Age- and Diet-Specific Effects of Variation at *S6 Kinase* on Life History, Metabolic, and Immune Response Traits in *Drosophila melanogaster*

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Life history theory hypothesizes that genetically based variation in life history traits results from alleles that alter age-specific patterns of energy allocation among the competing demands of reproduction, storage, and maintenance. Despite the important role that alleles with age-specific effects must play in life history evolution, few naturally occurring alleles with age-specific effects on life history traits have been identified. A recent mapping study identified *S6 kinase* (*S6k*) as a candidate gene affecting lipid storage in *Drosophila*. *S6k* is in the target of rapamycin pathway, which regulates cell growth in response to nutrient availability and has also been implicated to influence many life history traits from fecundity to life span. In this article, we used quantitative complementation tests to examine the effect of allelic variation at *S6k* on a range of phenotypes associated with metabolism and fitness in an age-, diet-, and sex-specific manner. We found that alleles of *S6k* have pleiotropic effects on total protein levels, glycogen storage, life span, and the immune response and demonstrate that these allelic effects are age, diet, and sex specific. As many of the genes in the target of rapamycin pathway are evolutionarily conserved, our data suggest that genes in this pathway could play a pivotal role in life history evolution in a wide range of taxa.

Introduction

MOST PHENOTYPIC TRAITS of organisms are quantitative, exhibiting continuous rather than categorical variation in populations. This variation is the product of genetic variation at many loci and their interaction with the environment (Tanksley, 1993). A fundamental goal of quantitative genetics is to determine the genetic basis of variation in quantitative traits and to understand the processes that maintain this variation. This is essential, not only for practical reasons such as assessment of disease risk in human medicine and selective breeding in agriculture, but also for understanding of the evolution of phenotypic traits.

Among the most important set of quantitative traits from an evolutionary perspective are life history traits. As these traits determine the age schedules of birth and death, they are major components of fitness, and so primary targets of natural selection. Life history theory posits that differential allocation of energy among the competing demands of growth, development, reproduction, energy storage, and somatic maintenance and repair lead to trade-offs among traits (Stearns, 1991). Thus, genes controlling how energy is used and allocated should play key roles in the evolutionary divergence of life history strategies between species and contribute signifi-

cantly to variation in fitness within populations. Although it is clear that genes influence energy allocation, we know very little about the genes controlling this process. As such, identification of the genes that contribute to natural variation in life history variation is a major goal of evolutionary biology.

One particular area of life history research in which age-specific genetic effects play a central role is the study of the genetic basis of longevity. A central assumption of the two leading evolutionary theories of aging, mutation accumulation (Medawar, 1952), and antagonistic pleiotropy (Williams, 1966), is that senescence evolves through the action of alleles that have age-specific phenotypic effects on fitness. Under the mutation accumulation model, alleles that are neutral with respect to fitness, but deleterious at later ages, can accumulate in a population over time. An alternative, but not mutually exclusive, theory is the antagonistic pleiotropy model of senescence (Williams, 1957). Under this model, an allele with positive effects on fitness at early age but deleterious effects at later age will accumulate in the population over time. Under both models, accumulation of alleles with late-acting deleterious give rise to senescence and limit life span. Both of these theories rely on the fact that selection against deleterious alleles gets progressively weaker after the onset of reproduction (Hamilton, 1966). This is because individuals that

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reproduce before the age at onset of the deleterious effects will have already passed their alleles on to the next generation, thus making it difficult for natural selection to remove them. Although the assumption that alleles with age-specific effects are central to life history theory and evolutionary models of senescence, few studies have attempted to characterize the age-specific effects of alleles at defined loci on traits associated with fitness.

Environmental influences also have a strong effect on life history traits in general (Yang *et al.*, 2008) and senescence in particular, both directly (e.g., Brakefield *et al.*, 2005; Masoro, 2005; Valenzano *et al.*, 2006) and indirectly by modifying the genetic influences on life history traits (Hoffmann and Merila, 1999; Vieira *et al.*, 2000; Charmantier and Garant, 2005). Because environmental variation is ubiquitous in natural populations, it is also important to evaluate how age-specific allelic effects on fitness traits are modulated by ecologically relevant environmental variation. Such knowledge would provide a more complete picture of the genetic basis of variation in life history traits and aging because the relative influence of genes contributing to the phenotype may vary depending on the environmental conditions experienced. An efficient approach to this problem is to first identify the genes that contribute to phenotypic variation in traits known to exhibit age-related changes, and then characterize the age-specific effect of alleles at these loci under different environmental conditions.

In an earlier study, De Luca *et al.* (2005) carried out a quantitative trait loci (QTL) mapping experiment with *Drosophila melanogaster* to identify chromosomal regions (QTL) producing variation in triacylglycerol levels, an important storage lipid in all organisms (McManus and Travis, 1998; Meex *et al.*, 2009; Petschnigg *et al.*, 2009). One candidate gene identified by this study was *S6 kinase* (*S6k*), a key component of the target of rapamycin (TOR) signaling pathway. Notably, *S6k* was also identified as a candidate gene contributing to variation in life span by a previous QTL study that used the same set of lines (Nuzhdin *et al.*, 1997). Genes regulating the TOR pathway are ideal candidates to test for their influence on age-specific metabolism, as the TOR pathway regulates protein synthesis and glucose homeostasis in cells in response to nutrient availability (Arsham and Neufeld, 2006). Downstream components of this pathway are also influenced by insulin and insulin growth factor (IGF) signaling, and mutations in genes in the TOR pathway including TOR and insulin receptor have been shown to influence life span, reproduction, and metabolism (Grewal, 2009).

TOR itself is a kinase that responds to nutrient availability by phosphorylating and activating *S6k*. *S6k* is a critical gene in this pathway as it phosphorylates a number of potential substrates, including ribosomal protein *s6* (Rp6) and elongation initiation factor 4B. The exact regulatory control of translation by the *S6k* protein is not known, but experimental evidence suggests that phosphorylation of Rp6 by *S6k* downregulates protein synthesis, whereas phosphorylation of elongation initiation factor 4B upregulates protein synthesis (Ruvinsky and Meyuhah, 2006; Ma and Blenis, 2009). In this way, *S6k* may act to fine-tune the rate of protein production.

