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Deciphering life history transcriptomes in different environments

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Abstract

We compared whole transcriptome variation in six pre-adult stages and seven adult female ages in two populations of cactophilic *Drosophila mojavensis* reared on two host plants to understand how differences in gene expression influence standing life history variation. We used singular value decomposition (SVD) to identify dominant trajectories of life cycle gene expression variation, performed pairwise comparisons of stage and age differences in gene expression across the life cycle, identified when genes exhibited maximum levels of life cycle gene expression, and assessed population and host cactus effects on gene expression. Life cycle SVD analysis returned four significant components of transcriptional variation, revealing functional enrichment of genes responsible for growth, metabolic function, sensory perception, neural function, translation and ageing. Host cactus effects on female gene expression revealed population- and stage-specific differences, including significant host plant effects on larval metabolism and development, as well as adult neurotransmitter binding and courtship behaviour gene expression levels. In 3- to 6-day-old virgin females, significant upregulation of genes associated with meiosis and oogenesis was accompanied by downregulation of genes associated with somatic maintenance, evidence for a life history tradeoff. The transcriptome of *D. mojavensis* reared in natural environments throughout its life cycle revealed core developmental transitions and genome-wide influences on life history variation in natural populations.

Keywords: ageing, development, *Drosophila*, gene expression, life history, microarray

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Introduction

Understanding life history evolution requires knowledge of the forces shaping correlated suites of fitness characters in response to patterns of age-specific mortality (Williams 1957; Hamilton 1966; Stearns 1977; Reznick 1982; Roff 2002; Reznick *et al.* 2004). Therefore, it is necessary to integrate how life history traits are expressed across environments (Gupta & Lewontin 1982; Caswell 1983; Etges 1993; Scheiner 1993) and standing levels of genetic variation in fitness components (Istock *et al.* 1976; Gustafsson 1986; Price & Schluter 1991; Walsh & Blows 2009) with patterns of demographic and environmental variability (Orzack & Tuljapurkar 1989; Tuljapurkar 1989; Caswell 2009; Steiner & Tuljapurkar 2012). To predict life history patterns, we must also examine the genetic architecture of life history variation and the unfolding of organismal developmental programs over the life cycle (Levitis 2011). In particular, we need to understand the number and kind of genes responsible for life history differences, how coordinated groups of genes are expressed at different life cycle stages and how environmental effects on genes influence internal and external buffering and

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genotype by environment (GxE) interactions (Arbeitman *et al.* 2002; Stolc *et al.* 2004; Koutsos *et al.* 2007; Fiedler *et al.* 2010).

Currently, large gaps remain in our understanding of how genomic expression throughout the life cycle is influenced by relevant ecological variables. In organisms where expression of genetic differences in life histories depends upon local ecological variation, examination of the sensitivity of gene expression, as well as gene expression–environment interactions, is necessary to evaluate the adaptive significance of life history variation in response to ecological variability. Environmental variation can maintain genetic polymorphism in populations, directly influence gene expression leading to GxE interactions (Gillespie & Turelli 1989), and be limited in its selective effects if alleles are neutral in some environments but not others (Anderson *et al.* 2013). Limits to plasticity of genome expression (Zhou *et al.* 2012) are of direct concern to organismal persistence in changing environments unless standing levels of genetic variability are high enough to allow short-term microevolutionary change. Although levels of genetic variation in components of fitness, as well as fitness itself, are sometimes low (Gustafsson 1986), it is essential to understand the nature of genome expression throughout the life history (Gibson 2008; Hodgins-Davis & Townsend 2009).

Here, we examine transcriptional profiles throughout the life cycle in *Drosophila mojavensis*, a cactophilic species endemic to the deserts of northwestern Mexico and southwestern USA, using whole transcriptome microarrays to document patterns and sensitivity of gene expression in populations characterized by genetically differentiated life history differences. We assessed transcriptional variation from embryogenesis to four-week-old female adults to characterize the range of variation in gene expression and gene function in inter-related groups of genes. We focused on pre-adult stages and revealed expression shifts related to development, while analyses across female adult life stages revealed expression changes underlying maturation, senescence and trade-offs between reproduction and somatic maintenance in different environments.

