Genome-wide association of foraging behavior in *Drosophila melanogaster* fails to support large-effect alleles at the *foraging* gene

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ABSTRACT

Thirty four years ago, it was postulated that natural populations of *Drosophila melanogaster* are comprised of two behavioral morphs termed "rover" and "sitter", and that this variation is caused mainly by large-effect alleles at a single locus. Since that time, considerable data has been amassed that compares the behavior and physiology of these morphs. Contrary to common assertions, however, published support for the existence of common large effect alleles in nature is quite limited. To further investigate, we quantified the foraging behavior of 36 natural strains, performed a genome-wide association study, and described patterns of molecular evolution at the *foraging* locus. Though there was significant variation in foraging behavior among genotypes, this variation was continuously distributed and not significantly associated with genetic variation at the *foraging* gene. Patterns of molecular population genetic variation at this gene also provide no support for the hypothesis that *for* is a target of long term balancing selection We propose that additional data is required to support a hypothesis of common alleles of large effect on foraging behavior in nature. Genome-wide association does support a role for natural variation at several other loci, including the *sulfateless* gene, though these associations should be considered preliminary until validated with a larger sample size.

INTRODUCTION

Sokolowski (1980) described a now classical difference in behavior between two mutant strains of *Drosophila melanogaster.* When placed in a food patch, larvae of one strain would stay there and eat ("sitters") while the larvae of the other strain would crawl around while eating and visit other food patches ("rovers"). It was later determined that the difference between a strain with the sitter trait and a strain with the rover trait could be explained mostly by variation at a single locus containing the *foraging* gene, and that transgenic manipulations of this gene phenocopied this variation (Osborne *et al.* 1997). Though the original variation was described in strains that had been reared in the lab for decades, variation in foraging behavior was also found in natural populations, and a hypothesis was put forth that this behavioral variation is maintained by balancing selection in the wild (Sokolowski 1980, Bauer and Sokolowski 1984, Sokolowski, Pereira and Hughes 1997). In the 34 years since the original description of this variation, a considerable research effort has been focused on "reference" rover and sitter strains, thought to be representative of two natural morphs. These reference strains have been repeatedly compared and found to differ in many traits in addition to larval foraging, including adult movement patterns (Pereira and Sokolowski 1993, Edelsparre *et al*. 2014), energy storage (lipid vs. carbohydrates, Kent *et al.* 2009), glucose homeostasis (Kaun, Chakaborty-Chatterjee and Sokolowski 2008), thermotolerance (Chen *et al.* 2011), resistance to sleep deprivation (Donlea *et*

al. 2012), relative strength of short term vs. long term memory (Kaun *et al.* 2007, Mery *et al.* 2007), use of "retroactive inference" (Reaume, Sokolowski and Mery 2011), use of public vs. private information (Foucaud *et al.* 2013), and more. The picture painted is one in which *D. melanogaster* populations are composed of two coexisting behavioral "morphs", differentiated in many phenotypic dimensions, that coexist through some type of balancing selection. The only comparable case we are aware of is variation at the *npr-1* gene in *Caenorhabditis elegans* (though there may be non-recombining supergenes with such diverse effects [Lawson, Vander Meer and Shoemaker 2012; Thomas *et al.* 2008]). Similar to the *foraging* case in *D. melanogaster*, the *npr-1* allele from the N2 strain was found to cause solitary foraging, in contrast to the group foraging observed in other strains (de Bono and Bargmann 1998). It has recently been postulated, however, that this large-effect allele arose as an adaptation to lab culture, and may not be maintained by selection in natural populations (Rockman and Kruglyak 2009; McGrath *et al.* 2009). We feel that, based on published data, this same scenario cannot be ruled out in the case of variation at *foraging*. We first critique the existing evidence supporting the "common alleles of large effect" hypothesis, and then present additional data which fails to support this hypothesis.

Evidence for bimodal trait distributions in natural populations

If the distribution of trait variation is bimodal, this supports a hypothesis that there is a factor of large effect involved (genetic or environmental). In the case of foraging behavior in *D. melanogaster*, several bimodal distributions have been reported. An impressively bimodal distribution is shown in a highly cited review that features the *foraging* story (Sokolowski, 2001). The source of these data is not reported in the review, but the distribution appears identical to figure 1 from de Belle, Hilliker and Sokolowski (1989). If this is indeed the source of these data, this bimodality does not address whether there is a common factor of large effect in natural populations. The bimodal distribution in de Belle, Hilliker and Sokolowski (1989) is a comparison of only two "reference" strains: the EE ($ebony^{11}$) strain (one mode) and the B15 strain (the other mode; see below for a further description of these genotypes). The variance around each mode is variation among replicate individuals of the same genotype.

A bimodal distribution is also reported in a note published by the Drosophila Information Service (Sokolowski, 1982). This distribution shows variation in foraging behavior in larvae collected from fallen pears near Toronto. The distribution of foraging path lengths for these larvae is bimodal, with one mode between 60-80 mm (named "rovers") and the other mode at 0-20 mm (named "sitters"). The bimodality of this distribution supports a hypothesis that there is a factor of large effect in the data, but this factor is not necessarily genetic. These larvae were sampled from nature and assessed directly, so variation among them could be due to genes, the environment, and/or gene-by-environment interactions. Indeed, major effects of environmental factors were later quantified in laboratory assays (Graf and Sokolowski. 1989), and it was later noted that "a carefully controlled environment is required to minimize the phenotypic overlap between distinct genotypes" (de Belle, Hilliker, and Sokolowski, 1989). It therefore seems plausible that the alternative possibility—individuals of the same genotype behaving differently due to environmental influence—cannot be excluded. Indeed, in Sokolowski (2001), it is stated that "flies with rover alleles can be made to behave as sitters after a short period of food deprivation".

Evidence for a genetic effect on foraging distance was reported in the original foraging paper, but not for genotypes recently sampled from nature (Sokolowski 1980). Larvae of one

strain, carrying a *white*^{blood} mutation affecting eye color, traversed a much larger area when feeding ("foraging") than the other strain, which carried a mutant *ebony*^{11} allele affecting body color. This behavioral difference did not seem to be caused by either of the pigmentation mutations, as *ebony* is on the third chromosome, *white* is on the X, and the behavioral difference mapped almost entirely to the second chromosome. Individuals with the second chromosome from the *ebony*^{11} strain were termed "sitters", and those with the *white*^{blood} second chromosome were termed "rovers". Though given the same names as the larvae sampled from nature, no evidence is reported that supports this inference. Differences between these lab stocks could have arisen during lab culture. Flybase.org lists the first reference to the *ebony*^{11} allele as a paper from 1926 (Stern, 1926): 54 years of lab culture is likely equivalent to over 1000 generations since this strain was sampled from nature. The earliest reference we could find to the *white*^{blood} strain was published in 1945 (Ephrussi and Herold, 1945), which is also a considerable interval.