In this article we use quantitative complementation tests (Pasyukova *et al.*, 2000, 2004; De Luca and Leips, 2007) to test the influence of natural alleles of *S6k* age-specific fecundity, immune response, total protein, glycogen and triacylglycerol

(TAG) levels, and life span. We chose these traits as they should reflect energy investment in reproduction, maintenance and repair, and storage, all key elements that should compete for acquired energy. Because we wished to examine the effect of environmental variation on the allelic effects of this gene, we reared flies on one of two diets, a regular diet of cornmeal molasses and yeast (STD diet) and under dietary restriction, which is identical to the regular diet but with a 60% reduction in yeast. Yeast is the primary source of protein and cholesterol for this species, and therefore reduction in yeast should have far-reaching effects on many phenotypic traits. We also used an independent mutant stock of *S6k* to verify the influence of this gene on the immune response as well as to assess mechanistically how this gene might affect immunity. To do this we used a modification of a standard phagocytosis assay to examine the role of this gene on the ability of blood cells to engulf bacteria.

Materials and Methods

We carried out quantitative complementation tests (Pasyukova *et al.*, 2000) to evaluate the effects of alternative alleles of *S6k* on live weight, life span, and the following age-specific traits: glycogen content, triacylglycerol levels, total protein, fecundity, and immune response.

Flies

For the quantitative complementation test we used two isogenic laboratory strains, *Oregon* and *2b* (described in detail in Hughes and Leips, 2006), which were the parental strains of the recombinant inbred lines used in the initial QTL mapping experiment for lipid storage (De Luca *et al.*, 2005).

We used two mutant stocks of *S6k* for the experiments described in this study. For the quantitative complementation test we used Bloomington Stock P{PZ}S6k⁰⁷⁰⁸⁴ ry⁵⁰⁶/TM3, ry^{RK} Sb¹ Ser¹ (Stock #11713), which has a P-element insert in the 5' region (~48 bp upstream of the first ATG site in the coding region). For the phagocytosis experiments we used the w¹¹¹⁸ stock from the Bloomington Drosophila Stock Center (#6326) and a hypomorphic *S6k* mutant, PBac{WH}S6k¹⁰⁷⁶²² stock from the Exelixis Drosophila Stock Collection at the Harvard Medical School. The w¹¹¹⁸ stock was used as the control in the phagocytosis experiment since the mutant strain was derived from w¹¹¹⁸ and should be genetically identical except for the piggyBac insertion (Thibault *et al.*, 2004). Unlike the stock with the P-element insert that was used in the quantitative complementation test (#11713), this mutant stock is viable as a homozygote. The PBac stock has the lepidopteran transposon piggyBac inserted into the 3' UTR section of *S6k*.

Crossing design for quantitative complementation tests

Ten males of each parental strain, *2b* and *Ore*, were collected as virgins and mated with 20 females of the *S6k* mutant strain separately in 500 mL population bottles. The opening of each bottle was covered by egg laying chamber (Petri dish containing 2% agar brushed with food and sprinkled with yeast) and inverted. From each dish, 50 first to second instar larvae were collected and placed in vials containing 10 mL of standard fly food to standardize larval density.

Virgin offspring were collected and sorted into one of four genotypes: *Ore/Balancer*, *Ore/S6k* mutant, *2b/Balancer*, and *2b/S6k* mutant. Flies with the balancer chromosome were distinguishable because they had the *Stubble* bristle mutation, which is on the balancer chromosome. Virgin males and females of each genotype were separated and placed in vials containing either the STD diet or yeast-restricted diet (DR), 5–6 to a vial.

For all phenotypes for which we made age-specific measurements, we measured early and late age traits on different individuals because in all but the fecundity assay, the assays themselves were destructive. Individuals held until 4 weeks were transferred onto new food at least once a week until the phenotypic measurements were made.

Live weight, total protein, glycogen, and TAG

Three replicate groups of 20 flies per age, sex, diet, and genotype were anesthetized, weighed, and homogenized using the homogenization protocol described in Clark and Keith (1988). Briefly, adults were homogenized on ice using 25 μ L of homogenization buffer (0.01 M KH_2PO_4 , 1 mM EDTA pH 7.4). The homogenates were centrifuged in a microcentrifuge at 2000 rpm for 2 min at 4°C. The lipid layer on the surface was resuspended with the supernatant in 1.5 mL tubes. TAG levels were assayed at the Clinical Nutrition Research Center at University of Alabama at Birmingham using the Vitros DT60 II reader (Johnson and Johnson Clinical Diagnostics, Rochester, NY) and Vitros TRIG DT slides. The Vitros TRIG DT Slide is a multilayered film that contains all the reagents necessary to determine TAG levels in the homogenate. The analyzer calculates the amount of TAGs in the sample by measuring the amount of light emitted from a colored dye produced in the last of a series of chemical reactions that start with the conversion of TAG molecules into glycerol and fatty acids. A precision test was carried out at each run using quality-control materials at various concentrations of TAGs. Each sample was assayed twice and the mean used in the analysis. Total proteins were measured for each homogenate using a standard Lowry protein assay.

Glycogen content was determined separately for the thoraces and the abdomens of flies. Glycogen was measured using a modification of the protocol described in Clark and Keith (1988). Briefly, aliquots of 1.67 μ L of homogenate were added to 250 μ L of a reagent containing 0.1 U/mL of amyloglucosidase, 5 U/mL of glucose oxidase, 1 U/mL of peroxidase, and 0.04 mg/mL of *O*-dianisidine dihydrochloride. After a 30-min incubation period at 37°C, OD540 was measured. Concentration of glycogen was determined from glucose and glycogen standards run with each replicate. Each sample was assayed twice and the mean used in the analysis. Previous studies have shown that this protocol accurately reflects glycogen concentration and that endogenous glucose present in the flies contributes only negligibly to the results (Clark and Keith, 1988). Total proteins were also measured for each sample.

Virgin fecundity

There were two reasons to measure virgin fecundity in this study. The first was that the eggs of *Drosophila* contain a significant amount of glycogen (Gutzeit *et al.*, 1994) and we wanted to be able to assess the extent to which variation in

glycogen levels might be explained by variation in fecundity. The second was that age-specific fecundity often exhibits a trade-off with life span (Rose, 1991), although this is typically observed in mated flies, and we wanted to examine the potential for trade-offs among these traits in our study. Thirty-five females per genotype were maintained separately in vials containing one of the two food treatments. We counted eggs laid by individual females over 10 consecutive days beginning at 1 and 4 weeks of age. Two groups of females were used: one group was used to obtain 1 week fecundity data, whereas the second group was used to determine 4 week fecundity. At the end of each egg counting period females were frozen for thorax measurements. Thoraces were measured using a stereomicroscope fitted with an ocular micrometer. Females that laid no eggs during this time period were not included in the final analysis.

