LETTERS

Maintaining a behaviour polymorphism by frequency-dependent selection on a single gene

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Accounting for the abundance of genetic variation in the face of natural selection remains a central problem of evolutionary biology[1,2]. Genetic polymorphisms are constantly arising through mutation, and although most are promptly eliminated[3], polymorphisms in functionally important traits are common. One mechanism that can maintain polymorphisms is negative frequency-dependent selection on alternative alleles, whereby the fitness of each decreases as its frequency increases[4,5]. Examples of frequency-dependent selection are rare, especially when attempting to describe the genetic basis of the phenotype under selection. Here we show frequency-dependent selection in a well-known natural genetic polymorphism affecting fruitfly foraging behaviour. When raised in low nutrient conditions, both of the naturally occurring alleles of the *foraging* gene (for^s and for^R) have their highest fitness when rare—the hallmark of negative frequency-dependent selection. This effect disappears at higher resources levels, demonstrating the role of larval competition. We are able to confirm the involvement of the *foraging* gene by showing that a sitter-like mutant allele on a rover background has similar frequency-dependent fitness as the natural sitter allele. Our study represents a clear demonstration of frequency-dependent selection, and we are able to attribute this effect to a single, naturally polymorphic gene known to affect behaviour.

Negative frequency-dependent selection is often described as being one of the most powerful selective forces that can maintain balanced polymorphisms[1,4,5]. Although it is believed to be a common phenomenon, attempts to link naturally occurring allelic variation with negative frequency-dependent selection are surprisingly few. Several studies report frequency-dependent selection mainly at the level of the phenotype[6,7]. Other studies report frequency-dependent selection at polymorphic allozyme-encoding genes[8,9], but precise interpretation is confounded by the fact that the observed effects may be due to alleles of other genes in linkage disequilibrium with the alleles of the allozyme genes. A flower pigmentation polymorphism in the morning glory, *Ipomoea purpurea*, is maintained by frequency-dependent selection on the *W* locus[10]. Negative frequency-dependent selection maintains allelic variation at the self-incompatibility *S* locus of plants, and two genes within the locus, *SRK* and *SLG*, are candidates for involvement in this selection[11].

We investigated the potential for negative frequency-dependent selection in the maintenance of a stable behavioural polymorphism directly linked to naturally occurring functional alleles of the *foraging* (*for*) gene. Now an established model system in behavioural genetics, the naturally dimorphic foraging behaviour of larval fruitfly (*Drosophila melanogaster*) is largely influenced by allelic variation in *for* (refs 12, 13). 'Rover' (for^R) larvae move more than 'sitter' (for^s) larvae when foraging within a food patch and they are also more likely to explore new food patches than are sitters[13]. *for* encodes a cGMP-dependent protein kinase (PKG), and rovers have higher *for* messenger RNA transcript levels and PKG activity levels than sitters[12]. The fitness consequences associated with the rover/sitter polymorphism are largely unknown, although density-dependent selection during the larval stage can lead to changes in allelic frequency[14]. Despite the marked effects of this polymorphism on foraging, an explanation of its maintenance in natural populations has yet to be uncovered.

We proposed that the rover/sitter polymorphism of *D. melanogaster* might be maintained by negative frequency-dependent selection during bouts of resource competition in the larval stage. Previous studies have emphasized the importance of larval resource competition in life history evolution in *Drosophila*[15–18]. Variation in fitness associated with the rover/sitter polymorphism is likely to arise from the resulting inherent differences in foraging behaviour, leading to differential intra- and inter-morph competition. To test our hypothesis, we reared the morphs together over a range of frequencies, at lower and higher food levels, and assayed their fitness. Densities were held constant to disentangle food level effects from any density effects.

To differentiate the morphs, we marked either homozygous rovers or sitters with a green fluorescent protein (GFP), by substituting the third pair of chromosomes that is shared by our rover and sitter lines with one that carries a GFP marker. Foraging path lengths of the GFP-marked rovers (mean ± s.e.m., 5.228 ± 0.214 cm, $N = 55$) do not significantly differ from those of unmarked rovers (4.648 ± 0.216 cm, $N = 54$); the path lengths of marked sitters (3.080 ± 0.251 cm, $N = 40$) do not significantly differ from unmarked sitters (3.460 ± 0.207 cm, $N = 59$); and, as expected, rovers have higher foraging path lengths than sitters (single factor analysis of variance (ANOVA), $F_{3,204} = 19.946$, $P < 0.0001$, Tukey–Kramer test). Below, we focus on experiments using the marked rovers, but similar results were attained using marked sitters (see Supplementary Fig. 1).