The strongest evidence for the existence of a common allele of major effect in nature, to the best of our knowledge, was presented by Sokolowski, Pereira, and Hughes (1997). An outbred laboratory population was founded from 500 flies, again collected from a Toronto orchard, and 500 individual larvae were assayed from this population after a year of lab culture. The trait distribution of these individuals was once again bimodal, (figure 1, Sokolowski, Pereira, and Hughes, 1997), and appears very similar to the distribution of larvae measured directly from nature (Sokolowski, 1980; Sokolowski, 1982). Moreover, the differences between "rover" and "sitter" larvae from this population failed to complement the sitter mutation from the *ebony*^{11} lab strain, suggesting that there was variation at the same locus. Together, these data do support a hypothesis of common, large-effect alleles in nature. However, these data on their own still allow room for doubt, especially in light of the unprecedented nature of the foraging story. Possible critiques of these data include the complications of epistasis when performing complementation tests in uncontrolled genetic backgrounds (Service, 2004). In addition, the genotypes found to be bimodal were not isolated independently from nature. Instead, a large outbred population was adapted to the lab for a year, then individuals from this population were assayed. This means that allele frequencies could evolve between the sampling and measurement, so that a rare sitter allele (for example) could become more common. Indeed, the same paper proposes that these alleles can rapidly change in frequency in lab culture due to density-dependent selection. A more accurate procedure for investigating the frequency of natural genotypes in *Drosophila* is to sample individual females and use them to found independent isofemale or inbred lines. This method was used by Bauer and Sokolowski (1984), who show little support for the common alleles of large effect hypothesis. Fifteen fertilized females were collected from nature (source population not stated), and an isofemale line was started from each female's descendents. Path lengths were found to vary significantly among these 15 lines, implicating heritable (likely genetic) variation. However, the trait values of all lines shown would lead to their classification as rovers based on the previous definition. The data presented appear to vary continuously from ~50-85 mm, without two modes (based on visual inspection of figures 1 and 3 from Bauer and Sokolowski 1984). Thirteen of these lines were not classified as either rover or sitter, but the line (B1) with the shortest path length was classified as a sitter. This appears arbitrary, as the behavior of this line appears more similar to the "roving" *white*^{blood} stock than to the "sitting" *ebony*^{11} stock (Sokolowski, 1980). The strain with the longest path length (B15) was designated a "rover". The B1 and B15 lines were referred to as "extreme", but they do not appear to be statistical outliers compared to the other 13 lines (figure 1, Bauer and Sokolowski 1984). The primary support for a hypothesis that the genetic difference

between B1 and B15 was related to the difference between *white*^{blood} and *ebony*^{11} was that the mutation(s) causing the difference between the B1 and B15 strains were later mapped mainly to the second chromosome (Bauer and Sokolowski 1985). This is consistent with a large-effect allele, but it should be noted that the second chromosome comprises ~2/5 of the euchromatic *D. melanogaster* genome, and could therefore harbor causal variants in many genes (Adams *et al.*, 2000). The strain with the longest path (B15) appears to be the strain that was later used as the "reference" rover strain, renamed the *for*^{R} strain (sometimes also referred to as R, B15B15 or BB). The strain with the shortest path, however, does not appear to be the reference sitter strain (EE, S, or *for*^{s}) that was used in subsequent mapping experiments. Instead, strains with the second chromosome from B15 were compared to strains with the second chromosome from the *ebony*^{11} lab stock in later work (see below).

Other genotypes have also been compared, but these cases do not support the common allele of large effect hypothesis either. For example, artificial selection was performed on a *sepia* mutant stock collected by Timothy Prout near the University of California, Riverside, and kept in the lab for 15 years before the start of the experiment (Sokolowski and Hansell, 1983). The trait values of this stock before selection would classify them as "rovers" based on the previous definition (Sokolowski 1980, Sokolowski 1982), as the average path lengths were between 60 and 110 mm (Sokolowski and Hansell, 1983; Sokolowski, Hansell, and Rotin, 1983). Selection was successful in both directions, indicating that there was heritable variation in path length in this stock (Sokolowski, Hansell, and Rotin, 1983). However, this is not evidence that the stock was segregating a "sitter" allele of large effect. After selection, in fact, the population selected to be sitter-like would still have qualified as "rovers" based on the definitions provided (visual inspection of figure 3 from Sokolowski, Hansell, and Rotin [1983] suggests a range of ~40-80 mm after selection). These data are therefore consistent with multiple alleles of small or moderate effect.

Additional collections were made from the Toronto pear orchard in 1986, and laboratory stocks collected from different regions of a single pear were found to differ in average path length (Sokolowski *et al*, 1986). Only the mean values are shown, however, and no evidence of a bimodal distribution is presented. Stocks with the second and third chromosomes from the *ebony*^{11} strain (now called EE) and *white*^{blood} (now WW) stocks (and stocks derived from them) were reassayed for comparison to this fresh collection, and the newly sampled short-path-length strains had intermediate trait values compared to the EE and WW lab strains (figure 2 from Sokolowski *et al*, 1986; note the difference in scale between 2a and 2b). The shorter path strains were called sitters, and the longer path strains were called rovers, but this appears arbitrary compared to previous definitions. The only additional report of a bimodal distribution we are aware of is for a trait correlated with larval foraging distance, but this appears to be a truncation artifact, as flies given a larger area displayed continuous variation with a single mode (figure 2, Nagle and Bell 1987). Nearly all other work on rover and sitter that we are aware of focused only on the "reference" strains, discussed further below, and therefore cannot speak to the frequency of *foraging* alleles in nature. We therefore feel that these data, though they raise the interesting possibility of common behavioral morphs in nature, are insufficient to strongly support such a hypothesis.

Connecting genotype and phenotype

Regardless of whether or not there are common alleles of large effect at the *foraging* gene in natural populations, considerable evidence connects variation at *foraging* to variation in some

behaviors. Efforts to connect genotype and phenotype have focused nearly exclusively on three reference strains: *for*^{r}, *for*^{s}, and *for*^{s2}. This is understandable, due to the labor-intensive nature of behavioral characterization, but limits inference regarding natural populations. First, it is not clear that the *for*^{s} strain used has an allele recently isolated from nature, as is sometimes asserted (Kent *et al*. 2009; Edelsparre *et al*. 2014). Efforts to map the genetic basis of path length to a sub-chromosomal level were first published in de Belle, Hilliker, and Sokolowski (1989): the sitter allele used in these efforts is the one isolated from the *ebony*^{11} lab stock. The sitter strain, before being renamed *for*^{s}, is described as "EE". Based on Sokolowski (1980) and Sokolowski *et al*. (1986), this strain seems to have the second and third chromosomes, and therefore the *foraging* allele, from the *ebony*^{11} stock. The rover strain, renamed *for*^{R}, is also referred to as B15B15, and therefore appears to have the second and third chromosomes from the B15 strain (Bauer and Sokolowski 1984). This B15 or R stock is referred to as *for*^{R} by de Belle, Sokolowski, and Hilliker (1993), and appears to have become the reference rover strain. Supporting this inference is the common reference to the *for*^{R} strain as the parent of the irradiated strain *for*^{s2}, produced from B15B15 by de Belle, Hilliker, and Sokolowski (1989). However, de Belle, Sokolowski, and Hilliker (1993) also clearly indicate that the strain renamed *for*^{s} is the EE strain, which has the *foraging* allele from the *ebony*^{11} stock. The final identification of the gene *dg2* (a cGMP-dependent protein kinase, subsequently renamed *foraging*) as the gene harboring the *for*^{R} and *for*^{s} alleles does not state the source of the *for*^{s} allele, but the *ebony*^{11} stock seems likely as it was a continuation of the earlier mapping work, and uses the same stock name (Osborne *et al*., 1997). Other sitter-like chromosomes that failed to compliment the *ebony*^{11} allele have also been referred to as *for*^{s} in the literature (Sokolowski, Pereira, and Hughes 1997), so it is possible that other alleles were used.

