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Quantitative evolutionary genomics: differential gene expression and male reproductive success in *Drosophila melanogaster*

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We combined traditional quantitative genetics and oligonucleotide microarrays to examine within-population genetic variation in a trait closely related to fitness. The trait, male reproductive success under competitive conditions (MCRS), is of central importance to both life-history and sexual-selection theory. We identified 27 candidate genes whose expression levels were associated with within-population variation in MCRS. 'High' MCRS was associated with low expression of a cytochrome P450 that causes pesticide resistance, suggesting a fitness cost to resistance. Two groups of metabolic proteins (glutathione transferases and phosphatases) were significantly over-represented, and a large portion of the candidates are genes involved in oxidative stress resistance, energy acquisition or energy storage. Genes expressed in accessory glands and testes were not over-represented among differentially expressed genes, but testis-expressed genes were significantly more likely to be upregulated in high MCRS genotypes. Finally, nine candidate genes that we identified had no previous functional annotation, and this experiment suggests that they play a role in male reproductive success.

Keywords: microarrays; gene expression; male mating success; quantitative genetics; *Cyp6g1*; antioxidants

1. INTRODUCTION

Two of the central questions in evolutionary genetics are: how much genetic variation exists within populations and how is that variation maintained (Lewontin 1974; Charlesworth 1987; Barton & Turelli 1989; Lynch *et al.* 1998; Charlesworth & Hughes 2000)? These questions are especially difficult to answer for polygenic (quantitative) traits, because the genes contributing to the variation are not usually known. The traditional approach to the question has been to use breeding experiments (artificial selection or analysis of individuals of known relatedness) to measure additive, non-additive and non-genetic components of phenotypic variation. Parameters derived from these experiments can be used to test some models of the maintenance of genetic variation (cf. Houle *et al.* 1996; Lynch *et al.* 1998; Charlesworth & Hughes 2000; Hughes *et al.* 2002). However, the traditional tools of quantitative genetics are not very useful for measuring the numbers of genes that contribute to trait variation, for identifying candidate genes or for elucidating the molecular mechanisms that underlie the variation (Toma *et al.* 2002).

Integration of molecular approaches and quantitative genetics has enormous power to address these fundamental questions (Jansen 2003). Microarray experiments measure the level of messenger RNA (mRNA) abundance (gene expression) of thousands of genes at once by using either the entire transcribed sequence of each gene (complementary DNA arrays) or unique 25–70 bp regions (oligonucleotide arrays) as probes. Microarray experiments can be used to discover 'candidate' genes, those that have transcriptional differences associated with trait variation, and to assess molecular mechanisms that underlie variation. This genomic molecular technique has been advocated as a tool for identifying candidate genes for quantitative variation; it can be especially useful for identifying (and simultaneously suggesting functions for) genes with no previous functional annotation (Gibson 2002).

Although microarrays have many advantages, they also have limitations that should be kept in mind. Microarrays indicate which genes are differentially expressed, but those genes may or may not contain DNA sequence variation (polymorphism) that causes genetic variation. Expression variation can be caused by sequence variation in a different gene, and tracing regulatory pathway connections can be difficult (Montooth *et al.* 2003). However, sequence variation in the regulatory region of a gene can lead to differential expression of that gene, and such regulatory polymorphism may play an important role in evolution (Townsend *et al.* 2003). A recent analysis of both mice and maize indicates that transcriptional variation can often be a result of polymorphism in the gene itself (Schadt *et al.* 2003).

That microarrays can be used to directly identify candidate genes for complex phenotypes was recently illustrated by a study of behavioural variation in *Drosophila melanogaster*. Toma *et al.* (2002) identified candidate genes for locomotor behaviour in strains of flies selected for positive and negative geotaxis. The causal relationship between expression of the candidates and geotaxis was cconfirmed using mutant and transgenic lines. Thus, can-

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didate genes were identified directly, without complex crosses and mapping experiments. Microarrays have also been used to detect candidate genes within chromosomal regions identified by linkage analysis (Wayne & McIntyre 2002; Tabakoff *et al.* 2003).

