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GENOMIC BASIS OF AGING AND LIFE-HISTORY EVOLUTION IN *DROSOPHILA MELANOGASTER*

Silvia C. Remolina,[1,2] Peter L. Chang,[2,3] Jeff Leips,[4] Sergey V. Nuzhdin,[3] and Kimberly A. Hughes[5,6]

[1]*Department of Animal Biology, University of Illinois, Urbana-Champaign, 505 S. Goodwin Ave, Urbana, Illinois 61801*

[2]*These authors contributed equally to this work*

[3]*Section of Molecular and Computational Biology, Department of Biological Sciences, University of Southern California, 1050 Childs Way, Los Angeles, California 90089*

[4]*Department of Biological Sciences, University of Maryland Baltimore County, 1000 Hilltop Circle, Baltimore, Maryland 21250*

[5]*Department of Biological Science, Florida State University, Tallahassee, Florida 32306-4295*

[6]*E-mail: kahughes@bio.fsu.edu*

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Natural diversity in aging and other life-history patterns is a hallmark of organismal variation. Related species, populations, and individuals within populations show genetically based variation in life span and other aspects of age-related performance. Population differences are especially informative because these differences can be large relative to within-population variation and because they occur in organisms with otherwise similar genomes. We used experimental evolution to produce populations divergent for life span and late-age fertility and then used deep genome sequencing to detect sequence variants with nucleotide-level resolution. Several genes and genome regions showed strong signatures of selection, and the same regions were implicated in independent comparisons, suggesting that the same alleles were selected in replicate lines. Genes related to oogenesis, immunity, and protein degradation were implicated as important modifiers of late-life performance. Expression profiling and functional annotation narrowed the list of strong candidate genes to 38, most of which are novel candidates for regulating aging. Life span and early age fecundity were negatively correlated among populations; therefore, the alleles we identified also are candidate regulators of a major life-history trade-off. More generally, we argue that hitchhiking mapping can be a powerful tool for uncovering the molecular bases of quantitative genetic variation.

KEY WORDS: **Fecundity, pleiotropy, quantitative genetics, selection—experimental, senescence, trade-offs.**

Eukaryotes exhibit enormous diversity in patterns of aging and other life-history traits (Roff 2002). Even closely related populations of the same species can differ dramatically in life span, age-specific reproductive rate, and age-related decline in health (Austad 2005; Ricklefs 2010). These differences can be particularly informative for investigating the genetic basis of variation because they can be large relative to within-population differences, but they occur within organisms that possess similar genetic backgrounds and inhabit similar environments. Moreover, population divergence can be generated in organisms sharing a common environment by application of selective breeding, a technique known as experimental evolution (Rose 1984; Lenski et al. 1991; Partridge and Fowler 1992).

Experimental evolution has been used to investigate aging and other life-history phenotypes, beginning with the seminal work of Rose and Charlesworth (1980, 1981). A general pattern

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emerging from these experiments is that increased selection on late-life performance results in increased life span and late-age reproduction and that this increase is often accompanied by decreased early fecundity (Rose and Charlesworth 1980; Luckinbill et al. 1984; Rose 1984; Service et al. 1988). This pattern is interpreted as evidence for the involvement of alleles with antagonistic pleiotropic effects in the evolution of aging. Antagonistic effects of individual alleles are also believed to underlie other life-history trade-offs that are observed both within and between species (Roff 2002). The existence of trade-offs between different components of fitness is a cornerstone of evolutionary theory, yet the molecular basis of these trade-offs is not understood (Leroi et al. 2005; Hughes 2010b).

One approach to uncovering the genetic and genomic bases of phenotypic divergence is to use experimental evolution and apply deep whole-genome sequencing to uncover the genomic changes that accompany evolutionary change (Barrick et al. 2009; Burke et al. 2010; Turner et al. 2011). This approach has become feasible only recently with the advent of highly parallel sequencing technologies and well-annotated reference genome sequences for many different organisms. In multicellular organisms, only body-size and maturation-rate evolution have been investigated in this way (Burke et al. 2010; Turner et al. 2011). We have extended this approach by (1) combining deep whole-genome sequencing with extensive gene-expression profiling to characterize functional variation, (2) developing new analytical methods to identify the location and frequency of causal genetic variants, and (3) focusing on aging and age-specific fertility in a system that demonstrates a classic life-history trade-off.

In our experiment, life-history differences between populations were produced by the response to 50 generations of selection on age at first reproduction in *Drosophila melanogaster*. By selectively breeding only from flies that survived and were fertile at old (or at relatively young) age, we imposed selection on both late-age fertility and life span. Genes and pathways that contributed to the response to selection under these conditions are therefore candidates for modifying aging and fertility in wild-type noninbred organisms. Conducting three independent selection experiments allowed us to determine the extent to which replicates were alike or different in genetic changes underlying life-history evolution.

Methods

SELECTED POPULATIONS

We applied selection on survival and late-age reproduction in replicated, paired selection (S) and control (C) populations of *D. melanogaster*. The ancestral population was derived from ~8000 offspring of 400 wild-caught females collected in New Jersey in 1998. This population was maintained in the laboratory at large population size (>1000) until 2004, when we obtained >1600 individuals from Dr. Alan Gibbs. We then maintained the population with overlapping generations (flies transferred to new food every 14 days, but without clearing of parental flies) until the beginning of the selection experiment in late 2005. During that period, we maintained the population in vials (100 vials, >25 flies per vial) and on media identical to those used during the selection experiment.

