doi:10.1111/evo.12082

TRANSCRIPTOME-WIDE EXPRESSION VARIATION ASSOCIATED WITH ENVIRONMENTAL PLASTICITY AND MATING SUCCESS IN CACTOPHILIC *DROSOPHILA MOJAVENSIS*

Gilbert Smith,[1,2] Yongxiang Fang,[3] Xuan Liu,[3] John Kenny,[3] Andrew R. Cossins,[3] Cassia Cardoso de Oliveira,[4] William J. Etges,[5] and Michael G. Ritchie[1,6]

[1]*School of Biology, University of St Andrews, St Andrews, Fife KY16 9TH, United Kingdom*

[2]*gilbert.smith@st-andrews.ac.uk*

[3]*Centre for Genomic Research, Institute of Integrative Biology, University of Liverpool, Biosciences Building, Crown Street, Liverpool L697ZB, United Kingdom*

[4]*Math and Science Division, Lyon College, 2300 Highland Road, Batesville, Argon 72501*

[5]*Program in Ecology and Evolutionary Biology, Department of Biological Sciences, University of Arkansas, Fayetteville, Arkansas 72701*

[6]*E-mail: mgr@st-andrews.ac.uk*

Received August 22, 2012
Accepted January 23, 2013

Ecological speciation occurs with the adaptation of populations to different environments and concurrent evolution of reproductive isolation. Phenotypic plasticity might influence both ecological adaptation and reproductive traits. We examined environment-specific gene expression and male mating success in cactophilic *Drosophila mojavensis* using transcriptome sequencing. This species exhibits cactus-dependent mating success across different species of host plants, with genotype-by-environment interactions for numerous traits. We cultured flies from egg to eclosion on two natural cactus hosts and surveyed gene expression in adult males that were either successful or unsuccessful in achieving copulation in courtship trials. We identified gene expression differences that included functions involved with metabolism, most likely related to chemical differences between host cactus species. Several epigenetic-related functions were identified that might play a role in modulating gene expression in adults due to host cactus effects on larvae, and mating success. Cactus-dependent mating success involved expression differences of genes implicated in translation, transcription, and nervous system development. This suggests a role of neurological function genes in the mating success of *D. mojavensis* males. Together, these results suggest that the influence of environmental variation on mating success via regulation of gene expression might be an important aspect of ecological speciation.

KEY WORDS: **Ecological speciation, gene expression, phenotypic plasticity, transcriptome sequencing.**

Environmentally induced phenotypic variation is an under-studied aspect of speciation, particularly its role in the evolution of reproductive isolation (Butlin et al. 2012). However, phenotypic plasticity provides a way for the same genotype to produce different traits according to environmental context and thus may be an important process facilitating ecological adaptation and speciation (Thibert-Plante and Hendry 2011). Assessment of the role of phenotypic plasticity in ecological adaptation is difficult

EVOLUTION
INTERNATIONAL JOURNAL OF ORGANIC EVOLUTION

due to a poor understanding of the developmental processes producing plastic traits and how these link to environmental adaptation (Ghalambor et al. 2007; Scoville and Pfrender 2010). If an organism possesses the developmental flexibility necessary for a plastic response then ecological adaptation may result in particularly rapid divergence due to ecological selection on relevant traits (Schluter 2001; Rundle and Nosil 2005). When such traits diverge, speciation may follow if reproductive isolation evolves as a direct or indirect consequence (Funk et al. 2006).

Speciation may be more likely when ecological and sexual selection interact (Ritchie 2007; Van Doorn et al. 2009; Sobel et al. 2010; Maan and Seehausen 2011), if traits that influence mating success are under divergent ecological selection. Determining how intimate any such associations are (e.g., if this involves pleiotropy or covariance between traits) is central to understanding debates about the likelihood of so-called "magic trait" speciation (Servedio et al. 2011) or how extensive traits with multiple effects are (Smadja and Butlin 2011). Plasticity of ecological and reproductive traits may be important for adaptation and speciation if selection in an environment facilitates genetic population divergence perhaps via genetic accommodation (West-Eberhard 2005a,b), or adaptive plasticity could simply allow persistence for long enough to allow genetic adaptation (Crispo 2008). There are multiple ways in which plasticity could either accelerate or inhibit speciation (Pfennig et al. 2010), but most current methodologies to detect genes involved in ecological adaptation ignore expression variation (Pavey et al. 2010).

Drosophila species are commonly used for both gene expression and plasticity studies (Levine et al. 2011). The cactophilic species, *Drosophila mojavensis*, is an excellent candidate for such studies due to the use of different host cacti during development, and has been used as a model system for divergence during speciation. This species is endemic to northwestern Mexico and the southwestern United States and its range is comprised of four allopatric regions with little evidence of contemporary gene flow between them (Ross and Markow 2006; Machado et al. 2007; Reed et al. 2007). Populations of *D. mojavensis* that occupy Baja California and the mainland Mexico-Arizona regions are of particular interest as they demonstrate a significant level of environmentally influenced premating sexual isolation between them, mediated by divergent epicuticular hydrocarbon profiles (CHCs), which act as contact pheromones and courtship songs (Etges et al. 2007, 2009). This premating isolation is seen only between the Baja and mainland populations, with little evidence of postzygotic isolation between any population pair (Ruiz et al. 1990). The Baja California population is thought to be ancestral based on genetic and ecological evidence (Ruiz et al. 1990; Wasserman 1992; Matzkin and Eanes 2003; Matzkin 2004), with all populations diverging from an ancestral group around 230–270,000 years ago (Smith et al. 2012). Colonization of the mainland Mexico involved a host-plant shift from the favored pitaya agria cactus, *Stenocereus gummosus*, to organ pipe cactus, *Stenocereus thurberi*, that is distributed in the southern half of Baja California, mainland Sonora and Sinaloa, and southern Arizona (Heed 1982; Etges et al. 1999). A suite of phenotypic changes in life history and reproductive traits accompanied this host shift and many of these phenotypes demonstrate plasticity when flies are raised on differing cactus hosts (Etges et al. 2007, 2009; Etges et al. 2010). Therefore, *D. mojavensis* has undergone ecological selection with concurrent divergent selection of reproductive traits, and shows plastic expression of many key traits across cactus hosts.

Plasticity due to gene expression variation is potentially an important component of adaptation to a varying environment and can act alongside gene–environment interactions in determining levels of adaptation during ecological specialization. A series of quantitative trait locus (QTL) studies revealed the genetic architecture of life-history traits involved in host-plant adaptation and sexual isolation in *D. mojavensis* (Etges et al. 2007, 2009, 2010). F_2 males from crosses between Baja and mainland populations were reared on either agria or organ pipe cactus and QTLs were identified for mating success, courtship songs, CHCs, and egg to adult development time. All traits showed evidence of genotype-by-environment interactions (G×Es) influencing their expression. Hence this system displays extensive genetic variability influencing viability and mating success. The extent of variation in gene expression due to rearing cactus (or other environmental variation) in *D. mojavensis* is only beginning to be studied. Microarray analyses revealed approximately 1500–3000 genes with cactus-specific expression in third instar larvae in Baja and mainland populations reared on agria versus organ pipe cacti (Matzkin et al. 2006; Matzkin 2012), thousands of genes in adults under dessication stress (Rajpurohit et al. *in press*), and thousands of genes assayed across the entire life cycle (W. J. Etges, unpubl. data). Many of these genes were involved in metabolism and detoxification pathways as would be predicted due to host-plant chemical differences (Fogleman and Danielson 2001), as well as fatty acid biosynthesis and olfaction (Matzkin et al. 2006; Matzkin 2012).

Here we describe an RNA sequencing study of gene expression variation associated with different cactus substrates in adult *D. mojavensis* that explicitly examines the link between ecological variation and courtship behavior. We examined gene expression in adult males from a mainland population reared from egg to eclosion on either organ pipe or agria cactus after identifying the first males to succeed in mating trials with mainland females. Our aim was to (1) enumerate and identify genes or functional gene networks that showed plastic expression responses to host cactus, (2) identify expression variation associated with rapid mating

success, and (3) examine the interaction between cactus and mating success variation. The latter is particularly important for identifying genes and functional pathways involved in cactus-dependent mating success; for example, pleiotropic linkage between cactus adaptation and mating behavior would predict that the same genes are involved in both traits. Note that we reared flies to fermenting cactus from egg to eclosion and surveyed expression differences in adults; here, we use the term "epigenetics" to include induced gene expression changes during development (e.g., Chittka et al. 2012) and when used does not imply that we have identified trans-generational effects.

Methods

FLY MAINTENANCE

All experiments were performed with a population of *D. mojavensis* from Organ Pipe National Monument (OPNM), Arizona, collected in 2002 by T. Markow. This multifemale stock was reared en mass on banana food in 8 dr shell vials at ambient temperature. Although this population was known to be homokaryotypic for gene arrangements on the second and third chromosomes (Etges et al. 1999), we made multiple pair-mated lines and cytologically verified that no inversions were segregating. We then sib-mated these lines for five generations and one inbred line was selected for the mating trials described below. Flies from this inbred line were derived from an isofemale line established in 2004. This is the same line used in the QTL crosses analyzed recently (Etges et al. 2007, 2009, 2010). We chose to analyze the mainland line because this derived population has successfully performed a host shift, and females from the mainland are more discriminating in mate choice (Markow 1991; Etges 1992). Flies were reared on banana food at moderate larval densities in half-pint bottles in an incubator at 27°C during the day and 17°C at night on a 14-h light:10-h dark cycle. Emerging adults were aged until sexually mature (10–12 days), then placed into oviposition chambers (approximately 400 adults per chamber), allowed to mate, and then oviposit for 10 h each day. Eggs were washed in deionized water, 70% ethanol, and again in sterile deionized water. Groups of 200 eggs were transferred to a 1 cm^2 piece of sterilized filter paper and then placed on fermenting cactus tissue, either agria, *S. gummosus*, or organ pipe cactus, *S. thurberi*, in an incubator programmed as above. Experimental flies were reared on each cactus species from egg to eclosion, and thereafter on banana food until sexual maturity.

