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Epistatic interactions attenuate mutations affecting startle behaviour in *Drosophila melanogaster*

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Summary

Epistasis is an important feature of the genetic architecture of quantitative traits. Previously, we showed that startle-induced locomotor behaviour of *Drosophila melanogaster*, a critical survival trait, is highly polygenic and exhibits epistasis. Here, we characterize epistatic interactions among homozygous *P*-element mutations affecting startle-induced locomotion in the *Canton-S* isogenic background and in 21 wild-derived inbred genetic backgrounds. We find pervasive epistasis for pairwise combinations of homozygous *P*-element insertional mutations as well as for mutations in wild-derived backgrounds. In all cases, the direction of the epistatic effects is to suppress the mutant phenotypes. The magnitude of the epistatic interactions in wild-derived backgrounds is highly correlated with the magnitude of the main effects of mutations, leading to phenotypic robustness of the startle response in the face of deleterious mutations. There is variation in the magnitude of epistasis among the wild-derived genetic backgrounds, indicating evolutionary potential for enhancing or suppressing effects of single mutations. These results provide a partial glimpse of the complex genetic network underlying the genetic architecture of startle behaviour and provide empirical support for the hypothesis that suppressing epistasis is the mechanism underlying genetic canalization of traits under strong stabilizing selection. Widespread suppressing epistasis will lead to underestimates of the main effects of quantitative trait loci (QTLs) in mapping experiments when not explicitly accounted for. In addition, suppressing epistasis could lead to underestimates of mutational variation for quantitative traits and overestimates of the strength of stabilizing selection, which has implications for maintenance of variation of complex traits by mutation–selection balance.

1. Introduction

Epistasis – the dependence of the allelic effects at one locus on the genotype at another locus – is an important feature of the genetic architecture of quantitative traits (Anholt & Mackay, 2004; Flint & Mackay, 2009; Mackay *et al*., 2009). Assessing the magnitude and nature of epistatic interactions is important from several perspectives. First, epistasis causes bias in estimates of allelic effects from quantitative trait locus (QTL) mapping studies when it is present but not accounted for (Carlborg *et al*., 2006; Phillips, 2008). Second, epistatic interactions can be used to infer genetic networks affecting complex traits (Phillips, 2008). Finally, epistasis can affect predictions of long-term response to artificial and natural selection (Wright, 1977; Carlborg *et al*., 2006; Phillips, 2008).

Epistasis is not easy to detect in experiments designed to map QTLs. Genome scans for pairwise epistatic interactions with n markers require $n(n-1)/2$ statistical tests, which impose a severe multiple testing penalty and concomitantly low significance thresholds, such that only very large interactions are detectable. Further, all possible two locus genotypes may not be present unless mapping populations are very large; this is a particular problem for closely linked loci.

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Nevertheless, epistatic interactions have been reported between QTLs affecting *Drosophila* bristle number (Long *et al*., 1995; Gurganus *et al*., 1999; Dilda & Mackay, 2002), wing morphology (Weber *et al*., 1999, 2001), lifespan (Leips & Mackay, 2000, 2002; Mackay *et al*., 2006) and startle-induced locomotor behaviour (Jordan *et al*., 2006). In mice, epistasis has been documented between QTLs affecting many traits related to growth, body weight and morphometry (Brockmann *et al*., 2000; Cheverud *et al*., 2001; Klingenberg *et al*., 2001; 2004; Workman *et al*., 2002; Yi *et al*., 2006). Epistasis also features prominently in the genetic architecture of growth rate of chickens (Carlborg *et al*., 2006), *Arabidopsis* (Kroymann & Mitchell-Olds, 2005) and yeast (Steinmetz *et al*., 2002; Sinha *et al*., 2008). Epistatic effects can be as large as main QTL effects, and can occur in opposite directions between different pairs of interacting loci and between loci without significant main effects on the trait. Epistatic effects can also occur between closely linked QTLs (Steinmetz *et al*., 2002; Kroymann & Mitchell-Olds, 2005; Sinha *et al*., 2008) and even between polymorphisms at a single locus (Stam & Laurie, 1996).

Epistasis is more readily detectable in crosses among lines in which genetic heterogeneity is reduced and the genetic background is controlled precisely. Diallel crosses have revealed extensive epistasis between introgression lines of tomato for yield-associated traits (Eshed & Zamir, 1996), and between *Drosophila P*-element mutations in a common isogenic background affecting olfactory (Fedorowicz *et al*., 1998; Sambandan *et al*., 2006), climbing (van Swinderen & Greenspan, 2005) and startle (Yamamoto *et al*., 2008) behaviour. Clark & Wang (1997) constructed all nine possible two-locus genotypes for several pairs of *P*-elements and identified extensive epistasis affecting metabolic activity in *Drosophila*.

Other powerful designs for detecting epistatic interactions are to construct a panel of chromosome substitution lines, in which single chromosomes from one strain are each substituted into the homozygous genetic background of a second strain; or even more precisely, to construct segmental introgression lines, in which small genomic segments from one strain are introgressed into the background of a second strain, such that the entire collection of introgression lines tile across the genome. In the presence of epistasis, the sum of the effects of all chromosome substitution lines or introgression lines is greater or less than the difference in the mean value of the trait between the two parental strains. Using these designs, substantial epistasis has been revealed for tomato yield traits (Eshed & Zamir, 1996), a battery of quantitative traits in mice (Shao *et al*., 2008) and *Drosophila* aggressive behaviour (Edwards & Mackay, 2009).

Drosophila melanogaster is an excellent model system for studies of epistasis affecting quantitative traits, due to the ease of constructing the relevant genotypes. Previously, we showed that startle-induced locomotor behaviour is a phenotype that is easily and reproducibly quantified, highly polygenic and exhibits epistasis both at the level of QTLs and *P*-element mutations in a diallel cross design (Jordan *et al*., 2006, 2007; Yamamoto *et al*., 2008). Here, we characterize epistatic interactions among homozygous *P*-element mutations affecting startle-induced locomotion in the *Canton-S* isogenic background. In addition, we combine mutational analysis with the chromosome substitution design to assess the effects of naturally occurring modifiers of mutations affecting the startle response. We accomplish this by substituting second or third *Canton-S* chromosomes with or without *P*-element insertion mutations that impair the startle response into 21 wild-derived inbred genetic backgrounds. We show that epistasis is pervasive both for pairwise combinations of homozygous *P*-element insertional mutations and for mutations in wild-derived backgrounds; that in all cases the direction of the epistatic effects is to suppress the mutant phenotypes; and that there is variation in the magnitude of epistasis among the wild-derived genetic backgrounds. We discuss the implications of these results for genetic networks affecting startle-induced locomotion, phenotypic robustness and maintenance of genetic variation for the startle response in natural populations.

