Genetic Architecture of Natural Variation Underlying Adult Foraging Behavior That Is Essential for Survival of *Drosophila melanogaster*

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Abstract

Foraging behavior is critical for the fitness of individuals. However, the genetic basis of variation in foraging behavior and the evolutionary forces underlying such natural variation have rarely been investigated. We developed a systematic approach to assay the variation in survival rate in a foraging environment for adult flies derived from a wild *Drosophila melanogaster* population. Despite being such an essential trait, there is substantial variation of foraging behavior among *D. melanogaster* strains. Importantly, we provided the first evaluation of the potential caveats of using inbred *Drosophila* strains to perform genome-wide association studies on life-history traits, and concluded that inbreeding depression is unlikely a major contributor for the observed large variation in adult foraging behavior. We found that adult foraging behavior has a strong genetic component and, unlike larval foraging behavior, depends on multiple loci. Identified candidate genes are enriched in those with high expression in adult heads and, demonstrated by expression knock down assay, are involved in maintaining normal functions of the nervous system. Our study not only identified candidate genes for foraging behavior that is relevant to individual fitness, but also shed light on the initial stage underlying the evolution of the behavior.

Key words: genome-wide association study, foraging behavior, Drosophila melanogaster, inbreeding depression.

Introduction

Maintaining energy balance is critical for the survival and reproduction of an animal (Burger et al. 2007; Rion and Kawecki 2007). Foraging behavior, which comprises of both energy expenditure (searching and working for food) and energy intake (food consumption), is accordingly central to individual fitness (Stephens and Krebs 1987). It is often assumed that various physiological and neural processes (e.g., energy sensing, physiological state, reward learning, memory, decision-making, motor functions) influence foraging behavior (Glimcher 2002; Itskov and Ribeiro 2013; Stephens and Krebs 1987), and it is conceivable that mechanisms underlying these different components have been under selection to increase individual fitness. Furthermore, evolution of foraging behavior is dependent upon the evolvability of the behavior, in which the underlying genetic variation within populations is a critical component.

However, most genetic studies of foraging behavior focus on specific aspects of the trait (e.g., energy sensing Burke and Waddell 2011; Dus et al. 2011, food search Masse et al. 2009; Montell 2009; Yarmolinsky et al. 2009), and/or the molecular mechanisms of a limited number of candidate genes

(Sokolowski et al. 1997; Sokolowski 2001; Chen et al. 2012). Lack of efforts in investigating the genetic variation for foraging behavior and the associated genetic elements in natural populations have stagnated the understanding of the critical early stage underlying the evolution of foraging behavior. Furthermore, studies focusing on specific aspects of foraging behavior rarely investigate how genetic variation in foraging behavior may ultimately influence the fitness of an individual (Glimcher 2002). We thus know little about the forces that shape the variation of foraging behavior. A genome-wide, unbiased study that aims to identify genetic variants contributing to the variation of foraging behavior and, accordingly, individual fitness is a natural step bridging the gaps between the understanding of neurobiological mechanisms and the evolutionary forces shaping variation of foraging behavior.

In *Drosophila*, variation at a single locus (*foraging*) was suggested as the sole contributor to bimodal variation in larval foraging behavior in wild populations ("rover" vs. "sitter", Belle et al. 1989; Osborne et al. 1997; and reviewed in Sokolowski 2001, but see Turner et al. 2015). However, unlike larvae, adult flies are intermittent eaters and their foraging behaviors are expected to be much more complex (Masek et al. 2014; Qi et al. 2015). Compared with larvae, adult flies forage in higher dimensional space and have access to more diverse arrays of food. In addition to maintaining energy and nutrient homeostasis, adults also have other essential behaviors (e.g., searching for suitable mates) that are in trade-off relationships with foraging. It is expected that adult foraging behavior may have a more complex genetic basis, depending on multiple small-effect genetic variants.

Natural populations harbor a wealth of genetic variation, which is a powerful resource to identify the association between phenotypic variation and the underlying genetic variants (King et al. 2012; Mackay et al. 2012; Huang et al. 2014). Drosophila Genetic Reference Panel (DGRP), which consists of highly inbred *Drosophila melanogaster* strains established from a wild North American population (Mackay et al. 2012; Huang et al. 2014), has been successfully applied to identify the genetic basis of a wide range of phenotypes (Magwire et al. 2012; Dembeck et al. 2015; Unckless et al. 2015a, 2015b; Battlay et al. 2016; Vonesch et al. 2016), including several behavior traits (Harbison et al. 2013; Gaertner et al. 2015; Shorter et al. 2015). However, it is commonly assumed that inbred *Drosophila* strains would suffer inbreeding depression, in which offspring of genetically related parents have lower survival rate and/or fertility (reviewed in Charlesworth and Charlesworth 1987; Charlesworth and Willis 2009). This phenomenon is expected to substantially influence studies focusing on life history traits, which directly relate to the survival and/or fertility of animals. Whether DGRP, whose inbred strains were established by multiple-generation brother–sister mating, is prone to the effect of inbreeding depression and would be less ideal for mapping the genetic basis of life history traits has not been evaluated.

To understand the little known first step underlying the evolution of foraging behavior, we investigate the genetic variation of adult foraging behavior that is critical for individual fitness. We used wild-derived *D. melanogaster* DGRP strains and measure their variation in the ultimate functional consequence of foraging behavior (i.e., the differences in survival rate of adult flies in a foraging environment). We designed a foraging environment that was modified from the Capillary feeder (CAFE) assay (Ja et al. 2007). In our assay, liquid food is constantly provided through a thin capillary tube, representing a foraging environment with hard to locate and consume, but unlimited, food source. Survived flies are thus those that can correctly sense their energy need, successfully locate the food, and/or well balance their energy usage between food searching and food gathering. Despite being such an essential trait, we found substantial variation of survival rate among DGRP strains in a foraging environment. Neither body condition nor starvation resistance could explain the great variation of foraging behavior we observed. Importantly, for the first time, we evaluated the potential caveats of using inbred DGRP strains to identify genetic basis of life-history traits, and concluded that inbreeding depression is unlikely a major contributor for the observed large variation in adult foraging behavior. We found a strong genetic component for our measured phenotype and, unlike larval foraging behavior (Sokolowski et al. 1997; Sokolowski 2001), adult foraging behavior depends on multiple loci. Identified candidate genes are enriched with those that have high expression in adult heads and/or have neurobiological significance. Our study provides a prioritized gene lists for future investigations on the neurophysiology mechanisms of foraging behavior that is critical for animal fitness in nature.

