Genet. Res., Camb. (2012), **94**, *pp.* 9–20. © Cambridge University Press 2012
doi:10.1017/S001667231200002X

Extensive epistasis for olfactory behaviour, sleep and waking activity in *Drosophila melanogaster*

SHILPA SWARUP[1,2], SUSAN T. HARBISON[1,2], LAUREN E. HAHN[1], TATIANA V. MOROZOVA[2,3], AKIHIKO YAMAMOTO[2,3], TRUDY F. C. MACKAY[1,2] AND ROBERT R. H. ANHOLT[1,2,3]*

[1] *Department of Genetics, North Carolina State University, Raleigh, NC 27695-7614, USA*
[2] *W. M. Keck Center for Behavioral Biology, North Carolina State University, Raleigh, NC 27695-7617, USA*
[3] *Department of Biology, North Carolina State University, Raleigh, NC 27695-7617, USA*

(*Received 3 November 2011; revised 21 December 2011; accepted 5 January 2012*)

Summary

Epistasis is an important feature of the genetic architecture of quantitative traits, but the dynamics of epistatic interactions in natural populations and the relationship between epistasis and pleiotropy remain poorly understood. Here, we studied the effects of epistatic modifiers that segregate in a wild-derived *Drosophila melanogaster* population on the mutational effects of *P*-element insertions in *Semaphorin-5C* (*Sema-5c*) and *Calreticulin* (*Crc*), pleiotropic genes that affect olfactory behaviour and startle behaviour and, in the case of *Crc*, sleep phenotypes. We introduced *Canton-S B* (*CSB*) third chromosomes with or without a *P*-element insertion at the *Crc* or *Sema-5c* locus in multiple wild-derived inbred lines of the *Drosophila melanogaster* Genetic Reference Panel (DGRP) and assessed the effects of epistasis on the olfactory response to benzaldehyde and, for *Crc*, also on sleep. In each case, we found substantial epistasis and significant variation in the magnitude of epistasis. The predominant direction of epistatic effects was to suppress the mutant phenotype. These observations support a previous study on startle behaviour using the same *D. melanogaster* chromosome substitution lines, which concluded that suppressing epistasis may buffer the effects of new mutations. However, epistatic effects are not correlated among the different phenotypes. Thus, suppressing epistasis appears to be a pervasive general feature of natural populations to protect against the effects of new mutations, but different epistatic interactions modulate different phenotypes affected by mutations at the same pleiotropic gene.

1. Introduction

Epistasis is an integral feature of the genetic architecture of quantitative traits (Anholt & Mackay, 2004; Flint & Mackay, 2009; Mackay *et al*., 2009). Epistasis occurs when the effect of variation at one locus is suppressed or enhanced by the genotype at another locus. Epistatic interactions can bias estimates of the effects of quantitative trait loci (QTLs) in mapping populations when present but not accounted for (Carlborg *et al*., 2006); enable inferences of genetic networks affecting complex traits (Phillips, 2008); and affect predictions of long-term response to artificial and natural selection (Carlborg *et al*., 2006; Phillips, 2008). Epistasis is difficult to detect in classical quantitative genetic analyses based on resemblance between relatives in outbred populations (Falconer & Mackay, 1996), and epistatic interactions contribute largely additive genetic variation in outbred populations when the contributing alleles are rare (Hill *et al*., 2008). However, epistatic interactions are common in experiments designed to examine their effects on trait means in QTL mapping populations. For example, in *Drosophila* epistatic interactions have been reported between QTLs affecting bristle number (Long *et al*., 1995; Gurganus *et al*., 1999; Dilda & Mackay, 2002), wing morphology (Weber *et al*., 1999), lifespan (Leips & Mackay, 2000, 2002) and startle-induced locomotor behaviour (Jordan *et al*., 2006). In mice, epistasis has been reported between QTLs affecting growth, body weight and morphometry (Brockmann *et al*., 2000; Cheverud *et al*., 2001; Workman *et al*.,

* Corresponding author: Robert R. H. Anholt, Department of Biology, Box 7617, North Carolina State University, Raleigh, NC 27695-7617, USA. Tel: (919) 515-1173. Fax: (919) 515-1801. E-mail: anholt@ncsu.edu

2002; Klingenberg *et al.*, 2004; Yi *et al.*, 2006). Epistasis is also a prominent feature of the genetic architecture of growth rate in *Arabidopsis* (Kroymann & Mitchell-Olds, 2005), chickens (Carlborg *et al.*, 2006) and yeast (Steinmetz *et al.*, 2002; Sinha *et al.*, 2008).

Although epistatic interactions have been detected in QTL mapping experiments, it is easier to study epistasis in crosses among lines with reduced genetic heterogeneity in largely homozygous genetic backgrounds (Eshed & Zamir, 1996; Clark & Wang, 1997; Sambandan *et al.*, 2006). *Drosophila melanogaster* is an excellent model system to study epistasis affecting quantitative traits due to the ease of constructing chromosome substitution and introgression lines, and generating mutations in a common homozygous genotype. Epistasis has been documented for aggressive behaviour by constructing chromosome substitution lines in which small segments of one genotype were introgressed into a different genetic background (Edwards & Mackay, 2009). Epistasis for aggression was also evident from behavioural and whole genome transcriptional analyses of an ensemble of co-isogenic hyper-aggressive *P*-element insertion lines (Zwarts *et al.*, 2011). Epistasis for metabolic activity was revealed by constructing all possible two-locus genotypes for several pairs of *P*-element insertion mutations (Clark & Wang, 1997). Diallel cross analysis of co-isogenic *P*-element insertion lines enabled identification of epistatic networks of genes affecting negative geotaxis (Van Swinderen & Greenspan, 2005), olfactory avoidance behaviour (Fedorowicz *et al.*, 1998; Sambandan *et al.*, 2006), aggression (Zwarts *et al.*, 2011) and startle behaviour in *Drosophila* (Yamamoto *et al.*, 2009).

Previously, Yamamoto *et al.* (2009) created pairs of chromosome substitution lines in which isogenic *Canton*-S *B* (*CSB*) chromosomes with *P*-element insertions in genes affecting startle behaviour and their *P*-element free co-isogenic control chromosomes were substituted into different homozygous wild-derived *D. melanogaster* genotypes. This design enables the quantification of the extent to which naturally segregating variants modify the effects of single mutations, as well as the magnitude of variation of epistasis among the different lines. This study reported widespread suppressing epistasis of naturally segregating modifiers on startle behaviour. Since the magnitude of suppressing epistasis was proportional to the magnitude of the mutational effect of the *P*-element insertion on startle behaviour, it was concluded that suppressing epistasis buffers the effects of new mutations in natural populations.

P-element insertions at genes previously implicated in startle behaviour, *Semaphorin-5C* (*Sema-5c*) and *Calreticulin* (*Crc*) also affect olfactory behaviour (Sambandan *et al.*, 2006) and in the case of *Crc*, sleep phenotypes (Harbison & Sehgal, 2008). The objective of the present study was to ask whether suppressing epistasis by naturally segregating modifiers on behavioural traits is a general principle or unique to the startle response, and, moreover, to assess whether the effects of the same *P*-element insertion on different phenotypes is modulated by the same or different epistatic modifiers.