2. Materials and methods

(i) Drosophila *stocks*

The 15 *P*-element insertion mutants affecting locomotor startle response used in this study have been described previously (Yamamoto *et al*., 2008). They contain single *P*[*GT1*] insertions (Lukacsovich *et al*., 2001) and were generated in the same isogenic w^{1118}; *Canton-S B* background as part of the Berkeley *Drosophila* Gene Disruption Project (Bellen *et al*., 2004). All *P*-elements were inserted within or in close proximity to a well-annotated gene: *tramtrack* ($ttk^{BG01491}$), *extramacrochaetae* ($emc^{BG00986}$), $HLHm7^{BG02029}$, *longitudinals lacking* ($lola^{BG02501}$), *Calreticulin* ($Crc^{BG01724}$), *neuralized* ($neur^{BG02542}$), *Laminin A* ($LanA^{BG01389}$), *roundabout* ($robo^{BG01092}$), $Sema\text{-}1a^{BG02570}$, $Sema\text{-}5c^{BG01245}$, *Darkener of apricot* ($Doa^{BG02498}$), *Cysteine string protein* ($Csp^{BG01863}$), $CG8963^{BG02470}$, $mir\text{-}279^{BG01888}$ and $mir\text{-}317^{BG01900}$.

Four of the genes tagged by *P*[*GT1*] insertions were on the second chromosome (*Sema-1a*, *CG8963*, *robo* and *lola*) and 11 were on the third chromosome (*ttk*, *emc*, *HLHm7*, *Crc*, *neur*, *LanA*, *Sema-5c*, *Doa*, *Csp*, *mir-279* and *mir-317*). We created all 44 possible

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chromosome 2–chromosome 3 double homozygous genotypes of these *P*-element insertions in the *Canton-S* background.

Inbred lines were derived by 20 generations of full-sib mating from isofemale lines that were collected from the Raleigh, NC Farmer's Market in 2003 (Ayroles *et al*., 2009). We selected 21 Raleigh inbred lines of which males caused no obvious hybrid dysgenesis with *Canton-S* females when their F_1 hybrids were examined for ovarian development at 28·9 °C. We chose three *P*[*GT1*]-tagged genes on the second chromosome (*Sema-1a*, *robo* and *lola*) and seven on the third chromosome (*emc*, *HLHm7*, *Crc*, *neur*, *Sema-5c*, *Csp* and *mir-317*) for further analyses, and constructed chromosome substitution lines in which a *Canton-S* chromosome carrying a *P*[*GT1*]-element insertion or its co-isogenic *P*-element free counterpart was substituted in each of the Raleigh inbred line genetic backgrounds, using balancer chromosomes to prevent recombination, and minimizing any remnant *P*-element activities by using flies with natural population-derived chromosomes as female progenitors (Supplementary Figures 1 and 2). We were able to generate all homozygous genotypes of combinations between each of the 10 *P*[*GT1*]-tagged genes and 21 Raleigh inbred lines except in two cases (RAL-765 and *mir-317* and RAL-765 and *Crc*).

All flies were reared on cornmeal–agar–molasses medium at 25 °C under controlled density and aged 3–7 days post-eclosion prior to the behavioural assay.

(ii) *Behavioural assay*

We measured startle behaviour as described previously (Jordan *et al*., 2006, 2007; Yamamoto *et al*., 2008). We placed single flies in a plastic culture vial with medium the day before measurement. The next day, we subjected the flies to a mechanical disturbance by a gentle sudden tap of the vial, which was then immediately placed horizontally under a fluorescent lamp. We quantified startle-induced locomotion as the number of seconds each fly was active in the 45 s period immediately following the disturbance. All tests were done at 25 °C between 8 am and 12 pm. The sample size for all experiments was $N=20$ measurements/sex/genotype. For the *P*-element mutations in the *Canton-S* background, we measured two males and two females from each of the 44 double homozygous lines, 15 single homozygous parental genotypes and the progenitor *Canton-S* strain each day for 10 days ($N=2400$). We measured two males and two females from each of the second chromosome substitution lines (84 genotypes) each day for 10 days ($N=3360$) and one male and one female from each of the third chromosome substitution lines (166 genotypes) each day for 20 days ($N=6640$).

(iii) *Analysis of epistasis in the* Canton-S *background*

We performed three-way fixed effects analysis of variance (ANOVA) of the startle-induced locomotion scores of the double homozygous genotypes using the model

$$Y=\mu+C2+C3+S+C2\times C3+C2\times S+C3\times S+C2\times C3\times S+\varepsilon,$$

where Y is the observed value, μ is the overall mean, $C2$ is the effect of the second chromosome insertions, $C3$ is the effect of the third chromosome insertions, $C2\times C3$ is the effect of the epistatic interaction between the second and third chromosome insertions, $C2\times S$ and $C3\times S$ represent the sex interaction terms between the second chromosome and third chromosome effects, respectively, $C2\times C3\times S$ is the three-way interaction between *P*-element insertion effects on the second chromosome, the third chromosome and sex, and ε is the error term.

We estimated $2a$, the deviation of the mean startle-induced locomotion score of the homozygous mutations from the mean of the *Canton-S* strain (Falconer & Mackay, 1996), for each of the 15 homozygous *P*-element mutations. We then computed the expected startle-induced locomotion score of the double homozygous genotypes from the deviation of $(2a_i+2a_j)$ from the mean of the *Canton-S* strain, where the subscripts i and j denote the respective homozygous genotypes. The estimate of the epistatic effect (I) for each double homozygous genotype is then the difference between the expected and observed scores. We assessed the significance of the $C2\times C3$ epistatic interactions by performing the same ANOVA models described above to the four relevant genotypes (*Canton S*, second chromosome homozygote *P*-element insertion, third chromosome homozygous *P*-element insertion, and the double chromosome 2–chromosome 3 *P*-element insertion) for each of the 44 double homozygotes.