To assess the role of frequency-dependence, we raised 32 larvae from the first instar to pupation in one of three ratios (3:1, 1:1 and 1:3) of marked rovers (+; for^R; *Ubi*-GFP) to unmarked sitters (+; for^s; +). We used food limitation to impose resource competition among larvae. Larvae were reared in 50-ml vials that contained 6 ml of yeast–sugar–agar media with a substantial reduction in the nutrient components of the food and larval density relative to our standard rearing conditions (see Methods). We used a 75% reduction ('higher nutrient abundance') of the yeast and sugar concentrations relative to our standard rearing media as our first level of food limitation and an 85% reduction ('lower nutrient abundance') as our second level of food limitation. These conditions were chosen because they impose larval competition while maintaining relatively high survivorship. Our estimate of fitness was the proportion that survived to pupation—a commonly used metric[19] that is highly correlated with survival to adulthood ($r^2 = 0.887$, $P < 0.0001$, $N = 71$).

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The results of this experiment strongly supported our predictions of negative frequency-dependent selection owing to larval resource competition (Fig. 1). When reared on the higher nutrient abundance media the fitnesses of rovers and sitters were unaffected by their frequencies (Fig. 1a, two-factor ANOVA, frequency × genotype $F_{2,114} = 1.544$, $P = 0.218$). However, when reared on the lower nutrient abundance media, a clear signature of negative frequency-dependent selection was detected. We found that rovers attained their highest relative fitness when rare in the population (outnumbered 3 to 1 by sitters); sitters attained their highest relative fitness when they were similarly rare (Fig. 1b, frequency × genotype $F_{2,2} = 96.789$, $P = 0.010$; see also Supplementary Table 1). This experiment was conducted in two blocks, and therefore we included block in the analysis. Both blocks revealed a similar pattern, as indicated by the lack of a significant block × frequency × genotype interaction ($F_{2,228} = 0.398$, $P = 0.672$).

As with previous experiments[8,9], the possibility of linkage disequilibrium between alleles at other loci and alleles at *for* limits our ability to attribute the frequency-dependent selection we observed to the *for* alleles. To confirm the involvement of *for*, we substituted the for^{s} strain for a sitter mutant that was generated on a rover genetic background (for^{s2}; refs 20, 21) and replicated the low resource experiment. The for^{R} and for^{s2} strains differ only at the mutated site within *for* (refs 12, 21) and thus markedly reduce the possible confounding effect of linkage disequilibrium. Using the for^{R} (+; for^{R}; *Ubi*-GFP) and for^{s2} (+; for^{s2}; +) strains, we similarly observed negative frequency-dependent selection, with each genotype attaining the highest fitness when at the lowest frequency (Fig. 2, frequency × genotype $F_{2,114} = 15.362$, $P < 0.0001$).

We also raised unmarked rovers (+; for^{R}; +) and unmarked sitters (+; for^{s}; +) under the same rearing conditions and frequencies as above. These strains share the third pair of chromosomes from rover. We distinguished rover from sitter survivors using a restriction fragment length polymorphism (RFLP) in the coding region of *for*, which segregates within our rover and sitter strains (see Methods). In this experiment, the metric of fitness was survival to adulthood. Genomic DNA from single adult flies was extracted for RFLP genotyping. After genotyping, we again found that each genotype attained the highest fitness when at the lowest frequency (Fig. 3, frequency × genotype $F_{2,52} = 7.508$, $P = 0.001$).

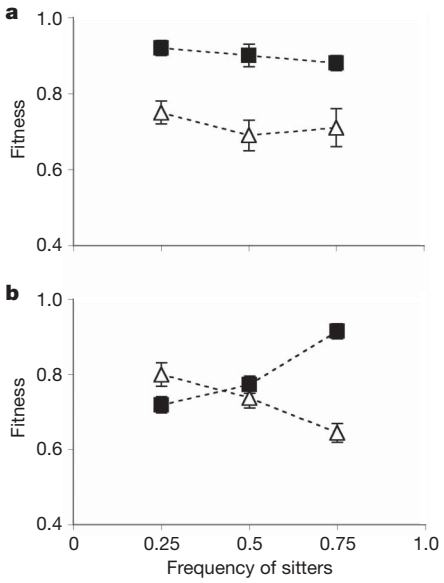

Figure 1 | The effects of frequency and nutrient level on rover and sitter fitness. The *x* axis, plotted as the frequency of sitters, is inversely proportional to the frequency of rovers. **a**, **b**, Rover (for^{R}, squares) and sitter (for^{s}, triangles) morphs were reared together under a range of frequencies (3:1, 1:1, 1:3) and on either higher (**a**) or lower (**b**) nutrient abundance media. To facilitate counts, rovers were marked with GFP. Fitness was estimated using the proportion that survived to pupation (mean ± s.e.m.). Sample sizes were 20 (**a**) and 40 (**b**) vials per treatment.