Regardless of the source of the *for*^{s} allele, the *for*^{R} and *for*^{s} strains were created by chromosome extraction (Sokolowski 1980; Sokolowski *et al*. 1986; de Belle, Sokolowski, and Hilliker, 1989), and therefore differ at a very large number of loci. As a result, it is not possible to associate traits with *foraging* alleles simply by measuring the phenotype of these strains (e.g. MacPherson *et al*. 2004; Foucaud et al. 2013). Thankfully, most studies also use the *for*^{s2} strain (Kaun et al. 2007; Mery et al. 2007; Kaun, Chakaborty-Chatterjee & Sokolowski 2008; Kent et al. 2009; Chen et al. 2011). This sitter-like strain was created from the reference rover strain using high doses of gamma radiation (5000 rad; de Belle, Hilliker and Sokolowski 1989). This strain therefore shares a common background with *for*^{R}, though it seems likely to have secondary mutations, despite assertions that it differs from *for*^{R} only at *foraging* (Mery et al. 2007; Reaume, Sokolowski and Mery 2011; Edelsparre et al. 2014). We note, however, that the association with larval foraging behavior is supported by more substantial evidence (Osborne *et al*., 1997), and some trait associations have additional support from transgenic manipulation (Kaun et al. 2007; Mery et al. 2007; Donlea et al. 2012; Edelsparre et al. 2014). To our knowledge, however, the genome sequences of the reference strains have not been reported.

It therefore seems that, although variation at the gene *foraging* can clearly affect interesting behaviors, additional evidence is required to support a hypothesis of large-effect alleles at high frequency in nature, which affect many aspects of physiology and behavior, and thereby create two behavioral "morphs" maintained by balancing selection. To collect additional data, we have quantified larval foraging behavior on a set of inbred lines isolated from a wild population and attempted to associate trait variation with genotype. Our analysis fails to support the common-alleles of large effect hypothesis nor were we able to map a causative nucleotide difference segregating at the *for* locus. Our analyses of patterns of variation at the locus also do

not support that hypothesis that *foraging* is a target of balancing selection in the *Drosophila* genome.

METHODS

Fly strains. The reference for^{r}, for^{s}, and for^{s2} strains were provided by Marla Sokolowski. We also used inbred lines from the Raleigh (RAL) collection, provided by the Bloomington Drosophila Stock Center. These lines were collected by the Mackay lab in Raleigh, North Carolina, and each line underwent full-sibling inbreeding for 20 generations to eliminate most genetic variation (Ayroles *et al.* 2009; Mackay *et al.* 2012). All lines were maintained in 25x95 mm vials on molasses medium in standard *Drosophila* incubators, at 25˚C under a 12-hr light/dark cycle.

Behavioral measurement. The rover/sitter phenotype was measured for each line similarly to previously published methods (deBelle *et al*. 1987). Oviposition bottles were prepared with grape juice agar plates with yeast paste added. Parents were allowed to lay eggs freely for 1 hour at 25°C, after which the plates were incubated overnight at 25°C. Twenty-four hours later, individual newly hatched larvae were carefully removed from the oviposition plates using forceps and transferred to Petri plates containing 35 mL standard *Drosophila* food. No more than 50 larvae were grown in each plate to avoid overcrowding. They were allowed to grow in these plates until 96 hours post-hatching. At this time, each third instar larva was removed from the food and individually tested for foraging behavior. Only larvae found within the food were tested. Any larvae on the food surface or on the surface of the Petri plate were not used. For the behavior test, a thin layer of yeast paste was applied to a custom-built plastic plate using a rubber squeegee. A larva was placed into the middle of the yeast paste, the yeast paste was covered with half a Petri plate, and the larva moved freely for five minutes. After this time, the larval trail, visible in the yeast paste, was traced by pen onto the covering Petri plate. The marked plate was photographed, and the length of the larval path was measured using the ImageJ software package. Sample sizes per line varied from 28-81 (median n=54); raw data are available as supplementary data. The R software package was used to further analyze the results.

Genome-wide association study. Associations were conducted using the $\log_{10}$ transformed mean path length for each line. Transformed trait values for the 36 lines were uploaded in December 2013 to the Drosophila Genetic Reference Panel (DGRP) webtool available at dgrp.gnets.ncsu.edu. This tool, created by the Mackay lab (Mackay et al. 2012) performs associations between 1,303,322 genetic variants and trait vales using ANOVAs of the form $Y = \mu + M + \varepsilon$, where M is the effect of a genetic variant. Associations were done with only 35 of the 36 genotypes, as no genome was available for strain RAL-765. Full results are available as supplementary data.

Molecular Population Genetics. All population genetic analysis was performed on the complete data from Mackay et al. (2012). To test for selection acting at the *for* locus we performed a lineage-specific McDonald-Kreitman test (McDonald and Kreitman 1991) and compared results from that test to lineage-specific McDonald-Kreitman tests from every other protein coding locus throughout the genome using *D. simulans w501* and *D. yakuba* genomes (Begun et al. 2007) as outgroups for polarization of fixed differences. A similar test was also performed using

unpolarized comparisons. For summary statistics of patterns of polymorphism and divergence we calculated nucleotide diversity (Tajima 1983), Tajima's *D* (Tajima 1989), and the ratio of nucleotide diversity to divergence on the *D. melanogaster* lineage ignoring repetitive regions masked by RepeatMasker (repeatmasker.org). These statistics were also calculated on synonymous and nonsynonymous sites separately. For nearly every summary statistic, we calculated the same statistic for the longest transcript from each protein coding locus throughout the genome to establish the genome-wide empirical distribution from which to compare population genetic summaries at *for*. All population genetic analysis was performed using scripts written in Ruby or Python and are available upon request.

RESULTS

Foraging path lengths were measured in the reference rover and sitter strains as well as 36 natural isolates of *D. melanogaster* from the RAL collection (figure 1). These strains were captured in Raleigh, North Carolina, and underwent full-sib mating for 20 generations (Ayroles *et al.* 2009). This procedure produces an inbred genotype while allowing very little opportunity for selection (though some alleles, like recessive lethals, are purged).