(iv) *Analysis of epistasis in the Raleigh inbred line backgrounds*

We estimated the expected effect of the *P*-element mutants in each wild-derived inbred line background by subtracting the estimate of $2a$ for each mutation in the pure *Canton-S* background from the observed startle-induced locomotion score of the chromosome substitution lines with wild-type *Canton-S* chromosomes. The estimate of the epistatic effects (I) for each inbred line genetic background is thus the difference between the expected effect and the observed startle-induced locomotion score of the line in which the mutant *Canton-S* chromosome was substituted. We assessed the significance of each of the 208 epistatic

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interactions by three-way fixed effect performing ANOVAs using the model:

$$Y=\mu+G+R+S+G\times R+G \times S+R\times S+G\times R\times S+\varepsilon,$$

where Y and μ are defined above, G is the effect of the presence or absence of the *P*-element, S is the effect of sex, R is the effect of Raleigh inbred line versus *Canton-S* genetic backgrounds, $G\times R$, $G\times S$, $R\times S$ and $G\times R\times S$ are the interaction terms and ε is the environmental variance. Significance of the $G\times R$ and/or $G\times R\times S$ interaction terms indicates significant epistasis. In addition, we determined whether there was variation in epistatic effects among the 21 wild-derived inbred lines by performing the mixed model ANOVAs of the same structure described above across all Raleigh line genotypes, treating the Raleigh genotypes and interactions with Raleigh genotypes as random effects.

3. Results

(i) *Epistasis between* P[GT1] *mutations affecting startle behaviour in the* Canton-S-*background*

Based on our previous observations of extensive epistasis among double heterozygotes (Yamamoto *et al.*, 2008), we predicted that defects in startle-induced locomotor reactivity should be aggravated in double homozygotes and epistatic interactions would also be prevalent. We generated all 44 possible double homozygous combinations of four second chromosomal and 11 third chromosomal *P*[*GT1*] insertion lines which had been used in the previous double heterozygote analysis, and measured their startle-induced locomotion.

When all 44 genotypes of the combinations of four *P*-element-tagged genes on the second chromosome and 11 on the third chromosome were analysed by three-way ANOVA, the main effects of the second and third chromosome insertional mutations were highly significant, as expected (Table 1). The interaction between the second and third chromosome mutations was also highly significant overall (Table 1), which indicates epistatic interactions among these mutations.

The mean startle response scores of homozygous *P*[*GT1*] insertional mutations, estimates of 2*a* (the difference between the mean startle response score of the co-isogenic *Canton-S B* strain and the *P*[*GT1*] mutation), observed and expected double homozygous mutant scores, and estimates of the epistatic interactions between chromosome 2 and chromosome 3 mutations are given in Supplementary Table 1. Since the overall main effect of sex and its interaction terms were not significant (or only marginally significant for the interaction with chromosome 2 mutations,

Table 1. *ANOVA of startle-induced locomotion scores of double homozygous genotypes between* P[GT1] *insertions in a common isogenic background*

Source of variation	d.f.	MS	*F*	*P*
Chromosome 2 *P*-elements (*C2*)	3	19051·2	112·27	****
Chromosome 3 *P*-elements (*C3*)	10	2024·1	11·93	****
Sex	1	472·6	2·79	ns
$C2\times C3$	30	571·0	3·37	****
$C2\times$ Sex	3	833·7	4·91	**
$C3\times$ Sex	10	242·4	1·43	ns
$C2\times C3\times$ Sex	30	160·5	0·95	ns
Error	1672	169·7		

d.f., degrees of freedom; MS, mean square. **** $P<0{\cdot}0001$; ** $0{\cdot}01<P<0{\cdot}001$; ns $P>0{\cdot}05$.

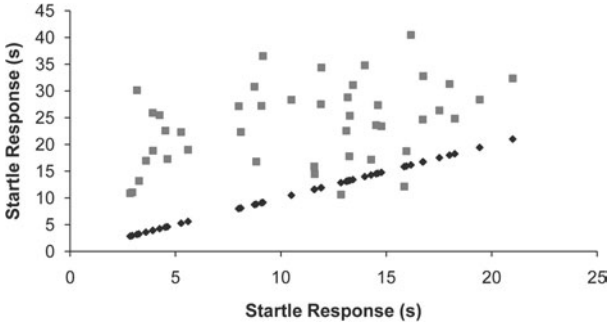

Fig. 1. Plot of observed (squares) and expected (diamonds) startle-induced locomotion scores of 44 double homozygous *P*-element mutations in the *Canton-S* genetic background.

Table 1), we present these data averaged across both sexes. The average startle response of the 15 mutations was 27·59 s, 33% lower than the wild-type *Canton-S B* score. The average startle response of the 44 double homozygous genotypes was 23·87 s, lower than that of the parental homozygotes from which they were derived, as expected from combining mutations which affect the trait in the same direction, but not as low as predicted from additive combinations of homozygous mutations. Thus, the interactions indicated by the overall ANOVA (Table 1) must predominantly involve suppressing epistasis, such that the double homozygotes are less mutant (i.e. more wild-type) than expected. This trend is clear from the plot of observed versus expected startle response of the 44 double homozygous genotypes (Fig. 1).

Widespread suppressing epistasis was indeed confirmed from estimates of individual epistatic effects for each of the 44 pair wise interactions, of which 35 were at least nominally significant and negative, and 20 had *P*-values (assessed from the significance of the $C2\times C3$ interaction term) that survived a

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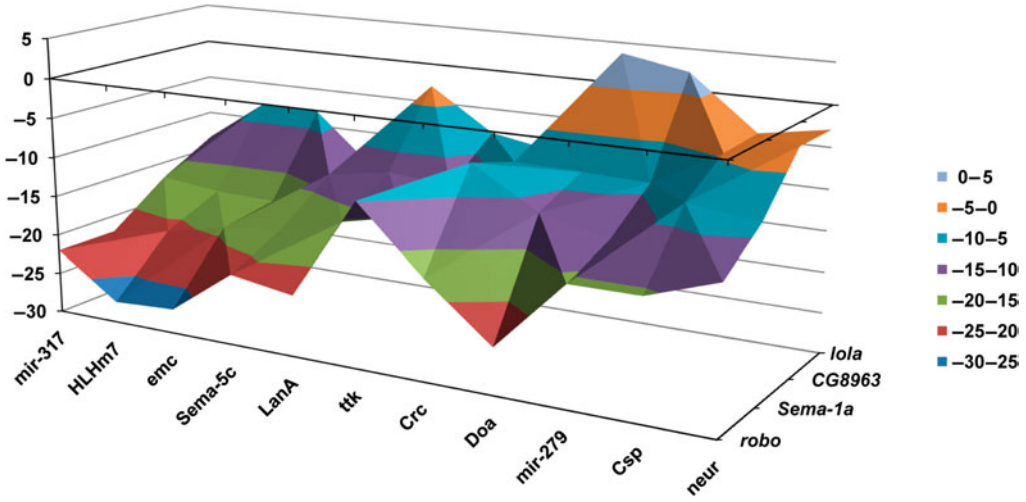

Fig. 2. Epistatic interactions for startle-induced locomotion among 44 double homozygous *P*-element mutations in the *Canton-S* genetic background. The plot depicts the estimates of the epistatic effect, *I*, for each double homozygote genotype. The colours indicate different values of *I*. Most estimates of *I* are negative, indicating widespread suppressing epistasis.