The above studies used microarrays to identify candidates for differences between selection lines or inbred lines. So far, only a few studies have used this technique to study within-population variation on a genomic scale. Townsend *et al.* (2003) found widespread differences in gene expression among natural isolates of *Saccharomyces cerevisiae*, and reported that much of the variation was potentially important to fitness. Bochdanovits *et al.* (2003) measured expression differences in *D. melanogaster* that correlated with geographical variation and developmental plasticity in body size. They found 19 genes that varied significantly with latitudinal and temperature-induced variation in adult size. The studies of Townsend *et al.* (2003) and Bochdanovits *et al.* (2003) demonstrate that microarray experiments are sensitive enough to detect meaningful within-population variation. A critical question remaining is whether the technique can be used to identify genes associated with segregating genetic variation within populations.

Here, we describe quantitative genetic and microarray experiments that examine within-population genetic variation in a trait closely related to fitness. We evaluated genotypes of *D. melanogaster* that were derived from a single population and were known to differ in male reproductive success under competitive conditions (MCRS). Because MCRS is a major component of male fitness, identifying and understanding genes causing variation is of central importance to both life-history theory (Hughes 1995; Cordts & Partridge 1996; Prowse & Partridge 1997) and sexual-selection theory (Rowe & Houle 1996; Wyckoff *et al.* 2000; Agrawal 2001; Kotiaho *et al.* 2001; Houle & Kondrashov 2002). We found that microarray analysis provided an efficient means of identifying candidate genes for MCRS. One candidate gene is known to cause pesticide resistance and other forms of stress resistance, and our results suggest that increased resistance leads to decreased male fitness in the absence of pesticides. Functional classification of the other candidate genes indicated that two groups of metabolic proteins were over-represented and that variation in oxidative stress resistance, energy acquisition and energy storage are correlated with MCRS. Finally, nine candidates were predicted genes without previous functional annotation, and our results suggest that they are involved in a male's ability to reproduce under competitive conditions.

2. METHODS

(a) *Experimental organisms*

We created 14 lines that were isogenic for the II and III chromosomes from a large, randomly mating, laboratory adapted population of *D. melanogaster* (the Ives population). Additionally, the X chromosomes in the lines were isogenic for (on average) 50% of the loci, and were segregating for at most two alleles at the remainder of the loci; any residual X-linked variation would contribute to within-line variance and would increase the error variance in ANOVA models (see Hughes *et al.* (2002) for detailed description of the crosses). To examine the genetic variation from the Ives population represented among these lines, we made full diallel crosses among subsets of them. Diallel 1 consisted of all possible crosses among 10 different lines, yielding 90 different wild-type non-inbred genotypes and 10 inbred genotypes. Diallel 2 was made using four of the lines used in diallel 1 and four additional isogenic lines (these lines were not chosen on the basis of their effects on male fitness), producing 56 different non-inbred genotypes and eight inbred genotypes. Four (diallel 1) or nine (diallel 2) replicate crosses were made per genotype from two (diallel 1) or three (diallel 2) different sets of parents. To minimize any maternal and/or density effects on fitness we conducted two generations of controlled-density rearing. First, within each isogenic line, we set up eight-dram vials with seven males and seven females; we then collected virgin offspring to be the parents for the diallel crosses. Second, the F_1 offspring from the diallel crosses were raised at a constant larval density of 25 per vial and collected as virgins to be used as the experimental males in the MCRS assay.

