DOI: 10.1534/genetics.104.032631

Flexibility in a Gene Network Affecting a Simple Behavior in *Drosophila melanogaster*

Bruno van Swinderen and Ralph J. Greenspan[1]

Neurosciences Institute, San Diego, California 92121

Manuscript received June 21, 2004
Accepted for publication January 12, 2005

Context and interaction are of the essence.
LEWONTIN (1974)

ABSTRACT

Gene interactions are emerging as central to understanding the realization of any phenotype. To probe the flexibility of interactions in a defined gene network, we isolated a set of 16 interacting genes in Drosophila, on the basis of their alteration of a quantitative behavioral phenotype—the loss of coordination in a temperature-sensitive allele of *Syntaxin1A*. The interactions *inter se* of this set of genes were then assayed in the presence and in the absence of the original *Syntaxin1A* mutation to ask whether the relationships among the 16 genes remain stable or differ after a change in genetic context. The pattern of epistatic interactions that occurs within this set of variants is dramatically altered in the two different genetic contexts. The results imply considerable flexibility in the network interactions of genes.

CONTEXT is the hallmark of biological processes. Among the various categories of biological context, much attention has been paid to environment and to historical contingency. These factors have been approached experimentally in studies of norms of reaction and by means of laboratory selection. The discovery early in experimental genetics of epistasis established genetic context as another important factor and gave rise to the study of interactions among genes.

Interactions among genes are key to understanding the realization of any phenotype. Although the term "epistasis" has meant different things to different people (PHILLIPS 1998), the study of gene interactions and networks has been integral to population genetics (FISHER 1918; WRIGHT 1931, 1968), quantitative genetics (COCKERHAM 1954; HIRSCH and ERLENMEYER-KIMLING 1962; COHAN *et al.* 1989; CLARK and WANG 1997), classical mutant analysis (HERSKOWITZ and HAGEN 1980), molecular genetic analysis (SCOTT and CARROLL 1987), and more recently the interpretation of DNA microarray results (DAVIDSON *et al.* 2003). In its more recent molecular incarnations (SCOTT and CARROLL 1987; DAVIDSON *et al.* 2003), this viewpoint has assumed that the elements of a gene network are specific in their interactions and that the relationships among them are stable (GREENSPAN 2001), specific in the sense that their molecular interactions and the phenotypic consequences of those interactions are predominately dedicated and restricted and stable in the sense that the propensity for interactions among these elements is predominately fixed and predictable. The first assumption—specificity—has come under increasing scrutiny as more and more genes are found to be pleiotropic, a property that appears to be particularly prevalent in genes implicated in neural function and behavior (HALL 1994; GREENSPAN 2001; SOKOLOWSKI 2002). In this report, we challenge the second assumption—the stability of relationships in a gene network—in an analysis of interactions among a set of genes affecting loss of coordination in *Drosophila melanogaster*. Unlike previous studies of gene networks, we have predefined a gene network in terms of functional interactions, rather than common phenotype, common biochemical function, or covariation of gene expression patterns. A classical analysis of epistasis was performed on 16 mutations isolated on the basis of their interaction with a mutation of *Syntaxin-1A* (*Syx1A*), a component of the machinery of secretion and synaptic transmission (RICHMOND and BROADIE 2002). The functional relationships among the 16 genes are shown to vary with their genetic context: they change dramatically depending on the presence or absence of the *Syx1A* mutation, indicating a potential for network flexibility beyond that predicted by standard, molecular biological models of gene interactions.

MATERIALS AND METHODS

***D. melanogaster* cultures and stocks:** Flies were cultured at 22°, 50–60% humidity, 12 hr:12 hr light:dark cycle, on brewer's yeast, dark corn syrup, and agar food, slightly modified from the original (BENNETT and VAN DYKE 1971). Flies were aged 2–5 days prior to behavioral testing. $Syx1A^{3\text{-}69}$ was obtained from

[1]*Corresponding author:* Neurosciences Institute, 10640 John Jay Hopkins Dr., San Diego, CA 92121. E-mail: greenspan@nsi.edu

T. Littleton (Massachusetts Institute of Technology). Wild-type flies are from the Canton-S strain. The w^{1118} strain, a non-isogenized laboratory strain maintained in vials, was obtained from the Berkeley Drosophila Genome Project, as were the 200 chromosome 2 EP lines (Rorth *et al.* 1998). All other strains were obtained from the Bloomington Drosophila Stock Center. New EP lines were generated by crossing w^{1118}/Y; *TM6 Sb* Δ*2-3/TM3 Ser* × w^{1118}/w^{1118}; *EP409/EP409* females, collecting w^{1118}/Y; *TM6 Sb* Δ*2-3/EP409* progeny, and crossing them to w^{1118}/w^{1118}; *TM3 Ser/Syx1A*$^{3\text{-}69}$, from which w^{1118}/Y; *EP*; TM6 Sb/Syx1A*$^{3\text{-}69}$ flies, representing jumps to either chromosome 1 or 2, were collected. (Representative schemes for these crosses can be found in Greenspan 2004.) These were then crossed with w^{1118} females to establish stocks. A total of 268 such lines were tested, from which 15 were saved, yielding two unique inserts (EP2 and EP7, see Table 2) whose flanking sequences and genomic position were identified by inverse PCR (Dalby *et al.* 1995). For all subsequent analysis, *Syx1A*$^{3\text{-}69}$ and all EP lines were placed onto the w^{1118} background by backcrossing for a minimum of six generations.

The eight additional lines used as a control matrix of EP, not isolated on the basis of an interaction with *Syx1A*$^{3\text{-}69}$ (EP2221, EP2367, EP2534, EP3417, EP2162, EP2508, EP2505, and EP3171), were obtained in a screen for variants altering locomotor patterns.

Interactor screen: Flies tend to climb upward, especially when tapped or heated, but *Syx1A*$^{3\text{-}69}$/+ flies display a "bottom-dwelling" phenotype when placed in glass test tubes at 39°. In contrast, wild-type flies will still be able to run up (and down) the sides of the glass tube for several minutes, before also becoming uncoordinated. In the initial screen for genes interacting with *Syx1A*$^{3\text{-}69}$, EP lines were crossed to the w^{1118}; *Syx1A*$^{3\text{-}69}$/*Syx1A*$^{3\text{-}69}$ strain, resulting in EP/+; *Syx1A*$^{3\text{-}69}$/+ females, which were tested behaviorally, in batches of 10 flies per genotype. Flies were placed in 16 × 150-mm borosilicate glass test tubes (Fisher Scientific, Pittsburgh), which were stoppered with foam plugs and submerged in a 39° water bath up to the plug levels. After 4 min of exposure to heat, all tested flies were allowed into a "refuge" at room temperature (22°) for each tube, provided some were still able to climb the walls of the glass tube. This was achieved by raising the foam plug 3 cm above the waterline in the partially submerged test tubes. Strains with any flies in the refuge were selected and subsequently retested as described below.

Behavioral assay: Nine flies per genotype were loaded into 16 × 150-mm borosilicate glass test tubes, plugged with foam stoppers, and lowered into a 39° circulating water bath such that the chambers containing the flies were completely submerged. The instantaneous number of flies found in the bottom third of the tube was scored at 10-sec intervals for a total of 10 min, four tubes at a time. Four sets of flies were tested for each genotype. The data for each strain thus yielded four sets of 60 time points, for which a $t_{1/2}$ score could be calculated by logistic nonlinear regression (Waud 1972).

Curve fitting: The proportion (normalized to a maximum of 1) of bottom-dwelling flies was averaged for each time point (four replicates per time point and 60 time points) and the averaged data were fit by logistic nonlinear regression (Waud 1972) to provide a $t_{1/2}$ statistic for each genotype (the time required for half the flies to become bottom dwellers). Different genotype scores were compared for significance against one another by a simultaneous curve-fitting approach (DeLean *et al.* 1978), where all of the data are considered rather than just the $t_{1/2}$ statistic. In the case of calculating the significance of deviations for observed data from predicted curves (see below), the simultaneous curve-fitting protocol compared the observed data points to a predicted curve of 60 points. Microcal Origin 5.0 (Northampton, MA) software was used for these curve-fitting calculations.

Diallel design and statistical analyses: Two diallel designs were used. In the first (matrices I and II), two sets of eight EP interactor strains (with or without *Syx1A*$^{3\text{-}69}$/+) were intercrossed for all pairwise combinations *within* each set (2 × 28 combinations). In the second design (matrix III), each EP interactor strain from one set (with or without *Syx1A*$^{3\text{-}69}$) was crossed with each strain from the other set (64 combinations), to tie both smaller matrices together. An additional matrix of eight entirely different EP strains (28 combinations) was also analyzed for the coordination phenotype in the absence of *Syx1A*$^{3\text{-}69}$, for comparison to the *Syx1A*$^{3\text{-}69}$-interacting EP strains. Average EP effects were derived from each matrix separately, and the "tie-in" matrix (matrix III) provides an independent assessment of the average effects calculated from matrices I and II. These calculations provide the material for calculating predicted values for each *trans*-heterozygote pair, with which observed data can be compared and epistasis identified. These calculations are somewhat different for either diallel design (matrices I and II *vs.* matrix III). For matrices I and II, the general combining ability (GCA) for each strain and the specific combining ability (SCA) for each pair were calculated exactly as described in Griffing (1956) and Fedorowicz *et al.* (1998). The calculations were performed for data at each of the 60 time points, resulting in 60 separate GCA and SCA calculations that were in turn fit by nonlinear regression, as described above, to yield genotypic data. For matrix III, which involves all pairwise combinations of the two separate matrices, the GCA calculations were modified as

$$\mathrm{GCA}_i = T_i/(n-1) - \sum T/2(n^2 - n),$$

where T_i is the sum of mean bottom-dwelling proportions at each time point of heterozygotes with the ith EP element, $\sum T$ is two times the sum of mean proportions of all heterozygotes at each time point, and n is the scale of the matrix (the number of EP lines on one side). The SCA effects for matrix III were then calculated as

$$\mathrm{SCA}_{ij} = x_{ij} - (T_i + T_j)/(n-1) + \sum T/2n^2,$$

where x_{ij} is the observed mean proportion at each time point, and $\sum T/2n^2$ is the grand mean for matrix III. For all matrices, significance was determined by simultaneous curve fitting of the data in x_{ij} *vs.* (GCA_i + GCA_j + grand mean) for all time points. An *F*-variance ratio was found by

$$((\mathrm{SSR2} - \mathrm{SSR1})/(\mathrm{d.f.\ 2} - \mathrm{d.f.\ 1}))/\chi^2 1,$$

where SSR2 and SSR1 are the sums of squares resulting from a logistic fit of either set of data together or separately, respectively, d.f. are degrees of freedom, and $\chi^2 1$ is the chi-square statistic for the two curves treated separately (DeLean *et al.* 1978). Significance was set at $P < 0.05$.