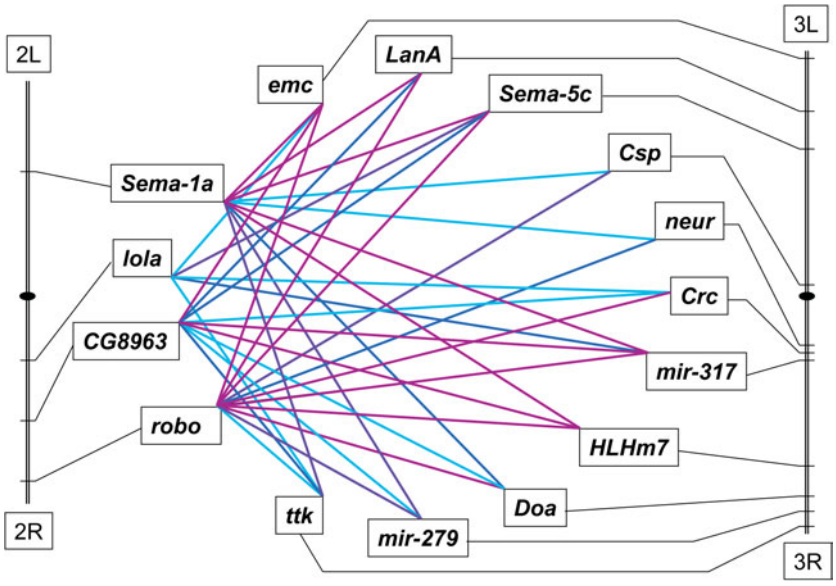

Fig. 3. Epistatic interaction network of startle-induced locomotion of double homozygous *P*-element insertions in a *Canton-S* genetic background. The approximate locations of *P*-element insertion sites on the left (*L*) and right (*R*) arms of the second and the third chromosomes are illustrated. The colours of lines connecting genes represent significance of epistasis at $P<0{\cdot}05$ (teal), $P<0{\cdot}01$ (blue), $P<0{\cdot}001$ (violet) and $P<0{\cdot}0001$ (purple).

Bonferroni correction for multiple tests (Supplementary Table 1). Graphical depictions of these epistatic interactions are given in Figs 2 and 3. Particularly prominent and abundant epistatic interactions involve *robo* and *Sema-1a* on the second chromosome, and *emc*, *Sema-5c*, *mir-317* and *HLHm7* on the third chromosome.

(ii) *Epistasis between* P[GT1] *mutations affecting startle behaviour and wild-derived genetic backgrounds*

We substituted ten *Canton-S* chromosomes with *P*[*GT1*] mutations that affect startle behaviour as well as the wild-type *Canton-S* chromosomes into 21 wild-derived Raleigh inbred lines (Supplementary Tables 2 and 3) and measured the startle-induced locomotion behaviour of each of the resulting 250 genotypes. Similarly to the analysis of double homozygous mutations, the observed startle responses of the wild-derived chromosomes in which a *P*-element mutation had been substituted was much less than predicted based on the estimate of 2*a* in the *Canton-S* background (Fig. 4). That is, naturally segregating alleles suppress the mutational effects of the *P*-element mutations on startle-induced locomotion. We quantified the epistatic effects of each mutant allele in the

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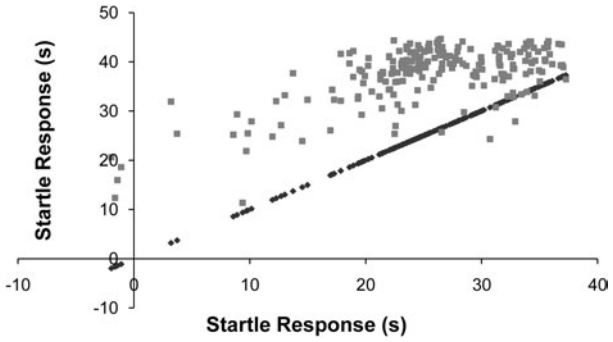

Fig. 4. Plot of observed (squares) and expected (diamonds) startle-induced locomotion scores of 208 chromosomes with *P*-element mutations affecting startle response substituted into 21 Raleigh inbred line genetic backgrounds.

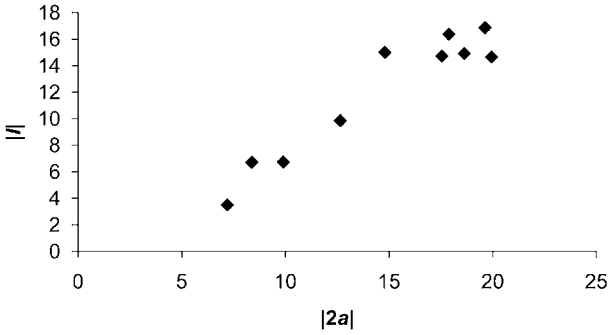

Fig. 5. Scatter plot of the relationship between the absolute value of the difference between the mean startle induced locomotion score of the *P*-element mutations and wild-type in the *Canton-S* background ($|2a|$, *x*-axis) and the estimate of the absolute value of the epistatic interaction in the Raleigh wild-derived inbred lines ($|I|$, *y*-axis). The correlation coefficient is $r = 0{\cdot}952$.

wild-derived backgrounds (Supplementary Table 4). The loci exhibiting the largest epistatic effects in the wild-derived genetic backgrounds were the same as those showing the greatest epistasis in double homozygotes in the *Canton-S* genetic background: *robo*, *Sema-1a*, *mir-317*, *emc*, *Sema-5c* and *HLHm7*. However, the *lola* mutation, which displayed little epistasis in the *Canton-S* double homozygotes, also had large epistatic effects in the Raleigh inbred line backgrounds.

When we examined the correlation between main effects and epistatic effects, we found that, remarkably, the magnitude of the epistatic effect in the wild-derived lines is nearly perfectly correlated with the magnitude of the main effect of the mutation in the *Canton-S* background ($r = 0{\cdot}952$, $t_8 = 8{\cdot}8$, $P < 0{\cdot}0001$, Fig. 5). That is, the more extreme the effect of the mutation, the greater the degree of suppression in the Raleigh inbred lines. Suppressing epistasis may be a general mechanism for conferring phenotypic robustness.

