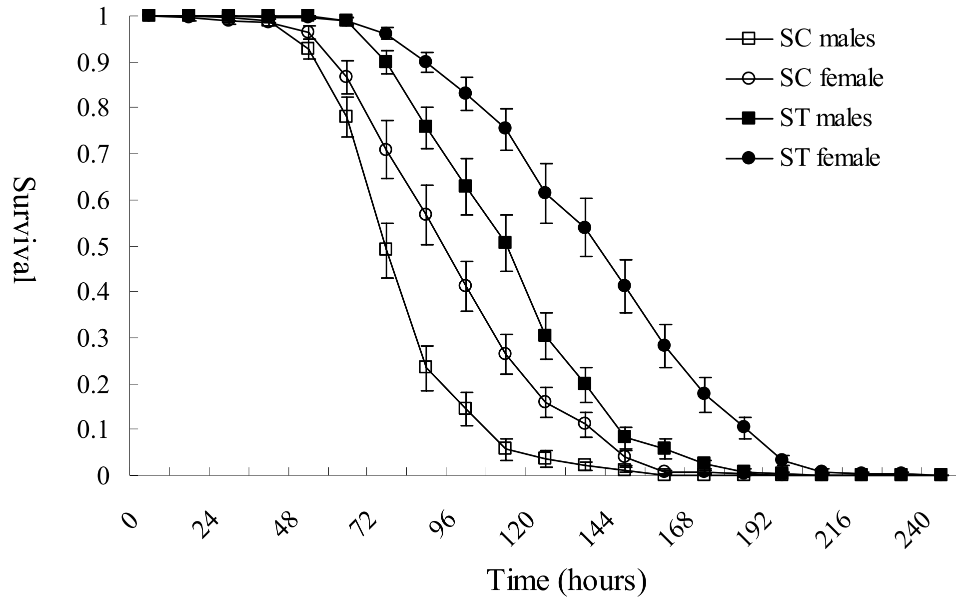
(a) Survival curves for control lines and starvation selected lines subjected to starvation stress. Each point on the survival curve is the mean percent survival rate ±SE of replicate vials. All flies were allowed to mate for 3 days and seven-day old flies in both sexes were used in starvation stress assay. (n=54)

(b) Survival curves for control lines and starvation selected lines. Each point on the survival curves is the mean percent survival rate ±SE of replicate vials. All flies were seven-day old when we did the longevity cages assay. (n=36)

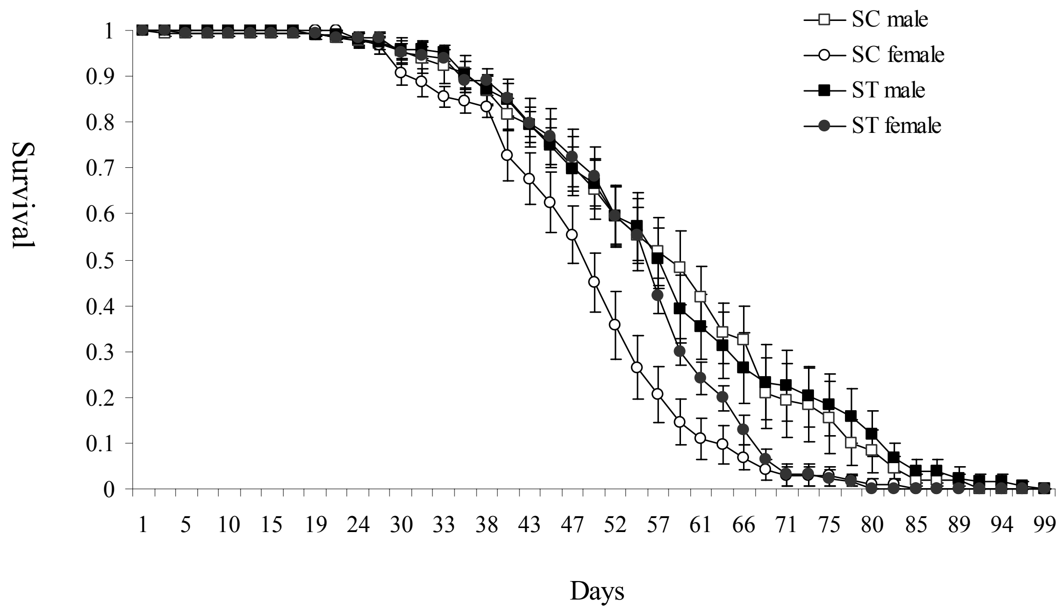
المنارة للاستشارات

www.manaraa.com

(a)



(b)



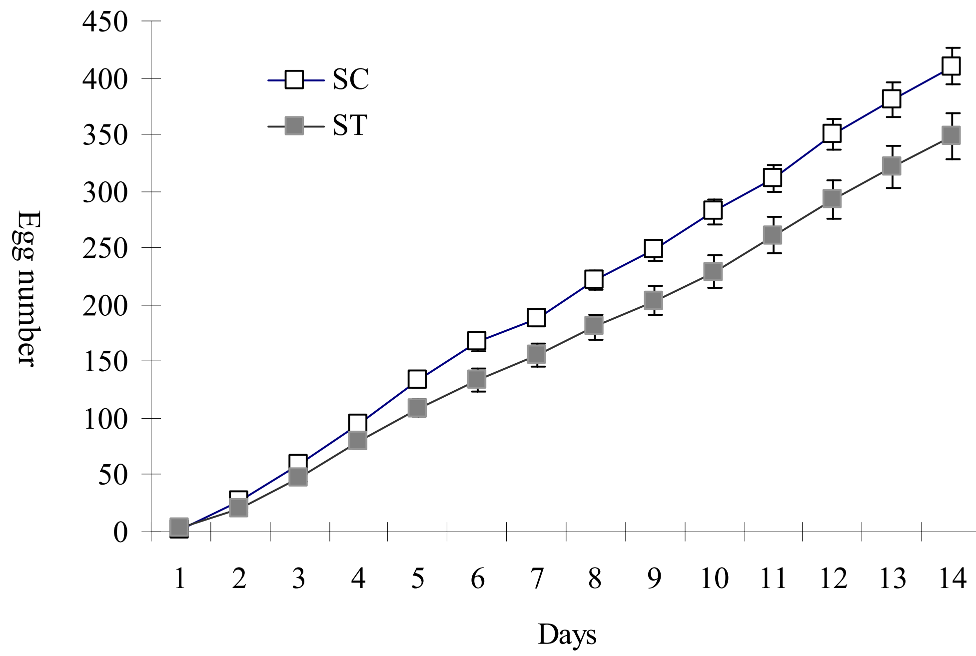
www.manaraa.com

Figure 2

The accumulated fecundity of control females and starvation selected females. Each point represents the mean (±SE) accumulated egg numbers of single female of replicate vials. White square is control females and gray square is selected females. (n=54)

المنارة للاستشارات

www.manaraa.com



www.manaraa.com

Figure 3

(a) Stage-10 to stage-14 oocyte distribution for control and selected lines at different starvation time points (0 hour or 36 hours). Each bar represents the mean ±SE of specific oocyte stage from single female. (n=48)

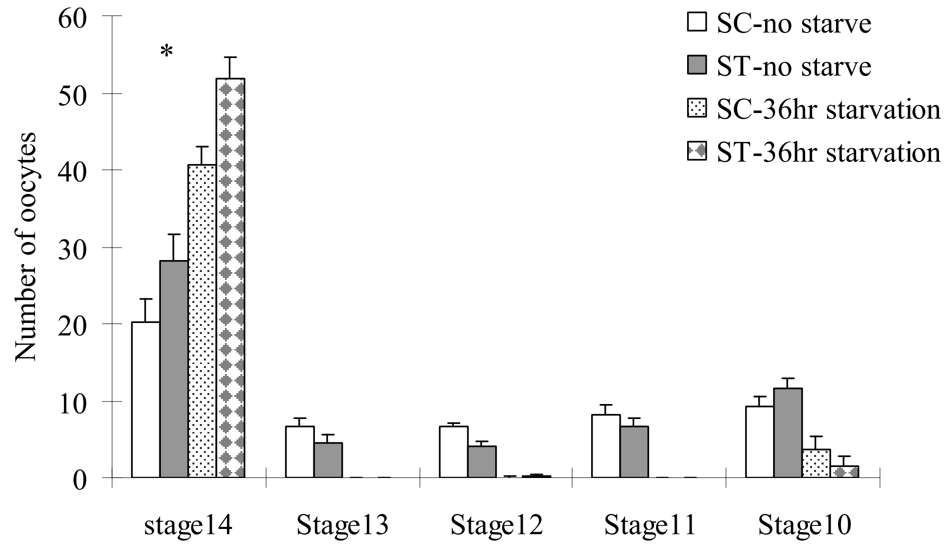
(b) The total number of stage-14 oocytes in control and selected females at different starvation time points (0 hour or 36 hours). Each bar represents the mean ±SE of total stage-14 oocytes from single female. (n=48)