Fermenting cactus cultures were set up in half pint bottles with 75 g of aquarium gravel covered with a 5.5-cm diameter piece of filter paper. Bottles were autoclaved, 60 g of either agria or organ pipe tissues were added then autoclaved again for 8 min at low pressure (Etges 1998). After cooling to room temperature, each culture was inoculated with 0.5 mL of a pectolytic bacterium, *Erwinia cacticida* (Alcorn et al. 1991), and 1.0 mL of a mixture of seven yeast species common in natural agria and organ pipe rots (Starmer 1982): *Dipodascus starmeri*, *Candida sonorensis*, *Candida valida*, *Starmera amethionina*, *Pichia cactophila*, *Pichia mexicana*, and *Sporopachydermia cereana*. Inoculation was performed to ensure that microorganisms found in nature, rather than those present in the laboratory environment, populated the necrotic tissue, and differential growth of the inoculants will generate variation between cactus types. Eggs were then added and, once eclosed, adults from four replicate cactus bottles were separated by gender and kept on banana food in vials in the incubator until sexually mature at 10–12 days.

MATE CHOICE EXPERIMENTS

We identified males who were successful in mating trials to assess the influence of preadult cactus rearing on the mating behavior of male flies. Males used in the mate trials were reared on agria or organ pipe cactus, whereas all females were reared only on organ pipe cactus to identify the effects of rearing substrates on male mating success. Organ pipe cactus reared females are more discriminating in mate choice trials than agria cactus-reared females (Etges 1992). A total of four treatments (two rearing cacti and two mating statuses) were performed, with four replicates per treatment.

Mate choice trials were carried out using a multiple-choice design (Etges 1992; Etges and Ahrens 2001). Twenty female and male virgin adults 12–16 days old were used in each trial. A 50 mL Erlenmeyer flask was used as a mating chamber and changed after each trial. Each trial lasted until half of the pairs copulated or for a maximum of half an hour. All trials were performed at room temperature (18–20°C) in the morning (10:00 a.m. to 12:00 p.m.; lights on at 6 a.m.) over no more than a 2-h time period. We define the first mating males as "successful" and the nonmating as nonsuccessful, so our measure of mating success potentially includes measures of male mating speed and vigor as well as female discrimination. However, previous studies suggest that male mating success is due overwhelmingly to female choice and not male–male interaction or unwillingness to mate (Havens et al. 2011). In mating trials with cactus-reared adults, usually almost all flies mate by the end of the trials.

Copulating pairs were observed for at least 10 sec to avoid any pseudo-copulating pairs (Markow et al. 1983), then flash frozen in liquid nitrogen and stored in RNA later at −20°C prior to RNA extraction. After half of the flies had mated, the remaining unmated flies were also flash frozen. Males from two mate choice trials, that is, 20 male whole bodies, were pooled together per sample.

RNA SEQUENCING AND ANALYSIS

RNA was extracted from each pooled sample using a Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA), and the high quality of the RNA was confirmed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). Library preparation was carried out at the Centre for Genomic Research, University of Liverpool and included poly(A) tail selection using the Dynabeads messenger RNA (mRNA) purification kit (Invitrogen, Paisley, UK). A total of 100 ng of the resulting mRNA was used as input for library production using the SOLiD Total RNA-Seq kit (Life Technologies, Paisley, UK). Sequencing was performed on a SOLiD 4 sequencer (Life Technologies, Paisley, UK), generating reads of 50 bp length. The sequence data have been deposited in the European Nucleotide Archive (ENA) at the European Bioinformatics Institute (EBI, accession ERP002218).

Reads were filtered for quality and mapped on to the *D. mojavensis* genome (Clark et al. 2007) using the Tuxedo suite (Bowtie/Tophat v1.4.0), with only uniquely mapped reads retained for analysis. HTSeq (Anders 2010) was used to quantify read counts for two different types of features, genes and exons. This enabled a quantitative examination of expression levels at the level of whole genes as well single exons within a gene.

Raw count data were normalized across sequenced libraries and a generalized linear model was fitted with a negative binomial distribution using the EdgeR package in R (Robinson et al. 2010). The full model was $Y = g^{-1}(AX) + \varepsilon$, where Y is a vector of normalized count numbers for one gene, $g^{-1}(.)$ is the inverse link function, A is the model matrix, and X incorporates the model parameters. X includes an intercept and effects of factors, cactus (organ pipe or agria), mating success (success or fail), and an interaction effect, whereas ε represents the random noise of sampling a negative binomial population. A likelihood ratio test was used to compare a full model to models with each term removed to test for significance of each treatment effect. Gene specific P values were corrected for multiple testing using the false discovery rate (FDR) approach of Storey and Tibshirani (2003) with significance taken at 10%. A heatmap was constructed for significantly differentially expressed (DE) genes overall and clusters of coregulated genes were obtained using the k-means clustering (Hartigan and Wong 1979) from the R package.

Analysis at the level of exons can identify those genes where alternative exon expression arising from alternative transcript initiation, alternative splicing, or alternative polyadenylation occurs due to treatment (Griffith et al. 2010). For this, we used a modified version of the function *spliceVariants* from the EdgeR package. This fits a negative binomial generalized linear model for each gene, given the counts for the exons within that gene. The same approach was adopted as for the gene-level testing except that term X (see previous paragraph) now includes terms reflecting the difference among the exons, treatment effects, and interaction of exon and treatment. Thus, the model becomes $E(Z) = g^{-1}(B\beta) + \varepsilon$, where ε is random noise, Z is a vector of normalized count numbers for all exons within one gene, $g^{-1}(\cdot)$ is the same as stated before, B is the model matrix, β is the vector of model parameters which includes the intercept and terms reflecting the difference among the exons, treatment effects, and interaction of exon and treatment. Because exon expression is modeled according to the other exons within the same gene, significance represents independent responses of exons by treatment or alternative expression (AE). That is, the test reveals genes for which there is an exon-specific signature across treatments or interactions between exons and treatments. Significant AE was taken for each gene at 10% FDR.

FUNCTIONAL ENRICHMENT

Functional enrichment or overrepresentation analysis aims to detect common functions within the DE or AE gene sets. For categorizing genes, we used Gene Ontology (GO) annotations from the *D. mojavensis* entries in QuickGO (Binns et al. 2009), as well using corresponding GO terms for orthologous genes in *Drosophila melanogaster* FlyBase entries (vFB2012_01, *D. melanogaster* release 5.43) taken from the ortholog conversion tool (McQuilton et al. 2012).

Two test methods were employed and each were undertaken separately for both DE and AE genes across the three contrasts (cactus, mating success, and their interaction) using FDR $< 10\%$. First, we developed a "rank mean test" written in R (*marrayRankTest*, available from Y. Fang), which ranks genes by P value and takes the mean of the rank for members of gene sets (i.e., biological process GO categories, downloaded from QuickGO; Binns et al. 2009) as the test statistic for enrichment. This method employs a corrected normal distribution that more accurately estimates P values for enrichment with small gene sets, which can occur with poorly annotated genomes.

Second, *D. melanogaster* orthologs of the responding DE and AE genes (FDR $< 10\%$) were analyzed in DAVID v6.7 (Database for Annotation, Visualization and Integrated Discovery v6.7; Dennis *et al.* 2003; Huang et al. 2009). DAVID uses Fisher's exact test to identify significantly enriched GO categories. A "Fuzzy" clustering algorithm then groups annotation terms into functional clusters of genes. Clusters are considered significant with an enrichment score >1.3 (the geometric mean of annotation P values; Dennis et al. 2003; Huang et al. 2009).

Several small, noncoding RNAs (ncRNAs) were identified as significantly DE. For each, the orthologous ncRNA in *D. melanogaster* was found through a BLASTn search on the NCBI website, using the nucleotide database (Altschul et al. 1997). Hits were called as having significant orthology when the E-value was $<$10e-6, and GO annotations for each ncRNA were obtained from FlyBase.

Table 1. Number of genes with significant cactus, mating success, and interaction effects. The number of differentially expressed (DE) and alternatively expressed (AE) genes in *Drosophila mojavensis* are presented, along with the number of genes that had confirmed *Drosophila melanogaster* orthologs and the total number of unique genes across all effects.

Test	Cactus	Mating success	Interaction	Total unique gene models
DE *D. mojavensis*	111	19	147	212
DE *D. melanogaster*	71	11	92	144
AE *D. mojavensis*	64	28	48	115
AE *D. melanogaster*	51	24	38	100

Results

The experimental design consisted of a pooled sample of 20 males for each of the two factors (mating success) and two treatment (rearing cactus diet) groups. Four biological replicates were produced independently from rearing to mating success producing 16 RNA samples each of which was subjected to RNA-Seq. Fragment data were analyzed by analysis of variance (ANOVA) for DE gene and AE, each generating three contrasts between rearing cacti, differential mating success, and their interaction. The number of reads obtained per sample was typically 45 million reads of 50 bp length. Of these, approximately 30% mapped uniquely to the reference genome, producing a final average of 15.3 million reads per biological replicate. The number of DE genes was relatively small with 111 showing main effects of cactus, 19 associated with mating success, and 147 due to their interaction (Table 1), all from 212 unique gene models. Fewer genes were generated by the AE analysis, with 64 and 48 for cactus and interaction effects, respectively. However, in contrast to DE genes, there was a greater involvement of AE in male mating success (28; Table 1).