We established three independent S-C pairs by taking three independent samples of founders from the ancestral population. Offspring of 320 single-pair matings were used to establish both the S and the C population within a replicate. A different set of 320 pairs was used to establish each S-C pair, such that each pair represented an independent sample of the genetic variation existing within the progenitor population. After the initial generation, the S and C lines of a pair were maintained independently for the remainder of the experiment. Each generation, S and C populations were propagated from 220 to 320 single-pair matings. Parents were cleared after 7 days, but we collected offspring on days 10–11, to ensure that they were derived from eggs laid early in the mating stage. Equal numbers of offspring were collected from each pair and allowed to age in single-sex vials until 14 days of age for C lines or N days of age for S lines (at the beginning of the experiment $N = 28$; N was gradually increased to 40 as flies evolved longer reproductive life spans). At the appropriate age, flies were paired with other surviving individuals from their population, but from a different family, to produce single-pair matings for the next generation. That is, in S populations, only flies that survived and were fertile at >N days of age contributed to the next generation; in C populations, flies that survived and were fertile at 14 days contributed to the next generation. We chose reproduction at 14 days for C populations to mimic average age at reproduction in the overlapping-generation rearing protocol for the ancestral population and to avoid artifactual selection for too-early reproduction (Linnen et al. 2001). Approximately 50% of individuals in S populations survived and contributed offspring to the next generation. We avoided differential selection on development time by collecting newly emerged adults within a 24-h period beginning 10 days after initiation of egg laying. In early 2009 (~ generation 35), the single-pair mating strategy was changed to three males and three females per vial to increase productivity of S lines. Because of the different generation times, S and C lines were in the experiment for different numbers of generations. The genomic and phenotypic data reported here are based on the 50th generation of selection in the S lines (about 80 generations of selection in the C lines). S- and C-line generations were not perfectly synchronized, but phenotypic assays of the S and C lines within a pair were begun within 7 days of each other.

The selection scheme was chosen to minimize directional selection in C lines relative to the laboratory conditions under

which the founding population had been maintained, but we do not claim that no selection was imposed on C lines during laboratory evolution in this experiment. Our goal was to impose differential selection on age at first reproduction in S and C lines and then to identify genomic regions that diverged during the period of differential selection.

PHENOTYPE ASSAYS

Life span

We collected 900 virgin female and male flies from each C and S line using light CO_2 anesthesia. Virgin vials (100 each sex) were initiated with six flies; mated vials (100) were initiated with three males and three females. Flies were kept at 25°C on a 12L:12D light cycle and transferred to fresh cornmeal medium every week. Number of flies remaining and any escapees were recorded at each transfer. Log-rank tests were used to determine differences in survival.

Age-specific fecundity

We measured offspring production at 7, 14, 35, 44, and 60 days of age, using 50 vials of three virgin females and three virgin males of the appropriate age for each population-by-age combination. Adult flies were allowed to mate for 24 h and then discarded. Any females that died or escaped before the end of the mating period were recorded, and all offspring eclosing within 16 days after egg laying were counted. We used a generalized linear mixed model with negative binomial distribution and log link function to test for effects of selection regime, age, and regime-by-age interaction. Replicate within regimes was treated as a random effect.

SEQUENCING

To evaluate effects of selection, we prepared genomic DNA from 100 female flies for each of the six populations into six libraries (one per population) and sequenced them as paired-ended 74-bp reads on an Illumina GAII. Each library was individually barcoded and used to create a pooled sample of barcoded DNA. Use of an aliquot of the pooled sample in each of six lanes of the GAII minimized lane effects. This process generated 514 million reads and 3.8×10^{10} bp of sequence. Sequenced reads were aligned as pairs, with BWA 0.5.7 (Li and Durbin 2009), to the complete *D. melanogaster* 5.29 genome build downloaded from Flybase. Reads were allowed up to six mismatches throughout the 74 bp per end, and unique reads were mapped to the genome. All other BWA alignment parameters were set to default values. Approximately 80% of the sequencing reads mapped uniquely and with high stringency to the Flybase 5.29 genome build, resulting in ~244× coverage of 126 Mb of the *D. melanogaster* genome (95% of euchromatic and 52% of heterochromatic DNA). Median coverage ranged from 40 to 50× across all six population samples. Sequence data are archived at the NCBI Short-Read Archive under accession number SRA038471/SRP007248.

ALLELE FREQUENCY DIFFERENCES

Initially, reads from all six populations were pooled to identify multiallelic sites across the genome. For this comparison, 2,106,064 positions were considered, all having at least two alleles observed in the pooled data with each allele called at least five times with frequency of at least 5% in the pooled data. To identify genomic positions with differences in allele frequencies at these sites, we used a Fisher exact test to compare the counts of all base calls at each position between S and C populations (either as independent replicate pairs or pooled). We applied a Bonferroni cutoff of 0.05 to call allele differences significant.

SIGNATURES OF SELECTIVE DIVERGENCE

We compared the sequences of selection and control lines to detect genome regions that had differentiated significantly. Differences in allele frequency between S and C populations could arise from selection, from the stochastic effects of genetic drift, or from binomial sampling error. To detect regions where divergence was more probably driven by selection in S populations, we scaled the heterozygosity in S populations

$$H_S = \left(1 - \sum_i p_i^2\right), \text{ where } p_i \text{ is the frequency of the } i\text{th allele}$$

a measure of allelic variation, by the allelic divergence between S and C populations (D), where D was calculated as the average number of pairwise differences between two populations. This statistic is analogous to the HKA test of deviation from neutral evolution (Hudson et al. 1987); it is expected to be small in genome regions that experienced directional selection on a sequence variant in the S but not the C population. Selection on a sequence variant should carry the variant (along with flanking genome regions) to high frequency, increasing D and decreasing Hs in a "selective sweep." H_S/D is therefore expected to be small in regions that experienced a sweep in S populations. Although we designed the experiment to minimize evolution of C lines during the selection phase, this method of evaluating divergence of allele frequencies between S and C populations does not depend on any assumptions about selection or neutrality in the C populations.