An asterisk (*) indicates a significant difference

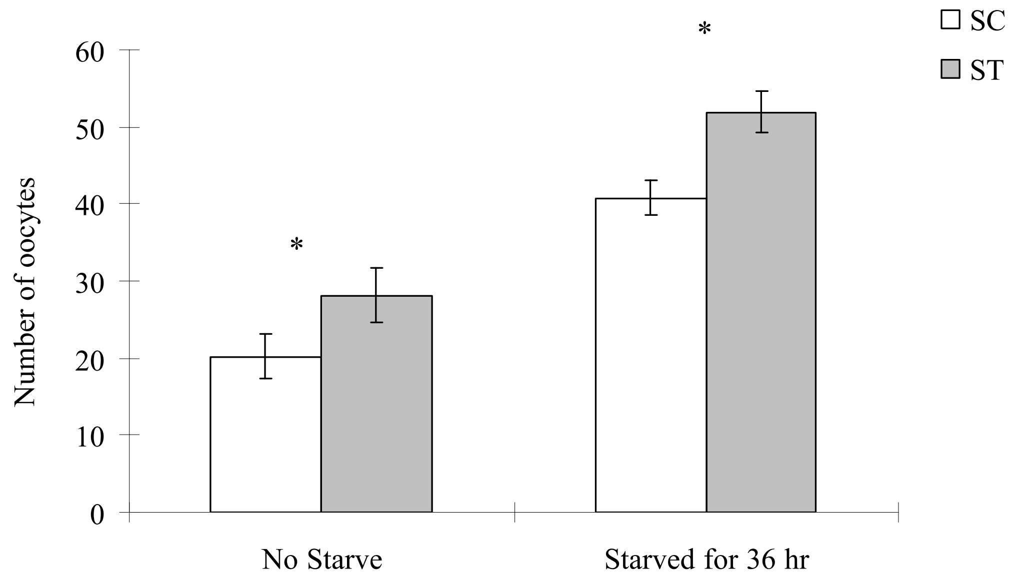
المنارة للاستشارات

www.manaraa.com

(a)



(b)



المنارة للاستشارات
www.manaraa.com

Figure 4

(a) Total lipid contents at three different stages. Flies that had been emerged for one hour. seven day old flies, seven day old flies with another 36 hours of starvation. Each point is the mean ±SE of total lipids of single females. (The total lipid protocol measured 3 flies and this figure is modified to show the lipid content of single flies) (n=36)

(b) Triglyceride levels at different starvation time points. Females from control and selected lines were starved for 0 hour, 8 hours, 16 hours, 24 hours or 32 hours. Each point is the mean ±SE of triglycerides of single female. (The triglyceride protocol measured 5 flies and this figure is modified to show the triglyceride content of single flies) (n=54)

(c) Triglyceride abundance of males from SC or ST population. (n=12)

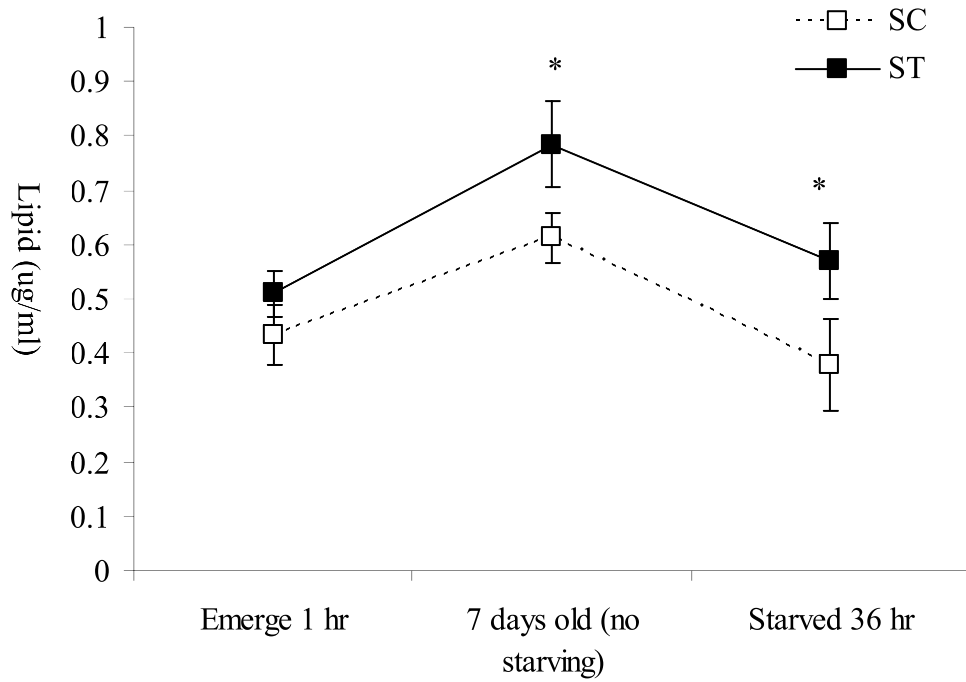
SC: starvation control lines; ST: starvation selected lines

An asterisk (*) indicates significant difference

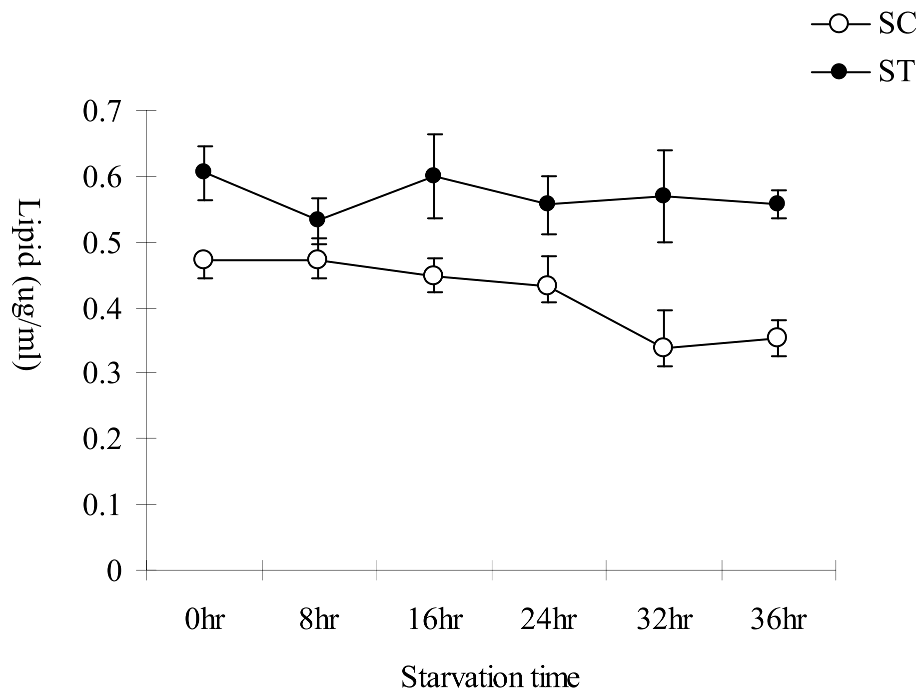
المنارة للاستشارات

www.manaraa.com

(a)

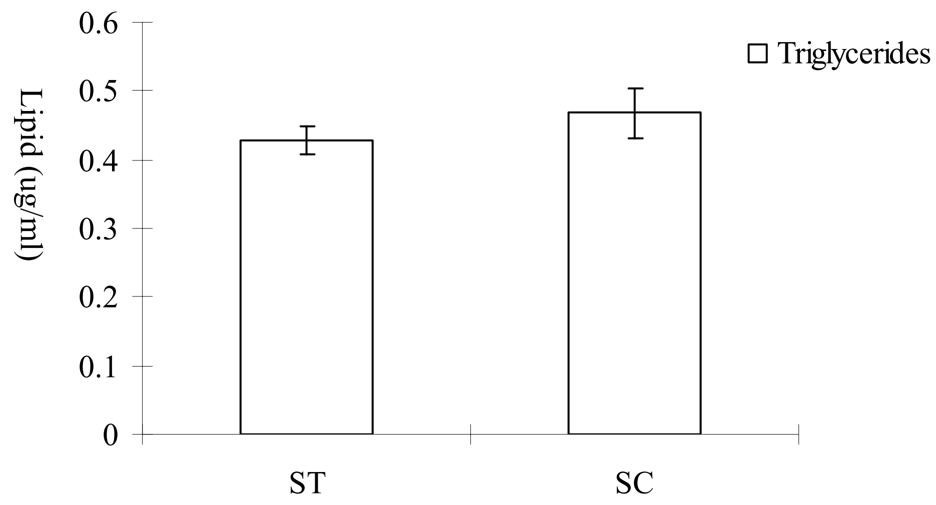


(b)