The for^{s} and for^{s2} strains were found to have shorter average path lengths (1.30 and 1.71 cm) than the for^{R} strain (2.66 cm), as expected. The foraging paths of the for^{s} strain were significantly shorter than the for^{s2} strain (t-test p=0.002), and both are significantly shorter than the for^{R} strain (t-test p=1.8E-6 and p=0.016, respectively). All reference strains traveled shorter distances compared to published data. In Pereira and Sokolowski (1993), for example, for^{s2} larvae average 10.89 cm and for^{R} 17.83 cm in the same period of observation.

Data from the 36 inbred RAL strains are distributed with only one mode. The mean path length among lines is significantly non-normal (Shapiro-Wilk's Test p=0.015), but not after $\log_{10}$ transformation (Shapiro-Wilk's Test p=0.075). Only three strains had shorter paths than the for^{s} strain, while only five strains traveled farther than the for^{r} strain: the majority of strains fell between these two reference points (figure 1).

To investigate whether any of the phenotypic variation among RAL strains may be due to alleles at the *foraging (for)* gene, we performed a genome-wide association study, and also an association study at the *for* gene only. In the latter case, the *a priori* expectation of an association at this locus reduces the loss of power from multiple testing. As annotated at flybase.org (genome assembly version 5.54) the *for* gene is found on the left arm of chromosome 2 from base pairs 3,622,074-3,656,953. We analyzed the 607 single nucleotide polymorphisms (SNPs) that are mapped to this interval in our 35 sequenced lines; 5,000 bp to either side were also included as potential regulatory regions. The most significant association in this interval had a p-value of only 0.018, uncorrected for multiple tests (figure S1). With 607 parallel tests, this variant is not close to the Bonferroni-corrected significance value of 8.24E-5. Less conservative corrections also fail to support an association at this locus : there is no excess of low p-values in this interval compared to the null expectation (figure S2).

At the genomic level, there is a slight excess of low p-values compared to the neutral expectation (figure S2 and figure 2). No SNPs are significant after Bonferroni correction for 1,303,332 tests. However, there are 4 SNPs with p-values less than 1E-6, where only one is expected by chance (false discovery rate estimate of 25%). All four of these SNPs are within or adjacent to genes that could plausibly affect foraging behavior. The most significant SNP

(p=8.14E-8) is in an intron of the gene *sulfateless* (figure 3). *Sulfateless* is the homolog of the mammalian enzyme glucosaminyl N-deacetylase-N-sulfotransferase, involved in the synthesis of heparan sulfate (Ren *et al.* 2003, Kamimura *et al.* 2013). Loss of function mutations at *sulfateless* in *D. melanogaster* cause locomotory defects in larvae by altering synaptic transmission at the neuromuscular junction (Ren *et al.* 2009). The second most significant SNP (p=1.39E-7) is in an intergenic region between *HSP60B* and *Excitatory amino acid transporter 2* (*Eaat2*). *Eaat2* is expressed in both the central and peripheral nervous system of third instar larvae (Besson *et al.* 2011). *Eaat2* is a glutamate:sodium symporter, and glutamate is the excitatory neurotransmitter at the neuromuscular junction (Jan and Jan 1976). Though loss-of-function mutations at *Eaat2* do not seem to impair larval locomotory performance, these mutations do affect how larvae respond to chemosensory stimuli such as salt and propionic acid (Besson *et al*. 2011). If the putatively significant SNP near *Eaat2* affected the expression of this gene, it could plausibly alter how larvae sense and respond to food in their environment. The third most significant SNP (p=2.28E-7) is in the 3' UTR of the gene *Shaker cognate w* (*Shaw*). *Shaw* is a voltage-dependent potassium channel in the Kv3 family expressed broadly in the nervous system, and affects neuronal excitability when perturbed (Hodge *et al.* 2005). This SNP is only 70.4 Kb from the *for* gene -- though there are 27 other genes annotated between *Shaw* and *for*. Finally, the fourth most significant SNP (p=7.26E-7) is within an intron of the uncharacterized gene *CG32204*. FlyAtlas gene expression data indicate that this gene is strongly expressed in the larval and adult nervous system, with little expression detected in other tissues (Chintapalli *et al*. 2007).

Patterns of Polymorphism and Divergence

To investigate whether there is any signature of balancing or frequency dependent selection acting at the *for* locus (Fitzpatrick *et al.* 2007), we examined patterns of polymorphism and divergence at *for* using a large set of genome sequences derived from a North American population of *D. melanogaster* (Mackay *et al.* 2012). If some flavor of balancing selection was acting at this locus we would expect to see elevations in polymorphism relative to divergence either throughout the locus or in a particular region of the locus that is the target of such selection. The *for* locus, which encodes a rather long protein, has 18 nonsynonymous polymorphisms and 76 synonymous polymorphisms in the DGRP sample. Accounting for the total length of the longest transcript at *for* this amounts to 0.0063 nonsynonymous sites per base pair (56^{th} percentile among all genes) and 0.028 synonymous sites per base pair (89^{th} percentile). Nucleotide diversity paints a rather similar picture. Locus-wide π=0.0072, which is greater than median diversity but only at the 84^{th} percentile among all genes. Nucleotide diversity at synonymous and nonsynonymous sites appears no different; π_N=0.00061 (59^{th} percentile genome-wide) and π_S=0.017 (91^{th} percentile genome-wide). Thus while *for* shows above average diversity, it does not seem to be a strong outlier as might be expected from an ancient balanced polymorphism.

To formally test the neutral model at the *for* locus we looked at the site frequency spectrum of polymorphism and comparisons of polymorphism and divergence. Under a model of balancing selection one expects to see a skew in the site frequency spectrum towards intermediate frequency polymorphisms. Using Tajima's *D* statistic, this would translate into a strongly positive value of *D*. At *for* we find that $D = 0.6074$, which when tested against coalescent simulations under the standard neutral model yields a p-value of $p = 0.204$ in a one-side test. Further, compared to the genome-wide empirical distribution of *D* statistics for protein

coding genes *for* is at the 74th percentile. This observation suggests that the site frequency spectrum at *for* is not particularly unusual or strongly skewed towards intermediate frequencies.

Another strong prediction under a model of balancing selection is that the ratio of polymorphism and divergence should be skewed in favor of too much polymorphism relative to divergence under neutrality. We tested this prediction in three different ways. First we used an unpolarized McDonald-Kreitman (MK) test to examine patterns of polymorphism and divergence at synonymous and nonsynonymous sites at *for* using *D. simulans* as an outgroup. The unpolarized MK test (Table 1) is non-significant (p=0.30; Fisher's exact test) and has a Neutrality Index (N.I.) of 0.64 (Rand and Kahn 1996). Comparing the N. I. of *for* to the empirical distribution from all protein-coding genes in the genome, we find that *for* is near the middle of the distribution (31st percentile). The second test of polymorphism and divergence we performed was a polarized MK test where we examined only fixations along the *D. melanogaster* lineage. The results are qualitatively similar in this case (p=0.26; N.I.=0.53; 16th percentile). Thus neither polarized nor unpolarized MK tests can reject the neutral model.