Materials and Methods

Drosophila Strains

Strains of DGRP (Mackay et al. 2012; Huang et al. 2014), GAL4-driver, UAS-RNAi, and eight African strains (Emerson et al. 2008) used in the study and their sources are in supplementary table S6, Supplementary Material online. Flies were reared under standard culture conditions (cornmeal–molasses–agar medium, 25 °C, 60–75% relative humidity, 12 h light/dark cycle). Foraging behavior and starvation resistance assays were conducted under the same condition. Four DGRP strains (49, 355, 596, and 642) did not breed well in our assay condition. Because our focused trait is "survival" in foraging environment, we excluded these four strains from our analysis.

Foraging Assay

We modified the CAFE method (Ja et al. 2007) with the following procedures (see supplementary fig. S6, Supplementary Material online). We used 24-well flat bottom tissue culture plates to individually house 24 flies. Each well has a thin layer

of 1% agar (1 mL per well, to maintain humidity) and a small circular opening of radius 1.5 mm at the top, through which we inserted capillary tubes (calibrated glass micropipettes 5 μl, VWR) filled with 4% (w/v) sucrose (dyed to a concentration of 0.05% (Spectrum FD&C Blue #1) for visualization). Capillaries were replaced as needed. For each strain/genotype, we quantified the survival of 20–24 one-day-old males for five consecutive days. For each 201 DGRP strain, the survival rate of 20–24 male flies is considered as one replicate. For DGRP338 and DGRP566, we performed additional replicates (four and seven, respectively) and found the standard deviation of our foraging index is 18.41% and 15.57%. See table S7, Supplementary Material online for Day 5 survival rate in foraging environment of each DGRP strain.

We chose sucrose as food source to limit the influence of variation in olfactory sensing. *Drosophila* senses sugars primarily using gustatory receptors located on their mouthparts and legs (Amrein and Thorne 2005), suggesting that olfactory sensing plays a lesser role. We also compared the survival rate of flies with mutant *Orb83b*, a broadly expressed odorant receptor that is essential for *Drosophila* olfaction (Larsson et al. 2004), with wildtype flies. All *Orb83* mutant ($n = 22$) and wildtype flies ($n = 31$) survived the five-day foraging assay with sucrose as food source, suggesting that variation in olfactory sensing unlikely accounts for the observed variation of foraging index.

Starvation Assay

A group of 10 flies were transferred into individual vials containing 5 mL 1% agar at the bottom, which maintains the humidity. We counted survived flies for two consecutive days. These assays were performed in the same environmental condition as the foraging assay. See table S7, Supplementary Material online for Day 2 survival rate in starvation resistance assay of individual DGRP strain.

Generation of Outcrossed F1

We randomly crossed DGRP strains that have high/low Day 5 survival rate to generate F1. Strains used and crosses performed in our assay are listed in supplementary table S3, Supplementary Material online. For each cross, we collected 20–24 one-day-old adult males and recorded their survival rate in the foraging apparatus. To avoid the influence of variation in environmental and/or other unforeseen factors, we repeated foraging assay for parental strains at the same time as our assays with F1.

Heritability Estimation, Principal Component Analysis, and Genome-Wide Association

We used single nucleotide polymorphisms (SNPs), SNPs annotations, and inversion status reported in DGRP freeze 2 (Huang et al. 2014), which is based on *D. melanogaster* genome release 5. Coordinates and gene names reported in our study are also based on release 5. We used GCTA 1.02 (Yang et al. 2011) to estimate genetic covariance matrix of SNPs, followed by using REML (restricted maximum likelihood) implemented in GCTA to estimate narrow sense heritability (V_G/V_P). To evaluate the influence of population structures on our observed phenotype, we used the SmartPCA program of Eigensoft 5.0.2 (Patterson et al. 2006) to identify top principle components (PCs) from the SNP data. We then used the projection length of each strain on the first and second PCs to test if our observed phenotypic variation is due to cryptic population structures.

We used PLINK (Purcell et al. 2007) to perform association analysis for SNPs that have minor allele frequency above 15% (1,023,674 SNPs). Day5 survival rates in foraging assay (foraging index) and Day 2 survival rate in starvation assay (starvation resistance index) were regressed on each SNPs and covariates. Covariates include starvation resistance index and inversion status (*In(2L)t* and *In(3R)Mo*). See Result sections for reasons of including different covariates. Regression model used includes:

Foraging index ~ genotype,
Foraging index ~ genotype + starvation resistance index,
Foraging index ~ genotype + inversion status,
Foraging index ~ genotype + starvation resistance index + inversion status, and
Starvation resistance index ~ genotype.

Go enrichment analysis was performed using GOWINDA (Kofler and Schlötterer 2012). GOWINDA was run with "gene" mode, including SNPs upstream/downstream 2,000 bp to a gene, minimum gene number 3, and with 100,000 simulations. Functional annotations of genes were downloaded from FuncAssociate (http://llama.mshri.on.ca/funcassociate/ (last accessed July 2015), Berriz et al. 2009).

Genome-Wide Expression Analysis

We used modEncode tissue-specific expression data (Brown et al. 2014). For each gene, we ranked its expression in 19 adult tissues from the highest (rank 1) to the lowest (rank 19), using "average" as tie-breaker. We then calculated the average rank of nine adult head tissues. To assay the significance of our observation, we sampled the same number of genes from the genome as the number of candidate genes used for our analysis and calculated their average rank of nine adult head tissues. Larger genes naturally leads to more associated SNPs and thus more likely to be identified as "significant candidate" genes with our analysis. To avoid this potential confounding factor, we categorized genes into four equal bins with respect to their gene length and control for this factor while sampling genes. Sampling of genes from the same bin of lengths used "matching" R package of R.