RESULTS

The goal of this study was to probe the flexibility of interactions in a defined gene network. A set of 16 such interacting genes was isolated, on the basis of their alteration of a quantitative behavioral phenotype as double heterozygotes with a starting mutation. The interactions *inter se* of this gene set were then assayed in the presence and in the absence of the starting mutation. The question is whether the relationships among the 16 genes remain stable or change in a different genetic context (*i.e.*, presence *vs.* absence of the original mutation).

Flies of the genotype *Syx1A*$^{3\text{-}69}$/+ lose coordination when their temperature is raised from 25° to 39° (Littleton *et al.* 1998). A count of the number of uncoordinated flies (see materials and methods), every 10 sec over a 10-min period following the temperature shift, provides a sensitive, quantitative measure of the heat-response kinetics. In both the presence and the absence of *Syx1A*$^{3\text{-}69}$, time at 39° can be treated like a "dosage" effect to yield dose-response curves with pharmacological (sigmoidal) characteristics. The loss of coordination data (proportion of uncoordinated flies at 60 time points following the temperature shift) can be fit by nonlinear regression (Waud 1972) to yield a $t_{1/2}$ for the tested fly strain. Although the $t_{1/2}$ statistic assigns a shorthand for the phenotype, a fuller representation of the strain's phenotype is described by the entire curve, which is used in all subsequent statistical tests. The strain used as the genetic background for this study, w^{1118}, was found to have a $t_{1/2}$ of 233 ± 4.4 s (± standard error of the estimate), as compared to 103 ± 2.7 sec for w^{1118}; *Syx1A*$^{3\text{-}69}$/+. The significantly lower $t_{1/2}$ for the w^{1118}; *Syx1A*$^{3\text{-}69}$/+ strain indicates a semidominant effect of temperature at the level of synaptic release (*Syx1A*$^{3\text{-}69}$ homozygotes "pass out" completely in seconds at 39°). The *Syx1A*$^{3\text{-}69}$/+ semidominant phenotype provided the starting material for identifying genes interacting with *Syntaxin1A*. The set of 16 mutations suppressing the mutant *Syx1A*$^{3\text{-}69}$/+ phenotype (increasing the $t_{1/2}$) was isolated in a screen of 486 EP insertions (Rorth *et al.* 1998), tested as heterozygotes with *Syx1A*$^{3\text{-}69}$ on the white (w^{1118}) background (Table 1). Although we are not primarily concerned in this study with their mechanism of interaction, the putative identities of the EP lines suppressing the synaptic mutation phenotype are shown in Table 2. They suggest a wide range of functions, from transcription to metabolism.

To measure the interactions among the 16 *Syx1A* interactors, we divided them arbitrarily into two groups of eight and constructed a matrix of double heterozygotes for each group in the presence of the original *Syx1A*$^{3\text{-}69}$ mutation (a classical diallel cross). Coordination phenotypes were scored at 39° for each of the pairwise combinations within each group, average data was fit by nonlinear regression, and a $t_{1/2}$ statistic was derived to describe the coordination phenotype of each *trans*-heterozygote (see Figure 1A, red curve, for a sample *trans*-heterozygote phenotype on a *Syx1A*$^{3\text{-}69}$/+ background). These data are shown (by $t_{1/2}$ statistics) as the top entry for each genotype in Table 3, where matrix I (top left 28 pairs) corresponds to one set of eight interactors and matrix II (bottom right 28 pairs) corresponds to the other. To relate the two matrices to each other, and to provide an independent assessment of EP effects, matrix III (top right 64 pairs) represents the results of pairwise combinations made between the members of each set, also on a *Syx1A*$^{3\text{-}69}$/+ background. To assess the effect of genetic context, the same three matrices were also done in the absence of the *Syx1A*$^{3\text{-}69}$ mutation (Table 4, top entries, and Figure 1B, red line, for an example).

The diallel cross design (the "matrix") can be used to identify genetic interactions among a set of genes. These interactions are uncovered in *trans*-heterozygote phenotypes, which deviate significantly from the phenotype predicted by the additive effect of their combined components. The average effect of each EP element (GCA; see materials and methods and Table 1) is calculated as a deviation from the grand mean of the matrix. In this study, each EP element is described by 60 average effects (at each of 60 time points), which are the deviations from the mean curve for the matrix. To generate a predicted curve for a *trans*-heterozygote on the basis of additive effects, the average effects of each component are added to the grand mean data at each of the 60 time points, and the resulting data are fit by nonlinear regression to produce a predicted $t_{1/2}$ statistic (Tables 3 and 4, bottom entry for each genotype, and Figure 1, A and B, blue lines). If the predicted and observed curves for a *trans*-heterozygote are significantly different from each other (see materials and methods and Figure 1B for an example), this indicates an emergent phenotype dependent on the specific combination of elements in that strain, or epistasis. In the *Syx1A*$^{3\text{-}69}$/+ background that was used to isolate these suppressors, EP pairings identified a large number of epistatic interactions among the suppressor elements (Table 3, entries in boldface type). This high level of epistasis was fairly evenly divided among the three matrices examined (matrix I, 13 of 28 pairs; matrix II, 10/28; matrix III, 21/64) for a grand total of 44 significant interactions among 120 EP combinations on the *Syx1A*$^{3\text{-}69}$/+ background. The relative connectivity among these EP elements can be illustrated in a formal diagram indicating (with a line connecting the two elements) the significant deviations from additivity (Figure 2, A, C, and E for matrices I, II, and III, respectively).

In further probing the character of this network, the *Syx1A*$^{3\text{-}69}$ mutation used as the original basis for isolation of the EP interactors was removed from each double-heterozygote combination, and the three matrices of EP pairs were rescored on a (nonmutant) *Syx1A*$^{+}$ background (Table 4). Predicted phenotypes were calculated from combined average effects of each EP as before on this novel genetic background. As for the previous crosses performed in the presence of the *Syx1A*$^{3\text{-}69}$ mutation, the observed phenotypes (curves) of many *trans*-heterozygotes also differ significantly from their predicted values, indicating that epistasis was prevalent among these EP elements even when the mutation they originally suppressed individually was removed. The number of nonadditive interactions found was similar to the number found in the presence of the *Syx1A*$^{3\text{-}69}$ mutation (matrix I, 10 of 28 pairs; matrix II, 9/28; matrix III, 24/64), adding to a grand total of 43 significant

TABLE 1

"Bottom-dwelling" kinetics of *Syx1A*$^{3\text{-}69}$ interactor EPs

EP strain	EP; *Syx1A*/+	EP/+	GCA *Syx*$^-$ from MI and II	GCA *Syx*$^-$ from MIII	GCA *Syx*$^+$ from MI and II	GCAs *Syx*$^+$ from MIII
			Matrix I			
2	162 ± 3.4	318 ± 4.7	−50	−16	−2	−14
548	136 ± 3.5	410 ± 5.9	+15	+40	+46	+63
364	176 ± 4.9	260 ± 6.0	−9	−17	−47	−5
718	195 ± 3.5	315 ± 3.3	+20	+9	−15	+1
598	165 ± 2.8	201 ± 4.0	+1	−29	−34	−1
563	206 ± 4.7	220 ± 4.5	+2	+21	+4	+27
547	180 ± 3.3	374 ± 6.3	+7	−12	+49	−44
1244	182 ± 3.8	258 ± 4.6	+14	+4	−1	−27
			Matrix II			
638	146 ± 2.6	178 ± 4.2	+6	+8	−46	+7
315	156 ± 3.1	356 ± 5.4	+20	+5	−2	−16
2096	125 ± 3.1	347 ± 3.6	+7	−14	+55	+80
386	156 ± 3.2	186 ± 6.0	−37	−23	−70	−115
701	128 ± 2.5	206 ± 5.2	+32	+24	−15	−31
7	126 ± 0.95	349 ± 6.7	−7	+25	+96	+74
704	145 ± 3.6	404 ± 4.7	−21	−18	+5	+25
454	184 ± 4.0	201 ± 4.9	0	−7	−23	−24

	Syx1A$^-$			*Syx1A*$^+$		
	MI	MII	MIII	MI	MII	MIII
Grand means	218 ± 2.6	215 ± 2.4	166 ± 1.5	265 ± 3.1	235 ± 3.7	227 ± 2.4

Background strains	
w^{1118}	233 ± 4.4
Syx1A$^-$/+	111 ± 3.2
w^{1118}; *Syx1A*$^-$/+	103 ± 2.7
Canton-S[a]	251 ± 6.7

The coordination kinetics for the 16 EP lines, with and without the mutant *Syx1A* allele, are expressed as $t_{1/2}$ scores (in seconds ± standard errors of the estimate). The EP lines were arbitrarily placed into two groups, matrix I (MI) and matrix II (MII). GCAs were calculated within either matrix for all 60 time points. Shown here for both allelic backgrounds are the $t_{1/2}$ deviations (GCA) from the grand means, which sum to zero for each matrix. Grand means for each matrix (MI, MII, and MIII) are shown below. These statistics were then calculated again from a completely different source, matrix III (MIII, all intercrosses between I and II), yielding an independent estimate of average effects. Although the $t_{1/2}$ statistics shown provide an estimate of effects, they are not the actual data used in our analysis. For calculating predicted effects and determining significant deviations, we used the entire fitted curve of 60 individual GCA statistics. In this way, any characteristic effect on the shape of the curve is included in the analysis, yielding a more accurate assessment than a single $t_{1/2}$ score.