ANOVAs confirmed highly significant variation among the Raleigh inbred line backgrounds for all 10 *P*[*GT1*]-tagged genes; the line term was highly significant in all cases (Table 2). Genotype, i.e. absence or presence of a *P*[*GT1*]-tagged gene, was also significant for most genes. The one exception was *Sema-1a*, with an average deviation from the control across all Raleigh inbred lines of only 0·19 s (Supplementary Table 2): the large mutant effect observed in the *Canton-S* background was eliminated in the wild-derived backgrounds. The Raleigh inbred lines also vary in the extent to which they suppress the effects of mutations (Table 2, Fig. 6), as indicated by the significant $G \times R$ and/or $G \times R \times S$ interactions for most of the genes (Table 2). Thus, there is evolutionary potential for enhancing or suppressing effects of single mutations.

4. Discussion

We observed substantial suppressing epistasis among 15 *P*-element insertion mutations with large homozygous effects on locomotor startle response in the *Canton-S* genetic background. The only previous evidence for genetic interactions among these loci is our diallel cross analysis of locomotor startle response of all 105 double heterozygotes (Yamamoto *et al*., 2008) among the 15 mutant lines. The diallel cross analysis implicated both suppressing and enhancing epistatic interactions among these loci, rather than the pervasive suppressing epistasis observed in this study. There are two, non-mutually exclusive, explanations for this discrepancy. First, epistasis in the diallel cross analysis was measured relative to the mean startle response of all 105 double heterozygotes and not relative to the startle response of the *Canton-S* progenitor strain, as is done in this study. Second, the analysis for double heterozygotes detects dominance by dominance epistasis, and the analysis for double homozygotes detects additive by additive epistasis: the two sources of epistatic interaction are independent (Falconer & Mackay, 1996).

Epistasis indicates that the interacting loci affect the same genetic network or pathway leading to the quantitative trait phenotype. Suppressing epistasis occurs when the network is buffered against deleterious mutations, such that the trait phenotype resulting from the combination of two mutations is less than additive. An understanding of the mechanism underlying this buffering, or canalization (Waddington, 1957) is required if we are to predict individual phenotypes from knowledge of their genotypes at loci affecting the trait, as well as to predict the response to artificial and natural selection. Can we derive clues about the underlying network affecting locomotor startle response from knowledge of the molecular functions of the 15 interacting loci in this study? The

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Table 2. P *values from mixed model ANOVAs of the effects of wild-type* Canton-S *chromosomes and co-isogenic chromosomes with* P*-element mutations affecting startle response in 21 wild-derived genetic backgrounds*

	Chromosome 2 or 3 genotype									
Source	*robo*	*Sema-1a*	*lola*	*mir-317*	*emc*	*Sema-5c*	*HLHm7*	*Csp*	*Crc*	*neur*
Genotype (*G*)	***	ns	***	**	****	*	***	**	*	****
RAL Line (*R*)	****	****	****	****	****	****	****	****	****	****
Sex (*S*)	**	**	*	ns	ns	ns	ns	ns	ns	*
$G \times R$	**	****	****	*	****	****	****	****	****	****
$G \times S$	ns	ns	ns	ns	**	ns	ns	ns	ns	ns
$R \times S$	****	****	****	*	*	**	****	ns	****	*
$G \times R \times S$	ns	*	**	ns	ns	ns	ns	ns	ns	*

The alleles of the genes are *robo*^{BG01092}, *Sema-1a*^{BG02570}, *lola*^{BG02501}, *mir-317*^{BG01900}, *emc*^{BG00986}, *Sema-5c*^{BG01245}, *HLHm7*^{BG02029}, *Csp*^{BG01863}, *Crc*^{BG01724} and *neur*^{BG02542}. **** $P<0{\cdot}0001$; *** $0{\cdot}0001<P<0{\cdot}001$; ** $0{\cdot}001<P<0{\cdot}01$; * $0{\cdot}01<P<0{\cdot}05$; ns $P>0{\cdot}05$.