Ecology and evolution of D. mojavensis

Throughout the arid lands of the New World, over half of the ca 100 species in the large *D. repleta* group use fermenting cactus tissues to carry out their life cycles (Heed 1982; Wasserman 1992; Filchak *et al.* 2005; Oliveira *et al.* 2012). Within the *mulleri* species complex, *D. mojavensis* and its two closest relatives, *D. arizonae* and *D. navojoa*, form a monophyletic group endemic to Mexico and the southwestern United States (Ruiz *et al.* 1990). *Drosophila mojavensis* became isolated in present-day peninsular Baja California from its closest relative, *D. arizonae*, on the mainland due to tectonic drift and changing sea levels (Gastil *et al.* 1975). Natural populations of *D. mojavensis* from the Sonoran and Mojave Deserts and adjacent arid lands use different host cacti across their range, including pitaya agria cactus, *Stenocereus gummosus*, on the peninsula and organ pipe, *S. thurberi*, and sina cactus, *S. alamosensis* in mainland Mexico and Arizona (Heed & Mangan 1986; Etges *et al.* 1999). In the Mojave Desert in southern California and central Arizona, barrel cactus, *Ferocactus cylindraceus*, is a major host and populations of *D. mojavensis* on Santa Catalina Island near Los Angeles, California use *Opuntia* cactus. Southern California populations likely split from mainland Sonora and southern Arizona populations ~117–135 kya with little recurring gene flow (Smith *et al.* 2012).

Natural populations of *D. mojavensis* show considerable genetic variation in life histories, including host plant-influenced differences in adult mortality rates (Jaureguy & Etges 2007). Baja California populations express shorter egg-to-adult development times, higher viabilities and smaller thorax sizes than mainland populations when reared on fermenting agria vs. organ pipe cactus in common garden experiments suggesting adaptation to these hosts in nature (Etges & Heed 1987; Etges 1990; Etges *et al.* 2010). Mainland Sonoran Desert *D. mojavensis* are characterized by larger body sizes (Etges 1992; Etges & Ahrens 2001), higher metabolic rates, more ovarioles (W. Heed, unpublished) and higher lifetime fecundities, but earlier ages at first reproduction than Baja populations (Etges & Klassen 1989). Genetic variation in development time and thorax size in both Baja and mainland populations, as well as significant GxE interactions when reared on different host plants, and positive across-host genetic correlations suggested ongoing life history evolution and evidence for ecological generalism (Etges 1993). Baja California and mainland populations also harbour significant genetic variation for adult longevity and average numbers of eggs laid per day, as well as a genetic trade-off between early and late-life fecundity (Etges & Heed 1992). Together, these data suggest that as *D. mojavensis* colonized mainland Mexico and Arizona by switching host cacti, new life histories evolved in these derived populations (Etges 1993), with correlated shifts in reproductive isolation (Etges 1998; Etges *et al.* 2010).

We measured whole-genome transcriptional responses of *D. mojavensis* from two populations exposed to fermenting tissues of two host cacti, that is agria, *S. gummosus*, and organ pipe cactus, *S. thurberi*, in pre-adult stages and adults of increasing age to reveal whole transcriptome responses to different host plants

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over the life cycle. We approached the analysis of our data with two distinct goals in mind. First, we assessed effects of stage/age independent of population and diet by generating a pooled data set composed of mean expression levels for all genes (averaged across populations, diets and biological replicates) at each stage/age. We used this averaged data set to investigate highly conserved trajectories of gene expression across the *D. mojavensis* life cycle, independent of diet or population effects. To identify clusters of genes with similar age trajectories of expression, we performed a singular value decomposition (SVD) of total genome expression (Alter *et al*. 2000; Alter 2006) on this pooled data set. The SVD cluster analyses revealed continuous changes difficult to observe with simple pairwise comparisons between stages and ages. We then considered as correlated gene clusters those sets of genes whose expression closely correlated with the dominant trajectories revealed in the SVD analysis. We also performed pairwise comparisons, for example, comparing expression at adjacent stages/ages, with the primary aim of mapping gene expression levels into functional domains as in previous studies (Pletcher *et al.* 2002; McCarroll *et al.* 2004; Kim *et al*. 2005; Koutsos *et al.* 2007; Remolina *et al.* 2012).

