ABSTRACT

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Quantitative Molecular Genetics of Longevity in *Drosophila melanogaster*.
(Under the direction of Trudy F. C. Mackay.)

Limited life span and senescence are universal phenomena, controlled by genetic and environmental factors whose interactions both limit life span and generate variation in life span between individuals, populations and species. To understand the genetic architecture of aging it is necessary to know what loci affect variation in life span, what are the allelic effects at these loci and what molecular polymorphisms define quantitative trait locus (QTL) alleles. Here, quantitative complementation tests were used to determine whether candidate life span genes such as *Superoxide dismutase* (*Sod*), *Catalase* (*Cat*), heat shock proteins, DNA repair enzymes, glucose metabolism or male accessory gland proteins interact genetically with naturally occurring QTL affecting variation in life span in *Drosophila melanogaster*. Inbred strains derived from a natural population were crossed to stocks containing null mutations or deficiencies uncovering the above genes. Life span of the heterozygous progeny was assayed. A significant cross (mutant versus wild-type allele of the candidate gene) by inbred line interaction term from analysis of variance of the life span data indicates a genetic interaction between the candidate gene allele and the naturally occurring life span QTL. Of the sixteen candidate regions and genes tested, *Df(2L)cl7*, *Df(3L)Ly*, *Df(3L)AC1*, *Df(3R)e-BS2*, and *α-Glycerol phosphate dehydrogenase* showed significant failure to complement wild-type alleles in both sexes, and an *Alcohol dehydrogenase* mutant failed to complement in females. Several genes known to regulate life span (*Sod*, *Cat*, and *rosy*) complemented the life span effects of alleles, suggesting little natural variation affecting longevity at these loci, at least in this sample of alleles. Quantitative complementation tests are therefore useful for

identifying candidate genes contributing to segregating genetic variation in life span in nature.

Mutations in most vital genes can potentially affect life history traits, but it is not known what subset of these loci harbor naturally occurring variation affecting the rate of aging and the ability to resist stress. While the gene *Punch* (*Pu*) was not significant in the quantitative complementation test, it has been implicated in starvation resistance. As there is a direct relationship between stress resistance and longevity, *Pu*, which encodes GTP cyclohydrolase (GTPCH), is a candidate gene for associating molecular variation and variation in life pan. GTPCH regulates the catecholamine biosynthesis pathway by catalyzing the formation of tetrahydrobiopterin, the rate-limiting molecule, and by regulating tyrosine hydroxylase, a key enzyme in the pathway. The extent to which molecular variation at *Pu* contributes to phenotypic variation was assessed by associating single nucleotide polymorphisms (SNPs) at *Pu* with longevity. Nucleotide variation was determined for ten *Pu* alleles. Genotypes of 28 SNPs were determined on a sample of 178 isogenic second chromosomes sampled from the Raleigh, USA population and substituted into the highly inbred *Samarkand* background. Life span was determined for the chromosome substitution lines and the association between longevity phenotype and SNP genotype was assessed for each polymorphic marker. Three SNPs were significantly associated with life span (C6291A, $P = 0.0183$; A6389T, $P = 0.0466$; G6894C, $P = 0.0024$). None of these SNPs was significant individually following a permutation test accounting for multiple tests and partially correlated markers. However, the three SNPs associated with life span were in global linkage disequilibrium. Haplotypes of these SNPs were highly significantly associated with variation in longevity ($P < 0.0001$), and accounted for 13.5 % of the genetic variance and 1.86 % of

the phenotypic variance in longevity attributable to chromosomes *2*. As *Pu* is a regulator of the catecholamine biosynthetic pathway, these findings suggest the importance of the production of biogenic amines in determining variation for longevity.

Quantitative Molecular Genetics of Longevity in *Drosophila melanogaster*

by
GRETCHEN LINDSAY GEIGER THORNSBERRY

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Chair of Advisory Committee

DEDICATION

During my years in school, I had the privilege of being taught by many great teachers. Four of these stand out in my mind. Dr. Glenda George taught biology at Irmo High School in Columbia, South Carolina. She introduced me to biology and started me on the path that I have taken. While I attended the College of Charleston, Dr. Rob Dillon taught me genetics and supervised my research and bachelor's essay. He always took the time to answer my questions, even after my graduation and during his sabbatical. Dr. Dillon always told me that I should go to graduate school and not medical school. He was right. Mrs. Betsy Martin and Dr. Rick Heldrich were two of my chemistry professors and teaching mentors. I learned a great deal as their student but even more as their teaching assistant. It is because of these four remarkable teachers that I want to teach. As this stage of my education ends, I want to thank them for all they have done. I owe them more than I can say and dedicate this work to them.

BIOGRAPHY

I was born in Columbia, South Carolina on July 14, 1974 to Paulette and Frank Geiger. Irmo High School is where I developed an interest in two things: biology and technical theater. After graduation in 1992, I enrolled at the College of Charleston. When not studying, I spent my time tutoring chemistry and biology, serving as a teaching assistant for introductory, organic, and honors quantitative analysis chemistry labs, and volunteering backstage at theater productions. My interest in genetics started during a required course in my sophomore year. It became my favorite subject. My undergraduate research under Dr. Rob Dillon involved recreating part of the original *Drosophila* second chromosome linkage map. I graduated from the College of Charleston honors program with a Bachelor of Science in biology and a minor in chemistry in December 1995.

In June 1996, I started medical school at the Medical University of South Carolina. I decided to withdraw after two weeks. While leaving medical school was one of the most difficult things I have ever done, it was the right decision. Within a few weeks I had decided to pursue a degree in genetics. Meanwhile, I worked as a departmental secretary at the College of Charleston, and with a little help from Dr. Rob Dillon, taught myself population and quantitative genetics. Dock Street Theater became a second home for me, and I went from being a theater technician to being a stage manager. Just before leaving Charleston, I had the opportunity to work as an assistant with the Woodrow Wilson Institute, a program in which college master teachers mentor high school teachers. I knew I wanted to teach.

I enrolled as a doctoral student in the Department of Genetics at North Carolina State University in August of 1997. Under the guidance of Dr. Trudy Mackay, I continued

working on *Drosophila*, but this time focused on genotype environment interaction affecting bristle number. I decided to concentrate the rest of my research on the quantitative genetics of longevity in *Drosophila* after the completion of the bristle experiment. While at State I taught one semester of introductory genetics recitation with Dr. Ted Emigh and one semester of introductory genetics laboratory under Dr. Betty Gardner. I participated in the Preparing the Professorate program, enrolled in a teaching course and completed several workshops presented by the Faculty Center for Teaching and Learning to improve my teaching skills.

While in graduate school, I met my husband Jeff Thornsberry. We married on December 1, 2001. There is not as much time to volunteer at the theater as in the past, but I still spend several evenings a month at Raleigh Little Theater. Jeff and I will be moving to Missouri in August where he will be teaching at Northwest Missouri State University.

ACKNOWLEDGMENTS

Longevity experiments in *Drosophila* require an enormous amount of fly food. I would like to thank the fly food chefs of the Mackay lab for the thousands upon thousands of fly vials that made my experiments possible. These are Brant Hackett, Chad Burris, Michelle Bennett, Jeremy Locklear, Paul Gilligan, Will Martin, and Grace Jordan. Thank you also to Faye Lawrence who keeps our lab up and running.

Longevity experiments can also take months and months of scoring vials at forty-eight hour intervals. The longevity crew of Jeff Leips, Rhonda Wilson, Paul Gilligan, Mike Magwire, and Christine Varnum deserve many thanks for taking care of my flies during times when I was unable to score the longevity flies. Thank you for helping me keep my sanity when dealing with the immortal flies. Thank you also to Richard Lyman who created chromosome two extraction lines used in my work and to Susan Harbison and Katie Jordan for helping me in "re-stocking" sick stocks.

When I moved from the fly lab to the molecular lab, another set of helpers was there. Donghui Tan runs the molecular lab and helped with my introduction to pyrosequencing. Maria De Luca tweaked the pyrosequencing protocol and was invaluable in helping get the project up and running. Stephanie Rollmann was there to get me started again whenever things stopped running. I would also like to thank Ted Morgan and Mary Anna Carbone who critiqued my writing.

Graduate school, particularly during preliminary exams and thesis writing, can be stressful. Having good friends is not important; it is essential. Stefanie Heinsohn and the lab mates listed above have been wonderful colleagues and friends. My classmates Amy

Lawton-Rauh, Michael Buck, Christy Dilda Carringer, and Chui-Yueh Huang have enriched my experience at NC State as well. Graduation is a bittersweet time as we have accomplished so much together but now start separate adventures. Thank you also to my friends at Raleigh Little Theater, a sort of second home for me, and to the McCarthy family, a sort of second family.

No thesis would be complete without a heartfelt thank you to the major advisor. I was truly lucky that Dr. Trudy Mackay offered to take me into her lab. Accepting was one of my easiest decisions. She is not only a great scientist but also a great mentor. Trudy is a leader in quantitative genetics. She can teach, write grants and articles, and supervise a large lab all at one time and still find time for a life outside Gardner Hall. Thank you for all that you have done for me.

I must also thank my graduate committee members: Dr. Bruce Weir, Dr. Michael Purrugannan, Dr. Greg Gibson, Dr. Dahlia Nielsen, and Dr. Gail Wilkinson. My committee members travel a great deal and they have bent over backwards to be here for my defense. Thank you. Changing your schedules to accommodate my defense has allowed me to move to Missouri with Jeff without having to come back to Raleigh alone.

Next I would like to thank my family. My parents, Paulette Geiger and Frank Geiger, put me through college and have supported me in every thing I have done. Thank you also to my grandmother, Elizabeth Blume, and my sister and brother-in-law Jennifer and Michael Fermin. My thanks also goes out to two friends who might as well be family: Randal Davis and Mary Peyton Davis. Randal has been calling me Dr. Geiger since we were undergraduates. Now it is official, although the last name has changed. Mary Peyton has

been with me through everything good and bad during the last six years. Thankfully there has been much more good than bad.

The one deserving most of my appreciation is my husband Jeff. We started dating during my preliminary exams, so he is accustomed to seeing me under stress. He takes great care of me and knows how to keep me calm even under the most stressful of times. He has shown a great deal of patience with me particularly while answering my science questions. I am looking forward to starting the next stage of our life together in Missouri.

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INTRODUCTION

As the world human population rapidly grows older, population aging will become one of the most important social and health problems in the coming half century. Almost nightly a news broadcast will claim that someone has found the key to slowing the aging process. While progress is being made in the treatment of some age-related illnesses, the genetic basis of neither the aging process nor age-related diseases such as Alzheimer's disease and Parkinson's disease are well understood. Why do organisms age? How do organisms age? What factors control aging and prevent an organism or a cell from living forever? How do these factors interact to determine life span? While advances in the study of aging are becoming more and more frequent, the work on determining what factors influence aging is just beginning.

Aging is a complex series of events that prevent an organism from being able to maintain homeostasis. In humans, mortality rates are high in the first year of life but decrease greatly by age ten (Austad, 1997). As growth and development slow and the onset of puberty occurs, mortality rates begin to rise again. By the early twenties, muscle coordination has peaked, and by the early thirties stamina has peaked marking the maximum physiological capabilities of the body (Hayflick, 1994). However, at the same point in life, mortality rates have increased to be the same as an infant. Above age thirty the risk of dying doubles every eight years (Austad, 1997). Typical hallmarks of aging include decreases in muscle strength, decreases in mental acuity and increased susceptibility to diseases such as influenza, cancer and Parkinson’s disease. It is inevitable that the deterioration will continue until the body can no longer function and dies. Why does our body begin to give way to age?

Why do muscles become weaker? Why does the mind start to falter? Why are the elderly stricken more often and with higher death rates for illnesses than younger individuals? What is normal aging?

Limited life span and senescence are universal phenomena, controlled by genetic and environmental factors whose interactions both limit life span and generate variation in life span between individuals, populations and species. Genetic and environmental factors combine to influence the age-related symptoms each individual will have, the onset and severity of those symptoms, and ultimately, the length of life and cause of death. As no two people age in the same manner, teasing apart the genetic and environmental factors and their interactions is a complex task. There are two approaches to address these questions: the study of genes regulating life span and variation in life span in model organisms and the study of age-related diseases both in humans and in model systems.

Model systems, such as the fruit fly *Drosophila melanogaster,* the nematode *Caenorhabditis elegans,* and the mouse are invaluable for teasing apart relationships between genes and life span. Longevity was first shown to be genetically determined in *Drosophila* because life span can be increased as a correlated response to selection for postponed senescence (Rose, 1984; Luckinbill *et al.*, 1984) or by directly selecting for increased life span (Zwaan *et al.*, 1995). *Drosophila* are useful in aging studies because of the wide variety of mutants available, the short generation time, a short life span, and a great deal of naturally occurring genetic variation affecting variation in life span (Nuzhdin *et al.*, 1997). Knowing the fate of every cell in the organism as well as a vast collection of information on metabolism and how it effects stress response and life span is one advantage of studying

aging using *C. elegans* (Guarente and Kenyon, 2000). Mice are generally used in studies of mutations and their relationship to disease as they are more closely related to humans than the other two organisms listed. It is plausible that mutations in many genes will lead to decreased life span; indeed, it has been hypothesized that no fewer than one-hundred genes directly affect naturally occurring variation in *Drosophila* longevity (Deckert-Cruz *et al.*, 1997). There is no reason to believe that longevity in humans would be controlled by a more simplistic genetic regulation. Further, genes that regulate longevity in model organisms are likely to have human orthologues, with similar functions. In humans, however, it is the genetic mechanisms that cause age-related degeneration and disease that are of primary interest.

Current research has focused on the genetic basis of age-related diseases, such as Parkinson's and Alzheimer's disease, in hopes of finding ways to prevent and ultimately, cure them. Association tests in families who have certain diseases can help determine which mutations are associated with a particular disease, although careful consideration is required in the design and interpretation of these experiments. Association studies have greatly advanced the study of Alzheimer's, Parksinson's and Huntington's diseases (Joyce *et al.*, 1998; Turjanski *et al.*, 1995). Studies of both normal and cancerous cell cultures from humans aid in the study of cellular senescence and age-related changes in gene expression (Chen *et al.*, 1995; Smith and Pereira-Smith, 1996; Komano *et al.*, 1999). Finally, experiments looking at age-related disease in organisms such as mice are useful in understanding the way in normal aging goes awry to create age-related diseases when these

experiments cannot be done in humans (Baik *et al*, 1995) . It is thought that by knowing what has gone wrong to cause a disease, the normal process of aging can be untangled.

THEORIES OF AGING: THREE REPRESENTATIVES

Several theories on the mechanisms of aging have been advanced in recent years. While Austad (1997) states that there are no fewer than 55 different theories of aging, this review will focus on the three most common theories: the mitotic clock, antagonistic pleiotropy, and mutation accumulation. Many of the other theories are derivatives or combinations of these three theories. These theories are not mutually exclusive and some genetic mechanisms may fall under more than one theory. Furthermore, one must remember aging is a result of a combination of genetic and environmental mechanisms.

THE MITOTIC CLOCK THEORY

The mitotic clock theory, also called the Hayflick phenomenon, is the idea that some biological process within each cell weaken with each division until the cell reaches the point where it is no longer able to divide. Two possible mechanisms for this are the changes in gene expression due to the age-related decrease in methylation of genes and the ever-shortening of telomeres until a critical minimum length is reached. In the former, age-related changes in the pattern of methylation affect the expression of genes, thereby causing aging. In the latter, the shortening of the ends of the chromosomes sends a signal to the cell telling it to cease division. According to this theory, a newly created cell has a program that causes the cell to divide a finite number of times. When division ceases, the cell dies. In yeast and

other budding organisms, the clock is set to produce a particular number of daughter cells. While this theory is frequently thought of in terms of single cell organisms, it may also hold true for more complex species. In higher eukaryotes, the clock would reside in each and every cell. For example, non-cardiac muscle cells divide much more frequently than do cells of the central nervous system. Cell death in muscles occurs much sooner in life than does neural degeneration. Hence, the loss in strength in individuals of moderate age.

LIFE HISTORY EVOLUTION THEORY

The evolutionary explanation for the phenomenon of aging is based on the decline of the force of natural selection with age (Charlesworth, 1994). Thus germ-line mutations that have late-age specific effects, such as those causing late-age-of-onset diseases (Huntington's Disease, Alzheimer's Disease) cannot be effectively selected against, and increase in frequency (Medawar, 1952). Mutations that confer a high fitness during development and reproduction phases but lower fitness later in life (*i.e.,* antagonistic pleitropy) will also accumulate and remain segregating in populations (Williams, 1957; Rose, 1984). Antagonistic pleiotropy is best thought of as a series of biological trade-offs (Martin *et al.,* 1996). A classic example of antagonistic pleiotropy in humans deals with the prostate. The prostate gland is necessary for reproduction, but growth of the prostate does not cease after reproduction stops. This hyperplasia seen in older men can lead to cancer. In fact, it is currently estimated that seventy-percent of men aged seventy and over show some cancerous changes in the prostate. The two main areas to be considered in this hypothesis are how reproduction and development time are correlated with life span.

THE SOMATIC MUTATION ACCUMULATION THEORY

As an organism ages, the DNA is exposed to increasing numbers of opportunities for mutation either through exposure to mutagens coming from outside the cell or created within the cell by toxic by-products of metabolism. Mutations will build up and affect the levels of gene expression and the efficacy of the proteins produced. It is thought that much of the mutation and cellular damage is attributable to the highly reactive oxygen species which are the byproducts of cellular respiration. Changes in gene expression due to a mutation, mitochondrial function, caloric restriction and metabolic rate are all included among mechanisms for oxidative damage because the underlying source of the change is the same. Skin cancer is one example of this mechanism for aging (Crawford and Shields, 2000). DNA everywhere in the body is subjected to damage from reactive oxygen species and mistakes in DNA replication, while skin cells are subjected to damage from ultraviolet light. In young cells, DNA repair mechanisms will almost always repair the DNA damage before a cell divides, ensuring that the daughter cells will have two copies of the correct DNA. An older cell may not be able to make a timely repair. If the mutation is not corrected before the cell divides, one of the daughter cells will carry the mutation which cannot be corrected as the new cell cannot distinguish between the normal and mutant DNA. Another mutation in a tumor suppressor gene in a single cell allows cell division to be unchecked leading to cancer. By coupling the number of opportunities for DNA to be mutated over a life time with the aged cells decrease in DNA repair ability, it is possible to see why certain types of cancer are more common in the elderly.

ENVIRONMENTAL FACTORS THAT AFFECT AGING

Recently there has been considerable progress towards understanding the environmental factors that regulate life span across diverse taxa (Guarente and Kenyon, 2000; Finch and Ruvkun, 2001; Partridge and Gems, 2002). Environmental factors affecting life span include reproduction and caloric restriction. Caloric restriction extends life span in mammals (Weindruch and Walford, 1988), *Drosophila* (Pletcher *et al.,* 2002) and yeast (Lin *et al.,* 2000), while reproduction shortens longevity in humans (Westendorp and Kirkwood, 1998), *Drosophila* (Partridge and Farquar, 1981; Partridge *et al.*, 1987; Chapman, 1992; Chapman *et al.,* 1995) and *Caenorhabditis elegans* (Gems and Riddle, 1996).

REPRODUCTION AND AGING

The idea of antagonistic pleiotropy is based on a trade off between two life-history traits. In several species this has been found to be true, as a negative genetic correlation exists between reproduction and life span. In *C. elegans* it has been shown that life span can be increased by using lasers to ablate cells that produce the germ cell lines (Hsin and Kenyon, 1999). By removing the possibility of reproduction, the life span of the nematode was increased. In both mice and humans, reproduction and life span have been correlated as well. In these cases, however, longer life span is not linked to lack of reproduction, but reproduction at a later age. This was found true in strains created from wild-caught mice (Biddle, *et al.*, 1997). In humans women who had children at an early age died sooner on average than did women who had children late in life (Westendorp and Kirkwood, 1998).

The greatest volume of work associating reproduction and life span has been in the fruit fly. Several independent studies have produced lines with increased longevity by selecting for older parents (Rose, 1984; Pletcher *et al.,* 1997; Partridge *et al.,* 1999). In some long-lived selection lines, there was a correlated response in fertility late in life (Rose, 1984), but not always (Partridge *et al.,* 1999). However, most lines selected for postponed senescence did have decreased reproduction in early life as expected under the theory of antagonistic pleiotropy (Rose, 1984; Partridge *et al.,* 1999). Furthermore, Partridge, Green and Fowler (1987) showed that female life span could be extended by reducing egg laying, again showing a trade-off between life span and reproduction. Reduction in male life span occurs when male sexual activity increases, a trade-off that was previously only expected in the maternal parent (Partridge and Farquhar, 1981).

Trade-offs between reproduction and life span were also observed in an experiment investigating the relationship of temperature to body size and fecundity in female *Drosophila melanogaster*. Specifically, flies with the greatest early fecundity had the shortest life span (Nunney and Cheung, 1997). Service (1993) investigated the relationship between male mating ability and life span. He found that older males from lines selected for longer life span had superior late-life mating ability but inferior early-life mating ability when compared with flies from lines that undergo normal senescence (Service, 1993). In an experiment selecting for late-life fitness, long-lived males mated faster than short-lived males, but long-lived females were slower mating than short-lived females (Pletcher *et al.,* 1997). While the former results are expected, the latter are not, but this may be attributable to the less effective selection for the females (Pletcher *et al.,* 1997). Again these experiments provide support for

the antagonistic pleiotropy theory of aging. How do these trade-offs occur? Djawdan *et al.* (1996) showed there is a relationship between metabolism and life history trade-offs: flies with longer life spans had increased lipid and carbohydrate stores and speculated that this might be due to the decrease in early reproduction. While the total amount of energy accumulated was not enough to account for the level of energy consumption used by short-lived flies that produced more eggs, the difference was striking enough to give some insight into how choice regarding energy expenditure could be responsible for the trade-off between longevity and reproduction.

In *Drosophila*, accessory proteins (Acps) are secreted from the accessory gland in the male reproductive tract and are passed to the female during reproduction, causing physiological and behavioral changes in the female. Acps are responsible for decreasing attractiveness to males, decreasing receptivity to repeated mating, increasing egg-laying and directing the storage and usage of sperm after mating (Wolfner, 1997). Fowler and Partridge (1989) saw a decrease in female life span when the females were mated and the egg-laying behavior and exposure to males was controlled. A decrease in life span of mated females was seen regardless of whether the males were normal or deficient in sperm production, indicating that the protein portion of the semen was associated with the decrease in life span (Chapman, 1992; Chapman *et al*., 1993). To further localize the causative agent, transgenic flies lacking secretions from the accessory gland were created. Females mated to transgenic males had a life span comparable that of virgin females, while females mated to normal males had a shorter life span than the controls, localizing the causative agents to the accessory gland (Chapman *et al.*, 1995). EST studies have now identified 83 predicted

accessory proteins which include prohormone precursors, glycoproteins, proteases, protease inhibitors and lipases as well as many novel proteins (Wolfner 2002).

While the function of some of these proteins is not known, the protease inhibitor Acp62F plays a direct role in determining the life span of females after mating. Lung *et al.* (2002) ectopically expressed the secreted forms of eight different accessory proteins and found only Acp62F caused toxicity. Sequence and structural homology show that Acp62F is a trypsin protease inhibitor similar to those found in the intestinal parasite *Ascaris* which serve to protect the parasite from the enzymes of the host's digestive tract (Goodman and Peanasky, 1982). It is hypothesized that protease inhibitors prevent damage to healthy sperm by lessening the proteolytic activity of enzymes released when acrosomes of other sperm are prematurely released (Lung *et al.*, 2002). Protease inhibitors also prevent loss of sperm from the female reproductive tract by preventing proteolysis that would result in liquefication of the semen (Lung *et al.*, 2002). While the protease inhibitor is necessary for male reproductive success, it is detrimental to the females. Approximately ten percent of the Acp62F is absorbed from the female posterior reproductive tract into the hemolymph (Wolfner, 2002). Lung and others (2002) had observed that expression of Acp62F was much more toxic to developing flies than to adults. They hypothesize that the protease inhibitor in the hemolymph interferes with the cleavage needed in the developmental cascades of the young as well as the cascades necessary for immune system response in adults, concluding that disruption of immunity contributes to the shortening of female life span after mating (Lung *et al.*, 2002). At the present time, Acp62F is the only accessory protein shown to have a direct effect on aging, although there are seven other putative protease inhibitors that have

not been thoroughly investigated. Furthermore, decreases in RNA levels for two accessory proteins affecting egg-laying and sperm storage have been noted in an aging microarray experiment (Zou *et al*., 2000). Further study is needed to determine how or if other accessory proteins directly affect life span.

While accessory proteins have been extensively studied in *Drosophila*, a relationship between reproduction and aging has been seen in organisms other than fruit flies. The life span of *C. elegans* can be extended by ablation of the germ line cells (Hsin and Kenyon, 1999), but a similar response was not observed upon ablation of the sperm, oocytes, or meiotic precursor cells (Arantes-Oliveira *et al.*, 2002). It is thought that the germ-line cells affect either the production of a steroid hormone or its regulation which in term acts downstream to extend life span. Male longevity in the nematode is also affected by the presence of other males. All male groups have shorter life span than solitary males while hermaphrodite life span was unaffected by grouping (Gems and Riddle, 2000). The effect of interactions between individuals on longevity is thus dependent on the sex of the nematode in question, and as well on the alleles expressed in the strain (Gems and Riddle, 2000).

Experiments regarding mammalian reproduction and senescence have also been undertaken. Biddle and colleagues (1997) were able to use a selection regime to delay reproduction in mice, and they expect a corresponding increase in life span although they have not completed an aging curve for the selected strain (Biddle *et al.*, 1997). Castration of cats (Hamilton, 1965; Bronson, 1981-a) and dogs (Bronson, 1981-b) has been shown to increase life span. Castrated mentally handicapped men also live longer than intact mentally retarded men, suggesting a relationship between testosterone and life span (Hamilton and

Mestler, 1969). There is also a trend in which the age at first child birth is directly correlated with life span of women, and a negative correlation between the number of children and life span (Westendorp and Kirkwood, 1998). A similar trade-off between life span and the number of offspring regardless of the sex of the parent has been seen by looking at 1,200 years of genealogical data from European aristocrats (Gavrilova *et al.*, 1998). It is evident that reproduction, which may be considered an environmental effect, causes changes in the organism that contribute to the determination of longevity.

CALORIC RESTRICTION

Research by Djawdan and colleagues (1996) has shown a relationship between energy stores and life span. The contribution of caloric restriction to the slowing of the aging process is being investigated because multiple experiments have shown a decrease in caloric intake can increase life span by decreasing the rate of oxidative damage (Sohal and Weindruch, 1996). Extension of life span through caloric restriction has been shown in many organisms including fish, spiders, and rodents. How does a reduction in caloric intake actually work to increase life span? One possible mechanisms is that caloric restriction is tied to oxidative damage and mitochondrial activity which will be discussed later in this review. The rate of mitochondrial activity is tied to the metabolic rate of the cell. This theory states that by restricting caloric intake, the metabolic rate is slowed, and hence the levels of superoxide anion and hydrogen peroxide generation are reduced, lowering the level of oxidative damage. Rodents placed on a restricted diet have lower levels of superoxide anion and hydrogen peroxide generation rates and lower rates of mutation accumulation due

to oxidative damage (Sohal *et al.,* 1994). Mice placed on a caloric restriction regimen at one year of age have lower rates of lymphoma than ad lib fed mice (Weindruch and Walford, 1988). Rodents on a restricted diet have lower blood glucose and insulin levels, show an increased sensitivity to insulin and a marked decrease in body temperature, all of which can reduce the metabolic rate (Sohal and Weindruch, 1996). The decrease in body temperature is due to both a decrease in metabolic rate and lower stores of carbohydrate and lipid stores in organisms fed only the minimum amount of calories necessary for survival. Increased starvation resistance is also found in flies selected for longer life and is attributable to a decrease in metabolic rate caused by starvation (Chippindale, Chu and Rose, 1996). Caloric restriction may increase life span by reducing metabolic rate that in turn reduces the amount and accumulation of oxidative damage.

Recent studies have implicated caloric restriction in modifying normal age-related gene expression. In one study, the expression profiles of 6,347 genes were studied in the gastrocnemius muscle of control 5-month and control 30-month mice as well as 30-month mice that had been subjected to caloric restriction without nutritional deficiency (Lee *et al.*, 1999). The caloric restriction treatment produced great changes in the expression profile of the mice with 29% of age-related changes being prevented and 34% suppressed to some degree (Lee *et al.,* 1999). Pletcher found similar result with once-mated female *Drosophila melanogaster* with 885 transcriptional changes by age and 827 changes by caloric restriction, of which 448 were common between the two treatments (Pletcher *et al.,* 2002). Furthermore, there was a direct relationship between the direction of the expression change in both treatments (Pletcher *et al.,* 2002). Genes down regulated by caloric restriction included those

involved in DNA repair and replication, cell cycle control, protein metabolism and oogenesis (Pletcher *et al.,* 2002).

Another study focused on expression profiles of 11,000 genes in the liver of young and old mice subjected to either a control, short term, or long term caloric restriction. The experiment was designed to be able to tease apart changes due to aging versus those due to aging in combination with caloric restriction. They first compared gene expression in the young and old mice, finding that of the genes with increased expression in old mice, 40% were involved in inflammation and 25% were involved with stress response or molecular chaperones (Cao *et al.*, 2001). Of the genes with lowered expression in the old mice, 26% where involved in cell cycle control or DNA replication indicating the cells inability to regulate cell division leading to hepatoma, a form of liver cancer (Cao *et al.,* 2001). Comparison of the affect of long-term caloric restriction to the profiles for control young and control old mice, showed the restricted diet negated the normal age-related increase in 75% of the genes whose expression increases with normal aging (Cao *et al.,* 2001). Genes included in this set encode stress response proteins and chaperones (Cao *et al.,* 2001). A corresponding increase in the time of onset of hepatoma was also seen (Cao *et al.,* 2001). When short term and long term caloric restriction are compared, the short treatment (4 weeks) shows approximately a 30% decrease in the effects of the gene expression that increases with long-term caloric restriction. Kayo and colleagues (2001) did a similar experiment by analyzing the expression profiles of skeletal muscle from young ad lib fed, aged ad lib fed, and aged calorically restricted rhesus monkeys. While others had found that

80% of age-related changes in transcriptional expression could be suppressed by early-onset caloric restriction (Lee *et al.,* 1999), Kayo and colleagues (2001) did not see any expression changes after adult-onset caloric restriction that suppressed the normal aging profile and therefore, hypothesize that caloric restriction must begin earlier in life to be beneficial. Furthermore, aging up-regulated transcripts in inflammation and oxidative stress pathways as noted in the mouse experiment, but down-regulated genes involved in mitochondrial electron transport (Kayo *et al.,* 2001). Caloric restriction increased transcripts of structural and cytoskeletal protein coding genes while also decreasing the levels of gene transcripts involved in mitochondrial energy production (Kayo *et al.,* 2001). Combining the results of the microarray experiments discussed above leads to the conclusion that caloric restriction mitigates age-related stress by increasing the transcripts of genes involved in either heat stress or oxidative stress as well as decreasing mitochondrial activity, thereby protecting the cell from damage due to metabolism related oxidative stress.

Caloric restriction has another mechanism by which it can delay the onset of aging -- neuroprotection. Astrogliosis is a condition in which the brain is damaged due to increased concentrations of glial fibrillary acidic protein (GFAP) in the astrocytes of the brain (Nichols *et al.,* 1995). While levels of GFAP mRNA are similar in two groups of young rats, maximum transcript levels for rats fed an unrestricted diet occurred at 25 months, compared to 33 months for rats on a calorically restricted diet (Nichols *et al.,* 1995). These levels correspond to mean life span of 23.4 months for rats on an unrestricted diet, while the restricted rats lived 35 months on average (Nichols *et al.,* 1995). Nichols and colleagues concluded that increased GFAP mRNA levels could serve as a biomarker of aging, and that

food restriction provides neuroprotection by delaying the age-related deposits of GFAP in the rat hypothalamus.

While there are beneficial effects of caloric restrictions, there are also detrimental effects. Patel and Finch (2002) note that caloric restriction is associated with increased life span and provides a form of neuroprotection in the brain, but it also increases the level of blood corticosterone (CORT) which can result in cognitive impairment and atrophy of the hippocampus. During normal aging there is a slow but progressive increase in the levels of CORT found in the blood, which is concurrent with a decrease in cognitive function and memory in humans, and a decrease in maze performance and atrophy of the hippocampus in rodents (Patel and Finch, 2002). These decreases in brain function can be as mild as decreasing the speed of information processing to as severe as full-blown dementia seen in Alzheimer's patients (Patel and Finch, 2002). Normal aging pathology in rats is marked by a decrease in blood flow and microvasculature to the brain which can be overcome by dilating the blood vessels through a caloric restriction regimen (Goldman *et al.,* 1987; Smith *et al.,* 1980). However the stress of caloric restriction increases CORT levels, causing a decrease in glucose uptake which leads to hypoxic and hypoglycemic damage to the brain (Tombaugh *et al.,* 1992). Patel and Finch (2002) hypothesize that the beneficial effects of caloric restriction including maintenance of the microvasculature of the brain, decreasing oxidative stress through the activation of a stress response pathway, as well as neural protection through other stress proteins far outweigh any negative effect of increased CORT levels during caloric restriction .

METABOLISM, INSULIN SIGNALING AND AGING

Metabolism and factors affecting metabolism have been shown to affect aging in yeast, flies, mice and worms. In yeast, the shift in metabolism from glycolysis to gluconeogenesis and energy storage can be used as a biomarker for aging (Lin *et al.,* 2001). The shift in metabolism of yeast can be affected by caloric restriction and by a retrograde response (Jazwinski, 2000). When yeast mitochondria dysfunction, the proteins RTG1, RTG2 and RTG3 move into the nucleus and, with modulation from RAS2, stimulate a retrograde response pathway (Jazwinski, 2000; Kirchman *et al.*, 1999). When the pathway is initiated, energy production is shifted away from the Krebs cycle and into the glyoxylate cycle where Krebs intermediates can be produced from acetate which conserves energy (Jazwinski, 2000). When calories are restricted and RAS2 is functional, mitochondria have difficulty producing energy and trigger the retrograde response pathway. The shift in metabolism conserves carbon, but allows NAD to be available which is then used by Sir2. Sir2 is an NAD-dependent histone deacetylase which may affect gene expression by modifying chromatin structure (Guarente, 2000). Sir2 is implicated in telomere function and DNA repair, both of which play a role in regulation of gene expression and will be discussed later (Gottschling *et al.,* 1990). The relationship between increased longevity and the function of Sir2 and the availability of NAD has been confirmed in a yeast caloric restriction experiment (Lin *et al.,* 2000). During aging, as RAS2 and RTG expression drops, the retrograde pathway cannot be initiated, NAD is used in glycolysis, and Sir2 is prevented from maintaining proper chromatin structure. Now inappropriate gene expression has been allowed which leads the loss of homeostasis and death for the yeast cell.

Drosophila metabolic pathways have been studied mainly from the point of insulin-like signaling. The insulin-like growth factor (IGF) pathway of flies starts with an insulin-like receptor (InR). Females carrying hypomorphic mutations of InR are dwarf and deficient in juvenile hormone but have an 85% extension in life span (Tatar *et al.,* 2001). It is thought that the relationship between receptor and JH may extend life span through decreasing metabolism thereby slowing growth and development (Tatar *et al.,* 2001). InR also interacts with CHICO, the insulin receptor substrate (Clancy *et al.,* 2001). Homozygous mutations at *chico* increase female life span by 48% while heterozygous mutations cause a 36% increase without producing dwarf flies and while maintaining fecundity (Clancy *et al.,* 2001). Phosphatidylinositol-3-kinase (PI3K) and protein kinase B also play a role in insulin signaling, but their characterization is best described in the dauer larva formation pathway of *C. elegans.* Another factor plays a role in the IGF pathway of *Drosophila*. The cleverly named I'm-Not-Dead-Yet (*Indy*) gene shows homology to a mammalian sodium dicarboxylate co-transporter which transports succinate, citrate, and alpha-keto-glutarate produced during the Krebs's cycle (Rogina *et al.,* 2000). *Indy* expression is greatest in the fat body and mid-gut of flies which are the sites of highest metabolic activity (Rogina *et al.,* 2000). Mutations in this gene produce a decreased metabolic state similar to the one in caloric restriction which increases life span maximally in heterozygotes and to some extent in homozygotes (Rogina *et al.,* 2000). There are no decreases in fertility, fecundity, or behavior in these mutants indicating that the decrease in metabolic rate increases longevity directly rather than being caused by a trade-off in another life history trait (Rogina *et al.,* 2000).