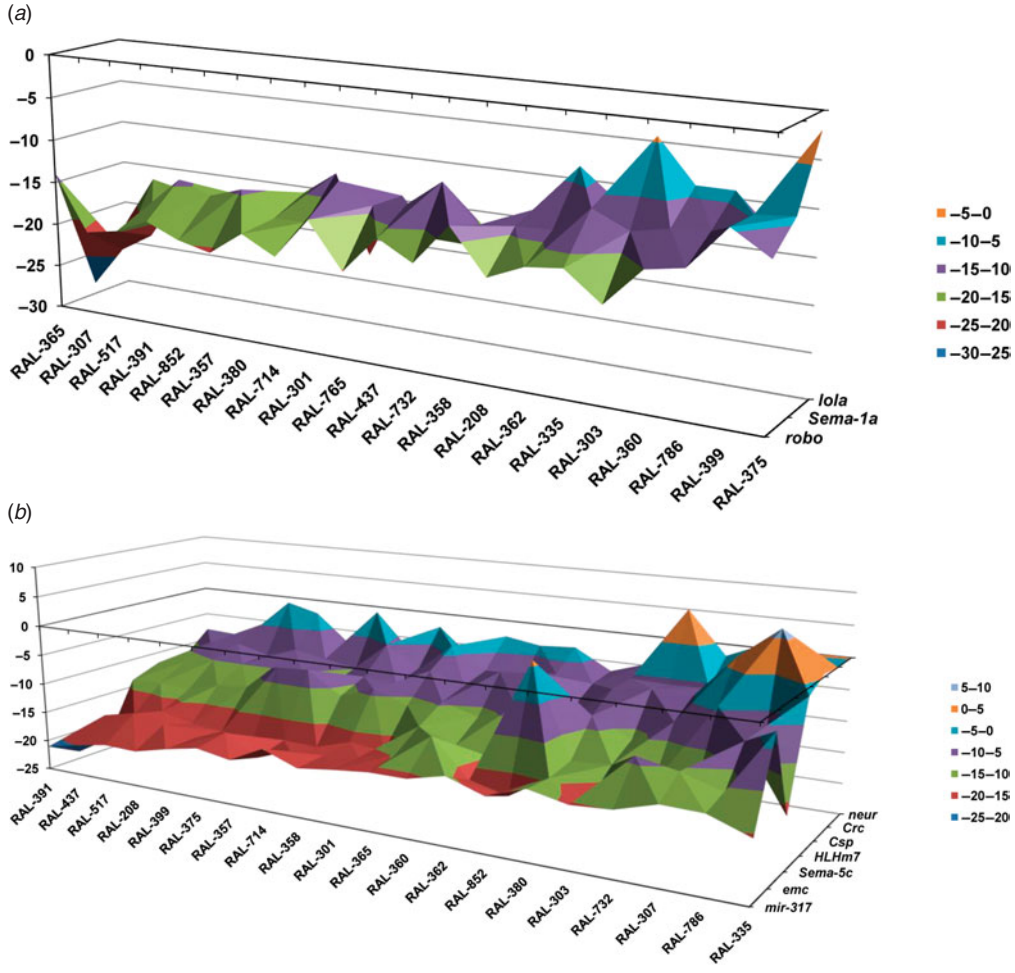

Fig. 6. Landscape of epistatic interactions for startle-induced locomotion among (*a*) second and (*b*) third chromosome homozygous *P*-element mutations in Raleigh inbred line genetic backgrounds. The plots depict the estimates of the epistatic effect, *I*, of the mutations in the different inbred line backgrounds, colour-coded to indicate different values of *I*. Most estimates of *I* are negative, indicating widespread suppressing epistasis.

genes tagged by the *P*-elements include transcriptional regulators (*ttk*, *emc*, *HLHm7* and *lola*), a calcium binding chaperone (*Crc*), an ubiquitin protein ligase (*neur*), an extracellular matrix protein (*LanA*), axon guidance molecules (*robo* and *Sema-1a*), a protein threonine/tyrosine kinase (*Doa*) and microRNAs (*mir-279* and *mir-317*), as well as a gene of unknown function (*CG8963*). However, with the sole exception

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of *mir-279*, all of these genes have been implicated in the development and function of the central and/or peripheral nervous system (Tweedie *et al*., 2009), consistent with their role in modulating startle-induced locomotion.

We have shown that suppressing epistasis is prevalent among 15 loci affecting startle response, but have not generated all 105 possible double homozygotes necessary to determine their relationships in the underlying network. Further, locomotor startle response is highly polygenic. Yamamoto *et al*. (2008) observed significant effects on startle response for 267 of 720 (37%) *P*-element insertions tested. This analysis does not approach a saturation mutagenesis screen. Even so, a complete analysis of all of these mutations would require synthesis and testing of 35 511 double homozygous genotypes, which ignores the possibility of higher order interactions. An alternative strategy for dealing with the issue of dimensionality when making inferences about epistasis and networks is to take advantage of the multifactorial perturbations of the network from naturally occurring variation. There is substantial naturally occurring variation for locomotor startle response (Jordan *et al*., 2006, 2007; Ayroles *et al*., 2009), and comparison of estimates of genetic variation from realized heritability and variation among inbred lines implicates a large contribution of epistasis to the genetic architecture of this trait (Jordan *et al*., 2007). Thus, we should be able to use single *P*-element mutations as molecular probes to discover interacting loci without fully exploring the universe of potentially interacting mutations.

We substituted 10 chromosomes containing *P*-element mutations affecting startle response in the *Canton-S* background and the wild-type *Canton-S* chromosomes into each of 21 distinct wild-derived inbred backgrounds. On average, the effects of *P*-element insertions on startle-induced locomotion were nearly five times greater in the *Canton-S* background than in the introgression lines (13·17 s compared to 2·76 s). Suppressing epistasis was observed for all 10 mutations, but was very strong for *robo*, *Sema-1a*, *mir-317*, *emc*, *Sema-5c*, *HLHm7* and *lola*, and weaker for *Csp*, *Crc* and *neur*. Therefore, wild-derived lines harbour modifiers that attenuate the effects of the homozygous *P*-element insertions on the startle response. More importantly, there is genetic variation among the lines in the extent to which modifiers suppress the mutant effects. The 21 inbred lines used for this study are a subset of the 192 inbred line Drosophila Genetic Reference Panel (DGRP), for which whole genome sequences are being obtained (Mackay *et al*., 2008). With the future availability of complete polymorphism data for the DGRP and the ability to construct larger sets of chromosome substitution lines, we will be able to use genome-wide association analysis to map the modifiers suppressing the mutant effects.

Rapid avoidance of sudden and adverse environmental disruptions is essential for survival and, hence, startle-induced locomotion is plausibly associated with fitness. Whereas impairment of startle behaviour may compromise survival, excessive startle behaviour might also be detrimental in that it would interfere with normal behavioural functions, including feeding, mating, and defence of territory; and would be energetically wasteful. Thus, startle behaviour is likely to be under stabilizing selection for an intermediate optimum.

Waddington (1957) suggested that traits under strong stabilizing selection would be strongly genetically canalized; i.e. the phenotype would be robust to deleterious mutations. The mechanism underlying genetic canalization is suppressing epistasis. There has recently been much theoretical attention to the evolution of genetic canalization in terms of developmental and genetic networks (Siegal & Bergman, 2002; Hermisson *et al*., 2003; Hermisson & Wagner, 2004; Proulx & Phillips, 2005; Gros *et al*., 2009). Indirect evidence for the evolution of suppressing epistasis comes from experiments demonstrating an increase in genetic variance when mutations are introduced into different wild-derived genotypes (Gibson & Wagner, 2000; Flatt, 2005), as expected if the mutation is in a gene for which the wild-type allele suppresses the effects of segregating variation at other loci, thus releasing previously hidden cryptic genetic variation. Our observations provide direct experimental support for widespread suppressing epistasis in natural populations, as well as natural variation in the magnitude of epistatic modulation. *P*-element mutations have pleiotropic effects on multiple quantitative traits (Rollmann *et al*., 2006; Sambandan *et al*., 2006; Yamamoto *et al*., 2008); therefore, it will be interesting in the future to evaluate a similar panel of chromosome substitution lines for a suite of quantitative traits to determine whether suppressing epistasis is a general feature of the genetic architecture of most traits; and, if so, whether the same modifiers modulate the effects on multiple traits.

Widespread suppressing epistasis has implications for gene mapping studies of common human diseases and more generally for maintenance of quantitative genetic variation in natural populations. The main effects of individual loci will be underestimated if suppressing epistasis exists but is not accounted for in a mapping study. The collective result of many highly powered whole genome association studies for human common diseases and complex traits is that the main effects of significant loci are small, and together explain very little of the estimated heritable variance for these traits (Altshuler *et al*., 2008). Suppressing epistasis is one of several explanations

for this observation. The consequence of genetic variation in suppressing epistasis is that a particular variant affecting the likelihood of developing the disease will have a greater or lesser effect depending on the individual's genetic background with respect to other loci.

