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Environment-Dependent Survival of *Drosophila melanogaster:* A Quantitative Genetic Analysis

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Abstract

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

Keywords: environmental stress, genetic correlations, lifespan, QTL, starvation

Introduction

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

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

Recombinant inbred lines (RILs) can be used to identify QTLs as regions of the genome that account for a significant component of the segregating genetic variation and characterize genetic correlations between traits. One set of RILs derived from laboratory stocks has been used to map quantitative trait loci for longevity and stress-related traits in female and male *D. melanogaster* (Nuzhdin et al., 1997). Using a dense molecular map, a genome-wide screen was conducted for QTLs that affect the lifespan of individually held virgin males and females. Five QTLs were identified that had a significant effect on longevity, but they affected lifespan in only one sex or the other. Vieira et al. (2000) extended this study by measuring the lifespan of virgin females or males under high and low temperature conditions and after heat shock. In addition, survival was determined under starvation conditions. Seventeen QTLs were identified that affected lifespan under different conditions as well as starvation survival. The QTLs typically were sex- and condition-

dependent and there was no statistically significant positive covariation between starvation survival and longevity QTLs. Longevity QTL studies based on mixed sex cohorts have produced different results in that no sex-specific QTLs were observed in the same panel of RILs (Reiwitch & Nuzhdin, 2002) or in a different set of RILs (Curtsinger & Khazaeli, 2002). Only in one study has positive QTL covariation between longevity and stress resistance (oxidative stress resistance) been reported (Curtsinger & Khazaeli, 2002). In this case, the RILs were derived from a laboratory selection experiment for extended longevity. Given that the lines had been maintained for many generations in the laboratory before inbreeding to generate the RILs, it was not clear that the QTL represented naturally occurring positive genetic covariation between longevity and stress resistance.

The purpose of the present study was to investigate the genetic relationship between longevity and starvation resistance in *D. melanogaster* using recombinant inbred lines that were recently derived from the field. The RILs employed were derived from heterozygous individuals taken from a natural population (Kopp et al., 2003). The following questions were of particular interest given that our RIL lines are relatively representative of natural genetic variation:

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

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

Results

Analysis of variance

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

correlation between longevity and starvation survival was positive and significant within sexes, but only marginally significant across sexes (table 1).

Table 1. Pearson correlation coefficients (*r*, below the diagonal) and probability (*P*, above the diagonal) that the absolute value of *r* is greater than zero for female and male survival with (lifespan = "Life") and without (starvation condition = "Starve") food present

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

QTL analyses

Single marker analysis

Table 2 presents probabilities and genetic variance components accounted for by the strongly supported markers that are bolded when they exceed the whole genome permutation threshold. Across the centromere of chromosome 2 (approximately corresponding to cytological positions 38A–41F as described, Kopp et al. , 2003), there are markers that are associated with longevity and starvation survival (table 2, 38A and 41CE). A nearby region of the genome (table 2, 42D), has a strongly supported QTL longevity as well as starvation survival. Other markers that exceed permutation threshold in relationship to male longevity and female survival under starvation conditions are listed in table 2. Other regions (48A and 48D) are associated with male and female longevity, but not starvation resistance of either sex. Importantly, alleles of the most significant QTLs account for approximately half genetic variation in both traits.

Table 2. Single marker analysis for female and male survival traits. The proportion of variance accounted by marker segregation is shown for each sex and condition corresponding to the cytogenetic region of the marker (for example, 38C). The probability (*P*) reported is that marker segregation is not associated with trait segregation. The values in bold type indicate marker effects whose probability is less than the permutation threshold (life male = 0.000461, life female = 0.000489, starve male = 0.000523, starve female = 0.000468). "Life" refers to survival when food is present and "Starve" refers to survival when no food is present.