DIFFERENTIAL EXPRESSION

Figure 1 indicates the fold-change responses of all DE genes as a heatmap across the three ANOVA contrasts. *K* means clustering generated eight clusters; clusters three to eight were upregulated by the organ pipe relative to agria diets, and clusters five, six, three, and eight were upregulated and one, two, and seven were downregulated by successful relative to unsuccessful mating. Full details of all DE and AE genes and all functional enrichment results are given in Supplementary Tables S1–S6, and GO term annotations for genes within each heatmap cluster in Supplementary Table S7.

Functional enrichment of cactus-specific DE genes showed functions for several processes linked to metabolism (Fig. 2). These included glycerol ether metabolic processes, the tricarboxylic acid (TCA) cycle, and cell redox homeostasis. Annotations for protein modification were also significant, specifically protein ubiquitination and ubiquitin-mediated protein catabolism. Other significant annotations included immune response, methylation, tRNA processing, and calcium-based signaling. Genes showing expression differences by male mating success were enriched for two terms, translation and glycerol ether metabolic process (Fig. 2). The interaction of cactus and mating success produced only one significant annotation, for translation (Fig. 2), despite having the greatest number of significantly DE genes.

Functional enrichment of DE genes using *D. melanogaster* orthologs produced several significant terms for cactus and the interaction effects, although there was no main effect for mating success. Cactus effects produced a single cluster, containing several different terms involved in immune response (Fig. 2). Several terms were individually significant across cacti, although not as a cluster. These included four genes for olfactory behavior, chemosensory behavior, and cognition (Fig. 2; *Obp99A*, *Or83A*, *Gr94A*, and *drk*). Two functional clusters were enriched in the interaction between rearing cactus and mating success. The first included terms for ribosome, ribonucleoprotein complex, and translation. The second had an enrichment score <1.3, but included significant single annotations for protein targeting to mitochondria.

Several ncRNAs were DE due to both cactus and interaction effects (Table 2). These were small nuclear RNAs (snRNAs) and small nucleolar RNAs (snoRNAs). snoRNAs are often located in the introns of genes, particularly those associated with ribosome structure, and are excised from introns by the spliceosome. Alternatively they are transcribed as polycistronic transcripts by RNA poymerase II and processed into multiple RNAs (Terns and Terns 2002). The majority of snoRNAs in Table 2 originated from the introns of protein coding genes. Transcription of these genes by RNA polymerase II means that both snRNAs and snoRNAs would have survived size selection from the RNA extraction protocol and poly(A) tail selection, and thus were accurately quantified. These ncRNAs do not have detailed functional annotations and thus were not part of the enrichment analyses. However, of the DE protein-coding genes, 14 were identified as known constituents of ribonucleoprotein complexes. Most of these were ribosomal but one, *U2af38*, forms part of the spliceosome, attaching to the 3′ splice site during alternative splicing. Both this and another significantly expressed gene, *LSm-4*, have roles in alternative splicing.

Consistent upregulation of gene expression in one treatment group over another is often associated with an increased functional importance. The majority of significantly DE genes were upregulated on organ pipe cactus, the host used in nature in this

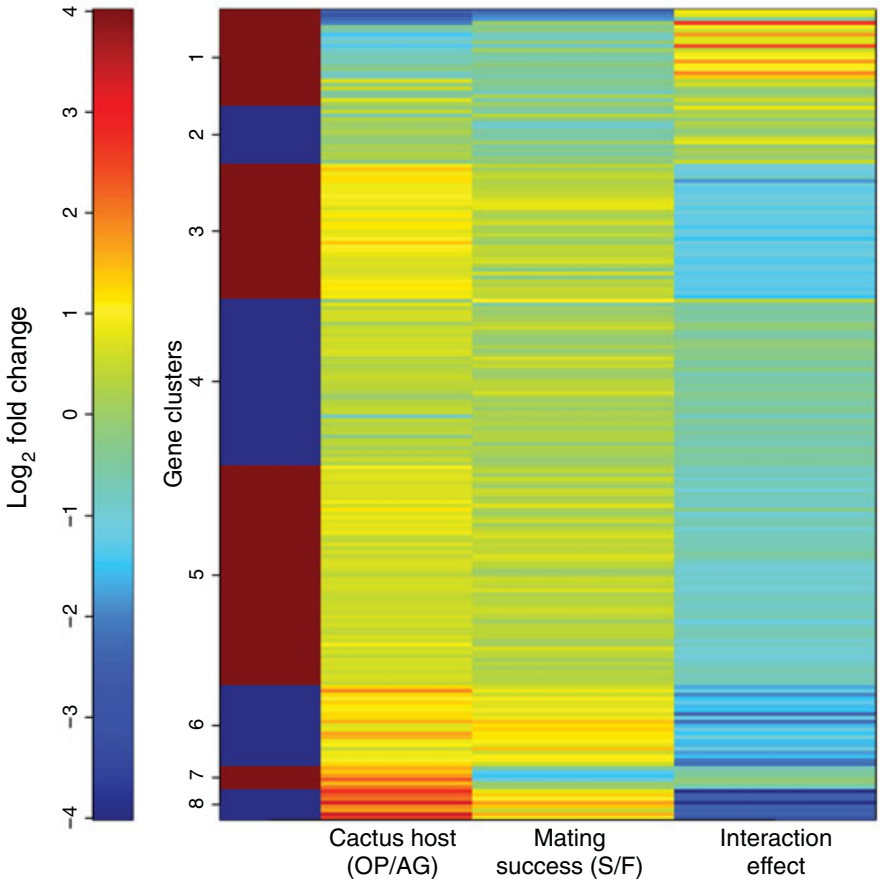

Figure 1. **Heat map displaying all differentially expressed genes across the three contrasts indicated. The color key representing the log_2 fold change values for each gene in each contrast is shown to the left, and the grouping of genes into eight clusters indicated in the main panel. S and F denote successfully and unsuccessfully mated male treatments, respectively, OP is the organ pipe cactus host treatment and AG is the agria cactus treatment.**

population, as were genes involved in methylation (Fig. 3). The methylation GO term (GO:0032259) involves the attachment of a methyl group to a molecule and is not limited to DNA methylation, but includes RNA and protein methylation. Interestingly, the most consistently upregulated genes in successfully mated males were involved in methylation, the other DE categories surprisingly being downregulated in successful males. Most glycerol ether metabolism and translation genes were also upregulated.

ALTERNATIVE EXPRESSION

Fewer AE genes were detected than DE genes, except in the case of mating success (Table 1). Surprisingly, the interaction effect showed only one significant annotation; however, more functions were identified using the most significant *D. melanogaster* orthologs in DAVID.

Rank mean test enrichment suggested functions for cellular signaling and ion transport genes differed across cactus hosts (Fig. 4). Annotations included signal transduction and G-protein coupled receptor-signaling pathways that function in the cellular response to extra-cellular signals. The end point of cellular signaling pathways is often the regulation of transcription, and this term was also significantly enriched due to cactus effects. Ion transport and sodium ion transport were seen, along with calcium ion transport via voltage-gated channels. These terms are often associated with synaptic transmission. Histone deacetylation genes were also AE across cacti, indicating a potential epigenetic response to cactus.

Functional annotations for both transcription, ion transport and chitin metabolic process were seen in the mating success contrasts, along with multicellular organismal development (Fig. 4). Lastly, the interaction effect contained only one significant functional annotation, for the regulation of calcium ion transport via voltage-gated channels.

DAVID analysis of AE orthologs revealed a similar set of functional annotations to the rank mean test, with the exception of mating success which showed no significantly enriched GO terms (Fig. 4). Cactus-specific genes were enriched for one cluster that included terms for transmission of nerve impulses and