For each replicate cross of each genotype, we measured MCRS by placing three virgin experimental males (wild-type) together in a vial with three virgin *ebony* (*e*/*e*) males and three virgin *e*/*e* females. *Ebony* flies were all 3 days old and had been reared at constant adult density from an outbred stock derived from the Ives population, in which the *ebony* mutation arose spontaneously. Because the *ebony* females were genetically diverse, MCRS measured the average success of each male genotype over many different female genotypes. Wild-type and *e*/*e* flies were left together in the vial for 7 days, after which the adult flies were discarded. Ten days later, all progeny emerging from the vials were counted and scored for phenotype. Any wild-type offspring were the progeny of the wild-type males; *ebony* offspring were the progeny of *e*/*e* males. Offspring were counted 17 days after the experimental flies were introduced into the vials to maximize the number of F_1 offspring counted, while avoiding inclusion of any F_2 offspring (9 days is the minimum egg-adult development time in this population at 25 °C).

MCRS values for each genotype were calculated as the least-square means from a linear mixed model that included genotype, block and replicate within block. To assess differences in gene expression associated with MCRS, we chose three genotypes with consistently high MCRS (H genotypes) and three genotypes with consistently low MCRS (L genotypes) from the non-inbred genotypes. Thus the H and L genotypes differed in total male reproductive output over a 7 day period. The potential for differences in offspring larval viability to bias MCRS values was independently assessed by mating additional experimental males from each H and L genotype to virgin *e*/*e* females and raising 60 +/*e* larvae with 60 *e*/*e* larvae per vial, eight vials per genotype, which approximated the larval density in the MCRS assay. No significant variation in larval viability was detected ($F_{5,42} = 1.73$; $p = 0.15$); any true larval viability differences undetectable by our assay (i.e. power of less than 80%) could only account for, at most, 12% of the differences in MCRS values between the H and L genotypes. Thus, variation in MCRS reflected variation in adult male reproductive success, and not variation in offspring viability.

The H and L genotypes also met the following requirements: (i) low standard error among replicates; and (ii) no significant difference between MCRS in reciprocal genotypes; the genotype produced by crossing line X females to line Y males is the reciprocal of the genotype produced by crossing line Y females by line X males. Only two of the genotypes shared an isogenic parental line; however, one was an H genotype, and one was an L genotype.

Therefore, sharing of a parent would not have contributed to an association between MCRS category and gene expression.

Subsequent to the experiment, we discovered that our Ives population carries the *Wolbachia* endosymbiont, which can cause weak cytoplasmic incompatibility (i.e. reduced hatching success) in *D. melanogaster* when infected males mate with uninfected females (Hoffmann 1988; Hoffmann *et al.* 1994). Although three parental isogenic lines from diallel 2 were later found to lack the endosymbiont, none of the genotypes created from these lines was used in the microarray study because they did not rank among the highest or lowest for MCRS. Thus, all experimental and e*bony* flies in the microarray analysis had *Wolbachia* and therefore incompatibility should not have contributed to among-genotype variation.

(b) *Messenger RNA expression assays*

Tissue for mRNA extraction was collected from flies that had experienced exactly the same protocol used for measuring the MCRS phenotype. Three sets of three males were pooled per replicate, and two independent replicates were made per genotype. Flies were anaesthetized under light CO_2 and snap-frozen in liquid nitrogen. Total RNA was extracted using the standard TRIzol protocol (TRIzol Reagent, Life Technologies), and absorbance of RNA was checked at 260 and 280 nm for determination of sample concentration and purity; A_{260}/A_{280} was between 1.9 and 2.1 for each sample. We checked for integrity of the total RNA by visualizing the bands on a 0.8% agarose gel stained with SYBR Gold (Molecular Probes). mRNA was labelled using the MessageAmp aRNA kit (Ambion) and biotintylated ribonucleotides (Enzo Biochemicals); we followed the standard protocol (MessageAmp™ manual, v. 0201), except we precipitated the double-stranded cDNA by adding 0.5 vol of 5 M NH_4OAc and 2.5 vol of 100% ethanol. We checked the quality of the labelled mRNA both before and after fragmentation by gel electrophoresis as above.