Real-Time RT-PCR Analysis

We extracted RNAs from 2 to 6-day-old (which is the duration of our behavior experiment) adult males. At least 40 heads were dissected for one replicate between 2 and 3 PM and

stored in RNAlater (Qiagent) in -20° until performing RNA extraction. RNA extraction was performed using Qiagent RNeasy mini kit. Extracted RNAs were digested with DNAase I (Invitrogen) to remove DNAs and reversed transcribed to cDNA using SuperScript III Reverse Transcriptase (Invitrogen) following manufacture's protocol. Real-time RT-PCR was performed using iTaq Universal SYBR Green Supermix (Bio-rad), following manufacture's protocol. The expression level of each candidate gene was measured with at least ten individuals for both alleles of significant SNPs. Primers used for qPCR are listed in supplementary table S8, Supplementary Material online.

We performed analysis of variance (ANOVA) with the following model: Gene expression ~ genotype + batch + genotype*batch interaction. Note that none of the interaction terms (between genotype and batch) is significant, suggesting that the directionality of expressional differences is consistent across batches (i.e., survival-lowering alleles have lower gene expression).

Expressional Knock down of Candidate Genes

We used RNAi strains of TRiP transgenic RNAi project (http://www.flyrnai.org/; last accessed July 2014) that have no predicted off-target (s19 = 1). We crossed strong and weak *elav-GAL4* drivers with UAS-RNAi strain to knock down the expression of candidate genes (see supplementary table S6, Supplementary Material online for strains information). 20–24 one-day-old adult males of F1 as well as parental strains (GAL4 drivers and UAS-RNAi strains) were used to perform foraging assay. At least three replicates were performed with F1s. The crosses were performed in both direction and no significant differences were observed (see results).

Results

Substantial Variation in adult Foraging behavior among DGRP Strains

Our main goal is to identify variants that are involved in *Drosophila* foraging behavior and play a critical role in individual fitness. Accordingly, instead of measuring different components of the foraging behavior, we focused on the end result of foraging behavior- survival rate with hard-to-locate, but unlimited, food source. We used strains from DGRP, which consists of highly inbred *D. melnoagster* strains that were collected in a fruit market in Raleigh, NC, USA in 2003 (Mackay et al. 2012; Huang et al. 2014). For each strain, we measured the survival rate of 20–24 one-day-old male flies over five days in our foraging assay arena (see Materials and Methods).

Given effective foraging is critical for survival and our study used strains that were derived from natural population recently, we were surprised to observe great variation of survival rate among the DGRP strains (fig. 1). The survival rate at Day 5 ranges from zero (no flies survived) to one (all flies survived), which is greatest among the five observed days. We thus used Day 5 survival rate (referred to as "foraging index" in the following text) to perform our analyses. It is expected that, despite unable to effectively locate and consume food, strains that are more resistant to starvation might have higher survival rate in our assay. However, under the same environmental condition, no flies survived over three days in our starvation assay (see Materials and Methods). Accordingly, we used Day 2 survival rate as an index for starvation resistance for our analyses. There are no obvious correlations between foraging efficiency (Day 5 survival rate with food) and starvation resistance (Day 2 survival rate without food, see supplementary fig. S1, Supplementary Material online) and only around 10% of the variation of the former might be attributed to the variation of the latter (*Spearman Rank* $\rho = 0.355$, $P = 1.836 \times 10^{-7}$). This suggests that our observed phenotype is not merely driven by variation in starvation resistant among strains. It is worth mentioning that the correlation between the starvation resistance of our study and previously reported male starvation resistance (Mackay et al. 2012; Huang et al. 2014) is significantly, but not particularly strong (*Spearman Rank* $\rho = 0.271$, $P = 9.054 \times 10^{-5}$). In addition, our foraging behavior phenotype is not correlated with previously reported starvation resistance (*Spearman Rank correlation test*, $P = 0.603$). This suggests that one should be aware of the variation created by differences in environmental condition between studies.

Foraging strategy depends on not only the energy status but also the nutrient balance of an animal (Waldbauer and Friedman 1991; Simpson et al. 2004). Accordingly, we tested if variation in nutrient conditions among DGRP strains contributed to our observed substantial variation in foraging index. We used nutrient condition (glucose, glycogen, glycerol, triglycerides, and protein) as well as body weight measured for a subset of DGRP strains (Unckless et al. 2015a). We found that our foraging index is not correlated with any of these nutrient conditions (*Spearman Rank correlation test*, $P > 0.1$ for all. See supplementary table S1, Supplementary Material online). Accordingly, variation in body condition is unlikely a major contributor for the observed variation in foraging behavior among strains.

To investigate whether any cryptic population structure contributed to the observed variation in foraging behavior, we used GCTA to identify major principal axis of genetic variation of DGRP strains (Yang et al. 2011). There were no obvious clusters of strains that have different foraging index on the first two principle axes (see supplementary fig. S2, Supplementary Material online). However, we also observed a marginally significant difference in the first principal component between the strains with different foraging index (*Kruskal–Wallis test*, $P = 0.0259$, see supplementary fig. S3, Supplementary Material online), but not the second principal component (*Kruskal–Wallis test*, $P = 0.991$). The pattern associated with first principal is mainly driven by three outlier strains with low foraging index and having inversion *In(3R)Mo* in their genomes. *Drosophila melanogaster* harbors a wealth