An unresolved problem in evolutionary quantitative genetics is the reconciliation of the twin observations of strong stabilizing selection ($s \sim 0{\cdot}01$) with large amounts of segregating variation for most quantitative traits, such that heritabilities are of the order of 0·5, and hence genetic variation (V_G) is approximately the same as environment variation (V_E). Theory predicts that the equilibrium genetic variance maintained by a balance between stabilizing selection and the input of variation from new mutations (V_M) is $V_G = V_M/s$. Estimates of V_M are consistently $0{\cdot}001 V_E$. However, evaluating the equation for $V_G = V_E$ and $V_M = 10^{-3} V_E$, gives $s = 0{\cdot}001$, which is not a strong stabilizing selection. Alternatively, evaluating the equation for $V_G = V_E$ and $s = 0{\cdot}01$ gives $V_M = 10^{-2} V_E$, which is too large (Falconer & Mackay, 1996). However, with suppressing epistasis, it is possible that the strength of stabilizing selection is overestimated, and/or the input of mutational variation is underestimated. Support for this hypothesis comes from long-term mutation accumulation experiments on sensory bristle number in which the divergence among inbred lines (Mackay *et al*., 1995) or among lines selected for divergent trait values from an inbred base population (Mackay *et al*., 1994) attenuate over time, and the effects of mapped mutations exceed the divergence among lines (Mackay *et al*., 2005), as would be expected if new mutations exhibit suppressing epistasis.

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References

Altshuler, D., Daly, M. J. & Lander, E. S. (2008). Genetic mapping in human disease. *Science* **322**, 881–888.

Anholt, R. R. H. & Mackay, T. F. C. (2004). Genetic analysis of complex behaviours in *Drosophila*. *Nature Reviews Genetics* **5**, 838–849.

Ayroles, J. F., Carbone, M. A., Stone, E. A., Jordan, K. W., Lyman, R. F., Magwire, M. M., Rollmann, S. M., Duncan, L. H., Lawrence, F., Anholt, R. R. H. & Mackay, T. F. C. (2009). Systems genetics of complex traits in *Drosophila melanogaster*. *Nature Genetics* **41**, 299–307.

Bellen, H. J., Levis, R. W., Liao, G., He, Y., Carlson, J. W., Tsang, G., Evans-Holm, M., Hiesinger, P. R., Schulze, K. L., Rubin, G. M., Hoskins, R. A. & Spradling, A. C. (2004). The BDGP gene disruption project: single transposon insertions associated with 40% of *Drosophila* genes. *Genetics* **167**, 761–781.

Brockmann, G. A., Kratzsch, J., Haley, C. S., Renne, U., Schwerin, M. & Karle, S. (2000). Single QTL effects, epistasis, and pleiotropy account for two-thirds of the phenotypic F_2 variance of growth and obesity in DU6i × DBA/2 mice. *Genome Research* **10**, 1941–1957.

Carlborg, O., Jacobsson, L., Ahgren, P., Siegel, P. & Andersson, L. (2006). Epistasis and the release of genetic variation during long-term selection. *Nature Genetics* **38**, 418–420.

Cheverud, J. M., Vaughn, T. T., Pletscher, L. S., Peripato, A. C., Adams, E. S., Erikson, C. F., & King-Ellison, K. J. (2001). Genetic architecture of adiposity in the cross of LG/J and SM/J inbred mice. *Mammalian Genome* **12**, 3–12.

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

Dilda, C. L. & Mackay, T. F. C. (2002). The genetic architecture of *Drosophila* sensory bristle number. *Genetics* **162**, 1655–1674.

Edwards, A. C. & Mackay, T. F. C. (2009). Quantitative trait loci for aggressive behaviour in *Drosophila melanogaster*. *Genetics* **182**, 889–897.

Eshed, Y. & Zamir, D. (1996). Less-than-additive epistatic interactions of quantitative trait loci in tomato. *Genetics* **143**, 1807–1817.

Falconer, D. S. & Mackay, T. F. C. (1996). Introduction to Quantitative Genetics, 4/e. Reading, MA: Addison Wesley Longman.

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

Flatt, T. (2005). The evolutionary genetics of canalization. *Quarterly Review of Biology* **80**, 287–316.

Flint, J. & Mackay, T. F. C. (2009). Genetic architecture of quantitative traits in mice, flies and humans. *Genome Research* **19**, 723–733.

Gibson, G. & Wagner, G. (2000). Canalization in evolutionary genetics: a stabilising theory? *BioEssays* **22**, 372–380.

Gros, P. A., Le Nagard, H. & Tenaillon, O. (2009). The evolution of epistasis and its links with genetic robustness, complexity and drift in a phenotypic model of adaptation. *Genetics* **182**, 277–293.

Gurganus, M. C., Nuzhdin, S. V., Leips, J. W. & Mackay, T. F. C. (1999). High resolution mapping of quantitative trait loci affecting sternopleural bristle number in *Drosophila melanogaster*. *Genetics* **152**, 1585–1604.

Hermisson, J., Hansen, T. F. & Wagner, G. P. (2003). Epistasis in polygenic traits and the evolution of genetic architecture under stabilising selection. *American Naturalist* **161**, 708–734.

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

Jordan, K. W., Carbone, M. A., Yamamoto, A., Morgan, T. J. & Mackay, T. F. C. (2007). Quantitative genomics of locomotor behaviour in *Drosophila melanogaster*. *Genome Biology* **8**, R172.

Jordan, K. W., Morgan, T. J. & Mackay, T. F. C. (2006). Quantitative trait loci for locomotor behaviour in *Drosophila melanogaster*. *Genetics* **174**, 271–284.

Klingenberg, C. P., Leamy, L. J. & Cheverud, J. M. (2004). Integration and modularity of quantitative trait locus effects on geometric shape in the mouse mandible. *Genetics* **166**, 1909–1921.

Klingenberg, C. P., Leamy, L. J., Routman, E. J. & Cheverud, J. M. (2001). Genetic architecture of mandible

shape in mice: effects of quantitative trait loci analyzed by geometric morphometrics. *Genetics* **157**, 785–802.

Kroymann, J. & Mitchell-Olds, T. (2005). Epistasis and balanced polymorphism influencing complex trait variation. *Nature* **435**, 95–98.

Leips, J. & Mackay, T. F. C. (2000). Quantitative trait loci for lifespan in *Drosophila melanogaster*: interactions with genetic background and larval density. *Genetics* **155**, 1773–1788.

Leips, J. & Mackay, T. F. C. (2002). The complex genetic architecture of *Drosophila* life span. *Experimental Aging Research* **28**, 361–390.