Immune response assay—bacterial clearance

One measurement of the immune response was the rate of clearance of a bacterial load of *Escherichia coli* by flies at each age. We used a procedure modified from McKean and Nunney (2001), along with a control of sham-infected flies, injected with only Ringer's solution. A streptomycin-resistant strain of *E. coli*, strain HB101, was suspended in *Drosophila* Ringer's solution and diluted until an OD₆₀₀ reading of 1.0 ± 0.1 was reached (Eppendorf BioPhotometer [Hauppauge, NY], Beckman DU-65 Spectrophotometer [Brea, CA]). Three microliters of the bacterial or Ringer's solution was loaded into pulled glass capillary needles and connected to a microinjector (Eppendorf FemtoJet, Westbury, NY). The microinjector was set at a compensation pressure of 0.49 psi, injection pressure of 0.49 psi, and injection time of 0.2 s.

Flies were injected in the abdomen under CO₂ anesthesia. Twenty-four hours after injection each surviving fly was placed into 250 μ L of 20% glycerol solution in a 1.5 mL centrifuge tube and homogenized with a pestle and motorized homogenizer (Fisher Scientific, Pittsburgh, PA). We plated 25 μ L of this solution as well as two other serial diluted aliquots of this solution to obtain plates with countable colonies. Solutions were plated onto LB agar plates containing 100 μ g/mL streptomycin and incubated for 15–18 h at 37°C. Bacterial colonies were manually counted, and this colony count served as the immune response phenotype for that replicate. We obtained colony counts from a minimum of 30 flies per genotype per age sex and dietary condition. Sham-infected flies were also homogenized and plated as above. We observed no bacterial growth on plates containing homogenates of sham-injected flies.

Lifespan assay

Male and female flies were placed five or six to a single-sex vial containing one of the two diets. Vials were monitored every other day for dead flies and placed on new food once a week.

Immune response assay—phagocytosis assay

We modified a newly developed procedure developed by Dr. Louisa Wu (University of Maryland College Park) for measuring phagocytosis in larval blood cells, summarized here. We injected third instar larvae of the w^{1118} control and

S6k stock (PBac{WH}*S6k*^{f07622}) with heat-killed fluorescent bacteria (*E. coli* labeled with tetramethylrhodamine conjugate, Invitrogen #E2862 [Carlsbad, CA]). Individual larvae were held for 30 min and then bled onto microscope slides. Blood cells were fixed in 4% formaldehyde for 10 min. We then applied 5 μ L of Oregon Green 488 phalloidin in 200 μ L phosphate-buffered saline +1% bovine serum albumin for 20 min. Blood cells were stained with phalloidin because this toxin binds actin at the cell cortex and so made it possible for us to distinguish cell boundaries. Cells were washed two to three times in phosphate-buffered saline and then we added Prolong + DAPI (Invitrogen # P36930). The Prolong was added as an antifade agent, and the nuclear stain DAPI was used to ensure that we were counting bacteria in viable cells. We counted the number of engulfed bacteria per blood cell using the 40 $\times$ objective of a Zeiss Axio Imager Z1 microscope with AxioVision software. Blood cell counts were obtained from seven mutant larvae and six controls, with the number of cells counted per individual ranging from 8 to 60. This range was due to the fact that the total number of countable blood cells varied among individuals. We also counted the total number of cells containing bacteria between the control and mutant strains per larvae but found no difference between the genotypes in this measure of phagocytosis (data not shown).

Statistical analyses

Main effects of sex, diet, and age. We used either analysis of variance (ANOVA) or analysis of covariance (ANCOVA) (detailed for each trait below) to assess the main effects of diet, sex, and age on each phenotype where appropriate. For these analyses we used reduced models, pooling data from all genotypes. All analyses were carried out in SAS using Proc GLM (SAS V9.1; Cary, NC).

Live weight. We used ANOVA to test for the effects sex, diet, and age on live weight using the model $y = c + s + a + d$ plus all interactions, where y = live weight of individual flies in milligrams, c is a constant, s = sex (male or female), a = age (1 or 4 weeks posteclosion), and d = diet (STD food or DR). All effects in this model were fixed effects.

Total protein, glycogen, and triacylglycerol. We analyzed the main effects of sex, diet, and age on these traits in ANCOVA. For total protein content we used the model $y = c + lw + s + a + d + \text{all interactions} + \text{error}$, where y = total protein, lw = live weight, a covariate to correct for differences in body size and all other variables as above. We analyzed glycogen content in the abdomen and thorax separately and only analyzed triacylglycerol in the abdomen. For glycogen and triacylglycerol we used the model $y = c + p + s + a + d + \text{all interactions} + \text{error}$, where y = glycogen or triacylglycerol and p = tissue-specific protein content, a covariate to account for differences in amounts of extracted tissue. In each full model tested, none of the interactions involving covariates (live weight or total protein) and the main effects were significant, and thus these interaction terms were dropped in the final analysis. Total protein and glycogen data were transformed to natural logs before analysis to satisfy assumptions of ANOVA.

Immune response—bacterial clearance. We used ANOVA to test for the main effects of sex, age, and diet on

the ability to clear infection using the model $y = c + s + a + d + \text{all interactions} + \text{error}$, where y = colony count 24 h after infection and all other parameters as above. We used the natural log of the colony count for individual to satisfy assumptions of ANOVA.

Virgin fecundity. We used an ANCOVA to test the effect of age and diet on fecundity using the model $y = c + tl + a + d + \text{all interactions} + \text{error}$, where y = the total eggs laid per female over a 10-day period, and tl = thorax length. In the full model, none of the interactions between thorax length and the main effects were significant, so interaction effects involving live weight were dropped from the model in the final analysis.

Life span. We used mixed model ANOVA to test for the fixed main effects of sex and diet on lifespan using the model $y = c + s + d + s*d + v(s\ d) + \text{error}$, where y = lifespan in days, and $v(s\ d)$ is the random effect of vial nested within sex and food.

Quantitative complementation tests. All complementation analyses were done separately for each sex, age, and diet combination. The general model for each trait was $y = c + \text{line} + \text{geno} + \text{line*geno} + \text{error}$, where *line* was *2b* or *Ore*, *geno* = mutant or balancer, and *line*geno* was the interaction term. In the quantitative complementation test significant phenotypic differences between the *Ore/S6k* and *2b/S6k* genotypes can arise from two mechanisms, by differences in the additive effects of the *Ore* and *2b* alleles at the *S6k* locus on the trait and due to the phenotypic effects of allelic differences between the parental strains outside of the locus being tested (Pasyukova *et al.*, 2000). To control for the effects of allelic differences between the *Ore* and *2b* strains on the trait elsewhere in the genome, we compare the trait values of the *Ore/Balancer* and *2b/Balancer* genotypes with those of the *Ore/S6k* and *2b/S6k* mutants using ANOVA. In this analysis a significant interaction term would indicate a failure to complement. In addition, to use this test to support the interpretation that alternative alleles at *S6k* produce variation in the phenotype tested, the difference in the average phenotypes of each line over the deficiency (the *Ore/Def-2b/Def*) must be greater than the difference between the average phenotypes of each line over the balancer (*Ore/Bal-2b/Bal*).