Mammals have a more complex insulin signaling pathway with two distinct receptors as well as a third of unknown function (Clancy *et al.,* 2001). Mice engineered to have the fat-specific insulin receptor knocked out (FIRKO) show a reduction in fat mass although their food intake is normal (Blüher *et al.,* 2003). FIRKO mice are protected against obesity, changes in glucose tolerance and metabolic abnormalities associated with age and show an 18% increase in mean life span when compared to wild-type mice (Blüher *et al.,* 2003). It is hypothesized that the changes produced in the insulin-signaling pathway by FIRKO mice results in increased life span (Blüher *et al.,* 2003). Mice with a mutation at the *klotho* locus have very little white adipose, but maintain their brown adipose tissue and have increased glucose tolerance and insulin sensitivity (Mori *et al.,* 2000). Such mice have a lower body temperature indicating a decrease in metabolic rate which is similar to what occurs during caloric restriction (Mori *et al.,* 2000). The longevity of these *klotho* mutant mice has not been measured, but one would predict an extension in life span.

It is clear that metabolic rate affects life span regardless of whether the changes are due to the retrograde response pathway in yeast, or insulin signaling in flies or mice. In *C. elegans,* longevity is inversely correlated with metabolic rate, strengthening the relationship between the two factors (Van Voorhies and Ward, 1999). Caloric restriction or mutations can cause shifts in metabolism. It follows that lowering metabolism extends life in some organisms as it decreases oxidative damage and even changes gene expression which overlaps the metabolic pathway with aging pathways. But there are exceptions and complexities as well. Bats live up to ten times as long as rodents, but they have similar

metabolic rates, indicating that there are more factors involved (Guarente, 2000). Let us now consider a system that combines environmental stress resistance as well as insulin signaling.

DAUER LARVA FORMATION

C. elegans normally undergoes four stages of larval development before reaching adulthood, but there is also an alternate larval form that enables the worms to survive in stressful environments. When the temperature is high, food supplies are low and larval density is high, *C. elegans* will shift its metabolism to store energy and become dauer larvae (Riddle and Albert, 1997). Dauer larvae are developmentally arrested, do not feed and do not reproduce giving them a unique morphology and physiology compared to that of normal adults (Lithgow, 1996). Mutations in the dauer formation (*daf*) pathway also provide a protective mechanism and have increased uv, heat and oxidative stress resistance (Lithgow, 1996). Individuals that have been through a dauer stage show an increase in life span that is equivalent to the time spent in the arrested stage. Specifically worms that go through the dauer stage can live up to eight times longer than animals that do not arrest developmentally (Tissenbaum and Ruvkun, 1998). However, the time spent in the dauer stage does not affect the post-dauer life span in most cases, but rather, the time in the dauer stage is added to the normal life span (Klass and Hirsch, 1976).

There are two types of *daf* mutants. There are constitutive *daf (daf-c*) mutations that become dauer forms even though conditions are favorable. These mutants arrest in the dauer stage even though the environment is lacking the stress needed to trigger normal dauer development. The other mutations are dauer defective (*daf-d*) and will not arrest

developmentally even under the harshest conditions. While there are many *daf* genes which form a complex web of interactions, three of these genes have been characterized extensively: *daf-16, daf-2* and *age-1*, all of which play a role in insulin/IGF signaling.

Daf-16 is a dauer-defective gene which prevents dauer formation during stress conditions (Larsen *et al.,* 1995). A mutation screen uncovered 24 different alleles of *daf-16,* all of which suppress the phenotype of *daf-2* mutants (Lin *et al.,* 1997). Worms carrying a transgene coding for DAF16 grow and reproduce more slowly than control worms, but have much greater stress resistance (Henderson and Johnson, 2001). DAF-16 accumulates in the nucleus and increases life span under stresses including starvation, heat stress, and oxidative stress (Henderson and Johnson, 2001) as well as when the germline cells are ablated or the sensory neurons are stimulated (Lin *et al.*, 2001). As DAF-16 is a HNF-3/forkhead transcription factor (Lin *et al.,* 1997), nuclear accumulation may signal changes in gene expression which are prevented under non-stress conditions by the presence of the *daf-2* gene product (Lin *et al.,* 2001). However, simply changing the phosphorylation pattern of DAF-16 causes nuclear accumulation but has no effect on life span, but life span extension through the insulin/IGF pathway cannot occur without expression of *daf-16* (Lin *et al.,* 2001).

Daf-2 codes for the only insulin-like receptor in *C. elegans* and shows homology to *InR* in *Drosophila* (Murakami and Johnson, 2001; Clancy *et al.,* 2001). At permissive temperatures, *daf-2 (e1670)* mutants do not enter the dauer phase, have normal fertility and viability and have a life span twice that of wild-type worms (Kenyon *et al.,* 1993). At non-permissive temperatures, the *daf-2 (e1670)* mutant still has extended life span but have reduced fertility and viability (Tissenbaum and Ruvkun, 1998). Complete lack of DAF-2

activity causes worms to remain in the dauer stage (Lin *et al.*, 1997). While different *daf-2* alleles can cause a great deal of phenotypic changes, it is thought that the primary function of DAF-2 is to suppress the function of DAF-16 (Riddle *et al.*, 1981). RNAi experiments have identified the ligand of DAF-2 as the insulin/IGF-like homolog encoded by the gene *Ceinsulin-1* (Kawano *et al.*, 2000). Upon binding its ligand, normal DAF-2 activates a kinase which changes the phosphorylation of DAF-16, thereby stopping nuclear localization of DAF-16 and shortening life span (Lin *et al.*, 2001). When DAF-16 is present, and the activity of DAF-2 is reduced, life span can be extended up to two times that of a wild-type worm (Gerisch *et al.*, 2001). Furthermore, if stress detection by sensory neurons is blocked, DAF-2 levels decrease and life span is extended (Guarente and Kenyon, 2000).

Age-1 was initially discovered in a screen for genes which increased worm survivorship at various ages and temperatures (Friedman and Johnson, 1987). At 20^0, *age-1* mutants can increase average life span by 40% and maximum life span by 60%; at 25^0 the same mutation can further extend mean life span 15% and maximal life span 50% (Friedman and Johnson, 1987). Furthermore, *age-1* mutants show increased resistance to heat stress, oxidative stress and uv damage (Litghow, 1996). It is now known that *age-1* encodes the nematode PI3K (Murakami and Johnson, 2001), and is also known as *daf-23* (Hekimi *et al.*, 1998). The wild-type function of both *age-1* and *daf-2* causes the worm to enter the dauer stage regardless of the presence of stress pheromone, and are therefore known as constitutive dauer formation genes. Like *daf-2*, but unlike any other genes who cause constitutive dauer formation, mutants of *age-1* can increase adult life span (Kenyon *et al.*, 1993).

Now let us consider how these factors work together. RNAi studies have shown that

factors which extend life span must act downstream of *daf-2, age-1* and the kinase responsible for the proper phosphorylation of DAF-16 (Dillin *et al.,* 2002-b). While the model is not complete, combining the results of the experiments above, gives a partial pathway. CEINSULIN-1 binds to the insulin/IGF-1 homolog DAF-2. DAF-2 may signal for the function of PI3K (*age-1* gene product) which changes the phosphorylation pattern of DAF-16. This change prevents nuclear accumulation of DAF-16. When the environment is right, phosphorylation of DAF-16 would be restored, and the transcription factor would be able to change gene expression to respond to stress. Murakami and Johnson (2001) have shown that *daf-16* can regulate the expression of *old-1* which induces stress resistance leading to life span extension. There may also be regulation in the opposite direction as it is thought that *daf-16* may regulate gene expression during stress by triggering a molecular messenger to be released from cells in the insulin/IGF signaling pathway which bind to neuronal DAF-2 (Gems and Partridge, 2001). This is an incomplete and much simplified model of a very complex pathway as there are many other *daf* genes which will not be discussed here. A study of the expression profiles of 11,130 genes showed that 2,618 were only detected in non-dauer larvae, while 2,016 transcripts where only detected in dauers giving a very complex gene expression profile (Jones *et al.,* 2001).

Daf mutants not only play a role in insulin/insulin-like signaling, but also have a wide variety of effects. Mutants in this pathway show increased resistance to stress, increased levels of superoxide dismutase, lower metabolic rate and consequently, lower rates of protein carbonyl accumulation (Yasuda *et al.,* 1999). This lends support to the mutation accumulation theory of aging with the mutations being caused by oxidative stress. However,

another experiment has separated life span from metabolic rate. Wolkow and colleagues (2000) used different promoters to express either *daf-2* or *age-1* mutants in neurons, muscle or intestine cells of worms. Their findings indicate that restoration of the insulin/IGF-1 signaling pathway in neurons could restore life span, but restoring the pathway solely in muscle cells rescued metabolic defects but not life span, uncoupling life span and metabolic rate (Wolkow *et al.*, 2000). It is evident that genes in the dauer formation pathway confer a longer life span than is seen in non-dauer larva due to changes in the physiology of the arrested stage of development including up-regulation of antioxidant mechanisms, lower metabolic rate and slower accumulation of oxidative damage. In a subtractive library experiment, five of six genes that were up-regulated more than 3-fold in dauer larvae also showed up-regulation of the same magnitude during aging (Cherkosova *et al.*, 2000). Dauer larvae formation and insulin/IGF pathways are complex and overlap with aging pathways in *C. elegans*.

GENETIC MECHANISMS OF AGING

As both environmental and genetic factors contribute to aging, next we shall consider the genetic mechanisms of aging which have been determined through the study of mutations at candidate genes. Mutations which decrease life span indicate genes that are required for normal life span, while mutations which increase life span indicate genes that normally limit life span. Studies of mutated genes which affect aging include those encoding proteins which protect the cell by methylating or repairing DNA, maintain the length of the

telomeres, provide energy for the cell in the mitochondria or protect the cell from oxidative, thermal or other stresses.

METHYLATION

As DNA is replicated, methyl groups are added to specific cytosine residues on some regions of the new strand. Regions of chromosomes, rather than specific genes, undergo methylation with the relationship between chromatin structure, methylation and gene expression being complex. Heterochromatic regions of the chromosome are characterized by abundant methylation and no transcription, whereas euchromatic regions are under-methylated and transcriptionally active (Razin, 1998; Cedar, 1988). For example, transcription of housekeeping genes is integral for the survival of every cell, and consequently these under-methylated genes are found in euchromatic regions of the chromosome. Specifically, DNA methylation causes histone deacetylation resulting in the heterochromatic structure that prevents transcription (Bird and Wolffe, 1999). A 5-methylcytosine followed by a guanine pattern constitutes the specific pattern of methylation which is thought to change the conformation of DNA (Catania and Fairweather, 1991). Furthermore, the protrusion of methyl groups into the major groove of DNA changes its conformation interfering with the binding of many transcription factors.

Any change in the relationships of DNA and transcription factors will affect gene expression. The levels of methylation fall during cellular aging. This decrease may be due to changes in the enzyme responsible for methylation, C5m-transferase, which shows a decrease in efficiency during cellular aging (Catania and Fairweather, 1991). As levels of 5-

methylcytosine fall, genes will be turned on when they should not be functioning, a change with the potential to affect life span. As the number of cell divisions increases, the levels of 5-methylcytosine decrease, triggering cellular senescence. This hypothesis can be tested by preventing methylation, by providing the drug 5-azacytidine to proliferating cells, which will be incorporated instead of cytosine into newly synthesized DNA strands. The functional group at the 5' end of 5-azacytidine cannot be removed, preventing methylation. In such experiments, a twenty-percent drop in methylation levels decreases the average life span of human fibroblast cells by twenty-five percent (Holliday, 1986; Fairweather *et al.*, 1987). Gradual loss of 5-methylcytosine upon multiple rounds of cell division may contribute to the mitotic clock that signals cellular senescence. Noting that levels of gene expression are not connected to methylation in all organisms is important. In Dipteran insects such as *Drosophila*, DNA is not methylated, leading to the conclusion that age-related changes in gene expression must be controlled by other systems. As of yet, this mechanism is unknown.

Recent interest in studies of methylation has been in connecting patterns of methylation with disease, specifically cancer. Cancer cell DNA is under-methylated compared to normal cells, leading to hyper-expression of some genes (Feinberg and Vogelstein, 1983). Also, there is hyper-methylation of tumor suppressor genes compared to that of normal cells which allows hypertrophy of cancer cells (Baylin and Herman, 2000). DNA is methylated by DNA methyltransferases (DNTMs) with DNTM1 being responsible for duplicating the pattern of the old strand onto the new strand during replication (Szyf, 2001). Given the problems with hyperactive cell division in tumorogenesis, regulation of DNTM1 is a natural candidate for stopping transformation of normal cells into cancer cells.

As of yet, no changes in cell cycle regulation have been noted in embryonic stem cells which are null at the *Dnmt1* locus (Li *et al.*, 1992), and there are conflicting reports on the concentration of DNTM1 in cancer cells versus non-transformed cells (El-Deiry *et al.*, 1991). The concentration, activity and functions of DNTM1 in cancer cells must be very complex. Inhibiting DNMT1 by direct or pharmacological means or by lowering the *Dnmt1* expression in knockout mice has been shown to have anti-tumorogenic effects (Laird *et al.*, 1995; Bigey *et al.*, 1999). Szyf (2001) hypothesizes that changing the pattern of DNA methylation through pharmacological action on DNMTs may be a significant advancement in the treatment of cancerous tumors. The emphasis on genetic factors in anti-cancer medicine is in its infancy; it will be fascinating to see the future developments.

TELOMERES

Telomeres are the highly conserved complexes of DNA and protein found at the termini of linear chromosomes. The ends of chromosomes cannot be fully replicated, leading to a shortening of the chromosome with each cell division. The loss of the telomeres may function as a mitotic clock. Shortening of the telomere is due to the activity of DNA polymerase III. During DNA replication one new strand will be synthesized in the normal 5' to 3' manner and will be as long as the original molecule. However, the lagging strand is synthesized through Okazaki fragments. An RNA primer with a free hydroxyl group is needed at the 3' end to start polymerase activity, but the primer will not be placed at the very end of the second newly replicating strand. This leaves an overhang of the old strand. When cell division occurs, one daughter cell will receive the normal DNA molecule while the other

will receive the one with the 3' overhang. The exposed end is then vulnerable to digestion by nucleases resulting in the loss of part of the telomere. Losing some length from the telomere does not affect the cell since telomeres consist of up to twenty-five kilobases of short, tandomly repeated sequence which is conserved throughout evolution. It is estimated that between fifty and one-hundred base-pairs of telomere is deleted per cell generation in human fibroblasts (Sedivy, 1998). After many generations of cell division, at least one chromosome will have a critically shortened telomere, stopping further cell division. However, the shortening of some chromosomes is not associated with aging. In humans, the shortening of 17p is not associated with cellular senescence, but shortening of the telomeres on chromosomes 1p, 5p and 22p are associated with senescence (Blasco *et al.*, 1999). This finding is particularly interesting in that the telomere region of 17p is initially shorter than the others studied (Martens *et al.*, 1998).

It has long been known that the shortening of the telomeres triggered senescence, but the mechanism for this change remained elusive. The cascade is now known. Progressive telomere loss leads to shortening of the chromosomes resulting in telomere fusions between chromosomes. End-to-end fusion produce dicentric or circular chromosomes which line up along the cell midline during mitosis. These unusual chromosome structures are seen at a frequency of 30 to 70% in senescent cells (Allsopp, 1996). At anaphase, these chromosomes are broken producing more single-stranded ends which repeat the cycle (Hackett *et al.,* 2001). Eventually a catastrophic loss of gene function or chromosome loss will trigger a DNA damage pathway resulting in up-regulation of the tumor suppressor p53 (Blasco, 2003). From there, p53 up-regulates p21 and other inhibitors of cyclin dependent kinases resulting in

the hypophosphorylation of pRB (Vaziri and Benchimol, 1996). The cell cycle cannot progress from G1 to S phase without proper activity of pRB. Lack of phosphorylation impedes pRB activity, triggering cellular senescence and, in some cases, apoptosis (Vaziri and Benchimol, 1996; Blasco, 2003).

Cell senescence and apoptosis can also be triggered by the telomeres regardless of the length of the telomeric regions. Telomere function depends on the maintenance of the tandomly repeated sequences as well as proper function of a group of proteins which bind to this sequence. In the yeast telomere, Cdc13p and Est1 help recruit telomerase, while Rap1 binds to the telomere and interacts with Rif1, Rif2 and dimer of Ku70/80 as well as three different Sir proteins (Blasco *et al.*, 1999). The Sir proteins, Rap1, and the the Ku70/80 dimer have been shown to play roles in repairing DNA breaks in the region, while Sir proteins may also participate in protecting the single-stranded ends from exonculease activity (Blasco *et al.*, 1999). In mice, mutations in telomere binding proteins TRF2, Ku86, and DNA-PK_{cs} causes telomere dysfunction even when the telomeres have not been shortened to a critical length (Blasco, 2003). Similar results were found when Van Steensel and colleagues over-expressed two truncated forms of TRF2 in human tissue culture cells. Truncated TRF2 lacking a C-terminal Myb domain caused end-to-end fusions, while TRF2 lacking an N-terminal basic domain did not produce fusions (Van Steensel *et al*, 1998). The end-to-end fusion chromosomes produced by the C-terminal mutant TRF2 contained telomeric sequences, indicating that TRF2 maintains proper conformation of telomeres and may be as important as the number of repeats remaining (Van Steensel *et al.*, 1998).

Further evidence is given by experiments that look not at the effects of shortening telomeres, but the effects of lengthened telomeres. Telomerase is an enzyme that has reverse transcriptase activity and can, therefore, increase the length of the telomere. In yeast and slime molds, telomerase appears in every cell and is responsible for maintaining a balance between telomere elongation and deletion. Because these organisms reproduce by budding, all their cells are germ cells and must have the proper length of telomeres. If telomere length is not maintained in the mother cells, the daughter cells would be generated with shortened telomeres and would reach replicative senescence prematurely. Even with normal levels of expression, the telomeres will be critically shortened after many cell generations, and the mother cell will no longer divide.

In yeast, telomerase activity is coupled with chromosomal replication in the S phase of the cell cycle, lengthening the telomere by up to fifteen base pairs per generation (Blasco *et al.,* 1999). Furthermore, a feedback inhibition mechanism must be in place as telomerase activity has been shown to decrease as the number of repeats increases (Blasco *et al.,* 1999). Telomerase also plays a role in regulating the mutation rate by inhibiting chromosome instability (Hackett *et al.*, 2001). Hackett and colleagues (2001) noted that the mutation rate in the CAN1 gene of yeast increased between 10 and 100 fold as telomerase became dysfunctional resulting in the end-to-end fusions discussed above. Perhaps the dysfunction of telomeres is not just responsible for the mitotic clock but also for the increase in mutations accumulated during aging of the yeast cell.

Telomerase activity is also important in mammalian germ cell lines, as it has been shown that telomerase deficient mice have extremely low fertility (Herrera *et al.,* 1999).

Mice null for telomerase could only be bred for four generations with half of the mice in the final generation dying at only five months of age (Herrera *et al.,* 1999). Besides infertility, mice with a null mutation at telomerase in a genetic background of shortened telomeres showed atrophy of the spleen, immune system failure, abnormal hematology as well as an increase in tumorogenesis (Herrera *et al.,* 1999).

The role of telomerase in humans is not as clear. The presence of telomerase was first noted in human sperm and tumor cell lines including the cervical carcinoma line HeLa and a line of metastatic ovarian carcinoma cells (Allsopp *et al*, 1992; de Lange, 1994; Harley, 1991). The cancer cell lines never reach the critical minimum telomere length and are said to be immortal, but most normal somatic cells have insufficient telomerase activity and will eventually suffer from the effects of shortened telomeres (Blasco, 2003). Human fibroblast cells culture lack telomerase and have a finite number of cell division before senescence (Bodner *et al.,* 1998), but 90% of human tumors have telomerase activity allowing for continued cell division (Hiyama and Hiyama, 2001).

Inhibition of telomerase activity in cancer cells may arrest cancer growth, while activation of telomerase in normal aged cells may be a way to increase the life span of such cells and decrease the incidence of age-related disease (Bodner *et al.,* 1998). The latter hypothesis has been tested on cell lines cultured from patients with dyskeratosis congenita (DKC), a disease which presents initially as a skin pigmentation and nail growth disorder and progresses to common age-associated disorders such as pulmonary disease, osteoporosis, and cancers of the skin and blood (Marciniak *et al.,* 2000). Human telomerase requires a catalytic subunit, a telomerase reverse transcriptase and RNA template (hTR). It has been shown that

telomeres are unusually short and hTR concentration is low in DKC patients (Marciniak *et al.,* 2000). The shortening of telomeres during subsequent rounds of division and the lack of telomerase activity in somatic cells may be one mechanism contributing to the senescence attributable to the mitotic clock as modeled through DKC.

OXIDATIVE STRESS

It has been shown that reactive oxygen species (ROS) and free radicals can damage DNA and protein, and are therefore implicated in senescence and age-related diseases. An estimated two to three percent of oxygen used in the cell will be turned into ROS, which can attack other regions of the cell (Sohal and Weindruch, 1996). During cellular respiration, electrons are donated to O_2 to form the reactive superoxide anion $O_2^{-}\cdot$. These ROS will migrate through the cell trying to find a place to relieve their charge. Exchange of charge between the ROS and either DNA or protein will cause mutations in the former and may reduce or destroy the efficacy of the latter. The cell must have an enzymatic system to take away the toxicity of the reactive species generated by cellular respiration. The following reactions compose the cell's antioxidant mechanisms. First, the superoxide anion is reduced to hydrogen peroxide (H_2O_2) by the enzyme superoxide dismutase (SOD). Hydrogen peroxide is less toxic than the superoxide anion, but in the presence of a transition metal ion, it can form the extremely toxic hydroxyl radical, $OH\cdot$. Further reduction of hydrogen peroxide to water is critical and is accomplished by the enzyme catalase. Glutathione peroxidase can also undertake this final reduction. The enzymes catalase, superoxide dismutase and glutathione peroxidase are the cell's greatest weapons in reducing the threat of

damage by ROS. Evidence for the role of oxidative stress in aging comes from an experiment in which human diploid fibroblast cells where grown under either 3% oxygen (the normal physiological level) or 20% oxygen (the normal oxygen concentration of air). The cells grown at the lower oxygen concentration showed a 50% increase in population doubling compared with cells maintained at 20% oxygen (Chen *et al.,* 1995). Chen and others also noted a dose-dependent increase in population doubling with the application of the antioxidant alpha-phentyl-tetra-butyl nitrone, which supports the hypothesis that oxidative stress is a key component in the cessation of division in human fibroblast cells (Chen *et al.,* 1995). Furthermore, it has been noted that extracellular over-expression of SOD improves the acquisition, reacquisition and retention time of aged mice in a maze, indicating a neural protective role for SOD (Levin *et al.,* 2002). In humans, a neuroinflammatory response increases the level of nitric oxide in the brain causing an increase of oxidative damage to both neural proteins and DNA (Floyd and Hensley, 2002). This damage is cumulative and leads to an increased risk of stroke, impairment of cognitive function, and in some cases, the plaque deposits seen in Alzheimer's and other age-related diseases (Floyd and Hensley, 2002).

Many studies have been undertaken to explore the relationship of antioxidant enzymes with life span. In one experiment, strains of *Drosophila melanogaster* selected for increased longevity had greater than normal levels of the product of the antioxidant gene, Cu-Zn SOD (Arking, 1998). Other researchers have determined that lack of a functioning *Sod* allele greatly reduces life span in fruit flies (Parkes *et al.*, 1998-a). In addition, transgenic flies were engineered to over-express human cytosolic SOD1 in the motorneurons. The

authors found that over-expression led to a forty percent increase in life span, and expression of the transgene could rescue the life span of a *Sod* loss of function mutation (Parkes *et al.,* 1998-b). Expression of both the *Sod1* and catalase transgenes has also been shown to increase life span up to thirty-percent (Orr and Sohal, 1994). The Sohal laboratory has also shown that *Drosophila* which over-express CAT as well as Cu-Zn SOD show slower rates of mitochondrial hydrogen peroxide production but increased metabolic capacity than in control flies, providing a mechanism for how expression of CAT and SOD can increase life span (Sohal *et al.*, 1995-b). While over-expression of CAT and SOD increases life span in *Drosophila*, we cannot know how this would affect the human system. Glutathione peroxidase has been implicated in human longevity for some time because it has enzymatic capabilities similar to that of catalase, but there is no experimental evidence to support this hypothesis.

In *Drosophila melanogaster*, three other genes affect life span through oxidative stress mitigation: *rosy, methuselah,* and *thioredoxin reductase.* The first two were tested, not by over-expression, but by knocking out gene expression and exposing the flies to hyperoxic conditions. *rosy* is the structural gene for xanthine dehydrogenase. This enzyme catalyzes the formation of urate, a scavenger of oxygen radicals. Flies lacking a functional *rosy* allele have very short life spans when exposed to paraquat, which is a source of exogenous free radicals (Hilliker *et al.*, 1992). Corresponding tests have been conducting using the gene *methuselah* with similar results. The line containing the *methuselah* mutation has a life span thirty-five percent greater than the parental strain and has increased resistance to oxidative stress as measured by the paraquat assay (Lin *et al.*, 1998). The Methuselah

protein is currently under investigation, but it is thought to be a regulator of a G-protein associated with stress response and aging (Lin *et al.,* 1998). The *dmtrxr-1* gene codes for thioredoxin reductase (TrxR), originally thought to be the *Drosophila* homolog to glutathione reductase and provides antioxidant function by transferring reducing equivalents from NADPH to both thioredoxin and glutathione disulfide rather than directly participating in the detoxification of the superoxide anion (Missirlis *et al.,* 2001). Flies carrying null mutations of *dmtrxr-1* show an increased free radical induced damage with decreased adult life span (Missirlis *et al.,* 2001).

Another gene is implicated by a study of mice with a mutant allele at the methionine sulfoxide reductase locus, which show reduced life span under both normal and hyperoxic conditions, and have increased levels of oxidative protein damage (Moskovitz *et al.,* 2001). It is important to note that this enzyme functions as a repair enzyme for methionine residues in proteins and not as a method of detoxifying the superoxide anion. Oxidative stress is also connected to insulin based metabolism as mice heterozygous for a mutation of the insulin-like growth factor type 1 receptor (IGF-1R) lived longer than their wild-type siblings but had normal energy metabolism and increased oxidative stress resistance (Holzenberger *et al.,* 2003).

There is great interest in how oxidative stress affects gene expression. Rogina and Helfand (2000) showed that the temporal pattern of gene expression at the *wingless* locus in *Drosophila melanogaster* is accelerated in flies homozygous for a null mutation of Cu-Zn SOD. First, the experiment shows a decrease in life span in the *Sod* mutant flies (Rogina and Helfand, 2000). Then by using a *wingless* enhancer-trap stock and plotting β-gal expression

as a function of percent of life span, they observed the Sodn1/Sodn1 curve to be identical to the control, indicating that *wingless* is expressed in the same manner but at an earlier time in the mutant flies (Rogina and Helfand, 2000). Further evidence for the role of oxidative stress in aging is provided by a microarray study in which transcript levels of aging flies were compared with that of flies subjected to a paraquat treatment (Zou *et al.*, 2000). While expression of some genes was affect by either aging or hyperoxic conditions, expression patterns for 42 genes were changed by both aging and paraquat treatment. These genes encode proteins as diverse as protease inhibitors involved in protein turnover and accessory proteins involved in reproductive potential (Zou *et al.*, 2000). Decreases in protein turnover and reproduction are hallmarks of aging, indicating that changes in transcription levels in response to oxidative stress are similar to those seen in the normal aging process. The proteasomal system is responsible for removing damaged proteins from the cell, but Grune (2000) observed a decrease in activity of proteasome subunits under oxidative stress, leading to a decrease in protein turnover. A review by Allen and Tresini (2000) lists over 100 genes or proteins that show changes in expression or activity when stimulated by hydrogen peroxide or paraquat or when the expression of antioxidant genes are changed. This finding implicates oxidative stress as a significant factor in not only aging but in pathways from development, to metabolism, to hormone signaling.

MITOCHONDRIA

Mitochondria, the powerhouse of the cell and the location of cellular respiration, have the greatest potential for age-related oxidative damage. The mitochondrial genome of many

species contains two types of genes: a set that is responsible for converting toxic reactive oxygen species (ROS) and free radicals into less harmful derivatives and another set of genes responsible for creating the products needed for cellular respiration. Manganese superoxide dismutase belongs to the former category for it changes the toxic O_2^- into the less harmful product hydrogen peroxide, as discussed previously. In the latter group are the structural genes for cytochrome C oxidase, cytochrome B and the other enzymes necessary for oxidative phosphorylation. From an evolutionary standpoint, this makes perfect sense. The products encoded by mitochondrial genes are responsible for creating the highest levels of ROS, while other mitochondrial encoded proteins are responsible for eliminating them. However, levels of expression of the anti-oxidant mechanisms are not great enough to counteract the levels of ROS production.

Mitochondrial DNA is not protected by histones (Wei *et al.,* 1998), and produces 90 % of the ROS in the cell (Berdanier and Everts, 2001). Age-related accumulation of mutations in the mitochondria may occur in two ways. Deletion mutations are common as there is slippage when repeated sequences of mitochondrial DNA are replicated (Madsen *et al.,* 1993). Secondly, during aging the expression of mitochondrial proteins dramatically decreases (Calleja *et al.,* 1993), dropping levels of SOD to the point where it can no longer remove free radicals (Berdanier and Everts, 2001). The mitochondrial genome encodes all the proteins necessary for base excision repair within the mitochondria (Mandavilli *et al.,* 2002), but they too are subjected to damage by ROS. Having one mutated mitochondria out of many will not disrupt any cell function, but it is possible for the cell to go from one or a few mutant mitochondria (heteroplasmy) to all mutant mitochondria (homoplasmy), which is

fatal for the cell. Normal mitochondrial DNA is replicated five to ten times faster than nuclear DNA, which decreases the likelihood that all mitochondrial DNA repairs will be completed before replication (Bernadier and Everts, 2001). Furthermore, it has been suggested that the shortened strands of deleted mitochondrial DNA may be replicated with an even higher frequency than normal mitochondrial DNA, leading to homoplasmy (Poulton *et al.,* 1993). Regardless of the method by which it occurred, mitochondrial dysfunction will eventually lead to energy depletion and apoptosis (Mandavilli *et al.,* 2002).

The relationship between mitochondrial activity and life span for *C. elegans* was established by determining that over-expression of the *clk-1* mutant increases mitochondrial activity and greatly shortens life span (Felkai *et al.,* 1999). The *clk-1* locus encodes a mitochondrial hydroxylase whose activity is required for synthesis of ubiquinone (Jonassen *et al*, 2001.) Mutants of the nuclear encoded *daf-2* show increased life span as *daf-2* up-regulates expression of sod-3, which is a nuclear encoded Mn-SOD (Honda and Honda, 1999). Similarly a mutation affecting the iron sulfur protein (*isp-1*) of the mitochondrial complex III, confers a similar life span to mutants of *daf-2* and has lower oxygen consumption and decreased ROS sensitivity compared to wild-type worms (Feng *et al,* 2001). Worms containing both mutant *isp-1* and *daf-2* did not have a greater life span than single mutant strains leading to the conclusion that they are members of the same pathway regulating life span (Feng *et al.,* 2001). The rate of mitochondrial activity in worms is decreased by rearing worms on a lawn of bacteria that express an RNAi clone for ATP synthase (Dillin *et al.,* 2002-a). Worms reared from hatching on this media have smaller body size due to decreased energy production and increased longevity due to lower ROS

production by the mitochondria (Dillin *et al.*, 2002-a). Furthermore, another RNAi screen has shown that a mutation in the mitochondrial encoded leucyl-tRNA synthetase gene (*lrs-2*) confers an increase in life span and decreased mitochondrial function with lower ATP production and oxygen consumption (Lee *et al.,* 2003). However, these mutant nematodes were still sensitive to paraquat treatment, indicating that a decrease in oxidative damage may not be the sole mechanisms for mitochondria to influence life span in the nematode (Lee *et al.,* 2003).

In other experiments, comparisons of the rate of ROS production, hydrogen peroxide levels and rates of protein carbonyl formation (an indicator of oxidative damage to protein), have shown that life span is inversely related to the rate of damage and directly related to the activity of cytochrome C oxidase (Sohal *et al.,* 1995). The same has been shown in houseflies where life span is directly related to the amount of damage to the citric acid enzyme aconitase that is directly targeted by oxidative damage (Yan *et al.,* 1997). Mitochondrial dysfunction also plays a role in mouse aging. Mice which are homozygous for a null allele of the mitochondrial Mn-SOD called *Sod2* show severe mitochondrial defects in newborns; heterozygous mice have some mitochondrial dysfunction which worsens with age (Kokoszka *et al.,* 2001). The increased dysfunction in the heterozygous mouse was identical to that of wild-type aged mice. The decline in both the wild-type and the heterozygous mice was the typical pattern of mitochondrial ROS production, oxidative stress, dysfunction and ultimately cell death with the only difference being the time of onset (Kokoszka *et al.,* 2001). Similarly, the skeletal muscle of rhesus monkeys has been shown to have lowered expression of transcripts of genes important for oxidative phosphorylation and mitochondrial electron

transport which may lead to mitochondrial loss of function (Kayo *et al*, 2001). In humans, there is interest in the role of mitochondrial dysfunction and age-related diseases. In their 2001 review, Berdanier and Everts implicate mutations in the mitochondrial genome with premature aging, some forms of cancer, Parkinson's disease and Alzheimer's disease. However, a study of the DNA from autopsies of human hearts did not show a connection between mitochondrial dysfunction and cardiomyopathy, although some hearts did show rearrangements of mitochondrial DNA (Kajander *et al.,* 2002). Though the pathways and pathologies may vary somewhat between different species and different individuals within a species, proper mitochondrial function is critical for maintenance of cell function.

DNA REPAIR AND AGING

It has been shown that oxidative damage is tied to aging through the accumulation of mutations. It follows that cells which can repair such damage would be able to contribute to the maintenance of homeostasis. Cortopossi and Wong (1996) showed that there is good, but not excellent, correlation between DNA repair activity and maximal life span. There is a six fold range of DNA repair activity with rodents having shorter life spans and lower activity and humans and gorillas being on the high end of repair activity and maximal longevity. While there are many types of DNA repair and over 130 known human DNA repair genes, this review will focus on three methods and corresponding genes: base excision repair, nucleotide excision repair, and non-homologous end joining. Base excision repair (BER) occurs when oxidative or hydrolytic decay of DNA bases are excised and replaced (Lindahl and Wood, 1999). Nucleotide excision repair (NER) removes and replaces regions of DNA

where the helix is distorted by uv lesions, oxidative lesions, as well as chemical adducts that protrude into the helical grooves (De Laat *et al.,* 1999). Non-homologous end joining (NHEJ) involves identifying breaks in the chromosome and modifying the ends before rejoining (Bohr *et al.,* 2001). There are also two other mechanisms which should be addressed. Organisms can also repair DNA through homologous recombination, but this system is much stronger in yeast than in higher eukaryotes (Liang *et al.,* 1998). DNA repair is also coupled with transcription in some cases. It has been shown that transcribed DNA is repaired more rapidly than other regions of mammalian genomes (Bohr *et al.,* 1985). Furthermore, the strand of DNA that is transcribed is repaired more rapidly than the other strand, a mechanism called transcription coupled repair (Mellon *et al.,* 1987). Failure of some of these DNA repair mechanisms have been tied to aging in that the associated disease phenotypes resemble premature aging such as Werner's syndrome, xeroderma pigmentosum, Cockayne syndrome and trichothiodystrophy.