2. Materials and methods

(i) Drosophila *stocks*

P-element insertion lines for *Crc* and *Sema-5c*, which were generated as part of the Berkeley *Drosophila* Gene Disruption Project (Bellen *et al.*, 2004), contain single *P*[*GT1*] insertions generated in the isogenic w^{1118}, *CSB* background. *Crc* and *Sema-5c* have pleiotropic effects on olfactory avoidance of benzaldehyde (Sambandan *et al.*, 2006; Rollmann *et al.*, 2007), bristle number (Norga *et al.*, 2003) and startle response (Yamamoto *et al.*, 2008, 2009), and *Crc* also has pleiotropic effects on sleep traits (Harbison & Sehgal, 2008). Both *Crc* and *Sema-5c* are located on chromosome *3* (C*3*). The construction of chromosome substitution lines carrying either a *CSB* C*3* or the *Crc* and *Sema-5c P*[*GT1*] mutations on the same *CSB* C*3* in inbred lines of the *Drosophila melanogaster* Genetic Reference Panel (DGRP; Mackay *et al.*, 2012), derived from a Raleigh (North Carolina) population of wild *D. melanogaster*, has been reported previously (Yamamoto *et al.*, 2009). Thirteen chromosome substitution lines with *P*-element insertions at *Sema-5c* and 14 chromosome substitution lines with *P*-element insertions at *Crc* and the corresponding controls were used in this study (Fig. 1). All flies were reared in large mass cultures on cornmeal/molasses/agar medium at 25 °C and a 12 h light/12 h dark cycle (lights on at 6 : 00 am; lights off at 6 : 00 pm).

(ii) *Behavioural assays*

We measured olfactory behaviour for C*3* substitution lines with *Crc* and *Sema-5c* mutations, and the corresponding *CSB* C*3* substitution lines contemporaneously using a modification of the 'dipstick' assay (Anholt *et al.*, 1996), as described previously (Swarup *et al.*, 2011). We measured olfactory behaviour of single-sex groups of 50 flies/replicate and three replicates/sex for each line. Assays were conducted between 2 : 00 and 4 : 00 pm using 0·3 % (v/v) benzaldehyde (Sigma-Aldrich, St. Louis, MO). Replicate measurements on individual lines were collected over multiple days to account for environmental variation. Flies between 4 and 7 days old were collected a day prior to the assay and food deprived for 2 h in a 50 ml conical tube containing a cotton wool swab tip

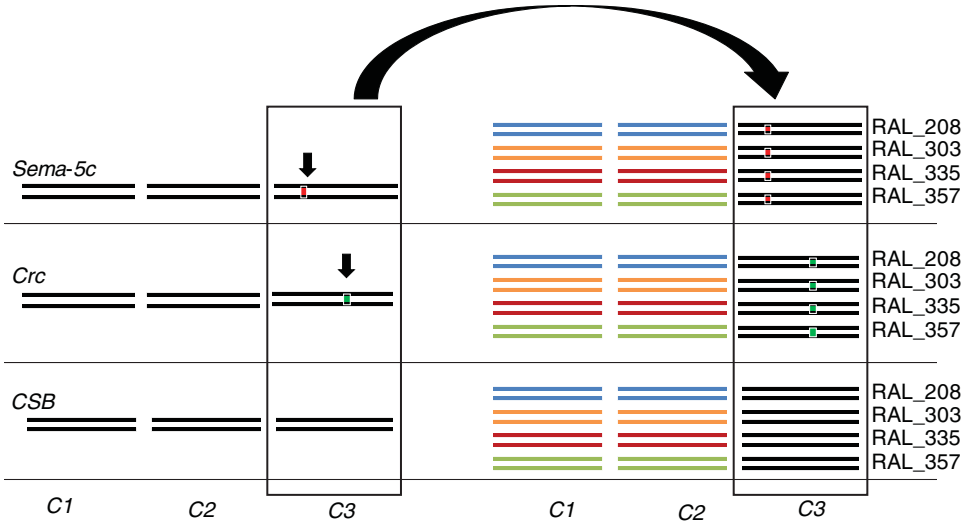

Fig. 1. Generation of co-isogenic *CSB* C*3* substitution lines in inbred DGRP genetic backgrounds. The left side of the diagram illustrates the three major *D. melanogaster* chromosomes in co-isogenic *CSB* lines, with arrows indicating the locations of *P*-element insertions in *Sema-5c* and *Crc*. The right side of the diagram illustrates the introduction of *CSB* C*3* with or without *P*-element insertions into different DGRP lines, indicated with different colours (Yamamoto *et al.*, 2009).

(referred to as 'odour tube'). The measurement is initiated by depositing 0·1 ml of odorant solution on the cotton wool swab tip in the odour tube. The odour tube is then connected to a collection tube and flies are given 2 min to partition between the tubes. At the end of the assay, a response index (RI) is calculated as follows: RI = number of flies in the collection tube/ total number of flies. An RI of 1 indicates the highest avoidance response to benzaldehyde, while 0 indicates indifference (or attraction) to the odorant.

We measured sleep and waking activity of the *Crc* chromosome substitution lines and their respective controls by recording locomotion of virgin male and female flies for seven continuous days using the *Drosophila* Activity Monitoring (DAM) System (Trikinetics, Waltham, MA). Each fly was housed separately in an activity monitor tube. The DAM system uses an infrared beam to detect movement in the monitor tube; the movement is recorded as activity counts in 1-min intervals. We eliminated flies that died after 7 days of recording from the analysis. We used a custom C^{++} program to compute day and night sleep duration in minutes, and waking activity as counts per waking minute. Sleep is defined as inactivity lasting 5 min or longer (Hendricks *et al.*, 2000; Shaw *et al.*, 2000; Huber *et al.*, 2004, Ho & Sehgal, 2005).

(iii) *Mutational effects and epistatic interactions*

We estimated the effects (2*a*) of each mutation on olfactory behaviour and sleep phenotypes in the *CSB* background as the deviation of the mean phenotypic value of the homozygous mutant from that of the *CSB* control (Falconer & Mackay, 1996). We used Student's *t* tests to assess the significance of the difference in phenotypic values between mutant and control.

We estimated the epistatic interaction for each DGRP line as the difference between the expected and observed phenotypic values. There are two chromosome substitution lines for each DGRP line, one with a mutant C*3* and the other with a wild-type C*3*. The observed phenotypic value of each DGRP line is the mean of the line with the mutant C*3*. The expected phenotypic value of each line is the difference between the mean of the line with the wild-type C*3* and the estimate of *2a* for the appropriate mutation obtained in the pure *CSB* background. We assessed the significance of epistatic interactions in each DGRP line background by performing three-way fixed effect analyses of variance (ANOVAs) using the model: $Y = \mu + G + L + S + G \times L + G \times S + L \times S + G \times L \times S + \varepsilon$, where Y is the observed value, μ is the overall mean, G is the effect of the presence or absence of the *P*-element, S is the effect of sex, L is the random effect of the DGRP line versus *CSB* genetic backgrounds, $G \times L$, $G \times S$, $L \times S$ and $G \times L \times S$ are the interaction terms, and ε is the environmental variance between replicates. A significant interaction term ($G \times L$ and/ or $G \times L \times S$) indicates epistasis. To assess variation in epistatic effects among DGRP lines, we performed similar mixed model ANOVAs across all genotypes, treating the DGRP genotypes and interactions with them as random effects. Finally, to determine the significance of epistatic interactions among different wild-derived genetic backgrounds, we estimated individual epistatic effects for each background and tested for significance using ANOVA.

3. Results

(i) *Effects of* Crc *and* Sema-5c *mutants on olfactory behaviour*

To assess the effects of naturally segregating epistatic modifiers on *P*-element insertional mutations that affect olfactory behaviour, we selected *P*-element insertions in the *Sema-5c* and *Crc* genes that have large effects on olfactory avoidance behaviour towards benzaldehyde (Sambandan *et al.*, 2006; Rollmann *et al.*, 2007). We verified the previously reported effects on responsiveness to benzaldehyde using our modified behavioural assay. To analyse the data, we used a two-way ANOVA model, $Y=\mu+L+S+L\times S+E$, where μ is the overall mean, L is the fixed effect of line, S is the fixed effect of sex, $L\times S$ is the line × sex interaction term and E is the environmental variance.