Quantitative complementation tests—triacylglycerol. For TAG analyses we used the model: $y = c + p + \text{line} + \text{geno} + \text{all interactions} + \text{error}$. Terms in the model are as described above except that y = triacylglycerol level (assessed from the abdomen only) from 5 individuals pooled from each treatment combination. We also included total protein (p) as a covariate in the model. Any interactions involving the covariate that were not significant were dropped from the model in the final analysis.

Quantitative complementation tests—glycogen. For glycogen analyses we used the model $y = c + p + \text{line} + \text{geno} + \text{all interactions} + \text{error}$. Terms in the model are as described above except that y = glycogen level (assessed separately from the abdomen and thorax) from five individuals pooled from each treatment combination. We also include total

protein (*p*) as a covariate in the model. Any interactions involving the covariate that were not significant were dropped from the model for the final analysis.

Quantitative complementation tests—total protein. For total protein analysis we used the model $y = c + lw + line + geno + \text{all interactions} + error$. Terms in the model are as described above except that in this model y = total protein level (whole body protein obtained from five individuals pooled from each treatment combination per replicate). We include live weight as a covariate in the model. Any interactions involving the covariate that were not significant were dropped from the model for the final analysis.

Quantitative complementation tests—immune response—bacterial clearance. For the bacterial clearance analysis we used the model $y = c + line + geno + \text{all interactions} + error$. Terms in the model are as described above except that in this model y = the total number of colony forming units in an individual fly 24 h after artificial infection.

Quantitative complementation tests—virgin female fecundity. For analysis of fecundity we used the model $y = c + tl + line + geno + \text{all interactions} + error$. Terms in the model are as described above except that in this model y = the total number of eggs laid in a 9-day period per female. We also include thorax length (*tl*) as a measure of body size as a covariate in the model. We did not use weight, as this variable could be confounded with egg load. We checked for significant interactions between covariate and the main effects. Any interactions involving the covariate that were not significant were dropped from the model in the final analysis.

Immune response—cellular phagocytosis assay. For analysis of the effects of *S6k* on phagocytic ability, we carried out a nested ANOVA using the model $y = c + line + ind(line) + error$, where y = the transformed number of bacteria per cell (transformed to the square root of the number per cell + 1 square root of the number per cell to satisfy assumptions of ANOVA, Snedecor and Cochran, 1980), and *ind(line)* = individuals nested within line. This was necessary as we measured several cells per individual, and thus individual cell counts were not independent of individuals.

Results

Live weight

Main effects of age/sex/diet. We found significant differences in live weight between the sexes and between ages. Males were significantly smaller than females (the average live weight for males was 0.77 mg, the average weight of females was 1.03 mg, $F_{1,396} = 477.4$, $p < 0.0001$), and older flies were significantly lighter than younger flies (average weight of 1-week-old flies was 0.91 mg, the average weight of 4-week-old flies was 0.89 mg, $F_{1,396} = 6.51$, $p = 0.006$). Neither diet nor any interactions among the main effects significantly affected live weight.

Quantitative complementation test: effects of variation in *S6k*. We found no effects of variation at *S6k* on the live weight of males or females at any age or on any diet.

Total protein

Main effects of age/sex/diet. Total protein was significantly influenced by both age ($F_{1,395} = 568$, $p < 0.0001$) and diet ($F_{1,395} = 89$, $p < 0.0001$), with age having the most dramatic effect. Total protein decreased by 60% from week 1 to 4 (the least squared mean corrected for differences in live weight for 1-week-old flies was 54.7 $\mu\text{g}/\text{fly}$, and for 4-week-old flies was 22.1 $\mu\text{g}/\text{fly}$), and the effect of age explained 41% of the variation in the data. Reduction in protein content with age in *Drosophila* has previously been reported (Johnson and Butterworth, 1985). We suspect that the magnitude of decline in total protein observed in our study was due to age-associated decrease in muscle mass, although this remains to be confirmed. The effect of diet was also substantial as flies on DR had 33% less total protein than those on the STD diet (the least squared mean for flies on the STD diet was 46.1 $\mu\text{g}/\text{fly}$, and for those on DR was 30.7 $\mu\text{g}/\text{fly}$). As yeast is the primary source of protein for *Drosophila*, the effect of DR on total protein levels is predictable. Indeed, in a study of the effect of dietary composition on metabolic traits in *Drosophila*, Skorupa *et al.* (2008) report that flies maintained on a diet of high yeast also had a high protein body composition compared with diets with reduced yeast levels. Additional effects on protein level could be due to different feeding rates of flies on the different diets, but this remains to be evaluated in future studies. After accounting for differences in live weight, there was no difference between males and females in total protein and there were no significant interactions among the main effects.

Quantitative complementation test: effects of variation in *S6k*. Variation in *S6k* influenced total protein levels, but the allelic effects at this locus were age, sex, and diet specific, with significant effects only identified in 4-week-old females on DR ($F_{1,30} = 5.0$, $p = 0.04$, Fig. 1). In this case, females with the *2b* allele had 20% more protein than female flies with the *Ore* allele.

FIG. 1. Results of complementation test for total protein. Results are for females on the yeast-restricted diet (DR) at 4 weeks of age. Values shown are the means of each genotype $\pm$ one standard error.

TABLE 1. RESULTS FROM ANALYSIS OF COVARIANCE ON ABDOMINAL GLYCOGEN LEVELS (WITH PROTEIN AS THE COVARIATE)

Source	Df	Type III SS	F _{1,395}	p	cd
Protein	1	19.495	61.95	<0.0001	0.12
Sex	1	4.161	13.23	0.0003	0.03
Age	1	24.133	76.7	<0.0001	0.16
Diet	1	11.816	37.55	<0.0001	0.08
Sex*Age	1	8.776	27.89	<0.0001	0.06
Sex*Diet	1	0.005	0.02	—	—
Age*Diet	1	18.789	59.71	<0.0001	0.12
Sex*Age*Diet	1	31.88	101.32	<0.0001	0.20

cd, coefficient of determination.