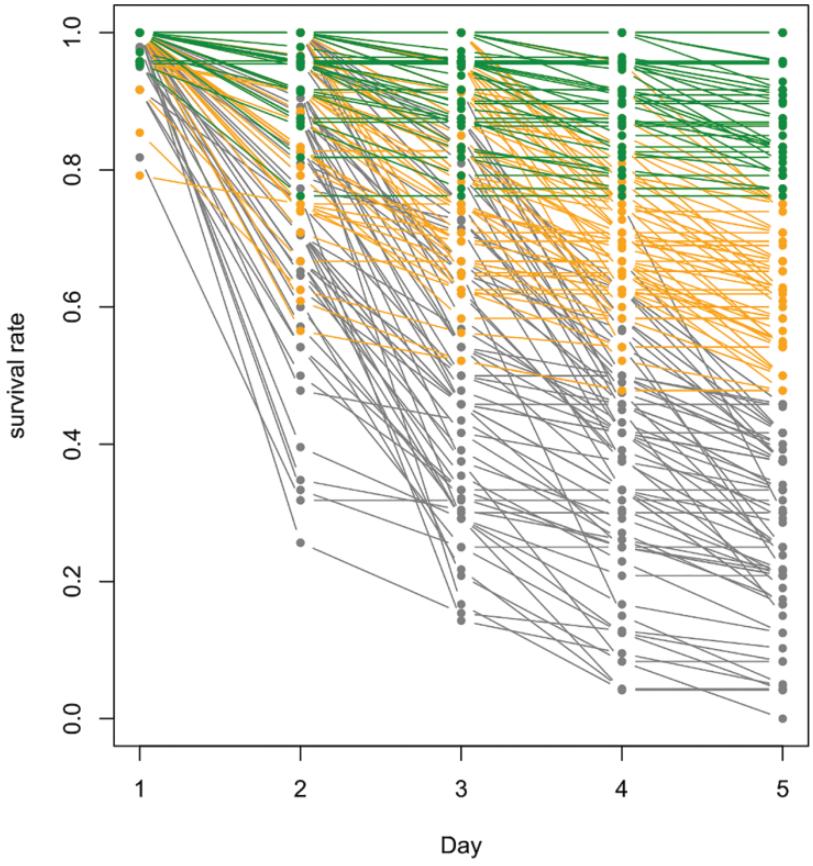

Fig. 1.—Survival rate of 201 DGRP strains over five days in a foraging environment. Strains were categorized into three roughly equal-sized bins according to their Day 5 survival rate (low—gray, intermediate—orange, high—green).

of segregating inversions (Corbett-Detig and Hartl 2012), which have been implicated to play important roles in population subdivisions and/or adaptation (reviewed in Kirkpatrick and Barton 2006; Hoffmann and Rieseberg 2008). We systematically tested the correlations between genotyped inversions of the DGRP strains and the first principal component of genetic variation, and found that only inversion status of *In(2L)t* and *In(3R)Mo* have strong effects (see supplementary table S2, Supplementary Material online). Similarly, the status of these two inversions, but not other inversions, is associated with the variation of our observed phenotype (see supplementary table S2, Supplementary Material online). Accordingly, we also included inversion status as covariates in our association analysis (see below).

Observed Great Variation in adult foraging behavior Is Not Due to Inbreeding Depression

A widely known genetic phenomenon is that offspring of parents that are highly genetic related have lowered survival rate and/or fertility, a phenomenon known as inbreeding depression (reviewed in (Charlesworth and Charlesworth 1987; Charlesworth and Willis 2009). Outcrossed offspring of two inbred strains has been observed to have higher fitness than individual parents. Part of this could be attributed to the fact that inbreeding makes recessive deleterious variants, which are usually segregating in heterozygous states in natural populations, homozygous. Because DGRP strains used in this study were generated with many generations of brother–sister mating, it is of general concern that the observed significant variation in surveyed phenotype is due to inbreeding depression for some or many of the strains. Particularly, we measured survival, which is expected to be especially sensitive to the effect of inbreeding depression.

Genomic analyses have found that the degrees of inbreeding vary among DGRP strains (Cridland et al. 2015; Lack et al. 2015). We focused on 40 DGRP strains whose genome-wide level of residual heterozygosity were reported (Cridland et al. 2015), and found that did not correlate with our observed foraging index (*Spearman Rank correlation test*, $P = 0.3168$; *Pearson correlation test*, $P = 0.353$). To empirically test if inbreeding depression contributed to our observed substantial variation, we generated outcrossed F1 between strains that have low Day 5 survival rate as well as between strains that have high Day 5 survival rate (see supplementary table S3, Supplementary Material online).

For F1 of strains with lowest Day 5 survival rate, we found that their Day 5 survival rate is not significantly different from their respective parents (*Mann–Whitney U test* $P = 0.8055$ [comparing offspring observation to parents observation, without pairing], *Paired-Sign Rank test* $P = 1$ [comparing offspring observation to observation of corresponding parents], fig. 2, see supplementary table S3, Supplementary Material online). This was also observed for F1 of strains with high Day 5 survival rate (*Mann–Whitney U test* $P = 0.9507$ [comparing offspring observation to parents observation, without pairing], *Paired-Sign Rank test* $P = 0.625$ [comparing offspring observation to observation of corresponding parents], fig. 2, see supplementary table S3, Supplementary Material online). Our result suggests that inbreeding depression is not a major contributor to the variation of our observed Day 5 survival rate.

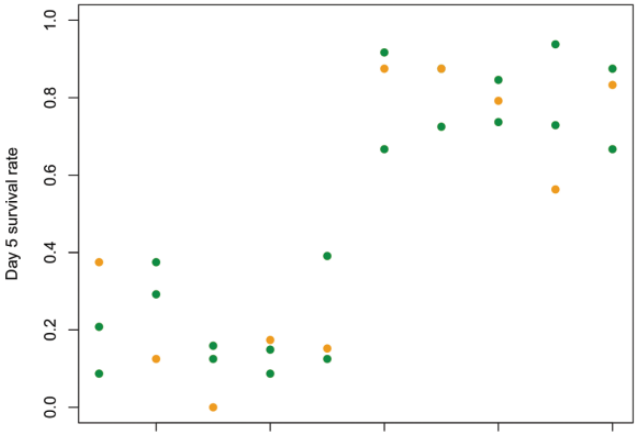

Fig. 2.—F1 of strains with extreme foraging behavior phenotype shows similar Day 5 survival rate as their parental strains. Each cross is represented with one vertical line. Green dots are parents and orange dots are the F1 of corresponding cross. Crosses on the left side are crosses between strains with low Day 5 survival rate whereas those on the right side are from crosses between strains with high Day 5 survival rate.

Genome-Wide Association Analysis Found SNPs Associated with the Phenotype

We used SNPs of DGRP freeze 2 (Huang et al. 2014) and estimated 51.79% (standard error 24.11%) of the observed variation in foraging behavior can be attributed to additive genetic variation (by using GCTA; Yang et al. 2011). Genome-wide association (GWA) that used Day 5 survival rate in foraging assay (foraging index) found, at nominal *P*-value threshold 10^{-5}, 90 significant SNPs (see supplementary table S4, Supplementary Material online). None of the identified SNPs leads to changes in amino acid sequences. Majority of the significant SNPs (63.33%) is located in intron, which is significantly higher than that of all the SNPs used in the study (50.47%, *Fisher's Exact test*, $P = 0.0153$).