To identify genomic signatures of selection, we calculated expected H_S/D for all nonoverlapping 1-kb windows. To calculate likelihood scores for each statistic, we summed individual scores across 100 1-kb regions and across all three replicate pairs and used the empirical genome-wide distribution of this statistic to calculate the probability of observing more extreme values by

chance across 100-kb windows using equation 1:

$$LL = \max\left\{-\sum_{i=1}^{100}\sum_{j=1}^{3}\log_{10}P(h \le H_{ij}|F), -\sum_{i=1}^{100}\sum_{j=1}^{3}\log_{10}P(h \ge H_{ij}|F)\right\}, \quad (1)$$

where F is the empirical genome-wide distribution of the appropriate statistic h and $\mathbf{H} = (H_{1j}, H_{2j}, \ldots, H_{100j})$ are the 100 individual 1-kb measure of h within a 100-kb window for the jth replicate. Because the overlapping 100-kb regions were not independent, we used permutations to identify a significance level. Bootstrap resampling of 100 random 1-kb regions for each replicate indicated that between 2.5% and 4.6% of samples had scores that exceeded 100. We chose this cutoff of 100 and assumed this false positive rate. After identifying 100-kb regions with scores greater than 100 for each statistic, we collapsed overlapping regions.

Although the H_S/D statistic is sensitive to classic selective sweeps, it is not sensitive to "incomplete sweeps," in which selection in S lines favored an allele that was initially at low frequency and carried it to intermediate frequency during selection (Przeworski et al. 2005). Under this scenario, genome regions with high H_S would also exhibit increases in D. To identify these incomplete sweeps, we therefore scaled H_S (expected to be high) by H_C (expected to be low), using a likelihood calculation equivalent to that in equation 1.

We also assessed divergence among replicate C lines and among replicate S lines. No genomic regions had log likelihood (LL) > 17 in any of these comparisons (results not shown). We therefore restrict our attention here to differentiation between the two selection regimes.

GENE EXPRESSION

To identify genes within sweep regions that also diverged in gene expression, we evaluated mRNA abundance using microarrays. Comprehensive analysis of genome-wide expression patterns will be described in a future publication. We extracted RNA from two independent groups of female flies for each population/age/tissue combination (six populations, five ages, two tissues for a total of 120 samples). Each replicate consisted of tissue from seven individual flies. We used the PicoPure RNA isolation kit (Molecular Devices, Sunnyvale, CA); RNA was amplified with Ambion's MessageAmpII aRNA Amplification Kit (Ambion, Austin, TX) and reverse transcribed to cDNA by the SuperScript III protocol (Invitrogen Life Technologies, Grand Island, NY). Samples were labeled by means of Roche-Nimblegen's One Color DNA Labeling Kit (Madison, WI), and cDNA from each replicate was hybridized to its own partition of Roche-Nimblegen's *D. melanogaster* 12 × 135K expression arrays. Each array consisted of 12 partitions, and each partition contained 16,637 target genes with eight probes per gene (DM 5.7 genome build). Arrays were scanned with a GenePix 4000B Scanner and Roche NimbleScan software.

For analysis, we used GCRMA-normalized expression values as the dependent variable in gene-specific mixed models (Chu et al. 2002) implemented in SAS Proc Mixed version 9.2. Selection regime, age, and selection-by-age interaction were fixed effects; population within selection regime was treated as the level of independent replication. Biological replication within population-age-tissue combinations was treated as a random effect nested within population-age-tissue to account for the expected correlation between samples drawn from the same population. Chip (array) was also treated as a random blocking effect. Except as noted, we applied a false-discovery-rate cutoff of 0.05 to call effects significant (Benjamini and Hochberg 1995). Gene enrichment analysis was conducted with DAVID (Dennis et al. 2003), and statistical tests of enrichment were performed within FlyMine (Lyne et al. 2007). We determined over- and underrepresentation of ontology terms by comparing the number of genes falling within each category to the number of transcripts represented on the Nimblegen *Drosophila* array that fall into that category. FlyMine uses a hypergeometric test to determine whether an ontology category is significantly overrepresented in a list of differentially expressed genes. Ages at which gene expression was measured did not correspond exactly to ages at which fecundity was measured because we wished to capture gene expression in newly eclosed (prereproductive) flies and because budgetary constraints prevented us from assaying expression at more ages. For logistical reasons, the tissue used in expression analysis was extracted from a different generation than the tissue used for DNA analysis. Expression data was based on flies from generation 40 of selection. Microarray data are archived at NCBI Gene Expression Omnibus under accession number GSE38106.

Results

PHENOTYPIC RESPONSE TO SELECTION

Figure 1 illustrates the results of 50 generations of selection in the S populations. In each replicate, S flies had longer virgin and mated life spans (all log-rank tests $P < 0.005$; mean over all replicates ± standard error: S virgin females = 69.0 ± 0.4 days, C virgin females = 60.5 ± 0.4, S virgin males = 72.0 ± 0.5, C virgin males = 62.2 ± 0.4;) and longer mated life spans (S mated females = 34.1 ± 0.5; C mated females = 29.9 ± 0.4, S mated males = 44.2 ± 0.6; C mated males = 40.7 ± 0.7). A generalized linear model fit to fecundity data collected from five adult ages (7, 14, 35, 44, and 60 days after eclosion) indicated highly significant differences between selection regimes ($\chi^2 = 15.5$, $P < 0.0001$)