Werner's syndrome (WS) is homozgyous recessive condition characterized by symptoms that resemble aging: wrinkling of the skin, graying hair, cataracts, brittle bones, hardening of the arteries and increased cancer susceptibility (Bohr *et al.,* 2001). Werner's patients have the onset of symptoms at puberty and generally do not reach age fifty (Bohr *et al.,* 2001). WS cell cultures have given insights into the mechanisms of this disease. Bohr and colleagues (2001) created various types of DNA lesions and looked for repair by two WS cell lines and a control line. WS cell lines were proficient at BER, NER, mitochondrial DNA repair, as well as NHEJ, but DNA deletions occurred twelve times as often during NHEJ for WS versus the control line (Bohr *et al.,* 2001). The Werner protein (WRN) forms a

heterodimer with the telomeric protein Ku (Cooper *et al.,* 2000) and is necessary to process the ends of double strand breaks before the repair can be made (Bohr *et al.,* 2001). WRN has ATP-dependent helicase activity and 3' to 5' exonuclease activity (Gray *et al.,* 1997; Huang *et al.,* 1998) with homology to the DNA helicase SGS1 of *S. cerevisiae* (Gangloff *et al.,* 1994). There is a consequence due to functional differences in the yeast and mammalian proteins: NHEJ results in deletion of genomic ribosomal DNA in mammals but formation of extrachromosomal ribosomal DNA circles (ERCs) in yeast (Park *et al.,* 1999). Mutations at the *sgs1* locus increase sensitivity to uv and peroxide, decrease life span by 60%, cause genomic instability and cause greatly increased rates of recombination in the rDNA (Park *et al.,* 1999; Saffi *et al.,* 2001). Hyperrcombination in the rDNA leads to formation of ERCs. The release of a single circle greatly reduces yeast life span (Park *et al.,* 1999). Saffi and colleagues (2000) measured toxin sensitivity in mutations in the helicase or C-terminal domains of *sgs1* to determine that helicase function was necessary to repair all of the lesions which could be repaired by SGS1, although function SGS1 is not sufficient for correcting all types of DNA lesions. When SGS1 does not function properly, ERCs are formed which triggers the release of the telomeric Sir3p protein leading to eventual apoptosis (Park *et al.,* 1999).

Lack of helicase function also plays a role in some of the diseases caused by the genes of the xeroderma pigmentosum (XP) complementation groups. There are seven major complementation groups labeled XPA through XPG which have been connected to DNA repair, but only some groups have been associated with disease. Most are subunits of the transcription factor II H complex (De Boer *et al.,* 2002). XPA and XPC recognize damage

and stabilize TFIIH, XPB and XPD open the helix and XPF and XPG are nucleases that cut around the damaged site (Taylor *et al.*, 1997). While XP may be caused by mutations affecting the XPA and XPC proteins which are necessary for transcription coupled repair, most human disorders resulting from this complex are attributable to mutations in *XPB* or *XPD* (Brooks, 2002). Mutations causing changes in the structure or activity of XPB are extremely rare (in only three families) as it is essential for both transcription and repair (Coin *et al.*, 1999). XPD can have a wider range of mutations and is associated with Xeroderma pigmentosum (XP), Cockayne syndrome (CS), and trichothiodystrophy (TTD) with a complex relationship between the pathologies and associated mutations.

XP is the most studied of the three XPD diseases. Patients with this disease are extremely sensitive to the sun, show skin symptoms such as freckling, have a 2000 fold increased risk of developing skin cancer and the most severe cases show neurological disorders due to neuronal death (Lehmann, 2001). Patients with TTD show scaly skin, brittle hair, abnormal facies, and both physical and mental retardation but do not show an increase risk of skin cancer although some TTD patients are sun-sensitive (Broughton *et al.*, 1994). CS patients show photosensitivity without an increased risk of skin cancer or pigmentation changes, as well as dwarfism, mental retardation, and retinal and skeletal abnormalities (Nance and Berry, 1992). Neurological disorders as a result of CS are due to demyelination rather than by neuronal death as seen in XP (Lehmann 2001). Furthermore, there are patients that have a combination of diseases or symptoms of multiple diseases (Broughton *et al.*, 1995). How can one gene be responsible for such diverse pathologies? The answer lies in the location of the mutations which is difficult to analyze as all of the patients studied have

been compound heterozygotes of 5 mutations (Broughton *et al.*, 1996;Taylor *et al.*, 1997; Lehmann, 2001).

First, it has been shown that all defective phenotypes of XPD cells can be restored by inserting wild-type XPD cDNA into the cells (Marionnet *et al.,* 1996) indicating that the *XPD* is the causative gene. Taylor and colleagues (1997) found many mutations clustered near the C- and N-termini of XPD and were able to identify mutations responsible for TTD or XP, but suprisingly, there were no domains specific for either condition. De Boer and colleagues (2002) generated mice carrying a point mutation shown to be associated with TTD in humans. These mice show premature aging and have a partial repair defect but have varying degrees of transcription coupled repair. When an XPA null mutant was added to the mouse, cellular sensitivity to oxidative damage increased, NER was absent and the mice showed an even greater rate of aging (De Boer *et al.,* 2002). Mutations in the ATP-binding site which affect the helicase activity do not interfere with transcription coupled repair but disrupt NER producing uv sensitivity and symptoms similar to XP (Lehmann, 2001). The current model is that TTD is due to varying degrees of defect in transcription and transcription coupled repair, while XP is caused by failure of TFIIH to repair DNA damage (Bootsma and Hoeijmaker, 1993; Broughton *et al.*, 1994; Lehmann, 2001). While this model explains XPD and TTD, little work has been undertaken to look at the genetic mechanisms that underlie CS. Nevertheless, it is evident that functional DNA repair mechanisms help to maintain the cell, while dysfunction in DNA repair lead to cellular aging.

HEAT STRESS RESISTANCE AND AGING

When organisms are selected for postponed senescence, a correlated response may be seen in their ability to cope with stresses including starvation, dessication, and temperature stress. Perhaps the most widely studied stressor is temperature. When a critical temperature is surpassed, some cellular processes are disrupted due to protein denaturation. Improperly folded proteins activate transcription factors which are responsible for the transcription of heat shock genes (Freeman *et al.*, 1999). Messenger RNA for heat shock proteins (Hsp) are preferentially translated and have greater stability at higher temperatures than do other mRNAs (Yost *et al.,* 1990). Once transcribed, these proteins are not denatured at the temperatures which can denature other proteins. These proteins serve as molecular chaperones which protect the cell against senescence caused by stress (Tatar *et al.*, 1997).

Heat shock proteins have been implicated in the extension of life span in organisms as diverse as yeast, nematodes and fruit flies. In yeast, a brief heat shock extends the life span if the gene products of *HSP104, RAS1,* and *RAS2* are expressed (Shama *et al.,* 1998). Previous studies have implicated HSP104 as being sufficient for recovery from stress (Sanchez and Lindquist, 1990) as well as being essential for survival at high temperatures (Lindquist and Kim, 1996). The *RAS2* gene product is needed to maintain life span during a prolonged heat shock, *RAS1* extends life span after a brief heat shock, and *HSP104* is necessary to increase life span regardless of the length of the heat shock (Shama *et al.,* 1998). Shama *et al.* (1998) also discovered a relationship between petite mutants and thermotolerance, implicating the mitochondria in heat stress response. The role of *Hsp90* is also important in yeast, although it functions in a different manner. Yeast with low Hsp90 activity have increased levels of

heat shock genes as well as increased thermotolerance as Hsp90 has been shown to down-regulate the activity of heat shock transcription factors (Duina *et al.,* 1998; Harris *et al.*, 2001). Reducing the concentration of Hsp90 activates the heat stress response pathway without being triggered by thermal stress and increases the time cells are viable after senescence without increasing the period to reach senescence (Harris *et al.,* 2001).

In *C. elegans* it has been shown that mutations in the genes *age-1, daf-2, daf-4, daf-7,* and *spe-26* increase life span as well as resistance to temperature stress (Lithgow *et al.,* 1995). The wild-type *age-1* worm produces a protein that interferes with thermotolerance as well as shortens life span, but *age-1* mutants are resistant to thermal stress, oxidative stress and show over-expression of SOD and CAT late in life, linking the two stress responses in the worm (Lithgow *et al.,* 1995; Larsen, 1993; Vanfleteren, 1993). Microarrays have also been used to analyze the expression pattern of 11,917 in genes in *C. elegans* during heat stress, finding 28 were up-regulated (GuhaThakurta *et al.,* 2002). Of the up-regulated genes, six were identified as heat shock proteins, one played a role in oxidative stress, fourteen were predicted coding sequence with no known function, while a few other genes were listed with an unknown connection to the response (GuhaThakurta *et al.*, 2002). Twenty-four of the up-regulated genes contained one of two *cis*-regulatory sequences: one that is conserved and is known to bind heat shock factors and a novel sequence (GuhaThakurta *et al.,* 2002). It will be interesting to see if this new regulatory element plays a role in stress responses other than thermotolerance.

In *Drosophila* there are six Hsps that have been studied in detail: Hsp22, Hsp23, Hsp26, Hsp27, Hsp70 and Hsp83 which are named for their sizes. Hsp22 is the least

abundant of the heat shock proteins during development and during heat stress. (King and Tower, 1999). By comparing Hsp expression to the constant expression of ribosomal protein 49 during aging, it was determined that Hsp26, Hsp27, and Hsp70 increased 3-fold, Hsp23 increased 5-fold, and Hsp22 increased 60-fold (King and Tower, 1999). Hsp83 is the least studied, although it is known that Hsp83 is constitutively expressed but is up-regulated by stress and modulates steroid hormone receptors (Parsell and Lindquist, 1993; Picard *et al.,* 1990.) *Hsp83* is a member of the *Hsp90* family (King and Tower, 1999). *Hsp70* is the most studied as well as the most highly conserved of the *Drosophila* heat shock genes. Strains reared at 28^0 for more than 20 years, showed lower levels of Hsp70 as well as lower inducible thermotolerance than strains reared at 18^0 or 25^0 for the same period of time (Bettencourt *et al.,* 2002). DNA variation between these strains as well as eleven populations showed such scant variation that it is hypothesized natural selection must act on the regulators of the gene rather than its sequence directly (Bettencourt *et al.,* 2002). What happens to Hsp70 at temperatures above 28^0? In flies a 36^0 Celsius heat shock produces a small, brief increase in the expression of Hsp70 which increases life span (Tatar *et al.,* 1997). When extra copies of *Hsp70* are inserted into the *Drosophila* genome and the flies are heat shocked, life span is increased by 7.9% (Tatar *et al.,* 1997). Tatar *et al.* (1997) originally hypothesized that the increase in survivorship was not caused by a trade-off in reproduction, as egg-laying remained constant, but was instead due to Hsp reducing age-related stress. However, a decrease in egg hatching after a maternal heat shock was later observed in the same transgenic flies, leading to the conclusion that there is a relationship between increasing life span through thermotolerance and decreasing fecundity, supporting the antagonistic

pleiotropy theory of aging (Silberman and Tatar, 2000). It is also thought that expression levels of the various heat shock proteins decreases with age, making the older organisms more susceptible to the effects of stress. For example, levels of Hsp70 decline in *Drosophila melanogaster* upon aging (Heydari, *et al.*, 1993). As aging progresses, the organism becomes less capable of coping with stress that can, in turn, increase the rate of aging.

CATECHOLAMINES

The genes which code for catecholamine biosynthetic enzymes are implicated in stress response as these neurotransmitters are required to signal stress in the brain of the organism. While different catecholamines play the most important role in different organisms, this section will focus on the genes involved in the synthesis of dopamine, the expression of dopamine receptors, and degeneration of dopaminergic neurons for three reasons. First, dopamine and noradrenaline are the primary catecholamines in the nervous system of insects where much of this work has been done (Evans and Gee, 1980; Anderson, 1979). Second, the dopamine system is tied to aging in that dopaminergic signaling shows a progressive decrease during aging (Roth and Joseph, 1994). Third, while the regulation of the factors involved is not completely understood, much attention has been given to individual genetic factors involved in catecholamine biosynthesis.

Production of dopamine may start with cytokine-induced expression of guanasine-tri-phosphate cyclohydrolase (GTPCH-1) in some tissues (Gross and Levi, 1992) or through the constitutive expression of GTPCH-1 in the brain and liver (Thöny *et al.*, 2000). GTPCH-1 catalyzes the formation of tetrahydrobiopterin (BH_4) which is a cofactor for phenylalanine

hydroxylase, tyrosine hydroxylase, tryptophan hydroxylase, and nitrous oxide synthase (Gross and Levi, 1992; Thöny *et al.*, 2000). BH_4 and phenylalanine are used by phenylalanine hydroxylase (PAH) to produce tyrosine. From there, BH_4 is needed for tyrosine hydroxylase (TH) to convert tyrosine to L-DOPA which is later converted to dopamine by dopa-decarboxylase. Serotonin production is also in this pathway as BH_4 and tryptophan are necessary for the production of 5-OH-tryptophan by tryptophan hydroxylase (TPH), the end product of which is serotonin (see www.bh4.org for models of dopamine production). Each gene and protein involved in the production of dopamine is a potential place for regulation and, in some cases, potential disease.

GTPCH-1 is the first protein in the pathway, and the first point of control. Increased concentration of BH_4 stops production of GTPCH-1 in the liver, while increased concentrations of phenylalanine increase GTPCH-1 levels (Harada *et al.*, 1993; Thöny *et al.*, 2000). GTPCH-1 deficiencies have been associated with the skin disorder vitiligo (De La Fuente-Fernández, 1997) as well as hyperphenylalaninaemia, a form of phenylketonuria that diet cannot control (Thöny *et al.*,2000). Ichinose and colleagues (1994) have identified mutations in human GTPCH-1 in over 200 patients that are associated with hereditary progressive dystonia with diurnal fluctuations. Onset of this disease is before puberty and causes Parksinson-like tremors and aggravation which are somewhat alleviated by rest and are responsive to treatment with L-DOPA, which bypasses the BH_4 deficiency (Ichinose *et al.*, 1994). However, studies of dopa-responsive-dystonia, a similar disease without diurnal fluctuations, have been complicated by association studies that found no mutations in the gene in some patients, indicating that other genetic factors may be contributing to the

phenotype (Bandmann *et al.,* 1996). They concluded that dopa-responsive dystonia may be due to autosomal dominant alleles of GTPCH-1 or autosomal recessive mutations of TH (Bandmann *et al.,* 1996).

GTPCH-1 in both flies and humans undergo alternate splicing (Togari *et al.,* 1992) with the human proteins differing at the 3' end, and the fly proteins differing at the 5' end (McLean *et al.,* 1993). Activity of both GTPCH-1 and TH are regulated by the product of the *Drosophila Catecholamines up* (*Catsup*) locus which changes the phosphorylation of the proteins (Stathakis *et al.,* 1999). Specifically, *Catsup* mutants lead to hyperactivation of TH leading to excessive levels of catecholamines and indicating that the wild-type *Catsup* protein is a negative regulator of TH (Stathakis *et al.,* 1999). Flies with increased levels of catecholamines due to a *Catsup* mutant are more resistant to dessication and starvation than are *Punch* (the fly GTPCH-1) mutants which have lower levels of catecholamines (Chaudhari *et al.,* 2003). Similar results were obtained when the mutant strains were exposed to hypergravity (Wang *et al.,* 2003). However, the effects of catecholamine levels may differ in humans and flies. Wei and colleagues (1997) have noted that homozygozity for one allele of human TH results in severe elevation of serum noradrenaline and hypothesized that the increase makes patients sensitive to stress, which may lead to illness. Furthermore, the regulation and interactions of GTPCH-1 and TH are complex as they are both subject to feedback inhibition (Harada *et al.,* 1993; Krumer and Vrana, 1996), regulation by *Catsup* (Stathakis *et al.,* 1999), alternate splicing (Krumer and Vrana, 1996) and post-translational modification (Thöny *et al.,* 2000). GTPCH-1 and phosphyorylated TH have been shown to co-immunoprecipitate indicating an interaction; however, the nature of this interaction is

unknown and currently being investigated (Xu *et al.*, 2000; Huang *et al.*, 2003).

Blockages of the various steps of dopamine synthesis are not the only causes of disease. Changes in dopaminergic neurons and dopamine receptor expression have been associated with a variety of age-related diseases. Loss of some types of the four dopamine receptors, named D_1 through D_4 in humans, is one pathology of the brains of patients with Huntington's disease (Cha *et al.*, 1998). Transgenic mice which express a mutant form of the Huntington disease genes have been shown to have losses of the D_1-like and D_2-like receptors long before the onset of symptoms. Similar associations have been made between these receptors and Huntington's disease with rigidity, while there was no association between the receptors and Huntington's disease without rigidity (Turjanski *et al.*, 1995). The D_2 receptor has been implicated in Parkinson disease as mice lacking this receptor have Parkinson-like tremor and mobility (Baik *et al.*, 1995). In humans, loss of 80% of the dopaminergic neurons in the substantia nigra and striatum is necessary to cause Parkinson symptoms (Hingtgen and Siemers, 1998). Furthermore, loss of D_2 receptors has been shown in patients who have Alzheimer's disease with Parkinson-like tremor, but not in patients with just Parkinson's or Alzheimer's disease although only 22 patients and 14 controls were used (Joyce *et al.*, 1998). However a study of 15 patients and 9 age-matched controls showed a decrease in D_2 receptor activity in the striatum of Alzheimer's affected individuals (Pizzolato *et al.*, 1996). Also, a study of mutations in all four dopamine receptor genes (*DRD1* through *DRD4*) showed that *DRD1* and *DRD3* polymorphisms are associated with psychosis and *DRD1* mutations are associated with aggressive behavior in 275 patients with probable Alzheimer's disease (Sweet *et al.*, 1998). It is clear that the dopamine system is important in Huntington's,

Alzheimer's and Parkinson's disease. Larger studies with more patients, analysis of mutations in all four receptor genes, and PET studies of the dopaminergic neurons of larger brain sections are needed to clarify the relationships between the genes and diseases.

DIRECTIONS FOR THE FUTURE OF AGING RESEARCH

Just as aging is a complex process, so is the task of teasing apart the causes of aging. The first complexity is that not all animals age in the same way. A female flounder doesn't age at all while her male counterpart does. Cessation of growth will lead to changes that kill the male flounder (Hayflick, 1994). Antichinus males die after reproducing; their mates live only long enough to wean the young. It is the spike in hormones tied to reproduction that leads to their deaths (Austad, 1997). Elephants and cows are more likely to die of starvation from losing their teeth than they are of failure of a metabolic system. Their deaths are caused by wear and tear on their bodies (Hayflick, 1994). Laboratory rodents generally die of cancer, although cancer rates are greatly dependent on their environment. Metabolic rate and environmental oxygen levels seem to contribute greatly to rodent deaths in the laboratory. And what about ourselves? Humans are not only the longest-lived mammals but also the most often stricken by age-related disease. Heart disease, cancers, and the neurodegeneration of Alzheimer's and Parkinson's disease are all common causes of death. There will not be a single answer as there is no single cause of death.

Several mechanisms for aging have been characterized in yeast, nematodes and fruit flies, but the underlying genetic mechanisms are poorly understood due to the complexities of interactions in multi-gene systems. Once single gene systems are understood, it will be

critical to look at epistatic interactions between genes that affect life span. In addition all of the research on model organisms has been done in a controlled setting, neglecting the environmental contribution to this quantitative trait. Careful association studies will lend further information about the causes of age-related diseases which may, in turn, help in determining what is normal aging. Such studies need to take into account multiple generations in many different families in order to avoid false associations. Microarray studies will also be a great help in deciphering the course of normal aging, for example by monitoring normal gene expression in many individuals over the course of their lifetimes. As the effects of genes are through protein function and activity, it will also be necessary to turn to proteomics for information about the underlying causes of aging and age-related disease.

During the last ten years great advancements have been made in understanding the causes of aging. However, in stark contrast to the wealth of knowledge accumulating regarding the loci and pathways regulating life span, virtually nothing is known about the genes causing naturally occurring variation in longevity. To understand the genetic architecture of aging, it is necessary to know not only which loci cause the aging process, but which subset of these loci affect variation in aging, and the actual molecular polymorphisms (quantitative trait nucleotides) causing the variation in longevity at these loci. Only then will it be possible to infer genetic interactions between loci affecting longevity.

Combining quantitative and molecular techniques used to assay the effects of candidate genes on longevity is a powerful method for associating molecular and phenotypic variation. When molecular information about a candidate gene is known, the quantitative genetic technique of linkage disequilibrium mapping can be used to determine if the

candidate locus is one of the multiple quantitative trait genes (QTG) responsible for the variation in phenotype (Lander and Schork, 1994). This technique is applicable to all organisms for which sequence information is available and will be more powerful once genome projects are completed.

In a sense, all genes are candidate longevity genes. However, whole genome linkage disequilibrium mapping is not currently technologically or economically possible in humans. Presently, the fruit fly *Drosophila melanogaster* can be used to determine which genes affect variation in aging. Recent analysis of the completed *Drosophila* genome sequence shows that over 60% of known human disease genes have homologues in *Drosophila* (Kornberg and Krasnow, 2000). Therefore, it is likely that genes affecting variation in *Drosophila* longevity will be relevant to human medicine. Manipulating the genetic background of *Drosophila* is simple, allowing the backcrossing of mutant alleles into a standardized background. The generation time is two weeks, and the average life span is relatively short. Further, there is substantial genetic variation for life span in *Drosophila*.

While many hundreds or more genes potentially regulate life span, it is likely that considerably fewer genes will have large effects on variation in longevity in nature. The first step of a candidate gene screen is to select genes that may cause significant variation in life span in natural populations. The genes discussed above are good selections for such screens as they have been associated with aging in other studies and have the potential to affect variation in life span. Several requirements must be met to ensure that the effect of a candidate gene on variation in longevity can be accurately quantified. First, a stock with either a null mutation at the gene of interest or a deletion covering the area of the gene must

be available, and these stocks should have a genetic background suitable for a backcross breeding scheme and the life span assay. Second, sequence information for the candidate locus must be available to facilitate later linkage disequilibrium mapping. The recent publication of the *Drosophila* genome sequence (Adam *et al.,* 2000) ensures the latter criterion is met for all *Drosophila* candidate genes. .

To facilitate comparisons of different mutations, mutations must be substituted into a standard inbred background. Thus, all mutations on the same chromosome have the same background genotype and are compared against the same control chromosome, although the genetic background of the chromosomes containing the mutant alleles of candidate genes are different for each candidate gene.

Quantitative complementation tests are used to assay the contribution of each locus to variation in life-span (Mackay, 2001). Each candidate gene stock has a null mutation or is deficient at the gene of interest, and the only functioning allele is on the control chromosome, usually a balancer chromosome. This stock is then crossed to a panel of inbred lines derived from a natural population, and which represent a range of life spans observed in nature.

The F_1 progeny of the cross are $m/+_i$ and $Bal/+_i$, where *m* is a mutation of the candidate gene, *Bal* is the balancer chromosome, and$+_i$ is one of the i wild-derived alleles of the candidate genes. The life span of virgin males and females is assessed for each of the genotypes and factorial analysis of variance (ANOVA) is used to partition variation between sexes (S), inbred lines (L), and mutant versus Balancer genotypes (G). Quantitative failure of the candidate gene to complement the wild-derived alleles is inferred if the L x G and / or the L x G x S terms from the ANOVA are significant. Observation of a significant failure to

complement cannot be attributed definitively to allelism or epistasis, or even to specific interactions with the mutation of interest. However, complementation is reason for exclusion of a candidate gene as contributing to naturally occurring variation in life span. In the past, the results of quantitative complementation tests have been completely consistent with results of association studies (Long *et al*, 1998; Lyman *et al*, 1999).

Having identified putative candidate genes from quantitative failure of wild type alleles to complement mutations at candidate genes, the next question to be addressed is to define the molecular polymorphisms at that gene that cause naturally segregating variation in longevity. To look for sequence variation, the wild-derived alleles at the candidate gene of interest are sequenced and aligned. Single nucleotide polymorphisms (SNPs) chosen for genotyping in the larger population must be present more than one time in the sample of lines and spaced at two-hundred base pairs intervals. Previous analysis of *Drosophila* shows decay of linkage disequilibrium over regions of five-hundred base pairs or greater in regions of normal recombination (Long *et al*, 1998). Life span is then assessed for a larger sample of alleles of the candidate gene, and SNP genotypes at the candidate genes are determined for each of these alleles. Association of the SNPs with longevity are then determined using ANOVA. If there are more significant associations than expected by chance, this implicates the candidate gene as a QTG responsible for the variation in phenotype (Lander and Schork, 1994), although the significant SNPS are not necessarily the causal QTNs.

In next chapter I present the results of quantitative complementation tests of sixteen candidate genes in longevity assays. In the third chapter, I show that molecular

polymorphisms at one of these candidate genes is associated with segregating variation in life span in a natural population.

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CHAPTER 2

CANDIDATE QUANTITATIVE TRAIT GENES AFFECTING VARIATION IN *DROSOPHILA* LONGEVITY

ABSTRACT

Limited life span and senescence are universal phenomena, controlled by genetic and environmental factors whose interactions both limit life span and generate variation in life span between individuals, populations and species. To understand the genetic architecture of aging it is necessary to know what loci affect variation in life span, what are the allelic effects at these loci and what molecular polymorphisms define quantitative trait locus (QTL) alleles. Here, quantitative complementation tests were used to determine whether candidate life span genes such as *Superoxide dismutase* (*Sod*), *Catalase* (*Cat*), heat shock proteins, DNA repair enzymes, glucose metabolism or male accessory gland proteins interact genetically with naturally occurring QTL affecting variation in life span in *Drosophila melanogaster*. Inbred strains derived from a natural population were crossed to stocks containing null mutations or deficiencies uncovering the above genes. Life span of the heterozygous progeny was assayed. A significant cross (mutant versus wild-type allele of the candidate gene) by inbred line interaction term from analysis of variance of the life span data indicates a genetic interaction between the candidate gene allele and the naturally occurring life span QTL. Of the sixteen candidate regions and genes tested, *Df(2L)cl7*, *Df(3L)Ly*, *Df(3L)AC1*, *Df(3R)e-BS2*, and *α-Glycerol phosphate dehydrogenase* showed significant failure to complement wild-type alleles in both sexes, and an *Alcohol dehydrogenase* mutant failed to complement in females. Several genes known to regulate life span (*Sod*, *Cat*, and *rosy*) complemented the life span effects of alleles, suggesting little natural variation affecting longevity at these loci, at least in this sample of alleles. Quantitative complementation tests are therefore useful for

identifying candidate genes contributing to segregating genetic variation in life span in nature.

INTRODUCTION

As the world human population rapidly grows older, population aging will become one of the most important social and health problems in the coming half century. While progress is being made in the treatment of some age-related illnesses, we have yet to completely understand the genetic basis of either the aging process or age-related diseases such as Alzheimer's disease and Parkinson's disease. To understand the genetic architecture of aging, it is necessary to know which loci cause the aging process, which subset of these loci affect variation in aging, and the actual molecular polymorphisms (quantitative trait nucleotides) causing the variation in longevity at these loci. Only then will it be possible to infer genetic interactions between loci affecting longevity.

Combining quantitative and molecular techniques used to assay the effects of candidate genes on longevity is a powerful method for associating molecular and phenotypic variation. When molecular information about a candidate gene is known, the quantitative genetic technique of linkage disequilibrium mapping can be used to determine if the candidate locus is the quantitative trait locus (QTL) responsible for the variation in phenotype (Lander and Schork, 1994). This technique is applicable to all organisms for which sequence information is available and will become increasingly powerful as more genome projects are completed.

In a sense, all genes are candidate longevity genes. However, whole genome linkage disequilibrium mapping is not currently technologically or economically possible in humans. Presently, the fruit fly *Drosophila melanogaster* can be used as a model system to determine which genes affect variation in aging. Recent analysis of the completed *Drosophila* genome sequence shows that over 60% of known human disease genes have homologues in *Drosophila* (Kornberg and Krasnow, 2000). Therefore, it is likely that genes affecting variation in *Drosophila* longevity will be relevant to human medicine. Manipulating the genetic background of *Drosophila* is simple, allowing the backcrossing of mutant alleles into a standardized background. The generation time is two weeks, and the average life span is short. Further, there is substantial genetic variation for life span in *Drosophila.*

While many candidate genes regulating life span have been found in *Drosophila* and other systems, it is not known whether polymorphisms at these loci segregate in nature, and hence, affect variation in life span. However, if null mutations are available for these candidate genes, quantitative complementation tests (Lyman and Mackay, 1998) can be used to assess whether the genes known to regulate life span also potentially affect variation in life span. If mutations are not available, complementation tests may be performed with small deficiencies uncovering the candidate gene, essentially fine-mapping a QTL containing one of more genes affecting variation in life span, one of which is the candidate gene (Pakyukova *et al.*, 2000).

Here, we apply the complementation test approach to address whether sixteen candidate genes potentially affect natural variation in *Drosophila* longevity. Six of the candidate genes are structural genes for metabolic enzymes: *Phosphoglucomutase* (*Pgm*),

Glucose-6-phosphate dehydrogenase (*G6pd*), *Phosphogluconate dehydrogenase* (*Pgd*), *α-glycerol phosphate dehydrogenase* (*Gpdh*), *Alcohol dehydrogenase* (*Adh*) and *Insulin receptor* (*InR*). In *C. elegans*, longevity is inversely correlated with metabolic rate (Van Voorhies and Ward, 1999). Caloric restriction or mutations can cause shifts in metabolism; therefore lowering metabolism extends life in some organisms. Increased starvation resistance is also found in flies selected for longer life and is attributable to a decrease in metabolic rate caused by starvation (Chippindale, Chu and Rose, 1996). Furthermore, gene expression analyses show that once-mated female *Drosophila melanogaster* have 885 transcriptional changes caused by aging and 827 changes caused by caloric restriction with 448 in common between the two treatments (Pletcher *et al.*, 2002). *Pgm* encodes for a glycolytic enzyme, an allele of which has been associated with an increase in mean *Drosophila* longevity (Deckert-Cruz *et al.*, 1997). *G6pd* and *Pgd* are, respectively, the first and last enzymes in the oxidative part of the pentose phosphate shunt pathway. *Gpdh* is essential for glycolysis. *Adh* confers resistance to ethanol. The *InR* locus encodes two subunits of the *Drosophila* insulin receptor and is homologous to the *daf-2* gene, which regulates longevity in *C. elegans* (Kimura *et al.*, 1997). In addition, hypomorphic mutations of *InR* produce dwarf flies that have a deficiency in juvenile hormone but have an 85% extension in life span (Tatar *et al.*, 2001). It is thought that the relationship between receptor and JH may extend life span by decreasing metabolism, thereby slowing growth and development (Tatar *et al.*, 2001). All of the aforementioned enzymes are implicated in life span as they are necessary for energy production and metabolism.

Superoxide dismutase (*Sod*), *Catalase* (*Cat*) and *rosy* (*ry*) alleviate oxidative stress. Reactive oxygen species (ROS) and free radicals can damage DNA and protein. An estimated two to three percent of oxygen used in the cell will be turned into ROS, which can attack other regions of the cell (Sohal and Weindruch, 1996). *Sod* and *Cat* act together to detoxify byproducts of metabolism, as the former catalyzes the reaction to change superoxide anions (O_2^-) to H_2O_2, and the latter converts H_2O_2 to H_2O and molecular oxygen (Lithgow, 1996). Transgenic expression of a Cu-Zn superoxide dismutase can restore a normal life span to fly strains lacking a functional *Sod* allele (Parkes *et al.*, 1998). In addition, strains of *Drosophila* selected for increased longevity had greater than normal levels of Cu-Zn Sod (Arking, 1998). Over-expression of both Catalase and Superoxide dismutase act synergistically to further increase life span and have slower rates of mitochondrial hydrogen peroxide production, although metabolic rate is increased (Orr and Sohal, 1994; Sohal *et al.*, 1995). While Sod and Cat detoxify ROS, the product of *ry*, xanthine dehydrogenase, catalyzes the formation of the oxygen scavenger urate. It is known that flies lacking a functional *ry* allele are hypersensitive to oxidative stress (Hilliker *et al.*, 1992). Further evidence for the role of oxidative stress in aging is provided by a microarray study in which transcript levels of aging flies were compared with that of flies subjected to a paraquat treatment (Zou *et al.*, 2000). While expression of some genes was affected by either aging or hyperoxic conditions, expression patterns for 42 genes were changed by both treatments, strengthening the relationship between oxidative stress and aging.

Genes involved in a variety of stress responses have also been implicated in the regulation of longevity, including the small heat shock proteins (*Hsp*), *Hsp22-Hsp27,*

clustered on chromosome *2*. These heat shock proteins are expressed only when the organism is exposed to temperature stress, thereby conferring stress resistance to the fly. These proteins serve as molecular chaperones which protect the cell against senescence caused by stress (Tatar *et al.*, 1997). By comparing Hsp expression to the constant expression of ribosomal protein 49 during aging, it was determined that Hsp26, Hsp27, and Hsp70 increased 3-fold, Hsp23 increased 5-fold, and Hsp22 increased 60-fold (King and Tower, 1999). While not implicated in heat stress, *Pu* encodes GTP cylcohydrolase, a key enzyme in catecholamine biosynthesis, and is, therefore, involved in stress response (Stathakis *et al.,* 1999). Catecholamine biosynthetic genes are implicated in stress response as the neurotransmitters they encode are required to signal stress in the brain of the organism. The dopamine system is tied to aging in that dopaminergic signaling shows a progressive decrease during aging (Roth and Joseph, 1994). Flies with increased levels of catecholamines due to a *Catsup* mutation are more resistant to dessication and starvation than are *Pu* mutants, which have lower levels of catecholamines (Chaudhari *et al.*, 2003).

Several other miscellaneous candidate genes were considered. The proteins encoded by *Accessory proteins 26A* (*Acp26A)* and *70A (Acp70A)* have an effect on female life span after mating. The protein encoded by *Acp26A* resembles a peptide pheromone precursor (Clark *et al.,* 1995). *Acp70A*, or the sex peptide protein, represses female receptivity after mating and stimulates oviposition (Chen *et al.,* 1988). Fowler and Partridge (1989) demonstrated that reproduction shortens female life span, and the reduction is not attributable to egg-laying or exposure to males. Sperm was eliminated as the causative agent as mated females have a shorter life span regardless of whether the males were normal or deficient in

sperm production (Chapman, 1992; Chapman *et al.*, 1993). Transgenic flies lacking secretions from the accessory gland were created and mated to virgin females. Females mated to transgenic males had a life span comparable that of virgin females, while females mated to normal males had a shorter life span than the controls, localizing the causative agents to the accessory gland (Chapman *et al.*, 1995). The protein encoded by the *period* (*per*) locus is essential for biological clock functions, although it has not been directly associated with life span. *Egfr* encodes the epidermal growth factor receptor homolog and was selected for its homology to a *let-23* a locus that regulates cell proliferation and differentiation in *C. elegans* (Aroian *et al.*, 1990). The gene *pre-senilin* (*psn*) shows homology to one of the genes implicated in the development of Alzheimer's disease in humans (Haass, 1997). Finally, *mutagen sensitive 306* (*mus306*), is sensitive to mutagens as it is defective in both excision and UV damage repair. While this individual mutant has not been associated with longevity, similar types of mutations have been implicated in other species (Lithgow, 1996). Cortopossi and Wong (1996) showed that there is good, but not excellent, correlation between DNA repair activity and maximal life span.