(c)

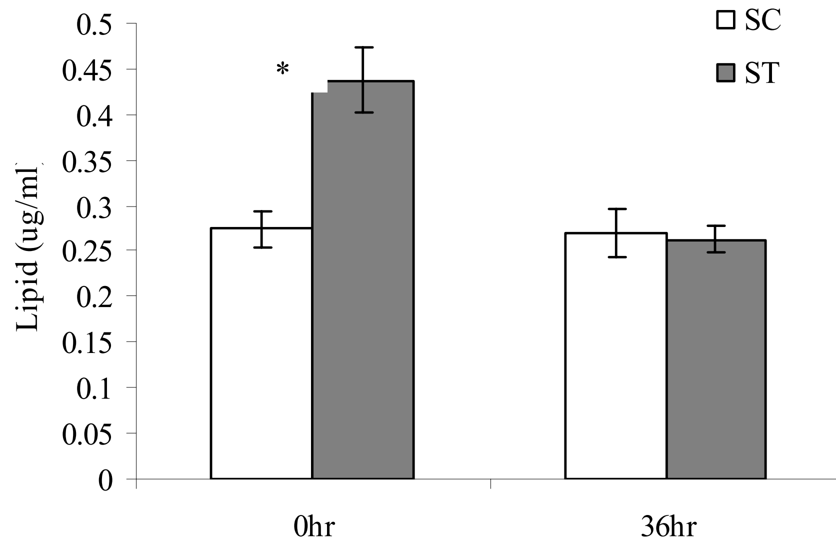
المنارة للاستشارات
www.manaraa.com



المنارة للاستشارات
www.manaraa.com

Figure 5
Total lipid contents of stage-14 oocytes. The mean ±SE of lipids of twenty stage-14 oocytes from control or selected females were measured at different starvation points: 0 hour or 36 hours. (SC: starvation control, ST: starvation selected) (n=48)
An asterisk (*) indicates significant difference

المنارة للاستشارات
www.manaraa.com



المنارة للاستشارات
www.manaraa.com

Reference

Bellamy, D. E., and D. N. Byrne. 2006. Quantification and ultrastructure of oosorption in *Eretmocerus eremicus* (Hymenoptera: Aphelinidae). Journal of Morphology 267:1066-1074.

Bownes, M. 1986. Expression of the genes coding for vitellogenin (yolk protein). Annual Review of Entomology 31:501-531.

Chippindale, A. K., T. J. F. Chu, and M. R. Rose. 1996. Complex trade-offs and the evolution of starvation resistance in *Drosophila melanogaster*. Evolution 50:753-766.

Djawdan, M., A. K. Chippindale, M. R. Rose, and T. J. Bradley. 1998. Metabolic reserves and evolved stress resistance in *Drosophila melanogaster*. Physiol. Zool. 7:584-594.

Gibbs, A. G. 1999. Laboratory selection for the comparative physiologist. Journal of Experimental Biology 202:2709-2718.

Harbison, S. T., S. Chang, K. P. Kamdar, and T. F. C. Mackay. 2005. Quantitative genomics of starvation stress resistance in *Drosophila*. Genome Biology 6:R36.

Harshman, L. G. 2003. Life span extension of *Drosophila melanogaster*: genetic and population studies. . Pp. 99-126 *in* J. R. Carey and S. Tuljapurkar, eds. Life span: Evolutionary, Ecological, and Demographic Perspectives, Population and Development Review, A Supplement to Volume 29 Population Council, New York.

Harshman, L. G., A. A. Hoffmann, and A. G. Clark. 1999. Selection for starvation resistance in *Drosophila melanogaster*. J. Evol. Biol. 12:370-379.

Harshman, L. G., and J. L. Schmid. 1998. Evolution of starvation resistance in *Drosophila melanogaster*: aspects of metabolism and counter-impact selection. Evolution 52:1679-1685.

Harshman, L. G., and A. J. Zera. 2007. The cost of reproduction: the devil in the details Trends in Ecology and Evolution 22:80-86.

Hoffmann, A. A., and L. G. Harshman. 1999. Desiccation and starvation resistance in *Drosophila*: patterns of variation at the species, population and intrapopulation

المنارة للاستشارات
www.manaraa.com

levels. Heredity 83:637-643.

Hoffmann, A. A., and P. A. Parsons. 1991. Evolutionary Genetics and Environmental Stress Oxford Science Publications, Oxford.

Isaac, P. G., and M. Bownes. 1982. Ovarian and fat body vitellogenin synthesis in *Drosophila melanogaster*. Eur. J. Biochem. 123:527-534.

King, R. C. 1970. Ovarian Development in Drosophila melanogaster. Academic Press, New York.

Lee, G., and J. H. Park. 2004. Hemolymph sugar homeostasis and starvation-induced hyperactivity affected by genetic manipulations of the adipokinetic hormone-encoding gene in *Drosophila melanogaster*. Genetics 167:311-323.

Leroi, A. M., W. R. Chen, and M. R. Rose. 1994. Long-term laboratory evolution of a genetic life-history trade-off in *Drosophila melanogaster* 2. Stability of genetic correlations. Evolution 48:1258-1268.

Leroi, A. M., A. K. Chippindale, and M. R. Rose. 1994. Long-term laboratory evolution of a genetic life-history trade-off in *Drosophila melanogaster* 1. The role of genotype-by-environment interaction. Evolution 48:1244-1257.

Messina, F. J., and C. W. Fox. 2001. Offspring size and number. Pp. pp. 113-127 *in* C. W. Fox, D. A. Roff and D. J. Fairbairn, eds. Evolutionary Ecology: Concepts and Case Studies. Oxford Univ. Press, New York.

Postlethwait, J. H., and P. D. Shirk. 1981. Genetic and endocrine regulation of vitellogenesis in Drosophila. Am Zool 21:687-700.

Prasad, N. G., and A. Joshi. 2003. What have two decades of laboratory life-history evolution studies on Drosophila melanogaster taught us? J. Genet 82:45-76.

Rauschenbach, I. Y., N. A. Chentsova, A. A. Alekseev, N. E. Gruntenko, N. V. Adonyeva, E. K. Karpova, T. N. Komarova, V. G. Vasiliev, and M. Bownes. 2007. Dopamine and octopamine regulate 20-hydroxyecdysone level in vivo in *Drosophila*. Insect Biochemistry and Physiology 65:95-102.

Rauschenbach, I. Y., N. E. Gruntenko, M. Bownes, N. V. Adonieva, J. Terashima, E. K. Karpova, N. V. Faddeeva, and N. A. Chentsova. 2004. The role of juvenile hormone in the control of reproductive function in *Drosophila virilis* under nutritional stress. J Insect Physiol 50:323-330.

المنارة للاستشارات
www.manaraa.com

Richard, D. S., N. L. Watkins, R. B. Serafin, and L. I. Gilbert. 1998. Ecdysteroids regulate yolk protein uptake by *Drosophila melanogaster* oocytes. J Insect Physiol 44:637-644.

Rion, S., and T. J. Kawecki. 2007. Evolutionary biology of starvation resistance: what we have learned from *Drosophila*. Journal of Evolutionary Biology 20:1655-1664.

Roff, D. A. 1992. The evolution of life histories. Chapman & Hall, New York.

Rose, M. R., and T. J. Bradley. 1998. Evolutionary physiology of the cost of reproduction. Oikos 83:443-451.

SAS. Institute Inc. 2003. Cary, NC: SAS Institute Inc.

Service, P. M. 1987. Physiological mechanisms of increased stress resistance in *Drosophila melanogaster* selected for postponed senescence. Physiological zoology 60:321-326.

Soller, M., M. Bownes, and E. Kubli. 1999. Control of oocyte maturation in sexually mature *Drosophila* females. Dev Biol 208:337-351.

Spradling, A. C. 1993. Developmental genetics of oogenesis. Pp. 1-70 *in* B. M. and M. A. A., eds. The Development of *Drosophila melanogaster*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Telonis-Scott, M., K. M. Guthridge, and A. A. Hoffmann. 2006. A new set of laboratory-selected *Drosophila melanogaster* lines for the analysis of desiccation resistance: response to selection, physiology and correlated responses. Journal of Experimental Biology 209:1837-1847.

Van Handel, E. 1985. Rapid determination of total lipids in mosquitoes. J. AM. Mosq. Control Assoc. 1:302-304.