Glycogen

Main effects of age/sex/diet. Abdominal glycogen: Abdominal glycogen differed significantly between sexes, ages, and diets, and we found significant interactions among all of the main effects except for the sex-by-diet interaction (Table 1). On average, females had 24% more abdominal glycogen than males (the least squared mean for males was 3.61 µg/fly, and for females 4.75 µg/fly). This difference between sexes may be due to a variety of factors, including sex-specific differences in metabolic rates, energy acquisition, or the presence of eggs in females (Gutzeit *et al.*, 1994). Older flies had 61% less abdominal glycogen than younger flies (the least squared mean for 1-week-old flies was 6.0 µg/fly, and for 4-week-old flies was 2.4 µg/fly). This cannot be explained by reduced egg load in females with age as female fecundity actually slightly increased with age (see Results for virgin female fecundity below). Glycogen is also a major component of the cells of the fat body, but age-related decline in glycogen as a proportion of the cellular constituents of fat body cells has only been previously reported in males (Johnson and Butterworth, 1985). Age-related decreases in glycogen in this study may be explained by any number of factors, including increased metabolic rates with age, a shift in the allocation of energy away from storage or reduced energy acquisition with age, or age-related decreases in feeding. These possibilities remain to be examined. Flies on the STD diet had 20% more glycogen than those on DR (the least squared mean for flies on the STD diet was 4.65 µg/fly, and for flies on DR was 3.73 µg/fly), which is to be expected given the higher nutritional value of the STD diet. The sex-

by-age interaction resulted from the fact that abdominal glycogen in females declined by a greater percentage with age (43%) than it did in males (34%, data not shown). The age-by-diet interaction was due to the fact that the drop in abdominal glycogen with age was much greater for flies on the DR (58%) than it was for those on the STD diet (15%). The interaction that explained the largest percent of the variation in the data was the sex-by-age-by-diet interaction, and was driven largely by the fact that males at 1 week of age had 40% more abdominal glycogen on the DR than they did on the STD diet (Table 2). This pattern was reversed in older males and it matched the pattern exhibited by females at all ages, being that females had higher glycogen on the STD diet.

Thoracic glycogen: All main effects and their interactions influenced thoracic glycogen levels (Table 3). The main effects of sex, age, and diet on thoracic glycogen were similar to that of abdominal glycogen: females had 17% more glycogen than males (the least squared mean for males was 0.95 µg/fly, and for females was 1.14 µg/fly), older flies had 65% less glycogen than younger flies (the least squared mean for 1-week-old flies was 1.55 µg/fly, and for 4-week-old flies was 0.54 µg/fly) and flies on standard food had 15% more glycogen than those on the RD (the least squared mean for flies on the STD diet was 1.08 µg/fly, and for those on DR was 1.04 µg/fly). The interaction effects explaining the greatest amount of variation in glycogen storage were the age-by-diet and the sex-by-age-by-diet interactions, together explaining 26% of the phenotypic variation. As was the case for abdominal glycogen, these interactions are largely due to the sex-specific influence of diet on glycogen storage in young flies, specifically young males increasing the level of glycogen stored under DR, with females exhibiting the opposite pattern (Table 4). As discussed above relative to the effects of age on glycogen storage, there are many possible explanations for the sex-, diet-, and age-specific interactions affecting glycogen storage, and these need to be investigated in future experiments.

Quantitative complementation test: effects of variation in *S6k*. Abdominal glycogen: Allelic variation at *S6k* affected female glycogen levels in an age-dependent manner (Table 5). Variation in *S6k* influenced abdominal glycogen only in 4-week-old females, and on both diets, females with the *2b* allele had significantly more glycogen than females with the *Ore* allele (Fig. 2) (STD diet: $F_{1,31} = 9.14$, $p = 0.01$; DR: $F_{1,30} = 7.33$, $p = 0.01$). Variation in *S6k* had no effect on gly-

TABLE 2. LEAST SQUARED MEANS OF ABDOMINAL GLYCOGEN LEVELS

Sex	Age (week)	Diet	Least squared mean $\pm$ standard error	% Change between diets
Female	1	STD	8.3 $\pm$ 0.5	58% decrease from STD to DR
Female	1	DR	3.5 $\pm$ 0.7	
Female	4	STD	3.7 $\pm$ 0.5	9% decrease from STD to DR
Female	4	DR	3.4 $\pm$ 0.5	
Male	1	STD	4.5 $\pm$ 0.6	40% increase from STD to DR
Male	1	DR	7.6 $\pm$ 0.6	
Male	4	STD	4.7 $\pm$ 0.5	100% decrease from STD to DR
Male	4	DR	$\sim 0 \pm 0.44$	

Glycogen values shown are adjusted mean values $\pm$ one standard error. % Change between diets is the change in % glycogen comparing flies on the two diets.

STD, standard food diet; DR, yeast-restricted diet.

TABLE 3. RESULTS FROM ANALYSIS OF COVARIANCE ON THORACIC GLYCOGEN LEVELS

Source	Df	Type III SS	F _{1,395}	p	cd
Protein	1	2.445	19.15	<0.0001	0.02
Sex	1	0.535	4.19	0.04	<0.01
Age	1	21.660	169.65	<0.0001	0.18
Diet	1	2.420	18.96	<0.0001	0.02
Sex*Age	1	3.659	28.66	<0.0001	0.03
Sex*Diet	1	2.807	21.99	<0.0001	0.02
Age*Diet	1	18.794	147.20	<0.0001	0.15
Sex*Age*Diet	1	14.763	115.62	<0.0001	0.11

cogen storage in the abdomens of males at any age on any diet.

Thoracic glycogen: Variation at *S6k* also affected female thoracic glycogen levels, but in contrast to the effect on abdominal glycogen, variation in *S6k* only affected young females and the allelic effects were reversed from that seen in the abdomen. In the thorax, female flies with *Ore* alleles had significantly more glycogen than those with *2b* alleles on both diets (Fig. 3, STD: $F_{1,31} = 5.60$, $p = 0.02$; DR: $F_{1,19} = 4.59$, $p = 0.04$). Variation at *S6k* also affected glycogen levels in male thoraces, but only when young and under DR. In this case, males with the *Ore* allele had 36% more glycogen in the thorax than males with the *2b* allele ($F_{1,31} = 10.66$, $p = 0.003$).