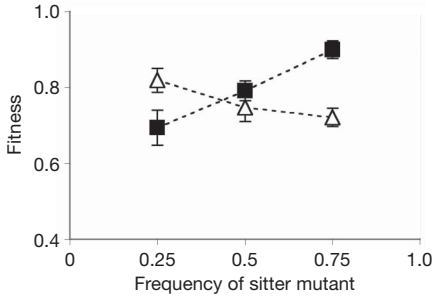

Figure 2 | The fitnesses of the rover strain and sitter mutants when raised in lower nutrient levels and over a range of allele frequencies (3:1, 1:1, 1:3). Rovers (squares) were marked with GFP. The sitter strain for^{s2} (triangles) carries a mutation in the *for* gene that was generated on a for^{R} genetic background. Fitness was estimated using the proportion that survived to pupation (mean ± s.e.m.). Sample sizes were 20 vials per treatment.

Our results demonstrate that this naturally occurring polymorphism in foraging behaviour can be maintained by negative frequency-dependent selection. Moreover, results from our experiment with the for^{s2} mutant allow us to link directly negative frequency-dependent selection to allelic variation at the *for* locus. To the best of our knowledge, this is the first study to directly show frequency-dependent selection acting on a single gene by way of demonstrating that both the natural and mutant alleles show similar fitness responses to the same selective forces. Thus, these results provide a rare example of negative frequency-dependent selection maintaining naturally occurring allelic variation at a single gene. The local frequency of alleles at the *for* locus has a marked effect on their fitness.

It is possible that additional forms of selection may also contribute to the maintenance of the rover/sitter polymorphism, including heterozygote advantage. Larvae that are heterozygous at the *for* locus behave similarly to rovers[22]; therefore, in our experimental setting, one might expect rovers and heterozygotes to have similar fitness when reared together and heterozygotes to have negative frequency-dependent fitness when reared with sitters. We tested these hypotheses directly (see Supplementary Fig. 2). These experiments demonstrated that heterozygotes do not have higher fitness than rovers when reared together; therefore, there is no evidence for a heterozygote advantage. Moreover, fitnesses of both heterozygotes and sitters were negatively frequency-dependent when reared together, and this pattern was independent of the way the reciprocal crosses were done (see Supplementary Information).

The mechanism underlying the negative frequency-dependent selection that we have observed seems to be competition for limiting food resources, where competition is most intense within morphs (Fig. 1). Although we have demonstrated that negative frequency-dependent

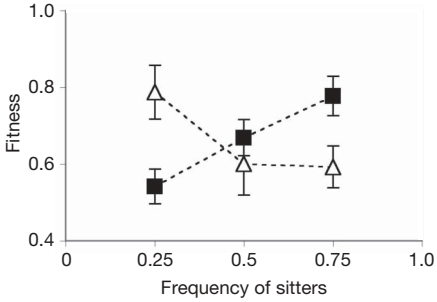

Figure 3 | The fitnesses of unmarked rovers and sitters when raised in lower nutrient levels and over a range of allele frequencies (3:1, 1:1, 1:3). Rovers (squares) and sitters (triangles) were not marked with GFP. Their frequency as adults was determined by genotyping using a restriction fragment length polymorphism. Fitness was estimated using the proportion that survived to adulthood (mean ± s.e.m.). Sample sizes were 10 vials per treatment.

selection is mediated by nutrient abundance, we are not yet able to be precise about the form of intra- and inter-morph competition. There are a number of possibilities, ranging from exploitation competition within morphs to facilitation between morphs. An important next step will be to identify the details of the interaction so that we can determine its role in maintaining this polymorphism in the wild. Monitoring changes in rover and sitter allelic frequencies over space and time in manipulation experiments (for example, manipulation of various stages of the life cycle, overlapping generations, predation and heterogeneous environments) will facilitate this aim.

The opportunity for food competition in nature is high[15], and therefore we expect that the phenomenon reported here is likely to occur in nature. Negative frequency-dependent selection may well occur during larval development in view of the fact that density, frequency, and age of rovers and sitters are likely to vary as the occupation of a food source intensifies. Food stress during larval development may be also affected by factors such as the accumulation of waste products[23], hypoxic conditions[24] and interspecific competition[18]. Although food stress may be negligible during the early occupation of rotting fruit, nutrient availability will decrease and larval competition will probably increase as the resource is exhausted.