Wang, M.-H., O. Lazebny, L. G. Harshman, and S. V. Nuzhdin. 2004. Environment-dependent survival of *Drosophila melanogaster*: a quantitative genetic analysis. Aging Cell 3:133-140.

Williams, G. C. 1966. Natural selection, the costs of reproduction, and a refinement of Lack's principle Am. Nat. 100:687-690.

Zera, A. J., and L. G. Harshman. 2001. Physiology of life-history trade-offs. Ann. Rev. Ecol. Syst. 32:95-126.

المنارة للاستشارات
www.manaraa.com

Zhao, Z. W., and A. J. Zera. 2002. ifferential lipid biosynthesis underlies a tradeoff between reproduction and flight capability in a wing-polymorphic cricket. PNAS 99:16829-16834.

Zou, S., S. Meadows, L. Sharp, J. L.Y., and J. N. Jan. 2000. Genome-wide study of aging and oxidative stress response in *Drosophila melanogaster*. Proc Natl Acad Sci USA:13726-13731.

www.manaraa.com

Chapter V:
Investigation of Differential Lipid Abundance in Starvation Resistance and Reproduction

المنارة للاستشارات

www.manaraa.com

Introduction

The increase of stress resistance is correlated with lower fecundity from the perspective of the evolution of life history. Increased stress resistance is typically associated with elevated accumulation of energy reserves, for example lipids or glycogen (Zera and Harshman, 2001; Zera and Zhao, 2003). Especially in insects, lipid reserves play an essential role in starvation survival and longevity. Varying abundance of lipid types is also associated with different life-history traits. Among all the different classes of lipids, triglycerides and phospholipids are the most abundance in insects. Triglycerides are preferentially allocated to somatic tissue for longer lifespan or starvation resistance and phospholipids, which are mostly allocated to ovaries, are important in egg development (Zhao and Zera, 2002).

In *Drosophila melanogaster*, higher starvation resistance is correlated with early fecundity. Higher starvation resistance is also found with high levels of lipids or triglycerides. Fecundity can be measured by egg or offspring numbers, but there have been few physiological studies conducted concerning fecundity. Also, there have been no studies done investigating phospholipid abundance for starvation resistance in fruit flies. Flight-capable Morph Crickets contain a substantially greater amount of total lipid and triglyceride, the main flight fuel of this species (Zera et al, 1999), than the flightless morph, whereas the reproductive morph contains a greater amount of phospholipids, a lipid class that is an important component of eggs (Grapes et al, 1989; Zhao and Zera, 2002). If there is existing trade-off between starvation resistance and early fecundity, it is possible to detect the trade-off between triglycerides and phospholipids in starvation resistant and non-starvation resistant flies.

Another valuable way to study trade-off between starvation resistance and reproduction cost is to use some females without any reproduction cost. Partridge (1989) identified *Drosophila melanogaster* costs of reproduction associated with egg production and with mating. Mutations in the orb gene (Lantz et al. 1992) are characterized by female sterility resulting from a failure of oocyte maturation at a previtellogenic oocyte stage. The sterile Orb female is a control because they can be exposed to agents that stimulate egg production, but they do not produce eggs (Salmon et al, 2001).

المنارة للاستشارات
www.manaraa.com

Most studies looking at trade-off between stress resistance and reproduction are static. For example, most of the literature concerning this topic has mostly been focused soley on phenotypic correlation of enhanced starvation resistance with other life-history traits but did not investigate extensively the reasons for these correlations. However, the physiological status of the fruit fly is dynamic. In other words, natural selection should favor genotypes capable of shifting their physiology towards greater starvation resistance. More starvation resistant flies are expected to have some degree of adaptive phenotypic plasticity. Harshman et al. (1999) found that starvation resistant flies were selected by prior exposure to lemon and this plastic response was absent in unselected controls. The starvation selection lines not only became more resistant to starvation, but also evolved a novel induced response.

Therefore, the more flexible physiological change appears to be advantageous when starvation resistant flies become more resistant. There has been no study conducted showing how starvation resistant flies' physiological traits are different from controls after a period of starvation. By comparing two different starvation conditions (nonstarved and starved), we can clearly observe if resistant flies show any plasticity at physiological levels. In this study, we are the first to compare the differential lipid distribution in somatic tissues and reproductive tissues in unstarved or starved populations.

The 'Y' model of resource allocation in life-history physiology reveals that the differential allocation of limited internal resources plays a central role in the cost of reproduction and other life-history tradeoffs (Townsend and Calow, 1981; Rose and Bradley, 1998; Zera and Harshman, 2001; Harshman and Zera, 2007). Food input is shown at the base of the 'Y' and energy resources are allocated to reproduction versus the rest of the somatic tissues. We use differential lipids (triglycerides and phospholipids) to examine the Y model or trade-off in starvation selected lines and control lines. The goal of this study is to identify the physiological and genetic causes of life-history variation and trade-off in starvation resistance and cost of reproduction in *D. melanogaster*. In order to demonstrate the trade-off of starvation resistance and reproduction by using differential lipid allocation, we are asking the following listed questions. Meanwhile, we want to study lipid dynamics to examine plasticity under two circumstances: lack of starvation or 36 hours of starvation.

المنارة للاستشارات
www.manaraa.com

(1) Does any trade-off occur between triglycerides and phospholipids in starvation selected females and is there a difference in control females?

(2) Do starvation selected females and control females have different ways of adjusting triglycerides and phospholipids levels before and after starvation?

(3) If trade-off of differential lipids in starvation resistant flies and control flies in their soma functions and reproduction, will *Orb* mutants (no reproduction cost) support this proposition?

(4) If control and selected lines have different lipid abundance and distribution, how does the activity of the lipid synthesis enzymes correspond to the changes?

المنارة للاستشارات
www.manaraa.com

Materials and Methods

Starvation selection stock

For laboratory selection, we established three selected and three control populations. All experimental lines (three independent control lines and three independent selection lines) were derived from a mass culture simultaneously. About 500 individuals (250 males and 250 females) were used as initiating stocks for each generation for each control line or selection line and the initial populations of adult flies were randomly sampled from a wild population cage. The control lines are designated SC (starvation control) and the selection lines are designated ST (starvation selection). Flies were reared at 25 °C under constant illumination. All flies were only cultured on a standard cornmeal, yeast, sugar, agar *Drosophila* medium in this study. This study was conducted from generations 1 to 23 of laboratory selection for starvation resistance in both sexes.

The regime of selection was based on differential survival of adult males and females in the absence of food. About 500 flies (males or females) were placed in two empty bottles per selection line. The cotton plug for each bottle was saturated with water and thus flies were held in a high-humidity environment in constant proximity to moisture. The plugs were inspected every 12 hours to ensure that they remained wet. After approximately 50% mortality, the survivors were transferred to laboratory medium to produce the next generation. The survivors included females and males.

After one round of starvation selection, selected flies or control flies were transferred to fresh food in bottles, where they laid eggs overnight. We used the same number of flies for generations ST1 and SC1, ST2 and SC2 and ST3 and SC3. For example, if there were 200 ST1 females that survived from selection, exactly the same number of flies from SC1 would be transferred to bottles for egg production and collection. Approximately 100 eggs were collected from each vial. Overall, 10 vials seeded with eggs were obtained from each selected and control line. These vials were placed at 25 °C in a 12h: 12h light and darkness incubator. Within 2-3 days after eclosion, flies were approximately 15 females and 15 males were collected and put into vials with fresh food for 2 days, providing sufficient time for sexual maturation, and then transferred to another vial containing fresh fly media for another 2 days.

المنارة للاستشارات
www.manaraa.com

Different sexes of flies were then separated under ether anesthesia and held for at least 24 hours on medium before being held in conducting the following experiments.

Body & Ovary Dissection

Ovaries were dissected in Ringer's solution (130 mM NaCl, 5 mM KCl, 2 mM CaCl2, 50 mM Na_2HPO_4, pH 7.0). Each female was dissected into two parts: body-no-ovary and ovary. For lipid assays, five body-no-ovaries or 10 pairs of ovaries were collected in 1 ml Chloroform/Methanol (2:1) Solution. Five body-no-ovaries or 10 pairs of ovaries from each line were collected in 250 µl of Tris-HCL pH 8.0 for soluble protein assay. All the samples were kept in a -20 °C freezer before triglyceride and phospholipids assays or protein assays.

Starvation treatment and survival assay

For starvation treatment, ten 7-days-old female flies were put into plastic vials without food but the cotton plug for each vial was saturated with water and thus flies were held in a high-humidity environment. After 36 hours of starvation, flies were transfered into 1.5 ml tubes and stored in a -20 °C or -80 °C freezer (enzyme assay).

For the starvation survival assay, ten flies of the same sex were placed in an empty plastic vial capped with a water-saturated cotton plug. The number of dead flies was determined every 12 hours until the time at which no flies remained alive.