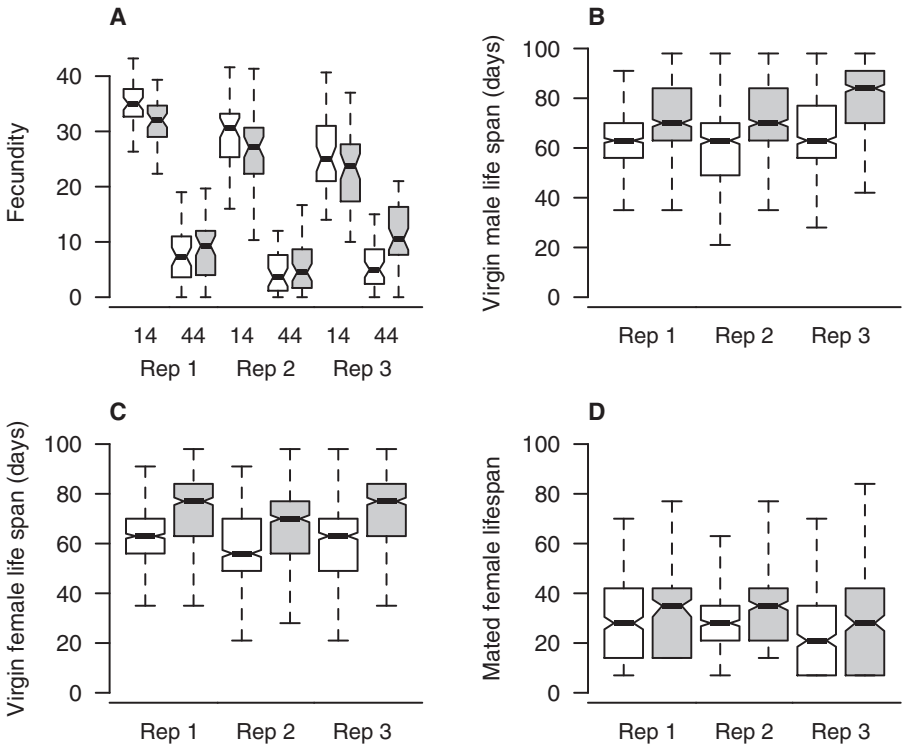

Figure 1. Differences between selected (S, filled symbols) and control (C, open symbols) populations of *Drosophila melanogaster* in age-specific fecundity and life span in days. Boxes show median and upper and lower quartiles; notches indicate ± 1.58 interquartile range/$\sqrt{n}$ and approximate 95% confidence intervals; whiskers are 2× upper and lower quartiles. (A) Offspring number at 14 and 44 days of age in three replicate S and C populations. (B–D) Age at death for virgin males (B), virgin females (C), and mated females (D) in replicate populations.

and ages ($\chi^2 = 1619.2$, $P < 0.0001$) and a highly significant interaction between selection regime and age (i.e., a difference in how S and C fecundity changed with age, $\chi^2 = 48.9$, $P < 0.0001$). Post-hoc tests indicated that S lines had higher fecundity at 44 days after eclosion (S = 8.6 ± 0.4, C = 6.1 ± 0.4, $P < 0.005$) and lower fecundity at 14 days after eclosion (S = 26.9 ± 0.7, C = 30.0 ± 0.8, $P < 0.01$). Means and standard errors for the complete fecundity data are shown in Table 1. These phenotypic results parallel those seen in other studies of selection on late-age reproduction (Rose and Charlesworth 1980; Rose 1984; Partridge et al. 1999), but see Partridge and Fowler (1992).

GENETIC DIVERGENCE AND TESTS FOR SELECTIVE SWEEPS

Figure 2A shows H_S/D calculated at 1-kb intervals across the genome and illustrates seven genome regions where the statistic is significantly below background levels in a comparison of all S and C populations (and two regions significantly above background, see Table S1 for *LL* values for genes within these regions). Regions with low values of H_S/D were similar in this pooled comparison and in each of the three S-C population comparisons individually, suggesting that the same regions responded to selection in each replicate (Fig. S1). Four of these sweep regions are near centromeres (on X, 2L, and 3L). We do not know of any methodological biases that could lead to this pattern. These regions fall within chromosomal neighborhoods characterized by low recombination rates (Fiston-Lavier et al. 2010) and might therefore be subject to greater hitchhiking effects.

We also observed several additional genome regions with H_S significantly elevated relative to H_C, consistent with the pattern expected under an incomplete sweep. Six chromosomal regions had H_S/H_C values significantly higher than background levels (Fig. 2B, which also shows three regions with values significantly above background *LL* values, see Table S1). Again, regions of extreme values for this statistic were similar in each replicate S-C pair of populations (Fig. S1). These regions also exhibited significantly reduced frequency in S populations of the most common allele in C populations and high F_{ST} values (Figs. 2C, D, and S1). This pattern is similar to that reported in a recent study of response to selection on development rate in *D. melanogaster* (Burke et al. 2010). These incomplete-sweep areas occupy relatively broad chromosomal regions, as would be expected if the sweeping allele derived from only one or a few ancestral haplotypes and adjacent loci evolved by genetic hitchhiking (Hudson 1994).

Table 1. Least-square mean fecundity at each age for control (C) and selected (S) populations of *Drosophila melanogaster.* S populations were selected for survival and late-age reproduction.

	C populations				S populations			
Age	Mean	SE	Lower CI	Upper CI	Mean	SE	Lower CI	Upper CI
7	28.1	0.7	26.6	29.7	25.0	0.7	23.6	26.5
14	30.0	0.8	28.5	31.7	26.9	0.7	25.5	28.5
35	19.3	0.6	18.0	20.6	19.1	0.6	17.9	20.4
44	6.1	0.4	5.4	6.9	8.6	0.4	7.8	9.5
60	0.2	0.1	0.1	0.4	0.8	0.1	0.6	1.2

CI = confidence interval.

Table 2. Biological function categories overrepresented (≥ 1.8-fold enrichment) among genes implicated in selective sweeps.

Biological process	Gene Ontology ID	Number of genes	Fold enrichment	*P* value
Defense response to fungus	0050832	6	6.7	2.3×10^{-6}
Proteolysis	0008236	15	2.2	1.2×10^{-2}
Ovarian follicle-cell development	0030707	7	1.8	5.2×10^{-3}