We observed that there is a significant, though weak, correlation between foraging index and starvation resistance (see above). To test whether our identified significant SNPs were the result of association with starvation resistance, we performed two analyses to address this issue. First, we performed GWA using starvation resistance as the phenotype. The correlation between *P*-values of two GWA is low (*Pearson correlation analysis* on log10 transformed GWA *P*-value, $\rho = 0.2026$, $P < 10^{-16}$) and we did not find SNPs that are significantly associated with both phenotypes (fig. 3). Also, we performed GWA using foraging index as phenotype and starvation resistance as a covariate. This analysis found strong correlations between *P*-values of GWA with and without starvation as a covariate (*Pearson correlation analysis* on log10 transformed GWA *P*-value, $\rho = 0.88795$, $P < 10^{-16}$, fig. 3).

Similarly, inversion status of *In(2L)t* and *In(3R)Mo* was found correlated with foraging index (see above). We performed GWA including the status of these two inversions as covariates and, again, found strong correlations of GWA *P*-values between analysis with and without inversions as covariates (*Pearson correlation analysis* on log10 transformed GWA *P*-value, $\rho = 0.7631$, $P < 10^{-16}$; fig. 3). Using both inversion status and starvation resistance as covariates to perform GWA with foraging index also found *P*-values that are highly correlated with analysis without these covariates (*Pearson correlation analysis* on log10 transformed GWA *P*-value, $\rho = 0.6839$, $P < 10^{-16}$; fig. 3). Most SNPs that have most significant *P*-values with regression without covariates also have significant *P*-values with regression with covariates (see supplementary table S4, figs. S4 and S5, Supplementary Material online for Quantile–Quantile plot and Manhattan plot for GWA with and without covariates).

Identified Candidate Genes Have High Expression in Brains and Enriched with Those Involved in Fundamental Neuronal Function and Alternative Splicing

We predicted that genes involved in foraging behavior are more likely to have an expression and/or high expression in nervous system, particularly in adult brains. To test if genes whose SNPs are significantly associated with foraging index are enriched with those that have high expression in adult brains, we used modEncode tissue expression data (Brown et al. 2014). modEncode measure genome-wide gene expression for 19 adult tissues, including 1-, 4-, and 20-day-old adult heads for males, virgin females, and mated females.

There are 49 protein-coding genes that have at least one SNP with significant ($P < 10^{-5}$) association with our foraging index, and they show a significant trend of having higher expression in adult heads (fig. 4). We found that, compared to randomly chosen gene set, our candidate genes have significantly smaller expression rank (i.e., higher expression) in adult heads (mean head expression rank 7.3889 (candidate genes) and 8.953 (all other genes), permutation *P*-

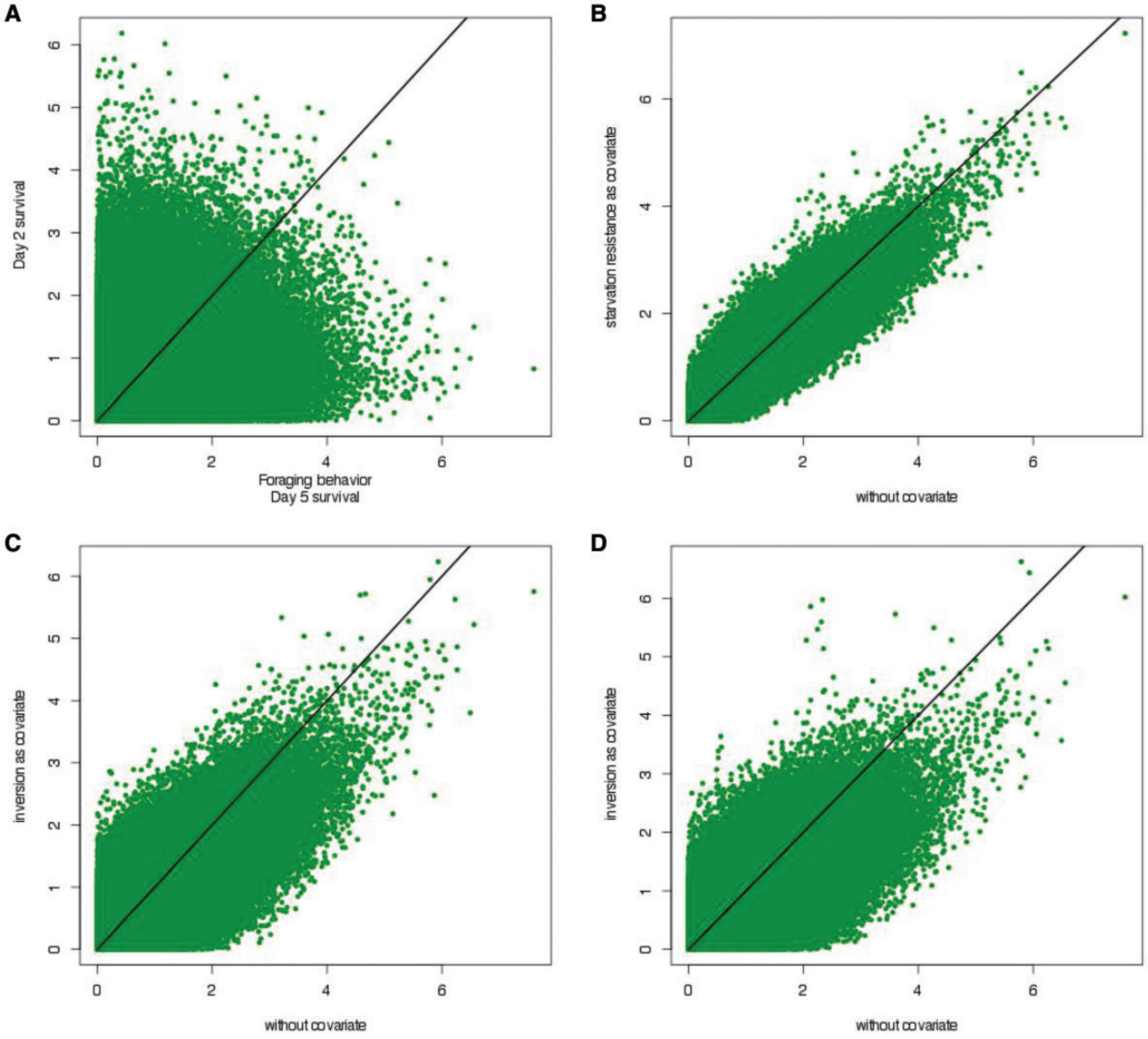

Fig. 3.—Correlation of *P*-values between GWAS. (*A*) Between GWA on foraging index (*x* axis) and on starvation resistance index (*y* axis), (*B*) between GWA on foraging index without covariate (*x* axis) and with starvation resistance as covariate (*y* axis), (*C*) Between GWA on foraging index without covariate (*x* axis) and with inversion status as covariate (*y* axis), and (*D*) Between GWA on foraging index without covariates (*x* axis) and with starvation resistance and inversion status as covariates (*y* axis). Both axes are on –log 10 scale and larger value suggests smaller (more significant) *P*-values.