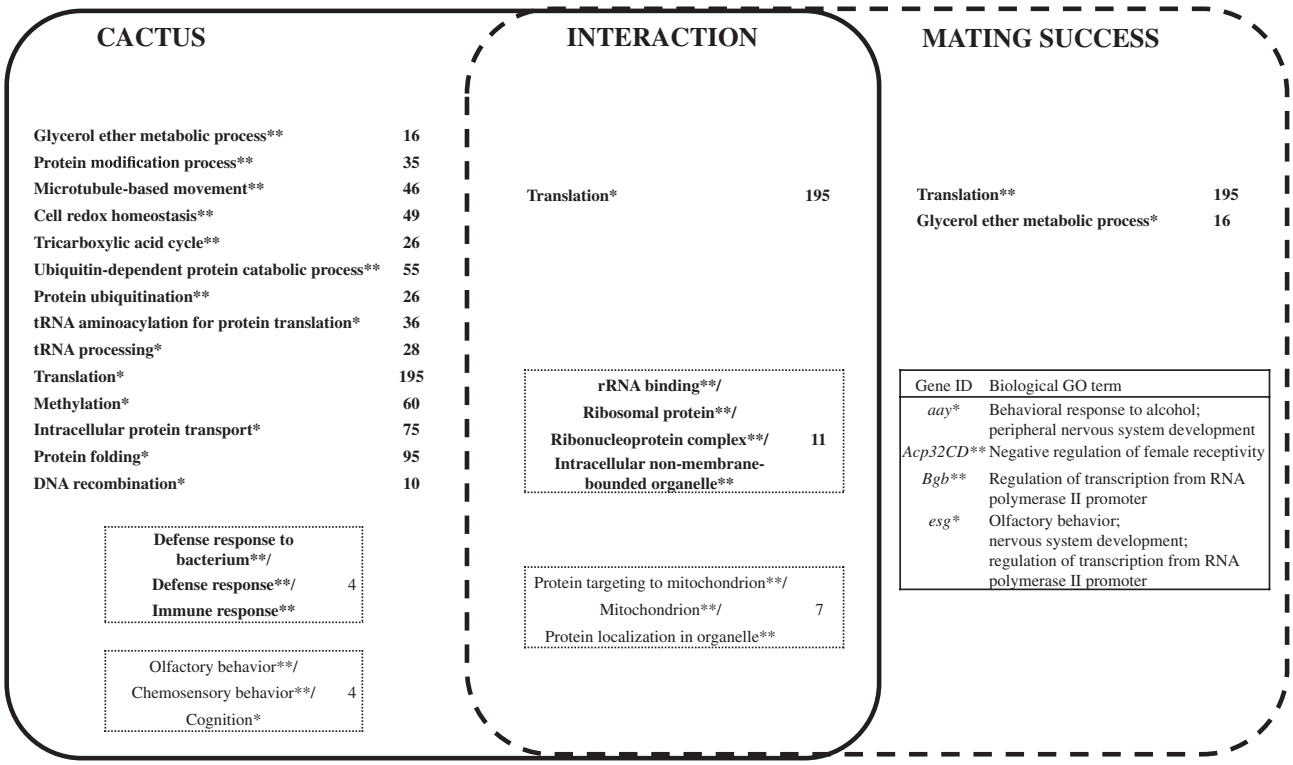

Figure 2. Summary of functional enrichment results for differentially expressed genes. Gene Ontology (GO) terms are grouped by experimental effect; cactus in large solid round box, mating success in large dashed round box, and their interaction. All GO terms are one per line with the number of associated genes indicated. Overlapping and root GO terms were removed for brevity. Rank mean test results are presented as unboxed, bold GO terms along with significance for each term (, FDR < 0.05 and *, FDR < 0.1). DAVID enrichment clusters are shown inside fine dashed boxes; bold and fine dashed boxed terms denoted significant DAVID clusters with an enrichment score of >1.3, fine dashed boxes represent clusters with enrichment score <1.3, yet, containing individually significant terms. Significance for each term from DAVID is indicated (**P < 0.05 and *P < 0.1). The solid table presents interesting significant genes (*Drosophila melanogaster* orthologs), and corresponding biological GO terms (**, FDR < 0.05 and *, FDR < 0.1). FDR = false discovery rate.**

synaptic transmission. Other individually significant terms were seen, including alternative splicing. The interaction of cactus and mating success contained two significantly enriched clusters of AE genes. The first included several terms for neuron-related development and axonogenesis, consistent with the results of the rank mean test. The second included terms for the regulation of transcription and chromatin modification and regulation. Interestingly, the *slowpoke* (*slo*) gene was AE in both the main cactus and interaction effects. This gene encodes an ion channel protein with biological functions in song production and structure and male courtship behavior, and is necessary for ethanol tolerance (Cowmeadow et al. 2005). It has also been shown to influence male courtship song in *D. melanogaster* (Peixoto and Hall 1998).

Discussion

Ecological speciation involves the adaptation of populations to new environments and concurrent evolution of reproductive isolation (Nosil 2012). Functional genetic links between genes involved in ecological adaptation and sexual behavior have rarely been examined. It is likely that environmental plasticity is common in ecological adaptation and thus traits potentially influencing both adaptation and isolation may often be influenced by coordinated changes in gene expression and plasticity (Thibert-Plante and Hendry, 2011). However, incorporating the analyses of such genes into studies of speciation is in its infancy. Here, using high-throughput transcriptome sequencing, we distinguished the gene expression changes due to both host-plant variation and mating success in cactophilic *D. mojavensis*, and identified the functions of genes involved in cactus-dependent mating success.

The *D. mojavensis* genome is poorly annotated with only 32% of all genes having at least one biological GO annotation, most of which are nonspecific (root) terms, and approximately 32% of *D. mojavensis* genes have no known orthologs in *D. melanogaster* (Tweedie et al. 2009). In comparison, 72% of *D. melanogaster* genes have been annotated, 67% of which are specific (nonroot) annotations (Tweedie et al. 2009). Here, approximately two thirds of the significant genes across effects

Table 2. **Significantly differentially expressed noncoding RNA products, their orthologs in *Drosophila melanogaster*, and their corresponding Gene Ontology (GO) functional annotations.**

Drosophila mojavensis ID	*D. melanogaster* ID	GO term annotations
Dmoj\snoRNA:GI25318	Dmel\snoRNA:Psi18S-110	Nuclear gene
Dmoj\snoRNA:GI25328	Dmel\snoRNA:Psi28S-2566	Nuclear gene; nucleolus
Dmoj\snoRNA:GI25330	Dmel\snoRNA:Psi28S-3327b	Nuclear gene
Dmoj\snoRNA:GI25333	Dmel\snoRNA:Me28S-G980	Nuclear gene
Dmoj\snoRNA:GI25343	Dmel\snoRNA:Psi18S-841d	Nuclear gene
Dmoj\snoRNA:GI25349	Dmel\snoRNA:Psi18S-841a	Nuclear gene; nucleolus
Dmoj\snoRNA:GI25354	Dmel\snoRNA:Me28S-C3420a	Nuclear gene
Dmoj\snoRNA:GI25358	Dmel\snoRNA:Me28S-G2703a	Nuclear gene
Dmoj\snoRNA:GI25368	No orthologous hits	-
Dmoj\snoRNA:GI25378	No orthologous hits	-
Dmoj\snoRNA:GI25382	Dmel\snoRNA:Me18S-C1096	Nuclear gene
Dmoj\snoRNA:GI25384	Dmel\snoRNA:Psi28S-2442b	Nuclear gene
Dmoj\snoRNA:GI25385	No orthologous hits	-
Dmoj\snoRNA:GI25391	No orthologous hits	-
Dmoj\snoRNA:GI25394	Dmel\snoRNA:Psi18S-1377d	Nuclear gene
Dmoj\snoRNA:GI25402	Dmel\snoRNA:Psi28S-3305b	Nuclear gene
Dmoj\snoRNA:GI25408	No orthologous hits	-
Dmoj\snoRNA:GI25409	Dmel\snoRNA:Psi28S-1060	Nuclear gene
Dmoj\snoRNA:GI25413	Dmel\snoRNA:Psi28S-1135a	Nuclear gene
Dmoj\snoRNA:GI25418	Dmel\snoRNA:Psi28S-1135f	Nuclear gene
Dmoj\snoRNA:GI25426	Dmel\snoRNA:U14:30Eb	Nucleolus; rRNA modification guide activity
Dmoj\snoRNA:GI25427	Dmel\snoRNA:Me18S-A1576	Nuclear gene; nucleolus
Dmoj\snoRNA:GI25433	No orthologous hits	-
Dmoj\snoRNA:GI25436	No orthologous hits	-
Dmoj\snRNA:U2:2	Dmel\snRNA:U2:34ABa	U2 snRNP; nuclear mRNA splicing, via spliceosome
Dmoj\snRNA:U4:2	Dmel\snRNA:U4:25F	U4 snRNP; nuclear mRNA splicing, via spliceosome

had no functional information (biological process GO terms) and one third of these genes had no discovered orthologs in the *D. melanogaster* genome. Functional enrichment analyses of poorly annotated genomes is thus challenging, especially when the number of significantly DE genes is small. Consequently, a combined approach was taken using two different enrichment methods to identify functional information. We uncovered clusters of coregulated gene sets (Fig. 1), and using functional enrichment tests with *D. mojavensis* annotated genes and *D. melanogaster* orthologs produced comparable results on the functions of these genes.

FUNCTIONAL ANALYSES

Cactus-specific differential gene expression showed significant enrichment for GO terms that were also detected in two microarray studies examining cactus-specific gene expression during larval development in *D. mojavensis* (Matzkin et al. 2006; Matzkin 2012). These included functions in immune response, metabolism, signal transduction, and the nervous system. Previous work has also shown that the transcriptomic response to cacti in larvae, and to desiccation, involve key metabolic pathways, including the TCA cycle (Matzkin and Markow 2009). Here, DE genes were seen that function in chemical metabolism, including glycerol ether metabolism, which aids in the assimilation of volatile alcohols. *D. mojavensis* is able to metabolize ethanol vapor, with consequent effects on life-history traits, such as longevity, life-time fecundity, and metabolic rates (Starmer et al. 1977; Etges and Klassen 1989). Cactus-specific effects also included genes involved in the TCA cycle and cell redox homeostasis. Cellular oxidative stress is known to accelerate cellular damage and shorten lifespan in *Drosophila* (Ruan et al. 2002), and is often linked to changes in metabolism. We studied sexually mature flies and found that the preadult rearing environment therefore has a carryover effect onto adult gene expression functioning in chemical metabolism. The chemical environment of columnar cacti is well documented. Alkaloids, medium chain