METHODS

(i) *Drosophila* stocks

Stocks with either a null mutation at the gene of interest or a deletion uncovering the candidate gene were obtained from the Bloomington *Drosophila* Stock Center (Bloomington, IN), the European *Drosophila* Stock Center (Umea, Sweden) and from Janis O'Donnell. The stocks are listed in Table 1. To facilitate comparisons of different mutations, the mutations

were substituted into the highly inbred *Samarkand* (*Sam*) genetic background (Lai *et al*, 1994; Lyman and Mackay, 1998). The three chromosome *1* mutations were backcrossed into *Sam* by crossing males of the mutant or deficiency stock (*m*) to *Sam; C(1)DX, y w f* (abbreviated *X^X*) females, and backcrossing male progeny to *X^X* females for 5 generations. After five generations of backcrossing, mutant males were crossed to both *Sam; FM4* and *Sam, X^X* females. *m/FM4* female and *m* male progeny were then crossed to produce the stock for complementation testing. On chromosome *2*, mutant males (*m/BalX*) were crossed to *Sam, Cy/Pm* females (*BalX* represents the initial balancer). Male *m/Cy* backcross progeny were crossed to *Sam, Cy/Pm* females. Subsequently female *Sam, m/Cy* progeny were crossed to *Sam, Cy/Pm* males for 4 generations, after which *Sam, m/Cy* males and females were crossed to produce the *Sam, m/Cy* stocks used in the complementation tests. The backcross scheme was the same for chromosome *3* mutations, except that backcrosses were to *Sam; TM3, Sb/TM6, Ubx*. After five backcross generations, the background genotype is expected to be 97% homozygous *Sam* (Falconer and Mackay, 1996). Thus, all mutations on the same chromosome had the same background genotype and are compared against the same balancer chromosome, although the genetic background of the chromosomes containing the mutant alleles of candidate genes are different for each candidate gene.

The 10 North Carolina (NC) inbred lines used in the quantitative complementation test were derived by 14 generations of full-sib inbreeding from isofemale lines collected in 1994 at the Raleigh Farmer's Market (Fry *et al.,* 1998). The NC lines show both a great deal of variation in life span ($P < 0.0001$) and a difference between the sexes ($P < 0.02$) for

Table 1. Candidate Gene Stock Information

	Balancer used in test	Breakpoints or cytological location	Number of genes uncovered
Df(2L)cl7 pr^{1} *cn*^{1}*/CyO* [c]	*CyO*	25E1-2; 26A7	73
Df(3L)Ly mwh^{1}*/TM1, jv** [d]	*TM6, Ubx*	70A2-3; 70A5-6	7
Df(3L)AC1 roe^{1} *p*^{p}*/TM3, Sb*^{1} [e]	*TM6, Ubx*	67A2; 67D11-13	125
Df(3R)e-BS2 rsd^{1}*/TM3* [f]	*TM6, Ubx*	93C3-6; 93F14	91
Df(1)JC19/FM7c [g]	*FM4*	2F6; 3C5	80
Df(3L)st-f13 Ki^{1} *roe*^{1} *p*^{p}*/Tm6B* [h]	*TM6, Ubx*	72C1-D1; 73A3-4	108
Df(3L)rdgC-co2 th^{1} *st*^{1} *in*^{1} *ri*^{1} *p*^{p}*/ TM6C, Sb, cu*^{1} *e*^{s} *Tb*^{1} [i]	*TM6, Ubx*	77A1; 77D1	45
Adh^{n1}	*CyO*	35B1	1
Cat^{n1}*/TM3, Sb*^{1} *Ser*^{1}	*TM6, Ubx*	75E1	1
Egfr^{f3}*/CyOO* [a]	*CyO*	57E9-F1	1
Gpdh^{n5-4}*/SM1*	*CyO*	26A3	1
mus306^{D1}	*TM6, Ubx*	86D2-90E1	1
Pgd^{n39} *Zw*^{n10} [a] [j]	*FM4*	2D4,18D13	2
Pu^{rAA1}*/SM1* [b]	*CyO*	57C7-8	1
ry^{506}	*TM6, Ubx*	87D9	1
Sod^{n1}*/TM3, Sb*^{1} *Ser*^{1}	*TM6, Ubx*	68A7	1

All stocks were obtained from the Bloomington *Drosophila* Stock Center (Bloomington, IN) except as marked. Genotypes and breakpoints are those provided by the stock center.

[a] Obtained from the European *Drosophila* Stock Center (Umeå, Sweden).

[b] Obtained from Janis O'Donnell at the University of Alabama.

[c] Deficiency uncovers *Acp26A*.

[d] Deficiency uncovers *Acp70*.

[e] Deficiency uncovers *Hsp22* through *Hsp28*.

[f] Deficiency uncovers *InR*.

[g] Deficiency uncovers *per*.

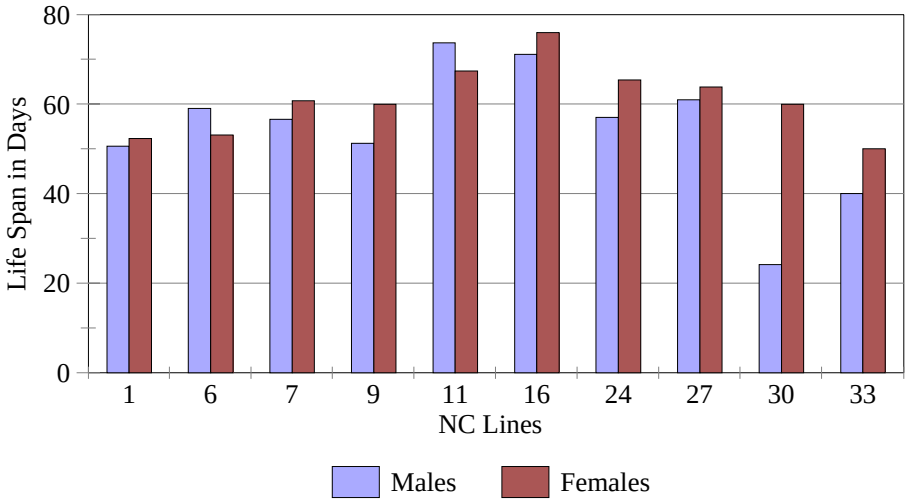
[h] Deficiency uncovers *Pgm.*

[i] Deficiency uncovers *psn*.

[J] No single mutant stocks were available. Tested together as *Pgd* and *Zw* (*G6pd*) are both in the pentose phosphate shunt pathway.

average longevity (Pasyukova *et al*, 2000). Furthermore, these ten lines represent a range of life spans observed in nature. The life spans of the NC lines are shown in Figure 1.

Figure 1. Life Span of NC Males and Females



(*ii*) Quantitative complementation tests

Quantitative complementation tests were used to assay the contribution of each locus to variation in life-span (Mackay, 2001). The stocks in which candidate gene mutations (or deficiencies uncovering the candidate gene) were substituted into *Sam* were each crossed to the 10 inbred lines derived from a natural population. The F_1 progeny of the cross are $m/+_i$ and $Bal/+_i$, where *m* is a mutation of the candidate gene, *Bal* is the balancer chromosome, and$+_i$ is one of the *i* wild-derived alleles of the candidate genes. Virgin flies were collected for 48 hours, and four replicates of six single-sexed flies were placed in shell vials containing approximately 5 ml of cornmeal-agar-molasses medium without yeast on the surface. Flies were placed in a 25^0 incubator for the duration of the assay. Flies were transferred to fresh

vials every two to six days as needed, and the number of live and dead flies was recorded every two days. Any escaped flies were noted and removed from the assay, dropping the total number of some genotypes to 18 rather than 24 flies.

(*iii*) Statistical analysis

Analysis of variance (ANOVA) of longevity was used to assess whether there was quantitative failure of alleles in the Raleigh lines to complement mutations at the candidate genes. The full model was: $y = \mu + L + G + S + L \times G + L \times S + G \times S + L \times G \times S + R(L \times G \times S) + E$ where L , G, and S are the fixed cross-classified effects of line, genotype, and sex, respectively, R is the random replicate vial, and E is the within vial variance. In the full models, significant L × G terms indicate failure to complement, and significant L × G × S interaction terms indicate a sex-specific failure to complement (Pasyukova *et al.*, 2000). Sexes were also analyzed separately using the reduced model $y = \mu + L + G + L \times G + R(L \times G)$.

Potential variation at the candidate gene locus (quantitative failure to complement) is indicated when a candidate gene has a significant L × G or L × G × S term in the full model or a significant L × G term in the reduced model. When quantitative complementation tests are conducted with only two wild-type lines ($+_1$ and $+_2$), the additional criterion that the difference in means between $m/+_1$ and $m/+_2$ is greater than that between $Bal/+_1$ and $Bal/+_2$ must be met (Pasyukova *et al.*, 2000). A greater difference in the Balancer background is not consistent with an allelic interpretation for failure to complement but rather indicates epistatic interaction of QTL affecting the trait in the wild-type lines with genes on the Balancer chromosome. Here, we have 10 wild-type lines. The analogous criterion is that variation in

longevity among the *Bal*/+$_i$ genotypes should not be significantly different from that among the *m*/+$_i$ genotypes. We computed the among-line variance component [Var(Line)] separately for each genotype and determined the ratio between Var(Line) *Bal*/+ : Var(Line) *m*/+. This ratio of two variances has an F distribution with 9, 9 degrees of freedom, with a significance of $P < 0.05$ corresponding to a ratio of 3.18. Therefore, if the ratio exceeded 3.18 there was significantly more variation among lines in the Balancer than the mutant background, and failure to complement was attributed to an epistatic interaction rather than allelism.

RESULTS AND DISCUSSION

Considerable variation in life span was observed in the quantitative complementation test. The mean life span for males was between 14 and 79 days, while the mean female life span was between 22 and 79 days. The line means and standard errors are presented in Supplemental Table 1. The full model for the analysis of variation includes the effects of line (L), sex (S), and genotype (G), as well as the interactions between these factors. The effect of line was highly significant for almost all of the candidate genes (Table 2). This result is expected, as there was significant variation among the 10 homozygous inbred lines, and indicates that the QTLs affecting natural variation in life span are not completely recessive. The genotype term represents the difference in life span between the balancer and mutant genotypes and was generally highly significant (Table 2). On average, the *m*/+ genotype lives longer than the *Bal*/+ genotype, although there are exceptions within lines. The last of the main effect terms is sex, which is highly significant in almost every analysis. The only

exception is *ry* (Table 2). Again, there are exceptions, but females tend to outlive males of the same line and genotype.

Table 2. Source of Variation

	L	G	S	L × S	G × S	L × G	L×G× S
Df(2L)cl7 [a]	<0.0001	0.0008	0.0447	0.0023	0.7769	0.0024	0.6877
Df(3L)Ly [b]	<0.0001	0.0689	<0.0001	0.2202	<0.0001	0.0057	0.1968
Df(3L)AC1 [c]	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0040	0.0215
Df(3R)e-BS2 [d]	<0.0001	<0.0001	<0.0001	0.0002	0.5486	0.0126	0.0998
Df(1)JC19 [e h]	0.0011	0.7223				0.4471	
Df(3L)st-f13 [f]	<0.0001	<0.0001	<0.0001	<0.0001	0.3623	0.0849	0.5602
Df(3L)rdgC-co2 [g]	<0.0001	<0.0001	<0.0001	<0.0001	0.0012	0.4352	0.0568
Adh^{n1}	<0.0001	0.0083	<0.0001	<0.0001	0.7836	0.1082	0.0846
Cat^{n1}	<0.0001	<0.0001	<0.0001	0.0002	0.8143	0.5067	0.1216
$Egfr^{f3}$	0.0042	0.0667	0.0002	0.0064	0.0038	0.3957	0.7129
$Gpdh^{n5-4}$	<0.0001	<0.0001	<0.0001	<0.0001	0.1279	0.0093	0.1149
$mus306^{D1}$	<0.0001	<0.0001	<0.0001	0.0115	0.2482	0.4225	0.0886
Pgd^{n39} Zw^{n10} [i]	0.3243	0.0367				0.0432	
Pu^{rAA1}	0.0120	0.7417	<0.0001	0.0041	0.0062	0.6844	0.0846
ry^{506}	0.0001	0.0001	0.7548	0.0829	0.0053	0.7166	0.1967
Sod^{n1}	0.0011	0.0001	0.0001	0.0015	0.0516	0.5583	0.8297

[a] Deficiency uncovers *Acp26A*.
[b] Deficiency uncovers *Acp70*.
[c] Deficiency uncovers *Hsp22* through *Hsp28*.
[d] Deficiency uncovers *InR*.
[e] Deficiency uncovers *per*.
[f] Deficiency uncovers *Pgm*.
[g] Deficiency uncovers *psn*.
[h] Mutation on chromosome 1; only female progeny.
[i] No single mutant stocks were available. Tested together as *Pgd* and *Zw* (*G6pd*) are both in the pentose phosphate shunt pathway.

The L × S and G × S term are the first of the interaction terms to be considered, as much genetic variance in life span is sex-specific. While females generally live longer than the males, the magnitude as well as the direction of the difference can vary from line to line within a candidate gene analysis. Hence, the L × S term is significant in all but one analysis. A *P* value < 0.05 in the G × S term indicates a difference in the genotype effects between the males and females. This term was significant for six of the complementation tests: to the deficiencies *Df(3L)Ly, Df(3L)AC1,* and *Df(3L)rdgC-co2,* and to the null mutation of *Egfr*, *Pu,* and *ry*. Figure 2 shows the differences between the males and females of each line in the mutant and balancer backgrounds for each of these genes.

A significant L × G or L × G × S term is the first criterion that must be met for quantitative failure to complement. Four candidate gene regions have a significant L × G term: *Df(2L)cl7, Df(3L)Ly, Df(3R)e-BS2,* and $Gpdh^{n5\text{-}4}$. *Df(3L)AC1* shows significance in both the L × G and the L × G × S term. The second criterion is that variation among lines in the balancer background is not greater than the variance among lines in the mutant background. *Df(2L)cl7, Df(3L)Ly, Df(3L)AC1,* and *Df(3L)e-BS2* met this criterion, with F ratios below 3. The ratio for $Gpdh^{n5\text{-}4}$ was 15.9, indicating that the failure to complement was more likely attributable to an epistatic rather than an allelic interaction. We therefore did not consider this gene to be significant. The differences between the life span of the mutant and balancer genotypes are shown for each significant candidate gene in Figure 3.

In the analyses run for sexes separately, two additional stocks, Pgd^{n39} Zw^{n10} and Adh^{n1}, show failure to complement in females (Table 3). However, the failure to complement for Pgd^{n39} Zw^{n10} is due to an increase in variance among the balancer chromosomes rather than

Figure 2. Sex Differences in Life Span for the Mutant and Balancer Backgrounds

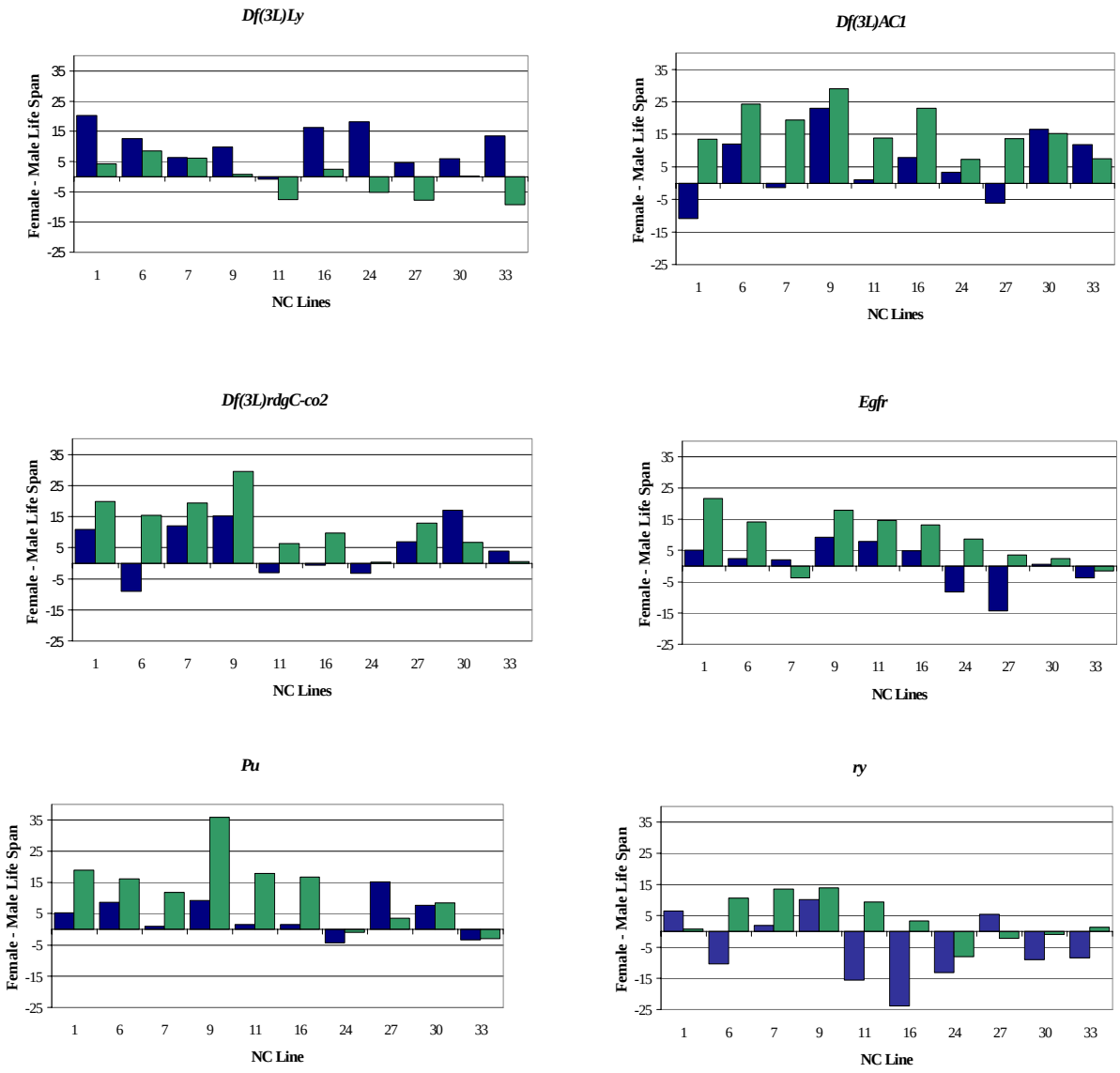


Figure 2 shows the difference between the life span of females and males for each genotype for each NC line. The difference in the life span of females and males of the mutant genotype is shown in blue, while that of the balancer genotype is shown in green.

Figure 3. Difference in Life Span Between Mutant and Balancer Backgrounds

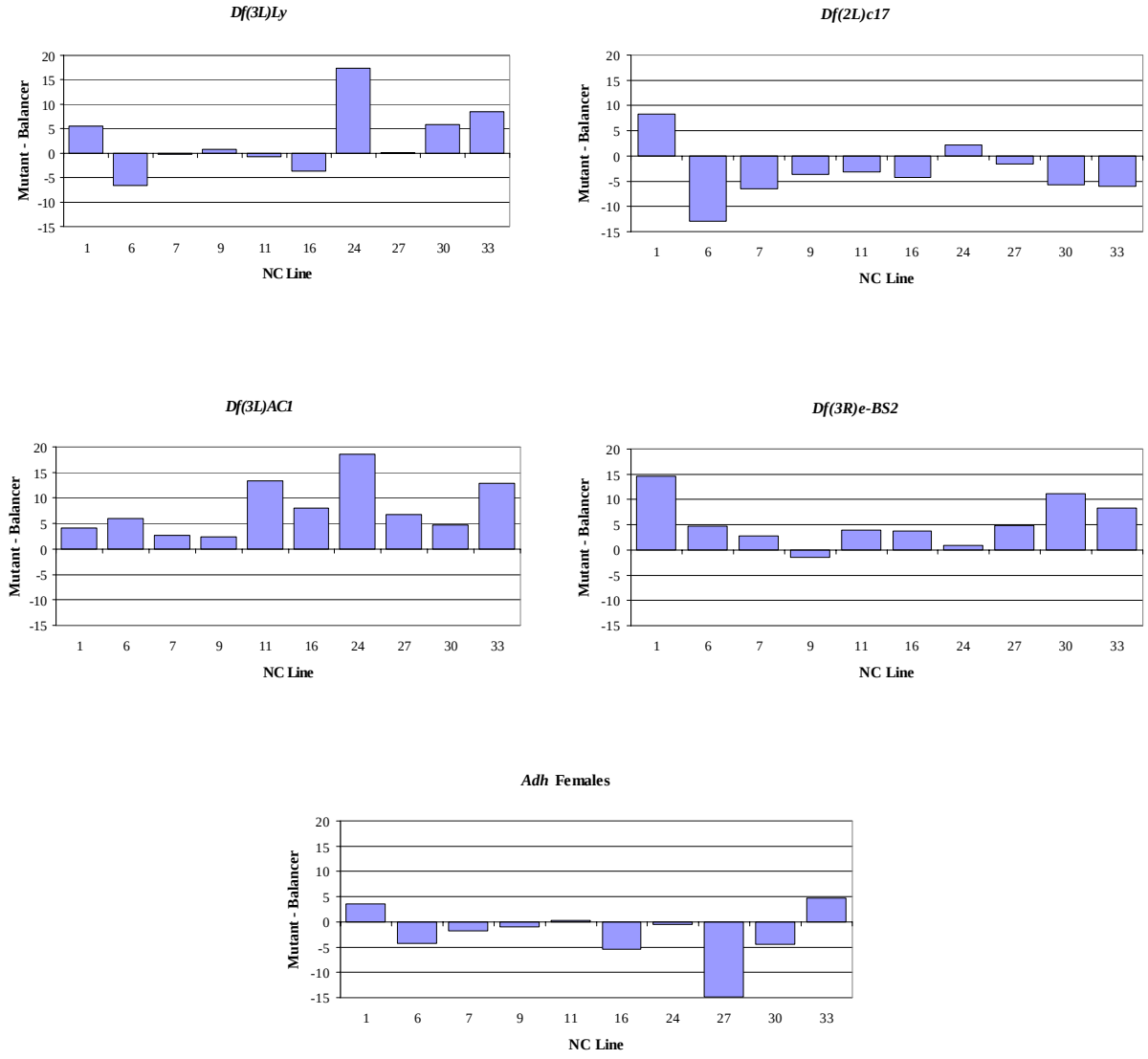


Figure 3 shows the difference between the life span of the mutant and balancer genotypes across the sexes for each NC line.

Table 3. Source of Variation by Sex

	Females			Males		
	LINE (L)	GENOTYPE (G)	L × G	LINE (L)	GENOTYPE (G)	L × G
Df(2L)cl7 [a]	<0.0001	0.0053	0.1115	0.0003	0.0317	0.0623
Df(3L)Ly [b]	<0.0001	<0.0001	0.0034	<0.0001	0.0252	0.3370
Df(3L)AC1 [c]	<0.0001	0.0486	0.0004	<0.0001	<0.0001	0.1856
Df(3R)e-BS2 [d]	<0.0001	0.0002	0.0155	0.0018	0.0008	0.1424
Df(1)JC19 [e h]	0.0011	0.7223	0.4471			
Df(3L)st-f13 [f]	0.5479	<0.0001	0.2543	<0.0001	<0.0001	0.2543
Df(3L)rdgC-co2 [g]	<0.0001	0.0020	0.0280	<0.0001	<0.0001	0.5905
Adh^{n1}	<0.0001	0.0304	0.0257	<0.0001	0.0853	0.1998
Cat^{n1}	<0.0001	<0.0001	0.3604	0.0001	<0.0001	0.2323
$Egfr^{f3}$	0.0293	0.0006	0.1172	0.0031	0.4683	0.9809
$Gpdh^{n5-4}$	0.0094	<0.0001	0.2152	<0.0001	0.1121	0.0170
$mus306^{D1}$	0.0001	<0.0001	0.2219	0.0032	<0.0001	0.2212
Pgd^{n39} Zw^{n10} [i]	0.3243	0.0367	0.0432			
Pu^{rAA1}/CyO	0.2373	0.0427	0.2337	0.0022	0.0588	0.3229
ry^{506}	0.0422	0.0001	0.6987	0.0001	0.0001	0.1480
Sod^{n1}	0.0724	0.0003	0.7714	0.0006	0.0001	

[a] Deficiency uncovers *Acp26A*.
[b] Deficiency uncovers *Acp70*.
[c] Deficiency uncovers *Hsp22* through *Hsp28*.
[d] Deficiency uncovers *InR*.
[e] Deficiency uncovers *per*.
[f] Deficiency uncovers *Pgm*.
[g] Deficiency uncovers *psn*.
[h] Mutation on chromosome 1; only female progeny.
[i] No single mutant stocks were available. Tested together as *Pgd* and *Zw* (*G6pd*) are both in the pentose phosphate shunt pathway.

the mutant chromosomes in the background of the wild lines. Therefore, we do not consider this interaction as consistent with allelism.

Observation of a significant failure to complement cannot be attributed definitively to allelism or epistasis, or even to specific interactions with the mutation of interest. However,

complementation can be used to exclude a candidate gene as contributing to naturally occurring variation in life span in the Raleigh lines from future studies. In the past, the results of quantitative complementation tests have been completely consistent with results of association studies (Long *et al*, 1998; Lyman *et al*, 1999; Robin *et al.*, 2002). The caveat here is that only 10 wild-derived lines were tested. With larger samples, capturing more of the variation in nature, it is possible that some of the genes that complement in this sample may exhibit failure to complement. For example, *Sod* (Orr and Sohal, 1994; Parkes *et al.*, 1998; Arking, 1998), *Cat* (Orr and Sohal, 1994; Sohal *et al.*, 1995) and *ry* (Hilliker *et al.*, 1992) have all been shown to regulate life span, but mutations in these genes complemented the longevity QTL in the 10 Raleigh lines. Possibly these essential genes are under strong purifying selection and do not harbor alleles affecting life span in nature. Alternatively, such variants may be rare, and were not included in the sample of alleles tested.

The candidate genes shown to affect variation in life span are *Adh* and the deficiencies *Df(2L)cl7* uncovering *Acp26*, *Df(3L)Ly* uncovering *Acp70*, *Df(34)e-BS2* uncovering *InR*, and *Df(3L)AC1* uncovering *Hsp22-Hsp28*. In analyses of candidate genes where there is a null mutation, the interpretation of the data is simplified by knowing the location of the mutation. For deficiencies, significant results localize QTL affecting variation in life span in a natural population to the region uncovered by the deficiency. Such deficiency regions are excellent candidates for high resolution recombination mapping or for a region of focus for linkage disequilibrium mapping in a large sample of alleles from nature. While a significant result from a deficiency indicates a genetic interaction, is may be due to another gene in the region rather than the gene of interest.

The significant null mutation and the candidate genes which were uncovered by deficiencies effect life span in various ways. *Adh* and *InR* encode products involved in metabolism. Alcohol dehydrogenase enables flies to metabolize ethanol, and its expression has been shown to be down-regulated during aging (Zou *et al.,* 2000). *InR* encodes an insulin-like receptor. Hypomorphic mutations of *InR* produce dwarf flies which have an 85% extension in life span (Tatar *et al.,* 2001). Furthermore, *InR* shows homology to the *C. elegans daf-2* gene (Clancy *et al.,* 2001; Murkami and Johnson, 2001) which affects life span in worms (Kenyon *et al.,* 1993; Tissenbaum and Ruvkin, 1998). The heat shock cluster of *Hsp22-Hsp28* has not been directly implicated in affecting longevity. However, *Hsp70* has been shown to affect longevity in *Drosophila* (Tatar *et al.,* 1997), and this gene shows a age-related decrease in expression (Heydari *et al.,* 1993). Expression of several of the small heat shock genes also vary by age with Hsp26, Hsp27, increasing 3-fold, Hsp23 increased 5-fold, and Hsp22 increased 60-fold (King and Tower, 1999). *Acp70a* and *Acp26A* are transferred to the females during mating. *Acp70A* stimulates oviposition, a behavior with the potential to affect life span (Chen *et al.,* 1988). *Acp26A* resembles a peptide pheromone precursor (Clark *et al.,* 1995). However, neither of these accessory proteins have been shown to directly affect life span in previous experiments. *Acp62F* is the only accessory protein shown to have a direct effect on aging (Lung *et al;* 2002), but the expression of the accessory protein genes *Mst57dc* and *Acp36DE* have been shown to change with age (Zou *et al.,* 2000).

Quantitative complementation tests are a powerful tool to determining whether candidate life span genes interact genetically with naturally occurring QTL affecting variation in life span in *Drosophila melanogaster*. The candidate genes and gene regions shown here

to fail to complement life span QTL segregating in nature are strong candidates for linkage disequilibrium mapping (Lander and Schork, 1994; Lai *et al.*, 1994; Long *et al.*, 1998; Long *et al.*, 2000; Robin *et al.*, 2002) in larger samples of alleles derived from nature to identify the actual molecular polymorphisms (quantitative trait nucleotides) causing the variation in longevity at these loci.

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Supplemental Table 1 – Line Means by Sex and Genotype

	NC line1				NC line 6				NC line 7			
	Males		Females		Males		Females		Males		Females	
	m	Bal	m	Bal	m	Bal	m	Bal	m	Bal	m	Bal
Df(2L)cl7 [a]	52 (2.9)	42 (2.8)	54 (2.2)	48 (1.6)	42 (2.7)	57 (2.5)	49 (2.2)	60 (1.4)	54 (3.8)	59 (2.3)	57 (1.0)	66 (1.8)
Df(3L)Ly [b]	38 (3.3)	41 (2.6)	58 (1.4)	45 (1.6)	31 (2.5)	39 (3.0)	43 (3.1)	48 (2.5)	39 (2.4)	39 (2.7)	45 (2.0)	45 (3.0)
Df(3L)AC1 [c]	50 (3.0)	34 (2.7)	39 (3.0)	47 (2.8)	53 (3.6)	41 (1.7)	65 (1.2)	65 (1.8)	53 (1.8)	39 (2.2)	52 (2.6)	58 (1.1)
Df(3R)e-BS2 [d]	59 (2.4)	42 (2.9)	65 (2.0)	52 (1.5)	46 (1.3)	41 (2.6)	65 (1.3)	61 (2.1)	45 (2.8)	47 (2.6)	74 (1.8)	66 (1.6)
Df(1)JC19 [e h]			56 (3.4)	58 (2.1)			73 (3.1)	68 (1.9)			64 (3.5)	67 (2.4)
Df(3L)st-f13 [f]	32 (1.9)	39 (1.6)	40 (2.8)	56 (2.3)	23 (1.8)	44 (2.4)	40 (0.8)	57 (3.2)	28 (3.1)	48 (2.6)	33 (1.1)	62 (1.8)
Df(3L)rdgC-co2 [g]	52 (2.5)	36 (3.2)	63 (1.8)	56 (3.8)	67 (2.4)	47 (3.1)	58 (1.2)	62 (1.0)	55 (2.5)	46 (3.2)	67 (1.7)	65 (2.3)
Adh^{n1}	48 (1.9)	44 (2.8)	67 (0.8)	63 (1.8)	56 (3.3)	59 (3.7)	71 (4.1)	76 (1.9)	56 (2.4)	51 (3.7)	69 (3.0)	70 (1.6)
Cat^{n1}	46 (5.4)	34 (2.1)	60 (2.3)	43 (1.7)	59 (2.1)	38 (2.1)	67 (2.7)	48 (2.7)	62 (3.2)	36 (1.6)	66 (1.6)	49 (3.0)
Egfr^{f3}	56 (2.5)	53 (3.9)	62 (3.0)	74 (2.8)	64 (2.8)	61(2.7)	66 (2.0)	75 (2.6)	69 (3.3)	70 (3.0)	71 (1.9)	66 (4.3)
Gpdh^{n5-4}			67 (1.7)	64 (2.0)			67 (1.9)	69 (1.5)			71 (2.3)	68 (1.9)
mus306^{D1}	48 (2.9)	45 (2.8)	60 (1.8)	71 (2.0)	55 (3.4)	53 (2.6)	66 (2.0)	70 (3.1)	69 (3.7)	64 (3.5)	62 (3.8)	75 (1.8)
Pgd^{n39} *Zw*^{n10} [i]	42 (2.3)	38 (2.5)	52 (2.1)	51 (1.7)	52 (2.4)	38 (2.0)	63 (1.6)	58 (0.9)	49 (3.4)	44 (2.6)	60 (2.2)	44 (2.5)
Pu^{rAA1}	56 (3.2)	54 (2.7)	62 (3.1)	73 (1.7)	59 (3.7)	58 (2.9)	67 (1.9)	74 (2.5)	66 (2.1)	63 (2.4)	67 (2.9)	74 (2.5)
ry^{506}	51 (2.0)	35 (3.1)	58 (2.0)	36 (4.8)	61 (2.0)	35 (2.4)	51 (2.5)	46 (3.1)	74 (4.0)	43 (2.9)	76 (3.0)	57 (3.2)
Sod^{n1}	49 (3.8)	40 (3.0)	66 (2.3)	65 (1.6)	59 (2.2)	47 (3.8)	61 (3.1)	56 (2.2)	67 (2.1)	58 (1.8)	73 (2.1)	62 (2.6)

[a] Deficiency uncovers *Acp26A*.
[b] Deficiency uncovers *Acp70*.
[c] Deficiency uncovers *Hsp22* through *Hsp28*.
[d] Deficiency uncovers *InR*.
[e] Deficiency uncovers *per*.
[f] Deficiency uncovers *Pgm*.
[g] Deficiency uncovers *psn*.
[h] Mutation on chromosome 1; only female progeny.
[i] No single mutant stocks were available. Tested together as *Pgd* and *Zw* (*G6pd*) are both in the pentose phosphate shunt pathway.