value = 0.0074). These observations are consistent with our a priori predictions that our identified candidate genes for adult foraging behavior should be enriched with those that have relatively high expression in heads.

We used GOWINDA, which is designed for GWAS and corrects for gene length and overlapping gene structures (Kofler and Schlötterer 2012), to perform GO enrichment analysis on genes that harbor at least one significant SNP (GWA *P*-value $< 10^{-5}$). Because of the rather restricted candidate gene list, none of the GO categories is significant after multiple test correction. However, top three GO categories identified are enriched with are all neurophysiology related (ensheathment of neurons, axon ensheathment, and calmodulin binding, see supplementary table S5, Supplementary Material online). In addition, there are four GO categories related to mRNA processing (regulation of alternative mRNA splicing, regulation of mRNA splicing, regulation of RNA splicing, and regulation of mRNA processing, see supplementary table S5, Supplementary Material online). Large repertoire of splice forms is common in *Drosophila* nervous systems (Venables et al. 2012), and candidate genes involved in regulation of alternative splicing may have a wide-spread effect mediated through their influences on the splicing of multiple downstream genes. Both of these observations are consistent

Table 1
Expressional Effects of Significant Variants

			Genotype		No. Observations		qPCR Expression Median		*ANOVA P*-value		
	SNP	SNP GWA *P*-value	Lower D5 Allele	Higher D5 Allele	Lower D5 Allele	Higher D5 Allele	Lower D5 Allele	Higher D5 Allele	Genotype	Batch	Genotype* Batch[1]
scrib	3R:22396347	2.51E-08	G	A	12	23	0.5725	0.712	3.50E-02	1.37E-08	9.85E-01
fray	3R:14404760	2.78E-07	T	G	15	21	0.8481	1	3.52E-03	1.95E-08	1.44E-01
CG7694	3R:14404760	2.78E-07	T	G	15	21	0.7809	0.658	5.03E-01	7.72E-03	4.06E-01
Cow	2L:5614896	8.78E-07	T	C	20	13	0.9869	0.9227	3.36E-01	5.10E-02	1.72E-01
DIP-η	3R:18929676	9.01E-07	C	A	15	11	0.803	1.137	7.63E-02	4.70E-05	5.42E-01

[1]genotype and batch interaction.

Table 2
Day 5 Survival Rates of Individuals with Expressional Knock Down of Candidate Genes in Neuronal Tissues

		Day 5 Survival Rate in Foraging Environment			
				RNAi Knock Down Offspring, UAS-RNAi Strain as	
UAS-RNAi strain	GAL4 Driver Strain	UAS-RNAi Strain	GAL4-Driver Strain	Maternal	Paternal
Scrib	Weak *elav*	0.91	0.90	0.92	1.00
Fray	Weak *elav*	0.96	0.90	0.36	0.22
CG7694			No appropriate RNAi line		
Cow	Strong *elav*	1.00	0.96	0.91	0.98
DIP-η	Strong *elav*	0.92	0.96	0.97	0.99

with our prediction that identified candidate genes have neuronal related functions.

Alternative Alleles of Significant SNPs Contributed to Divergent Expression in Adult Male Heads

Because our top significant SNPs are located in noncoding sequences (see supplementary table S4, Supplementary Material online), we hypothesized that their functional impacts are mediated through changes of gene expression. We chose to test the expression effect of four most significant SNPs that are in genes with high adult head expression in the modEncode study (table 1). We performed real-time RT-PCR (qRT-PCR) to measure the expression of these five candidate genes in adult male heads. For three out of five candidate genes, or three out of four tested significant SNPs, the expression levels of alleles associated with lower Day 5 survival rate are significantly (P-value < 0.05) or marginally significantly lower (P-value < 0.1) than the alternative allele (table 1). These results support our hypothesis that these significant SNPs influence the expression of candidate genes.

Expressional Knock Down of Candidate Genes in Neuronal Tissues Influences Foraging Behavior

According to our quantitative expression analysis, for significant SNPs, alleles that are associated with lower Day 5 survival rate in foraging assay also have lower expression than the alternative alleles. This suggests that the influence of these SNPs on foraging behavior may be mediated through lowering expression of corresponding candidate genes. We thus used RNA interference (RNAi) to knock down expression of these candidate genes and test their influence on foraging behavior. Particularly, we are interested in our hypothesis that these candidate genes influence foraging behavior through changes in their expression in neuronal tissues. We thus used pan-neuronal GAL4 driver (*elav*-GAL4) to perform our experiments. RNAi crosses using first *elav*-GAL4 driver for *scrib* and *fray* led to lethality (refer to as "strong" *elav*-GAL4), suggesting that the expression of these two genes in neuronal tissues have vital functions. We thus used weaker *elav*-GAL4 driver to perform RNAi cross for these two genes. For *DIP-η* and *Cow*, we used strong *elav*-GAL4 driver (see Materials and Methods).

We were able to validate the influence of expression knock down of one candidate gene (*fray*) on foraging behavior (table 2). The average Day 5 survival rate of *fray* knocked down individual (30.26%) is below 20 percentile of the DGRP strains (31.82%). This is also lower than the foraging index of strains that have survival-lowering allele of significant *fray* SNP (T at 3R:14404760; Day 5 survival rate, 47.83%) and much lower than the alternative allele (G at 3R:14404760; Day 5 survival rate, 70.80%). These suggest that expression of *fray* in neuronal tissue may indeed play a critical role in adult foraging behavior.