[a] Canton-S is not a background strain in this study, but is provided as a reference comparison.

interactions among 120 EP combinations on the *Syx1A*$^+$ background. The relative connectivity among the EP elements on the *Syx1A*$^+$ background is illustrated alongside the connectivity diagrams found for the same EP elements on the *Syx1A*$^{3\text{-}69}$ background (Figure 2, B, D, and F, for matrices I, II, and III, respectively). Strikingly, most of the interactions on the *Syx1A*$^+$ background differed from those on the *Syx1A*$^{3\text{-}69}$ background. Most of the interactions that were statistically significant on the *Syx1A*$^{3\text{-}69}$ background ceased to be significant, a few interactions reversed sign, and a whole new set of interactions emerged, even though the total number of interactions detected remained roughly the same.

One might have expected that these EP elements found to be suppressing the same mutation would have shown conserved synergistic effects on either background. Instead, phenotypes on either background are uncorrelated, even beyond the illustrative purposes of a diagram (which shows only significant effects, not quantitative tendencies). The observed phenotypes for matrices I and II in the absence of the *Syx1A*$^{3\text{-}69}$ mutation were not correlated with the observed phenotypes on the *Syx1A*$^{3\text{-}69}$ background

TABLE 2

***Syx1A*$^{3\text{-}69}$ interactor EPs**

EP2 (5B5)
Near CG15770—unlike anything, vague homology to mus309
Near MAPk-Ak2 (MAP kinase-activated protein kinase-2)
Near CG3097—some homology to *svr* carboxypeptidase (makes neuropeptides)
EP7 (23B1)
Disrupts CG9894—NLS motif, neural precursor gene
EP315 (21B4)—*P{EP}Gmd*^{EP315}
Gdp-mannose 4,6-dehydratase
EP364 (33D2)
CG6579—unknown function, domains
EP386 (54C7)—*P{EP}MESR4*^{EP386}
Misexpression interactor of ras 4 zinc finger, C2H2 type
EP454 (54C7)
Unclear which gene associated, no flanking sequence in database
Near *l(2)k00611*
l(2)rH280
EP547 (45D4)—*P{EP}Pdk*^{EP547}
Pdk—pyruvate dehydrogenase (lipoamide) kinase, mitochondrial matrix
EP548 (60E6)—*P{EP}CG3760*^{EP548}
CG3760—unknown function, domains
EP598 (22C3)
aop^{EP598}-anterior open—Ets-domain signature 1 and 2
EP563 (21B4)
Next to *kismet*
EP638 (50E6)—*P{EP}CG8415*
S12—component of the cytosolic small ribosomal subunit
EP701 (30E3)—*P{EP}CG5899*
ATP-dependent DNA helicase
EP704 (49D1) *P{EP}704*
Unclear which gene associated, flanking sequence resembles nothing
Near *l(2)k09328*
kelch-like—BTB/POZ-domain
EP718 (58F3)—*P{EP}CG13512*
Disrupts CG13512—unknown function, domains
EP1244 (12D3)—*P{EP}CG14756*
Very vaguely like Cecropin, otherwise unknown domains and protein
EP2096 (42A4)—*P{EP}Act42A*^{EP2096}
Actin 42A

Cytogenetic map position is in parentheses.

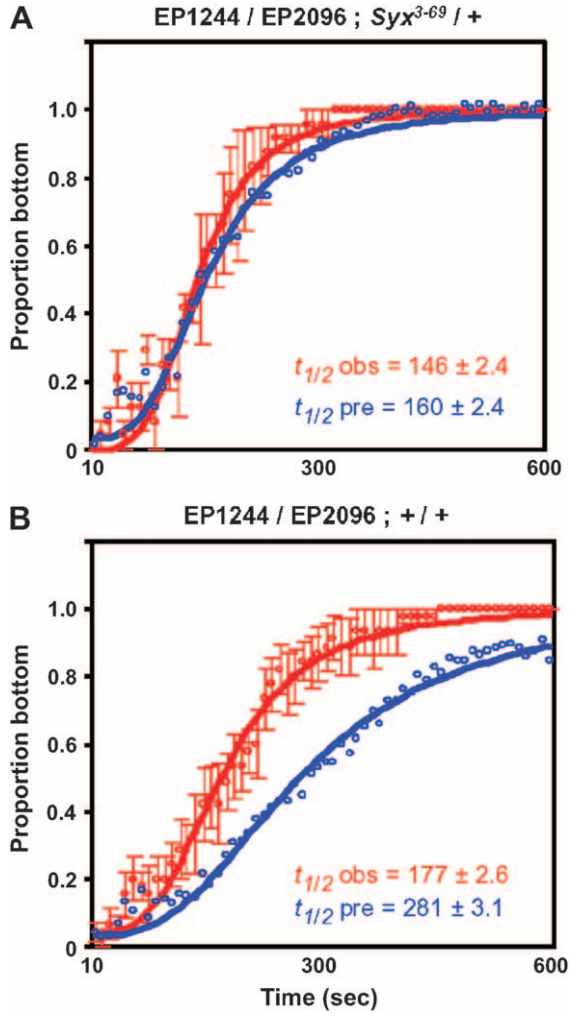

FIGURE 1.—Observed and predicted curves. The proportion of flies of a sample strain unable to climb the sides of a glass tube at 39° ("proportion bottom") is plotted against time and fit by a logistic equation to estimate a $t_{1/2}$ statistic ± a standard error of the estimate. Actual average data (60 red circles ± SEM) and fitted curves (red lines) are plotted alongside predicted phenotypes (blue circles) and curves (blue lines). (A) Data for a sample double-heterozyous EP pair, *EP1244/EP2096; Syx1A*$^{3\text{-}69}$*/+*, with the mutant *Syx1A* allele. (B) The same EP pair, *EP1244/EP2096; +/+*, on a *Syx1A*$^{+}$ background.

($r = 0.07$, $P = 0.60$, $n = 56$ combinations). Similarly, matrix III phenotypes in the absence of *Syx1A*$^{3\text{-}69}$ were not well correlated with measurements in the presence of the mutant ($r = 0.21$, $P = 0.09$, $n = 64$ combinations). Finally, the differences between observed and predicted $t_{1/2}$ values are also uncorrelated between matrices on either background ($r = 0.06$, $P = 0.65$, for matrices I and II, and $r = 0.13$, $P = 0.31$, for matrix III).

We questioned whether the strikingly different patterns of interactions among EP elements on either background might be due to some variability in our assay. To a certain extent, environmental variance is not an issue at this level because all significant epistatic effects were deemed as such following a statistical comparison between predicted curves and the variance (four separate curves) of a strain; if variance was a big problem, nothing would be significant by these measures. On the other hand, since the matrices were separate experiments done at different times, it remained possible that different environmental conditions produced the shifting patterns described above. We addressed this possibility in two ways: by investigating the stability of average effects and predicted phenotypes calculated for the same EP elements from completely different matrices (within the same background *Syx1A* allele) and by rescoring eight select EP pairs (on a *Syx1A*$^{3\text{-}69}$ background and backcrossed for an additional five generations to w^{1118}) after a hiatus of 3 years.

TABLE 3

Heterozygous interactions of EPs *inter se* in the presence of *Syx1A*$^{3\text{-}69}$