CANDIDATE GENES WITHIN SWEEP REGIONS

We identified the loci most strongly implicated in sweeps as those with the highest likelihood scores for H_S/D or H_S/H_C within each candidate region (Table S1), using an *LL* difference ≥ 2 to indicate a meaningful difference in scores. Using this criterion, we identified 156 genes, ranging from 1 to 21 genes per region. These genes are enriched for Gene Ontology biological-function categories related to oogenesis, immune response, and proteolysis (Table 2). In an attempt to identify candidate causal nucleotides, we evaluated these 156 genes' changes in coding regions. Fifteen genes exhibited nonsynonymous changes (or start-codon changes) that were unique to either S or C samples (Table 3). Moreover, nine of these 15 genes (60%) have been implicated in aging, reproduction, immune response, or proteolysis in previous studies: *CG12004* (response to microbial infection; Tanji et al. 2006); *Doc3* (age-related motor impairment; Jones et al. 2009), *Fhos* (response to microbial infection and aging; Boutros et al. 2002; Lai et al. 2007), *fs(1)M3* (oogenesis; Degelmann et al. 1990; Cernilogar et al. 2001), *Paramyosin* (oviduct and ovary response to mating; Apidianakis et al. 2005; Kapelnikov et al. 2008), *Tequila* (response to aging, oxidative stress, and microbial infection; Zou et al. 2000; Munier et al. 2004; Lai et al. 2007), *Ten-a* (a direct target of *doublesex;* Luo et al. 2011), and *CG6041* and *CG6048* (serine-type proteases). Because significant differential expression was observed for only two of these nine genes (*Ten-a* and *Paramyosin*, see below), the nucleotide differences in the other 13 genes may encode structural-protein variation that contributes to effects on late-life health and performance.

EXPRESSION AND PHENOTYPIC EFFECTS OF GENES IMPLICATED IN SWEEPS

We next looked for variation in the expression of candidate genes that could indicate cis-regulatory evolution. Of the 156 genes most strongly implicated in sweeps, 25 were differentially expressed between S and C populations at false discovery rates < 0.10 (Table S2). We found these differentially expressed genes within three of the seven complete-sweep regions and five of six incomplete-sweep regions. The differentially expressed genes included three with functions related to reproduction (*Cct1*, *karst,* and *Ten-a*), two encoding antimicrobial peptides (*dro-2* and *dro-4*) and one encoding a peptidase (*Psa*). Cis-regulatory change in these genes is therefore implicated in extended life span and late-age fertility of the selected lines. Functional studies support this hypothesis for at least two genes. *Cct1* encodes a membrane phospholipid and the rate-limiting enzyme in phosphatidylcholine synthesis. Experimental up-regulation of this gene increases fly life span by 8% (Landis et al. 2003). Our expression data are consistent with that result: *Cct1* was up-regulated in S relative to C flies at all ages beyond the day of eclosion (Fig. S2A). The aminopeptidase *Psa* has also been associated with effects on age-related health and performance in flies and in mammals. In humans, the PSA protein can protect brain cells from degeneration caused by aggregations of abnormal tau proteins, which is thought to have a causative role in Alzheimer's, Parkinson's, and other age-related neurodegenerative disorders (Karsten et al. 2006). In flies, experimental up-regulation of *Psa* protects against tau-induced neurodegeneration (Karsten et al. 2006). In our experiment, *Psa* was up-regulated in S flies relative to C flies at all

Table 3. Coding region changes unique to S or C lines among genes with strongest evidence for selective sweep.

Gene	Arm	Position	Aging/fertility phenotype	Other biological process	Coding change	Nucleotide change[1]
CG3726	X	5835226			NS SNP: T/S	C: 99% A
						S: 56% A, 43% T
CG6041	X	5844122		Proteolysis	NS SNP: L/I	C: 100% C
						S: 59% C, 40% A
CG6048	X	5846792		Proteolysis	NS SNP: T/M	C: 100% C
						S: 55% C, 44% T
fs(1)M3	X	5854443	Oogenesis, eggshell component (Degelmann et al. 1990; Cernilogar et al. 2001)		NS SNP: P/S, C/F, D/N, T/S, D/E	C: 100% G
						S: 57% G, 42% A
Ten-a	X		Maternal pair-rule gene (Rakovitsky et al. 2007)	Embryonic sexual development (Luo et al. 2011)	Start codon gain (–203 bp from TSS) in S	C: 0% with start codon
						S: 100% with start codon
CG12004	3L	1569354	Response to microbial infection (Tanji et al. 2006)	Cell adhesion, embryo segmentation	Start codon gain (–188 bp from TSS) in S	C: 62% with start codon
						S: 38% with start codon
CG6511	3L	8717122			Nonsynonymous SNP: C/G	C: 99% T
						S: 54% T, 39% G
Prm	3L	8731922	Enriched in oviduct and ovary in response to mating, microbial infection (Apidianakis et al. 2005; Kapelnikov et al. 2008)		Nonsynonymous SNP: M/L	C: 99% G
						S: 58% G, 41% A
CG13306	3L	8742671		tRNA metabolism, translation	Start codon lost in S	C: 100% with stop codon S: 59% with stop codon

Continued

Table 3. Continued.

Gene	Arm	Position	Aging/fertility phenotype	Other biological process	Coding change	Nucleotide change[1]
Fhos	3L	8754302	Up-regulated under microbial infection, down-regulated with age (Boutros et al. 2002)		Deletion with frameshift in C exon 2	C: 100% without frame-shift deletion
						S: 60% without frame-shift deletion
CG34426	3L	8795823			Nonsynonymous SNP: G/R, S/T, N/K	C: 100% G
						S: 61% G, 38% C
Doc3	3L	8998395	Gain-of-function mutation causes delay of age-related motor impairment (Jones et al. 2009)	Actin cytoskeleton organization	Nonsynonymous SNP: M/L	C: 100% A
						S: 57% A, 42% T
CG5194	3L	9002035		Polysaccharide metabolism	Nonsynonymous SNP: V/A, K/E	C: 99% A
						S: 59% A, 40% G
Doc1	3L	9034562		Heart development	Nonsynonymous SNP: M/L	C: 100% G
						S: 56% G, 43% T
Tequila	3L	9067903	Up-regulated with age and microbial infection, down-regulated with oxidative stress (Zou et al. 2000; Munier et al. 2004; Lai et al. 2007)	Long-term memory	Nonsynonymous SNP: L/I, M/L, N/D	C: 100% C
						S: 60% C, 39% A

[1]All these genes are in "incomplete sweep" regions.

Amino acid changes: C/S. NS = nonsynonymous; TSS = transcription start site.