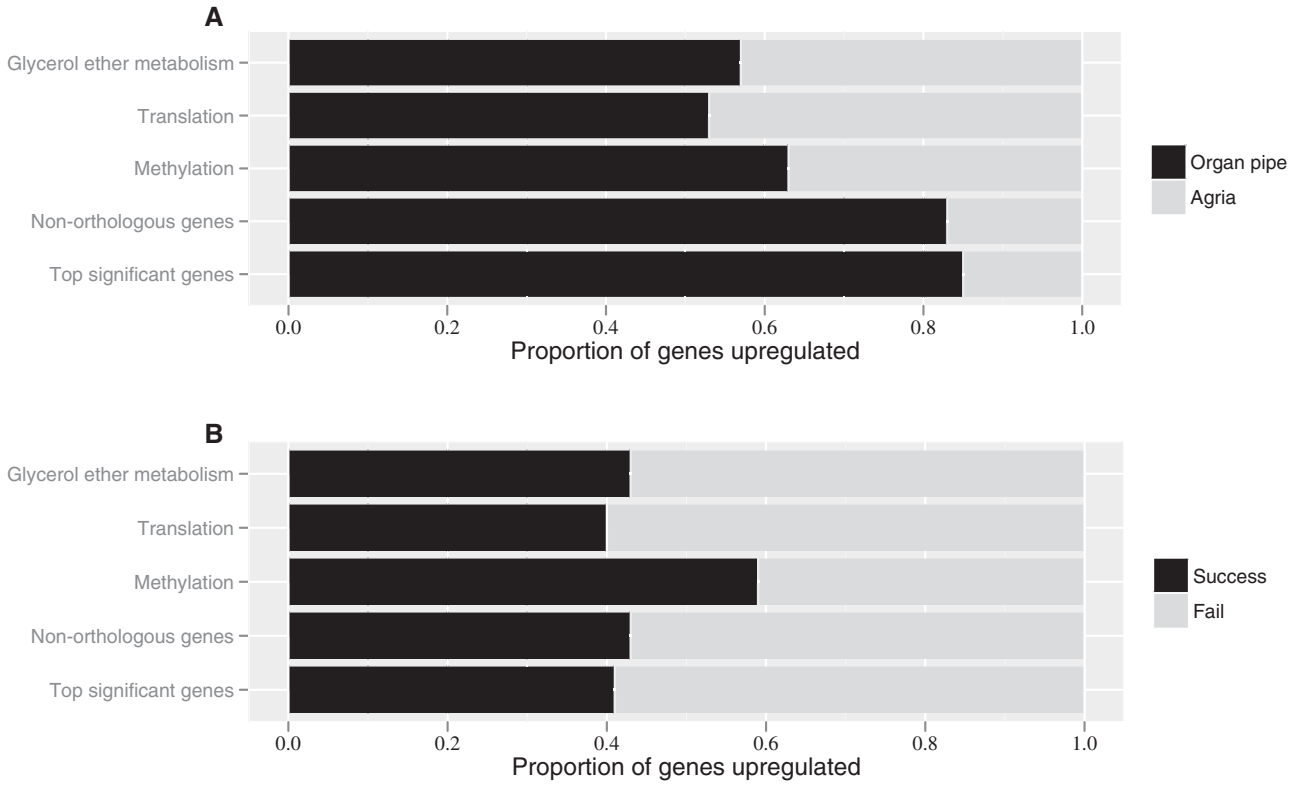

Figure 3. Proportion of genes upregulated on organ pipe cactus (A) and according to mating success (B). Genes are presented by several categories including all significantly differentially expressed genes, nonorthologous genes (i.e., no identified orthologs in *Drosophila melanogaster*), and three different functional categories of genes that were significantly enriched in the rank mean tests.

fatty acids, sterol diols, and triterpene glycosides have all been shown to be largely species-specific for Sonoran Desert cacti, and are causal factors in explaining patterns of host-plant use in cactophilic *Drosophila* (Fogleman and Danielson 2001). *D. mojavensis* is oligophagous due to its ability to metabolize medium chain fatty acids, sterol diols, and high levels of triterpene glycosides found in organ pipe and agria cacti (Fogleman and Danielson 2001). Organ pipe and agria differ in their triterpene glycoside content with agria containing higher levels than organ pipe cactus, however, it is not known if these compounds are differentially metabolized in Baja California versus mainland populations of *D. mojavensis*.

The fermenting cactus environment also caused differential expression of genes that function in protein modification, specifically protein ubiquitination in protein catabolism. Ubiquitination of proteins marks them for degradation by proteasomes and regulates protein levels for a host of critical cellular functions, including gene expression regulation (Pickart 2001; Shilatifard 2006). Ubiquitination also plays a role in stress and immune system responses and the latter term was significantly enriched under the rank mean test (Fig. 2).

Ecological links to speciation would predict a connection between adaptation to cactus and mating success, but relatively few enriched functional groups were found to differ between males who were successful and unsuccessful in mating, either directly or in interaction with cactus, regardless of the number of DE genes. Only one broad term, translation, was strongly enriched for the interaction effect using the rank mean test. Functional clustering of these genes showing interaction effects produced terms for ribosome function, ribonucleoprotein complex, and translation. These genes also included several ncRNAs and splicing factors. Such ncRNAs are involved in the production of mature mRNA, RNA modifications, and translation. They also included snRNAs, which are the backbone of the spliceosome, and snoRNAs, which modify snRNAs, rRNA, and mRNA (Kiss 2002). Further, it has been shown that snRNAs and snoRNAs can function in pre-mRNA processing through involvement in splice site selection (Kishore and Stamm 2006; Matera et al. 2007; Khanna and Stamm 2010). Two splicing factors were significantly DE, orthologous to *U2af38* and *LSm-4* in *D. melanogaster*, which regulate alternative splicing (Park et al. 2004; Tritschler et al. 2007). *U2af38* is a core splicing component that forms part of the spliceosome, attaching to the 3′ splice site during alternative splicing and Sm-like proteins, such as *LSm-4* associate with small RNA components of the spliceosome, influencing the AE of genes (Will and Lührmann 2011).

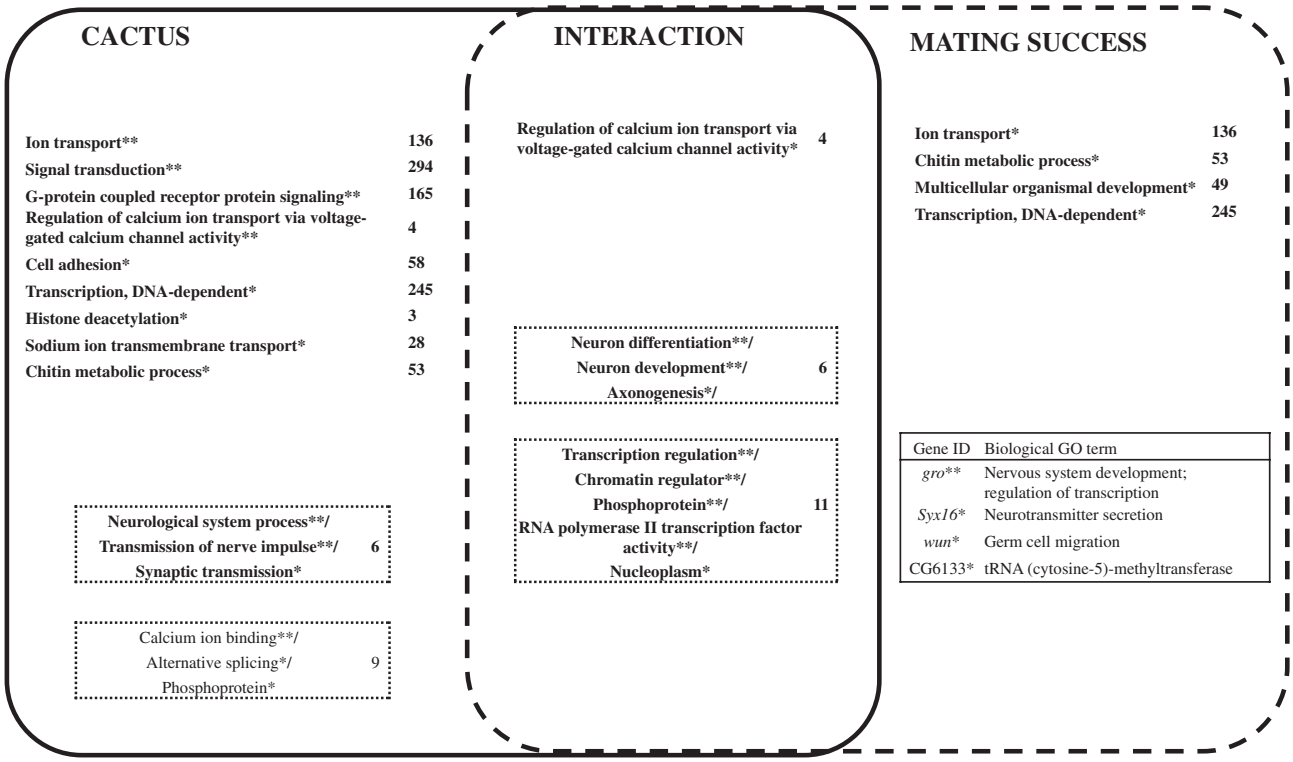

Figure 4. Summary of functional enrichment results for alternatively expressed genes. Figure details are the same as in Figure 2 above.

Alternatively expressed genes had roles in cellular signaling, neurological development, gene expression regulation, and organismal development. Cactus-specific functional enrichment implicated a role for intracellular signaling in response to extracellular cues. G-protein coupled receptor signaling pathways are a large family of cell surface molecules that act as receptors for a range of stimuli, including neurotransmitters, hormones, growth factors, odorant molecules, and light (Marinissen and Gutkind 2001). Other enriched functions among AE cactus-related genes included terms for the transmission of nerve impulses. These included calcium and sodium ion transport through voltage-gated channels, and synaptic transmission itself. Thus, rearing flies on differing hosts until eclosion had lasting effects on the expression of genes involved in extracellular signaling and transmission of nerve impulses and possibly adult behavior.