Triglyceride and Phospholipids assay

Homogenized samples for each treatment or line were dissolved in 1 ml Chloroform/Methanol (2:1) solution. Next, 250µl 0.88% KCl solution was added to extract lipid from the solvent and the solution was centrifuged for 5 minutes at 7000 rpm. The supernatant was removed and put into selective filtering columns contained in glass tubes followed by 2 ml of aHexane/Ethyl Ether (80% / 20%) solution. The purpose of this solution is to wash triglycerides through the column leaving behind other unwanted lipids such as phospholipids. Next the column was moved to a new glass tube and 2 ml of Chloroform/Methanol (65%/30%) was added to each column to extract phospholipids. Triolein (0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 2.0 and 3.0 mg/ml) in 2:1 chloroform: methanol was

المنارة للاستشارات
www.manaraa.com

used for standards. The triglyceride and phospholipids samples and standards were heated in a 85-90°C water bath for 15 minutes to evaporate the solvent before addition of sulfuric acid. Following this was an additional incubation in the 85-90ºC water bath for 10 minutes. The tubes were removed and allowed to cool to room temperature before addition 1 ml of vanillin-phosphoric acid reagent followed by gentle vortexing. Two hundred and fifty µl of each sample or standard was transferred to a well in a 96-well plate and read in a spectrophotometer at 525 nm.

Protein assay

Soluble protein was measured using the BCA Protein Assay Kit (Pierce Company, Rockford Illinois). Samples from different lines or treatments were homogenized in 250 µl of Tris-HCL pH 8.0. The homogenate was duplicated 1:3 in homogenizing buffer and 10 µl was added to each well (96-well plate) for The Microplate Assay Procedure. The following standard concentrations of protein were employed: 2 mg/ml, 1 mg/ml, 0.5 mg/ml, 0.25 mg/ml, and 0.125 mg/ml. The BCA reagents were added and after a 30-minute incubation at 37°C, the plate was read in a spectrophotometer at 562 nm.

Orb mutant cross procedure

Mutations in the *orb* gene (Lantz et al. 1992) are characterized by female sterility resulting from a failure in oocyte maturation at a previtellogenic oocyte stage. Female heterozygous orb/TM3 virgins from *orb* stock lines were crossed to wild type males which were from a base population from 20 inbred lines of *D. melanogaster* that had been maintained in the lab for approximately 2 years. These inbred lines were derived from individually inseminated females collected in the field (Wolfskill Orchards maintained by the University of California at Davis, Yolo County, CA, USA). The F1 females with orb/+ were then crossed to F1 males with genotype +/TM3. After the F2 hatched, we collected virgins with short-hair for both sexes. When the virgin status was confirmed, we did single pair matings (one female and one male) by using short-hair males and females. We did at least 40-50 single pairs matings. This procedure was done to find out the orb/TM3 female crossed to orb/TM3 male. The way to determine orb/TM3 female crossing to orb/TM3 male is to dissect their female progeny with long-hair (+/+,

المنارة للاستشارات
www.manaraa.com

orb/+, or orb/orb but orb/orb individuals can be produced only if both parents were orb/TM3). If dissected females carried degenerated ovaries, we could confirm that the female was *orb* homozygous. At least five females were dissected from each vial. If the five females all had degenerated ovaries, we knew that their parent's genotypes were both orb/TM3. We then collected virgins from these vials to start more crosses. We considered these crosses to be one round of crossing. The flies subjected to this study were derived from Round 6 and Round 9 crosses.

NADP+ -isocitrate dehydrogenase (IDH) & glucose-6-phosphate dehydrogenase (G-6PDH) enzymes assay

Specific activities of IDH (E.C. 1.1.1.42) and G-6PDH (E.C. 1.1.1.49) involved in lipid metabolism were quantified in homogenates of 30 female flies. The assay protocols and methods were modified from Zhao and Zera (2002). These two enzymes play a role in triglyceride synthesis. Their activities may be notable because an increase in lipid content can confer starvation resistance (Chippindale et al., 1996; Harshman and Schmid, 1998; Hoffmann and Harshman, 1999) and their activities may change under starvation. Enzymes were assayed using SC and ST females that were not starved or that were starved for 36 hours, which is the maximum length of starvation exposure reachable without significant mortality in controls.

المنارة للاستشارات
www.manaraa.com

Results

(1) Triglyceride & Phospholipids

Triglycerides are the main resource utilized upon starvation resistance and longevity and phospholipids are abundant in organisms with higher fecundity. Starvation selected females are found to have higher starvation survival and lower early fecundity. Did starvation selected females have more triglycerides in somal cells and less phospholipids in germ line cells? Female flies were dissected into two parts: body (no ovaries) and ovaries. In Figure 1a, starvation selected females had more triglycerides in their body compared to control females ($F_{1, 20}$=20.35, $P<0.001$). In addition, selected females had more triglycerides in their body and ovaries after 36 hours of starvation. On the contrary, SC females had more phospholipids in their bodies (Fig. 1b, $P<0.0001$). There was no phospholipid difference in ovaries or body cells even after 36 hours of starvation. Our results showed that starvation selected females had more triglycerides in their bodies before and after starvation compared to control females. Also, the ST females didn't even show a decrease in their triglycerides after starvation. SC females had lower triglycerides contrasted to ST individuals in soma and triglycerides in SC decreased after 36 hours of starvation. ST females had more triglycerides in their ovaries after 36 hours of starvation. Starvation resistant flies had more triglycerides in somatic tissues which seems to confer or contribute to some level of resist to famine.

SC females had more phospholipids in their bodies but not in their ovaries. However, ST females didn't demonstrate lower phospholipids in their ovaries. After starvation, both SC and ST females' phospholipid levels had decreased in their somatic tissues.

(2) Soluble Protein

A higher proportion of triglycerides might be correlated to weight in affected flies. High levels of triglycerides in ST females may be affecting their body weight because ST females are heavier than SC females. Therefore, we measured protein levels in order to examine how protein content compares to lipid content. For both SC and ST females, we used the exact same treatment (body-no-ovary and ovary; unstarved or after 36 hours of

المنارة للاستشارات
www.manaraa.com

starvation), as in the triglyceride and phospholipids assay, to extract proteins for determination of protein levels.

We measured these individuals' soluble protein mass to see if it positively correlated with triglyceride levels. The protein content was also measured from two different tissue sections: body with no ovaries and ovaries. In figure 2 that data shows that we didn't observe any difference between SC and ST females with respect to body or ovary protein levels (P= 0.1693). However, there was higher protein content in body tissues compared to ovaries in both populations ($F_{1, 28}$=28.47, P<0.001). ST females having a higher proportion of triglycerides did not have correlating protein levels. This leads to the idea that a higher concentration of triglycerides in ST females might not be affected by weight alone.

(3) Orb mutant evaluation

Another idea to consider is that reproduction cost may decrease starvation resistance or longevity in these flies. The cost of reproduction in *D. melanogaster* is associated with egg production. Mutations in the *orb* gene (Lantz et al. 1992) are characterized by female sterility resulting from a failure in oocyte maturation at a previtellogenic oocyte stage. *Orb* mutants have been found to have higher starvation resistance (Salmon et al., 2001), but they can not eliminate the inbreeding depression. We hypothesized that orb mutants have more triglycerides and less phospholipids relative to TM3/Orb individuals. If reproduction cost expends a significant amount of energy from females, it would be valuable to test the stress resistance and differential lipid distribution by using female mutants without ovaries. The *Orb* line in this study has been backcrossed to wild type for 9 generations to eliminate or reduce the inbreeding depression. The ovaries in TM3/Orb females were removed and compared to those of the *Orb* female mutants. Table 1 shows the average starvation survival time. The Orb mutant females were found to live longer under starvation compared to TM3/Orb individuals. This result supported the idea that decreasing the reproduction cost can extend starvation resistance. We also measured and compared the levels of triglyceride and phospholipids between Orb mutants and TM3/Orb individuals (the ovaries of TM3/Orb female were also removed). In figure 3, our results showed that *Orb* mutant females had more triglycerides than TM3/Orb females

المنارة للاستشارات
www.manaraa.com

($F_{1, 16}$=8.62, P=0.0188). This confirmed the notion that triglyceride abundance play an important role in starvation resistance. We didn't see any enhanced phospholipid abundance in TM3/Orb flies ($F_{1, 13}$=0.96, P=0.3461), but it may just be that the ovaries of TM3/Orb females were removed.