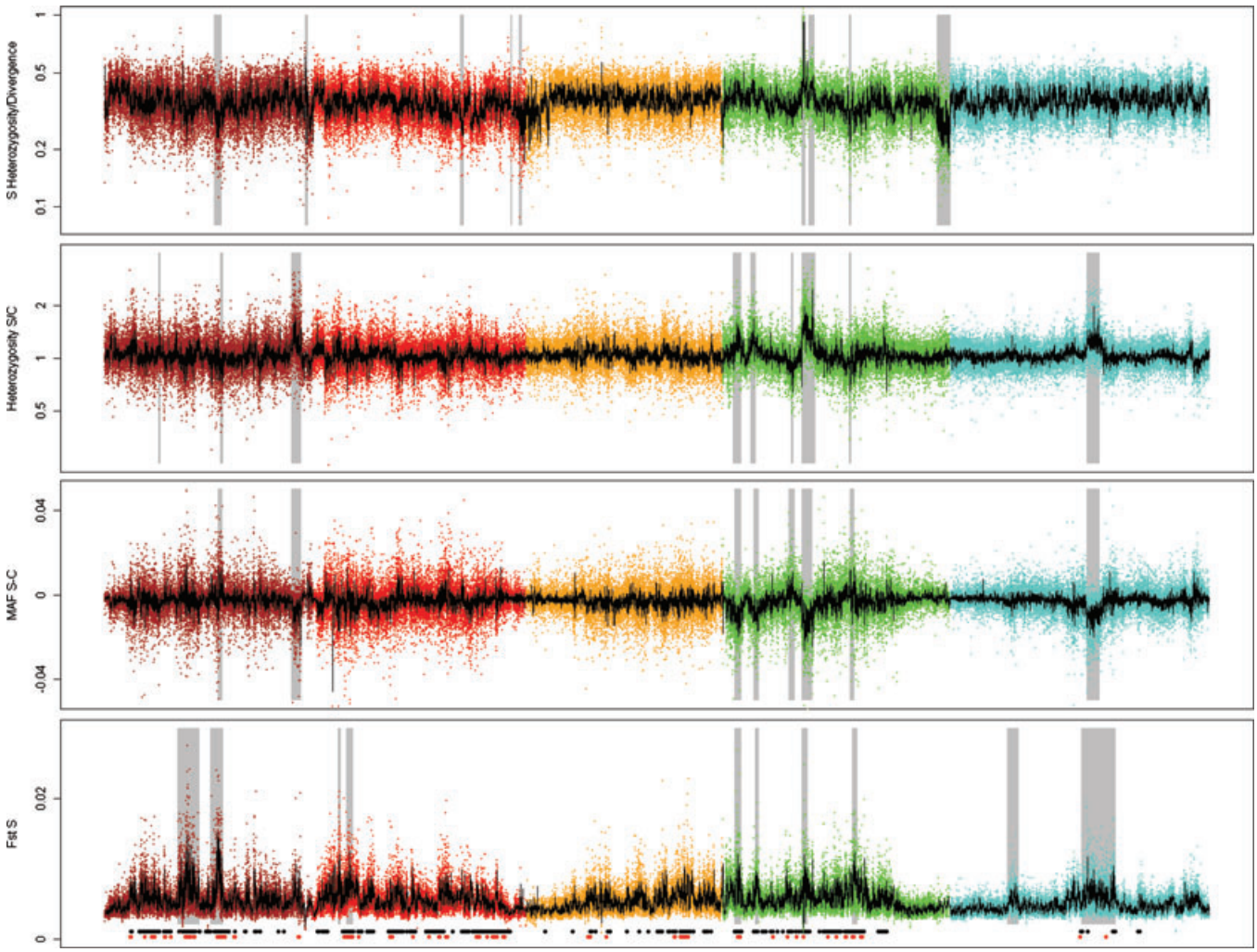

Figure 2. Signatures of selection. The horizontal axis is chromosomal position: X chromosome, maroon; 2L, red; 2R, yellow; 3L, green; 3R, blue. Colored dots and black lines in each panel represent the statistic average over 1 kb and 10 kb, respectively, for pooled S and C populations. Gray rectangles are centered on regions where log likelihood > 100 and extend in both directions until log likelihood < 50. Black and red dots at the bottom of the figure represent, respectively, 899 and 115 genes in which nucleotides in the S and C populations differ in frequency, identified with a Fisher's exact test or through D scores >0.8. (A) Expected heterozygosity in S populations divided by S-C divergence; the seven regions within gray bars that are characterized by low values of H_S/H_D (but not the two regions with high values) are interpreted as regions of near-complete selective sweeps. (B) Ratio of expected heterozygosity of S and C populations; the seven regions within gray bars that are characterized by high values of H_S/H_C (but not the two regions with low values) are interpreted as regions of incomplete sweeps. (C) Difference between S and C populations in the frequency of the most common allele in C populations. (D) F_{ST} between S and C populations.

ages except 50 days (Fig. S2B), suggesting that S flies would be less susceptible to tau-induced neural defects.

Discussion

NATURAL GENETIC VARIATION UNDERLYING LIFE-HISTORY EVOLUTION AND LIFE-HISTORY TRADE-OFFS

Using experimental evolution, high-throughput whole-genome resequencing, and transcriptome profiling, we found 156 genes that responded significantly to selection on improved late-life performance. By focusing on genes with strong signatures of selection and either (1) changes in nonsynonymous sites or (2) changes in gene expression, we constructed a narrower list of 38 candidate genes demonstrating structural and regulatory variation. Moreover, 14 of these genes (37%) have demonstrated effects on functions related to reproduction and aging (eight from the nonsynonymous change list, five from the differential expression list, and one gene, *Ten-a*, that appeared on both lists). Our focus on these genes does not mean that we exclude the other 118 strongly

differentiated genes as candidates, only that we were not able to identify obvious functional differentiation. We would not have found changes in noncoding regions that regulate mRNA stability or translocation or that influence posttranslational modifications.