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

Composite interval mapping

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

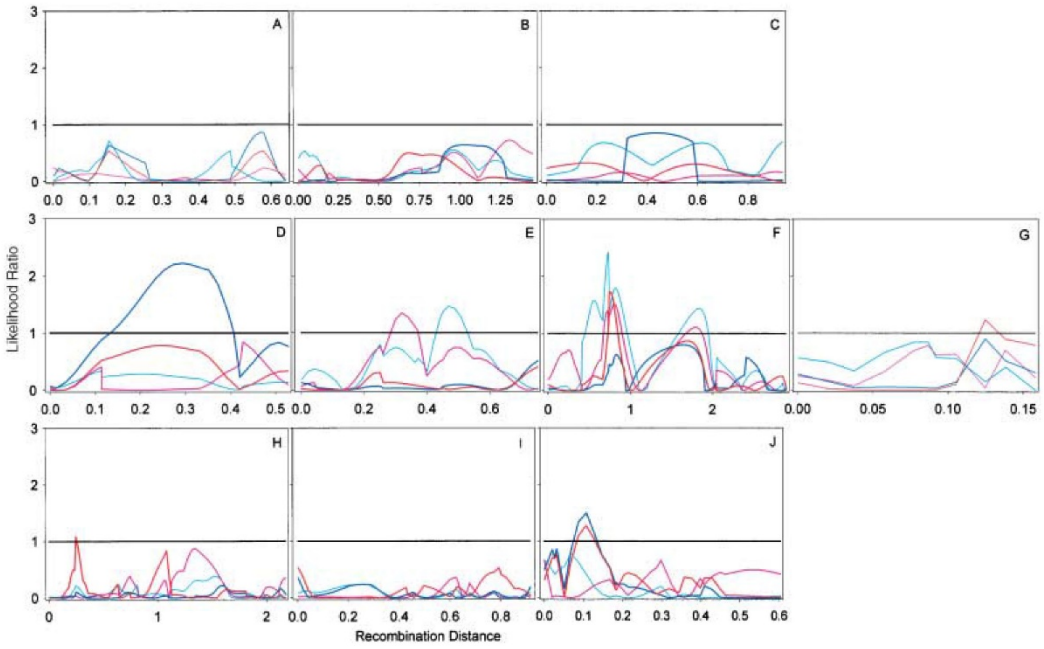

Figure 1. The QTL likelihoods for starvation of males (cyan) and females (pink), and the lifespan of males (blue) and females (red) on X chromosome panels (A–C), second chromosome panels (D–G) and third chromosome panels (H–J). The ordinate of each panel shows the strength of support for QTL regions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

Table 3. QTL effects on starvation survival and lifespan. QTL effects are indicated by positive or negative magnitude of table values and statistically significant effects are given in bold type. In the table, chromosome is followed by complementation group (chromosome-complementation), which is one of the four linkage groups in the starting population used to produce the RILs. "Life" refers to mean longevity and "Starve" refers to mean survival time when no food is present.

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

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

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

Discussion

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

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

Lifespan QTLs have previously been mapped in *D. melanogaster*. To form 98 RILs, Nuzhdin et al. (1997) crossed two homozygous laboratory strains with one allele at a variable site contributed by each of the parental strains. Using these RILs, five quantitative trait loci for longevity were identified for virgin flies. The genetic markers that comprise the QTLs account for approximately 50% of the genetic variation among the lines. The QTLs were sex-specific, in accord with the low level of genetic correlation between female and male longevity among the lines. However, the effects of two of the QTLs were in the same direction, positive or negative, in males and females. In this regard, Reiwitch & Nuzhdin (2002) and Curtsinger (2002) have pointed out that some of the sexual dimorphism attributed to QTLs might be due to limited statistical power. In Nuzhdin et al. (1997), one QTL was associated with a significant positive effect on males and a nonsignificant positive effect on females. This QTL was located in map position interval 38A to 44C, a region that encompasses the QTL for female and male longevity in the present study (38CD–41F).