(4) Enzyme activity

IDH did not show any difference in enzymatic activity without starvation and with 36 hours of starvation, but ST females had lower G-6P activity after 36 hours of starvation. Enzyme activities strongly paralleled profiles of triglyceride accumulation during adulthood. If ST females had more triglycerides, they might have relatively high enzyme activities or they might be reduced when flies are exposed to starvation. IDH and G-6-PDH activities were measured in SC and ST females that had not been starved or after 36 hours of starvation. There was no difference for IDH ($F_{3, 20}$=0.64, P=0.6003) but ST females had increased G-6-PDH activity after exposure to 36 hours of starvation ($F_{1, 7}$=3.98, P=0.05) (Fig. 5 a & b).

المنارة للاستشارات
www.manaraa.com

Discussion

(1) Physiology of starvation resistance: theoretical prediction and empirical evidence

In this study, the triglyceride concentration was observed to be higher in the somatic tissue of starvation selected females. Even after 36 hours of starvation, ST females didn't show any decrease in triglycerides. However, SC females showed decreased triglycerides in somatic cells after starvation. This result was similar to those of Wang et al (2007) who used whole fly extracts and demonstrated that ST flies didn't show decreased triglycerides after starvation but SC females did have lower amounts of triglycerides. In our study, we found that the decrease in triglycerides in control lines was from somatic tissues.

. Rion and Kawecki (2007) proposed three ways by which starvation resistant flies can survive famine: (1) increasing lipids reserves; (2) reducing the rate at which the reserves are used under starvation conditions; (3) lowering the minimal level of body energy content which allows survival. Our data suggested fairly robustly that the adaptations of ST females matched those laid out in the first two conditions mentioned above. In contrast, controls had less triglyceride reserves in somatic cells and consumed lipids faster than resistant flies. Our results helped explain the physiological mechanisms for starvation resistance in flies.

(2) Does trade-off between triglyceride and phospholipids accumulation in starvation selected lines and control lines occur

We hypothesized that ST females might have higher triglycerides and lower phospholipids (related to egg development) since starvation resistant females have greater stored energy and lower early fecundity. Looking back to the triglyceride and phospholipid results in somatic tissues and ovaries, ST females had higher triglycerides and lower phospholipids in somatic cells while SC females had less triglycerides and higher phospholipids in somatic cells. This indicates that there is trade-off between

للاستشارات المنارة
www.manaraa.com

triglyceride and phospholipid accumulation in starvation resistance. We didn't observe that ST females had less phospholipids relative to SC females. In fact, this result is not contradictory to lower early fecundity. In Wang et al. (2007), ST females were found to have lower early fecundity but they had more oocytes in their ovaries. In addition to ST females holding more oocytes, they do not lay them in their early life. This may explain why there is no difference in phospholipids in ovaries between ST and SC females. On the contrary, phospholipids were more abundant in somatic cells in SC females. Phospholipids are mainly found in cell membranes and lipoproteins, so SC females might have more phospholipid metabolism in somatic tissues.

After 36 hours of starvation, phospholipids in the soma in both SC and ST females decrease. Phospholipids have two fatty acids covalently bound to a glycerol or sphingosine backbone plus a phosphodiester bond linking a polar group to the molecule. Essentially, a phosphate group has replaced one fatty acid of a triglyceride. Triglycerides have three fatty acids hooked to a glycerol molecule. Energy is densely packed into these molecules due to their structure and hydrophobicity making them ideal for energy storage. Under starvation conditions, phospholipids in the soma might be the first resources broken down in surviving flies. However, lipid metabolism under starvation may be more complex than we think.

(3) Insights derived from the investigation of lipid synthesizing enzyme activities

A reduction in respiration rate might be a correlated response to selection for female starvation resistance. However, the laboratory selection experiment evidence for an association between reduced metabolic rate and stress resistance is not consistent. Hoffmann and Parson (1989) found that desiccation resistance was correlated with reduced metabolic rate independent of body size. Harshman and Schmid (1998) found that there was no association between starvation resistance and respiration rate, but they found that metabolism enzyme activities decreased in response to starvation in control line females. Harshman et al. (1999) found that the specific metabolic rate was lower in starvation selected lines and enzyme activities changed in response to selection.

The association between genetic variation for metabolic enzyme activity and lipid

المنارة للاستشارات
www.manaraa.com

storage in *D. melanogaster* has been found in some studies. The second chromosome replacement lines from Pennsylvania and California populations revealed an association between triacylgycerol abundance and G-6PDH (Clark and Keith, 1988; Clark, 1989). G-6PDH exists in various pathways involved in the production of NADPH, a cofactor required for reductive biosynthesis of fatty acids.

The ST females in this study were found to maintain stable triglycerides in somal cells after 36 hours of starvation. We further compared the triglyceride synthesis enzyme activity under unstarved and starved conditions. The activity of glucose-6-phosphate dehydrogenase (G-6PDH) was found to be lower in ST females after 36 hours of starvation while SC females still had similar activity. This implied that ST females may slow down their lipid metabolism under stressful environmental conditions which corresponds to the stable triglyceride levels in ST females after 36 hours of starvation. However, the details of the biochemical pathways leading to this adaptation are still unknown.

(4) The relationship between avoiding reproduction cost and increasing starvation resistance

Physiological reproduction costs are a particularly interesting area for study because the costs can be quantified and compared between disparate activities and functions (Sibly and Calow 1989; Rose and Bradley, 1998). It is a corollary of the cost of reproduction hypothesis that reproduction should be decreased with selection for increased adult survival. This result is indeed often observed (e.g. Luckinbill et al. 1984; Rose and Bradley1998), but not always (Partridge and Fowler 1992; Leroi et al. 1994).

Our main goal here is to use the *Drosophila* system in which increased adult survival is associated with decreased early reproduction under the evolutionarily appropriate conditions. We found that *Orb* female mutants had higher average starvation resistance than heterozygous orb females (TM3/Orb). It is undoubtedly suggested that reduced reproduction can raise starvation survival. In addition, Orb females mutants had more triglycerides in their bodies and similar amounts of phospholipids compared to TM3/Orb females whose ovaries were removed. Although, we didn't find any difference in phospholipid abundance in SC and ST ovaries, the Orb mutants and TM3/Orb females

المنارة للاستشارات
www.manaraa.com

somehow indirectly showed that phospholipids are more abundance during active reproduction.

The differential lipid distribution conforms to the Y model of resource allocation in either survival or reproduction. When flies were selected in starvation resistance, female flies responded by reducing early age reproduction in favor of survival under starvation conditions. Starvation selected females store more triglycerides and lower phospholipids levels in their bodies relative to control females. The plausible explanation is that starvation resistant females convert fatty acids into triglycerides instead of phospholipids since triglycerides are main storage lipids.

Harbison et al. (2004) identified 13 significantl loci contributing to starvation resistance. These loci included genes involved in oogenesis, metabolism and feeding behavior. This indicates that changes in metabolism, reproduction feeding behavior and resource allocation all contribute to improved starvation resistance. A recent study (Nuzhdin et al., 2007) contributed to the understanding of the genetics of direct and indirect starvation selection responses. The group estimated that there are approximate allele frequency differences of up to 40% between selected and control lines contributed to the selection response. Those candidate genes include some that are involved in metabolism, heat shock protein genes, and insulin-like growth factors. This study has proven that physiological evidence also supports the effects of associated genes on starvation resistance. *Drosophila* offers a unique opportunity for a comprehensive understanding and integration of the variety of aspects of functional and evolutionary response to starvation stress. In the future, the increase in reliability and number of molecular tools can help us to expand the studies of nutritional stress. Also, information garnered from Drosophila studies may help elucidate similar conserved physiological and cellular processes in other organisms, such as yeast, nematodes and mammals.

المنارة للاستشارات
www.manaraa.com

Table 1

The average of starvation survival time (±SEM) in Orb female mutants and TM3/Orb females.