As we observed a significant line effect ($P<0{\cdot}0001$), but no significant sex ($P=0{\cdot}99$) or line × sex effect ($P=0{\cdot}90$), measurements of sexes separately were pooled for analyses. The RI at 0·3% (v/v) benzaldehyde for the *CSB* control was $0{\cdot}98\pm0{\cdot}01$ ($n=3$ replicates/sex/genotype, 50 individuals per replicate), showing strong avoidance behaviour. RIs for the *Sema-5c* and *Crc* mutants were $0{\cdot}68\pm0{\cdot}03$ and $0{\cdot}56\pm0{\cdot}04$, respectively ($n=3$ replicates/sex/genotype, 50 individuals per replicate for each mutant), significantly lower than the *CSB* control ($P<0{\cdot}0001$; Fig. 2*a*).

(ii) *Effects of* Crc *mutations on sleep*

Like *Sema-5c*, the *Crc* locus is a hotspot for *P*-element insertions (Spradling *et al.*, 2011). Previously a *P*-element insertion allele at *Crc* ($Crc^{BG02566}$) was found to affect sleep phenotypes (Harbison & Sehgal, 2008), but this insertion was at a different site than the *P*-element insertion allele ($Crc^{BG01724}$) previously implicated in startle behaviour (Yamamoto *et al.*, 2009). Although both insertions are in the first exon, it is possible that different insertion locations may have distinct phenotypic effects (Rollmann *et al.*, 2006, 2008). Therefore, we assessed the effects of the $Crc^{BG01724}$ allele on day and night sleep and waking activity. There were significant differences between $Crc^{BG01724}$ and the co-isogenic control for night sleep in both sexes ($P<0{\cdot}0001$), for day sleep for males ($P<0{\cdot}0001$), and for waking activity in females ($P=0{\cdot}0024$) (Fig. 2*b–d*). There was a significant sex effect for day sleep and waking activity ($P<0{\cdot}0001$), and a significant sex × line interaction for day sleep ($P<0{\cdot}0001$, the mutation increases day sleep only in males).

(iii) *Epistasis between* Crc *and* Sema-5c *mutations and wild-derived DGRP backgrounds*

We used inbred DGRP lines in which C*3* has been replaced by a *P*-element free *CSB* wild-type C*3* or a

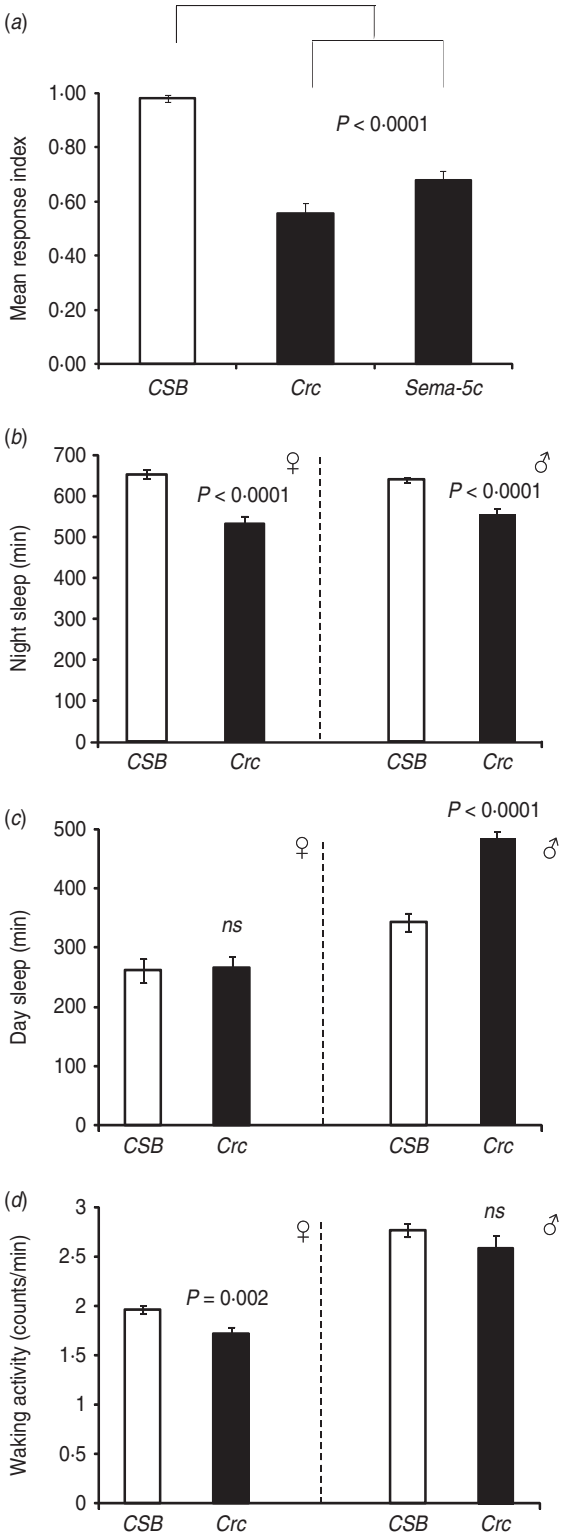

Fig. 2. Effects of *Crc* and *Sema-5c* mutations on olfactory behaviour and sleep phenotypes compared with the co-isogenic *CSB* control. (*a*) Olfactory behaviour. Bars represent mean response indices for pooled sexes; error bars are standard errors of the mean. (*b*) Night sleep. (*c*) Day sleep. (*d*) Waking activity. Bars represent mean day and night sleep and waking activity for males and females, separately, for the *CSB* control (open bars) or the *Crc* mutant (black bars); error bars are standard errors of the mean.