Supplemental Table 1, Continued – Line Means by Sex and Genotype

	NC line 9				NC line 11				NC line 16			
	Males		Females		Males		Females		Males		Females	
	m	Bal	m	Bal	m	Bal	m	Bal	m	Bal	m	Bal
Df(2L)cl7 [a]	51 (2.3)	57 (2.3)	60	61 (1.3)	63 (2.0)	66 (1.8)	60 (1.3)	63 (2.6)	49 (3.6)	57 (3.1)	62 (1.3)	63 (1.4)
Df(3L)Ly [b]	25 (3.4)	29 (3.1)	35 (1.7)	30 (3.3)	46 (2.5)	50 (2.1)	45 (3.6)	43 (3.8)	19 (3.0)	30 (4.7)	35 (1.4)	32 (3.4)
Df(3L)AC1 [c]	30 (3.1)	24 (2.9)	53 (1.8)	53 (1.2)	58 (1.8)	38 (2.2)	59 (0.9)	52 (1.8)	47 (2.6)	32 (1.7)	56 (1.9)	55 (1.2)
Df(3R)e-BS2 [d]	41 (2.3)	36 (2.6)	52 (3.4)	59 (0.9)	52 (2.1)	47 (1.9)	66 (1.6)	62 (1.0)	54 (2.8)	46 (3.2)	61 (1.6)	62 (2.0)
Df(1)JC19 [e h]			62 (4.4)	64 (1.8)			61 (3.7)	64 (3.4)			63 (2.2)	54 (3.5)
Df(3L)st-f13 [f]	24 (2.6)	64 (2.2)	40 (1.5)	62 (2.1)	36 (2.3)	53 (2.0)	44 (1.2)	58 (1.6)	31 (3.4)	51 (4.3)	41 (2.3)	53 (2.6)
Df(3L)rdgC-co2 [g]	48 (3.2)	33 (2.4)	63 (1.9)	62 (1.3)	63 (4.2)	51 (3.3)	60 (3.5)	57 (1.8)	34 (3.5)	56 (3.7)	63 (3.1)	65 (2.0)
Adh^{n1}	48 (2.7)	46 (3.9)	67 (2.3)	68 (1.2)	52 (3.4)	54 (3.6)	68 (1.9)	67 (1.6)	39 (3.4)	53 (2.8)	62 (1.2)	68 (3.2)
Cat^{n1}	51 (2.5)	33 (3.9)	74 (1.0)	51 (1.7)	66 (2.3)	39 (2.1)	60 (2.8)	42 (1.5)	50 (1.5)	41 (2.6)	74 (1.7)	48 (2.7)
Egfr^{f3}	65 (3.4)	56 (4.6)	74 (2.7)	74 (1.7)	67 (3.7)	65 (5.6)	75 (1.6)	79 (2.5)	67 (4.2)	64 (3.4)	72 (4.3)	77 (2.0)
Gpdh^{n5-4}			69 (1.6)	69 (1.4)			64 (2.7)	70 (1.2)			67 (2.2)	65 (1.4)
mus306^{D1}	54 (3.4)	48 (4.1)	68 (1.3)	72 (1.4)	68 (2.2)	56 (4.9)	70 (1.9)	73 (3.1)	60 (3.0)	79 (3.3)	66 (3.3)	79 (2.5)
Pgd^{n39} *Zw*^{n10} [i]	42 (1.8)	41 (4.2)	61 (2.0)	53 (2.5)	57 (2.1)	47 (2.6)	62 (1.3)	51 (2.2)	61 (3.0)	44 (2.5)	59 (2.6)	54 (2.5)
Pu^{rAA1}	61(2.3)	43 (3.0)	71 (3.4)	79 (2.6)	70 (2.3)	58 (4.0)	72 (2.5)	76 (2.1)	68 (3.6)	56 (2.4)	70 (1.6)	72 (2.1)
ry^{506}	48(3.0)	38 (4.2)	58 (3.9)	52 (3.9)	73 (4.8)	47 (2.1)	57 (3.2)	56 (1.7)	85 (4.9)	53 (2.6)	61 (3.9)	56 (3.8)
Sod^{n1}	48(4.0)	40 (3.1)	67 (3.0)	66 (2.6)	68 (3.7)	50 (3.6)	65 (2.3)	59 (3.1)	65 (3.2)	65 (3.1)	69 (3.7)	66 (2.2)

[a] Deficiency uncovers *Acp26A*.
[b] Deficiency uncovers *Acp70*.
[c] Deficiency uncovers *Hsp22* through *Hsp28*.
[d] Deficiency uncovers *InR*.
[e] Deficiency uncovers *per*.
[f] Deficiency uncovers *Pgm*.
[g] Deficiency uncovers *psn*.
[h] Mutation on chromosome 1; only female progeny.
[i] No single mutant stocks were available. Tested together as *Pgd* and *Zw* (*G6pd*) are both in the pentose phosphate shunt pathway.

Supplemental Table 1, Continued – Line Means by Sex and Genotype

	NC line 24				NC line 27				NC line 30			
	Males		Females		Males		Females		Males		Females	
	m	Bal	m	Bal	m	Bal	m	Bal	m	Bal	m	Bal
Df(2L)cl7 [a]	66 (2.0)	59 (3.4)	54 (2.4)	58 (3.1)	51 (2.1)	52 (2.0)	54 (2.0)	57 (3.1)	51 (3.5)	58 (3.5)	56 (2.1)	60 (2.1)
Df(3L)Ly [b]	33 (3.1)	27 (4.8)	51 (1.7)	22 (3.0)	14 (2.6)	29 (3.6)	37 (3.4)	21 (2.9)	40 (2.6)	38 (4.1)	46 (2.6)	38 (4.3)
Df(3L)AC1 [c]	57 (2.3)	34 (2.8)	57 (1.2)	42 (2.4)	56 (3.1)	39 (3.4)	50 (2.6)	53 (2.1)	48 (2.8)	44 (2.5)	65 (3.0)	59 (2.2)
Df(3R)e-BS2 [d]	43 (1.8)	47 (1.7)	49 (1.8)	45 (2.1)	52 (2.6)	46 (2.6)	58 (3.4)	54 (1.8)	51 (2.0)	45 (2.7)	68 (1.6)	52 (2.3)
Df(1)JC19 [e h]			56 (2.3)	46 (2.6)			63 (3.3)	59 (3.0)			58 (2.4)	61 (1.4)
Df(3L)st-f13 [f]	36 (2.2)	62 (1.5)	37 (2.5)	60 (0.9)	34 (2.2)	54 (2.3)	40 (2.2)	55 (1.2)	39 (2.4)	53 (2.8)	47 (1.3)	60 (2.4)
Df(3L)rdgC-co2 [g]	56 (2.9)	47 (2.4)	53 (2.4)	47 (2.0)	54 (2.8)	46 (3.0)	61 (1.8)	59 (2.5)	51 (2.5)	45 (3.1)	68 (2.6)	52 (1.7)
Adh^{n1}	60 (4.0)	66 (1.7)	63 (1.4)	64 (1.2)	58 (3.8)	55 (4.4)	49 (3.6)	64 (2.6)	51 (1.6)	63 (2.6)	66 (2.3)	70 (1.8)
Cat^{n1}	52 (3.3)	41 (2.7)	56 (1.9)	43 (2.4)	53 (2.9)	37 (3.6)	56 (2.6)	43 (2.1)	64 (1.7)	43 (2.1)	70 (1.7)	54 (2.7)
Egfr^{f3}	72 (3.0)	72 (5.2)	64 (3.4)	81 (2.2)	66 (2.7)	67 (3.8)	54 (4.5)	71 (2.1)	72 (3.3)	71 (2.4)	73 (2.0)	74 (2.3)
Gpdh^{n5-4}			71 (1.6)	55 (2.6)			64 (1.5)	65 (1.4)			70 (2.6)	66 (1.9)
mus306^{D1}	67 (3.6)	78 (2.2)	64 (2.8)	76 (2.2)	61 (3.4)	72 (3.5)	64 (1.9)	66 (1.8)	67 (4.0)	72 (3.4)	61 (2.7)	62 (2.1)
Pgd^{n39} *Zw*^{n10} [i]	59 (3.2)	45 (2.9)	59 (1.3)	47 (2.6)	47 (3.3)	40 (3.2)	53 (3.0)	43 (3.4)	58 (2.4)	40 (2.1)	59 (3.0)	55 (2.1)
Pu^{rAA1}	74 (3.8)	74 (3.3)	70 (2.1)	73 (1.6)	58 (4.0)	62 (4.2)	73 (2.0)	66 (2.3)	57 (3.2)	59 (3.7)	64 (3.0)	68 (2.9)
ry^{506}	67 (4.3)	53 (3.3)	54 (1.6)	45 (2.7)	51 (4.1)	43 (3.2)	57 (1.4)	40 (3.5)	71 (2.1)	50 (1.6)	62 (3.3)	49 (3.0)
Sod^{n1}	69 (3.6)	54 (3.0)	66 (1.9)	61 (2.8)	65 (1.9)	46 (2.9)	62 (2.7)	58 (2.1)	66 (3.0)	57 (2.7)	71 (2.1)	59 (2.0)

[a] Deficiency uncovers *Acp26A*.
[b] Deficiency uncovers *Acp70*.
[c] Deficiency uncovers *Hsp22* through *Hsp28*.
[d] Deficiency uncovers *InR*.
[e] Deficiency uncovers *per*.
[f] Deficiency uncovers *Pgm*.
[g] Deficiency uncovers *psn*.
[h] Mutation on chromosome 1; only female progeny.
[i] No single mutant stocks were available. Tested together as *Pgd* and *Zw* (*G6pd*) are both in the pentose phosphate shunt pathway.

Supplemental Table 1, Continued – Line Means by Sex and Genotype

	NC line 33			
	Males		Females	
	m	Bal	m	Bal
Df(2L)cl7 [a]	56 (2.3)	65 (2.7)	51 (2.1)	54 (2.2)
Df(3L)Ly [b]	44 (3.1)	47 (4.5)	57 (1.6)	38 (4.1)
Df(3L)AC1 [c]	48 (1.9)	38 (1.8)	60 (1.5)	45 (2.9)
Df(3R)e-BS2 [d]	42 (2.0)	39 (2.0)	57 (2.4)	43 (1.1)
Df(1)JC19 [e h]			55 (2.1)	61 (1.9)
Df(3L)st-f13 [f]	43 (1.5)	58 (3.2)	37 (2.5)	60 (3.0)
Df(3L)rdgC-co2 [g]	56 (2.5)	45 (2.0)	60 (1.9)	46 (1.3)
Adh^{n1}	66 (4.4)	75 (2.7)	66 (1.5)	62 (2.8)
Cat^{n1}	67 (2.5)	48 (1.7)	70 (1.0)	54 (1.9)
$Egfr^{f3}$	71 (1.9)	75 (4.1)	67 (2.1)	73 (2.7)
$Gpdh^{n5-4}$			68 (3.3)	61 (1.4)
$mus306^{D1}$	62 (4.2)	71 (5.9)	61 (1.9)	69 (1.5)
Pgd^{n39} Zw^{n10} [i]	58 (2.4)	43 (2.1)	63 (1.0)	58 (1.5)
Pu^{rAA1}	72 (3.6)	69 (2.3)	69 (1.6)	66 (2.3)
ry^{506}	68 (3.6)	44 (2.2)	60 (3.6)	46 (3.3)
Sod^{n1}	70 (4.1)	50 (2.6)	66 (2.7)	53 (2.5)

[a] Deficiency uncovers *Acp26A*.
[b] Deficiency uncovers *Acp70*.
[c] Deficiency uncovers *Hsp22* through *Hsp28*.
[d] Deficiency uncovers *InR*.
[e] Deficiency uncovers *per*.
[f] Deficiency uncovers *Pgm*.
[g] Deficiency uncovers *psn*.
[h] Mutation on chromosome 1; only female progeny.
[i] No single mutant stocks were available. Tested together as *Pgd* and *Zw* (*G6pd*) are both in the pentose phosphate shunt pathway.

CHAPTER 3

PUNCH AFFECTS VARIATION IN *DROSOPHILA* LIFE HISTORY TRAITS

ABSTRACT

Mutations in most vital genes can potentially affect life history traits, but it is not known what subset of these loci harbor naturally occurring variation affecting the rate of aging and the ability to resist stress. This question can be addressed using *Drosophila melanogaster* as a model system: over 60% of known human disease genes have *Drosophila* homologues. The *Drosophila* gene *Punch* (*Pu*; *2*-97) encodes GTP cyclohydrolase (GTPCH). GTPCH regulates the catecholamine biosynthesis pathway by catalyzing the formation of tetrahydrobiopterin, the rate-limiting molecule, and by regulating tyrosine hydroxylase, a key enzyme in the pathway. The extent to which molecular variation at *Pu* contributes to phenotypic variation in two life history traits was assessed by associating single nucleotide polymorphisms (SNPs) at *Pu* with starvation resistance and longevity. Nucleotide variation was determined for ten *Pu* alleles. Genotypes of 28 SNPs were determined on a sample of 178 isogenic second chromosomes sampled from the Raleigh, USA population and substituted into the highly inbred *Samarkand* background. Life span and resistance to starvation stress were determined for the chromosome substitution lines and the association between life history trait phenotype and SNP genotype was assessed for each polymorphic marker. One SNP was significantly associated with starvation resistance (G4148C, $P = 0.0070$), and three SNPs were significantly associated with life span (C6291A, $P = 0.0183$; A6389T, $P = 0.0466$; G6894C, $P = 0.0024$). None of these SNPs was significant individually following a permutation test accounting for multiple tests and partially correlated markers. However, the three SNPs associated with life span were in global linkage disequilibrium. Haplotypes of these SNPs were highly significantly associated with variation

in longevity ($P < 0.0001$), and accounted for 13.5 % of the genetic variance and 1.85 % of the phenotypic variance in longevity attributable to chromosome *2*. As *Pu* is a regulator of the catecholamine biosynthetic pathway, these findings suggest the importance of the production of biogenic amines in determining variation for life history traits.

INTRODUCTION

As the world human population rapidly grows older, population aging will become one of the most important social and health problems in the next 50 years. While progress is being made in the treatment of age-related illnesses, we have yet to completely understand the genetic basis of either the aging process or age-related diseases such as Alzheimer's disease and Parkinson's disease. To understand the genetic architecture of aging, it is necessary to know which loci cause the aging process, which subset of these loci affect variation in aging, and the actual molecular polymorphisms (quantitative trait nucleotides) causing the variation in longevity at these loci. Only then will it be possible to infer genetic interactions between loci affecting longevity.

Analyses of mutations affecting life span in model organisms have identified genes that regulate longevity in evolutionarily conserved signaling pathways affecting metabolism, nutritional control and stress resistance. Flies selected for postponed senescence live longer and have increased resistance to starvation stress, which is attributable to a decrease in metabolic rate (Chippindale *et al.*, 1996). Caloric restriction may increase life span by reducing metabolic rate or by modifying normal age-related gene expression. Comparing the transcriptional profiles of once-mated female *Drosophila melanogaster* on a calorie restricted

diet with once-mated aged females produces 885 transcriptional changes by age and 827 changes by caloric restriction, of which 448 were common between the two treatments (Pletcher *et al.,* 2002). Furthermore, there was a direct relationship between the direction of the expression change in both treatments (Pletcher *et al.,* 2002). Genes down regulated by caloric restriction included those involved in DNA repair and replication, cell cycle control, protein metabolism and oogenesis (Pletcher *et al.,* 2002).

Another study focused on expression profiles of 11,000 genes in the liver of young and old mice subjected to either a control, short term, or long term caloric restriction. Comparison of the affect of long-term caloric restriction to the profiles for control young and control old mice, showed the restricted diet negated the normal age-related increase in 75% of the genes whose expression increases with normal aging (Cao *et al.,* 2001). Genes included in this set encode stress response proteins and chaperones (Cao *et al.,* 2001). When short term and long term caloric restriction are compared, the short treatment (4 weeks) shows approximately a 30% decrease in the effects of the gene expression that increases with long-term caloric restriction. Combining the results of the microarray experiments discussed above leads to the conclusion that caloric restriction mitigates age-related stress by increasing the transcripts of genes involved in either heat stress or oxidative stress as well as decreasing mitochondrial activity, thereby protecting the cell from damage due to metabolism related oxidative stress.

Drosophila metabolic pathways have been studied mainly from the point of insulin-like signaling which is an important factor in aging. The insulin-like growth factor (IGF) pathway in flies starts with an insulin-like receptor (*InR*). Females carrying hypomorphic

mutations of *InR* are dwarf and deficient in juvenile hormone (JH), but have an 85% extension in life span (Tatar *et al.,* 2001). It is thought that the relationship between receptor and JH may extend life span through decreasing metabolism, thereby slowing growth and development (Tatar *et al.,* 2001). *InR* also interacts with *chico*, the insulin receptor substrate (Clancy *et al.,* 2001). Homozygous mutations at *chico* increase female life span by 48% while heterozygous mutations cause a 36% increase without producing dwarf flies (Clancy *et al.,* 2001). Phosphatidylinositol-3-kinase (PI3K) and protein kinase B also play a role in insulin signaling, but their characterization is best described in the dauer larva formation pathway of *C. elegans.* Another player in the IGF pathway of *Drosophila* is the eponymous *I'm-Not-Dead-Yet* (*Indy*) gene, which shows homology to a mammalian sodium dicarboxylate co-transporter which transports succinate, citrate, and alpha-keto-glutarate produced during the Krebs's cycle (Rogina and Helfand, 2000). *Indy* expression is greatest in the fat body and mid-gut of flies, which are the sites of highest metabolic activity (Rogina and Helfand, 2000). Mutations in this gene produce a decreased metabolic state similar to the one in caloric restriction which increases life span maximally in heterozygotes and to some extent in homozygotes (Rogina and Helfand, 2000).

As metabolism and nutritional control are tied to the aging process and oxidative stress, many studies have been undertaken to explore the relationship of antioxidant enzymes with life span. Strains of *Drosophila melanogaster* selected for increased longevity had greater than normal levels of the product of the antioxidant gene, Cu-Zn Sod (Arking, 1998). Lack of a functioning *Sod* allele greatly reduces life span in fruit flies (Parkes *et al.*, 1998-a). In addition, when transgenic flies were engineered to over-express human cytosolic SOD1 in

the motorneurons, there was a forty percent increase in life span, and the transgene rescued the life span of a *Sod* loss of function mutation (Parkes *et al.,* 1998-b). Expression of both the *Drosophila Cu-Zn Sod* and *Catalase* (*Cat*) transgenes increases life span up to thirty-percent (Orr and Sohal, 1994), slows rates of mitochondrial hydrogen peroxide production, and increases metabolic capacity relative to control flies (Sohal *et al.*, 1995).

Three other *Drosophila* genes affect life span through oxidative stress mitigation: *rosy, methuselah,* and *thioredoxin reductase.* The first two were tested, not by over-expression, but by knocking out gene expression and exposing the flies to hyperoxic conditions. *rosy* is the structural gene for xanthine dehydrogenase. This enzyme catalyzes the formation of urate, a scavenger of oxygen radicals. Flies lacking a functional *rosy* allele have very short life spans when exposed to paraquat, which is a source of exogenous free radicals (Hilliker *et al.*, 1992). Corresponding tests have been conducted using the gene *methuselah* with similar results. The line containing the *methuselah* mutation has a life span thirty-five percent greater than the parental strain and has increased resistance to oxidative stress as measured by the paraquat assay (Lin *et al.*, 1998). The Methuselah protein is currently under investigation, but it is thought to be a regulator of a G-protein associated with stress response and aging (Lin *et al.*, 1998). The *dmtrxr-1* gene codes for thioredoxin reductase (TrxR), originally thought to be th*e Drosophila* homolog to glutathione reductase, and provides antioxidant function by transferring reducing equivalents from NADPH to both thioredoxin and glutathione disulfide rather than directly participating in the detoxification of the superoxide anion (Missirlis, Phillips and Jäckle, 2001). Flies carrying null mutations of

dmtrxr-1 show increased free radical induced damage and decreased adult life span (Missirlis *et al.*,2001).

There is great interest in how oxidative stress affects gene expression. Rogina and Helfand (2000) showed that the temporal pattern of gene expression at the *wingless* locus in *Drosophila melanogaster* is accelerated in flies homozygous for a null mutation of Cu-Zn Sod. First, the experiment shows a decrease in life span in the *Sod* mutant flies (Rogina and Helfand, 2000). Then by using a *wingless* enhancer-trap stock and plotting β-gal expression as a function of percent of life span, they observed the Sod^{n1}/Sod^{n1} survival curve to be identical to the control, indicating that *wingless* is expressed in the same manner but at an earlier time in the mutant flies (Rogina and Helfand, 2000). Further evidence for the role of oxidative stress in aging is provided by a microarray study in which transcript levels of aging flies were compared with that of flies subjected to a paraquat treatment (Zou *et al.*, 2000). While expression of some genes was affected by either aging or hyperoxic conditions, expression patterns for 42 genes were changed by both aging and paraquat treatment. These genes encode proteins as diverse as protease inhibitors involved in protein turnover and accessory proteins involved in reproductive potential (Zou *et al.*, 2000). Decreases in protein turnover and reproduction are hallmarks of aging, indicating that changes in transcription levels in response to oxidative stress are similar to those seen in the normal aging process. The proteasomal system is responsible for removing damaged proteins from the cell, but Grune (2000) observed a decrease in activity of proteasome subunits under oxidative stress, leading to a decrease in protein turnover. Over 100 genes or proteins show changes in expression or activity when stimulated by hydrogen peroxide or paraquat or when the

expression of antioxidant genes are changed (reviewed by Allen and Tresini, 2000). This implicates oxidative stress as a significant factor in not only aging but in pathways from development, to metabolism, to hormone signaling.

We know that mutations in the genes of different stress response pathways affect life span. However, we do not know whether functional polymorphisms in the candidate genes identified in these studies affect population variation in life span and stress resistance, or whether analysis of naturally occurring variation will uncover new loci and novel pathways. In order to understand what loci and pathways affect naturally occurring variation in longevity and stress resistance, we need to map the quantitative trait loci (QTLs) causing variation in these traits, and identify the molecular polymorphism(s) that define QTL alleles. Knowledge of the causal genotypes – so-called quantitative trait nucleotides (QTNs, Long *et al.,* 1998) – is essential if we are to understand the functional relationship between the gene product and trait variation, and because gene frequency (rare or common) will give some insight as to the selective mechanism operating to maintain the variation.

Understanding the nature of the forces maintaining large amounts of segregating genetic variation for traits related to fitness in natural populations (Houle *et al.,* 1996) is one of the central, and unanswered, questions in evolutionary quantitative genetics. A balance between the creation of deleterious mutations and their elimination by natural selection will account for rare alleles affecting life history traits (Houle *et al.,* 1996). In addition, alleles at intermediate frequency can be maintained by balancing selection if they have opposite effects in males and females, different environments, or different life history traits (Barton and Keightley, 2002). For example, there is some evidence for antagonistic pleiotropy acting to

maintain variation for life span in *Drosophila*, in the form of a trade off between early fertility and longevity (Rose, 1984).

Previously, we mapped QTLs affecting resistance to starvation stress in *Drosophila melanogaster* in a population of recombinant inbred lines derived from the Oregon and 2b strains (Vieira *et al.,* 2000). High resolution deficiency complementation mapping followed by complementation tests to mutations implicated *Punch* (*Pu*) as a candidate gene affecting the difference in starvation resistance between these strains (Harbison *et al.,* 2003-a). Further, comparison of whole genome transcriptional profiles of flies undergoing starvation stress to fed controls revealed that *Pu* was highly significantly down-regulated in the starvation treatment ($P = 3.23 \times 10^{-11}$, Harbison *et al.,* 2003-b). *Pu* mutants are hyper-sensitive to starvation, dessication and hypergravity stress (Chaudhari *et al.,* 2003; Wang *et al.,* 2003) *Pu* encodes GTP cylcohydrolase (GTPCH-1) which catalyzes the formation of tetrahydrobiopterin (BH_4) which is a cofactor for phenylalanine hydroxylase, tyrosine hydroxylase, tryptophan hydroxylase, and nitrous oxide synthase (Gross and Levi, 1992; Thöny *et al.,* 2000). BH_4 and phenylalanine are used by phenylalanine hydroxylase (PAH) to produce tyrosine. From there, BH_4 is required by tyrosine hydroxylase (TH) to convert tyrosine to L-DOPA which is later converted to dopamine by dopa-decarboxylase. The dopamine is used for melanization and scleratization of the cuticle in addition to its function as the principle neurotransmitter in *Drosophila*. Serotonin production is also in this pathway as BH_4 and tryptophan are necessary for the production of 5-OH-tryptophan by tryptophan hydroxylase (TPH), the end product of which is serotonin (see www.bh4.org for models of dopamine production).

Pu is thus a strong candidate gene for resistance to starvation stress. Interestingly, *Ddc*, which is also in the catecholamine biosynthesis pathway, is associated with variation in *Drosophila* longevity (de Luca *et al.,* 2003). Further, longevity and starvation resistance appear to be positively genetically correlated in *Drosophila* (Service and Rose, 1985). Here we use linkage disequilibrium mapping to test whether molecular polymorphisms in *Pu* are associated with naturally segregating variation in longevity and starvation resistance, and find that *Pu* is a candidate gene affecting life history variation in *Drosophila*.

MATERIALS AND METHODS

(i) *Drosophila* stocks

Chromosome *2* substitution lines: Isofemale lines were constructed from wild-type gravid females collected at the Raleigh, NC Farmer's Market in 1999. A single second chromosome was extracted from each of 178 isofemale lines and substituted into the highly inbred *Samarkand* (*Sam*) background by standard techniques using balancer chromosomes (Lyman and Mackay, 1998). Stocks were maintained on cornmeal-agar-molasses medium at 25^0 C. Hemizygous flies for genotyping were produced by crossing the substitution lines to a deficiency stock *Df(2R)PF1, cn^1 bw^1 sp^1/SM1* uncovering the *Pu* locus, which was generously donated by Janis O'Donnell.

Inbred lines: A set of 10 inbred lines were derived by 14 generation of full-sibling inbreeding from isofemale lines collected from the Raleigh population in 1994 (Fry *et al.,* 1998).

All stocks were maintained in vials containing 10 ml of cornmeal-agar-molasses medium at 25^0.

(ii) Longevity

Virgin flies from each of the 178 chromosome *2* substitution lines were collected for 48 hours and six single-sex flies were placed in shell vials containing approximately 5 mL of cornmeal-agar-molasses medium without yeast on the surface. Flies were collected from one-third of the lines every week, with the groups being randomized every three weeks. The experiment was repeated four times in succession but randomizing the lines collected at any one time, thus all lines experienced the same temporal environmental variation across assays. Flies were placed in a 25^0 incubator for the duration of the assay. Flies were transferred to fresh vials every two to six days as needed, and the number of live and dead flies was recorded every two days. Any escaped flies were noted and removed from the assay, dropping the total number of some lines to 18 rather than 24 flies. A total of 6,421 flies were scored from 178 lines.

(iii) Starvation resistance

We also assayed the starvation tolerance of 153 chromosome substitution lines. We used 1.5% agar and 5 ml water in standard culture vials to provide moisture to the flies without providing nutrition. Flies were collected and separated by sex before placement on the starvation medium. Five two-to-seven day-old non-virgin flies were assayed per vial, with four replicate vials for each sex. Each vial was kept in an incubator at a constant temperature of 25^0 C, 60-75% relative humidity, and a 12-hour light-dark cycle. Flies were

scored for survival every twelve hours until all were dead. Starvation tolerance was calculated as the number of hours each fly survived.

(iv) *Pu* sequence

Qiagen DNA isolation kits (Qiagen, Valencia, CA) were used to extract the genomic DNA from the 10 reference inbred homozygous lines derived from the Raleigh population (Fry *et al.,* 1998; Geiger-Thornsberry and Mackay, 2003). PCR and sequencing primers were designed based on the published *Pu* sequence. To prevent polymerase error from being interpreted as sequence variation, three 45 ul PCR reactions from each primer pair were pooled and purified through Qiaquick columns before sequencing (Qiagen, Valencia, CA). The PCR products were sequenced directly from both strands using a set of 56 primers, designed at intervals of 500 base pairs, and using the ABI Big Dye terminator system. Twelve kilobases of sequence covering the *Pu* region, including approximately 500 base pairs upstream and 500 base pairs downstream of the coding region was obtained for each of the ten Raleigh lines. Primer sequences are available from the authors on request. Sequences were aligned using VectorNTI software (InforMax, Frederick, MD). Chromatograms were checked for singleton polymorphisms and manually edited where necessary.

(v) Polymorphism genotyping

DNA was extracted from all 178 chromosome substitution lines as described above. Twenty-seven SNPs were genotyped in each of the chromosome *2* substitution lines by Pyrosquencing (Ronaghi *et al.,* 1998), a quantitative method of micro-sequencing. Pyrosequencing reagents and protocols were supplied by Pyrosequencing AB. The SNP at

position 8147 was genotyped by direct sequencing of pooled PCR products. The sequences of the PCR and pyrosequencing primers are shown in Table 4.

(vi) Data analyses

Variance in life span and starvation resistance among the chromosome *2* substitution lines was partitioned according to the model $y = \mu + L + S + L \times S + R(L \times S) + E$, where L and S are, respectively, the random and fixed effects of line and sex, R is the variance between replicate vials, and E is the within vial variance. Assuming random mating and strict additivity, the genetic variance, V_G, for each trait was estimated as $\sigma_L^2/2 + \sigma_{SL}^2$, where σ_L^2 and σ_{SL}^2 are the among-line and sex x line variance components (Lyman and Mackay, 1998). Heritabilities were estimated as $V_G/[V_G + V_E]$ where the environmental variance, V_E, was estimated from the within line variance component.

The genetic correlation between longevity and starvation resistance was estimated as $cov_{LO, ST}/(\sigma^2_{LO} + \sigma^2_{ST})^{1/2}$. Here $cov_{LO, ST}$ was estimated from the covariance of line means. σ^2_{LO} and σ^2_{ST} are, respectively, the genetic variances among lines from ANOVA of longevity and starvation resistance separately, computed only for the lines for which both traits were measured. The same method was used to compute the cross-sex genetic correlations for each trait.

Estimates of θ_S (Watterson, 1975), θ_π (Nei and Tajima, 1981), θ_η (Fu and Li, 1993) and R (Hudson, 1987) and tests for departure from neutrality (Tajima, 1989; McDonald and Kreitman, 1991; Fu and Li, 1993) were applied to the ten Raleigh reference sequences

Table 4. Sequences of PCR, Sequencing Primers, and Pyrosequencing Primers

SNP	Pyrosequencing Primer	Upper PCR Primer	Lower PCR Primer
T145C	5'-CTGGCAGTGAGTCCGTAACATT-3'	*5'-GGAGGTCAGTAAGGAGC-3' [a b]	5'-TCAAAGTACTGGCCTACAA-3'
T185G	5'-TCGGTGTCCATCTCGTG-3'		
C1242T	5'-CACAATCATTATAGAACCCT-3'	5'-ACCCTGGTGTCCACCTACAC-3'	*5'-GACGGGCTAGTGTCATGGTAA-3'
T2150C	5'-CAATCAACCCTCCCAT-3' [c]	5'-CGACAAAAATCCAATGAGCA-3'	*5'-AATCCACACCCTCGGAGAC--3'
G2151A			
C2519T	5'-CTCCTCCGTGCTCAGGTT-3'	*5'-CCCAAGTGGATCCAAGAG-3'	5'-CTGCTCCATGTTCTCGAG-3'
C3012T	5'-TTGCTCTTGCAGTTGTATT-3'	5'-GGGCATGACTAACTTAACTG-3'	*5'-AGGATTCGGTTAAAGTATTCA-3'
C4009T	5'-CGGCAGAACTTTCGATTTTT-3'	*5'-CTTGTCTGGGGCTTCATGTT-3'	5'-GCCAAATACAAAAGTCC-3'
C4097T	5'-AACATGAAGCCCCAGACAAG-3'	*5'-ACCAGGAAGAGTTCCCGTTT-3'	5'-ACCAATCCCAGCACA-3'
G4148C	5'-AAGCGAGCAAAATGG-3'	5'-AACATGAAGCCCCAGACAAG-3'	*5'-ACCAGGAAGAGTTCCCGTTT-3'
C4769G	5'-CTAGATCGGGTTTACTG-3'	*5'-CGTTTGGTGATCTTCGTCAA-3'	5'-CGCCCCAACTAACCTATGAC-3'
C5441TA	5'-TGGCTAGTGGCTAAG-3'	*5'-TAAACCCCTTTGGCTTGTGA-3'	5'-CTCAAAACTGAGGGGCAATC-3'
C6291A	5'-AGAAAAGTGCTCGCGATAAA-3'	5'-CCTTCAACTCCATGCCATAAA-3'	5'-ATGTGAGGGAATGGTTGGAT-3'
A6389T	5'-CAATTGAAGCCCCACA-3'	5'-CCTTCAACTCCAATGCCATAAA-3'	*5'-ATGTGAGGGAATGGTTGGAT-3'

a * indicates the biotin label required for pyrosequencing
b SNPs separated by less than 250 base pairs are genotyped on the same amplicon if the direction for the optimal pyrosequencing primer is correct
c SNPs separated by less than 10 base pairs may, in most cases, by genotyped by a single pyrosequencing reaction

Table 4. Sequences of PCR, Sequencing Primers, and Pyrosequencing Primers

SNP	Pyrosequencing Primer	Upper PCR Primer	Lower PCR Primer
G6894C	5'-TCTTCGAGAACCACGGA-3'	5'-CGCTCTCTTCATCGAAGTAC-3'	*5'-CTCCGACTACTGTGATCCCT-3'
G7149A	5’-TTCAGGAAGTTCTTGATCAC-3’	5'-TTGGATCCCAAACCACTACC-3'	*5'-CCCTCCATTCATCTTGGAGA-3'
A8147G	BY DIRECT SEQUENCING[d]	*5'-AACAGCGTCTGCTCAACAAG-3'	5'-TACCTGGCGTTGTCGATGTA-3'
C8156G	5-CAGGAGCTGGCGC-3'		
C8205G	5'-GCCAACGGGACGAGA-3'		
A8226G	5'-GGCGGGGTCTCTG-3'		
G9113A	5'-TCCTATGAAATATGAGATTT-3'	5'-GACGCATCCAAAATCCATTC-3'	*5'-CTGCTGCTGTTGCTGTTGAT-3'
IN9496DEL	5'-CCCCTCCCCCACCA-3'	5'-AGTGAATAGCCTTCGGCATC-3'	*5'-GGAAAGCTTCAGTGCGTTTC-3'
G10069A	*5'-CGCACATGGAGAACATTTCA-3'	5'-CATTGGGCGAACTTTTCAAT-3'	*5'-AAATCACGTCGGGGTCA-3'
T10972A	5'-TGCATATAAAATTGAACAT-3'	*5'-TTTGAATACCGGTGTTGGGTA-3'	5'-CCCTGTCGAGGAACATTATGA-3'
C11540G	5'-TTCAAACAAAGCACTA-3'	*5'-CCCAAAAACAACAGGAGCTT-3'	5–CAGGCGAGCATTCTACCACT-3'
A11541G			
T11559C	5'-CGATGCTCTTGCTG-3'		
G11560CAT			

a * indicates the biotin label required for pyrosequencing
b SNPs separated by less than 250 base pairs are genotyped on the same amplicon if the direction for the optimal pyrosequencing primer is correct
c SNPs separated by less than 10 base pairs may, in most cases, by genotyped by a single pyrosequencing reaction
d Site 8147 was genotyped by direct sequencing using the lower PCR primer

using DnaSP Version 3.50 Software (Rozas and Rozas, 1999). Significance of pair-wise linkage disequilibrium was determined using Fisher's exact test.

Associations between SNP genotypes/haplotypes and life span and starvation resistance were assessed by ANOVA on line means according to the model $y = \mu + M + S + M \times S + E$ where M is the fixed effect of marker haplotype, and S is the fixed effect of sex. Significance of each term in the ANOVA was determined by F-ratios using the Type III mean squares using SAS procedures SAS (SAS Institute, Inc, 1988). Variance components of significant markers were estimated by ANOVA using the model above where marker is treated as a random effect.

RESULTS

Quantitative Genetic Analysis

We extracted 178 second chromosomes from the Raleigh population and substituted them into the highly inbred *Samarkand* background. These lines exhibit a wide range of life spans. Line means for males ranged from 10.5 to 50.6 days with an average of 32.5 and a standard deviation of 13.8 days. The females on average lived slightly longer than the males with the line means falling between 11.6 and 61.6 days with an average of 35.3 days and a standard deviation of 15.6. When averaged across sexes, the life span line means ranged from 16.1 to 52.0 days. The variation among lines for life span was highly significant ($P > 0.0001$), but the sex by line interaction term was not significant ($P = 0.0994$) in this sample of chromosomes (Table 5). The heritability of life span attributable to the second chromosome is 0.137 in these 178 lines.

The pattern of variation in the 153 assayed for starvation resistance was similar to that of longevity. Again, females survived longer without food than the males. The average female survival time was 68.9 hours with a standard deviation of 21.1 hours and a range between 27.6 and 106.8 hours. Males survived between 31.2 and 92.4 hours without food with a mean of 57.1 hours and a standard deviation of 19.3 hours. Averaged across sexes,

Table 5. ANOVA of Longevity

Source[a]	d.f.[b]	MS[c]	F	σ^{2} [d]
S	1	13,462.4	35.39****	Fixed
L	177	1,794.5	4.64****	39.93
S × *L*	159	387.0	1.16	2.18
R(S × *L)*	873	337.8	2.43****	38.15
Error	5210	138.9		139.08

**** : $P < 0.0001$

[a] See text for explanation.

[b] Several lines produced only males or females, leading to an unbalanced design.

[c] Mean Squares computed from Type III Sums of Squares.