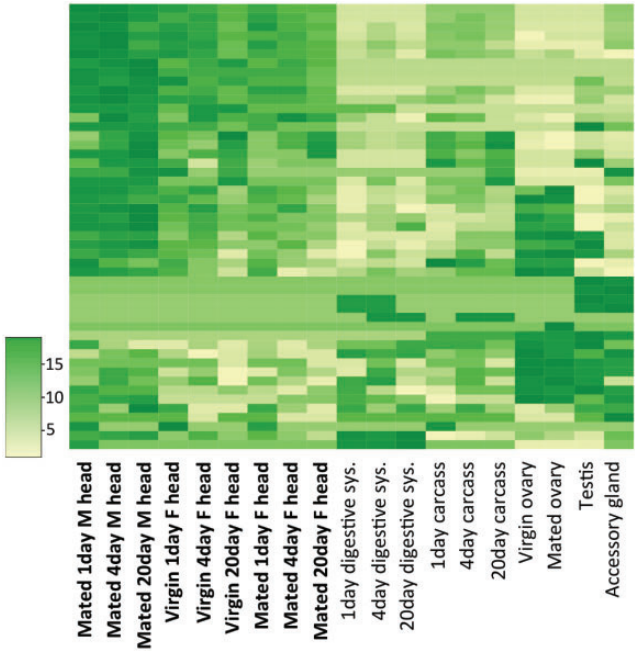

Fig. 4.—The expression of candidate genes in different adult tissues. Greener color represents smaller expressional rank (i.e., higher expression). Each row is one gene and each column is one tissue. The left nine columns are for adult head tissues.

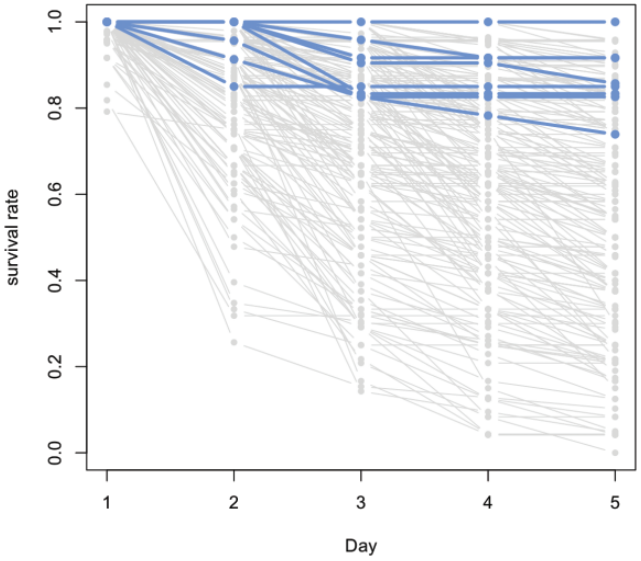

Fig. 5.—Survival rate of eight African and 201 DGRP strains over five days in a foraging environment. Blue lines are eight African strains whereas gray lines are DGRP strains (the same data as fig. 1).

Variation in Foraging Behavior Is Limited in African Population

Drosophila melanogaster is ancestrally distributed in sub-Saharan Africa (Lachaise et al. 1988; Veuille et al. 2004; Pool and Aquadro 2006) and colonized the North America relatively recently (around 200 years ago, Sturtevant 1920; Keller 2007). It has been widely observed that genetic variation of the North America population is significantly lower than that of the African population (Begun and Aquadro 1993; Langley et al. 2012). It is thus expected that African population may harbor more genetic variation for our focused phenotype, survival rate in foraging environment. We assayed the foraging behavior of eight African strains that are inbred and have wide geographic distributions (Emerson et al. 2008, see supplementary table S6, Supplementary Material online). Surprisingly, there is limited amount of variation in survival rate of African strains in foraging assay, especially when compared with the variation of North American strains (fig. 5). This striking difference is unlikely due to different degrees of inbreeding, because heterozygous offspring of low survival North American parents maintain the phenotype (see above).

Discussion

Variation in foraging behavior can critically influence the fitness of animals, and there are many important components of foraging behavior. In our foraging assay, while the amount of food is unlimited, the food was provided through a capillary tube that has a small opening. Flies need to be efficient in navigating and searching the space in order to locate the food source. In addition, there is energy cost associated for these activities. Flies need to correctly evaluate their energy condition and, based on that, decide the optimal foraging strategy in order to survive. Our foraging index, the Day 5 survival rate of flies in a foraging environment, is expected to capture the consequences of these various components and be highly relevant to the fitness of flies in nature. In addition, we investigated various potential confounding factors, including variation in starvation resistance, body condition, and inversion status, and concluded that they are not major contributors for our observed variation in foraging behavior. Importantly, we provided the first empirical evidence that inbreeding depression did not significantly contribute to our observed variation in survival rate in foraging assay. We expect that our approach of evaluating the potential influence of inbreeding, and the observed limited contribution of inbreeding depression to survival in foraging environment, will provide helpful guidelines for future similar studies using inbred *Drosophila* mapping panels.

We found that adult foraging behavior has a large genetic component: Around half of the phenotypic variation may be contributed by genetic variation (see Results). To the best of our knowledge, our study is the first effort to identify the genetic basis underlying *Drosophila* adult foraging behavioral variation on a genomic scale, which is critical for understanding the genetic divergence that leads to evolution of adult foraging behavior among species, such as those observed previously (Chen et al. 2012). Natural variation in foraging

behavior has been surveyed in *Drosophila* larvae (Sokolowski 1980; Bauer and Sokolowski 1984; Sokolowski et al. 1997), and studies have linked that the phenotypic variation to a single locus (*for*, Belle et al. 1989; Sokolowski et al. 1997; Sokolowski 2001, but see Turner et al. 2015). There, it is commonly taken that larval foraging behavior depends mainly on large-effect alleles. In contrast to larval foraging behavior, our observed continuous distribution of the adult foraging phenotype as well as identified large numbers of candidate SNPs suggest that the genetic determinant of adult foraging behavior is more complex than that of the larvae. Interestingly, despite previous reports that *for* also influences adult dispersal tendencies (Edelsparre et al. 2014) and adult response to food deprivation (Kent et al. 2009), SNPs of *for* are not significantly associated with our foraging index ($P > 10^{-4}$).