LINKS TO BEHAVIORAL PHENOTYPES

A potential link between larval cactus effects and adult behavioral phenotypes leads to an a priori expectation of an expressional response from behavioral loci. Several chemosensory behavior genes were DE across host plants, most likely relating to chemical differences between cacti. Such behavior includes sensing of volatile chemicals for host-plant detection (Fogleman and Danielson 2001), and evidence suggests that gustatory receptor genes, such as *Gr94A*, have neuronal links to the reproductive organs in *Drosophila* (Park and Kwon 2011). The AE of nervous system-related genes suggests that alternative exon use might be particularly important for behavioral plasticity. However, 10 significantly DE genes in the interaction effect were also annotated with neurogenesis/nervous system development functions. The expression of chemosensory and impulse transmission genes indicates cactus-specific differences in sensing and parsing of cues from the external chemical environment. This, in turn, has a potential influence on adult male mating success, through cactus-specific expression of nervous system development genes. The peripheral and central nervous systems both play a major role in *Drosophila* mating behavior (Villella and Hall 2008). Mutations in *Drosophila* ion channel genes are known to influence behavior, such as learning and olfaction, as well as courtship song production (Peixoto and Hall 1998; Gleason 2005). Ion channel genes are therefore good candidates for controlling mating behavior (Kyriacou 2002) and demonstrate complex expressional regulation through alternative splicing (Smith et al. 1996). Expression variation in ion channel and nervous system development genes, such as *slo*, which we found to be significantly AE, may have an influence on courtship behavior in flies. Further evidence of a role for *slowpoke* in *D. mojavensis* evolution comes from a QTL study in which *slo* was identified as a potential candidate gene underlying courtship song production (Etges et al. 2007). Thus, the expression of chemosensory and nervous system-related genes

across cacti and mating success treatments provide a potential link between cactus hosts and adult courtship behavior through nervous system development.

QTL studies have shown strong G×E effects between host cactus and traits involved in mating behavior in *D. mojavensis* through differences in epicuticular hydrocarbons (used as contact pheromones) and courtship song production (Etges et al. 2007, 2009, 2010). Surprisingly, few genes directly related to CHC production were seen here, although genes involved in metabolism, seen in both cactus and mating effects, might also play a role. Genes such as the Δ9 desaturases are thought to be important in pheromone production (Keays et al. 2011), were implicated in D. mojavensis QTL studies, and may influence reproductive isolation between *D. melanogaster* populations (Dallerac et al. 2000; Takahashi et al. 2001). However, no desaturase genes were significantly differentially or alternatively expressed in these analyses perhaps suggesting that any G×E effects of these genes do not involve an expression response. This suggests there may be a greater role for plasticity of neurological function, potentially involving courtship song and chemosensory behaviors in *D. mojavensis*.

GENE EXPRESSION CHANGES OVER THE LIFE CYCLE

We examined the effect of larval environmental manipulation on adult gene expression (rather than cross-generational effects) and found significant functional enrichment of several different types of epigenetic modifications. Because flies were raised on cactus hosts only during egg to eclosion, cactus-specific gene expression patterns may have been laid down during this period, and propagated through adulthood. Several processes that can play such a role were identified. Cactus-specific expression included genes functioning in methylation (e.g., ortholog of *pr-set7 D. melanogaster* gene), protein ubiquitination, and histone modification (e.g., orthologs of *Snp* and CG31703). The significant methylation GO term broadly includes any attachment of a methyl group to protein, DNA, or RNA. Interestingly, a higher proportion of methylation-related genes were upregulated in successfully mated males and most other DE genes were downregulated (Fig. 3). Increased methylation is thought to repress gene expression (Wolffe and Matzke 1999), meaning that mating success might be particularly influenced by methylation-based control of gene regulation.

Epigenetic modifications of RNA and proteins rather than DNA might be particularly important in *Drosophila* as this group does not have the full complement of DNA methyltransferases commonly found in other organisms, having only retained one methyltransferase, orthologous to the human *Dnmt2* gene (Lyko and Maleszka 2011). *Dnmt2* is only thought to function in the methylation of tRNA (although this has been disputed; Goll et al. 2006; Krauss and Reuter 2011), meaning that the methylation GO term seen in this study will mainly involve RNA and proteins. tRNA processing was a significantly enriched term in the rank mean test of DE genes, and a tRNA methyltransferase, *Nsun2*, was significantly AE. *Nsun2* functions in spermatogenesis in *Drosophila* (Gerbasi et al. 2011) and splicing mutations within it can cause short-term memory loss, demonstrating the importance of correct splicing for function of this gene (Abbasi-Moheb et al. 2012). Alternative splicing has also been suggested as an important mechanism underlying phenotypic plasticity (Marden 2008) and histone modifications and chromatin remodeling are known to play a role in alternative splicing (Luco et al. 2010, 2011). Chromatin remodeling genes are important for temperature-related plasticity in *D. melanogaster* (Levine et al. 2011) and chromatin assembly genes were DE in cactus-specific larval plasticity in *D. mojavensis* (Matzkin et al. 2006).

Evidence for a suite of epigenetic processes associated with host-plant plasticity suggests that *Drosophila* employ gene regulatory mechanisms other than DNA methylation, and that these mechanisms might play a role in ecological adaptation. There has been some controversy surrounding the role of epigenetic mechanisms in *Drosophila* species, specifically whether DNA methylation routinely occurs in the *Drosophila* genome. Recent studies suggest that methylation does occur (Krauss and Reuter 2011), yet experimental evidence indicates this is at low levels genome-wide, and the functional significance remains unclear (Lyko et al. 2000). Our results suggest a potential role for RNA and protein methylation that links larval cactus plasticity with adult phenotypes. Recent evidence suggests an important role of chromatin modification in gene expression plasticity in *Drosophila* (Levine et al. 2011). Therefore, species lacking the core *Dnmt* genes, the "*Dnmt2* only" species (Krauss and Reuter 2011), might regulate their genome through mechanisms other than, or in addition to, DNA methylation. This might include RNA and histone protein modifications, and involve snRNAs and snoRNAs.

Potential links between ecological adaptation and reproductive success have rarely been addressed at a transcriptomic level. Agria cactus causes decreased mate discrimination and higher mating success, particularly for Baja California males, in multiple choice studies (Etges 1992). QTLs for mating success and the phenotypes involved often show G×Es (Etges et al. 2007, 2009). Few DE or AE genes were seen to be present in both the cactus and mating success main effects, indicating little evidence for a shared genetic basis or pleiotropy. However, models of ecological speciation include other modes of linking ecological adaptation and reproductive isolation, such as physical linkage (Rundle and Schluter 2004). Here, we found that larval host cactus influenced adult male mating success by modulating the expression of genes involved in translation, transcription, and nervous system development. Gustatory receptor genes, such as *Gr94A*, and nervous system genes, such as *slo*, have been linked to reproduction and

courtship behavior in *Drosophila* (Peixoto and Hall 1998; Park and Kwon 2011). The expression of such genes here suggests that the genetic basis of mating success in *D. mojavensis* is likely to involve nervous system development genes that link the cactus environment to reproductive behavior. Mainland populations diverged from an ancestral Baja California population around 230–270,000 years ago (Smith et al. 2012). This suggests that the adaptation of *D. mojavensis* to organ pipe cactus and the concurrent evolution of reproductive isolation has been fairly rapid, and that plasticity in gene expression may have played an important role in this. Examining the molecular architecture that underlies plasticity of gene expression is therefore an important step toward understanding the role of gene expression in ecological speciation (Pavey et al. 2010).

ACKNOWLEDGMENTS

We wish to thank T. Markow from whom flies were originally obtained and everyone at the Centre for Genomic Research at the University of Liverpool. WJE was supported by National Science Foundation grant EF-0723930 and MGR and GS by Natural Environment Research Council (NERC), U.K.

LITERATURE CITED

Abbasi-Moheb, L., S. Mertel, M. Gonsior, L. Nouri-Vahid, K. Kahrizi, S. Cirak, D. Wieczorek, M. M. Motazacker, S. Esmaeeli-Nieh, K. Cremer, et al. 2012. Mutations in NSUN2 cause autosomal recessive intellectual disability. Am. J. Hum. Genet. 90:847–855.

Alcorn, S., T. Orum, A. G. Steigerwalt, J. L. M. Foster, J. C. Fogleman, and D. O. N. J. Brenner. 1991. Taxonomy and pathogenicity of *Erwinia cacticida* sp. nov. Int. J. Syst. Bacteriol. 41:197–212.

Altschul, S. F., T. L. Madden, A. A. Schäffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25:3389–3402.

Anders, S. 2010. Htseq: analysing high-throughput sequencing data with Python. Available at http://www-huber.embl.de/users/anders/HTSeq/doc/overview.html.

Binns, D., E. Dimmer, R. Huntley, D. Barrell, C. O'Donovan, and R. Apweiler. 2009. QuickGO: a web-based tool for Gene Ontology searching. Bioinformatics 25:3045–3046.

Butlin, R., A. Debelle, C. Kerth, R. R. Snook, L. W. Beukeboom, R. F. C. Cajas, W. Diao, M. E. Maan, S. Paolucci, F. J. Weissing, et al. 2012. What do we need to know about speciation? Trends Ecol. Evol. 27:27–39.

Chittka, A., Y. Wurm, and L. Chittka. 2012. Epigenetics: the making of ant castes. Curr. Biol. 22:R835–838.

Clark, A. G., M. B. Eisen, D. R. Smith, C. M. Bergman, B. Oliver, T. A. Markow, T. C. Kaufman, M. Kellis, W. Gelbart, V. N. Iyer, et al. 2007. Evolution of genes and genomes on the *Drosophila* phylogeny. Nature 450:203–218.