[d] Restricted maximum likelihood estimates of variance components.

mean survival time was 63.2 hours, and the standard deviation was 21.1 hours. The variation among lines in starvation tolerance was highly significant ($P < 0.0001$), as was the sex x line interaction ($P < 0.0001$), indicating sex-specific genetic variation in starvation resistance (Table 6). The heritability of starvation resistance attributable to the second chromosomes was 0.310, and the cross-sex genetic correlation in starvation resistance was 0.8543.

Longevity and starvation resistance were positively associated in these lines, with longer lived lines being more resistant to starvation and shorter lived lines being less resistant to starvation (Figure 4.) Using only the 133 lines that were assayed for both traits in both

sexes, the estimate of the genetic correlation was 0.451. The results of the analyses of variance and the variance components for the common lines are shown in Table 7.

Table 6. ANOVA of Starvation Resistance

Source[a]	d.f.[b]	MS[c]	F	σ^{2} [d]
S	1	203,439.96	228.47****	Fixed
L	152	6,179.10	6.94****	139.13
S × *L*	138	890.45	2.40****	26.06
R(S × *L)*	876	370.34	1.74****	31.44
Error	4671	213.21		213.21

**** : $P < 0.0001$
[a] See text for explanation.
[b] Several lines produced only males or females, leading to an unbalanced design.
[c] Mean Squares computed from Type III Sums of Squares.
[d] Restricted maximum likelihood estimates of variance components.

Figure 4. Correlation between Longevity and Starvation Resistance

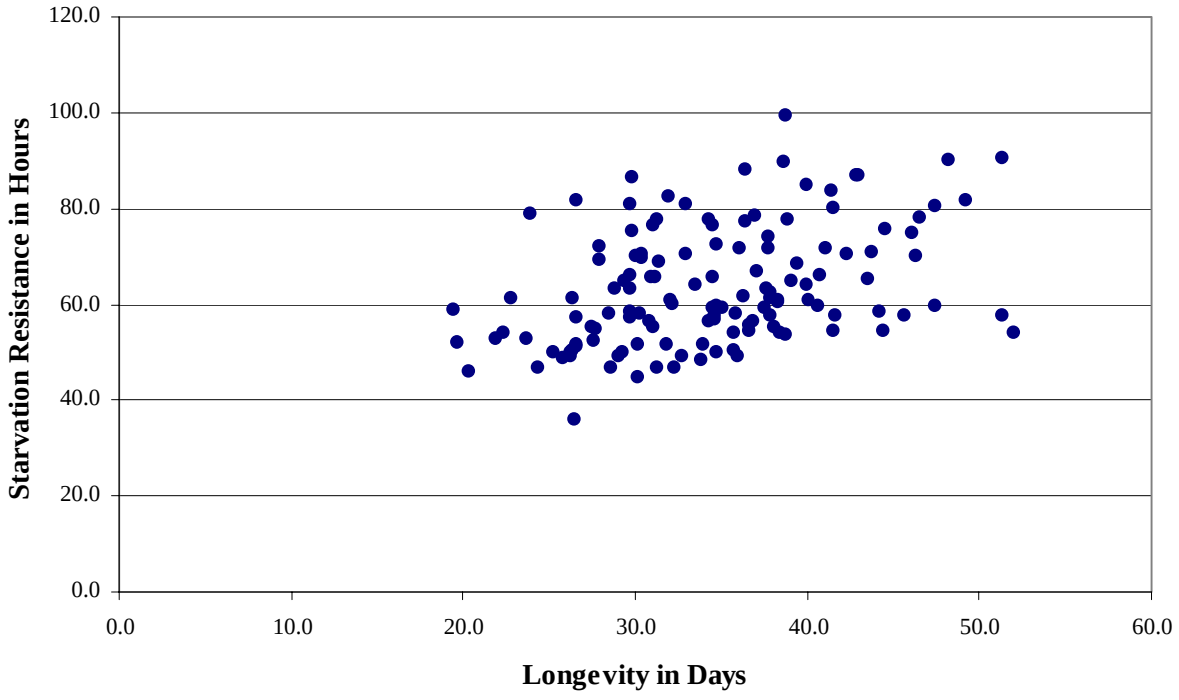


Table 7. ANOVA and Variance Components for
Lines in Common for Starvation Resistance and Longevity

	Longevity				Starvation Resistance			
Source[a]	d.f.	MS[b]	F	σ^{2} [c]	d.f.	MS[b]	F	σ^{2} [c]
S	1	13,834.9	35.75****	Fixed	1	193,251.9	227.3****	Fixed
L	132	1,939.9	4.97****	39.18	132	5,974.6	7.03****	128.11
S × *L*	132	390.0	1.15	2.03	132	850.1	2.43****	25.02
R(S × *L)*	733	342.5	2.53****	135.33	798	349.7	1.62****	26.77
Error	4327	135.2			4256	215.8		215.85

**** : $P < 0.0001$
[a] See text for explanation.
[b] Mean Squares computed from Type III Sums of Squares.
[c] Restricted maximum likelihood estimates of variance components.

Molecular population genetics of *Pu*

Pu covers almost 11,000 base pairs and is comprised of eleven exons (Figure 5). The three major transcripts differ at the 5' terminus but share the four common exons. Exon a is spliced to the common exons to produce a 273 amino acid protein. Isoforms B and C are alternatively spliced, with the C isoform being slightly longer at 325 amino acids, and isoform B having 308 amino acids (McLean *et al.*,1993). The putative transcript is thought to be formed by splicing the five D exons onto the common exons.

We sequenced 11,777 base pairs, including the *Pu* transcription unit, from 10 homozygous reference *Drosophila melanogaster* alleles from the Raleigh populations (Fry *et al.*, 1998). *Pu* displays the high degree of polymorphism typical of *Drosophila* genes. We observed a total of 255 single nucleotide polymorphisms (SNPs) and 15 insertion-deletion (indel) polymorphisms (Figure 5). Five of the SNPs caused amino acid replacement polymorphisms, G4148C, G8156C, C8502T, and A8226G and C8825T. Estimates of

nucleotide diversity based on the number of segregating sites (θ_s, Watterson, 1975), the average number of nucleotide differences between pairs of sites (θ_π, Nei and Tajima, 1981), and the number of singleton sites (θ_η, Fu and Li, 1993) were $\theta_s = 0.0059$, $\theta_\pi = 0.0059$, $\theta_\eta = 0.0060$ (± 0.0005 SD).

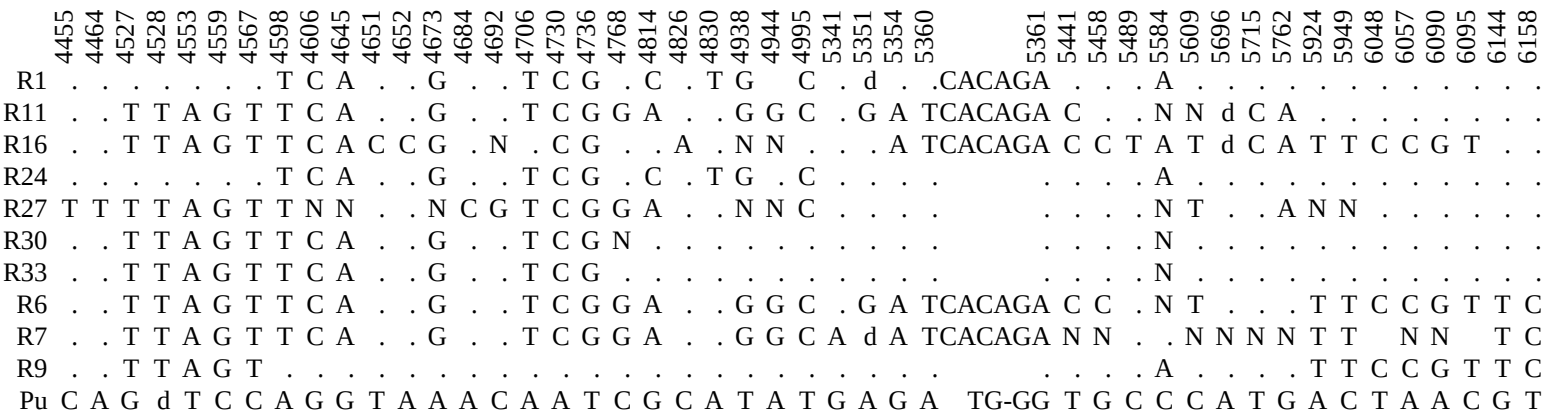
Application of several tests of selection to the 10 Raleigh *Pu* alleles failed to reveal significant departures from neutrality averaged over the whole region. Estimates of Tajima's (1989) D and Fu and Li's (1993) D* and F* statistics were D = 0.149 ($P > 0.10$); D* = 0.126 ($P > 0.10$) and F* 0.149 ($P > 0.10$). However, computation of those statistics using sliding window analyses revealed evidence both for more and for less variation than expected under neutrality (Figure 6). An excess of polymorphism relative to the neutral expectation, consistent with balancing selection, was observed for windows with midpoints of 5918, 6626, 7544, and 8347 of the aligned sequence. A reduction in polymorphism, consistent with a recent selective sweep of a favorable mutation or elimination of a highly deleterious mutation, was observed for windows with midpoints of 3228 and 9685-9789 of the aligned sequence. These analyses reveal the signature of complex evolutionary forces acting on the *Pu* region.

The estimate of Hudson's (1987) historical recombination parameter, *R*, was $R = 182$, or 0.0156 between adjacent sites. Linkage disequilibrium (LD) is thus expected to decay rapidly between sites separated by 64 bp as is typical for *Drosophila* genes in regions of normal recombination (Long *et al.*, 1998).

We determined the genotypes of 29 polymorphic sites (28 SNPs and 1 indel) in the 178 chromosome substitution lines. Four of the SNPs were amino acid polymorphisms

Figure 5. Structure and Sequence Variation in *Pu*

Figure 5. Structure and Sequence Variation in Pu



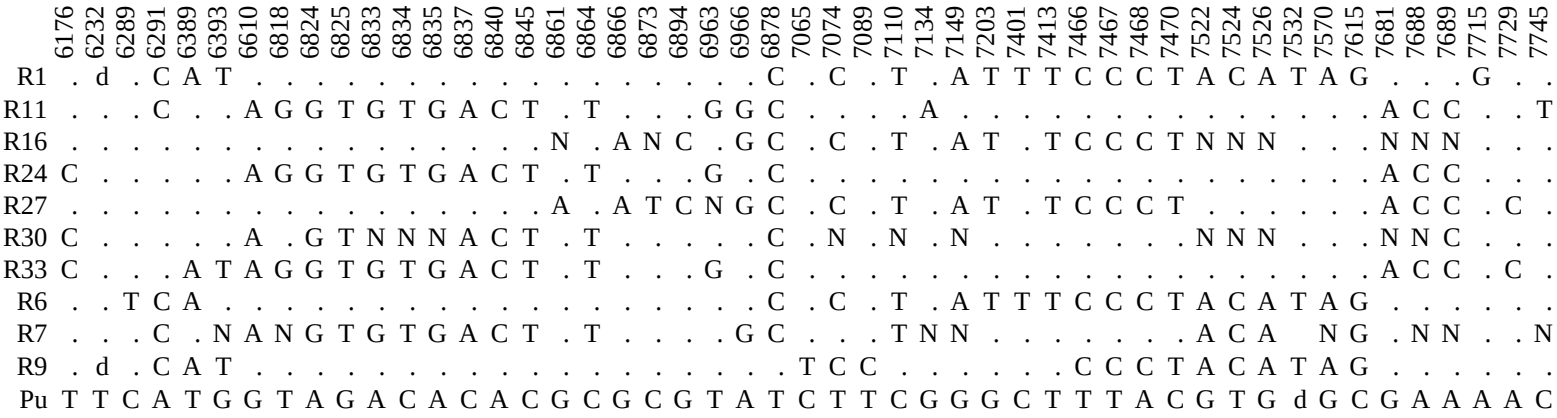


Figure 5. Structure and Sequence Variation in *Pu*

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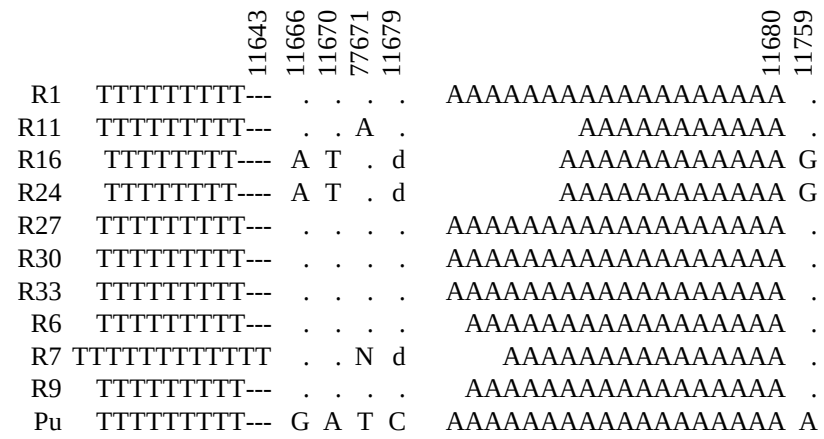


Figure 6. Sliding Window Analysis

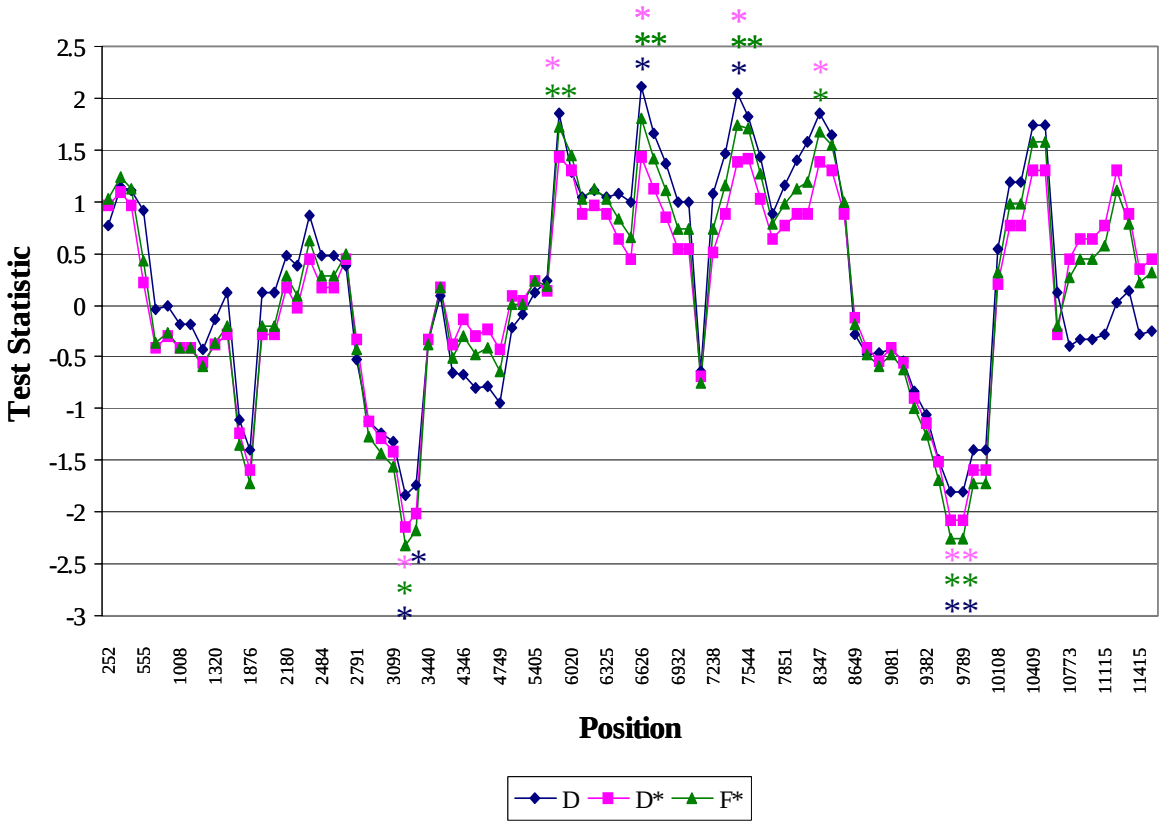


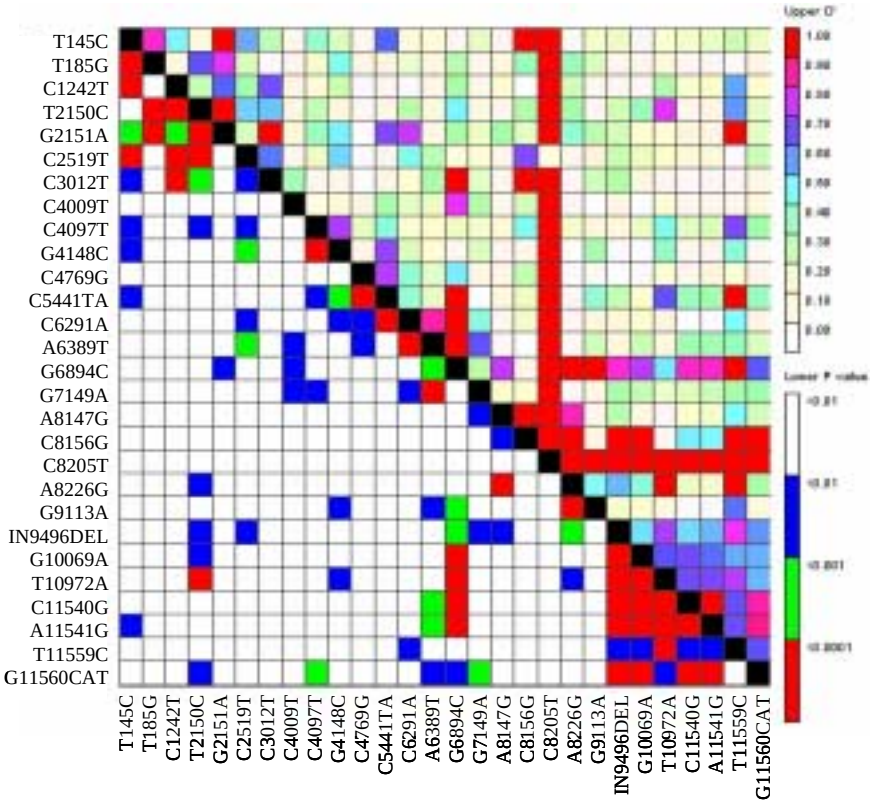
Figure 6 shows the value of Tajima's D, Fu and Li's D* and F* tests for selection for a sliding window. The mid-points of the 500 base pair windows are shown on the x-axis, and the increment was 100 base pairs. Significance for each test is indicated by asterisks in the same color as the test value. * indicates a *P*-value of less than 0.05, and ** indicates a *P*-value of less than 0.01

(G4148C, G8156C, C8205T and A8226G). SNPs were spaced at approximately 750 base pair intervals. One SNP, at position 6289 was present in one of the Raleigh alleles but only once in the larger sample, and was not considered further.

The pattern of linkage disequilibrium at *Pu* is illustrated in Figure 7. There are two large blocks of LD at the 5' and 3' ends of the region sequenced, extending from position 145-3012 and 9496-11,560, respectively. Those blocks are separated by an approximately

8.5 kilobase region with little significant LD, as expected given Hudson's estimate of historical recombination and the density of SNPs typed.

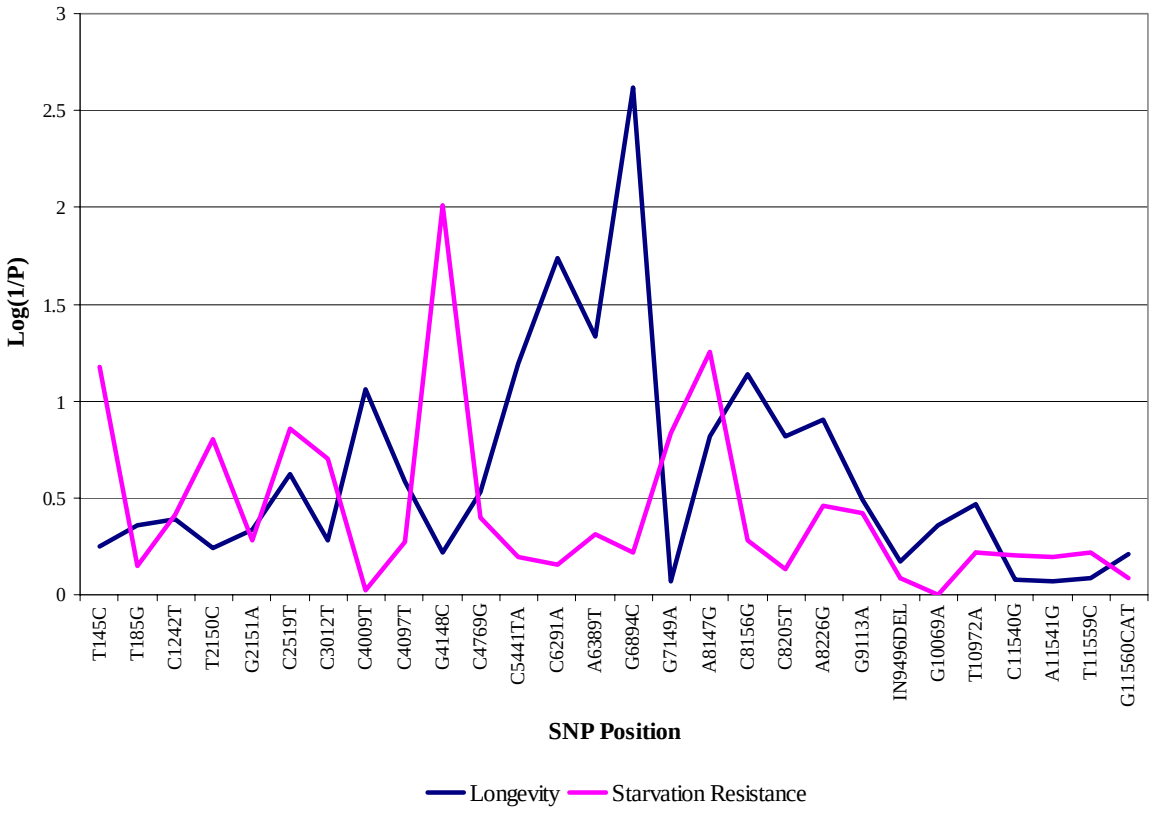
Figure 7. Linkage Disequilibrium Between Markers



Genotype - Phenotype Associations

ANOVA was used to assess associations of polymorphisms in *Pu* with variation in starvation resistance and longevity (Figure 8). One site, G4148C, a serine/threonine replacement polymorphism in exon A, was significantly ($P = 0.0070$) associated with variation in starvation tolerance. However, with 28 tests for association, one must take the multiple testing into account for an experimentwise significance threshold. This is $P = 0.0018$ using a Bonferroni correction. A permutation test gave a very similar Type I significance threshold of $P = 0.0020$. Thus, this SNP cannot formally be considered to be associated with variation in starvation resistance.

Figure 8. Log (1/*P*) for Starvation Resistance and Longevity ANOVA



Three closely linked SNPs in Intron 6 were nominally significantly associated with variation in longevity, C6291A (P = 0.0183), A6389T (P = 0.0466), and G6894C (P = 0.0024) (Figure 8). The frequency of C6291 is 0.79. The average life span of C6291 genotypes is 34.2, while the average life span of the A6291 haplotype is 31.7 days. The frequency of A6389 is 0.506. The average life span of A6389 genotypes is 34.5 days, compared for 32.8 days for T6389 genotypes. The frequency of G6894 is 0.929. Average life spans of G6894 and C6894 are 38.4 and 33.3 days, respectively. None of these SNPs are individually significant given Bonferroni or permutation based Type I significance thresholds, accounting for multiple tests (P = 0.0017 and P = 0.0020, respectively).

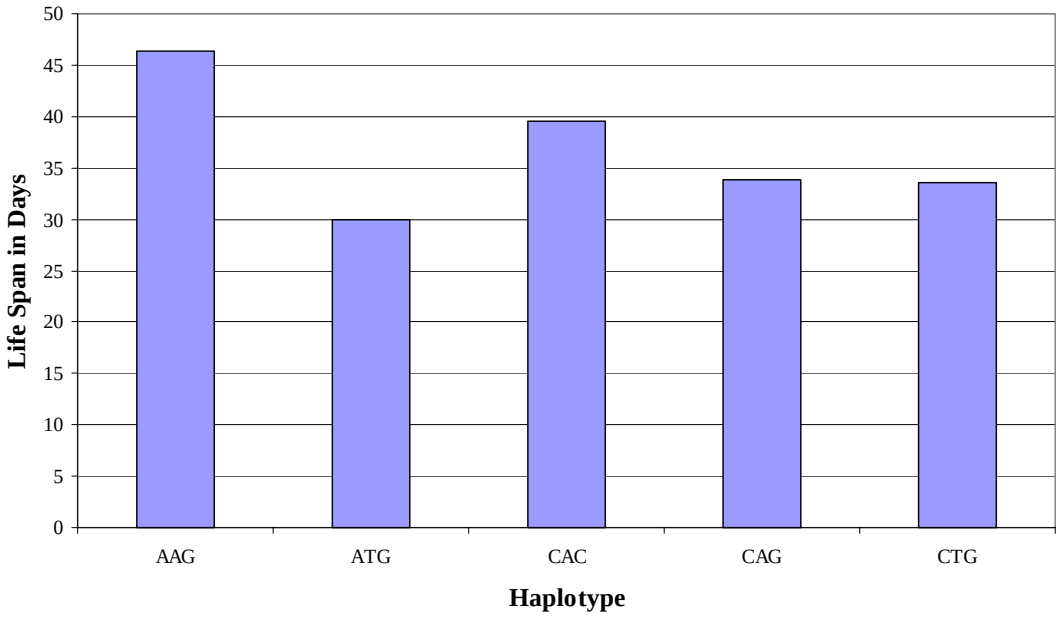
However, C6291A is in strong LD with A6389T ($P < 0.0001$), and A6389T is in strong LD with G6894C ($0.001 < P < 0.0001$) (Figure 7). These three SNPs fall into five haplotypes and were in very strong global LD ($X^2 = 54.09$, $P < 0.0001$) (Figure 7). ANOVA of life spans revealed highly significant variation among haplotypes (Table 8, Figure 9).

Table 8. ANOVA of Longevity Haplotypes

Source[a]	d.f.[b]	MS[c]	F	σ^{2} [d]
S	1	154.97	2.54	Fixed
H	4	401.20	6.59****	5.98
S × *H*	4	48.50	0.80	-0.43
Error	328	60.92		60.93

**** : $P < 0.0001$
[a] See text for explanation.
[b] Several lines produced only males or females, leading to an unbalanced design.
[c] Mean Squares computed from Type III Sums of Squares.
[d] Restricted maximum likelihood estimates of variance components.

Figure 9. Life Span of Haplotypes



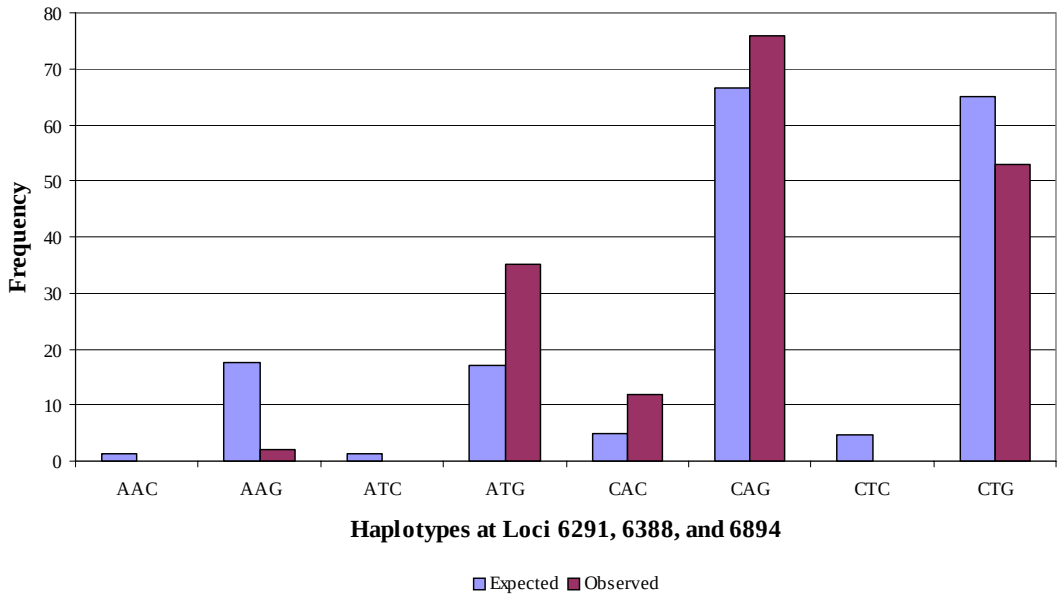
Variation among the *Pu* haplotypes accounted for 36 % of the genetic variance and 10 % of the phenotypic variance in longevity attributable to the second chromosome.

The haplotypes contributing most to the departure from linkage equilibrium were AAG, ATG and CAC, where the first, second and third base of the haplotypes are, repectively, the genotypes for SNPs C6291A, A6389T and G6894C.

The AAG haplotype is expected about 17 times in the set of lines, but only appears twice. It has the greatest life span of 46.4 days. The shortest lived haplotype is ATG at 30.0 days, and is present twice as often as expected. CAC is present 2.5 times more than expected and has an intermediate life span of 39.6 days. CAC is present 2.5 times more than expected and has the second longest life span of 39.6 days. These observations are not consistent with directional selection for increased life span, but indicate selection both for and against increased longevity. These SNPs may exhibit epistasis for life span, since the difference

between the A/T polymorphism for site 6389 is 16.4 days in the background of A6291 and G6894, but only 0.3 days in the background of C6291 and G6894 (Figure 10).

Figure 10. Observed and Expected Numbers of Haplotypes



DISCUSSION

Pu is a candidate gene affecting *Drosophila* life span, given the highly significant association of variation in life span with haplotypes of three SNPs in Intron 6. However, LD in *Drosophila* generally decays rapidly with physical distance in normal regions of recombination (Long *et al*. 1998; Robin *et al.* 2002), and this is the pattern observed at *Pu*. As for all association studies in which all polymorphisms are not genotyped, it is always possible that these SNPs are not the causal SNPs, but are in LD with the true causal polymorphisms that were not genotyped in this sample of alleles. The low level of LD in the region surrounding the significant SNPs is a highly favorable situation for mapping the QTNs causing the variation in life span in this sample of alleles, by complete sequencing of all

polymorphisms around the focal SNPs, extending sufficiently 5′ and 3′ of the region from position 6291-6894 of the aligned sequence for LD to decay across the sequenced region. Ultimately, formal proof that particular molecular polymorphisms at *Pu* cause variation in life span would require constructing all possible haplotypes and testing their effects on longevity when transformed into a *Pu* null mutant background (Stam and Laurie 1996).

Unfortunately, the small scale over which LD in *Pu* decays relative to the spacing of the genotyped polymorphisms means that it is very possible that other SNPs that were not genotyped are associated with variation in starvation resistance and longevity. There is strong evidence that *Pu* is a candidate gene for starvation resistance: *Pu* alleles are hypersensitive to starvation stress (Chaudhari, 2003) and fail to complement QTL for starvation resistance segregating between two wild type strains, Oregon and 2b (Harbison *et al*., 2003a). Further, transcription of *Pu* is highly significantly down-regulated in starving flies (Harbison *et al*., 2003b). Therefore, our failure to detect significant associations of naturally occurring variation in starvation resistance with polymorphisms at *Pu* is most likely attributable to the sparse spacing of SNPs. The power of association studies will be greatly increased when it is feasible to rapidly and economically genotype all polymorphisms in a candidate gene or region in a large sample of alleles.

The three SNPs that were associated with moderately large effects on longevity were present at intermediate frequencies. Intermediate allele frequencies are not consistent with maintenance of genetic variation for life span by mutation-selection balance, under which deleterious alleles are rare at equilibrium. Possibly these variants are effectively neutral, as predicted under the mutation accumulation hypothesis (Medawar, 1952), with deleterious

effects expressed so late in life that selection coefficients are of the order of the mutation rate. Alternatively, the SNPs could be maintained by antagonistic pleiotropy (Williams, 1957; Rose and Charlesworth, 1980; Kirkwood and Rose, 1991), with genotypes conferring longer and shorter longevity having, respectively, reduced and increased fitness early in life. These mechanisms might be distinguished by repeating the association study in different populations. If the polymorphisms are nearly neutral, they may be unique to the Raleigh population; if they are maintained by antagonistic pleiotropy or another balancing selection mechanism, they may be common to most populations.

There are two lines of evidence for complex selection acting on *Pu*. First, molecular population genetic tests for departure from neutrality revealed both regional excesses and deficiencies of polymorphisms from that predicted under mutation-drift equilibrium. Second, the SNPs associated with variation in longevity are not individually significant; it is only when joint haplotypes are considered that the association becomes highly significant. Thus, the SNPs act epistatically to affect longevity. Epistasis between multiple polymorphisms within a gene affecting variation in *Drosophila* quantitative traits has been observed previously: in the molecular dissection of the effect of variants at the *Adh* locus on Adh protein concentration (Stam and Laurie, 1996), and in the analysis of SNPs in *Dopa decarboxylase* (*Ddc*) affecting variation in life span (DeLuca *et al.*, 2003). It is possible that such epistasis is caused by selection. Indeed, the pattern of haplotype departures from linkage equilibrium and associations with longevity is consistent with maintenance of variation for longevity at *Pu* by balancing selection, with selection both for and against increased life span. Intriguingly, the SNPs jointly associated in longevity are all in the region exhibiting an excess

of polymorphism (Figure 7), indicative of maintenance of variation by balancing selection. Coupled with the evidence for selection for and against increased life span, we speculate that polymorphisms at *Pu* affecting longevity may be maintained by antagonistic pleiotropy, as predicted by evolutionary theory.

It is remarkable that exactly the same pattern of epistatic associations with longevity and evidence for balancing selection was observed at *Ddc*, another gene in the catecholamine biosynthesis pathway. In *Drosophila*, GTPCH is the first protein in this pathway, and is the rate limiting step in the synthesis of tetrahydropterin (BH_4), a co-factor for tyrosine hydroxylase (TH), encoded by the *Drosophila* gene, *pale*, which catalyzes the hydroxylation of tryosine to form DOPA. The decarboxylation of DOPA to form dopamine (DA) is catalyzed by Ddc. Two other major catecholamines, NADA and NBAD are produced from DA by NADA transferase (encoded by *Nat*) and NBAD transferease (encoded by *ebony*) (Stathakis *et al.*, 1999). Catecholamines are required for melanization and schlerotization of the cuticle, and aberrations in catecholamine pools have pleiotropic effects on development, female fertility, abnormal melanization and production of melanotic pseudotumors (Neckameyer, 1996, Stathakis *et al*., 1999). In addition, DA is required in *Drosophila* for fecundity and sexual receptivity in females (Neckameyer, 1996; 1998a); learning (Tempel *et al*., 1984; Neckameyer, 1998b); locomotion (Pendleton *et al*. 2002) and aggressive behavior (Baier *et al*., 2002). Dopamine concentrations decrease with age and are sexually dimorphic, with males having higher levels than females (Neckameyer *et al*., 2000). A plausible mechanism for antagonistic pleiotropy acting on *Pu* and *Ddc* might be through a beneficial effect of high levels of dopamine in development and early in life for peak fertility and

normal behaviors, but not later in life, where a high level of dopamine generates free radicals which damage dopaminergic neurons (Luo and Roth, 2000).

In the future, it will be important to assess associations of other genes in the catecholamine biosynthesis pathway with variation in life span, starvation resistance, and other life history and behavioral traits. Given that epistatic interactions among SNPs affecting quantitative traits have been observed within *Pu* and *Ddc*, it will be important to perform these analyses on the same set of lines, to allow assessment of potential epistatic interactions among loci in a common pathway. Further, the mechanism causing the variation in longevity is unknown, motivating future studies measuring differences in enzyme activity, transcript abundance, protein levels and catecholamine pools among the haplotypes associated with variation in longevity.