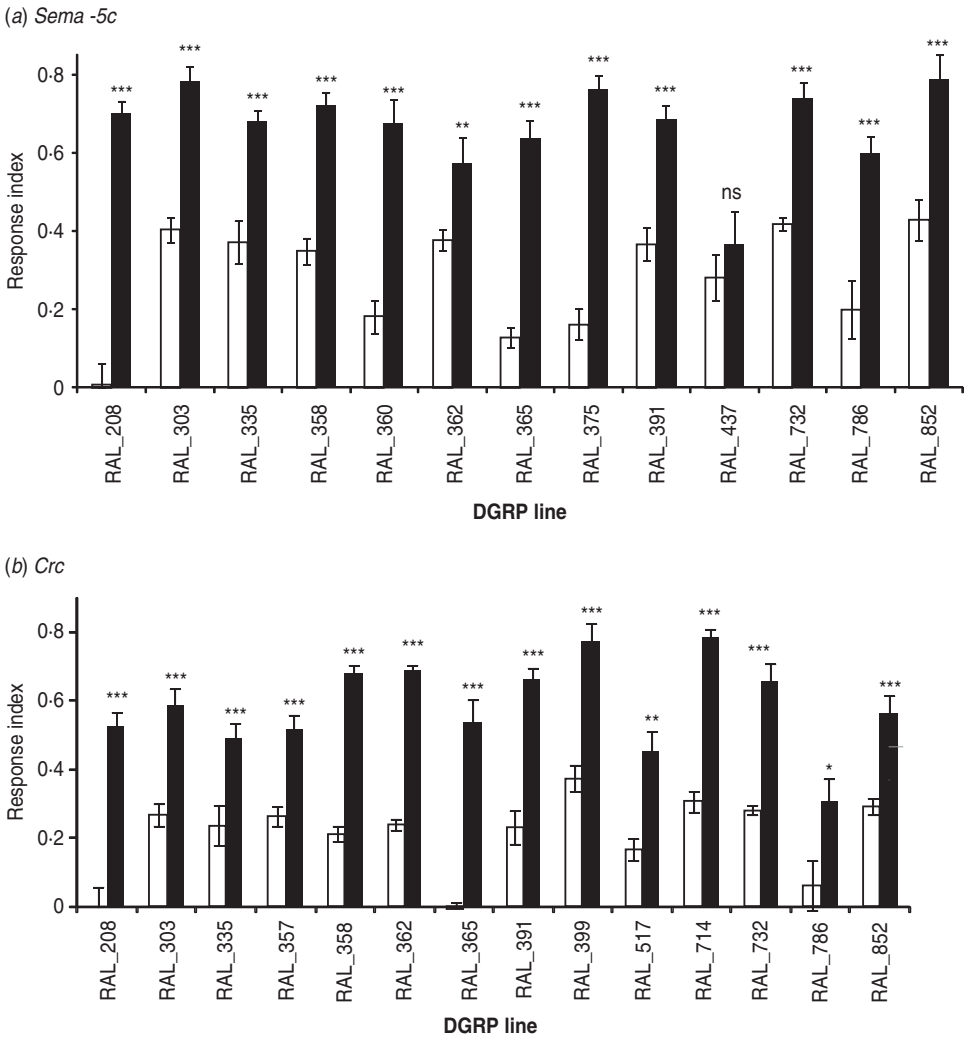

Fig. 3. Observed (closed bars) and expected (open bars) mean response indices for olfactory behaviour of (*a*) 13 DGRP C*3* substitution lines with a *P*-element insertion at *Sema-5c* and (*b*) 14 DGRP C*3* substitution lines with a *P*-element insertion at *Crc*. The error bars indicate standard errors of the mean for pooled sexes. ns, not significant, $*P<0{\cdot}05$, $**P<0{\cdot}01$, $***P<0{\cdot}001$.

co-isogenic C*3* carrying a *P*-element insertion in *Crc* or *Sema-5c* (Fig. 1; Yamamoto *et al*., 2009) to assess the effects of naturally segregating epistatic modifiers of the mutations on olfactory behaviour and sleep phenotypes. We measured olfactory behaviour for 13 DGRP lines in which C*3* was replaced by either an isogenic *CSB* C*3* or a co-isogenic *CSB* chromosome with a *Sema-5c* mutation. We also assessed olfactory behaviour and sleep phenotypes for 14 pairs of DGRP lines with wild-type *CSB* and co-isogenic *Crc* C*3*. Since there were no significant effects of sex or sex × line interaction in the analyses of olfactory behaviour among the chromosome substitution lines, whereas these terms were significant for night sleep, day sleep and waking activity, we report the results for olfactory behaviour pooled across sexes, and the sleep and activity data for males and females separately.

In the absence of epistasis, the expected phenotype of a DGRP line bearing a *Sema-5c* or *Crc* mutation is the difference between the effect (2*a*) of the mutation in the *CSB* background and the observed mean phenotype of the DGRP line with the *CSB* C*3*. Epistasis is implicated by a significant difference between this expected value and the observed mean phenotype of the DGRP line with a mutant C*3*. The significance of the estimate of the epistatic effect is given by the *P*-value of the genotype by line interaction in an ANOVA comparing the effect of the mutation in *CSB* and the DGRP line. Epistatic interactions that amplify the effect of the mutation are considered enhancer effects, whereas those that counteract the effect of the mutation are defined as suppresser effects.

We found significant epistasis for olfactory behaviour in all but one instance (*Sema-5c* in RAL_437) (Fig. 3, Table 1). In all cases where significant epistatic interactions were observed for olfactory behaviour, the epistatic effects were negative; i.e. the observed responses of the substitution lines to

Table 1. *Epistatic interactions for olfactory behaviour in DGRP chromosome substitution lines with* Sema-5c *or* Crc *mutations*

DGRP line	*Sema-5c*	*Crc*
RAL_208	−0·65***	−0·59***
RAL_303	−0·33***	−0·26***
RAL_335	−0·26***	−0·19**
RAL_357	nd	−0·20***
RAL_358	−0·33***	−0·41***
RAL_360	−0·45***	nd
RAL_362	−0·15*	−0·39***
RAL_365	−0·46***	−0·48***
RAL_375	−0·56***	nd
RAL_391	−0·28***	−0·37***
RAL_399	nd	−0·34***
RAL_437	−0·04 ns	nd
RAL_517	nd	−0·23**
RAL_714	nd	−0·42***
RAL_732	−0·28***	−0·31***
RAL_786	−0·36***	−0·19*
RAL_852	−0·31**	−0·21**

The values indicate estimated epistatic effects for olfactory RI of individual chromosome substitution lines with *Sema-5c* or *Crc* mutations. ***$P<0{\cdot}0001$; **$0{\cdot}0001<P<0{\cdot}01$; *$0{\cdot}01<P<0{\cdot}05$; ns, $P>0{\cdot}05$; nd, not determined.

benzaldehyde were greater than predicted based on the estimate of 2*a* in the *CSB* background (Table 1, Fig. 3). Since the effect of the mutations is to reduce the response to benzaldehyde in the *CSB* background, the negative difference between observed and expected olfactory behaviour in the DGRP lines indicates suppression of the mutant effect in wild-type backgrounds. We also found substantial and sex-specific epistasis between DGRP lines and the *Crc* mutation for night sleep, day sleep and waking activity (Fig. 4, Table 2). For night sleep, epistatic interactions were mostly suppressing, as for olfactory behaviour, with few exceptions (e.g. RAL_358 and RAL_852 for females and RAL_365 for males; Fig. 4*a* and *b*). The *Crc* mutation increases day sleep in males (Fig. 2*b*); thus, suppressing epistasis would counteract the *Crc* mutation by reducing day sleep duration. Interestingly, three genetic backgrounds showed epistatic interactions for day sleep for females, two of which were enhancer effects (Fig. 4*c*), indicating that mutations with no effects on a phenotype in one genetic background can have significant effects in other backgrounds (i.e. the effect of the mutation was suppressed in the *CSB* background). There were extensive epistatic effects for male day sleep (Fig. 4*d*). These effects were exclusively suppresser effects; that is, epistasis caused day sleep duration to be diminished in mutants that gave rise to prolonged day sleep. Few epistatic effects were observed for waking activity, with suppresser effects for both sexes in the RAL_365 background and enhancer effects for females in RAS_391 and males in RAL_517 (Fig. 4*e* and *f*).

We assessed whether there was significant variation in epistasis among the wild-type and mutant C*3* substitution lines for each trait, as indicated by a significant genotype (wild-type versus mutant) by line (DGRP line) interaction in the ANOVA. This term was significant for all traits (Tables 3 and 4). Thus, there is variation in the extent to which natural variants modify mutational effects.

(iv) *Pleiotropic epistatic effects*

In addition to their effects on olfactory behaviour (Sambandan *et al*., 2006; Rollmann *et al*., 2007), the *Sema-5c* and *Crc* mutations also show reduced startle behaviour (Yamamoto *et al*., 2008) and a mutation at *Crc* has been associated with reduced night and day sleep, and increased waking activity (Harbison & Sehgal, 2008). To assess whether the same epistatic modifiers affect the effects of the *Sema-5c* and *Crc* mutations on multiple traits, we first asked whether there was a correlation between the estimates of epistatic effects for olfactory behaviour and those of startle-induced locomotion, measured previously on the same lines (Yamamoto *et al*., 2009). We did not observe a significant correlation for either *Sema-5c* (Fig. 5*a*) or *Crc* (Fig. 5*b*). Similarly, epistasis of olfactory behaviour was not significantly correlated with epistasis for day sleep and night sleep for *Crc*, and the correlation with waking activity in males was only nominally significant ($P=0{\cdot}04$; Fig. 6). Epistasis of day time sleep, night time sleep or waking activity was also not correlated with epistasis of startle behaviour (Supplementary Fig. S1 available at http://journals.cambridge.org/grh). These results show that different naturally segregating epistatic modifiers modulate different phenotypes affected by mutations at the same pleiotropic gene.