Triacylglycerol

Main effects of age/sex/diet. Sex, age, and diet all significantly affected TAG levels, but age had the largest effect, explaining 14% of the total phenotypic variation (Table 6). Males had 3% more TAG than females (the least squared mean of males was 8.18 $\mu\text{g}/\text{fly}$, and for females was 7.91 $\mu\text{g}/\text{fly}$). Older flies had 9% more TAG (the least squared mean for 1-week-old flies was 7.65 $\mu\text{g}/\text{fly}$, and for 4-week-old flies was 8.43 $\mu\text{g}/\text{fly}$). Age-related increase in TAG levels are common in mammals (e.g., Tucker and Turcotte, 2003) and are one of the hallmarks of metabolic syndrome in aged humans (Kong *et al.*, 2006; Penninx *et al.*, 2009). Flies on DR had 2% less TAG than those on the STD diet (the least squared mean for flies on the STD diet was 8.12 $\mu\text{g}/\text{fly}$, and for those on DR was 7.96 $\mu\text{g}/\text{fly}$). Although the sex-by-age effect was significant, it explained very little of the phenotypic variation. Both males and females had higher TAG

TABLE 5. AGE-, DIET-, AND TISSUE-SPECIFIC INFLUENCES OF VARIATION AT *S6* KINASE ON GLYCOGEN IN FEMALES

Tissue	Age (week)	Diet	Allelic effect
Abdomen	1	STD	<i>Ore</i> = <i>2b</i>
Abdomen	1	DR	<i>Ore</i> = <i>2b</i>
Abdomen	4	STD	<i>2b</i> > <i>Ore</i> ^a
Abdomen	4	DR	<i>2b</i> > <i>Ore</i> ^b
Thorax	1	STD	<i>Ore</i> > <i>2b</i> ^b
Thorax	1	DR	<i>Ore</i> > <i>2b</i> ^b
Thorax	4	STD	<i>2b</i> = <i>Ore</i>
Thorax	4	DR	<i>Ore</i> = <i>2b</i>

Conditions under which allelic variation at *S6k* significantly affected glycogen level (^a0.001 < p < 0.01, ^b0.01 < p < 0.05).

2b, Russian *2b*; *Ore*, Oregon; *S6k*, *S6* kinase.

levels at 4 weeks, but females had a slightly higher increase with age compared with males (12% vs. 11% based on least squared means after accounting for differences in body weight).

Quantitative complementation test: effects of variation in *S6k*. We found no effects of variation at *S6k* on TAG levels in males or females at any age or on any diet.

Virgin fecundity

Main effects of age/diet. Both diet and age significantly affected virgin fecundity, and there was a significant age-by-diet interaction (Table 7). Females at 1 week of age actually laid fewer eggs than those at 4 weeks (the least squared mean for 1-week-old females was 1.0 eggs/day, and for 4-week-old females was 2.4 eggs/day). Flies on DR had fewer eggs per day than those on the STD diet (the least squared mean for flies on the STD diet was 2.6 eggs/day, and for those on DR it was 0.9 eggs/day). The age-by-food interaction resulted from the fact that females on the STD diet had a 70% increase in the number of eggs laid per day from weeks 1 to 4, whereas females in the DR treatment increased their daily egg production by 45% from week 1 to 4.

Quantitative complementation test: effects of variation in *S6k*. Variation at *S6k* had no effect on virgin fecundity at any age on either diet.

TABLE 4. LEAST SQUARED MEANS OF THORACIC GLYCOGEN LEVELS

Sex	Age (week)	Diet	Least squared mean $\pm$ standard error	% Change between diets
Female	1	STD	2.1 $\pm$ 0.1	28% decrease from STD to DR
Female	1	DR	1.5 $\pm$ 0.1	
Female	4	STD	0.5 $\pm$ 0.1	20% decrease from STD to DR
Female	4	DR	0.4 $\pm$ 0.1	
Male	1	STD	0.7 $\pm$ 0.1	60% increase from STD to DR
Male	1	DR	1.9 $\pm$ 0.1	
Male	4	STD	1.0 $\pm$ 0.1	80% decrease from STD to DR
Male	4	DR	0.2 $\pm$ 0.1	

Glycogen values shown are adjusted mean values $\pm$ one standard error. % Change between diets is the change in % glycogen comparing flies on the two diets.

FIG. 2. Results of complementation test for female abdominal glycogen on the DR at 4 weeks of age. Values shown are the means of each genotype $\pm$ one standard error.

Life span

Main effects of sex/diet. Averaged over all genotypes, there was no significant difference in life span between sexes or between diets, or any significant effect of the interaction between sex and diet on life span.

Quantitative complementation test: effects of variation in *S6k*. Variation in *S6k* did have a significant influence on life span, but only in one sex on one diet. Female flies with the *Ore* allele lived 24% longer than those with the *2b* allele but only on the STD diet ($F_{1,119} = 4.64$, $p = 0.03$, Fig. 4). Variation at *S6k* had no effect on the life span of males on either diet.

Immune response—bacterial clearance ability

Main effects of age/sex/diet. Both sex and diet had significant influences on the ability to clear bacterial infection (Table 8). The clearance ability of males was 10% greater than

FIG. 3. Results of complementation test for female thoracic glycogen on the DR at 1 week of age. Values shown are the means of each genotype $\pm$ one standard error.

TABLE 6. RESULTS FROM ANALYSIS OF COVARIANCE ON ABDOMINAL TAG LEVELS

Source	Df	Type III SS	$F_{1,395}$	p	Cd
Protein	1	62.352	225.16	<0.0001	0.31
Sex	1	5.318	19.20	<0.0001	0.03
Age	1	29.225	105.54	<0.0001	0.14
Diet	1	1.917	6.92	0.0009	0.01
Sex*Age	1	1.630	5.88	0.016	0.01
Sex*Diet	1	0.605	2.18	0.140	—
Age*Diet	1	0.202	0.73	0.394	—
Sex*Age*Diet	1	0.087	0.31	0.576	—

that of females (the least squared mean of the natural log of colony forming units in males was 5.10, whereas that of females was females 5.69). The relative ability of males to clear the infection compared to females is even more impressive when one considers that males are approximately 25% smaller than females (based on our live weight measurements). As such, male flies are receiving a significantly higher infection load per body mass than females. Although we do not know the physiological basis of this difference, we do not think that this result is due to differences in bacterial growth between the sexes. In our experience with females, *E. coli* does not grow in flies after infection (unpublished data).

Flies on DR had a 31% greater ability to clear infection than those on the STD diet (the least squared mean of the natural log of colony forming units for flies on the STD diet was 6.38, and for flies on DR 4.40) with differences due to diet explaining 33% of the total variation. Although there was no main effect of age, there was a significant sex-by-age interaction. This interaction was due to the fact that the clearance ability of males decreased by 5% with age, whereas the clearance ability of females actually increased. There was also a significant sex-by-diet interaction. In this case, although clearance ability of both males and females was improved on the restricted diet, dietary restriction was more beneficial to males (increasing clearance ability by 40%) compared with females (increased clearance ability by 22%).