Drosophila melanogaster GeneChip Arrays contain 14 probe pairs per gene for most genes, with a perfect match (PM) and a mismatch (MM) probe in each pair. Labelled complementary RNA was hybridized to Affymetrix GeneChip arrays using the University of Illinois Keck Center Affymetrix System. In pilot studies, we estimated the variance in expression levels from labelling and hybridizing using three sets of replicate aliquots from three independent pools of RNA; two aliquots of the same total RNA were independently labelled and hybridized to two different arrays (two sets of two aliquots), and a single aliquot of labelled RNA was hybridized to two different arrays. Basic statistical analysis of replicated chips using quantile-normalized PM values (Irizarry *et al.* 2002; Bolstad *et al.* 2003) indicated that our methods of labelling and hybridization generate little between-sample error (Pearson correlation coefficients: 0.994, 0.998 and 0.996, respectively; paired *t*-tests for differential gene expression: 99.4%, 99.9% and 99.7% of genes not significantly different). Additionally, all positive controls on the arrays were 'present' (PM > MM, paired *t*-test, one-sided $p < 0.05$) whereas none of the negative controls was present.

(c) *Microarray data analysis*

Each independent replicate was hybridized to a chip, and two replicates were made per genotype. To account for sources of non-biological variation between arrays, we normalized the PM and MM probes for all arrays using the quantile method (Irizarry *et al.* 2002; Bolstad *et al.* 2003). Not all of the 13 966 genes on the GeneChip will be expressed in adult males, therefore we tested each gene for presence of mRNA comparing every PM value to its complementary MM value in a paired *t*-test (one-tailed) that included all the probe pairs from either the H or the L genotype arrays; genes with $p < 0.05$ were considered significantly present. We excluded a gene if it was not significantly present on either the H arrays or L arrays; 9870 genes were present on at least one set of arrays and were analysed for differential expression.

To determine if mRNA expression differed significantly between H and L genotypes, we fitted a linear mixed-model to $\log_2$-transformed, normalized PM values (Chu *et al.* 2002) using SAS (release 8.02) PROCMIXED (Littell *et al.* 2002). The model was:

$$\log_2(\mathrm{PM}) = C + G(C) + P + C^{*}P + G(C)^{*}P + A + e,$$

where C is the category (H or L MCRS); G is genotype nested within category; P is the PM probe (14 per gene); A is the (random) array effect; and e is the residual error. We calculated external Studentized residuals from the fitted model and removed outliers where this value was greater than 4.0; 12 182 of 1 655 928 (0.7%) probe intensity values were removed as outliers. We then fitted the model to the remaining data. Significant association of mRNA expression with phenotype was given by the *p*-value of the category contrast (H versus L) calculated from the model. We controlled for multiple hypothesis testing by adjusting the expected number of false positives (the per-family error rate so that only one false positive was expected in a list of candidate genes (Ge *et al.* 2003)). At a nominal *p*-value of 0.0001, about one false positive was expected when conducting 9870 statistical tests: false positive discovery rate = $9870^{*}(0.0001) = 0.99$. Genes that met this criterion were also subjected to permutation tests to confirm the level of significance. For each gene, the probe level data were randomly permuted across the line and category effects, while retaining the appropriate 'probe' value (50 000 iterations). All genes with parametric *p*-values of less than 0.0001 also had permutation *p*-values of less than 0.0001. We also analysed gene expression in the H and L genotypes from each diallel separately to determine the robustness of candidate gene identification.

3. RESULTS

The difference in MCRS values between H and L genotypes was highly significant ($F_{1,22} = 33.6$, $p < 0.0001$; table 1). Figure 1 shows the mean difference in gene expression between H and L genotypes on the $\log_2$ scale ('effect size') plotted against the statistical significance of the test for differential expression; effect size is equivalent to $\log_2$(fold-change). Such plots have been called 'volcano plots' (Gibson 2002). The figure illustrates that significant differences were found across a wide range of effect sizes rather than being restricted to only the largest effect sizes.