Second, we searched for evidence of divergence in gene expression patterns at each stage and age in our four population X cactus treatment groups. By teasing out expression differences into shifts due to population, host plant, and their interactions, we revealed gene expression/regulatory changes potentially responsible for their recent divergence in life histories.

Materials and methods

Origin of stocks

Populations of *Drosophila mojavensis* were collected in nature by baiting over fermented bananas or by collecting adults emerged from cactus rots returned to the laboratory. A total of 465 adults were baited in Punta Prieta, Baja California in January 2008 and 1264 baited adults plus nine adults that emerged from sina, *S. alamosensis*, rots were collected from Las Bocas, Sonora in March 2009. All flies were returned to the laboratory, and each population was cultured on banana food (Brazner & Etges 1993) in 8-dr shell vials at room temperature until the experiments began in September 2009.

Preadult stage culture conditions

Thousands of adult flies from each population were introduced into separate population cages (12 720 cm^3) for 7–10 days and allowed to choose mates. Population cages were maintained in an incubator programmed for a 14:10 LD photoperiod and 27:17 °C. Flies were allowed to oviposit in cups containing fermenting agria or organ pipe cactus (see below). We used both cacti for egg oviposition because we were also interested in the effects of alternate cactus substrates on gene expression at all stages, including fertilized eggs. Thousands of eggs (~200 μg) were collected for 6 h and briefly rinsed in deionized water to remove cactus media, snap-frozen in liquid nitrogen and stored at −80 °C prior to RNA extraction. For larval and pupal stages, approximately 200 eggs were transferred to cups containing fermenting cactus media (see below) and allowed to develop to the stage of interest. Development times for the pre-adult stages were estimated from analysis of the duration of stage-specific differences in larval mouth hook morphology and pupal periods (D. White and W. J. Etges, unpublished results). A total of six pre-adult stages were used: fertilized embryos (6 h), first instar larvae (48 h), second instar larvae (144 h), third instar larvae (240 h), early pupae (288 h) and late pupae (384 h). Egg hatch is ca 24–25 h under these conditions. In addition to age in hours, we verified each larval and pupal stage morphologically and discarded individuals that were early or advanced for each developmental stage. Each sample of larvae consisted of thousands of individuals for the first and second instars and hundreds of individuals for the third instar. For early and late pupae, 30 individuals were used in each sample.

Cactus media for rearing pre-adult stages were prepared with 400 g of cactus (either agria or organ pipe), 600 mL of deionized water and 4 g of agar. First, fresh cactus tissue was blended using 2/3 of the water, boiled and then strained twice to remove large cactus fibres. These media were strained a third time using a fine mesh to remove excess fibres and the resulting liquid paste-like solution was added to the agar dissolved in boiling water. These media weres then boiled for 10 min, autoclaved for 8 min and poured into food cups. After the medium cooled, it was inoculated with a pectolytic bacterium, *Erwinia cacticida* (Alcorn *et al.* 1991), and a mixture of seven cactophilic yeasts: *Dipodascus starmeri*, *Candida sonorensis*, *C. valida*, *Starmera amethionina*, *Pichia cactophila*, *P. mexicana* and *Sporopachydermia cereana*. One mL of yeast and bacterial solution was injected into the cactus media every 48 h to yield constant fermentation of the cacti. The final media were soft enough to separate the larvae (especially the first and second instars) from the cactus media.

Adult culture conditions

Flies were raised for one generation in population cages (described above), and eggs collected from these cages

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were reared to eclosion on banana food at moderate larval densities in half-pint bottles. Emerged adults were transferred to 8-dr shell vials in small same sex groups containing banana food until they were sexually mature (8–10 days). Approximately 400 adults (200 females and 200 males) from each population were introduced into separate oviposition chambers and allowed to mate and oviposit for 10 h each day. Eggs were collected from a 5.5-cm-diameter Petri dish containing agar-cactus media attached to each oviposition chamber and washed in sterile deionized water, 70% ethanol, and again in deionized water. Eggs were counted into groups of 200, transferred to a 1 cm^2 piece of sterilized filter paper and placed in bottles containing 75 g of fermenting cactus tissue in the incubator described above. All unhatched eggs were counted to allow calculation of egg-to-adult viability. Eclosed adults from each replicate culture were counted daily, allowing determination of egg-to-adult development time, separated by sex and immediately transferred to vials containing fermenting cactus (see below) in same sex groups of 30 flies. All cultures were maintained in an incubator (described above).