4. Discussion

Previously, olfactory behaviour in *D. melanogaster* has been used as a model trait to dissect the genetic architecture of behaviour (Anholt, 2010) and dynamic epistatic networks of pleiotropic genes have been implicated as a major feature of the genetic ensembles that underlie the manifestation of this behavioural phenotype (Fedorowicz *et al*., 1998; Sambandan *et al*., 2006). *D. melanogaster* can also serve as a genetic model to study sleep (Hendricks *et al*., 2000; Shaw *et al*., 2000). While epistasis can be hypothesized from co-regulated gene expression networks (Harbison *et al*., 2009), no previous study has quantified the impact of epistasis on sleep in flies. In addition to modulation of behavioural phenotypes, suppressing epistasis may explain the paradox

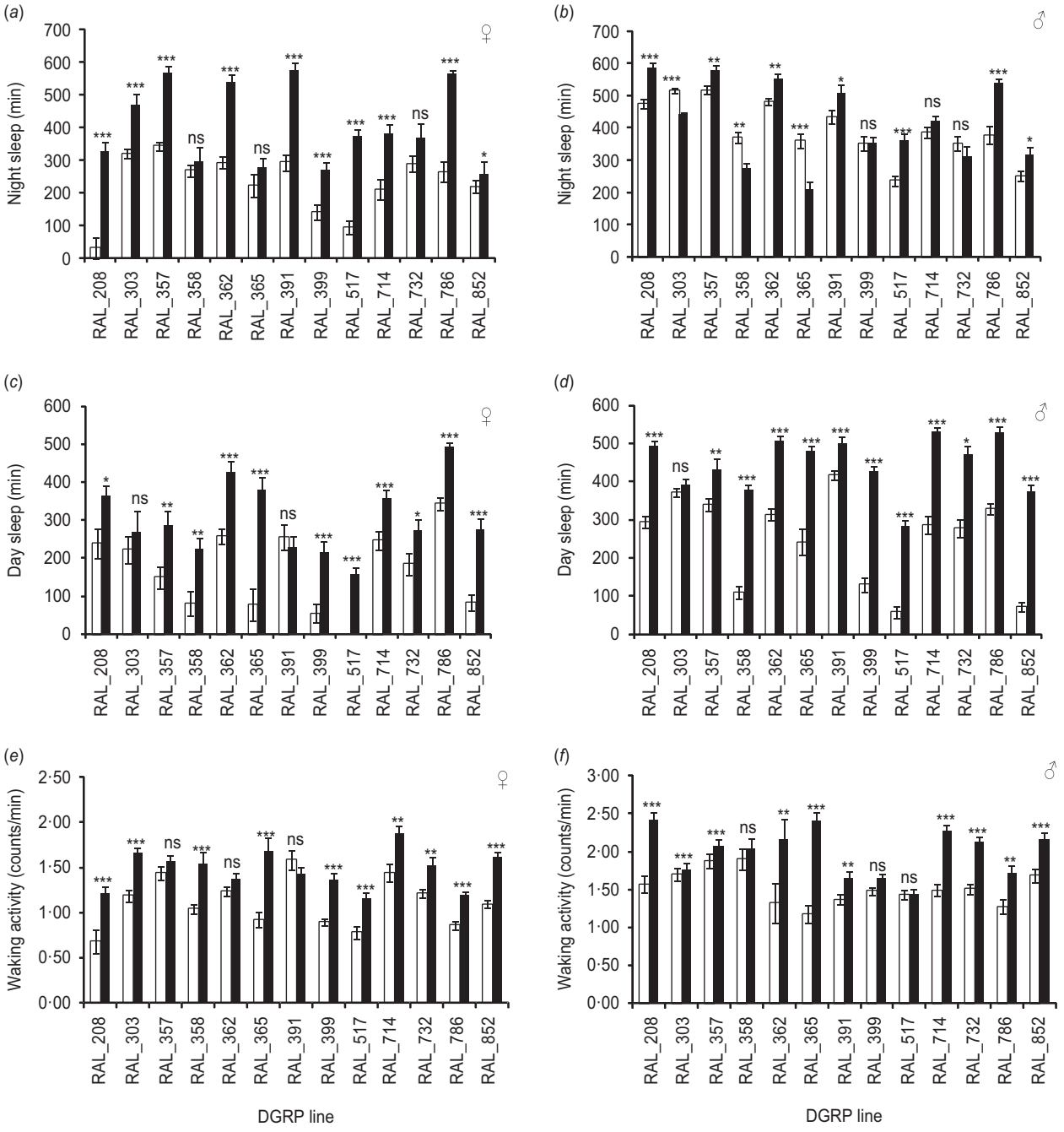

Fig. 4. Observed (closed bars) and expected (open bars) sleep phenotypes in DGRP C*3* substitution lines with a *P*-element insertion at *Crc*. (*a*) Night sleep in females. (*b*) Night sleep in males. (*c*) Day sleep in females. (*d*) Day sleep in males. (*e*) Waking activity in females. (*f*) Waking activity in males. The error bars indicate standard errors of the mean for sexes separately. ns, not significant, $*P<0{\cdot}05$, $**P<0{\cdot}01$, $***P<0{\cdot}001$.

between developmental robustness in the face of genetic variation, as illustrated by the effects of genetic background modifiers on mutations in *Sevenless* and *Drosophila Epidermal Growth Factor Receptor* that affect development of photoreceptors (Polaczyk *et al*., 1998).

The recent generation of a panel of diverse homozygous wild-derived chromosome substitution lines that carry the same homozygous *CSB* C*3* with or without a *P*-element insertion (Yamamoto *et al*., 2009) enables analyses of the effects of naturally segregating epistatic modifiers. We used chromosome substitution lines with *Sema-5c* and *Crc* mutations to analyse epistatic modulation of mutations that affect olfactory behaviour, sleep and waking activity in *Drosophila* in wild-derived genetic backgrounds. *Sema-5c* has been implicated in early development (Khare *et al*., 2000) and *Crc*, a calcium-binding

Table 2. *Epistatic interactions for sleep phenotypes in DGRP chromosome substitution lines with a* Crc *mutation*