Twenty-seven genes were differentially expressed ($p < 0.0001$), with only one false positive expected (table 2). The largest significant effect size was *ca*. 1 (twofold difference) in expression between H and L genotypes. Most of the significant differences in expression were less than 50%. Overall, more genes had higher expression (18) in H genotypes than lower expression (9), but this trend was not significant (two-tailed binomial test for deviation from equal occurrence, $p = 0.122$). When the two diallels were analysed separately, 14 out of the 27 genes were significant in both diallels ($p < 0.05$). These genes are indicated in bold in table 2.

The gene with the highest level of significance and second-largest effect size was a cytochrome P450 (*Cyp6g1*).

Table 1. H and L MCRS genotypes.

category	dam	sire	estimate[a]	s.e.m.	n[b]
H	7d	12b	1.3571	0.1411	4
H	14a	5e	1.3102	0.1411	4
H	3b	10c	1.1551	0.09410	9
L	10c	8b	0.6924	0.1411	4
L	4e	9a	0.6700	0.09410	9
L	15c	11f	0.4252	0.1996	4

[a] Least-square means of MCRS values standardized to a mean of 1.
[b] Number of replicate crosses.

Over-expression of *Cyp6g1* confers broad resistance to a variety of insecticides (Le Goff *et al.* 2003). In our population, males with greater expression of *Cyp6g1* had *low* MCRS, suggesting a fitness cost to over-expression.

Phosphoric monoester hydrolases (phosphatases) and glutathione transferases (Gst) were significantly over-represented compared with their frequency among genes expressed in adult males (figure 2). Both classes of genes showed complete concordance of their effects, with all four Gst genes being expressed more in H MCRS genotypes, and all four phosphatases being expressed less in the H genotypes.

Genes expressed in male reproductive tissues are thought to strongly affect male fitness (Wolfner 1997; Chapman 2001), and therefore might be expected to be over-represented among our candidates. To test this assumption, we first compared our genes with a list of testes-expressed genes from a comprehensive expressed sequence tag database search reported by Boutanaev *et al.* (2002). Twelve out of the 27 candidate genes are expressed in testes (table 2, right-most column). The relative proportion of testes-expressed genes was not significantly different from their proportional representation in adult males (proportion in adults $= 3270/9870 = 0.331$; proportion among significant genes $= 12/27 = 0.444$, two-tailed binomial test $p = 0.61$). However, all but one of the genes expressed in testes were upregulated in the H genotypes. This was a significant deviation from the overall proportion of upregulated versus downregulated genes (hypergeometric probability, $p = 0.018$). We then compared our candidate genes with a list of genes expressed in male accessory glands (MAG), which produce seminal fluid. This list was derived from a single-chip experiment in which we hybridized mRNA from 10 pairs of MAGs dissected from Ives males. Comparison of log-transformed PM and MM values showed that of the 9870 genes expressed in adult males, 4060 genes were significantly expressed in MAGs (one-tailed paired *t*-tests; $p < 0.05$). Among our differentially expressed genes, seven were expressed in MAGs (table 2, right-most column). The relative proportion of MAG-expressed genes was not significantly different from their proportional representation in adult males (proportion in adult males $= 4060/9870 = 0.41$; proportion among significant genes $= 7/27 = 0.26$, two-tailed binomial probability of seven or fewer $= 0.078$); five out of seven genes expressed in MAGs were upregulated in H genotypes (hypergeometric $p = 0.59$).