Finally we were interested in examining the ratio of polymorphism and divergence directly from the entire locus and from individual windows in a Hudson-Kreitman-Aguadé like test (Hudson et al. 1989). The ratio of nucleotide diversity to divergence (again using only fixations along the *D. melanogaster* lineage) for the whole *for* locus is equal to π/div=0.419. Comparing this ratio to that of all proteins throughout the genome, *for* is on the higher-end of the empirical distribution but not a strong outlier (91st percentile). We also examined sliding windows of the ratio of polymorphism to divergence (π/div) and the results from this analysis are shown in figure 4. This analysis revealed perhaps the only remarkable feature of genetic variation in the *for* region. While the ratio of polymorphism to divergence throughout the locus is below one and rather average for the genome, there is a strong peak in polymorphism relative to divergence approximately 8kb 3' of the *for* locus. Unfortunately this peak does not overlap any known regulatory sequences of *for* and moreover there are two proteins coding genes between this peak and the end of the *for* coding sequence. Thus while this may be an intriguing finding we have no ability to say whether this is associated with *for* or not. After observing that this region was unusual, we checked the GWAS data to see if any variants in this interval were associated with foraging behavior. Thirty-eight SNPs in this interval (3,613,450 - 3,613,450) were included in the GWAS. The most significant association of these is a G/T polymorphism at 6,614,038 (p=0.0047; p = 0.16 after correcting for 38 parallel tests).

DISCUSSION

There is little doubt that decades of work on variation at the gene *foraging* has proven interesting. Considerable mapping efforts cumulated in the connection of variation in larval behavior to the gene *foraging* (Osborne *et al*., 1997), and this catalyzed additional research into this interesting gene in other systems (Ben-Shahar *et al*. 2003). The prevalence of large-effect alleles in nature, however, is not well established, despite published claims that *D. melanogaster* populations are comprised of 70% rovers and 30% sitters (Osborne *et al* 1997; Sokolowski 2001). Published data are subject to alternative interpretation due to uncontrolled environmental variation (Sokolowski 1980; Sokolowski 1982) or mass culture (Sokolowski, Pereira & Hughes 1997). The only published data using independently sampled genotypes reared in a common environment, that we are aware of, does not appear to support a gene of large effect (Bauer & Sokolowski 1984). To gather additional data about variation in larval foraging behavior, we have

assayed a set of 36 inbred lines derived from flies collected in the Raleigh, North Carolina farmer's market. Distribution of larval foraging behavior among these lines is not bimodal, nor is trait variation associated with genetic variation at or near the *foraging* locus.

Using standard analyses from molecular population genetics we sought to detect any signature of balancing selection that may maintain two (or more) alleles at *for* in nature as has been suggested in the literature (Fitzpatrick *et al*. 2007). None of our analyses suggest that *for* is under strong balancing selection, nor do they suggest that patterns of variation at *for* are somehow unusual within the *D. melanogaster* genome. Given the very large sample of genomes in this analysis, we have no reason to believe that our statistical tests should be in any way underpowered. Thus our failure to reject the null model of neutrality for both patterns of polymorphism and divergence as well as the site frequency spectrum suggests that strong balancing selection at *for* is unlikely.

These negative results have several major caveats. One possibility is that the trait we measured is not a high-fidelity replication of the "foraging behavior" measured in previous publications. It is notable that, though the for^R strain had a path length more than twice as long as for^s in our data, the lengths of all paths are shorter than reported for these strains previously, despite careful replication of the phenotyping protocols published earlier. If subtle differences in larval condition or assay environment are crucial and different, the effect of *for* might fail to be expressed. Furthermore, we only assessed larval foraging distance in the presence of food. This is consistent with and directly comparable to most published data, including both published bimodal distributions. It seems possible that *foraging* variation would be more apparent if the difference in path length with and without food was assayed—especially if there were other genetic variation affecting overall motility. In contrast to the isofemale lines and outbred populations described previously, the RAL lines used here are inbred, and any recessive variation in overall performance could be a more substantial confounder. We also note that there could be genetic variants at the *for* locus that are not annotated in the RAL genome alignments. Though large-effect mutations have been found in nature for traits like pigmentation and insecticide resistance, it is still unclear when and why such large effects should be expected (Stern & Orgogozo 2008; Rockman 2012), and we hope that our negative results will motivate further work in this area.

Finally, despite the overall negative result, we report that larval path length is putatively associated with genetic variants at several loci other than *for*. Though the genes underlying these associations are intriguing, these associations should be considered preliminary until further replication is conducted. Attempting a genome-wide association study with only 35 lines is an ambitious scheme (Long & Langley 1999), and one that is unlikely to be successful unless trait variation is due primarily to a single gene (as previously postulated for larval foraging). Despite this fact, it is encouraging that there is a slight increase in low p-values compared to a null expectation of a uniform distribution from zero to one. This null expectation could be violated for reasons other than true positives, such as cryptic population structure or other violations of the assumptions of the technique. The four most significant associations are all in or near genes that could plausibly affect larval motility, and we hope this result will motivate further work on this reference collection of genotypes.

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REFERENCES

Adams, MD, Celniker SE, Holt RA, Evans CA, Gocayne JD 2000. The genome sequence of *Drosophila melanogaster*. Science 287: 2185-2195.

Ayroles, JF, Carbone MA, Stone EA, Jordan KW, Lyman RF *et al.* (2009) Systems genetics of complex traits in *Drosophila melanogaster*. Nat. Genet. 41: 299–307.

Bauer SJ, Sokolowski MB (1984) Larval foraging behavior in isofemale lines of *Drosophila melanogaster* and *D. pseudoobscura*. J. of Heredity 75: 131-134.

Bauer SJ, Sokolowski MB (1985) A genetic analysis of path length and pupation height in a natural population of *Drosophila melanogaster*. Genome 27(3): 334–340.

Begun DJ, Holloway AK, Stevens K, Hillier LW, Poh YP, *et al.* (2007) Population genomics: whole-genome analysis of polymorphism and divergence in *Drosophila simulans*. Plos Biology 5(11): e310.

Ben-Shahar Y, Leung H-T, Pak WT, Sokolowski MB, Robinson GE (2003) cGMP-dependent changes in phototaxis: a possible role for the foraging gene in honey bee division of labor. J Exp Biol 206, 2507-2515.

Chintapalli VR, Wang J, Dow JAT (2007). Using FlyAtlas to identify better *Drosophila* models of human disease. Nature Genetics 39: 715-720

de Belle JS, Sokolowski MB (1987) Heredity of rover/sitter: Alternative foraging strategies of *Drosophila melanogaster* larvae. 59: 73-83.

de Belle JS, Hilliker AJ, Sokolowski MB (1989) Genetic localization of *foraging (for)*: A major gene for larval behavior in *Drosophila melanogaster*. Genetics 123: 157-163.

de Belle JS, Sokolowski MB, Hilliker AJ (1993) Genetic analysis of the *foraging* microregion of *Drosophila melanogaster*. Genome. 36, 94–101.