Cowmeadow, R. B., H. R. Krishnan, and N. S. Atkinson. 2005. The *slowpoke* gene is necessary for rapid ethanol tolerance in *Drosophila*. Alcohol Clin. Exp. Res. 29:1777–1786.

Crispo, E. 2008. Modifying effects of phenotypic plasticity on interactions among natural selection, adaptation and gene flow. J. Evol. Biol. 21:1460–1469.

Dallerac, R., C. Labeur, J. M. Jallon, D. C. Knippie, W. L. Roelofs, and C. Wicker-Thomas. 2000. A delta-9 desaturase gene with a different substrate specificity is responsible for the cuticular diene hydrocarbon polymorphism in *Drosophila melanogaster*. Proc. Natl. Acad. Sci. USA 97:9449–9454.

Dennis, J. G., B. T. Sherman, D. A. Hosack, J. Yang, W. Gao, H. C. Lane, and R. A. Lempicki. 2003. DAVID: database for annotation, visualization, and integrated discovery. Genome Bio. 4:R60–R60.11.

Etges, W. J. 1992. Premating isolation is determined by larval substrates in cactophilic *Drosophila mojavensis*. Evolution 46:1945–1950.

———. 1998. Premating isolation is determined by larval rearing substrates in cactophilic *Drosophila mojavensis.* IV. Correlated responses in behavioral isolation to artificial selection on a life-history trait. Am. Nat. 152:129–144.

Etges, W. J. and M. A. Ahrens. 2001. Premating isolation is determined by larval-rearing substrates in cactophilic *Drosophila mojavensis.* V. Deep geographic variation in epicuticular hydrocarbons among isolated populations. Am. Nat. 158:585–598.

Etges, W. J. and C. S. Klassen. 1989. Influences of atmospheric ethanol on adult *Drosophila mojavensis*: altered metabolic rates and increases in fitness among populations. Physiol. Zool. 62:170–193.

Etges, W. J., W. R. Johnson, G. A. Duncan, G. Huckins, and W. B. Heed. 1999. Ecological genetics of cactophilic *Drosophila*. Pp. 164–214 *in* R. Robichaux, ed. Ecology of Sonoran Desert plants and plant communities. University of Arizona Press, Tucson, AZ.

Etges, W. J., C. C. de Oliveira, E. Gragg, D. Ortiz-Barrientos, M. A. F. Noor, and M. G. Ritchie. 2007. Genetics of incipient speciation in *Drosophila mojavensis*. I. Male courtship song, mating success, and genotype x environment interactions. Evolution 61:1106–1119.

Etges, W. J., C. C. de Oliveira, M. G. Ritchie, and M. A. F. Noor. 2009. Genetics of incipient speciation in *Drosophila mojavensis*. II. Host plants and mating status influence cuticular hydrocarbon QTL expression and G×E interactions. Evolution 63:1712–1730.

Etges, W. J., C. C. de Oliveira, M. A. F. Noor, and M. G. Ritchie. 2010. Genetics of incipient speciation in *Drosophila mojavensis*. III. Life history divergence in allopatry and reproductive isolation. Evolution 64:3549–3569.

Fogleman, J. C. and P. B. Danielson. 2001. Chemical interactions in the cactus-microorganism-*Drosophila* model system of the Sonoran Desert. Am. Zool. 41:877–889.

Funk, D. J., P. Nosil, and W. J. Etges. 2006. Ecological divergence exhibits consistently positive associations with reproductive isolation across disparate taxa. Proc. Natl. Acad. Sci. USA 103:3209–3213.

Gerbasi, V. R., J. B. Preall, D. E. Golden, D. W. Powell, T. D. Cummins, and E. J. Sontheimer. 2011. Blanks, a nuclear siRNA/dsRNA-binding complex component, is required for *Drosophila* spermiogenesis. Proc. Natl. Acad. Sci. USA 108:3204–3209.

Ghalambor, C., J. McKay, S. Carroll, and D. Reznick. 2007. Adaptive versus non-adaptive phenotypic plasticity and the potential for contemporary adaptation in new environments. Funct. Ecol. 21:394–407.

Gleason, J. M. 2005. Mutations and natural genetic variation in the courthship song of *Drosophila*. Behav. Genet. 35:265–277.

Goll, M. G., F. Kirpekar, K. A. Maggert, J. A. Yoder, C. L. Hsieh, X. Zhang, K. G. Golic, S. E. Jacobsen, and T. H. Bestor. 2006. Methylation of tRNAAsp by the DNA methyltransferase homolog *Dnmt2*. Science 311:395–398.

Griffith, M., O. L. Griffith, J. Mwenifumbo, R. Goya, A. S. Morrissy, R. D. Morin, R. Corbett, M. J. Tang, Y. C. Hou, T. J. Pugh, et al. 2010. Alternative expression analysis by RNA sequencing. Nat. Methods 7:843-U108.

Hartigan, J. A. and M. A. Wong. 1979. Algorithm AS 136: a k-means clustering algorithm. J. Roy. Stat. Soc. Ser. C. (Appl. Stat.) 28:100–108.

Havens, J. A., S. H. Orzack, and W. J. Etges. 2011. Mate choice opportunity leads to shorter offspring development time in a desert insect. J. Evol. Biol. 24:1317–1324.

Heed, W. B. 1982. The origin of *Drosophila* in the Sonoran Desert. Pp. 65–80 *in* J.S.F. Bark, and W.T. Starmer, eds. Ecological genetics and evolution: the cactus-yeast-*Drosophila* model system. Academic Press, Sydney.

Huang, D. W., B. T. Sherman, and R. A. Lempicki. 2009. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat. Protoc. 4:44–57.

Keays, M. C., D. Barker, C. Wicker-Thomas, and M. G. Ritchie. 2011. Signatures of selection and sex-specific expression variation of a novel duplicate during the evolution of the *Drosophila* desaturase gene family. Mol. Ecol. 20:3617–3630.

Khanna, A. and S. Stamm. 2010. Regulation of alternative splicing by short non-coding nuclear RNAs. RNA Bio. 7:480–485.

Kishore, S. and S. Stamm. 2006. The snoRNA HBII-52 regulates alternative splicing of the serotonin receptor 2C. Science 311:230–232.

Kiss, T. 2002. Small nucleolar RNAs: an abundant group of noncoding RNAs with diverse cellular functions. Cell 109:145–148.

Krauss, V. and G. Reuter. 2011. DNA methylation in *Drosophila*—a critical evaluation. Prog. Mol. Biol. Transl. Sci. 101:177–191.

Kyriacou, C. P. 2002. Single gene mutations in *Drosophila*: what can they tell us about the evolution of sexual behaviour? Genetica 116:197–203.

Levine, M. T., M. L. Eckert, and D. J. Begun. 2011. Whole-genome expression plasticity across tropical and temperate *Drosophila melanogaster* populations from Eastern Australia. Mol. Biol. Evol. 28:249–256.

Luco, R. F., Q. Pan, K. Tominaga, B. J. Blencowe, O. M. Pereira-Smith, and T. Misteli. 2010. Regulation of alternative splicing by histone modifications. Science 327:996–1000.

Luco, R. F., M. Allo, I. E. Schor, A. R. Kornblihtt, and T. Misteli. 2011. Epigenetics in alternative pre-mRNA splicing. Cell 144:16–26.

Lyko, F. and R. Maleszka. 2011. Insects as innovative models for functional studies of DNA methylation. Trends Genet. 27:127–131.

Lyko, F., B. H. Ramsahoye, and R. Jaenisch. 2000. DNA methylation in *Drosophila melanogaster*. Nature 408:538–540.

Maan, M. E. and O. Seehausen. 2011. Ecology, sexual selection and speciation. Ecol. Lett. 14:591–602.

Machado, C. A., L. M. Matzkin, L. K. Reed, and T. A. Markow. 2007. Multilocus nuclear sequences reveal intra and interspecific relationships among chromosomally polymorphic species of cactophilic *Drosophila*. Mol. Ecol. 16:3009–3024.

Marden, J. H. 2008. Quantitative and evolutionary biology of alternative splicing: how changing the mix of alternative transcripts affects phenotypic plasticity and reaction norms. Heredity 100:111–120.

Marinissen, M. J., and J. S. Gutkind. 2001. G-protein-coupled receptors and signaling networks: emerging paradigms. Trends Pharmacol. Sci. 22:368–376.

Markow, T. A. 1991. Sexual isolation among populations of *Drosophila mojavensis*. Evolution 45:1525–1529.

Markow, T. A., J. C. Fogleman, and W. B. Heed. 1983. Reproductive isolation in Sonoran Desert *Drosophila*. Evolution 37:649–652.

Matera, A. G., R. M. Terns, and M. P. Terns. 2007. Non-coding RNAs: lessons from the small nuclear and small nucleolar RNAs. Nat. Rev. Mol. Cell Biol. 8:209–220.

Matzkin, L. M. 2004. Population genetics and geographic variation of alcohol dehydrogenase (*Adh*) paralogs and glucose-6-phosphate dehydrogenase (*G6pd*) in *Drosophila mojavensis*. Mol. Biol. Evol. 21:276–285.

———. 2012. Population transcriptomics of cactus host shifts in *Drosophila mojavensis*. Mol. Ecol. 12:2428–2439.

Matzkin, L. M., and W. F. Eanes. 2003. Sequence variation of alcohol dehydrogenase (Adh) paralogs in cactophilic *Drosophila*. Genetics 163:181–194.