We imposed only 50 generations of selection, so the divergence we observed is likely to reflect changes in frequency of alleles that were already segregating in the ancestral population and not new mutations that arose during the course of the experiment. Alleles contributing to segregating variation have been filtered by natural selection and are maintained in a population either by some form of balancing selection or because weak purifying selection is balanced by recurrent mutation (Charlesworth and Hughes 2000). Either initially rare alleles (more likely to be transient alleles maintained by mutation-selection balance) or intermediate-frequency alleles (more likely to be maintained by balancing selection) could have contributed to near-complete or incomplete sweeps. Alleles that were at intermediate frequency in the ancestral population would have occurred on many different genetic backgrounds. Change in frequency of those alleles would not be expected to create broad chromosomal regions exhibiting sweep signatures, as would be expected for alleles that were rare in the ancestral population (Hermisson and Pennings 2005). The large sweep regions we observed, especially those in locations with high recombination rates (e.g., the middle of X, 2L, and 3L), therefore support the involvement of at least some rare alleles, but we cannot rule out the possibility that some balanced polymorphisms were initially rare in our founding population because of sampling or laboratory selection. Patterns consistent with contributions from both rare and common variants have also been reported from selection on body size in *D. melanogaster* (Turner et al. 2011).

The very broad region of high heterozygosity in S lines on chromosome arm 3R falls within the known breakpoints of the cosmopolitan inversion polymorphism *In(3R)Payne*, which could explain the large expanse of that signature of selection, but the other candidate sweeps are not associated with known inversion polymorphisms. Together, complete and near-complete sweep signals and broad and narrower sweep regions suggest that rare, formerly deleterious alleles and alleles that were maintained by balancing selection contributed to the life-history evolution that we observed.

Because selection on longevity and late-age reproduction resulted in increased life span, improved late-age fertility, and decline in early fertility, the candidate genes we identified are also candidates for regulating a major life-history trade-off between early and late-life fitness components. Such trade-offs are key components of life-history theory and of some models of the evolution of aging (Rose 1991; Roff 2002), yet genetic mechanisms underlying these trade-offs are virtually unknown (Charlesworth and Hughes 2000; Hughes 2010b). Our results suggest that genes regulating oogenesis, immune function, and proteolysis are important in conferring improved late-age performance at the cost of early age fecundity. Perhaps, the best candidate for directly mediating a trade-off between early fertility and life span is *Cct1*, which is known to participate in ovarian development (Gupta and Schupbach 2003) and has been shown to increase life span when up-regulated (Landis et al. 2003); effects of up-regulation on fecundity are not known, but the prediction based on our results would be that changes that lead to life-span increase should also lead to fecundity decline. *fs(1)M3* and *Prm* are involved directly in oogenesis and ovary function, respectively (Degelmann et al. 1990; Cernilogar et al. 2001; Apidianakis et al. 2005; Kapelnikov et al. 2008), but effects on life span have not been reported.

"REPEATABILITY" OF EVOLUTION

The extent to which evolution is "repeatable" is being actively debated (Arendt and Reznick 2008; Stern and Orgogozo 2008; Hughes 2010a; Rosenblum et al. 2010). The phenotypic response to selection was similar in all replicates, and the regions we identified as responding to selection were the same across three different replicate S-C pairs (Fig. S1). This pattern supports the hypothesis that evolution was repeatable at the molecular level in our experiment. The three population pairs were initiated from the same founding population but represent three independent samplings of the genetic variation present in that population. Results similar to ours have been reported in a recent study of natural populations of sticklebacks undergoing repeated adaptation to fresh water (Jones et al. 2012) and in a genome-wide sequencing study of *D. melanogaster* populations selected for development time (Burke et al. 2010). Together, these results suggest that repeated adaptation from a common ancestral population leads to similar underlying genetic changes, despite the potential influence of stochastic processes.

TARGETS OF SELECTION

That late-age female fertility was a direct target of selection in our S populations could account for the prominence of genes with oogenesis-related functions among the strongest candidates for causal sequence changes and differential expression. Late-age male fertility was also a target of selection, but genes related to male fertility were not overrepresented among candidates for causal sequence changes ($\chi_{[1]}^2 = 0.004$, $P > 0.9$) or among differentially expressed genes ($\chi_{[1]}^2 = 0.29$, $P > 0.5$). The *D. melanogaster* genome contains 2.5 times more genes annotated with functions related to oogenesis than to spermatogenesis (296 and 117, respectively), so failure to detect changes in male fertility genes might be due to underdescription of the genetic basis of spermatogenesis relative to that of oogenesis. If annotation is unbiased, then our results suggest that oogenesis presents more targets for selection and possibly higher levels of standing genetic

variation. Alternately, selection on male fertility might be weaker because of the ability of females to store sperm for long periods.

Although not a direct target of selection in this experiment, immune response has been linked to longevity and late-age morbidity in both fruit flies (Sarup et al. 2011) and humans (Bonafe et al. 2001; Franceschi et al. 2007). In flies, immune-response genes exhibit large increases in expression with age, and the expression of antimicrobial peptide reporters predicts subsequent life span in individual flies; long-lived individuals have lower expression of these genes when they are young (Landis et al. 2004). Again, this pattern is consistent with our results: drosomycin genes *dro-2* and *dro-4* were down-regulated in S relative to C flies at early and intermediate ages (Fig. S2C, D).

NOVEL CANDIDATES FOR REGULATING VARIATION IN AGING

Identifying and characterizing alleles responsible for natural variation in aging-related traits is relevant to biomedicine because these alleles are representative of the variation that segregates in human populations. Moreover, mechanisms of aging appear to be conserved in vertebrates and invertebrates (Partridge and Gems 2002), so human variation for late-age health and performance might be regulated by genes and pathways that are homologous to those we report here.