Quantitative complementation test: effects of variation in *S6k*. Variation in *S6k* influenced the immune response of both sexes, but in an age- and diet-specific manner. *S6k* only affected male immune response at 4 weeks of age on the STD diet (Fig. 4A, $F_{1,117} = 7.36$, $p = 0.007$). In this case, males with the *2b* allele had a higher bacterial clearance rate than males with the *Ore* allele. Variation at *S6k* influenced bacterial clearance rate of females under two conditions, at early age on the STD diet ($F_{1,120} = 5.58$, $p = 0.02$) and at late age on the restricted diet ($F_{1,138} = 5.94$, $p = 0.02$). In both cases, females with the *2b* allele had improved clearance ability compared to those with the *Ore* allele (Fig. 4B, C).

TABLE 7. RESULTS FROM ANALYSIS OF VARIANCE ON MAIN EFFECTS OF DIET AND AGE ON VIRGIN FECUNDITY

Source	Df	Type III SS	$F_{1,390}$	p	Cd
Age	1	78.701	105.7	<0.0001	0.15
Diet	1	110.537	148.5	<0.0001	0.21
Age*Diet	1	8.590	11.54	0.0008	0.02

FIG. 4. Results of complementation test of bacterial clearance ability (A) Males on the regular diet at 4 weeks of age. (B) Females on the regular diet at 1 week of age. (C) Females on the DR at 4 weeks of age. All values shown represent the means of each genotype $\pm$ one standard error.

Confirmation of effects of *S6k* on immune response and phagocytic ability

To independently assess the role of *S6k* on the immune response, we measured bacterial clearance ability of female flies with a hypomorphic mutation of this gene and females of a control strain, which were genetically identical except for the mutation in *S6k*. We used these same lines also to measure the phagocytic ability of blood cells of mutant and

TABLE 8. RESULTS FROM ANALYSIS OF VARIANCE ON BACTERIAL CLEARANCE ABILITY

Source	Df	Type III SS	F _{1,974}	p	Cd
Sex	1	83.566	46.18	<0.0001	0.03
Age	1	1.052	0.58	0.445	–
Diet	1	952.258	526.28	<0.0001	0.33
Sex*Age	1	11.383	6.29	0.012	0.01
Sex*Diet	1	76.036	42.02	<0.0001	0.02
Age*Diet	1	0.090	0.05	0.827	–
Sex*Age*Diet	1	0.326	0.18	0.671	–

control strains using a phagocytosis assay on larval blood cells and heat-killed bacteria. Bacterial clearance ability of the mutants on the STD diet was reduced by 40% compared with controls ($F_{1,52} = 16.47$, $p < 0.0002$). There was also a significant effect of *S6k* on the phagocytic ability of blood cells ($F_{1,435} = 18$, $p < 0.0001$). Surprisingly, the average number of bacteria per blood cell from the mutant flies was 12% greater than the number found in blood cells of the control strain (Fig. 5). A second replicate of this experiment yielded quantitatively similar results (not shown).

Discussion

We used quantitative complementation tests (Pasyukova *et al.*, 2000, 2004; De Luca and Leips, 2007) to test the age- and diet-specific effects of variation at *S6k* on life history, and metabolic and immune response traits in two laboratory strains, *Ore* and *2b*, of *D. melanogaster*. One objective of the study was to investigate whether genetically based trade-offs between traits were regulated by this locus. In this case, if a trade-off existed we would have expected one allele of *S6k* to have a positive effect on one fitness trait but a negative effect on another trait, and this was not observed for most traits (Table 9). However, one interesting finding was the apparent trade-off between bacterial clearance ability at 1 week of age and thoracic glycogen storage. Mounting a successful

FIG. 5. Comparison of the phagocytic ability of blood cells of the *S6k* mutant and *w¹¹¹⁸* control strain. Values shown are averages $\pm$ one standard error. *S6k*, *S6 kinase*.

TABLE 9. SUMMARY OF SIGNIFICANT PHENOTYPIC EFFECTS OF VARIATION AT *S6 KINASE* FROM THE QUANTITATIVE COMPLEMENTATION TESTS

Sex	Age (week)	Diet	Life span	Gly–Th	Gly–Ab	Protein	Imm R
Male	1	STD					
Male	1	DR		<i>Ore</i> > <i>2b</i> ^a			
Male	4	STD					<i>2b</i> > <i>Ore</i> ^a
Male	4	DR					
Female	1	STD	<i>Ore</i> > <i>2b</i> ^b	<i>Ore</i> > <i>2b</i> ^b			<i>2b</i> > <i>Ore</i> ^b
Female	1	DR		<i>Ore</i> > <i>2b</i> ^b			
Female	4	STD			<i>2b</i> > <i>Ore</i> ^b		
Female	4	DR			<i>2b</i> > <i>Ore</i> ^b	<i>2b</i> > <i>Ore</i> ^b	<i>2b</i> > <i>Ore</i> ^b

^a0.001 < *p* < 0.01; ^b0.01 < *p* < 0.05.

2b, *2b* allele; Gly, glycogen; IR, immune response (bacterial clearance ability).

immune response requires a significant energy investment (e.g., Demas *et al.*, 1997; Moret and Schmid-Hempel, 2000; Bonneaud *et al.*, 2003). There are a number of studies indicating an evolutionarily conserved link between immune function and insulin/IGF signaling (Diangelo *et al.*, 2009; Zeyda and Stulnig, 2009), which in turn regulates glycogen levels. In addition, *S6k* plays an essential role in pathways that render cells unresponsive to insulin (Manning, 2004). Based on these observations, we speculate that genotypic differences in the efficiency of the immune response between *Ore* and *2b* may reflect differences in allocation of glucose between traits associated with survival. Of course, given the number of traits we examined overall, the genetically based trade-off we observed here may be spurious. Future experiments are needed to see if this trade-off is maintained and if so to provide a mechanistic basis for the association between these traits.

For the most part, all significant allelic effects appeared to be maintained for a given trait, although it is interesting that flies with the *Ore* allele had higher thoracic glycogen, whereas flies with the *2b* allele had higher abdominal glycogen. Although eggs contain a significant amount of glycogen (Gutzeit *et al.*, 1994) the higher abdominal glycogen in older flies with the *2b* allele was not due to higher egg loads (at least as measured by the number of eggs laid) as we found no effect of variation at *S6k* on virgin egg production. We also found that natural variants of *S6k* have extensive pleiotropic effects on metabolic traits, immune response, and life span. Given the important role of *S6k* in regulating protein translation, it is not surprising to observe extensive pleiotropic effects, and indeed studies of *S6k* mutants have found effects on cell and body size (Shima *et al.*, 1998; Montagne *et al.*, 1999), cell survival (Harada *et al.*, 2001), autophagy (Scott *et al.*, 2004), egg production (Hansen *et al.*, 2005; Terashima and Bownes, 2005), hunger-driven behaviors (Wu *et al.*, 2005), life span (Kapahi *et al.*, 2004), and phagocytosis (Ganesan *et al.*, 2004; Stroschein-Stevenson, 2006). It is important to note, however, that *S6k* may act on a number of different substrates (Ruvinsky and Meyuhas, 2006), in addition to the Rp6, and thus the phenotypic effects of variation in this gene may be mediated through its effects on other processes.