Genotype	Starvation survival time (hours)
Orb/Orb	100.2±1.85
TM3/Orb	61.02±5.84

المنارة للاستشارات

www.manaraa.com

Figure 1

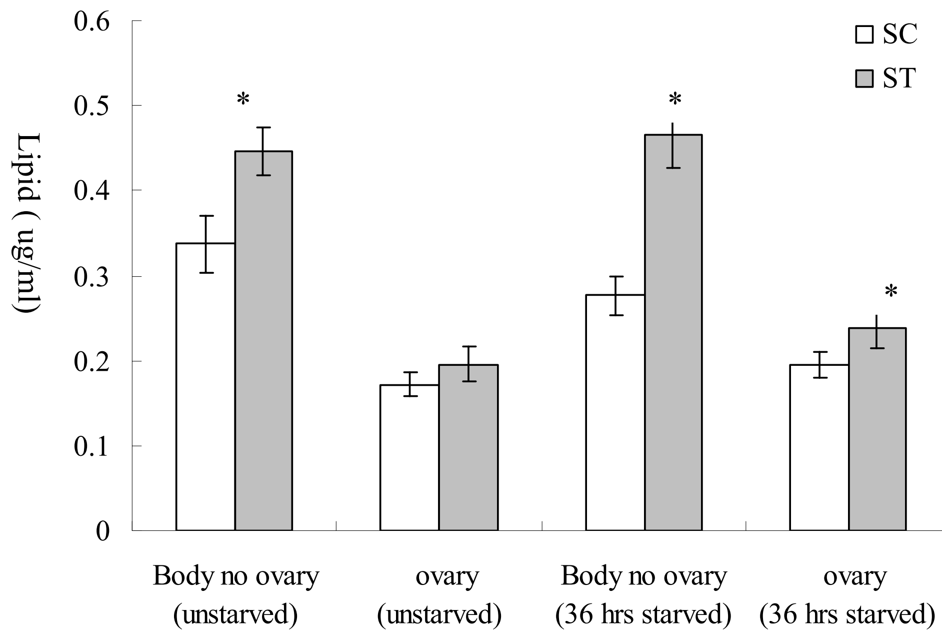
(a) Mean (±SEM) triglycerides per female of SC and ST lines in body-no-ovary and ovary in unstarved or 36 hours starved flies.

(b) Mean (±SEM) phospholipids per female of SC and ST lines in body-no-ovary and ovary in unstarved or 36 hours starved flies.

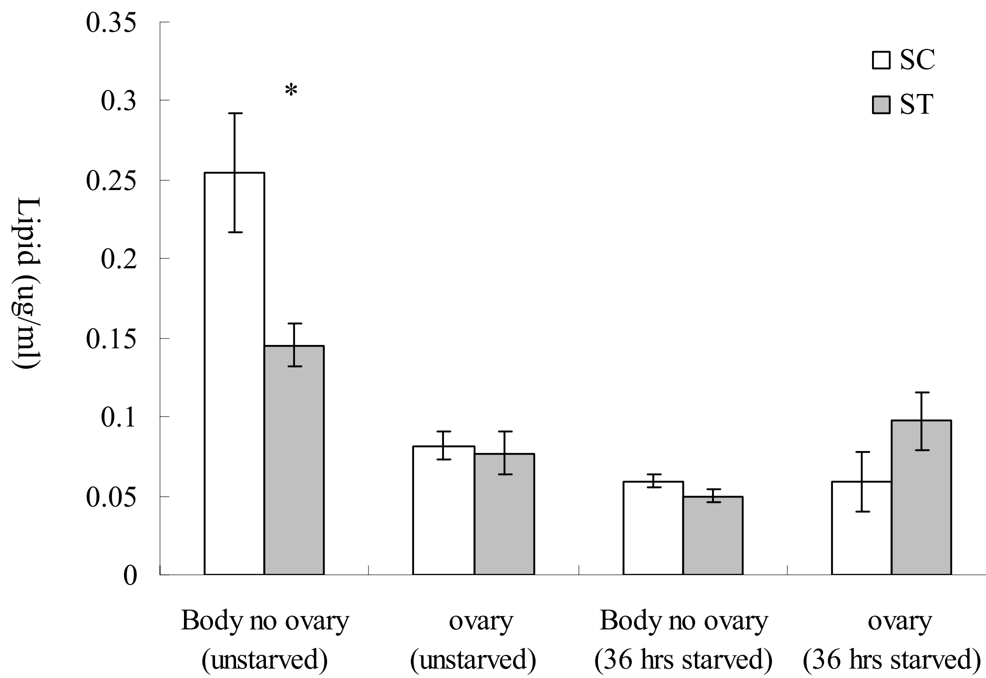
SC-Starvation control; ST-starvation selection
An asterisk (*) indicates significant difference

المنارة للاستشارات
www.manaraa.com

(a) Triglyceride level



(b)Phospholipids Level



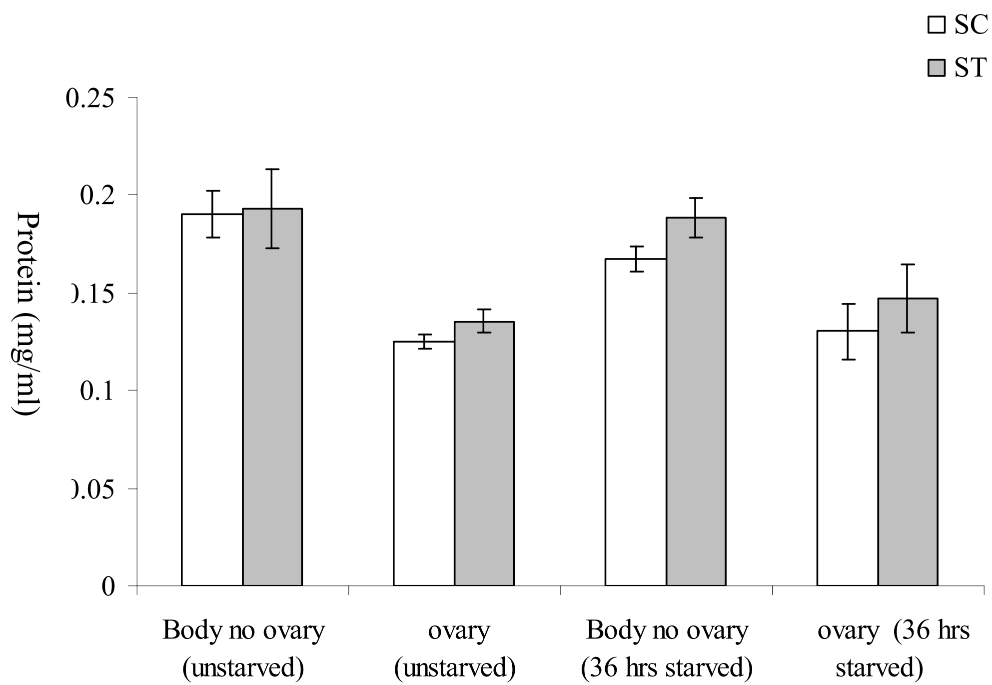
www.manaraa.com

Figure 2

Mean (±SEM) soluble protein per female of SC and ST lines in body-no-ovary and ovary at unstarved or 36 hours starved stages.

SC-Starvation control; ST-starvation selection

المنارة للاستشارات
www.manaraa.com



المنارة للاستشارات
www.manaraa.com

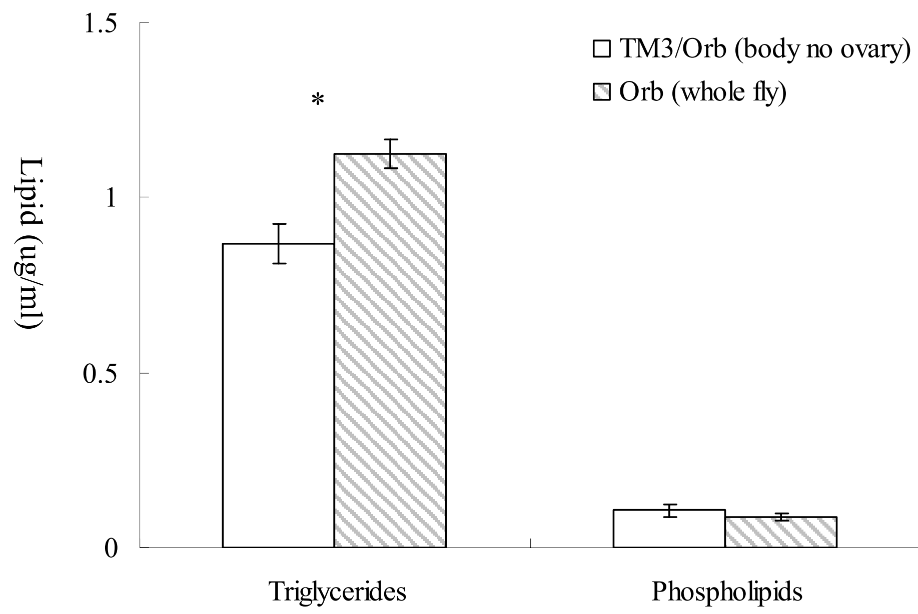
Figure 3

The average (±SEM) of Triglyceride and phospholipids concentrations in TM3/Orb females and Orb mutant females. The ovaries of TM3/Orb females were removed. Orb mutants had more triglycerides and there was no different in their phospholipids.