548	*364*	*718*	*598*	*563*	*547*	*1244*	*638*	*315*	*2096*	*386*	*701*	*7*	*704*	*454*	
			I								III				
168 ± 3.4	**241 ± 6.6**	207 ± 4.4	179 ± 4.5	**221 ± 7.6**	170 ± 3.9	143 ± 3.3	**190 ± 3.1**	160 ± 1.8	140 ± 2.3	**168 ± 4.8**	**142 ± 3.2**	199 ± 3.3	149 ± 2.2	**106 ± 1.3**	*2*
191 ± 39	**183 ± 3.5**	196 ± 3.9	182 ± 2.9	**187 ± 3.8**	188 ± 5.1	190 ± 3.5	**161 ± 1.8**	156 ± 1.8	146 ± 1.7	**136 ± 2.3**	**175 ± 2.0**	176 ± 2.5	139 ± 1.6	**151 ± 2.4**	
	221 ± 5.0	275 ± 8.9	**302 ± 9.6**	**157 ± 5.2**	**338 ± 6.9**	279 ± 6.3	250 ± 4.2	234 ± 6.1	**141 ± 3.6**	199 ± 4.8	**331 ± 6.6**	**158 ± 2.7**	204 ± 3.4	182 ± 1.8	*548*
	234 ± 5.1	260 ± 5.1	**239 ± 4.2**	**237 ± 4.3**	**267 ± 6.2**	257 ± 5.1	218 ± 2.4	222 ± 2.3	**188 ± 2.5**	181 ± 2.5	**256 ± 2.5**	**245 ± 2.8**	187 ± 2.3	199 ± 2.2	
		217 ± 6.3	191 ± 3.8	215 ± 4.6	**176 ± 2.7**	**297 ± 8.1**	172 ± 2.9	**126 ± 2.6**	**192 ± 3.2**	150 ± 2.8	154 ± 1.7	181 ± 3.9	**105 ± 1.0**	173 ± 1.8	*364*
		234 ± 5.5	216 ± 8.2	220 ± 5.4	**217 ± 2.6**	**231 ± 3.6**	158 ± 1.7	**155 ± 1.7**	**144 ± 1.7**	136 ± 2.1	173 ± 1.5	174 ± 1.9	**141 ± 1.3**	151 ± 2.1	
			152 ± 4.1	216 ± 4.6	**307 ± 7.2**	**311 ± 7.5**	174 ± 2.5	190 ± 9.6	172 ± 3.8	146 ± 2.9	**167 ± 3.4**	**230 ± 3.5**	152 ± 2.4	181 ± 3.9	*718*
			254 ± 5.1	242 ± 4.4	**256 ± 3.6**	**270 ± 4.6**	185 ± 2.0	182 ± 2.6	165 ± 2.1	158 ± 2.8	**201 ± 2.4**	**205 ± 2.5**	162 ± 2.0	175 ± 2.6	
				246 ± 5.1	**322 ± 11**	**418 ± 25**	**117 ± 2.2**	131 ± 1.4	137 ± 1.7	121 ± 2.9	**214 ± 7.4**	172 ± 3.7	130 ± 2.6	**174 ± 4.1**	*598*
				229 ± 5.3	**234 ± 5.2**	**250 ± 6.0**	**148 ± 1.9**	140 ± 2.0	134 ± 2.0	124 ± 2.4	**156 ± 2.5**	156 ± 2.5	130 ± 1.8	**139 ± 2.3**	
					232 ± 5.4	238 ± 6.6	217 ± 4.0	211 ± 6.9	169 ± 3.9	**127 ± 2.1**	225 ± 3.9	202 ± 4.3	188 ± 3.8	180 ± 3.3	*563*
					232 ± 3.5	242 ± 4.5	202 ± 2.1	199 ± 2.6	175 ± 2.1	**165 ± 2.4**	223 ± 3.6	226 ± 2.8	171 ± 1.9	184 ± 2.3	
						165 ± 2.9	165 ± 2.2	171 ± 3.5	152 ± 2.5	123 ± 2.8	173 ± 4.5	**131 ± 4.1**	161 ± 2.7	**193 ± 3.7**	*547*
						257 ± 3.2	164 ± 1.9	158 ± 2.5	147 ± 2.3	138 ± 2.9	174 ± 2.7	**181 ± 2.4**	144 ± 2.1	**153 ± 3.2**	
							165 ± 2.3	180 ± 4.8	146 ± 2.4	**185 ± 4.8**	180 ± 3.3	**242 ± 5.3**	175 ± 2.9	**128 ± 2.4**	*1244*
							183 ± 2.1	177 ± 3.0	160 ± 2.4	**150 ± 2.5**	197 ± 2.6	**203 ± 3.1**	156 ± 1.7	**168 ± 2.8**	
											II				
								284 ± 6.2	194 ± 8.2	222 ± 6.6	215 ± 6.2	**171 ± 6.2**	**261 ± 6.7**	216 ± 10	*638*
								243 ± 4.4	229 ± 3.9	187 ± 3.4	248 ± 4.5	**214 ± 3.8**	**198 ± 3.8**	219 ± 4.4	
									178 ± 6.6	**237 ± 6.3**	**414 ± 20**	196 ± 4.4	215 ± 6.1	220 ± 5.4	*315*
									245 ± 4.0	**194 ± 3.5**	**275 ± 4.7**	222 ± 4.6	217 ± 3.7	241 ± 4.6	
										137 ± 3.3	283 ± 7.2	**316 ± 12**	229 ± 4.4	**287 ± 6.8**	*2096*
										183 ± 2.7	267 ± 3.7	**215 ± 4.7**	208 ± 3.3	**228 ± 4.7**	
											169 ± 3.8	162 ± 4.2	200 ± 6.7	166 ± 3.6	*386*
											202 ± 3.2	174 ± 3.3	167 ± 2.7	179 ± 3.3	
												248 ± 4.7	187 ± 3.9	**292 ± 11**	*701*
												235 ± 3.6	219 ± 3.7	**247 ± 4.5**	
													179 ± 4.9	238 ± 7.2	*7*
													189 ± 3.7	208 ± 4.6	
														120 ± 5.2	*704*
														191 ± 3.7	

EP strain numbers are listed in the top row and right-most column. The top number of each entry is the observed time (in seconds) for half the flies of an EP pair to become uncoordinated, and the bottom number is the predicted time for each genotype, calculated from the general combining abilities (GCAs) of each strain (see MATERIALS AND METHODS). Scores are $t_{1/2}$ statistics ±SE of the estimate derived by logistic regression of at least 240 data points from 30–60 flies per genotype for the observed data, and 60 points for the predicted data. For analyses, the data are divided into three matrices (I, II, and III), as shown. All strains are heterozygous for the *Syx1A*$^{3\text{-}69}$ allele. Significant differences between observed and predicted curves ($P < 0.05$), determined by simultaneous curve fitting, are indicated by boldface type. Matrix I (top left) corresponds to one set of eight interactors and matrix II (bottom right) corresponds to the other. To relate the two matrices to each other, matrix III (top right) represents the results of pairwise combinations made between the members of each set.

TABLE 4

Heterozygous interactions of EPs *inter se*

548	*364*	*718*	*598*	*563*	*547*	*1244*	*638*	*315*	*2096*	*386*	*701*	*7*	*704*	*454*	
			I								III				
295 ± 7.2	206 ± 2.9	232 ± 4.0	**281 ± 6.0**	295 ± 7.7	293 ± 6.9	**342 ± 1.8**	221 ± 4.9	241 ± 5.3	**354 ± 9.1**	119 ± 1.6	**290 ± 4.7**	**220 ± 3.4**	**201 ± 3.6**	231 ± 5.9	*2*
330 ± 5.1	227 ± 3.1	258 ± 2.1	**236 ± 3.3**	279 ± 2.5	325 ± 5.4	**269 ± 3.4**	232 ± 3.3	215 ± 3.6	**293 ± 3.3**	124 ± 1.8	**203 ± 2.9**	**296 ± 4.3**	**251 ± 3.3**	207 ± 2.5	
	402 ± 16	344 ± 10	263 ± 6.4	320 ± 11	**579 ± 19**	**210 ± 4.0**	315 ± 10	276 ± 6.8	335 ± 7.5	123 ± 2.1	276 ± 6.1	**294 ± 8.0**	**575 ± 11**	291 ± 9.5	*548*
	261 ± 5.2	301 ± 6.1	277 ± 4.6	323 ± 6.7	**393 ± 10**	**316 ± 4.8**	308 ± 5.8	283 ± 5.8	397 ± 5.5	145 ± 5.0	247 ± 4.0	**402 ± 7.7**	**328 ± 5.1**	269 ± 4.8	
		263 ± 6.7	**152 ± 2.7**	291 ± 6.2	252 ± 6.3	**194 ± 3.7**	242 ± 6.6	219 ± 4.8	**379 ± 6.6**	154 ± 3.4	186 ± 3.5	324 ± 7.4	**201 ± 3.1**	205 ± 4.7	*364*
		221 ± 4.0	**200 ± 2.9**	241 ± 5.5	255 ± 4.8	**227 ± 3.5**	248 ± 2.8	229 ± 3.8	**311 ± 3.0**	128 ± 2.1	203 ± 3.1	304 ± 4.7	**255 ± 3.1**	210 ± 2.8	
			224 ± 9.2	247 ± 6.5	272 ± 6.3	266 ± 4.7	232 ± 6.6	**160 ± 3.8**	308 ± 4.9	112 ± 6.7	196 ± 4.0	327 ± 6.0	**337 ± 4.5**	206 ± 4.4	*718*
			225 ± 4.2	268 ± 5.0	295 ± 5.5	255 ± 3.6	248 ± 4.1	**229 ± 3.9**	315 ± 3.4	122 ± 2.2	208 ± 4.3	312 ± 6.0	**263 ± 4.3**	214 ± 2.6	
				242 ± 4.0	243 ± 6.6	**349 ± 6.8**	**194 ± 4.2**	183 ± 8.3	261 ± 6.4	**334 ± 7.0**	213 ± 4.6	292 ± 6.4	**212 ± 6.0**	193 ± 3.8	*598*
				245 ± 4.3	271 ± 3.6	**233 ± 3.0**	**246 ± 3.0**	221 ± 4.2	307 ± 3.7	**124 ± 2.6**	206 ± 2.7	311 ± 4.7	**259 ± 3.6**	213 ± 3.4	
					310 ± 6.6	250 ± 5.3	**383 ± 10**	221 ± 7.9	336 ± 9.1	**85 ± 1.1**	**306 ± 8.3**	348 ± 9.5	343 ± 6.1	229 ± 3.9	*563*
					316 ± 6.1	276 ± 4.1	**277 ± 6.0**	252 ± 4.5	362 ± 5.8	**130 ± 4.7**	**225 ± 4.4**	368 ± 9.1	300 ± 6.0	242 ± 3.7	
						364 ± 11	**163 ± 3.1**	**343 ± 9.5**	**334 ± 6.4**	110 ± 2.9	**121 ± 2.4**	379 ± 1.1	218 ± 3.9	139 ± 2.0	*547*
						310 ± 4.6	**202 ± 3.4**	**185 ± 3.8**	**268 ± 4.3**	104 ± 2.3	**176 ± 3.7**	261 ± 6.8	217 ± 4.2	176 ± 2.9	
							267 ± 8.1	190 ± 3.0	**177 ± 2.6**	**168 ± 6.6**	171 ± 3.4	261 ± 6.6	218 ± 6.0	**291 ± 5.3**	*1244*
							221 ± 3.7	204 ± 3.3	**281 ± 3.1**	**112 ± 1.9**	187 ± 4.4	273 ± 6.1	233 ± 4.4	**191 ± 2.6**	
											II				
								171 ± 6.4	205 ± 3.8	147 ± 4.8	204 ± 6.5	253 ± 6.8	195 ± 5.2	**245 ± 9.7**	*638*
								186 ± 9.9	237 ± 5.3	137 ± 4.1	189 ± 2.8	261 ± 5.8	205 ± 3.8	**180 ± 3.6**	
									304 ± 6.3	159 ± 10	199 ± 4.4	361 ± 14	296 ± 7.4	210 ± 5.0	*315*
									299 ± 5.1	175 ± 6.0	224 ± 5.4	337 ± 8.8	248 ± 5.2	218 ± 5.0	
										333 ± 9.2	250 ± 10	**288 ± 10**	337 ± 4.8	243 ± 9.0	*2096*
										217 ± 6.3	275 ± 9.7	**396 ± 7.0**	308 ± 3.7	278 ± 4.7	
											230 ± 6.8	**184 ± 9.6**	**134 ± 4.0**	154 ± 4.2	*386*
											165 ± 4.4	**244 ± 7.6**	**169 ± 3.9**	151 ± 3.6	
												366 ± 7.2	238 ± 6.3	**162 ± 2.7**	*701*
												303 ± 6.9	227 ± 3.5	**200 ± 3.7**	
													335 ± 9.2	**441 ± 13**	*7*
													353 ± 6.5	**308 ± 7.8**	
														222 ± 4.9	*704*
														226 ± 3.4	