Cactus media for rearing adults for RNA extraction were prepared by mixing cactus (agria or organ pipe), water and agar homogenized in a blender in the following proportions: 953 g cactus, 486 mL deionized water and 5 g agar. This mixture was autoclaved for 15 min, cooled and inoculated with bacteria and yeasts (see above). This cactus media were prepared 1 week prior to use and kept in an incubator at 37 °C to maximize microbial fermentation. These media were then loaded into individual cup-like 2.2–cm-diameter plastic barrel plugs (Alliance Express, Little Rock, AR, USA) that were pressed into one end of autoclaved 25 × 95 mm glass tubes. An additional inoculating loop containing a mixture of bacteria and seven cactophilic yeasts was added to the fermenting cactus tissue in each food cap to supplement nutrition. After adding 30 adult females or males to each tube, the other end of each tube was closed with a barrel plug that had been drilled with a 1.75 cm hole sealed with fine mesh to allow air circulation. Flies were fed atmospheric ethanol vapour by placing tubes in sealed desiccators containing 1 L of 4% ethanol (Etges 1989; Etges & Klassen 1989) from 8:00 AM to 6:00 PM in the incubator described above. For the remaining 14 h each day, all tubes were removed from each desiccator and kept in the incubator to minimize condensation inside the tubes. Plugs containing fermenting cactus were replaced every 4 days.

Adult females for RNA extraction were sampled at 8 time intervals: 0, 3, 6, 10, 14, 18, 24 and 28 days. Each adult sample consisted of 24 virgin females that were snap-frozen in liquid nitrogen and stored at −80 °C. Additional tubes of females and males sampled at each time interval were frozen at −20 °C and used for cuticular hydrocarbon analysis (Etges & de Oliveira 2014). Overall, we planned 24 treatment combinations for pre-adult stages (2 populations × 2 cacti × 6 stages) and 32 combinations (2 populations × 2 cacti × 8 ages) for adult females (Fig. 1). Each combination was replicated four times for RNA extraction and microarray analysis; however, samples of 28-day-old females were missing because few flies survived past 28 days in these conditions (Etges & Heed 1992; Jaureguy & Etges 2007), so we pooled them resulting in 7 ages sampled. A number of missing replicates resulted in 86 (pre-adult) and 86 (adult) samples (Table S1, Supporting information).

cDNA synthesis, hybridization and visualization

Total RNA was isolated from each sample using RNeasy mini-kits (Qiagen, Valencia, CA, USA) and stored at −80 °C until cDNA was prepared. Double-stranded cDNA was synthesized using Invitrogen Superscript Double-Stranded cDNA Synthesis kits, and cDNA concentrations were measured using a NanoDrop spectrophotometer (NanoDrop Technologies) to verify that all cDNA samples were 100 ng/μl, A260/A280 1.8, and A260/A230 1.8. All cDNA samples

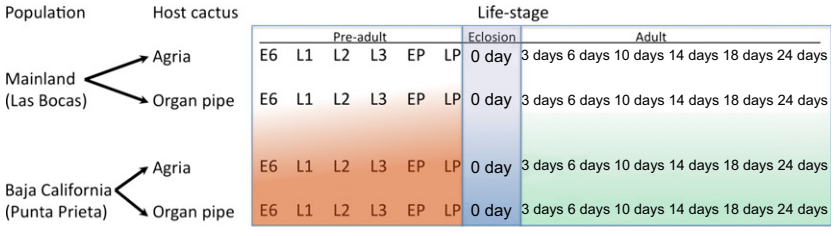

Fig. 1 Experimental design for RNA sampling of the two populations of *D. mojavensis* reared on two host plants throughout the life cycle where L1 = first instar, L2 = second instar, L3 = third instar, EP = early pupae, LP = late pupae, 0 D = adult day of emergence, 3 D = 3 day old adults, etc. Day 24 adults were pooled with Day 28 adults because so few Day 28 adults were available due to mortality.

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were Cy3 labelled using a NimbleGen One-Color DNA Labeling kit.