Matzkin, L.M., and T.A. Markow. 2009. Transcriptional regulation of metabolism associated with the increased desiccation resistance of the cactophilic *Drosophila mojavensis*. Genetics 182:1279–1288.

Matzkin, L. M., T. D. Watts, B. G. Bitler, C. A. Machado, and T. A. Markow. 2006. Functional genomics of cactus host shifts in *Drosophila mojavensis*. Mol. Ecol. 15:4635–4643.

McQuilton, P., S. E. St Pierre, J. Thurmond, and F. Consortium. 2012. FlyBase 101—the basics of navigating FlyBase. Nucleic Acids Res. 40:D706–D714.

Nosil, P. 2012. Ecological speciation. Oxford Univ. Press, Oxford, UK.

Park, J. H. and J. Y. Kwon. 2011. A systematic analysis of *Drosophila* gustatory receptor gene expression in abdominal neurons which project to the central nervous system. Mol. Cells 32:375–381.

Park, J. W., K. Parisky, A. M. Celotto, R. A. Reenan, and B. R. Graveley. 2004. Identification of alternative splicing regulators by RNA interference in *Drosophila*. Proc. Natl. Acad. Sci. 101:15974–15979.

Pavey, S. A., H. Collin, P. Nosil, and S. M. Rogers. 2010. The role of gene expression in ecological speciation. Ann. NY Acad. Sci. 1206:110–129.

Peixoto, A. A. and J. C. Hall. 1998. Analysis of temperature-sensitive mutants reveals new genes involved in the courtship song of *Drosophila*. Genetics 148:827–838.

Pfennig, D. W., M. A. Wund, E. C. Snell-Rood, T. Cruickshank, C. D. Schlichting, and A. P. Moczek. 2010. Phenotypic plasticity's impacts on diversification and speciation. Trends Ecol. Evol. 25:459–467.

Pickart, C. M. 2001. Mechanisms underlying ubiquitination. Annu. Rev. Biochem. 70:503–533.

Rajpurohit, S., C. C. de Oliveira, W. J. Etges, and A. G. Gibbs. 2012. Functional genomic and phenotypic responses to desiccation in natural populations of a desert drosophilid. Mol. Ecol.: *In press*.

Reed, L. K., M. Nyboer, and T. A. Markow. 2007. Evolutionary relationships of *Drosophila mojavensis* geographic host races and their sister species *Drosophila arizonae*. Mol. Ecol. 16:1007–1022.

Ritchie, M. G. 2007. Sexual selection and speciation. Annu. Rev. Ecol. Evol. Syst. 38:79–102.

Robinson, M. D., D. J. McCarthy, and G. K. Smyth. 2010. EdgeR: a bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics 26:139–140.

Ross, C. and T. Markow. 2006. Microsatellite variation among diverging populations of *Drosophila mojavensis*. J. Evol. Biol. 19: 1691–1700.

Ruan, H., X. D. Tang, M. L. Chen, M. A. Joiner, G. Sun, N. Brot, H. Weissbach, S. H. Heinemann, L. Iverson, C. F. Wu, et al. 2002. High-quality life extension by the enzyme peptide methionine sulfoxide reductase. Proc. Natl. Acad. Sci. USA 99:2748–2753.

Ruiz, A., W. Heed, and M. Wasserman. 1990. Evolution of the *mojavensis* cluster of cactophilic *Drosophila* with descriptions of two new species. J. Hered. 81:30–42.

Rundle, H. D. and P. Nosil. 2005. Ecological speciation. Ecol. Lett. 8:336–352.

Rundle, H. D. and D. Schluter. 2004. Natural selection and ecological speciation in sticklebacks. Pp. 192–209 *in* U. Dieckmann, M. Doebeli, J. Metz, and D. Tautz, eds. Adaptive speciation. International Institute for Applied Systems Analysis, Cambridge Univ. Press, Cambridge, UK.

Schluter, D. 2001. Ecology and the origin of species. Trends Ecol. Evol. 16:372–380.

Scoville, A. G. and M. E. Pfrender. 2010. Phenotypic plasticity facilitates recurrent rapid adaptation to introduced predators. Proc. Natl. Acad. Sci. USA 107:4260–4263.

Servedio, M. R., G. S. Van Doorn, M. Kopp, A. M. Frame, and P. Nosil. 2011. Magic traits in speciation: "magic" but not rare? Trends Ecol. Evol. 26:389–397.

Shilatifard, A. 2006. Chromatin modifications by methylation and ubiquitination: implications in the regulation of gene expression. Annu. Rev. Biochem. 75:243–269.

Smadja, C. M. and R. K. Butlin. 2011. A framework for comparing processes of speciation in the presence of gene flow. Mol. Ecol. 20:5123–5140.

Smith, G., K. Lohse, W. J. Etges, and M. G. Ritchie. 2012. Model-based comparisons of phylogeographic scenarios resolve the intraspecific divergence of cactophilic *Drosophila mojavensis*. Mol. Ecol. 21:3293–3307.

Smith, L. A., X. Wang, A. A. Peixoto, E. K. Neumann, L. M. Hall, and J. C. Hall. 1996. A *Drosophila* calcium channel alpha1 subunit gene maps to a genetic locus associated with behavioral and visual defects. J. Neurosci. 16:7868–7879.

Sobel, J. M., G. F. Chen, L. R. Watt, and D. W. Schemske. 2010. The biology of speciation. Evolution 64:295–315.

Starmer, W. T. 1982. Analysis of the community structure of yeasts associated with the decaying stems of cactus. I. *Stenocereus gummosus*. Microb. Ecol. 8:71–81.

Starmer, W. T., W. B. Heed, and E. Rockwood-Sluss. 1977. Extension of longevity in *Drosophila mojavensis* by environmental ethanol: differences between subraces. Proc. Natl. Acad. Sci. USA 74:387–391.

Storey, J. D. and R. Tibshirani. 2003. Statistical significance for genomewide studies. Proc. Natl. Acad. Sci. USA 100:9440–9445.

Takahashi, A., S. C. Tsaur, J. A. Coyne, and C.-I. Wu. 2001. The nucleotide changes governing hydrocarbon variation and their evolution in *Drosophila melanogaster*. Proc. Natl. Acad. Sci. USA 98:3920–3925.

Terns M. P., R. M. Terns. 2002. Small nucleolar RNAs: Versatile trans-acting molecules of ancient evolutionary origin. Gene Expr. 10:17–39.

Thibert-Plante, X. and A. Hendry. 2011. The consequences of phenotypic plasticity for ecological speciation. J. Evol. Biol. 24:326–342.

Tritschler, F., A. Eulalio, V. Truffault, M. D. Hartmann, S. Helms, S. Schmidt, M. Coles, E. Izaurralde, and O. Weichenrieder. 2007. A divergent Sm fold in EDC3 proteins mediates DCP1 binding and P-body targeting. Mol. Cell. Biol. 27:8600–8611.

Tweedie, S., M. Ashburner, K. Falls, P. Leyland, P. McQuilton, S. Marygold, G. Millburn, D. Osumi-Sutherland, A. Schroeder, R. Seal, et al. 2009. FlyBase: enhancing *Drosophila* Gene Ontology annotations. Nucleic Acids Res. 37:D555–D559.

Van Doorn, G. S., P. Edelaar, and F. J. Weissing. 2009. On the origin of species by natural and sexual selection. Science 326:1704–1707.

Villella, A. and J. C. Hall. 2008. Neurogenetics of courtship and mating in *Drosophila*. Adv. Genet. 62:67–184.

Wasserman, M. 1992. Cytological evolution of the *Drosophila repleta* species group. Pp. 455–541 *in* J. R. Powell, and C. B. Krimbas, eds. Inversion polymorphism in Drosophila. CRC Press, Inc., Bota Racon, FL.

West-Eberhard, M. J. 2005a. Developmental plasticity and the origin of species differences. Proc. Natl. Acad. Sci. USA 102:6543–6549.

———. 2005b. Phenotypic accommodation: adaptive innovation due to developmental plasticity. J. Exp. Zool. B Mol. Dev. Evol. 304:610–618.

Will, C. L. and R. Lührmann. 2011. Spliceosome structure and function. Cold Spring Harbor Perspect. Biol. 3:1–23.

Wolffe, A. P. and M. A. Matzke. 1999. Epigenetics: regulation through repression. Science 286:481–486.

Associate Editor: K. Dyer

Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Table S1. All significantly DE genes (FDR < 10%) separated by treatment contrast on different Excel sheets.

Table S2. All significantly AE genes (FDR < 10%) separated by treatment contrast on different Excel sheets.

Table S3. Full results of rank mean test for DE genes. Test results are presented by treatment contrast and each line shows a significant biological-process GO term, the number of genes associated with that term and the significance level.

Table S4. Full DAVID functional enrichment results for DE genes. Results are presented for cactus and interaction terms only (mating success genes did not show significant clustering). Cluster enrichment scores are shown with significance considered at >1.3, however some individual terms might show significance, as indicated. The numbers of genes are shown for each significant GO term.

Table S5. Full results of rank mean test for AE genes. Test results are presented by treatment contrast and each line shows a significant biological-process GO term, the number of genes associated with that term and the significance level.

Table S6. Full DAVID functional enrichment results for AE genes. Results are presented for cactus and interaction terms only (mating success genes did not show significant clustering). Cluster enrichment scores are shown with significance considered at >1.3, however some individual terms might show significance, as indicated. The numbers of genes are shown for each significant GO term.

Table S7. Details of clustered DE genes used in the heatmap (Fig. 1, see text for details). Biological-process GO terms for all clustered genes are included along with cluster membership and log fold change values.