The same experiment and analyses were performed as in Table 3, except that all doubly heterozygous EP combinations are *Syx1A*$^{+}$. Top number of each entry is the observed score, bottom number is the predicted value, calculated from the general combining abilities (GCAs) of each strain (see MATERIALS AND METHODS). Boldface type, $P < 0.05$. Matrix I (top left) corresponds to one set of eight interactors and matrix II (bottom right) corresponds to the other. To relate the two matrices to each other, matrix III (top right) represents the results of pairwise combinations made between the members of each set.

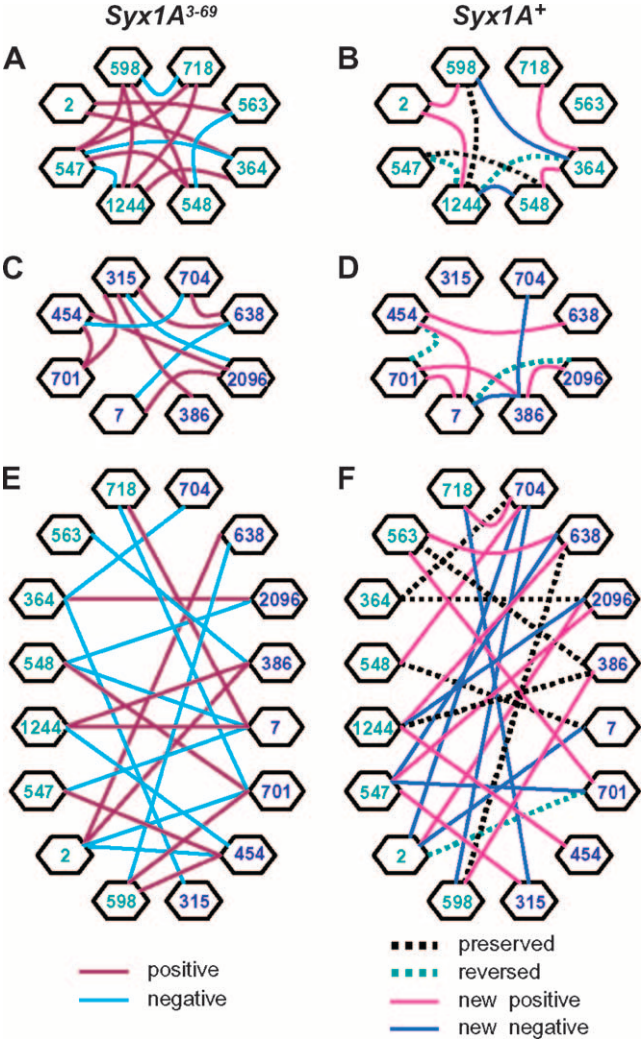

FIGURE 2.—Connectivity diagram of *Syx1A*$^{3\text{-}69}$ interactors. A, C, and E display interactions on a *Syx1A*$^{3\text{-}69}$ background. B, D, and F display interactions on a *Syx1A*$^{+}$ background. A–B and C–D are separate sets of EP strains and E–F is the interaction matrix between the two sets. Each set (A–B, C–D, and E–F) represents a separate experiment. The crosses in E–F are all performed between the two previous sets, not replicating crosses already done in A–B and C–D (hence the lack of any of the interactions seen above). EP numbers are displayed in green for the A–B set and in purple for the C–D set, to make them distinguishable in the combined matrix E–F. In A, C, and E, a significant interaction between two elements (see MATERIALS AND METHODS) is shown with a solid line, with the colors dark pink and turquoise indicating that the observed phenotype was higher or lower than predicted, respectively. In B, D, and F, preserved interactions from A, C, and E are shown with a dashed black line, interactions whose sign is reversed from that in A, C, and E are shown with a dashed green line, and new positive or negative interactions are shown as in A, C, and E with solid light pink or blue lines, respectively.

Our measure of epistasis assumes that the average additive effect of each element relative to other elements in a matrix is stable, regardless of some combinations producing unexpected phenotypic deviations. To test for the stability of average effects, and as part of our original design, we calculated GCAs for each EP element twice by including each element in two completely different diallel matrices (Tables 3 and 4; note that each EP is crossed to seven other EPs within matrix I or II, as well as to eight different EPs in matrix III). We were thus able to calculate two sets of average effects (GCA values) for each EP and two sets of predicted phenotypes for each EP combination in matrices I and II, from independent sources. On the *Syx1A*$^{+}$ background, both sets of predicted values ($n = 56$ combinations, *i.e.*, matrices I and II values derived from their own GCA calculations *vs.* matrices I and II values derived from matrix III GCA calculations) were highly correlated ($r = 0.65$, $P < 0.0000001$), as were the 16 EP phenotypic averages calculated from the different *Syx1A*$^{+}$ matrices ($r = 0.63$, $P < 0.01$). Similarly, on the *Syx1A*$^{3\text{-}69}$ background, average EP effects calculated twice from completely different sources were also found to be correlated ($r = 0.53$, $P < 0.05$, for the 16 EP phenotypic averages, and $r = 0.48$, $P < 0.001$ for the 56 predicted values). Thus, average EP effects on either background are stable (relative to one another) and can reliably be used to make phenotypic predictions, indicating that the deviations from additivity found throughout these matrices include genuine epistatic effects representing functional network phenomena. However, we did note some large environmental effects: on the *Syx1A*$^{3\text{-}69}$ background, matrix III displayed a significantly lower grand mean than matrices I and II, perhaps due to unidentified environmental conditions at the time of phenotyping matrix III in this background (see Table 1). Critically, such a phenotypic shift had little effect on the phenotypic averages relative to one another within the matrix (which is how epistasis was identified), as evidenced by the significant correlations between replicated average effects discussed above. The correlated phenotypes between matrix III and matrices I and II within the same genetic background are in marked contrast to the uncorrelated phenotypes between corresponding matrices on different *Syx1A* backgrounds. At the very least, this strengthens the argument that the dramatically altered pattern of epistasis on either background is not due to variance in average additive effects, but rather to altered functional relationships among the interacting genes when *Syx1A* is different. Results from retesting of eight selected EP pairs 3 years after the original experiment mirrored the preceding comparisons between matrices within a background. The correlation between phenotypes was high ($r = 0.69$ between replicates). This finding is particularly notable in view of the fact that the phenotypic mean was substantially different (270 sec originally *vs.* 180 sec for the repeats), thus underlining the robustness of our findings on the relationships among the EP elements *per se*, as opposed to the absolute phenotypes—a property for which diallel-type investigations are ideally suited.