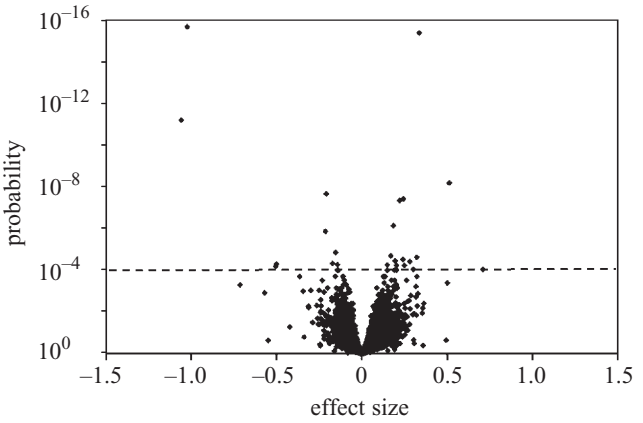

Figure 1. 'Volcano plot' of microarray results. The *p*-value of the 'category' contrast is plotted against the effect size of the contrast (average expression in H genotypes minus average expression in L genotypes). Negative values on the *x*-axis represent genes for which L genotypes had higher mRNA expression than did H genotypes, and vice versa. The *y*-axis shows the *p*-value on a logarithmic scale. The dashed line at $p = 10^{-4}$ shows the cut-off for genes considered significantly differently expressed in this study.

4. DISCUSSION

We have shown that quantitative-genetic microarray experiments can provide a powerful and straightforward way to identify candidate genes contributing to heritable fitness variation. Working with genotypes representing natural variation from a single population, we identified a moderate number of genes with transcriptional variation significantly associated with male fitness variation. The approach was very sensitive, detecting expression-level differences of less than 15%. Such sensitivity is necessary, because heritable transcriptional variation maintained within a randomly mating population is likely to be subtle.

The gene with the most significant effect on MCRS was a cytochrome P450 (*Cyp6g1*) that confers insecticide resistance when upregulated (Daborn *et al.* 2001, 2002; Le Goff *et al.* 2003). Our results indicate that modest (twofold) upregulation of this gene is associated with L MCRS in the absence of insecticides, suggesting a trade-off between insecticide resistance and male fitness. More generally, there might be a trade-off between stress resistance and male fitness, which could explain the maintenance of genetic variation in the Ives population.

Daborn *et al.* (2002) reported that a transposable element insertion in the promoter region of *Cyp6g1* was completely associated with upregulation of the gene and dichlorodiphenyltrichloroethane (DDT) resistance in a worldwide collection of *D. melanogaster*. This pattern indicates that the DNA sequence variation within the regulatory region of the gene itself (*cis* regulation) is probably responsible for transcriptional variation and DDT resistance. We are currently investigating whether there is a causal link between *Cyp6g1* allelic variation and MCRS in the Ives population.

Our results also implicate oxidative stress resistance and energy acquisition and allocation as important factors in male reproductive success. *Gst-E* genes are thought to be involved in resistance to oxidative stress (Sawicki *et al.* 2003), specifically in the metabolism of lipid peroxidation.

Table 2. List of candidate genes whose expression differed between H and L MCRS genotypes. Molecular functions were taken from the gene ontology terms for the genes listed on the Affymetrix (www.affymetrix.com) and Flybase (flybase.bio.indiana.edu) databases.