The rover/sitter polymorphism has become a classical example in behavioural genetics[25]. Recent studies of this polymorphism have focused on a mechanistic understanding of *for*'s effects on foraging[12,21], pleiotropic effects of the *for* gene[12,21,26], and the function of *for* orthologues in a variety of other taxa[27–29]. However, we still lack a full understanding of the evolutionary forces acting on rover and sitter alleles. The current study suggests that selection may well be acting to maintain the polymorphism in the wild. Further studies of selection in the laboratory and in the wild will allow for an evolutionary interpretation of the *for* gene, thus providing a rare opportunity for an understanding of natural variation in behaviour that ranges from genotype to phenotype, and from selection back to genotype.

METHODS SUMMARY

Strains and crosses. Rover and sitter strains are homozygous for the for^R and for^s alleles, respectively, on chromosome 2 and share co-isogenic third chromosomes from the for^R strain. The X chromosomes shared by these strains were mostly from the for^R background, and reciprocal cross analyses (this study, Supplementary Information) confirmed that the X chromosomes do not contribute to the fitness differences arising from larval competition on low nutrient resources. for^{s2} is a sitter mutant generated on the rover genetic background and does not differ from for^R except for the induced mutation within the *for* gene. The GFP-marked strain (w^{118}; P{*Ubi*-GFP.nls}3L1 P{*Ubi*-GFP.nls}3L2, P{FRT(w^{hs})}2A; Bloomington Stock Center) contains a *Ubiquitin* promotor fused with GFP inserted on chromosome 3. This chromosome was substituted into the for^R strain to produce the +; for^R; *Ubi*-GFP strain and into the for^s strain to produce the +; for^s; *Ubi*-GFP strain.
Nutrient and growth conditions. Our higher and lower nutrient abundance media were formulated with a 75% and 85% reduction of the yeast and sugar concentrations used in our standard rearing conditions (1,000 ml H_2O, 100 g sucrose, 50 g Fleischmann's yeast, 16 g agar, 8 g $C_4H_4KNaO_6$, 1 g KH_2PO_4, 0.5 g NaCl, 0.5 g $MgCl_2$, 0.5 g $CaCl_2$, 0.5 g $Fe_2(SO_4)_3$). The survivorship arising from the higher and lower nutrient level media were within the ranges obtained in our standard bottle populations. Preliminary studies demonstrated that when larvae were reared under low nutrient abundance conditions, relative to our standard well-fed food conditions, development was delayed by approximately 2 days (two-factor ANOVA, $F_{1,36} = 147.491$, $P < 0.0001$) and developmental times did not differ between rover and sitter genotypes ($F_{1,36} = 0.077$, $P = 0.782$). Furthermore, adult body sizes of rovers and sitters did not differ when reared under low nutrient conditions (two-factor ANOVA, $F_{1,56} = 0.414$, $P = 0.522$); as expected, sex affected body size ($F_{1,56} = 247.958$, $P < 0.0001$). All experiments were conducted in the same incubator under a 12/12 h light/dark cycle, 25 ± 1 °C, 70 ± 1% relative humidity.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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METHODS

Larval competition experiments. Thirty-two for^R and for^s first instar larvae ±2 h in age were gently placed onto the surface of the medium in each 50-ml vial (9.5 cm height, 3 cm diameter) in one of the following proportions: 3:1, 1:1 and 1:3. Each vial contained 6 ml of either the higher or lower nutrient abundance media. One-hundred vials were placed into each vial rack and the placement of vials within and between racks was arbitrarily randomized daily. We first performed a competition study under higher nutrient level conditions with $N = 20$ vials for each of the 3:1, 1:1 and 1:3 experimental conditions. This was conducted using GFP-marked for^R and unmarked for^s strains ($N = 20$ vials per frequency). We then conducted the following studies under the lower nutrient abundance condition. The first used GFP-marked for^R and unmarked for^s strains ($N = 40$ vials per frequency). This was done in two blocks with $N = 20$ vials per frequency per block. The second experiment used marked for^R and unmarked for^{s2} strains ($N = 20$ vials per frequency). The third used unmarked for^R and unmarked for^s strains ($N = 10$ vials per frequency) using RFLP mapping to distinguish between individual rover and sitter flies. Finally, to address heterozygote advantage, we raised rover/sitter heterozygotes with either GFP-marked rovers or sitters ($N = 10$ vials per frequency, see Supplementary Information). Rover/sitter heterozygotes were generated using reciprocal crosses.