Besson MT, Sinakevitch I, Melon C, Iché-Torres M, Birman S (2011). Involvement of the *Drosophila* taurine/aspartate transporter dEAAT2 in selective olfactory and gustatory perceptions. J. Comp. Neurol. 519(14): 273-257.

de Bono M, Bargmann C (1998) Natural variation in a neuropeptide Y receptor homolog modifies social behavior and food response in *C. elegans*. Cell 94(5): 679–689

Donlea J, Leahy A, Thimgan MS, Suzuki Y, Hughson BN, Sokolowski MB, Shaw PJ (2012) *foraging* alters resilience/vulnerability to sleep disruption and starvation in *Drosophila*. Proc. Natl. Acad. Sci. U.S.A. 109(7): 2613--2618.

Chen A, Kramer EF, Purpura L, Krill JL, Zars T, Dawson-Scully K (2011). The influence of natural variation at the foraging gene on thermotolerance in adult *Drosophila* in a narrow temperature range. J. Comp. Physiol. A, Neuroethol. Sens. Neural. Behav. Physiol. 197(12): 1113--1118.

Edelsparre AH, Vesterberg A, Lim JH, Anwari M, Fitzpatrick MJ. 2014. Alleles underlying larval foraging behaviour influence adult dispersal in nature. Ecology Letters, pre-published online Jan 6, 2014. DOI: 10.1111/ele.12234

Ephrussi B, Herold JL (1945) Studies of eye pigments of *Drosophila*. Genetics 30: 62--83

Fitzpatrick MJ, Feder E, Rowe L, Sokolowski MB (2007) Maintaining a behaviour polymorphism by frequency-dependent selection on a single gene. Nature. 447: 210-212.

Foucaud, J, Philippe AS, Moreno C, Mery F (2013) A genetic polymorphism affecting reliance on personal versus public information in a spatial learning task in *Drosophila melanogaster*. Proc. Biol. Sci. 280(1760): 20130588

Gioia A, Zars T (2009). Thermotolerance and place memory in adult *Drosophila* are independent of natural variation at the *foraging* locus. Journal of Comparative Physiology 195: 777-782.

Graf SA, Sokolowski MB (1989) Rover/sitter *Drosophila melanogaster* larval foraging polymorphism as a function of larval development, food-patch quality, and starvation. Journal of Insect Behavior 2(3) 301-313.

Griffith OL, Montgomery SB, Bernier B, Bernier B, Chu B, Kasaian K, et al. (2008) ORegAnno: an open-access community-driven resource for regulatory annotation. Nucleic Acids Res 36: D107-D113.

Hartigan, JA, Hartigan PM (1985) The dip test of unimodality. Annals of statistics 13(1): 70-84.

Hodge JJL, Choi JC, O'Kane CJ, Griffith LC (2005). *Shaw* potassium channel genes in *Drosophila*. J. Neurobiol. 63(3): 235-254

Hudson RR, Kreitan M, Aguade M (1987) A test of neutral molecular evolution based on nucleotide data. Genetics 116(1): 153-159.

Ioannidis JPA (2005) Why most published research findings are false. PLoS Med 2(8): e124.

Jan LY, Jan YN (1976) L Glutamate as an excitatory transmitter at the *Drosophila* larval neuromuscular junction. Journal of Physiology. 262(1): 215-236

Kamimura K, Ueno K, Nakagawa J, Hamada R, Saitoe M, Maeda N (2013) Perlecan regulates bidirectional Wnt signaling at the *Drosophila* neuromuscular junction. Journal of Cell Biology 200(2): 219-233.

Kaun KR, Chakaborty-Chatterjee M, Sokolowski MB (2008). Natural variation in plasticity of glucose homeostasis and food intake. J. Exp. Bio. 211: 3160-3166.

Kaun KR, Hendel T, Gerber B, Sokolowski MB (2007) Natural variation in *Drosophila* larval reward learning and memory due to a cGMP-dependent protein kinase. Learn. Mem. 14(5): 342-349.

Kent WJ, Sugnet CW, Furey TS, Roskin KM, Pringle TH, Zahler AM, Haussler D (2002) The human genome browser at UCSC. Genome Res. 12(6):996-1006.

Kent CF, Daskalchuk T, Cook L, Sokolowski MB, Greenspan RJ (2009) The *Drosophila foraging* gene mediates adult plasticity and gene–environment interactions in behaviour, metabolites, and gene expression in response to food deprivation. PLoS Genet 5(8): e1000609. doi:10.1371/journal.pgen.1000609

Lawson, LP, Vander Meer RK, Shoemaker D (2012) Male reproductive fitness and queen polyandry are linked to variation in the supergene Gp-9 in the fire ant *Solenopsis invicta*. Proc. R. Soc. B. 279(1741): 3217-3222.

Long AD, Langley CH (1999) The power of association studies to detect the contribution of candidate genetic loci to variation in complex traits. Genome Research 9: 720-731.

Mackay, TFC, Richards S, Stone EA, Barbadilla A, Ayroles JF, et al. (2012) The *Drosophila melanogaster* genetic reference panel. Nature 482: 173–178.

McDonald J, Kreitman M (1991) Adaptive protein evolution at the *Adh* locus in *Drosophila*. Nature 351: 652-654.

MacPherson MR, Broderick KE, Graham S, Day JP, Houslay MD, Dow JA, Davies SA (2004) The *dg2* (*for*) gene confers a renal phenotype in *Drosophila* by modulation of cGMP-specific phosphodiesterase. J. Exp. Biol. 207(16): 2769-2776.

McGrath PT, Rockman MV, Zimmer M, Jang H, Macosko EZ, Kruglyak L, Bargmann CI (2009) Quantitative mapping of a digenic behavioral trait implicates globin variation in *C. elegans* sensory behaviors. Neuron 61(5): 692-699.

Mery F, Belay AT, So AK, Sokolowski MB, Kawecki TJ (2007) Natural polymorphism affecting learning and memory in Drosophila. Proc. Natl. Acad. Sci. U.S.A. 104(32): 13051-13055.

Montgomery S, Griffith OL, Sleumer MC, Bergman CM, Bilenky M, Pleasance E, Prychyna Y, Zhang X, Jones SJ (2006) ORegAnno: an open access database and curation system for literature-derived promoters, transcription factor binding sites and regulatory variation. Bioinformatics 22: 637-640.

Nagle KJ, Bell WJ (1987) Genetic control of the search tactic of *Drosophila melanogaster*: An ethometric analysis of rover/sitter traits in adult flies. Behavior Genetics 17(4), 385-408.

Osborne KA, Robichon A, Burgess E, Butland S, Shaw RA, Coulthard A, Pereira HS, Greenspan RJ, Sokolowski MB (1997) Natural behavior polymorphism due to a cGMP-dependent protein kinase of *Drosophila*. Science 277(5327): 834-836

Pereira HS, Sokolowski MB (1993) Mutations in the larval foraging gene affect adult locomotory behavior after feeding in *Drosophila melanogaster*. PNAS 90:5044-5046.