QTLs for longevity and starvation survival have been identified using the set of 98 RILs (Vieira et al., 2000). In this study, starvation survival was investigated as well as longevity at low and high temperature, longevity after heat shock and longevity at the control environment. The genetic correlation between the starvation environment and other environments was not significantly different from zero, but it typically was negative. For example,

and of most relevance to the present study, the genetic correlation between starvation survival and longevity in the control environment was 0.021. There were multiple significant QTLs for starvation survival that were not significantly different from zero in terms of correlations with QTLs for longevity in the control environment. It might be noteworthy to mention that for one QTL (map region 38A–48D) starvation survival was positively associated with female longevity in the control environment but negatively associated with male longevity. This cytogenetic region includes significant QTL regions in the present study corresponding to starvation survival and survival with food present. Deficiency mapping in the 32F–44E map region of chromosome 2 delineated a relatively fine-scale longevity QTL for males in the 38–39 map region with evidence for line effects on male longevity and line-by-genotype interaction for both sexes (Pasyukova et al., 2000).

Results from the aforementioned studies (Nuzhdin et al., 1997; Vieira et al., 2000) overlap to variable degrees with subsequent QTL studies on *D. melanogaster* lifespan. Substantial differences are associated with the mating status of flies used for assays and the origin of the RILs employed. Using the panel of 98 RILs, longevity was assayed by holding males and females together in cages (Reiwitch & Nuzhdin, 2002). Lifespan declined in both sexes presumably due to courtship, mating, and increased gamete production. Generally, the location of longevity QTLs did not change, with the noteworthy exception of the loss of the lifespan QTL in the region 38E–43A. However, the sexual dimorphism characteristic of earlier studies was not observed in Reiwitch & Nuzhdin (2002). Similarly, results from a set of 120 RILs, derived from a longevity selection experiment, indicated an absence of sexual dimorphism associated with lifespan QTLs (Curtsinger & Khazaeli, 2002). In this study lifespan was tested with females and males housed together, and most of the genetic variation for longevity was found on the third chromosome. In contrast to the negative association between longevity and other traits reported in Vieira et al. (2000), the study by Curtsinger & Khazaeli (2002) observed a positive association between QTLs for longevity, midlife fertility and resistance to oxidative stress. A study of age-specific mortality in the RILs used in the present study, based on large sample size and mixed sex cohorts, has been conducted and the data analyzed (Nuzhdin et al., unpublished data). There is substantial overlap with lifespan QTLs in the present study, regardless of the fact that the diet and other environmental conditions were different in the two studies.

To date, RILs have been derived from three sources: established laboratory stocks (Nuzhdin et al., 1997), lines selected for extended longevity in the laboratory (Curtsinger & Khazaeli, 2002) and lines inbred immediately after collection from natural populations (Kopp et al., 2003). Laboratory populations evolve shortened lifespan (Promislow & Tatar, 1998; Hoffmann et al., 2001) and thus inbred lines from laboratory-adapted populations could represent a pattern of genetic variation for life history and related traits that deviates considerably from natural populations. Lines from natural populations could include the effect of ecological conditions that maintain genetic variation with pleiotropic effects on lifespan. However realistic in concept, lines recently derived from natural populations have only been assessed in laboratory environments and it remains to be determined if the results extend to natural environments. The assay conditions may also affect the results of lifespan QTL studies. Temperature, larval density, and genetic background can affect the location of QTLs that affect lifespan of virgin males and females (Vieira et al., 2000; Leips

& Mackay, 2000). Mating status changed whether sexual dimorphism was observed for lifespan QTLs (Nuzhdin et al., 1997; Reiwitch & Nuzhdin, 2002). Studies on virgin flies indicate that QTLs have antagonistic pleiotropic effects on lifespan in different environments (Vieira et al., 2000), whereas QTLs defined using mated flies exhibit positive genetic covariation between longevity and stress resistance or fecundity traits as shown in Curtsinger & Khazaeli (2002) and the present study.