gene name/ID[a]	effect size[b]	s.e.m.	d.f.	t	$p <$	location[c]	molecular function (known or inferred)	M/T[d]
Cyp6g1	−1.03	0.089	128	11.5	1×10^{-15}	2R,48E7	cytochrome P450	M
GstE5	0.34	0.035	130	9.5	1×10^{-15}	2R,55C7	glutathione transferase	M
CG18030	−1.06	0.140	128	7.6	1×10^{-11}	3R,99F6	chymotrypsin	
CG6271	0.51	0.082	126	6.2	1×10^{-8}	3R,97D14	triacylglycerol lipase	T
CG9449	−0.21	0.035	128	6.0	1×10^{-7}	3L,76B5	acid phosphatase	
CG4375	0.24	0.042	128	5.9	1×10^{-7}	2L,21E2		T
Tkr	0.22	0.038	130	5.8	1×10^{-7}	2R,60F3-5	DNA binding	
Pcd	0.19	0.036	128	5.2	1×10^{-6}	3R,99B9	4a-hydroxytetrahydrobiopterin dehydratase	
CG11699	−0.21	0.042	130	5.1	1×10^{-5}	X,10C7		M
CG11334	−0.15	0.034	128	4.5	2×10^{-5}	3R,100B8	translation initiation factor; protein biosynthesis	T
CG1561	0.17	0.038	130	4.4	2×10^{-5}	X,10C9		
rab3-GAP	0.32	0.073	128	4.4	3×10^{-5}	2L,33C-2	RAB GTPase activator	M,T
CG14391	0.24	0.056	128	4.3	3×10^{-5}	3R,87C5		T
CG3875	0.28	0.066	108	4.3	4×10^{-5}	2R,58F4	nucleic acid binding; ubiquinone biosynthesis	T
GstE8	0.20	0.046	128	4.3	4×10^{-5}	2R,55C7-8	glutathione transferase	
CG2680	−0.17	0.041	130	4.2	5×10^{-5}	3L,78F2-3	4-nitrophenylphosphatase	
CG13309	−0.50	0.119	128	4.2	5×10^{-5}	3L,66E1		
CG5693	0.15	0.036	128	4.2	6×10^{-5}	2L,36D2		T
CG2113	0.20	0.049	128	4.2	6×10^{-5}	3L,63A2		T
CG2241	0.25	0.060	130	4.2	6×10^{-5}	3R,99F9-10	ATPase activity	T
CG3290	−0.50	0.122	128	4.1	7×10^{-5}	2R,58D1	alkaline phosphatase	
GstE6	0.30	0.075	128	4.0	1×10^{-4}	2R,55C7	glutathione transferase	M
GstE1	0.71	0.175	128	4.0	1×10^{-4}	2R,55C6	glutathione transferase	M,T
CG15200	0.20	0.050	128	4.0	1×10^{-4}	X,10A10		T
CG16741	0.19	0.047	130	4.0	1×10^{-4}	2R,56F17		M,T
CG5171	−0.14	0.036	130	4.0	1×10^{-4}	2L,27F5	trehalose phosphatase activity	
CG17856	0.15	0.038	128	3.9	1×10^{-4}	3R,98C3	ubiquinol-cytochrome *c* reductase	

[a] Bold type, significant in both diallels when analysed separately.

[b] Mean difference in mRNA abundance between H and L genotypes on the $\log_2$ scale (equivalent to $\log_2$[fold-change]).

[c] Chromosome arm and cytological position.

[d] Tissue-specific expression: M, MAG; T, testis (see text for explanation).

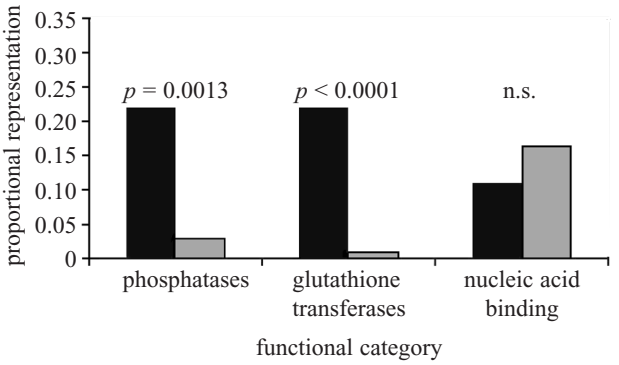

Figure 2. Over-representation of some enzymatic molecular function categories. Black bars show the proportional representation of the functional category among significantly differentially expressed genes with annotation ($n = 18$). Grey bars show proportional representation of the functional category among all annotated genes expressed in adult males ($n = 4749$). Phosphatases (phosphoric monoester hydrolases) and glutathione transferases were significantly over-represented among the differentially expressed genes as determined by binomial probability tests (p-values above bars). As a comparison, genes with nucleic-acid binding activity were proportionately represented among differentially expressed genes.