DGRP line	Sex	Night sleep (min)		Day sleep (min)		Waking activity (counts/min)	
RAL_208	f	−169·62	***	−24·46	ns	−0·23	ns
	m	−100·31	***	117·49	***	−0·33	ns
RAL_303	f	−19·19	ns	52·43	ns	−0·17	ns
	m	−37·75	ns	112·24	***	−0·18	ns
RAL_357	f	−96·62	**	−35·94	ns	0·18	ns
	m	−49·24	ns	224·17	***	0·32	ns
RAL_358	f	97·85	*	−42·37	ns	−0·21	ns
	m	108·11	***	47·16	ns	0·37	ns
RAL_362	f	−120·66	***	−66·80	ns	0·16	ns
	m	−59·56	**	122·30	***	−0·33	ns
RAL_365	f	73·22	ns	−199·98	***	−0·45	**
	m	162·15	***	76·78	*	−0·72	**
RAL_391	f	−153·71	***	125·73	**	0·46	**
	m	−63·63	**	233·48	***	0·21	ns
RAL_399	f	−2·58	ns	−61·95	ns	−0·17	ns
	m	9·90	ns	17·48	ns	0·33	ns
RAL_517	f	−154·34	***	−87·52	*	−0·07	ns
	m	−115·57	***	91·86	**	0·50	*
RAL_714	f	−40·84	ns	−11·99	ns	−0·13	ns
	m	−24·10	ns	71·39	*	−0·28	ns
RAL_732	f	47·79	ns	13·18	ns	−0·01	ns
	m	51·55	ns	123·30	***	−0·11	ns
RAL_786	f	−173·88	***	−48·61		−0·02	ns
	m	−132·34	***	116·61	***	0·08	ns
RAL_852	f	86·59	**	−91·57	*	−0·21	ns
	m	−55·06	*	14·67	ns	0·04	ns

The values indicate estimated epistatic effects of individual chromosome substitution lines with a *Crc* mutation. m, males; f, females; *** $P<0{\cdot}0001$; **$0{\cdot}0001<P<0{\cdot}01$; *$0{\cdot}01<P<0{\cdot}05$; ns, $P>0{\cdot}05$.

Table 3. *ANOVAs of olfactory behaviour among DGRP lines with* CSB *and* Sema-5c *or* Crc *mutant third chromosomes*

Mutation	Source of variation	df	SS	MS	*F*	*P*	
Sema-5c	Genotype (G)	1	0·330	0·330	23·97	<0·0001	***
	Line (L)	12	1·740	0·102	7·42	<0·0001	***
	Sex (S)	1	0·019	0·019	1·39	0·24	ns
	$S\times L$	12	0·127	0·007	0·54	0·9252	ns
	$G\times L$	12	0·929	0·077	5·62	<0·0001	***
	$S\times G$	1	0·005	0·005	0·36	0·5495	ns
	$S\times G\times L$	12	0·084	0·007	0·51	0·9059	ns
	Error	104	1·514	0·014	–	–	–
Crc	Genotype (G)	1	0·078	0·078	6·37	0·0129	*
	Line (L)	13	2·468	0·145	11·92	<0·0001	***
	Sex (S)	1	0·000	0·000	0·01	0·9206	ns
	$S\times L$	13	0·175	0·010	0·85	0·6371	ns
	$G\times L$	13	0·599	0·046	3·78	<0·0001	***
	$S\times G$	1	0·006	0·006	0·52	0·4729	ns
	$S\times G\times L$	13	0·173	0·013	1·09	0·3705	ns
	Error	112	1·362	0·012	–	–	–

df, degrees of freedom; SS: sums of squares (type III); MS, mean squares; ***$P<0{\cdot}0001$; *$0{\cdot}01<P<0{\cdot}05$; ns, not significant.

chaperone, is involved in intracellular protein transport, exocytosis and development of the nervous system in *Drosophila* (Prokopenko *et al.*, 2000). Mutations in *Sema-5c* reduce olfactory avoidance behaviour (Sambandan *et al.*, 2006; Rollmann *et al.*, 2007) and startle behaviour (Yamamoto *et al.*, 2008). Mutations in *Crc* result not only in aberrant chemosensory responses (Stoltzfus *et al.*, 2003;

Table 4. *ANOVAs of sleep phenotypes and waking activity among DGRP lines with* CSB *and* Crc *mutant third chromosomes*

Trait	Source of variation	df	SS	MS	*F*	*P*	
Night sleep	Genotype (*G*)	1	828 359	828 359	7·40	<0·0186	*
	Line (*L*)	12	5 493 950	457 829	2·10	0·0654	***
	Sex (*S*)	1	73 388	73 388	0·62	0·4462	ns
	$S \times L$	12	1 426 496	118 875	8·85	<0·0003	***
	$G \times L$	12	1 349 197	112 433	8·38	<0·0004	***
	$S \times G$	1	6658	6658	0·50	0·4942	ns
	$S \times G \times L$	12	161 098	13 425	1·77	0·0496	ns
	Error	707	5 368 009	7592·66	–	–	–
Day sleep	Genotype (*G*)	1	284 741	284 741	4·95	<0·0460	*
	Line (*L*)	12	5 543 930	461 994	4·69	<0·0002	***
	Sex (*S*)	1	4 724 142	4 724 142	83·57	<0·0001	***
	$S \times L$	12	681 402	56 783	3·53	<0·0190	*
	$G \times L$	12	693 050	57 754	3·59	<0·0179	*
	$S \times G$	1	1819	1819	0·11	0·7423	ns
	$S \times G \times L$	12	193 268	16 106	1·92	0·0295	*
	Error	707	5 939 596	8401·13	–	–	–
Waking activity	Genotype (*G*)	1	5·57	5·57	6·67	0·0240	*
	Line (*L*)	12	22·38	1·86	1·44	0·2656	ns
	Sex (*S*)	1	58·42	58·42	67·90	<0·0001	***
	$S \times L$	12	10·37	0·86	2·11	0·1051	ns
	$G \times L$	12	10·05	0·84	2·05	0·1136	ns
	$S \times G$	1	0·00	0·00	0·00	0·9660	ns
	$S \times G \times L$	12	4·90	0·41	2·74	0·0012	**
	Error	707	105·57	0·15	–	–	–

df, degrees of freedom; SS, sums of squares (type III); MS, mean squares; ***$P<0{\cdot}0001$; **$0{\cdot}0001<P<0{\cdot}01$; *$0{\cdot}01<P<0{\cdot}05$; ns, not significant.

Sambandan *et al*., 2006) and reduced startle behaviour (Yamamoto *et al*., 2008), but also reduce day and night sleep duration and increase waking activity (Harbison & Sehgal, 2008). We confirmed the effects of these *P*-element insertions in the *CSB* background on olfactory behaviour using a recently developed modified high throughput olfactory behavioural assay (Swarup *et al*., 2011; Fig. 2*a*) and confirmed the effects on sleep, using the same *P*-element insertion line in *Crc* previously implicated in startle behaviour (Yamamoto *et al*., 2009) and olfaction (Sambandan *et al*., 2006) (Fig. 2*b*).

We found that mutational effects were generally reduced in the chromosome substitution lines compared with the original effect observed in the *CSB* background. The presence of variation in epistatic effects for each phenotype for each *P*-element insertion indicates that different wild-derived genetic backgrounds harbour different segregating epistatic modifiers that alter the effect of the *P*-element mutation. Although phenotypic measurements of a larger number of chromosome substitution lines might reveal correlations in epistatic measures among olfactory behaviour, startle behaviour and sleep, the lack of correlation of epistatic effects across these phenotypes among the 27 lines that were available for our study (Figs 5 and 6) suggests that different

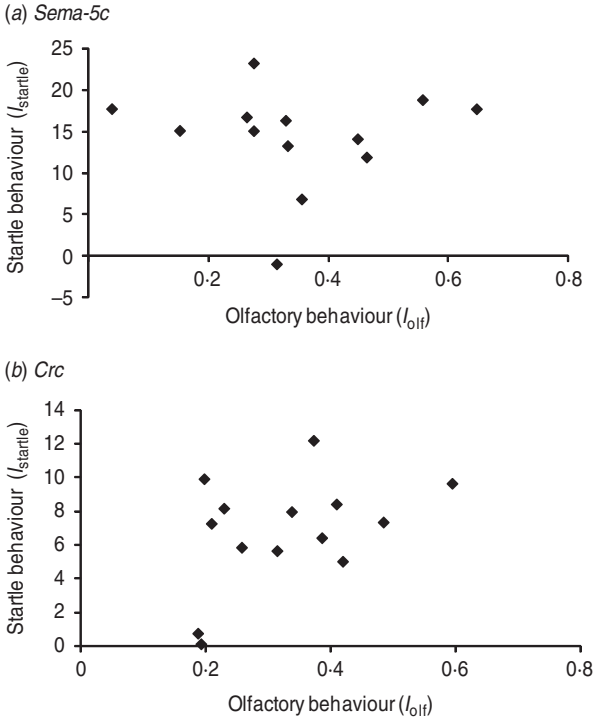

Fig. 5. Relationship between the estimates of epistatic interactions for olfactory behaviour (I_{olf}) and startle induced locomotion ($I_{startle}$) in DGRP C*3* substitution lines. (*a*) *Sema-5c*: $r^2=0$, $P>0{\cdot}05$. (*b*) *Crc*: $r^2=0{\cdot}186$, $P>0{\cdot}05$.