It was argued that stabilizing selection would remove variation underlying essential trait (Fisher 1930; Mousseau and Roff 1987; Roff and Mousseau 1987; Falconer 1989). The observed low variation in foraging behavior among African strains is consistent with this prediction. On the other hand, the substantial variation of foraging behavior in the DGRP samples, despite it being such an essential trait, is intriguing. Inbreeding depression is unable to explain such observation (see Results). Interestingly, by studying alleles that lower survival in a foraging environment, which is expected to be selected against, in the North American (DGRP) and an African (DPGP3, Lack et al. 2015) populations, we found a small, but highly significant correlation between the frequency differences of survival-lowering allele (DGRP minus DPGP) and the significant level of the SNP in GWA (*Spearman rank* $\rho = 0.0439$, $P < 10^{-16}$ [all SNPs]; $\rho = 0.0473$, $P < 10^{-7}$ [SNPs *P*-value < 0.01]). In other words, alleles that lower the survival rate in a foraging environment tend to have higher population frequencies in the North American than in the African population, consistent with the scenario that deleterious alleles arose in frequencies in the North American population. This observations within North American population maybe due to its unique recent demographic history (Lohmueller et al. 2008), local adaptation to traits in tradeoff to foraging behavior, and/or genetic interference (Hill and Robertson 1966; Felsenstein 1974)) from rampant selection in populations adapting to new habitat (Langley et al. 2012).

Alternatively, the observed substantial variation in foraging behavior may be due to the fact that foraging strategy could be environmental dependent (Beeler et al. 2010, 2012; Cervantes-Sandoval et al. 2016; Stephens and Krebs 1987). An environment with plenty unexplored food sources will be favored for individuals that spend most time exploring ("explorer"). On the other hand, an environment in which the known food source is unlimited while there are no other unexplored food sources will be favored for individuals that spend most time and energy on going to and consuming the known food source ("exploiters"). In our foraging assay, the food was provided through thin capillary tubes, which represents the latter case of the foraging environment. Accordingly, strains with low survival rate in our foraging environment might instead survive better in other foraging environment, a trade-off for foraging strategy.

We found that survival-lowering alleles of top candidate SNPs are associated with lower expression of candidate genes in adult heads than the homologous alleles, suggesting that changes in expression levels of candidate genes could have contributed to the variation in foraging behavior. Expression knock down of one candidate gene (*fray*) using pan-neuronal driver supports that its expression is important in adult foraging behavior. For the other three candidate genes tested, we did not observe significant differences in foraging behavior between wildtype and expression knock down flies. The right level of expression knock down that would recaptures naturally occurring variation may be hard to achieve. This may be especially true for *scrib*, the gene containing the most significant SNPs in GWA, because expression knock down of *scrib* using strong pan-neuronal driver leads to lethality while using weaker pan-neuronal driver has no obvious phenotype. Alternatively, expression of these candidate genes in other tissues, such as skeletal muscles, may instead play a bigger role in the foraging behavior. Still, it is expected that some of the identified significant SNPs in the GWA are false positives, which could happen to be these candidate genes we chose to functionally validate.

A further look into our top candidate genes provides promising directions for future molecular and neurophysiology investigations of adult foraging behavior. Our top candidate, *scrib* (*scribbled*), is an essential cell polarity gene (Bilder et al. 2000). Functional disruption of *scrib* causes neoplastic overgrowth and results in manifest neuron development (Albertson and Doe 2003). In addition, *scrib* also contributes to variation in age-specific fitness traits, olfactory memory, and olfactory perception (Durham et al. 2014; Arya et al. 2015; Walkinshaw et al. 2015; Cervantes-Sandoval et al. 2016). *DIP-η* (*Dpr-interacting protein η*) is one member DIP protein family (Özkan et al. 2013), whose interactions with DPR protein family were identified to form a network that is required for synaptic connectivity (Carrillo et al. 2015). Furthermore, the human homology of *Cow* (*Carrier of Wingless*) has been shown to play an important role in synapse assembly (Pazos Obregón et al. 2015). Finally, *fray* (*frayed*), whose expression knock-down at neuronal tissue was validated to lower adult survival in our foraging assay, encodes a protein with a serine-threonine kinase domain that has been implicated in olfactory memory formation (Walkinshaw et al. 2015). Null *fray Drosophila* larvae developed a nerve bulgings or swellings, which resulted in defective axonal ensheathment in larval peripheral nerves (Leiserson et al. 2000). This suggests that *fray* kinase has a role in late glial development. In addition, the mammalian ortholog of *fray* is a Ste20-like kinase (PASK; Leiserson et al. 2000,

2011), which has a role as a metabolic sensor that is required for energy balance in mice (Hao et al. 2007). Importantly, the energy-sensing role of PASK has been shown to be essential for the normal function of AMPK and mTOR/S6K1 (Hurtado-Carneiro et al. 2014).

In summary, all our top candidate genes are involved in the development and maintenance of normal functions of the nervous system. In particular, one of our top candidates, *fray*, was validated using neuron specific RNAi knockdown in our assay. Although published studies on *Drosophila fray* suggests its diverse functions in the nervous system, studies on its mammalian ortholog suggest that *fray* likely play a central role in energy sensing and in coupling energy sensing to downstream pathways in maintaining energy balance. While many sensory, motor, motivational, homeostatic, and metabolic processes may affect foraging, pathways that integrate energy sensing with downstream actions in maintaining energy balance will be most critical. It has been reported that energy-sensing can modify foraging-related behaviors in flies (Johnson et al. 2010) and in mice (Chen et al. 2005). Future studies focusing on these genes will help us bridge the gaps between the neurophysiology understandings of foraging behavior and the corresponding influence on animal's fitness in nature. Besides reporting candidate genes, we also provided, to the best of our knowledge, the first attempt to evaluate the potential caveats of using inbred *Drosophila* strains to perform GWA on life history related traits. Interestingly, we proposed that the recent demographic history, local adaptation, and/or the trade-off between life history traits might have brought the otherwise selected against survival-lowering alleles in foraging assay into higher population frequencies, resulting in our observed substantial variation in adult foraging behavior in the North American population.

Supplementary Material

Supplementary data are available at *Genome Biology and Evolution* online.

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