Rand DM, Kahn LM (1996) Excess amino acid polymorphism in mitochondrial DNA: contrasts among genes from *Drosophila*, mice, and humans. Mol. Bio. Evol. 13(6): 735-748.

Reaume CJ, Sokolowski MB, Mery F (2011). A natural genetic polymorphism affects retroactive interference in *Drosophila melanogaster*. Proc. Biol. Sci. 278(1702): 91-98.

Ren Y, Kirkpatrick CA, Rawson JM, Sun M, Selleck SB (2003) Cell type-specific requirements for heparan sulfate biosynthesis at the *Drosophila neuromuscular* junction: Effects on synapse function, membrane trafficking, and mitochondrial localization. The Journal of Neuroscience. 29(26): 8539-8550.

Renger JJ, Yao W-D, Sokolowski MB, Wu C-F (1999) Neuronal polymorphism among natural alleles of a cGMP-dependent kinase gene, *foraging*, in *Drosophila*. J of Neuroscience 19: RC28

Rockman MV, Kruglyak L (2009) Recombinational landscape and population genomics of *Caenorhabditis elegans*. PLoS Genet 5(3): e1000419.

Rockman, MV (2012) The QTN program and the alleles that matter for evolution: All that's gold does not glitter. Evolution 66(1): 1-17.

Service, PM (2004) How good are quantitative complementation tests? *Sci. Aging Knowl. Environ.* 12: 13.

Sokolowski MB (1980) Foraging strategies of *Drosophila melanogaster*: A chromosomal analysis. Behavior Genetics. 10:3, 291-302

Sokolowski MB (1982) Rover and sitter larval foraging patterns in a natural population of *D. melanogaster*. Dros. Inform. Serv. 58:138-139.

Sokolowski MB, Hansell RIC (1983) *Drosophila* larval foraging behavior. I. The sibling species, *D. melanogaster* and *D. simulans*. Behav. Genet. 13:159-168.

Sokolowski MB, Hansell RIC, Rotin D (1983) *Drosophila* larval foraging behavior. II. Selection in the sibling species, *D. melanogaster* and *D. simulans*. Behav. Genet. 13(2): 169-177.

Sokolowski MB, Bauer SJ, Wai-Ping V, Rodriguez L, Wong JL, Kent C (1986) Ecological genetics and behaviour of *Drosophila melanogaster* larvae in nature. Animal Behavior 34, 403-408.

Sokolowski MB, Pereira HS, Hughes K (1997) Evolution of foraging behavior in *Drosophila* by density-dependent selection. PNAS 94: 7373-7377

Sokolowski MB (2001) *Drosophila*: Genetics meets behaviour. Nature Reviews Genetics 2, 879-890.

Stern C (1926) Eine kreuzungsanalyse von korperfarbungen von *Drosophila melanogaster* verbunden mit drei neuen allelomorphen des faktors 'ebenholz'. Z. indukt. Abstamm.- u. VererbLehre 41: 198--215.

Stern DL, Orgogozo V (2008) The loci of evolution: How predictable is genetic evolution? Evolution 62(9): 2155-2177.

Tajima, F (1983) Evolutionary relationship of DNA-Sequences in finite populations. Genetics 105(2): 437-460.

Tajima, F (1989) Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. Genetics 123(3): 585-595.

Thomas JW, Cáceres M, Lowman JJ, Morehouse CB, Short ME, Baldwin ML, Maney DL, Martin CL (2008) The chromosomal polymorphism linked to variation in social behavior in the white-throated sparrow (*Zonotrichia albicollis*) is a complex rearrangement and suppressor of recombination. Genetics 179(3): 1455-1468.

Figure 1. Variation in larval foraging behavior. Boxplot of foraging path lengths for 36 inbred RAL strains (not individually labeled, for clarity) and the reference rover (r), sitter (s) and sitter 2 (s2) genotypes. The median for each line is shown, surrounded by a box (1st to 3rd quartiles), whiskers (range excepting outliers), and circles (outliers); sample sizes per line vary from 28 to 81 (median n=54).

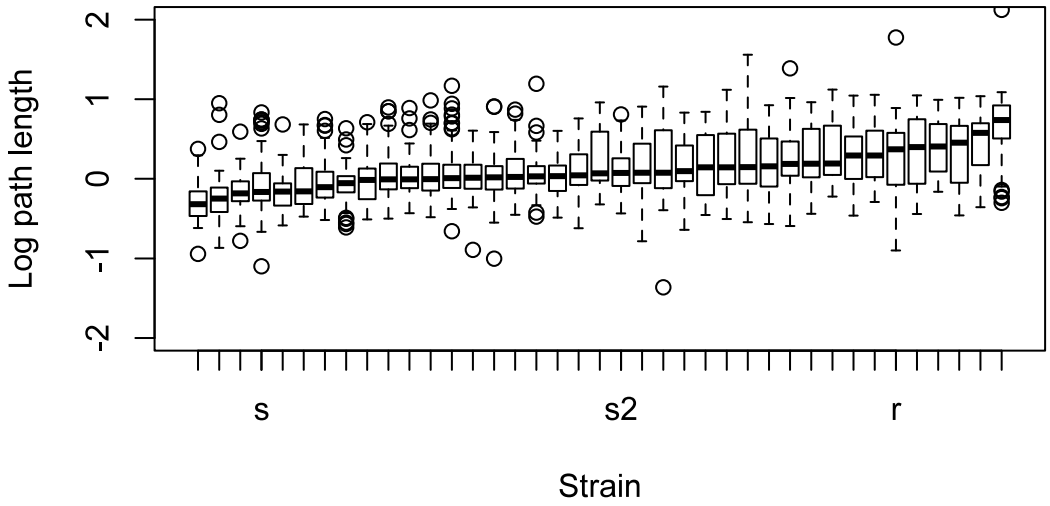

Figure 2. Genome-wide association study results. A Manhattan plot of *p*-values for all variants is shown. Chromosome arms are shown in alternating colors, and the four most significant variants are circled in red.

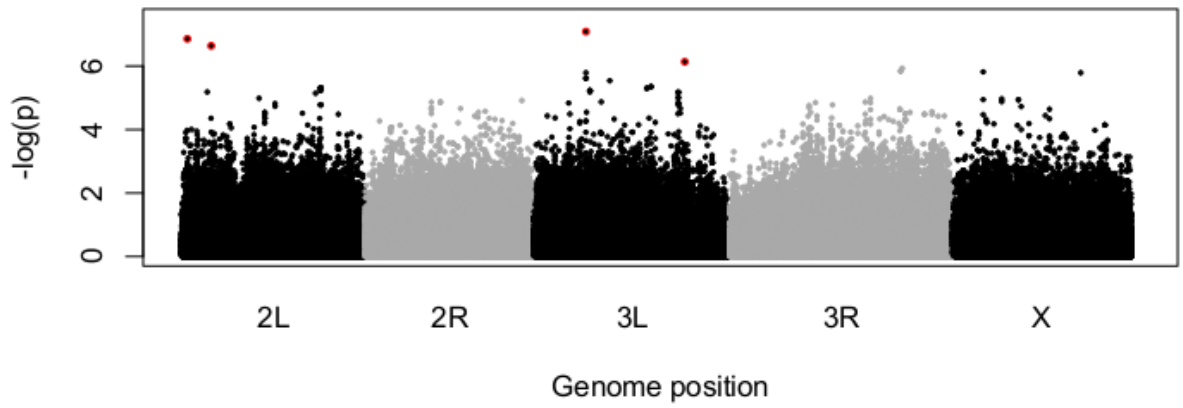

Figure 3. The most significant association in the genome. A G/A polymorphism on the left arm of chromosome 3 at base pair 6,537,335 was the most significant in the genome. Shown are the phenotypes of the 5 lines with a G and the 31 lines with an A. Associations were done with log transformed data, but non transformed data are shown for clarity.