Given the conservation between genes affecting key biological processes between *Drosophila* and humans, it is reasonable to speculate that human orthologs of other genes involved in the biosynthesis of specific neurotransmitters may determine variation in individual life span. Support for this hypothesis comes from the finding that a microsatellite polymorphism in the first intron of tyrosine hydroxylase, the rate-limiting enzyme in the synthesis of catecholamines, is associated with variation in human longevity (De Benedictis *et al.*, 1998; De Luca *et al*., 2001). Further, mutations in human GTPCH have been associated with the skin disorder vitiligo (De La Fuente-Fernández, 1997); hyperphenylalaninaemia (Thöny *et al.*, 2000); hereditary progressive dystonia with diurnal fluctuations (Ichinose *et al.*, 1994); and possibly dopa-responsive dystonia (Bandmann *et al.*, 1996). Possibly less severe polymorphisms could contribute to natural variation in human longevity; an hypothesis that

can be tested by including *Drosophila* candidate genes in future human association studies.

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Supplemental Table 2. Line Means and Genotypes

	Life span			Starvation resistance												
Line	Males	Females	Pooled	Males	Females	Pooled	145	185	1242	2150	2151	2519	3012	4009	4097	4148
2	36.4	43.9	39.9	76.2	94.2	85.2	T	T	T	T	G	T	C	C	C	G
4	25.0	27.5	26.1	.	69.6	.	T	T	T	T	G	C	C	T	T	G
5	.	31.6	.	.	60.6	.	T	G	C	C	A	C	C	C	T	G
9	28.0	33.6	30.9	62.4	69.0	65.7	T	G	C	C	G	C	T	C	T	G
11	49.2	46.3	47.4	.	31.8	.	T	T	C	T	G	T	C	T	T	G
16	33.0	41.0	37.2	.	.	.	T	T	T	C	G	C	C	T	C	G
17	24.4	27.9	26.2	42.2	58.2	50.1	T	T	T	C	G	C	T	C	C	G
25	31.4	38.4	35.1	58.8	60.0	59.4	T	T	T	T	G	C	C	C	T	G
26	22.0	18.4	20.3	34.2	58.2	46.2	C	T	T	T	G	C	C	C	T	G
31	21.8	.	.	.	.	.	T	T	T	T	G	T	T	C	T	G
33	39.0	35.3	36.8	55.2	57.6	56.4	T	G	C	C	G	T	C	T	C	C
40	50.4	52.1	51.3	45.0	70.8	57.9	T	G	C	C	G	C	C	C	T	G
43	42.0	19.2	29.3	.	.	.	T	G	C	C	G	C	T	C	T	G
44	26.2	33.8	30.2	61.2	55.2	58.2	T	T	T	T	G	C	C	T	C	G
46	39.6	48.6	44.2	52.2	64.8	58.5	T	T	T	T	G	T	C	T	T	G
49	25.0	27.7	26.4	31.2	41.4	36.3	C	T	C	T	G	T	T	T	C	C
58	32.2	36.0	34.2	60.0	95.4	77.7	C	T	C	C	G	C	C	C	T	G
72	17.8	29.4	23.9	71.4	86.4	78.9	T	T	T	C	G	C	C	T	C	G
73	31.1	34.3	32.7	42.0	56.4	49.2	T	T	T	T	G	C	C	T	C	C
74	42.0	34.1	38.3	51.0	71.4	61.2	T	T	T	T	G	C	C	T	C	C
76	32.0	29.5	30.7	46.8	66.0	56.4	T	T	C	T	G	T	C	C	C	G
77	29.6	42.2	35.9	44.4	54.0	49.2	T	T	C	T	G	T	C	C	C	G
79	30.6	31.5	31.1	52.8	78.6	65.7	C	T	C	C	G	C	T	C	C	G
80	24.5	30.9	27.7	45.0	64.8	54.9	C	T	C	T	G	C	C	T	C	G
81	32.9	41.6	37.8	43.8	72.0	57.9	C	T	C	T	G	C	C	T	C	G
87	45.8	49.0	47.4	66.6	94.8	80.7	T	G	C	C	G	T	C	C	T	G
88	32.9	42.8	38.0	51.0	59.4	55.2	C	T	C	T	G	C	T	C	C	C
89	40.5	47.1	43.8	61.8	80.4	71.1	C	T	C	C	G	C	T	C	C	C
97	31.6	37.5	34.4	61.8	70.2	66.0	C	T	C	T	G	C	C	C	C	C
100	44.2	52.7	48.2	92.4	88.2	90.3	T	T	T	C	A	C	C	C	C	G
104	.	26.4	.	50.4	54.0	52.2	C	T	T	T	G	T	C	T	C	G
105	.	30.0	.	66.0	77.4	71.7	T	T	T	T	G	C	C	T	C	C
109	23.8	35.2	29.7	52.2	62.4	57.3	C	T	C	C	G	C	C	T	C	C
110	37.5	37.6	37.5	49.8	69.0	59.4	T	T	T	T	G	T	C	C	T	C
113	34.6	42.3	41.0	63.0	80.4	71.7	C	T	C	C	G	C	C	T	C	G
115	50.6	52.2	51.3	92.4	89.4	90.9	T	T	C	T	G	T	C	C	C	G
117	.	32.0	.	.	79.2	.	C	T	C	C	G	C	T	T	T	G
119	35.8	35.5	35.7	48.0	60.6	54.3	T	G	T	C	A	C	C	C	C	G
120	28.6	32.9	31.0	49.2	61.8	55.5	C	T	C	C	G	C	C	T	C	C
122	41.1	40.2	40.6	53.4	66.0	59.7	T	G	T	C	A	C	C	C	C	G
125	32.0	38.9	35.8	51.0	65.4	58.2	C	T	C	T	G	C	T	T	C	C
129	31.0	38.4	34.6	46.2	54.0	50.1	T	G	C	C	G	C	C	T	C	G
130	35.5	40.6	38.2	57.0	64.2	60.6	T	G	C	C	G	C	C	T	C	G
135	26.7	25.0	25.7	45.0	52.2	48.8	T	T	T	T	G	T	C	C	C	G
146	.	30.1	.	81.0	100.2	90.6	T	G	T	C	A	T	C	T	C	G

Supplemental Table 2. Line Means and Genotypes, Continued

	Life span			Starvation resistance												
Line	Males	Females	Pooled	Males	Females	Pooled	145	185	1242	2150	2151	2519	3012	4009	4097	4148
154	31.3	21.6	26.5	49.8	52.8	51.3	C	G	C	T	G	C	C	C	C	G
155	45.3	49.7	47.4	50.4	69.6	60.0	C	T	T	C	G	C	C	C	C	G
161	34.0	30.1	32.0	57.0	71.4	61.2	T	T	C	T	G	T	C	T	T	G
166	40.4	41.0	40.7	55.2	77.4	66.3	C	T	C	C	G	C	C	C	C	G
168	25.1	33.4	29.3	49.8	80.4	65.1	T	T	C	T	G	T	C	C	C	G
171	35.1	43.1	38.8	65.4	90.0	77.7	C	T	C	C	G	C	T	C	C	G
177	26.9	49.1	37.8	46.2	76.8	61.5	T	G	T	C	A	C	C	T	C	C
179	42.5	37.1	38.3	39.0	69.0	54.0	C	T	C	C	G	C	C	C	T	G
180	47.8	44.5	46.3	67.2	73.2	70.2	C	T	C	C	G	C	C	C	T	C
181	29.7	38.6	34.5	48.0	67.8	57.9	T	G	C	C	G	T	C	T	C	G
183	30.4	25.2	27.9	58.2	88.4	72.3	T	G	T	C	A	T	C	T	C	G
188	29.2	31.0	30.1	58.2	45.6	51.9	C	G	C	T	G	C	C	T	T	G
194	.	33.2	.	52.8	89.4	71.1	T	T	C	C	G	C	C	T	C	C
200	35.1	38.0	36.6	47.4	64.2	55.8	T	T	T	T	G	T	C	T	T	G
201	28.6	30.8	29.7	76.8	85.2	81.0	T	T	T	T	G	T	C	T	T	G
203	23.6	28.9	26.3	43.2	57.6	50.4	C	T	C	C	G	C	C	C	C	C
205	10.5	31.5	26.3	44.4	54.6	49.5	C	T	T	C	G	C	C	C	C	G
207	34.7	47.6	41.5	47.4	61.8	54.6	T	T	T	T	G	T	T	C	T	G
215	22.4	33.0	27.9	64.2	74.4	69.3	C	T	C	C	G	C	T	C	C	G
216	49.4	37.4	33.8	.	.	.	T	T	T	T	G	T	C	T	T	G
222	40.3	38.4	39.4	64.2	73.2	68.7	T	G	C	C	G	T	C	T	C	G
223	24.0	26.8	36.6	50.4	58.8	54.6	T	G	T	C	A	T	C	T	T	G
224	38.3	42.9	40.5	.	.	.	T	G	C	C	G	T	C	T	C	G
225	31.9	31.8	31.9	80.4	84.6	82.5	T	T	C	T	G	T	C	C	T	G
226	27.3	20.1	23.6	51.0	54.6	52.8	T	T	C	C	G	C	T	T	C	C
228	22.7	32.0	27.4	47.4	63.6	55.5	C	T	C	C	G	C	T	T	C	C
232	26.7	32.4	29.8	88.2	85.2	86.7	T	T	T	C	G	C	C	T	C	G
233	26.2	11.6	21.9	45.0	61.2	53.1	T	T	C	T	G	T	C	C	C	G
234	29.6	41.1	29.6	51.6	.	.	T	T	T	T	G	T	C	T	T	G
243	41.1	42.3	41.6	52.2	63.6	57.9	T	T	T	T	G	C	C	C	T	G
244	24.3	40.1	32.2	42.0	51.6	46.8	T	T	T	T	G	T	C	T	T	G
247	26.5	31.7	29.2	48.0	52.2	50.1	T	G	T	C	A	C	C	C	C	G
248	33.6	25.8	29.7	.	73.8	.	T	T	T	T	G	T	C	T	C	C
249	27.2	32.2	29.8	67.2	83.4	75.3	C	T	T	T	G	C	C	T	C	G
257	38.4	41.6	40.0	61.2	61.2	61.2	T	T	C	C	A	T	C	C	T	C
258	.	33.2	.	.	.	.	T	T	T	T	G	T	C	T	C	C
260	.	40.5	.	40.8	55.8	48.3	T	T	T	T	G	T	C	C	T	G
266	30.7	32.5	31.9	.	.	.	C	T	C	C	G	C	C	T	C	G
268	22.4	28.2	25.3	45.0	55.2	50.1	C	T	C	C	G	C	C	T	C	C
271	26.0	27.9	26.6	45.0	58.2	51.6	T	G	T	C	A	C	C	C	C	G
273	39.7	33.4	36.3	49.2	74.4	61.8	T	T	T	T	G	T	C	T	T	G
274	19.8	29.8	26.3	.	.	.	T	G	T	T	G	T	C	T	C	G
277	43.3	43.8	43.5	55.2	75.6	65.4	T	G	T	C	A	T	C	T	C	G
278	23.3	21.9	22.4	49.2	58.8	54.0	T	G	C	C	G	C	T	C	T	G
281	22.0	22.8	22.3	.	.	.	T	G	T	C	A	C	C	C	C	G

Supplemental Table 2. Line Means and Genotypes, Continued

	Life span			Starvation resistance												
Line	Males	Females	Pooled	Males	Females	Pooled	145	185	1242	2150	2151	2519	3012	4009	4097	4148
282	.	34.3	.	.	58.8	.	T	T	T	T	G	T	C	C	C	G
284	27.2	28.2	27.7	.	.	.	T	T	C	T	G	T	C	C	C	G
285	28.7	26.4	27.5	44.4	60.6	52.5	C	T	T	T	G	C	C	T	C	C
294	43.8	54.5	49.2	72.6	91.2	81.9	T	G	C	C	G	C	T	C	C	G
300	26.2	44.8	32.4	.	61.8	.	T	T	T	T	G	C	C	T	C	C
308	31.3	30.8	31.0	68.4	85.2	76.8	T	T	T	T	G	T	C	C	C	C
309	27.7	30.0	28.8	57.0	69.6	63.3	T	T	C	C	G	C	C	C	C	C
311	38.0	37.2	37.6	62.4	64.8	63.6	T	T	T	T	G	T	C	C	T	G
314	27.7	30.6	29.0	.	.	.	T	T	T	T	G	T	C	T	T	G
316	28.5	39.0	38.7	48.6	58.8	53.7	T	G	T	C	G	T	C	C	C	G
317	25.2	27.4	26.3	59.4	63.6	61.5	T	G	C	T	G	C	C	C	C	C
325	40.7	43.9	42.3	65.4	75.6	70.5	C	T	C	T	G	C	C	T	C	G
327	40.0	48.2	44.5	77.4	74.4	75.9	C	T	C	C	G	C	C	C	T	G
334	31.8	32.6	32.2	57.6	63.0	60.3	C	T	C	C	G	C	C	C	C	C
336	38.6	41.2	39.9	60.6	67.8	64.2	T	T	C	T	G	C	C	C	T	G
337	.	16.1	.	33.6	27.6	30.6	T	T	T	C	G	C	C	T	C	C
339	44.0	32.2	35.6	.	.	.	T	T	T	T	G	T	C	T	T	G
340	21.8	23.2	22.5	.	.	.	T	T	C	C	G	C	C	C	C	C
341	42.8	50.5	46.5	62.4	94.2	78.3	C	T	C	T	G	C	C	C	C	C
343	.	25.7	.	.	55.2	.	T	G	T	C	A	C	C	C	T	G
345	39.0	36.6	37.8	53.4	72.0	62.7	T	T	T	T	G	C	C	C	C	C
347	28.5	31.8	30.1	45.6	44.4	45.0	T	G	C	C	G	C	T	C	T	C
351	41.5	61.5	52.0	56.4	52.2	54.3	C	T	C	T	G	T	C	C	T	G
354	24.1	33.3	28.6	45.0	48.6	46.8	T	T	C	C	A	C	C	T	C	G
355	29.7	26.1	27.7	48.0	.	.	T	T	T	T	G	T	C	T	T	G
361	26.6	33.3	30.0	66.0	74.4	70.2	T	T	C	T	G	T	C	C	C	G
363	36.4	48.2	42.3	55.8	.	.	C	T	C	C	G	C	T	T	C	C
367	27.7	31.7	29.7	52.2	64.8	58.5	T	G	C	T	G	C	C	T	C	G
368	29.7	22.5	29.9	.	61.2	.	T	G	C	T	G	C	C	T	C	G
369	22.5	42.4	31.3	37.2	57.0	47.1	T	G	C	C	G	C	T	C	T	G
374	.	28.8	.	.	61.8	.	T	T	T	T	G	T	C	T	T	G
376	29.2	39.3	34.7	57.0	63.0	60.0	T	G	C	T	G	C	C	T	C	G
382	37.1	31.6	34.5	57.0	61.8	59.4	T	G	C	C	A	C	C	C	T	G
383	31.6	36.3	33.8	46.2	51.0	48.6	T	T	T	T	G	C	C	T	T	G
385	21.7	30.4	25.9	.	.	.	T	T	T	T	G	T	C	T	T	G
387	19.4	28.7	24.3	43.2	51.6	47.0	T	T	C	C	G	C	T	C	T	G
388	36.7	39.2	38.3	.	.	.	C	T	T	T	G	T	C	T	C	G
390	48.8	44.0	46.6	.	.	.	T	G	T	T	G	C	C	C	C	C
396	37.2	36.8	36.9	73.8	83.4	78.6	T	T	T	T	G	T	C	C	T	G
402	.	30.4	.	.	.	.	C	T	C	C	G	C	T	T	C	G
405	18.5	20.3	19.4	51.6	66.0	58.8	T	G	C	C	G	C	C	C	C	G
406	27.8	31.3	29.6	60.0	72.6	66.3	T	G	T	C	A	C	C	C	C	G
407	37.0	34.3	35.6	39.0	62.4	50.7	C	T	C	T	G	C	C	C	C	C
408	28.3	28.7	28.5	50.4	66.0	58.2	C	T	C	C	G	C	C	C	T	G
415	30.1	38.0	34.2	49.8	63.6	56.7	C	T	T	T	G	T	C	C	T	G

Supplemental Table 2. Line Means and Genotypes, Continued

	Life span			Starvation resistance												
Line	Males	Females	Pooled	Males	Females	Pooled	145	185	1242	2150	2151	2519	3012	4009	4097	4148
418	30.2	35.4	33.9	.	.	.	T	G	T	C	A	C	C	T	C	C
427	32.6	34.3	33.5	66.6	69.6	64.1	T	T	T	T	G	T	C	C	T	G
429	37.0	40.9	39.0	58.2	72.0	65.1	C	T	T	T	G	T	T	C	C	G
434	42.7	45.8	44.3	47.4	51.2	54.6	C	T	C	C	G	C	C	C	C	C
437	34.2	44.5	39.5	.		.	C	T	C	C	G	C	T	T	C	C
440	21.5	31.8	26.5	55.8	58.8	57.3	T	T	C	T	G	T	C	C	C	G
444	40.0	45.9	43.0	82.8	91.2	87.0	T	G	T	C	A	C	C	C	C	G
453	29.5	16.2	22.7	56.4	66.6	61.5	T	T	T	T	G	C	C	C	T	G
455	24.7	.	.	.	.	.	C	T	C	C	G	C	T	C	C	G
456	29.4	33.2	31.4	61.8	76.2	69.0	T	T	C	T	G	T	C	T	T	G
458	37.0	32.9	34.5	50.4	63.6	57.0	T	T	C	T	G	T	C	C	C	G
460	43.8	39.2	41.5	78.6	81.6	80.1	T	T	T	T	G	T	C	C	T	G
461	32.2	28.5	30.3	73.2	66.6	69.9	T	G	T	C	A	C	C	C	C	G
464	44.3	38.3	41.3	78.6	88.8	83.7	T	T	T	T	G	C	C	T	T	G
466	23.0	32.4	29.0	52.2	46.2	49.2	C	T	T	T	G	T	C	C	C	C
467	37.0	35.6	36.3	79.8	96.6	88.2	T	T	T	T	G	T	C	C	C	G
470	37.4	48.6	42.9	78.6	95.4	87.0	T	T	T	C	G	T	C	C	C	G
472	33.4	40.5	37.0	53.4	80.4	66.9	T	T	T	T	G	T	C	C	T	G
473	33.0	40.1	36.4	72.6	82.2	77.4	T	G	C	C	G	C	T	C	T	G
477	20.2	19.2	19.7	56.4	48.0	52.2	C	T	T	T	G	T	C	C	C	G
479	16.7	25.2	22.0	.	43.2	.	T	T	T	T	G	T	C	C	T	G
481	15.2	26.4	21.0	.	.	.	C	T	T	C	G	C	C	T	C	C
489	22.9	29.8	26.6	82.2	81.6	81.9	T	T	T	T	G	T	C	T	T	G
492	34.2	42.4	38.7	92.4	106.8	99.6	T	G	C	C	G	T	C	T	C	G
498	34.2	29.2	31.6	.	.	.	T	T	T	T	G	C	C	T	T	G
977	.	25.5	.	.	.	.	T	T	C	T	G	T	C	T	C	C
981	25.5	39.7	37.6	68.4	80.4	74.4	T	G	C	C	G	C	T	C	T	G
985	31.7	34.4	33.0	67.8	73.2	70.5	C	T	C	C	G	C	C	C	T	G
9713	.	20.1	.	.	.	.	T	G	T	C	A	T	C	T	C	G
9726	41.0	54.2	45.6	51.6	63.6	57.6	T	G	T	C	A	T	C	T	C	G
9727	36.5	39.3	37.7	65.4	78.0	71.7	T	G	T	C	A	C	C	C	C	G
9729	30.3	30.2	30.2	.	.	.	T	G	C	C	G	C	C	C	C	C
9811	33.1	38.9	36.0	61.2	82.2	71.7	C	T	C	T	G	C	C	T	C	G
9814	31.3	28.7	29.6	46.8	80.4	63.6	T	T	C	C	G	C	C	T	C	C
9821	39.0	38.2	38.6	78.0	101.4	89.7	T	T	T	C	G	C	C	T	C	C
9825	37.8	32.3	34.7	63.0	82.2	72.6	C	T	C	C	G	C	C	C	C	C
9829	27.9	34.5	31.2	68.4	87.0	77.7	T	G	C	C	G	T	C	T	C	G
9830	45.6	46.5	46.1	72.0	78.0	75.0	C	T	T	T	G	T	C	C	C	G
9833	31.0	29.6	30.3	63.6	77.4	70.5	C	T	C	T	G	C	C	T	C	C
9835	35.8	33.4	34.5	70.8	82.2	76.5	T	T	T	T	G	T	C	T	T	G
9840	31.0	37.2	33.9	51.3	51.6	51.8	C	T	C	C	G	C	C	C	C	C
9841	40.7	25.5	32.9	76.2	85.5	81.0	T	T	T	T	G	T	C	T	T	G

Supplemental Table 2. Line Means and Genotypes, Continued

Line	4769	5441	6291	6388	6894	7149	8147	8156	8205	8226	9113	9496	10972	11540	11541	11559	11560
2	C	C	C	A	G	A	G	C	C	A	G	I	T	C	A	T	G
4	C	T	A	T	G	G	A	C	T	A	G	D	T	G	G	T	C
5	C	C	C	T	G	G	A	C	C	A	A	D	T	G	G	T	C
9	C	T	A	T	G	G	A	C	C	G	G	D	T	G	G	T	C
11	C	T	C	A	G	A	A	C	C	A	G	I	T	C	A	T	G
16	C	T	A	T	G	G	G	C	C	A	G	I	T	C	A	T	G
17	G	C	C	T	G	G	A	C	C	A	A	D	T	G	G	T	C
25	G	C	C	T	G	G	G	G	C	A	A	I	T	C	A	T	G
26	C	C	C	A	G	A	A	C	C	G	G	D	T	C	A	T	G
31	G	C	C	A	G	A	A	C	C	A	G	D	T	C	A	T	G
33	G	C	C	A	G	A	A	C	C	A	A	I	T	C	A	T	G
40	G	C	C	A	G	A	A	C	C	A	G	I	T	C	A	T	G
43	C	T	C	A	G	A	A	C	C	G	A	I	T	C	A	T	G
44	C	C	C	T	G	G	G	C	C	A	A	D	T	G	G	T	C
46	G	C	C	A	G	G	A	C	C	G	A	I	T	C	A	T	G
49	G	C	C	T	G	G	A	C	C	G	G	I	T	C	A	T	G
58	G	C	C	T	G	G	A	C	C	G	A	I	T	C	A	T	G
72	C	T	A	T	G	G	A	C	C	A	G	I	T	C	A	T	G
73	G	C	A	T	G	G	A	C	C	G	A	I	T	C	A	T	G
74	G	C	A	T	G	G	A	C	C	G	A	I	T	C	A	T	G
76	G	C	C	A	G	A	A	C	C	A	G	I	T	C	A	T	G
77	G	C	C	A	G	A	A	C	C	G	A	I	T	C	A	T	G
79	G	C	C	T	G	G	A	C	C	A	A	D	T	G	G	T	C
80	C	C	C	T	G	G	A	C	C	A	A	D	T	G	G	T	C
81	C	C	C	T	G	G	G	C	C	A	A	D	T	G	G	T	C
87	G	C	C	A	C	G	A	C	C	A	G	D	T	G	G	T	C
88	G	A	C	A	G	G	G	C	C	A	A	I	T	C	A	T	G
89	G	C	A	A	G	G	G	C	C	A	A	D	A	C	A	C	G
97	G	C	C	A	C	A	A	C	C	A	G	D	A	G	G	T	C
100	G	C	C	A	G	G	A	C	C	A	G	D	T	C	A	T	C
104	G	C	C	A	G	A	A	C	C	G	G	I	T	G	G	T	C
105	G	C	A	T	G	G	A	C	C	G	A	I	T	C	A	T	G
109	C	C	C	A	G	A	A	C	C	G	A	I	T	C	A	T	G
110	C	C	C	T	G	G	G	G	C	A	A	I	T	C	A	T	G
113	G	C	C	T	G	G	A	C	C	A	G	D	T	C	A	T	C
115	G	C	C	A	G	A	A	C	C	A	A	I	T	C	A	T	G
117	G	C	C	A	G	G	A	C	C	G	A	I	T	C	A	T	G
119	G	C	C	A	G	G	A	C	C	A	G	D	T	C	A	T	C
120	G	C	A	T	G	G	G	C	C	A	A	I	T	C	A	T	G
122	G	C	C	A	G	A	A	C	C	A	G	D	T	C	A	T	G
125	G	C	C	T	G	G	A	C	C	A	A	I	T	G	G	T	C
129	G	C	C	T	G	G	G	C	C	A	G	D	T	C	A	T	G
130	G	C	C	A	G	A	G	C	C	A	G	I	T	C	A	T	G
135	G	C	C	A	G	A	A	C	C	A	G	I	T	C	A	T	G
146	C	C	C	T	G	G	G	C	C	A	A	I	T	C	A	T	G

Supplemental Table 2. Line Means and Genotypes, Continued

Line	4769	5441	6291	6388	6894	7149	8147	8156	8205	8226	9113	9496	10972	11540	11541	11559	11560
154	G	C	C	T	G	G	A	C	C	A	G	I	T	C	A	T	G
155	C	C	C	A	C	G	A	C	C	A	G	D	T	G	G	T	C
161	C	T	C	A	G	A	G	C	C	A	G	I	T	C	A	T	G
166	C	C	C	T	G	G	G	C	C	A	G	I	T	G	G	T	C
168	G	C	C	A	G	A	G	C	C	G	G	I	T	C	A	T	G
171	G	C	C	T	G	G	G	C	C	A	A	D	A	G	G	T	C
177	C	C	C	A	G	G	A	C	C	A	G	D	A	C	A	T	C
179	G	C	C	A	G	A	G	C	C	A	G	I	T	C	A	T	G
180	G	C	C	A	C	A	A	C	C	A	G	D	A	G	G	T	G
181	C	C	C	T	G	G	G	C	C	A	A	I	T	C	A	T	G
183	C	C	C	T	G	G	A	C	C	A	G	I	A	C	A	T	G
188	C	C	C	T	G	G	A	C	C	A	G	I	T	G	G	T	C
194	G	C	C	T	G	A	G	C	C	A	A	I	T	C	A	T	G
200	C	C	C	T	G	G	A	C	C	G	G	I	T	G	G	T	C
201	C	C	C	A	G	A	G	C	C	A	G	I	T	C	A	T	G
203	C	C	A	T	G	A	G	C	C	A	A	D	A	G	G	C	C
205	C	T	A	T	G	G	A	C	C	A	G	D	T	C	A	T	G
207	G	C	C	T	G	A	G	C	C	A	G	D	T	C	A	T	G
215	G	C	C	T	G	G	G	C	C	A	A	D	T	G	G	T	C
216	C	A	C	A	G	A	G	C	C	A	G	D	T	C	A	T	C
222	G	C	C	T	G	G	G	C	C	A	G	D	T	G	G	T	C
223	C	C	C	A	C	A	A	C	C	A	G	D	A	G	G	T	C
224	C	C	C	A	G	G	A	C	C	A	G	D	A	G	G	C	C
225	C	C	C	T	G	G	A	C	C	A	A	D	T	G	G	T	C
226	G	C	C	A	G	G	A	C	C	A	G	D	T	C	A	T	C
228	C	C	A	T	G	G	G	C	C	A	A	D	T	G	G	T	C
232	C	T	A	T	G	G	A	C	C	A	G	I	T	C	A	T	G
233	G	C	C	A	G	A	G	C	C	A	G	I	T	C	A	T	G
234	C	T	A	T	G	G	A	C	C	A	G	I	T	C	A	T	G
243	C	C	C	A	G	A	A	C	C	G	A	I	T	C	A	T	G
244	C	T	A	T	G	G	A	C	C	G	A	I	T	C	A	T	G
247	G	C	C	T	G	G	A	C	C	G	G	I	T	C	A	T	G
248	G	C	C	A	G	A	G	C	C	A	G	I	T	C	A	T	G
249	G	C	C	A	G	G	A	C	C	A	G	D	T	G	G	T	C
257	C	C	C	A	C	A	G	G	C	A	G	I	A	G	G	T	G
258	G	C	C	A	G	G	A	C	C	A	G	D	T	G	G	T	C
260	G	C	C	A	G	G	G	C	C	A	G	I	T	C	A	T	G
266	C	C	A	T	G	G	G	C	C	A	G	D	T	G	G	T	C
268	G	C	A	T	G	G	G	C	C	A	A	I	T	C	A	T	G
271	G	C	C	A	C	A	A	C	C	A	G	D	A	G	G	T	C
273	G	C	C	A	G	A	G	C	C	A	G	I	T	C	A	T	G
274	G	C	C	T	G	G	G	C	C	A	A	D	T	G	G	C	C
277	C	C	C	T	G	G	G	C	C	A	G	I	T	C	A	T	G
278	C	T	C	A	G	A	A	C	C	G	A	I	T	C	A	T	G
281	G	C	C	T	G	G	A	C	C	A	G	D	T	G	G	T	C

Supplemental Table 2. Line Means and Genotypes, Continued

Line	4769	5441	6291	6388	6894	7149	8147	8156	8205	8226	9113	9496	10972	11540	11541	11559	11560
282	C	C	C	A	G	A	A	C	C	G	A	I	T	G	G	T	C
284	G	C	C	A	G	A	G	C	C	A	G	I	T	C	A	T	G
285	G	C	A	T	G	G	A	C	C	G	A	I	T	C	A	T	G
294	C	C	C	T	G	A	G	C	C	A	A	I	T	G	G	T	C
300	G	C	C	T	G	G	A	C	C	A	A	I	T	C	A	T	G
308	C	C	C	A	G	G	G	C	C	A	A	I	T	C	A	T	G
309	G	C	C	A	G	G	A	C	C	A	G	D	A	G	G	T	C
311	G	C	C	A	C	G	A	C	C	A	G	D	T	G	G	T	C
314	C	C	C	A	G	A	A	C	C	A	G	D	T	C	A	C	G
316	G	C	C	A	C	A	A	C	C	A	G	D	T	G	G	T	C
317	G	T	C	A	G	A	G	C	C	A	A	I	T	C	A	T	G
325	C	C	C	T	G	G	A	C	C	A	A	D	T	G	G	T	C
327	C	C	C	A	G	A	A	C	C	A	G	D	T	C	A	T	C
334	C	C	C	T	G	G	A	C	C	A	G	D	T	C	A	T	G
336	C	C	C	T	G	G	A	C	C	G	A	I	T	C	A	T	G
337	G	C	C	T	G	G	A	C	C	A	G	D	A	G	G	T	C
339	C	T	C	A	G	A	G	C	C	A	G	I	T	C	A	T	G
340	C	C	A	T	G	A	G	C	C	A	A	D	A	G	G	C	C
341	C	C	C	A	G	G	G	C	C	A	A	I	T	C	A	T	G
343	G	C	C	A	G	A	G	C	C	A	G	I	T	C	A	T	G
345	C	C	C	T	G	G	A	C	C	A	G	D	T	C	A	T	G
347	C	C	A	T	G	G	A	C	C	A	G	D	T	C	A	T	G
351	C	C	C	T	G	G	A	C	C	A	A	I	T	C	A	T	G
354	C	C	C	A	G	G	A	C	C	A	G	D	T	C	A	T	C
355	C	T	C	T	G	G	A	C	C	G	G	I	T	G	G	T	C
361	G	C	C	A	G	A	A	C	C	G	A	I	T	C	A	T	G
363	G	C	C	T	G	G	A	C	C	A	A	D	A	G	G	C	C
367	G	C	C	T	G	G	A	C	C	A	G	I	T	G	A	T	A
368	C	T	A	T	G	G	A	C	C	A	G	I	T	C	A	T	G
369	C	T	A	T	G	A	A	C	C	A	G	I	T	C	A	T	G
374	C	T	A	T	G	G	A	C	T	A	G	D	T	G	G	T	C
376	G	C	C	T	G	G	A	C	C	A	G	D	T	G	G	T	C
382	C	C	C	T	G	G	A	C	C	G	A	D	T	G	G	T	C
383	C	C	C	A	G	A	G	C	C	A	G	I	T	C	A	T	G
385	G	C	C	A	G	A	A	C	C	A	A	I	T	C	A	T	G
387	C	C	A	T	G	G	A	C	C	A	G	D	T	C	A	T	G
388	C	C	C	A	G	A	G	C	C	G	A	D	T	G	G	T	C
390	C	C	C	T	G	G	G	C	C	A	A	I	T	G	G	T	A
396	C	C	A	T	G	G	G	C	C	A	A	D	T	C	A	T	G
402	C	C	C	A	G	G	G	C	C	A	A	I	T	C	A	T	G
405	G	C	C	A	G	A	G	C	C	A	G	I	T	C	A	T	G
406	G	C	C	A	G	A	G	C	C	A	A	I	T	C	A	T	G
407	C	C	C	A	G	A	G	C	C	A	G	I	T	C	A	T	G
408	G	C	C	A	G	A	G	C	C	A	G	I	T	C	A	T	T
415	G	C	C	A	C	G	A	C	C	A	G	D	A	G	G	T	A

Supplemental Table, Continued

Line	4769	5441	6291	6388	6894	7149	8147	8156	8205	8226	9113	9496	10972	11540	11541	11559	11560
418	G	C	C	A	G	G	A	C	C	A	G	D	T	C	A	T	C
427	C	C	C	T	G	G	A	C	C	G	A	I	T	C	A	T	G
429	C	C	C	A	G	A	A	C	C	G	A	D	T	C	A	T	G
434	C	C	A	T	G	A	G	C	C	A	A	D	A	G	G	C	C
437	C	C	A	T	G	G	G	C	C	A	A	D	T	G	G	T	C
440	G	C	C	A	G	A	G	C	C	A	G	I	T	C	A	T	G
444	G	C	C	A	C	G	A	C	C	A	G	D	T	C	A	T	C
453	C	C	C	A	G	A	A	C	C	A	A	I	T	C	A	T	G
455	C	C	C	T	G	G	G	C	C	A	G	I	T	C	A	T	C
456	C	T	C	A	G	A	G	G	C	A	G	I	T	C	A	T	G
458	G	C	C	A	G	A	G	G	C	A	G	I	T	C	A	T	G
460	G	C	C	T	G	G	A	C	C	G	A	D	T	C	A	T	G
461	G	C	C	A	C	A	A	C	C	A	G	D	A	G	G	T	C
464	C	C	C	A	G	A	G	C	C	A	G	I	T	C	A	T	G
466	C	C	C	A	G	G	G	C	C	A	A	I	T	C	A	T	G
467	C	C	C	A	G	G	G	C	C	A	G	I	T	C	A	T	G
470	C	C	C	A	G	G	G	C	C	A	G	I	T	C	A	T	G
472	G	C	C	A	G	A	G	C	C	A	A	I	T	G	A	T	G
473	C	T	A	T	G	G	G	C	C	A	G	D	T	C	A	T	G
477	C	T	A	T	G	G	A	C	C	A	G	D	T	G	G	T	C
479	G	C	C	A	G	A	A	C	C	G	A	I	T	C	A	T	G
481	G	C	A	T	G	G	A	C	C	G	A	I	T	C	A	T	G
489	C	T	C	T	G	G	A	C	C	G	G	I	T	G	G	T	C
492	G	T	C	A	G	G	A	C	C	A	G	D	A	C	A	T	G
498	C	T	C	A	G	A	A	C	C	G	A	I	T	C	A	T	G
977	C	C	C	T	G	G	A	C	C	A	G	I	T	G	G	T	C
981	C	T	A	T	G	A	G	C	C	A	G	I	T	C	A	T	G
985	G	C	C	A	G	A	G	C	C	A	G	I	T	C	A	T	G
9713	C	C	C	T	G	G	A	C	C	A	A	I	T	C	A	T	G
9726	C	T	A	T	G	G	G	G	C	A	A	I	T	C	A	T	G
9727	C	C	C	T	G	G	A	C	C	G	A	I	T	C	A	T	G
9729	G	C	C	A	G	G	A	C	C	G	G	I	T	G	G	T	C
9811	C	C	C	T	G	G	A	C	C	A	A	D	T	G	G	T	C
9814	C	C	C	T	G	A	A	C	C	G	A	I	T	G	G	T	C
9821	G	C	A	T	G	G	A	C	C	G	A	I	T	C	A	T	G
9825	C	C	A	T	G	A	G	C	C	A	A	D	A	G	G	C	C
9829	G	C	A	T	G	A	G	C	C	A	A	I	A	G	G	C	C
9830	C	C	A	A	G	A	A	C	C	G	G	I	T	C	A	T	G
9833	C	C	A	T	G	G	A	C	C	G	G	D	T	G	G	T	C
9835	C	C	C	A	G	G	G	C	C	A	G	I	T	C	A	T	G
9840	C	C	C	A	G	G	G	C	C	A	A	I	T	C	A	T	G
9841	G	C	C	A	G	G	A	C	C	G	A	I	T	C	A	T	G

APPENDIX

ASSOCIATION OF SINGLE NUCLEOTIDE POLYMORPHISMS AT THE *DELTA* LOCUS WITH GENOTYPE-ENVIRONMENT INTERACTION FOR SENSORY BRISTLE NUMBER IN *DROSOPHILA MELANOGASTER*

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SUMMARY

The nature of forces maintaining variation for quantitative traits can only be assessed at the level of individual genes affecting variation in the traits. Identification of single nucleotide polymorphisms (SNPs) associated with variation in *Drosophila* sensory bristle number at the *Delta* (*Dl*) locus provides us with the opportunity to test a model of maintenance of variation for bristle number by genotype by environment interaction (GEI). Under this model, genetic variation is maintained at a locus under stabilizing selection if phenotypic values of heterozygotes are more stable than homozygotes across a range of environments, and the mean allelic effect is much smaller than the standard deviation of allelic effects across environments. Homozygotes and heterozygotes for two SNPs at *Dl*, one affecting sternopleural and the other abdominal bristle number, were reared in five different environments. There was significant GEI for both bristle traits. Neither condition of the model was satisfied for *Dl* SNPs exhibiting GEI for sternopleural bristle number. Heterozygotes for the abdominal bristle number SNPs were indeed the most stable genotype for two of the three environment pairs exhibiting GEI, but the mean genotypic effect was greater than the standard deviation of effects across environments. Therefore, this mechanism of GEI seems unlikely to be responsible for maintaining the common bristle number polymorphisms at *Dl*.