Four of these genes were over-expressed in high-fitness relative to low-fitness genotypes. Concordance of the expression of these genes might be a result of coregulation of tightly linked genes, because all four map to the same cytological region and appear to be part of a family of nine *Gst* genes in that region.

Energy acquisition and allocation are implicated because at least four of our candidates are directly involved in energy acquisition, storage or conversion. Trehalose phosphatase functions in conversion of glucose to trehalose, which is the principal energy storage sugar in insects. Chymotrypsin is one of the major protein-digesting enzymes and is found in the insect midgut. Triacylglycerol lipase breaks down triacylglycerols both in gut and in fat bodies. Finally, Ubiquinone-cytochrome b-c1 oxidoreductase is part of complex III of the mitochondrial electron transport chain.

There is a possible complicating factor in our interpretation that genetic variation between the H and L genotypes is the cause of MCRS and gene expression variation. Although all of the H and L genotypes and their *ebony* competitors had the *Wolbachia* endosymbiont, it is possible that variation in levels of *Wolbachia* could affect the MCRS phenotype (Hoffmann *et al.* 1998; Snook *et al.* 2000). To evaluate this possibility, we looked for effects of *Wolbachia* on MCRS in the offspring of reciprocal crosses from diallel 2 in which one parent carried the endosymbiont and the other did not. *Wolbachia* is transmitted through the egg cytoplasm (Hoffmann *et al.* 1998), so males inherit it only through their mothers. If *Wolbachia* affects MCRS, there should have been differences in MCRS between reciprocal crosses if only one parental line has the endosymbiont. There were three parental lines from diallel 2 that did not have the endosymbiont, probably as a result of treatment of these lines with antibiotics six generations before the MCRS assay. When we performed a *post hoc* examination of the reciprocal crosses involving these lines, we found no significant effects of *Wolbachia* state on MCRS ($F_{1,240} = 0.02$, $p = 0.88$), and no *Wolbachia*$*$line interaction ($F_{2,240} = 0.44$, $p = 0.64$). We also cured a subset of flies in each parental line that had been used in the microarray study and re-assayed MCRS for all H and L genotypes in both a *Wolbachia* + and *Wolbachia* − background ($n = 12$ per genotype per background). *Wolbachia* state had no effect on MCRS ($F_{1,125} = 0.07$, $p = 0.79$), nor was there a *Wolbachia*$*$genotype interaction ($F_{4,125} = 1.24$, $p = 0.30$). Given the lack of measurable differences in MCRS between infected and completely uninfected males of the same genotype, it appears unlikely that the large difference in MCRS between H and L genotypes could be a result of more subtle variation in *Wolbachia* infection level, and is instead probably caused by genetic variation among the male genotypes.

In conclusion, we found several unexpected results when we screened a population for genetic variation in male fitness and then searched for candidates using microarrays. *Cyp6g1* with its pesticide resistance function would not have been an obvious candidate for male reproductive success, but our results indicate that there may be trade-offs between these two traits. Direct tests of the causality of this relationship and how it changes with toxin stress can now be conducted using transgenic and mutant stocks, and the generality of the relationship can be investigated in other populations. In the same way, most of the other genes we identified would not have been chosen *a priori* as likely candidates, in particular the one-third that had no previous functional annotation at all. Conversely, genes expressed in MAGs or testes might have been chosen as good candidates because post-copulatory processes are thought to have major effects on male fitness (Wolfner 1997; Chapman 2001), but our results indicate that they are not disproportionately represented among differentially expressed genes, although testis-expressed genes were significantly more likely to be upregulated in H male fitness genotypes. These results confirm the utility of our experimental approach, which can be extended to provide more detailed insight into the causes of male fitness variation. In particular, experiments focusing on pre- and post-copulatory processes and on female fitness could address recent theories of sexual antagonism, sexual coevolution and the maintenance of genetic variation in fitness.

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