The breadth of $t_{1/2}$ phenotypes was large for EP pairs under both *Syx1A* backgrounds, partially due to many

large epistatic effects. This can be visualized by plotting predicted *vs.* observed phenotypes, where the deviation from a diagonal (representing equivalence) is a measure of the size of epistatic effects within a diallel cross (Figure 3). The data plotted this way provide an additional level of comparison between genetic backgrounds compared to that provided by network diagrams (Figure 2). Each EP line was isolated by virtue of its interaction with the *Syx1A*$^{3\text{-}69}$ mutation, so the "cloud" of epistatic effects seen among these functionally related elements may not be surprising when considered on a *Syx1A*$^{3\text{-}69}$ background (Figure 3A). However, when the mutant *Syntaxin1A* allele was exchanged for a wild-type form of *Syntaxin1A*, we still saw a large number of epistatic effects among the elements, which can be visualized by the even larger cloud of (blue and red) points around the diagonal in Figure 3B. We wondered whether the breadth of phenotypes on the *Syx1A*$^{+}$ background, including many significant epistatic effects, was peculiar to the group of EPs chosen, by virtue of their functional relatedness. It is possible that when the common interacting *Syx1A*$^{3\text{-}69}$ mutation is removed, these elements display larger than ordinary synergistic effects because they form part of a functional network. We investigated this possibility by analyzing eight other EP lines in a new diallel cross on the *Syx1A*$^{+}$ background. These EP lines were selected not because of any interaction with the *Syx1A*$^{3\text{-}69}$ mutation, but rather they were isolated for affecting a locomotor phenotype in a different behavioral study. As such, they are not functionally related to any common mutation and are less likely to be functionally related to one another. An analysis of this last matrix revealed much smaller deviations from additivity (Figure 3B, black points) and altogether fewer significant instances of epistasis (3/28 *vs.* 10/28 and 9/28 for matrixes I and II, respectively; $P < 0.001$ by chi-square comparison of ratios). Thus, the EPs isolated on the basis of interaction with *Syx1A*$^{3\text{-}69}$ show greater epistasis than an independently isolated set of EPs from an unrelated screen.

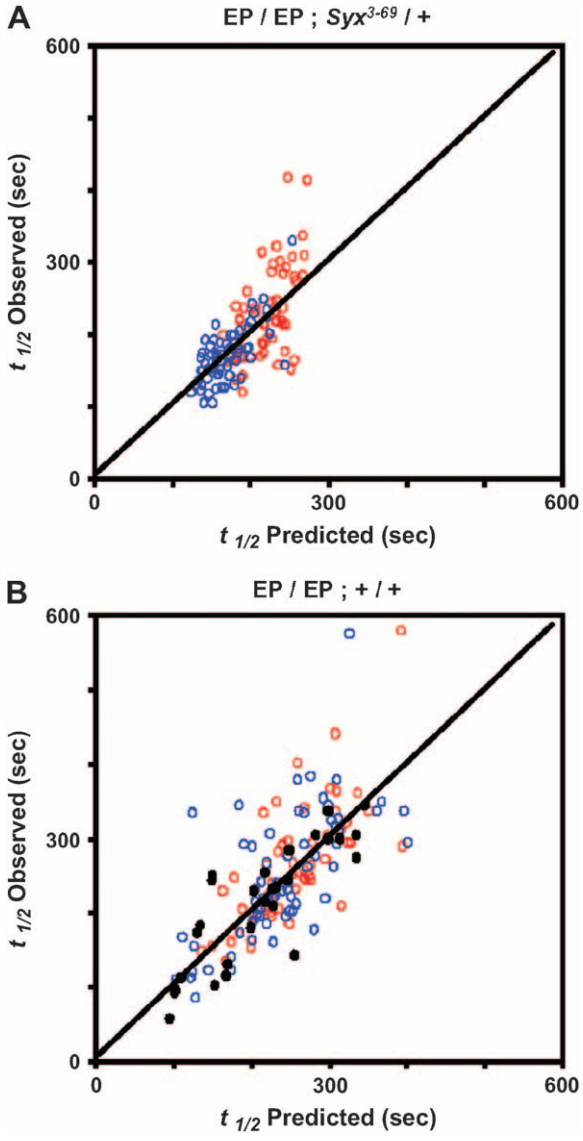

FIGURE 3.—Range of deviation of observed from predicted phenotypes in EP pairs. Data from Tables 3 (A) and 4 (B) on observed *vs.* predicted $t_{1/2}$ scores for loss of coordination of all pairwise genotypes are plotted against each other. Red circles represent the two different sets of eight EP lines (matrices I and II), blue circles represent the tie-in matrix (matrix III) between them, and the diagonal lines represent equivalence of observed and predicted values for each genotype (see text). An additional unrelated set of EP lines was analyzed for comparison in the *Syx1A*$^{+}$ background, and results are plotted as black circles in B. These lines are EP2221, EP2367, EP2534, EP3417, EP2162, EP2508, EP2505, and EP3171. Units on the ordinates and abcissas of A and B are time in seconds.

DISCUSSION

We set out to ask the question: How stable are gene interactions to changes in genetic context? The results suggest that they are less stable than expected. The pattern of epistatic interactions that are measurable within a set of variants is dramatically altered in the presence *vs.* the absence of an additional mutation. These results imply considerable flexibility and breadth in the network interactions of genes (GREENSPAN 2001).

Pleiotropy: The breadth of gene interactions revealed in the current study is reflected in the wide range of biological activities represented in the set of *Syx1A* interactors. While firm identifications of the genes affected in these insertion lines have yet to be made, the likeliest candidates (Table 2) cover a wide range of biological activities and, where their functions are well defined, reflect a high degree of pleiotropy—*e.g.*, DNA helicase, *Pdk*, *aop*, *Gmd*, *Act42A*, etc. This finding is in keeping with the well-documented extent of pleiotropy in genes affecting behavior in Drosophila (HALL 1994; GREENSPAN 2001; SOKOLOWSKI 2002). Wide-ranging genetic contributions to behavioral phenotypes in Drosophila have been seen previously in analyses of strains selected

for geotactic preference (Toma *et al.* 2002) and in sets of insertional mutations isolated for their effects on odor-guided behavior (Anholt *et al.* 2003) or for long-term memory (Dubnau *et al.* 2003).

The standard molecular biology model of gene interactions would have predicted that at least some of the *Syx1A* interactors would occur among the more intimate components of the vesicle secretion machinery. Their absence is likely to be accounted for by the fact that these are insertional mutations that affect expression levels, as opposed to amino acid sequence, and thus would be incapable of compensating for the altered conformation of the mutant $Syx1A^{3\text{-}69}$ protein (Littleton *et al.* 1998). Yet in spite of the far-flung activities of these genes, many of them exhibit network-like nonindependence as indicated by their epistatic interactions. (Note that even if the putative identifications of insertion sites in Table 2 prove incorrect, the neighboring two or three loci on either side of these sites are no more closely associated with vesicle secretion.) Our choice of *Syntaxin1A* as the central "node" for this network may have been conducive to uncovering such effects. Syntaxin, crucial for neurosecretion, is a highly promiscuous protein that has been shown to physically interact with at least a dozen other synaptic proteins (Jahn and Sudhof 1999). Each of these partners, in turn, is likely to interact with a number of other proteins, all of which are subject to regulation of their own. Such an exponential expansion of interactions quickly covers much of the genome, possibly explaining how such disparate elements can functionally interact to control a behavioral phenotype.

The epistatic paradox: The concept of epistasis is almost as old as that of the gene itself and its meaning has evolved with the discipline (Phillips 1998). Bateson (1909) first introduced the term to describe the ability of alleles at one locus to mask the effects of alleles at another locus. Fisher (1918) gave it a statistical form as the deviation from additivity and Wright (1931, 1968) made gene interactions the cornerstone of his evolutionary theory. Experimental analyses of epistasis have mirrored this bipartite origin. On one hand, comparisons of pairwise combinations of specific alleles at identified loci have been used to determine pathway order in studies of the specific actions of a gene on a particular phenotype. Although this method for detecting epistasis has a longer history, it has been practiced in earnest only relatively recently as part of the genetic and molecular analysis of development (*e.g.*, Avery and Wasserman 1992). These studies of epistasis are rarely, if ever, quantitative (see Gibson and van Helden 1997 and Polakzyk *et al.* 1998 for an interesting exception). On the other, statistical analyses of natural and selected strains and crosses among them have been used to infer the presence or absence of epistatic interactions among the unidentified genes contributing to a quantitative trait.

The two different approaches to epistasis, sometimes referred to as "physiological" *vs.* "statistical" epistasis (Cheverud and Routman 1995), have led to different assessments of its importance. The statistical sudies of natural and selected strains have often failed to detect significant epistasis (*e.g.*, Cohan *et al.* 1989; Hoffmann and Parsons 1989; Weber *et al.* 1999, 2001; reviewed by Mackay 2001). Its relative rarity in these contexts has contributed to a critique of Wright's "shifting balance" theory of evolution (Coyne *et al.* 1997, 2000), in which epistasis plays a central role. In recent times, a handful of quantitative studies have been made of epistatic interactions among individually defined genes. In Drosophila, these have focused on sets of independently isolated mutations that either have been selected for a common phenotype, such as defective olfactory behavior (Fedorowicz *et al.* 1998), or have been chosen at random and tested for a range of biochemical and physiological phenotypes (Clark and Wang 1997). Similar results have also been obtained with *Escherichia coli* (Elena and Lenski 1997; Remold and Lenski 2004). These and other studies (reviewed by Mackay 2001) have found evidence for extensive epistatic interactions. Furthermore, in molecular studies, whole-genome transcriptional profiles in Drosophila have revealed extensive nonadditivity at the level of transcription. This has been shown in an analysis of sex-specific gene expression in two inbred strains (Gibson and Dworkin 2004) and also in individual olfactory-defective mutants previously analyzed for epistatic interactions in behavioral phenotype (Anholt *et al.* 2003), as well as in this report.

One possible solution to this apparent paradox may be that it is simply a sampling problem. After all, some statistical studies do provide evidence for epistasis (*e.g.*, Cohan 1984; McGuire 1992; Blows and Sokolowski 1995; Wade and Goodnight 1998; Goodnight and Wade 2000; Mackay 2001; Li *et al.* 2001; Leamy *et al.* 2002) and not all of the specific genes tested for interactions with each other show epistasis (*e.g.*, Federowicz *et al.* 1998; and this report). Another possibility is that the natural or spontaneous variants present in the statistically analyzed natural or selected strains are generally less severe than those studied in the analyses of specific genes and that milder mutations are less likely to show epistatic interactions. This explanation fails to account for those natural cases that do show epistasis (*e.g.*, Cohan 1984; McGuire 1992; Blows and Sokolowski 1995; Wade and Goodnight 1998; Goodnight and Wade 2000; Li *et al.* 2001; Leamy *et al.* 2002) and it is not supported by our study in which many of the interacting EP variants show only very mild effects on their own (Table 1). A third, stronger possibility is that statistical measures of epistasis, in the absence of any knowledge of the relevant loci, may be relatively insensitive due to the assumptions that must be made (Cheverud and Routman 1995; Mackay 2001; Cordell 2002).