INTRODUCTION

The paradox of widespread segregating variation for quantitative traits in natural populations, despite strong stabilizing and directional selection which should eliminate

variation, is unresolved (Barton & Turelli, 1989). The problem is not a dearth of potential mechanisms that, in theory, lead to the maintenance of genetic variation, but rather a lack of empirical data with which to evaluate the theory. For example, some fraction of the variation for all quantitative traits must be that expected at equilibrium between the input of new deleterious alleles by mutation and their elimination by natural selection (Barton, 1990), and some fraction may be selectively neutral, with variation maintained by a balance between mutation and drift (Lynch & Hill, 1986). Overdominance of alleles associated with intermediate trait values (Robertson, 1967; Barton, 1990) and epistasis (Gimelfarb, 1989) will maintain variation at loci affecting traits under stabilizing selection. Variation can also be maintained when there is genotype by environment interaction (GEI) for fitness, if alleles have opposing (Levene, 1953) or variable (Fry *et al.*, 1996) effects on fitness in different environments, or if heterozygotes are less sensitive than homozygotes to environmental variation (Gillespie & Turelli, 1989).

These mechanisms are not mutually exclusive, and one might expect heterogeneity in mechanisms promoting genetic variation among different quantitative trait loci (QTL) affecting variation in a single trait, and even among different alleles at a single locus (Long *et al.*, 2000). (Here, QTL refers to the actual genetic locus at which alleles affecting the trait segregate in nature.) The difficulty in addressing this problem empirically is apparent: not only do we need to know what QTL affect variation in the trait of interest, in a range of ecologically relevant environments, but we need to know what molecular polymorphisms at the QTL actually cause the difference in trait phenotype (the ‘quantitative trait nucleotides’,

or QTN), their allele frequencies, and the fitness effects of different QTL genotypes, again in a range of environments.

Currently, no quantitative trait in any organism is understood at this level of detail. However, progress towards this goal has been made for sensory bristle number in *Drosophila melanogaster*. Abdominal and sternopleural bristle numbers have abundant genetic variation in natural populations and respond rapidly to artificial selection (e.g., Long *et al.*, 1995; Gurganus *et al.*, 1999). Bristle numbers are thought to be under strong stabilizing selection, because mean bristle numbers are relatively stable among natural populations despite the potential for divergence. However, efforts to deduce the relationship between bristle numbers and fitness in the laboratory have reached contradictory conclusions, with experiments supporting strong (Kearsey & Barnes 1970; Linney *et al.*, 1971; Nuzhdin *et al.* 1995), moderate (Clayton *et al.*, 1957; Latter & Robertson, 1962; García-Dorado & González, 1996) or very weak stabilizing selection (Spiers, 1974; Mackay 1985). Such inconsistencies could well reflect underlying heterogeneity in the relationship of genotypes at segregating bristle number QTL to fitness among the different populations used.

Several loci affecting peripheral nervous system (PNS) development are bristle number QTL, and molecular polymorphisms associated with QTN with large effects on bristle number have been identified at these loci (Long *et al.*, 1998; 2000; Lyman *et al.*, 1999). The fitness effects of the QTL genotypes are not known. However, molecular polymorphisms at intermediate frequency are likely to be associated with causal QTN that are also at intermediate frequency, and not with rare QTN with very large effects (Long *et al.*, 1998). Models for the maintenance of variation by mutation-selection balance predict that

equilibrium levels of genetic variance are reached when the mutant allele is at low frequency (Barton & Turelli, 1989; Barton, 1990); therefore, polymorphisms at intermediate frequency are not consistent with this mechanism. Intermediate frequency polymorphisms are consistent with maintenance by some form of balancing selection, or with selective neutrality.

For loci under 'real' stabilizing selection [i.e., selection based on the value of the trait, and not 'apparent' stabilizing selection due to overdominance of alleles associated with intermediate trait values (Robertson, 1967; Barton, 1990) or deleterious pleiotropic fitness effects of alleles causing extreme phenotypes (Barton, 1990; Keightley & Hill, 1990; Kondrashov & Turelli, 1992)], intermediate frequency polymorphisms can be maintained by GEI for the trait (Gillespie & Turelli, 1989). This model assumes stabilizing selection for a single phenotype that is optimal in all environments, constant fitnesses across environments, alleles with different additive effects on the trait in different environments, and that the mean and variance of allelic effects across environments are small. The latter assumption thus requires that the mean differences of average allelic effects must be very small relative to the standard deviation of allelic effects across environments. Under this model, heterozygotes will tend to have lower phenotypic variance than homozygotes, and higher mean fitness. Testing the model requires that we (1) identify a common molecular polymorphism at a QTL associated with variation in a trait that is under stabilizing selection, and (2) determine genotypic values of homozygous and heterozygous QTL genotypes across a range of environments. Here, we report the results of this test for single nucleotide polymorphisms (SNPs) at the *Delta* (*Dl*) locus that have been associated with variation for sternopleural and abdominal bristle number (Long *et al.*, 1998).

MATERIALS AND METHODS

(i) Construction of *Drosophila* stocks

Approximately 60 isogenic third chromosomes were derived from wild-caught flies and substituted into the inbred Samarkand *X* and second chromosome genetic background. Near-isoallelic lines containing the wild-derived *Dl* allele and an average of 10 cM flanking genomic fragment to either side, in an otherwise Samarkand chromosome *3* background, were constructed from each of the whole chromosome substitution lines by 10 generations of back-crossing. For further details, see Lyman & Mackay (1998).

(ii) SNP associations with sensory bristle number

Long *et al.* (1998) conducted a survey of restriction site variation in the *Dl* locus and examined the association of molecular variation with phenotypic variation in sternopleural and abdominal bristle number. Two SNPs in *Dl* were in significant linkage disequilibrium with bristle number QTN, as judged by a permutation test.

An *Hae*III restriction site polymorphism in the second intron (*Hae*III+8.6, represented here by H) was associated with a difference in sternopleural bristle number in both sexes. In the near-isoallelic lines, the mean sternopleural bristle numbers (± s.e.) of flies homozygous for the presence (H11) of this restriction site were 19.12 ± 0.14 in males and 20.16 ± 0.15 in females; and mean sternopleural bristle numbers in flies homozygous for the absence (H00) of this restriction site were 18.53 ± 0.10 in males and 19.36 ± 0.11 in females. Averaged over both sexes, the H11 genotype has approximately 0.7 more sternopleural bristles than does the H00 genotype.

An *Scr*FI restriction site polymorphism in the fifth intron (*Scr*FI+18.6, represented here by S) was associated with a difference in abdominal bristle number in females only. In the near-isoallelic lines, the mean abdominal bristle numbers (± s.e.) of flies homozygous for the presence (S11) of this restriction site were 19.22 ± 0.19 in males and 22.03 ± 0.27 in females; and mean abdominal bristle numbers in flies homozygous for the absence (S00) of this restriction site were 19.43 ± 0.17 in males and 23.21 ± 0.21 in females. In females, the S00 genotype has approximately 1.2 more abdominal bristles than does the S11 genotype.

(iii) Crosses among *Dl* near-isoallelic lines

Homozygous near-isoallelic *Delta* lines were grouped into four haplotypes according to the presence (1) or absence (0) of the H and S sites, respectively: 00, 01, 10, 11. Three lines of each haplotype were chosen at random for each of the bristle traits. The lines representing each haplotype are given in parentheses (see Long *et al.* 1998, Appendix): low sternopleural bristle number haplotypes 00 (33, 41, 51) and 01 (13, 22, 31); high sternopleural bristle number haplotypes 11 (15, 17, 84) and 10 (10, 50, 116); low abdominal bristle number haplotypes 11 (58, 111, 119) and 01 (19, 46, 95); high abdominal bristle number haplotypes 10 (10, 86, 116) and 00 (41, 53, 107).

The *Dl* near-isoallelic lines were crossed to generate F_1 genotypes that were homozygous or heterozygous for the focal SNPs, but randomized for heterozygosity at all other polymorphic sites at *Dl* and for the introgressed regions surrounding *Dl*. Thus, six low sternopleural bristle number genotypes (H00) were constructed by a round robin crossing scheme: $00_1 \times 00_2$; $00_2 \times 00_3$; $00_3 \times 01_1$; $01_1 \times 01_2$; $01_2 \times 01_3$; $01_3 \times 00_1$ (where the subscript denotes each of the three lines representing the designated haplotype). Progeny of all of these

crosses are homozygous H00, while progeny of the first two crosses are homozygous S00, progeny of the third and sixth cross are heterozygous S01, and progeny of the fourth and fifth cross are homozygous S11. Six high sternopleural bristle number genotypes (H11) were constructed similarly: $11_1 \times 11_2$; $11_2 \times 11_3$; $11_3 \times 10_1$; $10_1 \times 10_2$; $10_2 \times 10_3$; $10_3 \times 11_1$. Crosses to create heterozygous genotypes at the SNP associated with sternopleural bristle number (H10) were: $11_1 \times 01_1$; $11_2 \times 01_2$; $11_3 \times 00_1$; $10_1 \times 01_3$; $10_2 \times 00_2$; $10_3 \times 00_3$. The same logic was used to construct six lines representing each of the three abdominal bristle number SNP genotypes (S00, S01, S11); within each S genotype class, two genotypes were H11, two were H01, and two were H00.

(iv) Culture conditions and bristle number phenotypes

Two replicate vials for each of the 36 F_1 genotypes produced by the above crosses of *Dl* near-isoallelic lines were reared in each of five environments: standard cornmeal-agar-molasses medium at 18, 25, and 28 °C, cornmeal-agar-molasses medium brought to a final concentration of 9% ethanol at 25 °C, and tomato paste medium (Fry *et al.,* 1996) at 25 °C. These environments had previously been shown to affect a measure of competitive fitness (Fry *et al.* 1996). Ten males and ten females from each replicate vial were scored for either sternopleural (total number of bristles on the left and right sternopleural plates) or abdominal (the total number of bristles on the sixth abdominal sternite in females and the fifth sternite in males) bristle number, depending on whether the focal SNP was H or S, respectively. A total of 3,600 flies were scored for each bristle trait. The design was completely balanced.

(v) Statistical analyses

Distribution statistics, analyses of variance of bristle number and tests of significance

of *F*-ratios were estimated using SAS procedures MEANS and GLM (SAS Institute, 1988). Variance in bristle number was partitioned by three-way factorial analyses of variance (ANOVA) according to the full model:

$$Y = \mu + S + G + E + G\times S + G\times E + E\times S + G\times E\times S + L(G) + E\times L(G) + S\times L(G) + E\times S\times L(G) + R(E\times G\times L) + S\times R(E\times G\times L) + \text{Error}$$

where *S*, *G* and *E* represent the fixed cross-classified effects of sex, SNP genotype and culture environment, respectively; *L* and *R* are random effects of six different F_1 lines within each SNP genotype and replicate vial, respectively; and parentheses indicate nested effects. Reduced analyses by sex and/or by genotype, and for all possible pairs of environments, were also conducted as appropriate.

RESULTS

(i) Sternopleural bristle number

Table 9 shows the ANOVA of sternopleural bristle number for the three H SNP genotypes across both sexes and all five environments, and Figure 11 depicts the mean bristle numbers of each genotype in each environment. The main effect of genotype in the ANOVA was highly significant, confirming that this site is associated with variation in sternopleural bristle number. Rearing environment also had a highly significant effect on mean bristle number, with the highest mean bristle number at the lowest developmental temperature, and the lowest mean bristle number in the ethanol-supplemented medium (Figure 11). The SNP genotype by rearing environment interaction term was also highly significant, fulfilling the first criterion of Gillespie & Turelli's (1989) model.

Table 9. ANOVA for Bristle Numbers Across Environments

		Sternopleural bristles			Abdominal Bristles		
Source	d.f.	MS	*F*	*P*	MS	*F*	*P*
S	1	390.1	147	0.0001	7906	233	0.0001
G	2	1095	29.1	0.0001	1303	16.1	0.0002
E	4	226.2	55.7	0.0001	367.1	31.7	0.0001
G×S	2	6.370	2.41	0.1	227.0	6.70	0.008
G×E	8	17.50	4.31	0.0004	22.30	1.93	0.07
E×S	4	4.902	1.55	0.2	10.38	1.80	0.1
G×E×S	8	1.220	0.36	0.9	9.023	1.56	0.2
L(G)	15	37.65	10.7	0.0001	81.08	2.04	0.07
E×L(G)	60	4.058	1.08	0.4	11.57	1.80	0.03
S×L(G)	15	2.646	0.834	0.6	33.87	5.86	0.0001
E×S×L(G)	60	3.171	1.32	0.1	5.776	1.21	0.2
R(G×E×L)	90	2.994	1.25	0.2	5.400	1.13	0.3
S×R(G×E×L)	90	2.400	1.16	0.2	4.76	1.13	0.2
Error	3240	2.073			4.205		

Table 9. Analyses of variance of bristle number, pooled across sexes and all five environments. Sources of variation are sex (*S*), SNP genotype (*G*), environment (*E*), F_1 line cross (*L*) and vial replicate (*R*).

However, inspection of Figure 11 suggests that the pattern of GEI is not consistent with one of the requirements of the model, that the heterozygous genotype is more stable than the homozygous genotypes across environments. Rather, it appears that H11 homozygotes are the most stable genotype, and statistical analyses bear this out. ANOVAs were computed separately for each genotype, pooled across sexes and environments (data not shown). Both sex and environment were fixed effects. The effect of environment was significant for all

Figure 11. Mean Sternopleural Bristle Number of Each Genotype in Five Environments

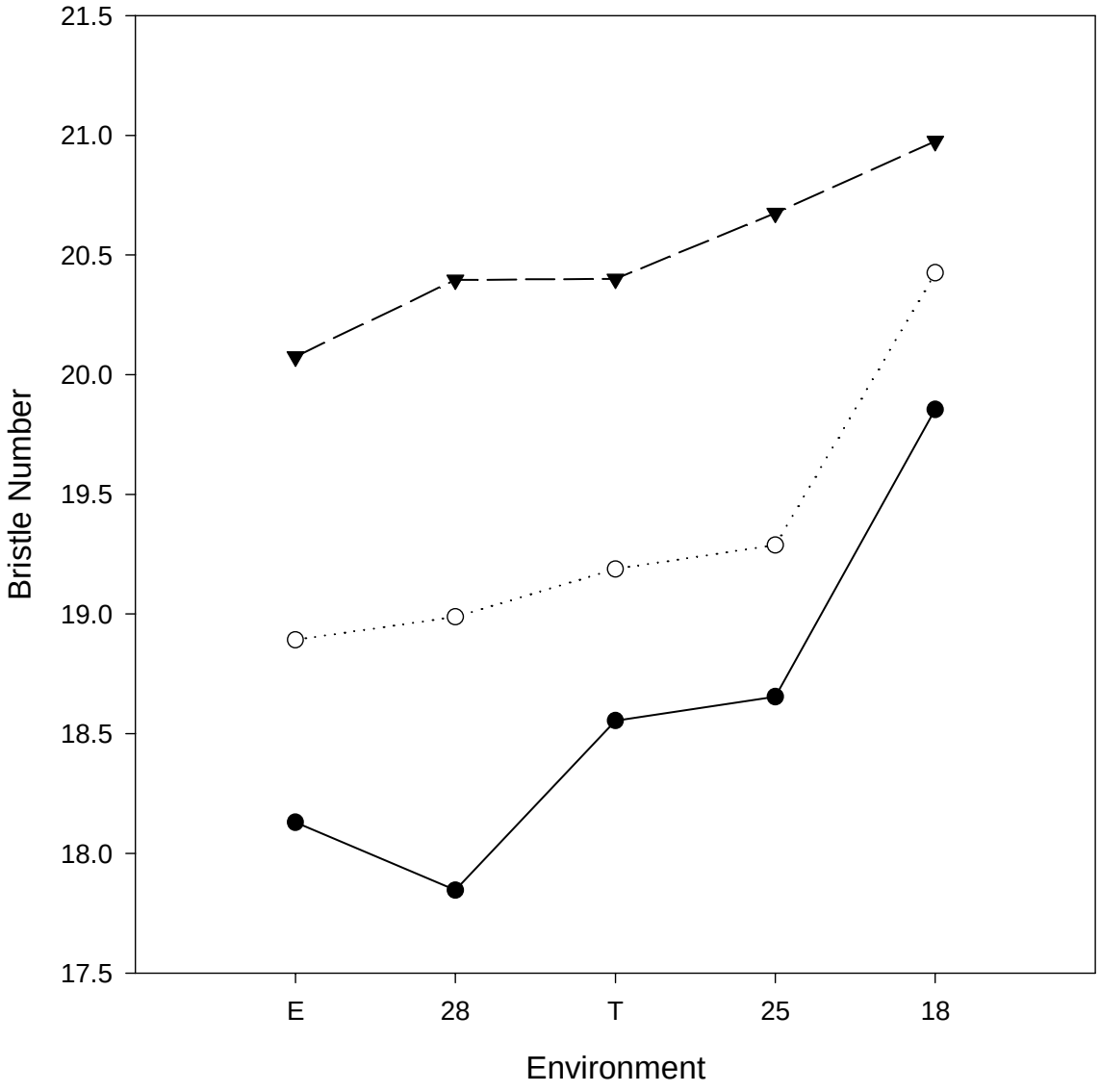

Figure 11 shows the mean sternopleural bristle number of H00 (•), H10 (◦) and H11 (▾) SNP genotypes for the *Dl* locus in five environments: standard culture medium at 18, 25 and 28 °C, standard culture medium supplemented with 9% ethanol (E), and tomato paste medium (T).

three genotypes: H11, $F_{4, 20} = 5.08$, $p = 0.005$; H10, $F_{4, 20} = 25.6$, $p < 0.0001$; H00, $F_{4, 20} = 44.5$, $p < 0.0001$. The proportion of the total sums of squares explained by the effect of environment was, however, much smaller for the H11 genotype (3.2%) than the H10 (11.1%) H11 (19.3%) genotypes.

It is possible that GEI is not significant for all pairs of environments, and that closer examination of the pairs that contribute to the overall significance of this term could reveal different patterns. Therefore, we computed separate ANOVAs for each of the 10 pair-wise environmental comparisons. There was significant GEI for sternopleural bristle number for five of these contrasts: 18° and 25° ($P < 0.0001$); 18° and 28° ($P = 0.0004$); 18° and ethanol medium ($P = 0.05$); 18° and tomato medium ($P = 0.03$); and 28° and tomato medium ($P = 0.002$). However, examination of the difference in mean bristle number in each of these environment pairs for each genotype (a measure of the environmental sensitivity) shows that in all cases, the H11 genotype was least sensitive and the H00 genotype the most sensitive, with the heterozygote intermediate (Table 10).

Further, the assumption that the mean genotypic effect across environments is much smaller than the standard deviation of effects across environments is not true for these data. Genotypic values (a) were estimated as one-half the difference between H11 and H00 homozygotes (Falconer & Mackay, 1996), averaged over sexes, within each environment. The mean genotypic value was 0.85, and the standard deviation across environments was 0.316. Thus, neither the mean nor variance of allelic effects across environments was small, and the mean effect was 2.7 fold greater than the standard deviation across environments, in violation of the model assumption.

Table 10. Differences Between Line Means for Pairs of Environments

	SNP Genotype (Sternopleural Bristles)		
Env. Pair	H00	H10	H11
18^0 - 28^0	2.008 (0.127)	1.438 (0.142)	0.579 (0.148)
18^0 - 25^0	1.200 (0.123)	1.138 (0.139)	0.300 (0.143)
18^0 - Ethanol	1.725 (0.120)	1.533 (0.133)	0.900 (0.128)
18^0 - Tomato	1.300 (0.124)	1.238 (0.121)	0.575 (0.132)
28^0 - Tomato	-0.708 (0.114)	-0.200 (0.131)	-0.004 (0.152)
	SNP Genotype (Abdominal Bristles)		
Env. Pair	S00	S10	S11
25^0 - 28^0	1.300 (0.276)	-0.233 (0.285)	-0.792 (0.284)
25^0 - Tomato	1.050 (0.308)	-0.008 (0.251)	-0.800 (0.274)
25^0 - Ethanol	0.117 (0.293)	-0.167 (0.299)	-1.058 (0.284)

Table 10. Differences between lines means (environmental sensitivities) for pairs of environments contributing to GEI for genotypes at the Dl locus. s.e. in parentheses.

(ii) Abdominal bristle number

The ANOVA of abdominal bristle number (Table 9) shows that there were highly significant differences in bristle number between the three SNP genotypes and among the five environments, but that the SNP genotype by environment interaction term was not significant, although it approached nominal significance ($P = 0.07$). However, the SNP genotype by sex interaction term was highly significant, which was not unexpected given the previous observation that this SNP had a female-specific effect on abdominal bristle number (Long *et al.*, 1998). We ran reduced ANOVAs for each sex separately (data not shown). In males, the effects of SNP genotype ($P = 0.06$) and SNP genotype by environment interaction ($P = 0.4$) were not significant. However in females, the effect of SNP genotype was highly

significant ($P < 0.0001$) and the SNP genotype by environment interaction term reached nominal significance ($P = 0.04$). Therefore, we restricted further analyses to females only.

Mean abdominal bristle numbers in females are shown for each genotype in each environment in Figure 12. The highest mean bristle number was at 18°, and the lowest at 28°. Analyses of environmental sensitivities of each genotype over all five environments (data not shown) do not indicate that the heterozygote is the most stable genotype. The effect of environment was significant for all three genotypes: S11, $F_{4, 20} = 16.104$, $P < 0.0001$; S10, $F_{4, 20} = 4.31$, $P < 0.01$; S00, $F_{4, 20} = 6.35$, $P < 0.002$. The proportion of the total sums of squares explained by the effect of environment was, however, much larger for the S11 genotype (15.8%) than the S10 (7.5%) or S00 (7.6%) genotypes.

Inspection of Figure 12 indicates that there may be some pairs of environments contributing to GEI for which the heterozygote is the most stable genotype. We again computed separate ANOVAs for each of the 10 pair-wise environmental comparisons. There was significant GEI for abdominal bristle number in females for three of these contrasts: 25° and 28° ($P = 0.02$); 25° and tomato medium ($P = 0.02$); and 25° and ethanol medium ($P = 0.04$). Further, examination of the environmental sensitivity for each genotype revealed that, for two of these pairs (25° and 28°; 25° and tomato medium), the S10 genotype was indeed less sensitive than either homozygote (Table 10).

However, the assumption that the mean genotypic effect is smaller than the standard deviation of effects across environments is not met for the abdominal bristle number polymorphism. The mean genotypic value was 1.45 over all five environments, and 1.39 over

Figure 12. Mean Abdominal Bristle Number of Each Genotype in Five Environments

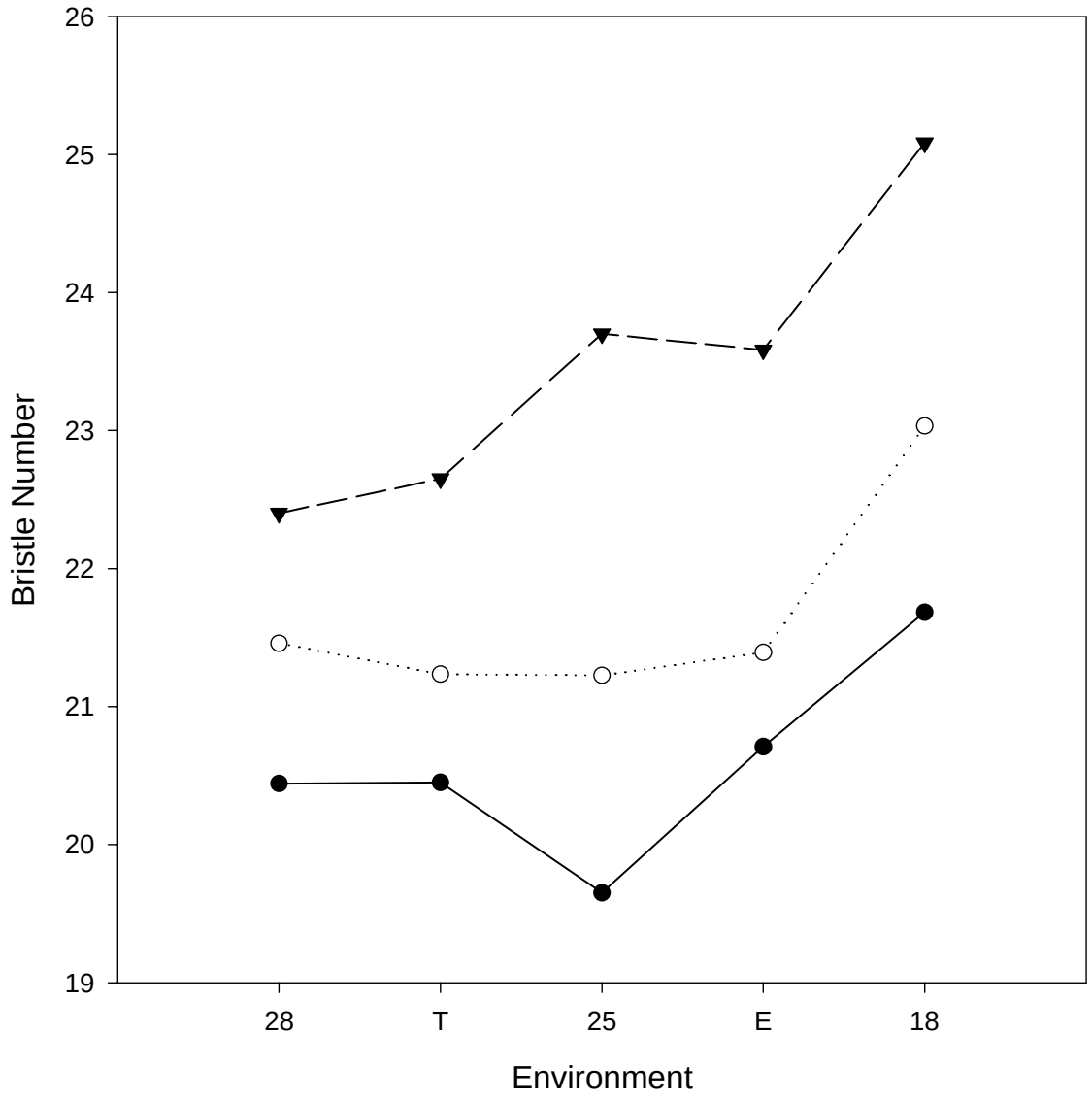

Figure 12 shows the mean female abdominal bristle number of S11 (•), S10 (◦) and S00 (▾) SNP genotypes for the *Dl* locus in five environments: standard culture medium at 18, 25 and 28 °C, standard culture medium supplemented with 9% ethanol (E), and tomato paste medium (T).

the four environments contributing to significant GEI. The standard deviation of allelic effects was 0.429 over all five environments, and 0.468 over the four environments contributing to GEI. Thus, the mean allelic effect is approximately three times as large as the standard deviation of effects across environments

DISCUSSION

Many fundamental questions in evolutionary quantitative genetics have remained unanswered because the theoretical underpinnings are in terms of fitness effects of individual genes, while observations have been at the level of collective properties of all genes affecting variation in traits of interest. As QTL for many traits begin to be resolved into discrete genetic loci and indeed into QTN at each of these loci, prospects are bright for empirically addressing such long-standing issues as the nature of the forces maintaining variation for quantitative traits, and the genetic basis of adaptation.

Drosophila sensory bristle numbers are classic examples of traits that are under presumed stabilizing selection, but for which large amounts of additive genetic variation segregates in natural populations (e.g., Long *et al.*, 1995; Gurganus *et al.*, 1999). Identification of SNPs in linkage disequilibrium with bristle number at several genetic loci corresponding to bristle number QTL (Long *et al.*, 1998; 2000; Lyman *et al.*, 1999) define, for the first time, genotypes of functional alleles affecting variation in bristle number. This in turn provides us with the opportunity to test, one locus at a time and in combination, what evolutionary forces act to maintain variation for bristle number. Here we have begun this process by evaluating the prediction of one particular model of maintenance of genetic

variation by GEI (Gillespie & Turelli, 1989): that phenotypic values of heterozygous genotypes are more stable across a range of environments than are phenotypic values of homozygous genotypes.

Homozygous and heterozygous genotypes for two SNPs at the *Dl* locus, one of which affects sternopleural and the other abdominal bristle number, were constructed by crossing six different near-isoallelic lines for the *Dl* gene region in such a manner as to randomize heterozygosity for other segregating sites within each SNP genotype class. When reared in five different environments, there was strong SNP genotype by environment interaction for sternopleural bristle number for males and females, and SNP genotype by environment interaction for abdominal bristle number in females only. While the rank order of the different genotypes was in both cases the same in the different environments, the additive effects of the bristle number SNPs varied over two-fold. For example, genotypic values ranged from 0.56 sternopleural bristle at 18° and 1.28 sternopleural bristles at 28°; and, in females, from 0.98 abdominal bristle at 28° to 2.03 abdominal bristles at 25°. Further, the degree of dominance, *d/a* (where *d* is the difference between the mean bristle number of the heterozygous SNP genotype and the average bristle number of the two homozygous genotypes; Falconer & Mackay, 1996), can also vary between environments. At 28°, the S polymorphism affecting female abdominal bristle number is additive ($d/a = -0.04$), but in the ethanol-supplemented culture medium, the low allele is partially dominant ($d/a = -0.52$). Thus, the amount of additive and dominance variance attributable to the QTNs at *Dl* will be different depending on the environment in which the flies were reared.

These observations are germane to the proposal to utilize SNP associations to determine the genetic basis of complex human diseases (Lander, 1996; Collins *et al.*, 1997). It is assumed that causal associations will replicate in different populations, while spurious associations produced by population admixture will not. However, GEI and sex-specific SNP effects reduce the power of association studies within populations and could lead to non-reproducibility of effects of causal SNPs across populations. Explicit inclusion of sex and common demographic factors in the experimental design of SNP association studies, with concomitant increases in sample sizes to give sufficient power to test all combinations of parameters, will be necessary to determine the full range of SNP effects, and to evaluate environmental risk factors.

Our data do not provide support for the maintenance of genetic variation for bristle number by the mechanism proposed by Gillespie & Turelli (1989). While heterozygotes for the abdominal bristle number polymorphism were more stable than homozygotes across two of the three environmental contrasts showing GEI for this trait, this was not true for the sternopleural bristle number polymorphism, where one of the homozygotes was least environmentally sensitive. In neither case was the model assumption of small means and variances of allelic effects across environments met.

This conclusion must be tempered by several caveats. First, and foremost, a fair test of this model, or any other model invoking GEI, requires that the means and variances of effects be measured in whatever range of environments is relevant to maintaining the observed variation in nature. As these conditions are not known, it is quite possible that the relevant factors were not included among the lab environments tested. Second, *Dl* is just one

of many loci affecting variation in bristle number, and the mechanisms maintaining variation may vary from locus to locus. Third, the model assumes real stabilizing selection acting on *Dl* through its effect on bristle number, for which we have no direct evidence. Fourth, we do not know that the SNPs associated with sternopleural and abdominal bristle number are themselves the causal QTN, all we know is that they are in linkage disequilibrium with the causal QTN. Thus, crosses to generate homozygote and heterozygote genotypes at the focal SNPs may not have produced homozygous and heterozygous genotypes at the causal QTN. This does not appear to be a problem, however, since the SNP genotypes recapitulate the expected differences in bristle number phenotypes. Despite these caveats, it is plausible that the assumptions of Gillespie & Turelli's (1989) model regarding small means and variances of allelic effects across environments are overly restrictive and compromise the generality of the model (Gimelfarb, 1990). A more realistic model of maintenance of variation by GEI would incorporate larger differences in mean allelic effects than the variance of effects across environments, as observed for the *Dl* polymorphisms.

Evaluation of other models for the maintenance of variation of the bristle number polymorphisms at *Dl* require that we estimate the fitnesses of the three genotypes at each polymorphic site. Knowledge of markers in strong linkage disequilibrium with the causal QTN (and, ultimately, the causal QTN themselves) opens up the possibility for applying the whole gamut of population genetic approaches that have been used to infer selection on allozyme and other polymorphisms, both in the wild (Endler, 1986) and in the laboratory, to the problem of selection on loci affecting variation for quantitative traits. Given the somewhat checkered history of such attempts, however, it may be unrealistic to presume that

we could directly measure fitness effects of all loci affecting variation in any trait. Selection acting on any one locus affecting a quantitative trait in any one environment at any point in time is likely to be quite weak, particularly if selection at the level of the trait is weak (Kingsolver *et al.*, 2001) or when there are large numbers of variable QTL to consider (Kimura 1983). In addition, as noted above, one needs to consider the whole range of environments that are ecologically relevant. However, there is a rich body of population genetics theory for inferring the action of historical selection from data on DNA sequence variation (Hartl & Clark, 1997; Wayne & Simonsen, 1998). When applied to sequences of cloned QTL, it will be possible to detect the signatures of purifying selection, selective sweeps, balancing selection and neutrally evolving polymorphisms; as exemplified by the demonstration that domestication of maize was accompanied by selection in the 5' regulatory region of *Teosinte-branched1* (Wang *et al.*, 1999).

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