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

Multiple stress resistance is an issue of considerable general interest in relationship to longevity. One long-lived mutant of *D. melanogaster* has been shown to be resistant to multiple forms of environmental stress (Lin et al., 1998), whereas a different long-lived mutation in an insulin signaling pathway gene was resistant to starvation but not to oxidative stress (Clancy et al., 2001). An extended question for the set of RILs used in the present study is whether there are QTLs that confer resistance to more than one environmental stress and whether such regions of the genome are associated with longevity in the absence of environmental stress. Starvation resistance might be especially salient because it presumably evolved from food scarcity, which might have been the selective agent producing a conserved mechanism that mediates aging and lifespan. In any case, QTL studies ultimately have the goal of identifying genes that are polymorphic for alleles that control genetic variation in populations (DeLuca et al., 2003) and this is also our goal with respect to the QTLs for extended starvation resistance and longevity.

Experimental procedures

Recombinant inbred lines (RILs) and genotypes

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

As markers, we have used positions of transposable elements that are highly heterogeneous in natural populations (see Charlesworth & Langley, 1989, for a review). Positions of the *roo* elements were determined by *in situ* hybridization to polytene salivary gland chromosomes, using a biotinylated DNA probe (Shrimpton et al., 1986). For use as probe, a plasmid carrying a full-length *roo* element was labeled with bio-7-dATP (BRL) by nick translation. Hybridization was detected using the Elite Vectastain ABC kit (Vector Laboratories) and visualized with diaminobenzidine. Element locations were determined from the images at the level of cytological bands on the standard Bridges' map of *D. melanogaster* polytene chromosomes (Lefevre, 1976). We genotyped five individuals per line. The marker was recorded as present if detected in all larvae, as absent if not detected in any larva, and as segregating otherwise. On the four homologous chromosomes per autosome, and similarly on the X chromosome, initial linkage of markers was inferred from residual linkage disequilibrium in the population of RILs (Proc CORR, SAS Institute, 1988). In some cases, *roo* elements either were uninformative (present in more than one complementation group) or gave a hybridization signal that was too weak to be scored reliably. Such elements were dropped from further analysis, leaving 152 markers that segregated between parental chromosomes (see fig. 2 in Kopp et al., 2003). Given that the genome size of *D. melanogaster* is approximately 170 Mb, the average density of markers is approximately 0.9 per Mb, but as indicated in Kopp et al. (2003), the markers are not evenly distributed. All chromosomes were homosequential, with the exception of an inversion on 3R that contains ~7% of the physical genome (approximately 89EF–96A). This inversion was not associated with either starvation or lifespan in either males or females (P = 0.69, 0.30, 0.78, 0.70) and is not discussed further.

Phenotypic measurements

Lifespan

For each measurement of lifespan, all RILs were expanded for one generation at a density of ten pairs of flies per vial and six vials per line to obtain a sufficient number of individuals for testing. In the following generation, ten pairs of flies were placed in each of ten vials per line for 3 days. Virgin males and females were collected at 8-h intervals over a 24-h period, 9 days after the parents were discarded. The following day, 25 males and females

per RIL were placed in population cages made from 500-mL plastic Dixie Cups with attached glass vials containing 8 mL of cornmeal-agar-molasses medium plus yeast. Dead flies were removed by aspiration and deaths recorded at 2-day intervals. The food was changed every fourth day.