We have also demonstrated that the allelic effects of variation in this gene are modified by diet and age and are not consistent across sexes. Although such context-dependent allelic effects are often found (Leips and Mackay, 2000; Vieira

et al., 2000) the age-specific nature of these allelic effects have not been investigated. The TOR pathway is involved in nutrient sensing and variation in nutrient levels are known to rapidly affect transcript levels of *S6k* (Arsic and Guerin, 2008). As such, genetic variation at this locus might produce differences in the degree to which *S6k* is expressed. Alternatively, the activity of S6K depends on how efficiently it becomes phosphorylated by kinases upstream in the TOR pathway (Long *et al.*, 2004). If alternative alleles produce functionally different S6K proteins, this could influence the activity of S6K, altering the efficiency of phosphorylation of its targets. Exactly how the context-dependent effects of these natural alleles manifest themselves is an important but unresolved issue.

Our finding that variation in *S6k* affected glycogen levels but not TAG is interesting given the fact that the original mapping study identified *S6k* as a gene regulating TAG levels. Although there are many potential explanations for the lack of congruence between the findings of this study and that of De Luca *et al.* (2005), the most likely is that *S6k* was not one of the genes that contributed to phenotypic variation in TAG in the earlier QTL mapping study. This is not necessarily unexpected as QTL studies identify regions of the genome affecting phenotypic traits, not the genes themselves. There were a large number of genes within the confidence interval of the original QTL, and further fine mapping experiments will be necessary to identify the actual genes giving rise to variation in TAG in this QTL. We did, however, confirm that variation in *S6k* contributes to variation in life span as suggested by the earlier QTL study of Nuzhdin *et al.* (1997).

We were able to confirm the effects of variation at *S6k* on bacterial clearance ability using an independent set of lines, so it is clear that this is an important gene regulating age-specific immunity. Interestingly, although the *S6k* mutant flies had a poorer bacterial clearance ability compared with the control, their phagocytic ability was significantly improved over that of the control. Immune response to bacterial infection in flies involves two processes: phagocytosis and antimicrobial peptide (AMP) production (Lemaitre and Hoffmann, 2007). Phagocytosis is executed by adult hemocytes (plasmatocytes), which are macrophage-like blood cells (Crozatier and Meister, 2007), whereas fat body tissue produces the bulk of AMPs (although other tissues also contribute) (Brennan and Anderson, 2004). Given the central role of *S6k* in protein translation (Ma and Blenis, 2009) it is conceivable that varia-

tion in *S6k* could influence both the production of AMPs as well as phagocytosis. Activation of the mammalian homolog of *S6k* (p70S6 kinase) has been shown to play a direct role in phagocytosis in mouse macrophages (Ganesan *et al.*, 2004), presumably by influencing the actin cytoskeleton (Berven *et al.*, 2004), remodeling of which is important for phagocytosis. The fact that flies with the hypomorphic mutation of *S6k* had an improved phagocytic ability may have more to do with an indirect effect of this mutation. *S6k* plays a central role in protein translation in response to nutrient availability, so dysregulation of this gene or reduced functional expression of this gene may mimic the effects of reduced nutrient availability to the cell. Mammalian macrophages reared in nutrient-limiting conditions in cell culture have enhanced phagocytic ability, which is presumably related to the enhanced autophagy that cells undergo when facing nutrient stress (Martinet *et al.*, 2009). Extending these observations to our study, one hypothesis is that flies with the mutation in *S6k* have reduced production of AMPs but enhanced phagocytic ability. As the difference in phagocytic abilities between control and mutant flies was small, relative to the difference in bacterial clearance ability between these genotypes, it could be that the reduced production of AMPs offset the benefits of enhanced phagocytosis. Of course, because our clearance phenotype is measured 24 h after infection, the relative influence of phagocytosis, production of AMPs, as well as other aspects of the fly physiology that might influence the immune response are confounded. Recent studies have shown an important interaction between the processes of phagocytosis and antimicrobial protein production in invertebrates, suggesting that phagocytosis is most important immediately after infection while the production of AMPs playing a role only after phagocytic cells are saturated (Elrod-Erickson *et al.*, 2000; Haine *et al.*, 2008). It should also be noted that the bacterial clearance assay was carried out on adults, but the phagocytosis assay was carried out using larval blood cells. Although we expect the phagocytic ability of larval and adult blood cells to be similar, given the fact that adult blood cells appear to be derived from larval blood cells (Minakhina and Steward, 2010), we cannot be certain of this without further study. Exactly how allelic variation in *S6k* influences the various traits that determine immunocompetence remains to be determined.

In summary, we have shown that natural alleles of *S6k* have extensive pleiotropic effects on traits related to fitness, and that these effects are highly dependent on the sex of the individual, the diet they are reared on, as well as age. A note of caution should be added with respect to this conclusion. Several of the quantitative complementation tests were only marginally significant; given that we tested a number of traits in different combinations of sex, age, and diet, we might expect to identify some significant relationships based on chance alone. However, for some traits we found a repeated association between variation at *S6k* and phenotypic variation in different treatment combinations (e.g., glycogen and the immune response assays), which greatly strengthens our conclusions for those traits. In addition, we were able to independently validate the influence of *S6k* on immune response traits. Future research is necessary to validate the effects of this gene on the other focal traits in this study.

An additional caveat is that we have not identified the actual causal polymorphism producing these different ef-

fects nor do we know for certain that alternative alleles of *S6k* with functional effects on the phenotypes evaluated are segregating in natural populations. The alleles tested here represent fixed differences in the *S6k* gene between two laboratory strains. Given the size of this gene (12 kb), there are likely to be many genetic differences between the tested strains at this locus alone, and thus it would be difficult to separate the effects of different variants with only two strains. Identification of the actual polymorphisms affecting these traits will require other approaches such as candidate gene association tests using a large number of individuals from a natural population or whole genome association tests, which may soon be possible in *Drosophila* using the *Drosophila* Genetic Reference Panel (http://service004.hpc.ncsu.edu/mackay/Good_Mackay_site/DBRP.html).

This is the first study that we are aware of to identify age- and diet-specific effects of natural alleles on fitness traits. As such, a great deal of future research is necessary to determine the mechanisms by which alleles exert environmental and age-specific effects on phenotypes. Such knowledge would enhance our understanding of the genetic basis of variation in life history traits and aging and provide a significant contribution to our understanding of phenotypic evolution.

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