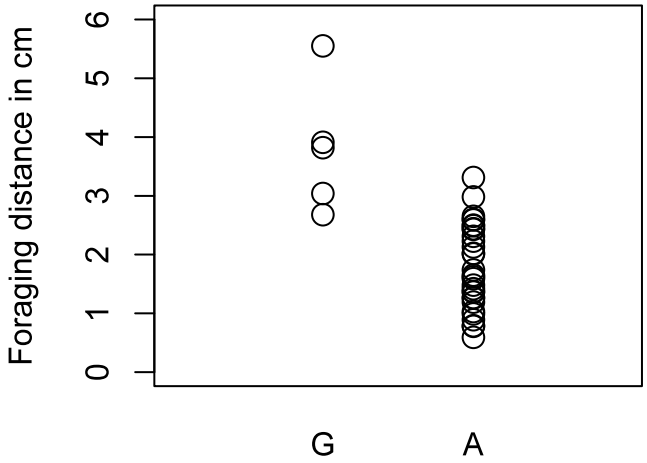

Figure 4. Polymorphism and divergence at the *for* locus. An image from the UCSC Genome Browser (Kent et al. 2002) showing the various isoforms of for and it's flanking regions. The ratio of nucleotide diversity to polarized divergence on the *D. melanogaster* lineage is shown in 1 kb windows sliding every 100 bp, labeled as piOverDiv. Regulatory elements from ORegAnno (Montgomery et al. 2006; Griffith et al. 2008) are also shown, as are repetitive elements from RepeatMasker (http://www.repeatmasker.org).

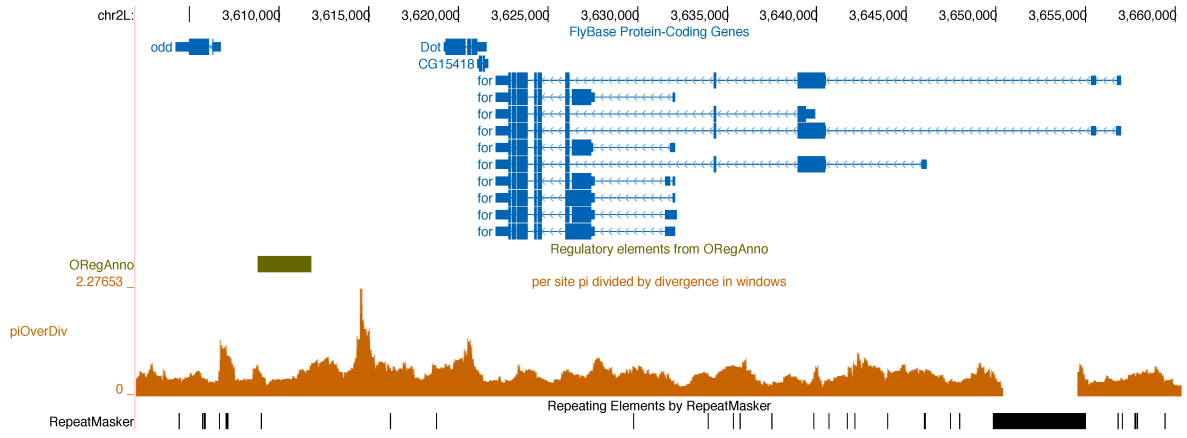

Table 1. Unpolarized McDonald-Kreitman test of *for*.

	Polymorphisms	Fixed differences
Nonsynonymous	18	14
Synonymous	76	38

Figure S1. Genome-wide association study at *foraging*. A Manhattan plot of *p*-values at the *foraging* locus are shown on the same scale as figure 2.

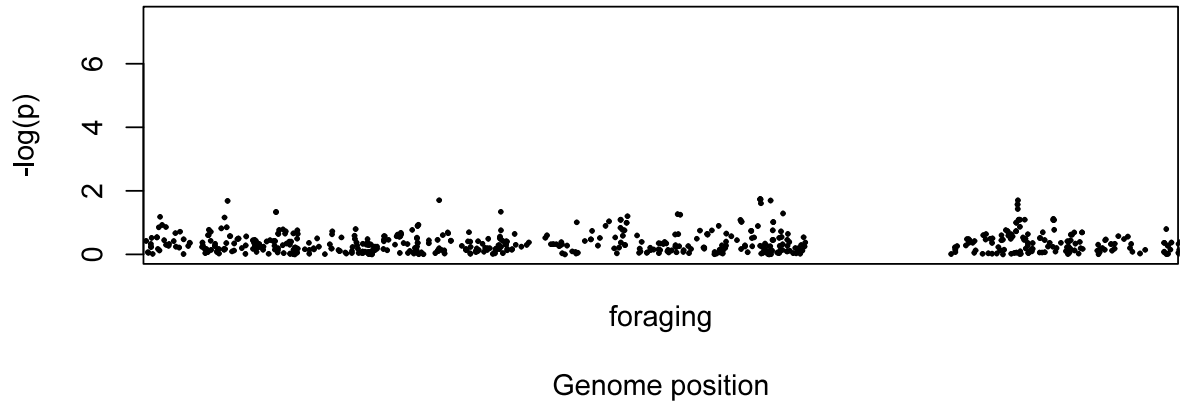

Figure S2. Distribution of *p*-values at *foraging*. The observed *p*-values for the 607 SNPs at *foraging* are shown against an expected (uniform) expectation.

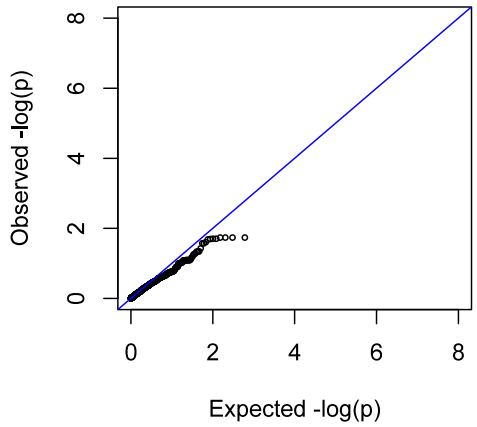

Figure S3. Distribution of *p*-values genome-wide. The observed *p*-values for the 1,301,321 SNPs genome-wide are shown against an expected (uniform) expectation.