Long, A. D., Mullaney, S. L., Reid, L. A., Fry, J. D., Langley, C. H. & Mackay, T. F. C. (1995). High resolution mapping of genetic factors affecting abdominal bristle number in *Drosophila melanogaster*. *Genetics* **139**, 1273–1291.

Lukacsovich, T., Asztalos, Z., Awano, W., Baba, K., Kondo, S., Niwa, S. & Yamamoto, D. (2001). Dual-tagging gene trap of novel genes in *Drosophila melanogaster*. *Genetics* **157**, 727–742.

Mackay, T. F. C., Fry, J. D., Lyman, R. F. & Nuzhdin, S. V. (1994). Polygenic mutation in *Drosophila melanogaster*: estimates from response to selection of inbred strains. *Genetics* **136**, 937–951.

Mackay, T. F. C., Lyman, R. F. & Hill, W. G. (1995). Polygenic mutation in *Drosophila melanogster*: non-linear divergence among unselected strains. *Genetics* **139**, 849–859.

Mackay, T. F. C., Lyman, R. F. & Lawrence, F. (2005). Polygenic mutation in *Drosophila melanogaster*: mapping spontaneous mutations affecting sensory bristle number. *Genetics* **170**, 1723–1735.

Mackay, T. F. C., Richards, S. & Gibbs, R. (2008). Proposal to Sequence a Drosophila Genetic Reference Panel: A Community Resource for the Study of Genotypic and Phenotypic Variation. Available from http://flybase.org/static_pages/news/wpapers.html

Mackay, T. F. C., Roshina, N. V., Leips, J. W. & Pasyukova, E. G. (2006). Complex genetic architecture of Drosophila longevity. In *Handbook of the Biology of Aging*, 6th edn (ed. E. J. Masaro & S. N. Austad), pp. 181–216. Academic Press, Burlington, MA, USA.

Mackay, T. F. C., Stone, E. A. & Ayroles, J. F. (2009). The genetics of quantitative traits: challenges and prospects. *Nature Reviews Genetics* **10**, 565–577.

Phillips, P. C. (2008). Epistasis – the essential role of gene interactions in the structure and evolution of genetic systems. *Nature Reviews Genetics* **9**, 855–867.

Proulx, S. R. & Phillips, P. C. (2005). The opportunity for canalization and the evolution of genetic networks. *American Naturalist* **165**, 147–162.

Rollmann, S. M., Magwire, M. M., Morgan, T. J., Özsoy, E. D., Yamamoto, A., Mackay, T. F. C. & Anholt, R. R. H. (2006). Pleiotropic fitness effects of the *Tre1/Gr5a* region in *Drosophila*. *Nature Genetics* **38**, 824–829.

Sambandan, D., Yamamoto, A., Fanara, J. J., Mackay, T. F. C. & Anholt, R. R. H. (2006). Dynamic genetic interactions determine odor-guided behaviour in *Drosophila melanogaster*. *Genetics* **174**, 1349–1363.

Shao, H., Burrage, L. C., Sinasac, D. S., Hill, A. E., Ernest, S. R., O'Brien, W., Courtland, H. W., Jepsen, K. J., Kirby, A., Kulbokas, E. J., Daly, M. J., Broman, K. W., Lander, E. S. & Nadeau, J. H. (2008). Genetic architecture of complex traits: large phenotypic effects and pervasive epistasis. *Proceedings of the National Academy of Sciences of the USA* **105**, 19910–19914.

Siegal, M. L. & Bergman, A. (2002). Waddington's canalization revisited: developmental stability and evolution. *Proceedings of the National Academy of Sciences of the USA* **99**, 10528–10532.

Sinha, H., David, L., Pascon, R. C., Clauder-Münster, S., Krishnakumar, S., Nguyen, M., Shi, G., Dean, J., Davis, R. W., Oefner, P. J., McCusker, J. H. & Steinmetz, L. M. (2008). Sequential elimination of major-effect contributors identifies additional quantitative trait loci conditioning high-temperature growth in yeast. *Genetics* **180**, 1661–1670.

Stam, L. F. and Laurie, C. C. (1996). Molecular dissection of a major gene effect on a quantitative trait: the level of alcohol dehydrogenase expression in *Drosophila melanogaster*. *Genetics* **144**, 1559–1564.

Steinmetz, L. M., Sinha, H., Richards, D. R., Spiegelman, J. I., Oefner, P. J., McCusker, J. H. & Davis, R. W. (2002). Dissecting the architecture of a quantitative trait locus in yeast. *Nature* **416**, 326–330.

Tweedie, S., Ashburner, M., Falls, K., Leyland, P., McQuilton, P., Marygold, S., Millburn, G., Osumi-Sutherland, D., Schroeder, A., Seal, R., Zhang, H. & The FlyBase Consortium. (2009). FlyBase: enhancing *Drosophila* gene ontology annotations. *Nucleic Acids Research* **37**, D555–D559.

Van Swinderen, B. & Greenspan, R. J. (2005). Flexibility in a gene network affecting a simple behaviour in *Drosophila melanogaster*. *Genetics* **169**, 2151–2163.

Waddington, C. H. (1957). *The Strategy of the Genes*. New York: McMillan.

Weber, K., Eisman, R., Higgins, S., Morey, L., Patty, A., Tausek, M. & Zeng, Z. B. (2001). An analysis of polygenes affecting wing shape on chromosome 2 in *Drosophila melanogaster*. *Genetics* **159**, 1045–1057.

Weber, K., Eisman, R., Morey, L., Patty, A., Sparks, J., Tausek, M. & Zeng, Z. B. (1999). An analysis of polygenes affecting wing shape on chromosome 3 in *Drosophila melanogaster*. *Genetics* **153**, 773–786.

Workman, M. S., Leamy, L. J., Routman, E. J. & Cheverud, J. M. (2002). Analysis of quantitative trait locus effects on the size and shape of mandibular molars in mice. *Genetics* **160**, 1573–1586.

Wright, S. (1977). *Evolution and the Genetics of Populations. Volume 3. Experimental Results and Evolutionary Deductions*. Chicago, IL: University of Chicago.

Yamamoto, A., Zwarts, L., Callaerts, P., Norga, K., Mackay, T. F. C. & Anholt, R. R. H. (2008). Neurogenetic networks for startle-induced locomotion in *Drosophila melanogaster*. *Proceedings of the National Academy of Sciences of the USA* **105**, 12393–12398.

Yi, N., Zinniel, D. K., Kim, K., Eisen, E. J., Bartolucci, A., Allison, D. B. & Pomp, D. (2006). Bayesian analyses of multiple epistatic QTL models for body weight and body composition in mice. *Genetical Research* **87**, 45–60.