Cryptic epistasis: In this study, we defined a gene set

on the basis of a physiological interaction—modification of *Syx1A*$^{3\text{-}69}$-induced loss of coordination—and then tested statistically for functional relatedness among them. Not only did they exhibit a significant level of interaction, but also the pattern of interaction changed depending on the genetic context. An extensively different set of interactions was obtained in the presence of *Syx1A*$^{3\text{-}69}$ than in its absence, indicating that the functional relationships among genes are plastic.

This finding of significant genetic and phenotypic effects due to sorting of a central network node (*Syx1A*$^{3\text{-}69}$ *vs. Syx1A*$^{+}$) provides a graphic example of cryptic genetic variation and is consistent with claims for the fundamental contribution of epistasis to cryptic genetic variation (Gibson and Dworkin 2004; Hermisson and Wagner 2004). Although the actual role of epistatic effects in evolution remains controversial (*e.g.*, Coyne *et al.* 1997, 2000), cryptic epistasis may nonetheless have evolutionary implications. Imagine a natural sequence of the same events that we contrived in the laboratory: (1) selection in favor of alleles that modify an existing variant and (2) segregation of the original variant away from the modifiers to produce (3) a new, emergent phenotype driven by rearranged genetic interactions. Although our experiments remain contrived laboratory paradigms, they lend some credence to a mode of phenotypic evolution involving shuffled genetic architectures rather than modular, piecemeal changes (Templeton 1980; Wade 2001; Hermisson *et al.* 2003). Epistatic interactions would build up like potential energy, without major phenotypic change, only to be released, with broad phenotypic consequences, following introgression or population bottlenecks. Phenotypic change, and evolution in general, would thus not be a consequence of single mutations *per se*, but of rearranged gene interactions. Our results support the contention that accumulation of cryptic genetic variation need not depend exclusively on the buffering action of specific genes (Bergman and Siegal 2003; Gibson and Dworkin 2004; Hermisson and Wagner 2004).

A major issue concerning the relevance of epistasis to evolution is the phenotypic magnitude of such effects, which appear small from statistical measures and large from physiological measures (see discussion above). Evaluation of this question is hampered, however, by our current lack of understanding of how gene networks function in the production of phenotypes and of the potential for small changes in these networks to have large, ramifying effects on phenotype. There is already good evidence that behavioral phenotypes are particularly sensitive to small genetic changes (Greenspan 1997), a property well illustrated by the *foraging* locus in *D. melanogaster*, in which a small change in the expression level of a cGMP-dependent protein kinase accounts for the majority of the variance in naturally polymorphic foraging behavior (Sokolowski 1980; deBelle *et al.* 1989; Osborne *et al.* 1997). Analogs of this property are also found in metabolic networks (*e.g.*, Cohen 1999), in the immune system (*e.g.*, Swain 2001), and in the nervous system (*e.g.*, Marder and Thirumalai 2002), where a network's output has been shown to shift substantially following subtly different conditions of stimulation. If networks have tipping points, where small alterations in their elements can have large effects on phenotype (in the appropriate context), then the evolutionary relevance of epistasis warrants reconsideration. These matters hinge on the fundamental nature and flexibility of gene networks.

Flexibility, robustness, and degeneracy: Flexible relationships among elements of a network are a major source of robustness (Wagner 2000; Siegal and Bergman 2002) as well as a source for the emergence of new properties (Bhalla and Iyengar 1999; Bergman and Siegal 2003). In contrast to the conventionally invoked mechanisms of local feedback or redundancy to account for such properties (Hartman *et al.* 2001; Davidson *et al.* 2003), the more far-flung interactions that we have uncovered may be better attributed to degeneracy, the wide-ranging ability of a system to produce the same output by different strategies. The many different genotypes that produce nearly identical behavioral scores (Tables 3 and 4) exemplify this property.

Degeneracy is a signature feature of biological systems in general (Edelman and Gally 2001) and of gene networks in particular (Greenspan 2001). Degenerate biological systems have many nonidentical elements (*e.g.*, genes) that are extensively interconnected, but that have nonuniform patterns of connectivity. The effective range of each gene is further enhanced by pleiotropy (see discussion above and Wright 1968). These properties endow biological systems with the ability to compensate for perturbations that may never have been encountered before. An important consequence of this property is that there is a great deal more latent potential in gene networks than has previously been revealed either by classical quantitative genetics, where the identities of interacting genes were not known, or by classical mutant analysis, where the scope of interaction was relatively narrow.

A shifting genetic landscape: An implication of our findings and of these ideas for mechanistic studies of gene action is that if the functional state of a gene network is perturbed when one of its elements is changed, then caution must be exercised in extrapolating back to a "normal" system state from mutant data. Moreover, it casts a shadow over the very concept of a normal system state, in much the same way that population geneticists have long questioned the notion of a "normal" individual (Hirsch 1963; Lewontin 1974).

If the functional relationships among genes change with each genetic perturbation, then the very feasibility of mapping a genetic landscape underlying a phenotypic landscape is called into question. The assumption in landscape mapping is that there is something stable

to map. If it shifts constantly with each perturbation, as if subject to a kind of genetic uncertainty principle, then there is no constant set of relationships to map. This property has its counterpart in the nervous system, referred to above, where the functional connections shift under different conditions of stimulation (Marder and Thirumalai 2002). Unlike the nervous system, gene networks lack a constant, underlying physical anatomy of connections to map. Thus, interaction mapping studies could potentially go on forever without ever producing a coherent system-wide set of relationships. Each result would be valid in its own context, but not necessarily in many others.

Confronting the conundrum: Degenerate biological systems can assume many states, some more functional and adaptive than others. A possible resolution to the conundrum of an endlessly shifting landscape of gene interactions may be found in defining boundary conditions for a particular phenotype between operationally defined different states (*e.g.*, viable *vs.* nonviable or coordinated *vs.* uncoordinated). Given the degenerate nature of gene networks, however, one should not necessarily expect an exclusive, common core set of gene interactions that characterizes each phenotype. Nonetheless, there may be generic similarities among the different states that supercede their degeneracy.

One way of investigating boundary conditions for a phenotype, and whether there are generic similarities (relating phenotype and patterns of epistasis), might be to further analyze the interactions found in this study by means of comprehensive gene or protein expression assays. This would allow us to see how the entire genome (or proteome) responds when we observe the various epistatic effects: physiological (the primary EP enhancer/suppressor interactions), statistical (the diallel crosses), or changes in genetic background (removal of the original *Syx1A* mutation). Such an approach might allow us to begin to reconcile genetic architecture with phenotype.

Helpful discussions and comments on the manuscript were provided by H. Dierick. Extremely helpful comments and criticisms were provided by two anonymous reviewers and by the corresponding editor, L. Harshman, for which we are very grateful. R.J.G. is the Dorothy and Lewis B. Cullman Fellow at The Neurosciences Institute, where this project was supported by the Neurosciences Research Foundation and by National Science Foundation Award No. CCF-0432063.

LITERATURE CITED

Anholt, R. R. H., C. L. Dilda, S. Chang, J.-J. Fanara, N. H. Kulkarni *et al.*, 2003 The genetic architecture of odor-guided behavior in Drosophila: epistasis and the transcriptome. Nat. Genet. **35:** 180–184.

Avery, L., and S. Wasserman, 1992 Ordering gene function: the interpretation of epistasis in regulatory hierarchies. Trends Genet. **8:** 312–316.

Bateson, W., 1909 *Mendel's Principles of Heredity.* Cambridge University Press, Cambridge, UK.

Bennett, J., and D. L. van Dyke, 1971 Improved food medium. Dros. Inf. Serv. **46:** 160.

Bergman, A., and M. L. Siegal, 2003 Evolutionary capacitance as a general feature of complex gene networks. Nature **424:** 549–552.

Bhalla, U. S., and R. Iyengar, 1999 Emergent properties of networks of biological signaling pathways. Science **283:** 381–387.

Blows, M., and M. B. Sokolowski, 1995 The expression of additive and nonadditive genetic variation under stress. Genetics **140:** 1149–1159.

Cheverud, J. M., and E. J. Routman, 1995 Epistasis and its contribution to genetic variance components. Genetics **139:** 1455–1461.

Clark, A. G., and L. Wang, 1997 Epistasis in measured genotypes: Drosophila *P*-element insertions. Genetics **147:** 157–163.

Cockerham, C. C., 1954 An extension of the concept of partitioning hereditary variance for analysis of covariance among relatives when epistasis is present. Genetics **39:** 859–882.

Cohan, F. M., 1984 Genetic divergence under selection. I. Similarity among populations of *Drosophila melanogaster* in their responses to artificial selection of modifiers of ci^{D}. Evolution **38:** 55–71.

Cohan, F. M., A. A. Hoffmann and T. W. Gayley, 1989 A test of the role of epistasis in divergence under uniform selection. Evolution **43:** 766–774.

Cohen, P., 1999 Identification of a protein kinase cascade of major importance in insulin signal transduction. Philos. Trans. R. Soc. Lond. B Biol. Sci. **354:** 485–495.