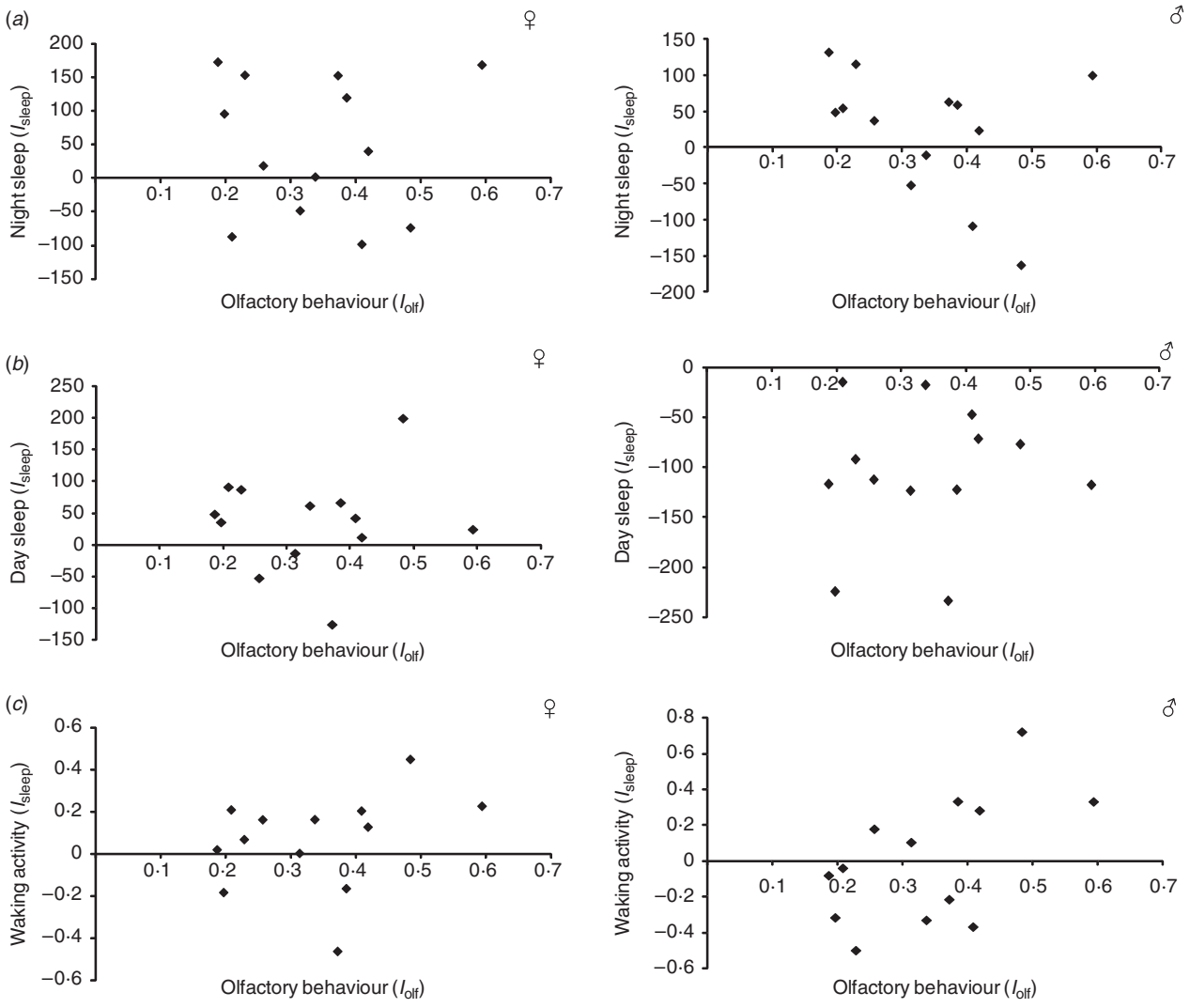

Fig. 6. Relationship between the estimates of epistatic interactions for olfactory behaviour (I_{olf}) and sleep phenotypes (I_{sleep}) in DGRP C*3* substitution lines with a *P*-element insertion at *Crc* (*a*) Night sleep. Females: $r^2=0{\cdot}001$, $P>0{\cdot}05$. Males: $r^2=0{\cdot}137$, $P>0{\cdot}05$. (*b*) Day sleep. Females: $r^2=0$, $P>0{\cdot}05$. Males: $r^2=0{\cdot}008$, $P>0{\cdot}05$. (*c*) Waking activity. Females: $r^2=0{\cdot}086$, $P>0{\cdot}05$. Males: $r^2=0{\cdot}319$, $P=0{\cdot}044$.

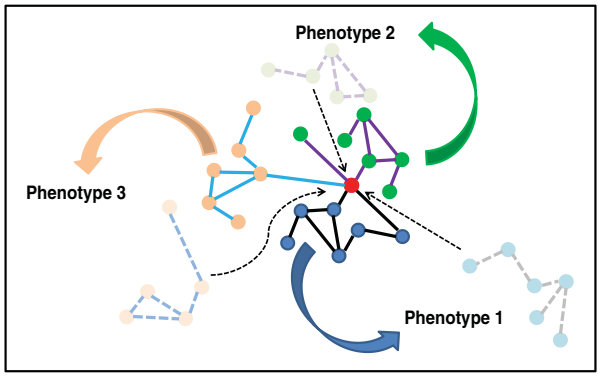

Fig. 7. Epistasis and pleiotropy. The diagram illustrates a focal *P*-element-tagged gene (red circle) that forms part of three genetic networks affecting different phenotypes, indicated by green, blue and orange colours, respectively. Gene ensembles that generate phenotype-specific epistatic interactions with the focal gene, indicated by the dotted arrows, are shown in corresponding muted colours.

epistatic modifiers are likely to interact with the same pleiotropic gene to modulate different phenotypes (Fig. 7). This complex genetic architecture is in line with previous conclusions that the manifestation of complex behavioural phenotypes can be altered by ensembles of epistatic genes (Sambandan *et al.*, 2006; Anholt, 2010; Zwarts *et al.*, 2011). Independent segregation of components of these ensembles in a natural population will result in variation in epistatic effects and these effects may express themselves differently for different pleiotropic phenotypes associated with the same causal variant.