The number of pupae within each vial was counted and recorded daily from 5 to 18 days after hatching. GFP-marked individuals were counted using a stereoscope affixed with a GFP attachment.

Analyses. Because we express fitness as a proportion, we used arcsine square root transformations to normalize the data for all statistical analyses[30]. Normality was assessed using Wilk–Shapiro statistics, and because there were no significant deviations from normality, we proceeded with parametric ANOVA. Our main interest was to determine whether there was an interaction between the factors frequency and genotype, as would be expected if there was frequency-dependent selection. Because we sampled only a subset of the possible frequencies between rovers and sitters, we consider frequency as a random effect in all statistical analyses. Analysis of the experiments under low nutrients reported in Fig. 1b included a third factor, block. Those analyses indicated a small but significant block effect ($F_{1,2} = 71.543$, $P = 0.014$), because survivorship to pupation was 6% higher in one of the blocks; however, there was a strong frequency × genotype interaction ($F_{2,2} = 96.789$, $P = 0.010$). The frequency × genotype interaction was consistent in the two blocks, as indicated by the lack of a significant frequency × genotype × block interaction ($F_{2,228} = 0.398$, $P = 0.672$), and in fact no interactions with block were significant (block × frequency $F_{2,2} = 0.310$, $P = 0.310$; block × genotype $F_{1,2} = 0.184$, $P = 0.710$).

Single-fly DNA extractions. Adult flies were stored in 95% ethanol at 4 °C. The flies were homogenized in 100 µl of TES (15.44 ml ddH_2O, 400 µl 1 M Tris-Cl, pH 7.5, 80 µl 0.5 M EDTA, 4 ml 10% SDS). The samples were incubated at 70 °C for 30 min. Fourteen microlitres of 8 M potassium acetate was added and the samples were placed on ice for 30 min, and were then centrifuged at 12,000 r.p.m. for 10 min at 4 °C. The supernatant was transferred to a new microfuge tube. Adding 50 µl isopropanol and placing the samples on ice for 5 min precipitated the DNA. The samples were then centrifuged at 12,000 r.p.m. for 5 min at room temperature. The pellet of DNA was washed in 75 µl 70% ethanol and then dried. Finally, the samples were re-suspended in 10 µl Tris-EDTA and stored at −20 °C.

***for*^{R} and *for*^{s} genotyping.** We use a polymorphism (R390C) that confers a change in the amino acid encoded by the for^R and for^s alleles. 'R' (arginine) is the amino acid encoded by for^R; '390' is the amino acid position in the *for*-T1 protein sequence[31]; and 'C' (cysteine) is the amino acid encoded by for^s. This is a C to T nucleotide change between for^R and for^s, respectively. The functional significance of this polymorphism is currently unknown; however, it segregates in our laboratory for^R and for^s stocks and provides a suitable marker for these experiments. Polymerase chain reactions (PCR) were conducted using the following primers to produce a 343-bp fragment of *for*: R390C_S, CTGGTGGATCCGAATTTCAT; R390C_AS, GGAGATACCCAATGCCCTCT. PCR cocktails comprised the following ingredients (50 µl each): 0.2 µl Taq polymerase (BioLase), 5 µl reaction buffer (BioLase), 1.5 µl 50 mM $MgCl_2$ (BioLase), 4 µl dNTPs (Promega, each 2.5 mM μl^{-1}), 1 µl each of the R390C primers (10 pmol each per µl), 2.5 µl genomic DNA, and 34.8 µl ddH_2O. Conditions were as follows: 94 °C for 4 min; 40 cycles of 94 °C for 45 s, 59 °C for 45 s, 72 °C for 60 s, followed by a final step of 72 °C for 7 min.

The restriction endonuclease *Hin*P1 I was used to digest the PCR products. The recognition site for the enzyme is G|CGC and it thus cleaved the for^R PCR product to produce two bands (286 bp and 57 bp), whereas the for^s PCR product was not cleaved, leaving a single 343-bp band. The primers were designed to include specifically only one potential endonuclease recognition site. The samples were digested at 37 °C for 2.5 h in the following 30-µl reactions: 1 µl *Hin*P1 I (New England Biolabs, R0124L), 3 µl NEBuffer 2 (New England Biolabs), 10 µl PCR product, and 16 µl ddH_2O. The digested and undigested PCR products were visualized using a 2% agarose gel ran at 115 mV for 60 min.

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