One surprising result of our experiment was the relatively weak support for involvement of the insulin/insulin-like signaling (IIS) or target-of-rapamycin (TOR) pathways in the evolution of late-life performance. Mutations in genes within these pathways can alter life span and fertility in flies and other organisms (Partridge and Gems 2002); natural genetic variation in expression of IIS/TOR-pathway genes has been reported to predict aging-related phenotypes (Nuzhdin et al. 2009), and natural clinal variation in the insulin receptor gene *InR* has been associated with variation in stress resistance and fecundity (Paaby et al. 2010). We therefore expected that some of these genes would contribute to the evolution of life span and late-life fecundity in our experiment. Only one gene previously annotated with the Gene Ontology biological function "determination of adult life span" (*Cct1*) was among the genes bearing the strongest signature of selection, no more than would be expected by chance (1/96 of the candidate genes that had some biological process annotation, compared to 116/10,792 of all genes with some biological-process annotation, $\chi_{[1]}{}^2 = 0.002$, $P > 0.96$). Genes annotated with the functions "aging" or "determination of adult life span" were also significantly underrepresented among differentially expressed genes (43/215 transcripts with these annotations had $P < 0.05$ for line or line-by-age effects, compared to 4488/13,258 of all annotated transcripts, $\chi_{[1]}{}^2 = 18.1$, $P < 0.0001$). Most of the genes we identified are therefore novel candidates for the regulation of life span and late-age performance.

These results do not necessarily contradict the empirical evidence that IIS/TOR pathways are key regulators of aging that are capable of sensing and responding to environmental conditions. The allele-frequency changes we observed necessarily reflect segregating variation that was present in the founding population. If genes in these pathways were functionally invariant in that population, then they could not contribute to the evolution of aging and fertility patterns that we observed. Functional variants could have been lost during the initial sampling from the wild, particularly if they segregated at low frequency in the wild population, or they could have been lost as a result of selection or drift during laboratory husbandry. Two other studies of natural variation have identified allelic variation in *InR* and associated the polymorphism with variation in life span (Geiger-Thornsberry and Mackay 2004; Paaby et al. 2010). We note, however, that both these analyses focused on phenotypic variation among inbred lines of flies, and we cannot be sure that homozygous effects of alleles predict their contribution to heritable variation in outbred populations. To our knowledge, the present study is the first to identify naturally occurring allelic variation associated with a life span–fecundity trade-off in outbred populations.

With the above caveat, our results support the hypothesis that genes in these critical metabolic pathways are under selective constraint in natural populations and might not be the most important determinants of naturally segregating variation (Hughes and Reynolds 2005; Partridge and Gems 2006). IIS/TOR pathways are thought to regulate longevity and aging in part by up-regulating response to stress and to immune challenge (Becker et al. 2010); they also participate in a negative feedback loop involving proteolysis (Rui et al. 2001). Our results are therefore more consistent with evolutionary change in downstream targets of IIS/TOR than with change in the upstream regulators of these pathways.

The high proportion of novel candidates that we identified is consistent with the results of a recent screen of lines homozygous for single P-element insertions (Magwire et al. 2010). Only one of 58 mutations that increased life span in that screen had been known previously to affect aging. That analysis also showed that a very large fraction of insertions affected life span and that mutations that increased life span had extensive pleiotropic, sex-specific, and epistatic effects, many of which were antagonistic. Although we focused on natural variants and Magwire et al. (2010) on P element insertions, taken together these data imply that the regulation of aging involves interacting networks of genes, many of which have not yet been identified.

Conclusions and Future Directions

The goal of the work reported here was to identify genomic regions and loci responsible for response to selection on a key life-history trait, age at first reproduction, and to assess the contributions

of low- and intermediate-frequency ancestral alleles to this response. The logical next steps in this program are to identify the specific nucleotide polymorphisms that contribute to phenotypic divergence and to determine the evolutionary forces (mutation-selection balance, antagonistic pleiotropy, genotype-environment interaction, etc.) responsible for the segregation of causal alleles in the ancestral population. This task will be challenging because of the high density of polymorphisms in *D. melanogaster* and the relatively large genome regions that responded to selection in this experiment. Nevertheless, despite recent arguments that identifying the molecular basis of complex-trait variation might be fruitless (Rockman 2012), we believe experimental evolution combined with hitchhiking mapping offers a promising way forward.

Traditionally, approaches like deletion mapping and quantitative complementation tests have been used to narrow candidate regions from quantitative-trait-locus mapping studies (see, e.g., Pasyukova et al. 2000, 2004), and association mapping of candidate polymorphisms has been used to identify the causal polymorphisms and characterize their effects (see, e.g., De Luca et al. 2003; Carbone et al. 2006). We envision several ways to improve the use of hitchhiking mapping approaches, however, that would speed progress in identifying and characterizing causal natural polymorphisms. First, we need tools in flies similar to human PolyPhen (Sunyaev et al. 2000; Ramensky et al. 2002) and similar programs. These tools would permit formation of a priori hypotheses about potential functional importance of each segregating polymorphism and focus on those most likely to affect a phenotype of interest. Second, to produce smaller target genomic regions, future experimental-evolution studies should increase the size of the founder population, up to several thousand (N. Barton, pers. comm.). Finally, genotyping of the founder population (Nuzhdin et al. 2007) rather than control populations would increase the power of future similar analyses. With these improvements, we feel that hitchhiking mapping techniques (Keightley and Bulfield 1993; Nuzhdin et al. 1993, 2007) would probably become superior to more traditional quantitative-trait-locus mapping and genome-wide association mapping approaches and enable the discovery of the loci underlying natural variation in complex phenotypes.

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Supporting Information

The following supporting information is available for this article:

Figure Sl. Signatures of selection in replicate S-C populations.

Figure S2. Age-specific transcript abundance of genes in regions implicated in selective sweeps, differentially expressed in S and C populations, and implicated in functional studies of aging.

Table S1. Genes within regions implicated in sweeps (see Fig. 2); bold type indicates the strongest candidates within each region.

Table S2. Line mean transcript expression values for 25 genes in regions implicated in sweeps and differentially expressed between S and C populations.

Supporting Information may be found in the online version of this article.

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