In conclusion, we have shown that epistasis appears to be a pervasive general feature of natural populations and our results suggest that epistatic interactions may protect against adverse effects of new mutations. Furthermore, different epistatic interactions modulate different phenotypes affected by

mutations at the same pleiotropic gene. The prevalence of epistasis in the genetic architecture of complex traits is relevant to the design and interpretation of genetic studies in human populations. Widespread suppressing epistasis may account for the 'missing heritability' for human traits, such as height (Manolio *et al*., 2009). Our study underscores the importance of *D. melanogaster* as a model system for the analysis of quantitative traits, as a similar detailed analysis of epistasis under conditions in which we can introduce a mutation in a range of tightly controlled genetic backgrounds would not be possible in human populations. Substitution of chromosomes with *P*-element insertions in DGRP backgrounds will enable future mapping of epistatic modifiers and, ultimately, genome-wide characterization of epistatic interactions between defined alleles and transposon-tagged sites that affect organismal phenotypes.

This work was supported by grants from the National Institutes of Health (GM45146, GM59469) to TFCM and RRHA.

References

Anholt, R. R. H. (2010). Making scents of behavioural genetics: lessons from *Drosophila*. *Genetics Research* (*Cambridge*) **92**, 349–359.

Anholt, R. R. H., Lyman, R. F. & Mackay, T. F. C. (1996). Effects of single *P*-element insertions on olfactory behavior in *Drosophila melanogaster*. *Genetics* **143**, 293–301.

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

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 behavior 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.

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

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

Harbison, S. T., Carbone, M. A., Ayroles, J. A., Stone, E. A., Lyman, R. F. & Mackay, T. F. C. (2009). Co-regulated transcriptional networks contribute to natural genetic variation in *Drosophila* sleep. *Nature Genetics* **41**, 371–375.

Harbison, S. T. & Sehgal, A. (2008). Quantitative genetic analysis of sleep in *Drosophila melanogaster*. *Genetics* **178**, 2341–2360.

Hendricks, J. C., Finn, S. M., Panckeri, K. A., Chavkin, J., Williams, J. A., Sehgal, A. & Pack, A. I. (2000). Rest in *Drosophila* is a sleep-like state. *Neuron* **25**, 129–138.

Hill, W. G., Goddard, M. E. & Visscher, P. M. (2008). Data and theory point to mainly additive genetic variance for complex traits. *PLoS Genetics* **4**, e1000008.

Ho, K. S. & Sehgal, A. (2005). *Drosophila melanogaster*: an insect model for fundamental studies of sleep. *Methods in Enzymology* **393**, 772–793.

Huber, R., Hill, S. L., Holladay, C., Biesiadecki, M., Tononi, G. & Cirelli, C. (2004). Sleep homeostasis in *Drosophila melanogaster*. *Sleep* **27**, 628–639.

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

Khare, N., Fascetti, N., DaRocha, S., Chiquet-Ehrismann, R. & Baumgartner, S. (2000). Expression patterns of two new members of the Semaphorin family in *Drosophila* suggest early functions during embryogenesis. *Mechanisms of Development* **91**, 393–397.

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.

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 life span 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.

Mackay, T. F. C., Richards, S., Stone, E. A., Barbadilla, A., Ayroles, J. F., Zhu, D., Casillas, S., Magwire, M. M., Cridland, J. M., Richardson, M. F., Anholt, R. R. H., Barrón, M., Bess, C., Blankenburg, K. P.,

Carbone, M. A., Castellano, D., Chaboub, L., Duncan, L., Han, Y., Harris, Z., Javaid, M., Jayaseelan, J. C., Jhangiani, S. N., Jordan, K. W., Lara, F., Lawrence, F., Lee, S. L., Librado, P., Linheiro, R. S., Lyman, R. F., Mackey, A. J., Munidasa, M., Muzny, D. M., Nazareth, L., Newsham, I., Perales, L., Pu, L.-L., Qu, C., Ràmia, M., Reid, J. G., Rollmann, S. M., Rozas, J., Turlapati, L., Worley, K. C., Wu, Y.-Q., Yamamoto, A., Zhu, Y., Bergman, C. M., Thornton, K., Mittleman, D. & Gibbs, R. A. (2012). The *Drosophila melanogaster* Genetic Reference Panel. *Nature* **482**, 173–178.

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.

Manolio, T. A., Collins, F. S., Cox, N. J., Goldstein, D. B., Hindorff, L. A., Hunter, D. J., McCarthy, M. I., Ramos, E. M., Cardon, L. R., Chakravarti, A., Cho, J. H., Guttmacher, A. E., Kong, A., Kruglyak, L., Mardis, E., Rotimi, C. N., Slatkin, M., Valle, D., Whittemore, A. S., Boehnke, M., Clark, A. G., Eichler, E. E., Gibson, G., Haines, J. L., Mackay, T. F. C., McCarroll, S. A. & Visscher, P. M. (2009). Finding the missing heritability of complex diseases. *Nature* **461**, 747–753.

Norga, K. K., Gurganus, M. C., Dilda, C. L., Yamamoto, A., Lyman, R. F., Patel, P. H., Rubin, G. M., Hoskins, R. A., Mackay, T. F. C. & Bellen, H. J. (2003). Quantitative analysis of bristle number in *Drosophila* mutants identifies genes involved in neural development. *Current Biology* **13**, 1388–1396.

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.

Polaczyk, P. J., Gasperini, R. & Gibson, G. (1998). Naturally occurring genetic variation affects *Drosophila* photoreceptor determination. *Development Genes and Evolution* **207**, 462–470.

Prokopenko, S. N., He, Y., Lu, Y. & Bellen, H. J. (2000). Mutations affecting the development of the peripheral nervous system in *Drosophila*: a molecular screen for novel proteins. *Genetics* **156**, 1691–1715.

Rollmann, S. M., Edwards, A. C., Yamamoto, A., Zwarts, L., Callaerts, P., Norga, K., Mackay, T. F. C. & Anholt, R. R. H. (2008). Pleiotropic effects of *Drosophila neuralized* on complex behaviors and brain structure. *Genetics* **179**, 1327–1336.

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.

Rollmann, S. M., Yamamoto, A., Goossens, T., Zwarts, L., Callaerts-Vegh, Z., Callaerts, P., Norga, K., Mackay, T. F. C. & Anholt, R. R. H. (2007). The early developmental gene *Semaphorin 5c* contributes to olfactory behavior in adult *Drosophila*. *Genetics* **176**, 947–956.

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

Shaw, P. J., Cirelli, C., Greenspan, R. J., & Tononi, G. (2000). Correlates of sleep and waking in *Drosophila melanogaster*. *Science* **287**, 1834–1837.

Sinha, H., David, L., Pascon, R. C., Clauder-Munster, 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.

Spradling, A. C., Bellen, H. J. & Hoskins, R. A. (2011). *Drosophila P* elements preferentially transpose to replication origins. *Proceedings of the Natural Academy of Sciences USA* **108**, 15948–15953.

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.

Stoltzfus, J. R., Horton, W. J. & Grotewiel, M. S. (2003). Odor-guided behavior in *Drosophila* requires calreticulin. *Journal of Comparative Physiology A. Neuroethology Sensory, Neural and Behavioral Physiology* **189**, 471–483.

Swarup, S., Williams, T. I. & Anholt, R. R. H. (2011). Functional dissection of *Odorant binding protein* genes in *Drosophila melanogaster*. *Genes Brain and Behavior* **10**, 648–657.

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

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.

Yamamoto, A., Anholt, R. R. H. & Mackay, T. F. C. (2009). Epistatic interactions attenuate mutations affecting startle behaviour in *Drosophila melanogaster*. *Genetics Research* (*Cambridge*) **91**, 373–382.

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 Natural Academy of Sciences 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.

Zwarts, L., Magwire, M. M., Carbone, M. A., Versteven, M., Herteleer, L., Anholt, R. R. H., Callaerts, P. & Mackay, T. F. C. (2011). Complex genetics architecture of *Drosophila* aggressive behavior. *Proceedings of the Natural Academy of Sciences USA* **108**, 17070–17075.