An asterisk (*) indicates a significant difference

المنارة للاستشارات

www.manaraa.com



www.manaraa.com

Figure 4

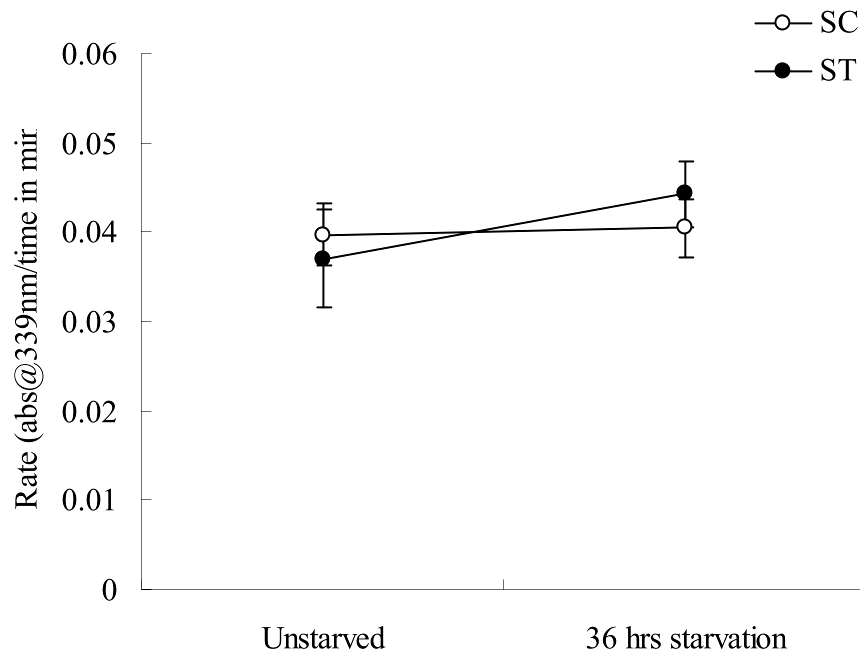
(a) The average (±SEM) activity of NADP+ -isocitrate dehydrogenase (IDH) in SC and ST lines at unstarved or 36 hours starved stages.

(b) The average (±SEM) activity of glucose-6-phosphate dehydrogenase (G-6PDH) in SC and ST lines at unstarved or 36 hours starved stages.

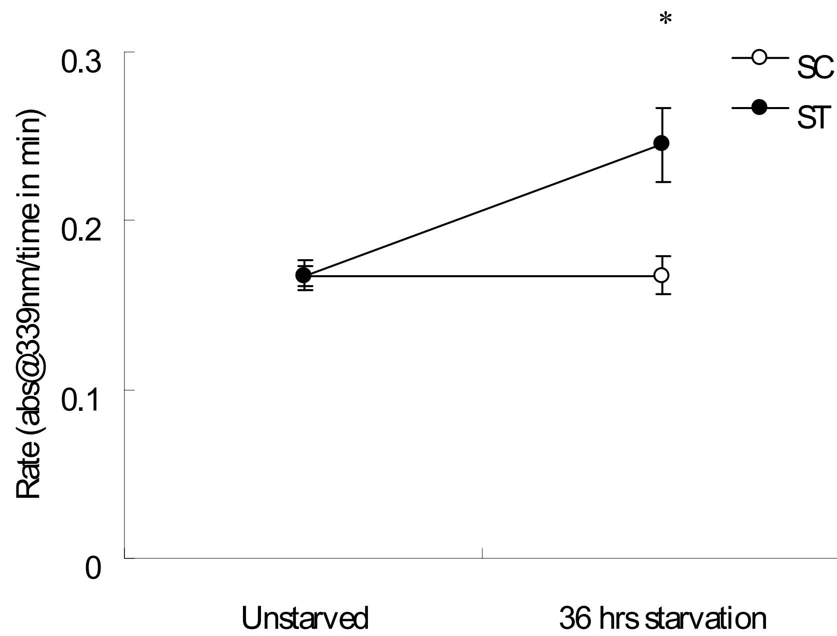
An asterisk (*) indicates a significant difference

المنارة للاستشارات
www.manaraa.com

(a)



(b)



www.manaraa.com

Reference:

Chippindale, A. K., T. J. F. Chu, and M. R. Rose. 1996. Complex trade-offs and the evolution of starvation resistance in *Drosophila melanogaster*. Evolution 50:753-766.

Clark, A. G. 1989. Causes and consequences of variation in energy storage in *Drosophila melanogaster*. Genetics 123:131-144.

Clark, A. G., and L. E. Keith. 1988. Variation among extracted lines of Drosophila melanogaster in triacylglycerol and carbohyrate storage. Genetics 119:595-607.

Grapes, M. P., P. Whiting, and L. Dinan. 1989. Fatty acid and lipid analysis of the house cricket, Acheta domesticus. Insect Biochem. 19:767-774.

Harbison, S. T., A. H. Yamamoto, J. J. Fanara, K. K. Norga, and T. F. C. Mackay. 2004. Quantitative trait loci affecting starvation resistance in *Drosophila melanogaster*. Genetics 166:1807-1823.

Harshman, L. G., A. A. Hoffmann, and A. G. Clark. 1999. Selection for starvation resistance in *Drosophila melanogaster*. J. Evol. Biol. 12:370-379.

Harshman, L. G., and J. L. Schmid. 1998. Evolution of starvation resistance in *Drosophila melanogaster*: aspects of metabolism and counter-impact selection. Evolution 52:1679-1685.

Harshman, L. G., and A. J. Zera. 2007. The cost of reproduction: the devil in the details Trends in Ecology and Evolution 22:80-86.

Hoffmann, A. A., and L. G. Harshman. 1999. Desiccation and starvation resistance in *Drosophila*: patterns of variation at the species, population and intrapopulation levels. Heredity 83:637-643.

Hoffmann, A. A., and P. A. Parsons. 1989. Selection for Increased Desiccation Resistance in *Drosophila melanogaster*: Additive Genetic Control and Correlated Responses for Other Stresses. Genetics 122:837-845.

Lantz, V., L. Ambrosio, and P. Schedl. 1992. The Drosophila orb gene is predicted to encode sex-specific germline RNA-binding proteins and has localized transcripts in ovaries and early embryos. Development 115:75-88.

Leroi, A. M., A. K. Chippindale, and M. R. Rose. 1994. Long-term laboratory

المنارة للاستشارات
www.manaraa.com

evolution of a genetic life-history trade-off in *Drosophila melanogaster* 1. The role of genotype-by-environment interaction. Evolution 48:1244-1257.

Luckinbill, L. S., R. Arking, M. J. Clare, W. C. Cirocco, and S. A. Buck. 1984. Selection for delayed senescence in *Drosophila melanogaster*. Evolution 38:996-1003.

Nuzhdin, S. V., L. G. Harshman, M. Zhou, and K. Harmon. 2007. Genome-enabled hitchhiking mapping identifies QTLs for stress resistance in natural *Drosophila*. Heredity 99:313-321.

Partridge, L. 1989. An experimentalist's approach to the role of costs of reproduction in the evolution of life-histories. Pp. 231-246 *in* P. J. Grubb and J. B. Whittaker, eds. Towards a more exact ecology. Blackwell Scientific, Oxford.

Partridge, L., and K. Fowler. 1992. Direct and Correlated responses to selection on age at reproduction in *Drosophila melanogaster*. Evolution 46:76-91.

Rion, S., and T. J. Kawecki. 2007. Evolutionary biology of starvation resistance: what we have learned from *Drosophila*. Journal of Evolutionary Biology 20:1655-1664.

Rose, M. R., and T. J. Bradley. 1998. Evolutionary physiology of the cost of reproduction. Oikos 83:443-451.

Salmon, A. B., D. B. Marx, and L. G. Harshman. 2001. A cost of reproduction in Drosophila melanogaster: stress susceptibility. Evolution 55:1600-1608.

Sibly, R. M., and P. Calow. 1989. A life-cycle theory of responses to stress. Biological Journal of the Linnean Society 37:101-116.

Townsend, C. R., and P. Calow. 1981. Physiological ecology: an evolutionary approach to resource use. Blackwell Scientific, Oxford.

Zera, A. J., and L. G. Harshman. 2001. Physiology of life-history trade-offs. Ann. Rev. Ecol. Syst. 32:95-126.

Zera, A. J., J. Sall, and K. Otto. 1999. Biochemical aspects of flight and flightlessness in Gryllus: flight fuels, enzyme activities and electrophoretic profiles of flight muscles from flight-capable and flightless morphs. J Insect Physiol 45:275-285.

Zera, A. J., and Z. W. Zhao. 2003. Life-history evolution and the microevolution of intermediary metabolism: Activities of lipid-metabolizing enzymes in life-history

المنارة للاستشارات
www.manaraa.com

morphs of a wing-dimorphic cricket. Evolution 57:586-596.

Zhao, Z. W., and A. J. Zera. 2002. ifferential lipid biosynthesis underlies a tradeoff between reproduction and flight capability in a wing-polymorphic cricket. PNAS 99:16829-16834.

www.manaraa.com