Cordell, H. J., 2002 Epistasis: what it means, what it doesn't mean, and statistical methods to detect it in humans. Hum. Mol. Genet. **11:** 2463–2468.

Coyne, J. A., N. H. Barton and M. Turelli, 1997 Perspective: a critique of Sewall Wright's shifting balance theory of evolution. Evolution **51:** 643–671.

Coyne, J. A., N. H. Barton and M. Turelli, 2000 Is Wright's shifting balance process important in evolution? Evol. Int. J. Org. Evol. **54:** 306–317.

Dalby, B., A. J. Pereira and L. S. B. Goldstein, 1995 An inverse PCR screen for the detection of P element insertions in cloned genomic intervals in *Drosophila melanogaster.* Genetics **139:** 757–766.

Davidson, E. H., D. R. McClay and L. Hood, 2003 Regulatory gene networks and the properties of the developmental process. Proc. Natl. Acad. Sci. USA **100:** 1475–1480.

DeBelle, J. S., A. J. Hilliker and M. B. Sokolowski, 1989 Genetic localization of the foraging (*for*) locus: a major gene for larval behavior in *Drosophila melanogaster.* Genetics **123:** 157–163.

DeLean, A., P. J. Munson and D. Rodbard, 1978 Simultaneous analysis of families of sigmoidal curves: application to bioassay, radioligand assay, and physiological dose-response curves. Am. J. Physiol. **235:** E97–102.

Dubnau, J., A. S. Chiang, L. Grady, J. Barditch, S. Gossweiler *et al.*, 2003 The *staufen/pumilio* pathway is involved in Drosophila long-term memory. Curr. Biol. **13:** 286–296.

Edelman, G. M., and J. A. Gally, 2001 Degeneracy and complexity in biological systems. Proc. Natl. Acad. Sci. USA **98:** 13763–13768.

Elena, S. F., and R. E. Lenski, 1997 Test of synergistic interactions among deleterious mutations in bacteria. Nature **390:** 395–398.

Fedorowicz, G. M., J. D. Fry, R. R. Anholt and T. F. Mackay, 1998 Epistatic interactions between smell-impaired loci in *Drosophila melanogaster.* Genetics **148:** 1885–1891.

Fisher, R. A., 1918 The correlations between relatives on the supposition of Mendelian inheritance. Trans. R. Soc. Edinb. **52:** 399–433.

Gibson, G., and I. Dworkin, 2004 Uncovering cryptic genetic variation. Nat. Rev. Genet. **5:** 681–690.

Gibson, G., and S. van Helden, 1997 Is the function of the Drosophila homeotic gene *Ultrabithorax* canalized? Genetics **147:** 1155–1168.

Goodnight, C. J., and M. J. Wade, 2000 The ongoing synthesis: a reply to Coyne, Barton and Turelli. Evol. Int. J. Org. Evol. **54:** 317–324.

Greenspan, R. J., 1997 A kinder, gentler genetic analysis of behavior: from dissection to modulation. Curr. Opin. Neurobiol. **7:** 805–811.

Greenspan, R. J., 2001 The flexible genome. Nat. Rev. Genet. **2:** 383–387.

Greenspan, R. J., 2004 *Fly Pushing: The Theory and Practice of Drosophila Genetics,* Ed. 2. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Griffing, B., 1956 Concept of general and specific combining ability in relation to diallel crossing systems. Aust. J. Biol. Sci. **9:** 463–493.

Hall, J. C., 1994 Pleiotropy of behavioral genes, pp. 15–28 in *Flexibility and Constraint in Behavioral Systems,* edited by R. J. Greenspan and C. P. Kyriacou. John Wiley & Sons, Berlin/New York.

Hartman, IV, J. L., B. Garvik and L. Hartwell, 2001 Principles for the buffering of genetic variation. Science **291:** 1001–1004.

Hermisson, J., and G. P. Wagner, 2004 The population genetic theory of hidden variation and genetic robustness. Genetics **168:** 2271–2284.

Hermisson, J., T. F. Hansen and G. P. Wagner, 2003 Epistasis in polygenic traits and the evolution of genetic architecture under stabilizing selection. Am. Nat. **161:** 708–734.

Herskowitz, I., and D. Hagen, 1980 The lysis-lysogeny decision of phage lambda: explicit programming and responsiveness. Annu. Rev. Genet. **14:** 399–445.

Hirsch, J., 1963 Behavior genetics and individuality understood. Science **142:** 1436–1442.

Hirsch, J., and L. F. Erlenmeyer-Kimling, 1962 Studies in experimental behavior genetics IV. Chromosome analyses for geotaxis. J. Comp. Physiol. Psychol. **55:** 732–739.

Hoffmann, A. A., and P. A. Parsons, 1989 Selection for increased desiccation resistance in *Drosophila melanogaster*: additive genetic control and correlated responses for other stresses. Genetics **122:** 837–845.

Jahn, R., and T. C. Sudhof, 1999 Membrane fusion and exocytosis. Annu. Rev. Biochem. **68:** 863–911.

Leamy, L. J., E. J. Routman and J. M. Cheverud, 2002 An epistatic genetic basis for fluctuating asymmetry of mandible size in mice. Evolution **56:** 642–653.

Lewontin, R. C., 1974 *The Genetic Basis of Evolutionary Change*. Columbia University Press, New York.

Li, Z.-K., L. J. Luo, H. W. Mei, D. L. Wang, Q. Y. Shu *et al.*, 2001 Overdominant epistatic loci are the primary genetic basis of inbreeding depression and heterosis in rice. I. Biomass and grain yield. Genetics **158:** 1737–1753.

Littleton, J. T., E. R. Chapman, R. Kreber, M. B. Garment, S. D. Carlson *et al.*, 1998 Temperature-sensitive paralytic mutations demonstrate that synaptic exocytosis requires SNARE complex assembly and disassembly. Neuron **21:** 401–413.

Mackay, T. F., 2001 The genetic architecture of quantitative trait loci. Annu. Rev. Genet. **35:** 303–339.

Marder, E., and V. Thirumalai, 2002 Cellular, synaptic and network effects of neuromodulation. Neural Networks **15:** 479–493.

McGuire, T. R., 1992 A biometrical genetic approach to chromosome analysis in Drosophila: detection of epistatic interactions in geotaxis. Behav. Genet. **22:** 453–467.

Osborne, K. A., A. Robichon, E. Burgess, S. Butland, R. A. Shoaw *et al.*, 1997 Natural behavior polymorphism due to a cGMP-dependent protein kinase of *Drosophila*. Science **277:** 834–836.

Phillips, P. C., 1998 The language of gene interactions. Genetics **149:** 1167–1171.

Polaczyk, P. J., R. Gasperini and G. Gibson, 1998 Naturally occurring genetic variation affects Drosophila photoreceptor determination. Dev. Genes Evol. **207:** 462–470.

Remold, S. K., and R. E. Lenski, 2004 Pervasive joint influence of epistasis and plasticity on mutational effects in *Escherichia coli*. Nat. Genet. **36:** 423–426.

Richmond, J. E., and K. S. Broadie, 2002 The synaptic vesicle cycle: exocytosis and endocytosis in Drosophila and *C. elegans*. Curr. Opin. Neurobiol. **12:** 499–507.

Rorth, P., K. Szabo, A. Bailey, T. Laverty, J. Rehm *et al.*, 1998 Systematic gain-of-function genetics in Drosophila. Development **125:** 1049–1057.

Scott, M. P., and S. B. Carroll, 1987 The segmentation and homeotic gene network in early Drosophila development. Cell **51:** 689–698.

Siegal, M. L., and A. Bergman, 2002 Waddington's canalization revisited: developmental stabiity and evolution. Proc. Natl. Acad. Sci. USA **99:** 10528–10532.

Sokolowski, M. B., 1980 Foraging strategies of *Drosophila melanogaster*: a chromosomal analysis. Behav. Genet. **10:** 291–302.

Sokolowski, M. B., 2002 Drosophila: genetics meets behaviour. Nat. Rev. Genet. **2:** 879–890.

Swain, S. L., 2001 Interleukin 18: tipping the balance towards a T Helper Cell 1 response. J. Exp. Med. **194:** F11–F14.

Templeton, A. R., 1980 The theory of speciation via the founder principle. Genetics **94:** 1011–1038.

Toma, D. P., K. P. White, J. Hirsch and R. J. Greenspan, 2002 Identification of genes involved in *Drosophila melanogaster* geotaxis, a complex behavioral trait. Nat. Genet. **31:** 349–353.

Wade, M. J., 2001 Epistasis, complex traits, and mapping genes. Genetica **112–113:** 59–69.

Wade, M. J., and C. J. Goodnight, 1998 Canalization of development and the inheritance of acquired characters. Evolution **52:** 1537–1553.

Wagner, A., 2000 Robustness against mutations in genetic networks of yeast. Nat. Genet. **24:** 355–361.

Waud, D. R., 1972 On biological assays involving quantal responses. J. Pharmacol. Exp. Ther. **183:** 577–607.

Weber, K., R. Eisman, L. Morey, A. Patty, J. Sparks *et al.*, 1999 An analysis of polygenes affecting wing shape on chromosome 3 in *Drosophila melanogaster*. Genetics **153:** 773–786.

Weber, K., R. Eisman, S. Higgins, L. Morey, A. Patty *et al.*, 2001 An analysis of polygenes affecting wing shape on chromosome 2 in *Drosophila melanogaster*. Genetics **159:** 1045–1057.

Wright, S., 1931 Evolution in Mendelian populations. Genetics **16:** 97–159.

Wright, S., 1968 *Evolution and the Genetics of Populations*. University of Chicago Press, Chicago.

Communicating editor: L. Harshman