Starvation resistance

For each measurement of survival under starvation conditions (no food present), all RILs were expanded in one generation using a low density of females and males in each vial. Adults emerging from these vials were used for egg production; approximately 75 eggs were transferred to each of three vials per RIL. A vial contained 8–10 mL of cornmeal-agar-molasses medium plus yeast. For the starvation assay, males and females emerging in the egg transfer vials were collected within a 24-h period for most lines, but some lines required another day to accumulate the target number of flies. Fifteen males and females were held together in vials for 3 days and then transferred to fresh food and this procedure was repeated a total of three times. Thereafter, females and males were separated into single sex vials for 2 days based on the expectation that this was enough time for the flies to have completely recovered from the brief exposure to ether used to separate them. First, this pre-assay fly housing regime allowed us to emulate the conditions of the mixed sex cohort used to assess longevity. It also allowed us to record the time interval of death of males and females in separate vials, which would have been difficult if the sexes had not been previously sorted into separate vials for starvation. Ten females or the same number of males were transferred to empty vials with a water-saturated fiber plug. Replicate vials were assayed for each sex for each line. Every 8 h, mortality was recorded in each vial until all flies died. Recording mortality every 8 h has been shown to be sufficient to differentiate populations that exhibit relatively small differences in starvation survival time (Harshman & Schmid, 1998). Both experiments were fully replicated twice.

Analysis of variance

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

Single marker analysis (SMA)

For each of the marker loci, the significance of the difference in the mean trait values was tested between two groups of lines, one with the marker allele containing the *roo* insert and the other with the marker allele having no *roo* insert (GLM procedure). An empirical distribution of the test statistics under the null hypothesis of no association between any of the markers and trait values was obtained by randomly permuting the trait data 1000 times and calculating the most significant probability for marker-trait associations across all markers for each permutation. Statistics from the original data that were exceeded fewer

than 50 times by those from the permutation are significant at $P = 0.05$ (Doerge & Churchill, 1996). The contribution of the QTL in the overall genetic variance was estimated with the VARCOMP procedure with the marker effect as random.

Composite interval mapping (CIM)

The standard QTL mapping software was not designed for the type of RILs used in the present study. These software packages fit a single difference in effects of alleles fixed between two isogenic parental stocks, which segregate among RILs. However, the standard mapping software has been adapted to the RILs used in the present study. For that, we test whether an allele from one homologous chromosome codes a trait value significantly different from the average trait due to combined effects of other alleles: two for the X or three for the second chromosomes. Except the chromosome tips, two of the parental third chromosomes appear identical (Kopp et al., 2003), and thus we modeled only three parental third chromosomes. For each of the parental chromosomes, we generate a separate likelihood profile: three for the X, four for the second, and three for the third chromosome. Note that the number of different allelic effects we can independently estimate is equal to the number of segregating chromosomes minus 1. With the above logic, likelihood profiles were generated with the CIM procedure in QTL Cartographer (Basten et al., 1994, 1999). QTL effects (table 3) were expressed as additive effect (a) on log transformed data, as estimated by QTL Cartographer software. Recombination distances between markers were reconstructed as described in detail elsewhere (Mezey, unpublished data). Note that recombination distances reflect history of recombination events during derivation of RILs, and thus for some chromosomes they are contracted and for others expanded in comparison with the standard *D. melanogaster* recombination map (fig. 1). The options were two (number of background markers), 30 (window size in cM), and two (Kosambi mapping function). Every parental chromosome has its own set of positions of *roo* markers. Only those were followed in the CIM analyses for a given chromosome; the other markers were disregarded. The significance of a QTL was tested with 1000 permutations for each trait and chromosome. Significance thresholds—likelihood ratios corresponding to 5% of the highest values obtained on permuted data sets—substantially varied (from 6.49 to 27.23), perhaps due to uneven sample sizes for different marker alleles. For convenience of comprehension, we scaled experimental likelihoods by permutation thresholds. These likelihood ratios show significant QTL when in excess of unity. Note that we performed ten tests for four traits, or 40 analyses in total. By chance alone, we expect to detect two QTLs, whereas in fact we detected 11 (fig. 1). We might have further adjusted significance thresholds for multiple tests to verify significance of every individual QTL. This, however, would substantially increase the type 2 error. As our composite interval mapping is exploratory, and intended to generate a list of candidate regions for the follow-up genetic analyses, we chose to make no such adjustment.

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