Our Roche NimbleGen microarray design contained a total of 14 528 unique transcripts based on the *D. mojavensis* genome (http://flybase.net/genomes/Drosophila_mojavensis/current/fasta/dmoj-all-transcript-r1.3.fasta.gz; 4/14/2009) with nine probes per transcript for a total of 130 705 probes (each microarray in the 12-plex design included 135K probes; see Gene Expression Omnibus entry GSE43220 for details). Hybridizations were performed with a NimbleGen Hybridization System (Hybridization System 4, BioMicro Systems, Inc.) and spot intensity scanning was carried out with a GenePix 4000B scanner (Molecular Devices) and GENEPIX PRO software. All hybridization intensities were normalized using quantiles (Bolstad *et al.* 2003) with NIMBLESCAN v2.5 software. Gene call files were generated using the Robust Multichip Average (RMA) algorithm as described by Irizarry *et al.* (2003).

Data analysis

Whole-data set analysis. We assessed time-series gene expression dynamics using SVD analysis (Alter *et al.* 2000, 2003). SVD is a linear transformation of expression data from genes x arrays space to a reduced 'eigengenes' x 'eigenarrays' space. In our case, the SVD took our 14528 gene × 13 stage/age data matrix and returned a 13 × 13 matrix where each row is an eigengene. Each of these eigengenes represents a consensus trajectory of gene expression, similar to a principal component, encompassing a proportion of the overall variation in gene expression over time. This application of SVD is closely analogous to its usual use in signal processing, with each eigengene representing a common trajectory of expression with a strong signal in the data. These eigengene profiles provide a way to cluster genes according to their correlation with these dominant trajectories of gene expression across the life cycle.

Singular value decomposition analysis was performed on an averaged *D. mojavensis* data set, consisting of mean within-life-stage gene expression values for all genes at each stage/age to evaluate overall gene expression variation changes. Preliminary analysis revealed a single eigengene representing steady-state expression that accounted for 99.6% of all variation in the data. The entropy of this data set was also low ($d = 0.012 \ll 1$), suggesting that stage-specific changes in expression were relatively small deviations from lifetime mean expression (Alter *et al.* 2000). We therefore mean-centred the data by filtering out this eigengene (Alter *et al.* 2000), and all further analyses were undertaken on the resulting normalized data set. After normalization, the stage-specific expression levels for each gene had values between −1 and 1, with positive relative expression levels indicating overexpression and negative expression indicating under-expression relative to the lifetime mean.

Singular value decomposition analysis contains an inherent sign ambiguity; thus, for each eigengene, its complementary (i.e. equal and opposite relative expression level at each stage and age) trajectory is equally significant. While heuristic methods do exist to try to work around this ambiguity, we chose to exploit it by treating significant eigengenes as paired sets of correlated and anticorrelated gene expression trajectories. For each significant eigengene pair, we arbitrarily designated the 'positive' eigengene to be the trajectory with positive relative expression in adult stages (Fig. 2). The corresponding 'negative' eigengene trajectory is a mirror image about zero of its complementary 'positive' eigengene. Thus, genes significantly correlated with a 'positive' eigengene will be significantly anticorrelated with the corresponding negative eigengene and vice versa.

Serial resampling of the biological replicates was used to assess variation within stage/age samples and its impact on eigengenes revealed by SVD analysis. 10 000 resampled data sets were created by randomly selecting one biological replicate from the available samples at each life stage/age. These resampled data sets were subjected to SVD analysis just as with the averaged data set and were used to form 95% confidence bounds around the original eigengenes (Ghosh 2002). To determine which transcripts were contributing most to each eigengene pattern, genes were sorted by their correlation with the eigengene's trajectory over the life history (top 10%), and then these transcripts were sorted again by the magnitude of their projection onto the eigengene (Alter *et al.* 2000) to arrive at 5% or 726 predicted genes. For simplicity, we included the top 750 genes with the highest ± rank in this sorting with respect to each eigengene at each stage and age for gene annotation and functional clustering.

Peaks and variance in gene expression

At each life stage and age, we calculated the mean and variance of expression for each transcript across populations and diets. We then determined when each gene was at its highest observed level of expression allowing us to characterize differences in maximum gene expression across the life cycle. We were also interested in the variability of gene expression across our replicate samples to determine whether gene expression may become less tightly controlled with age (cf. Pletcher *et al.* 2002). Thus, we plotted changes in genome-wide variances in gene expression characterized as the stage- or age-spe-

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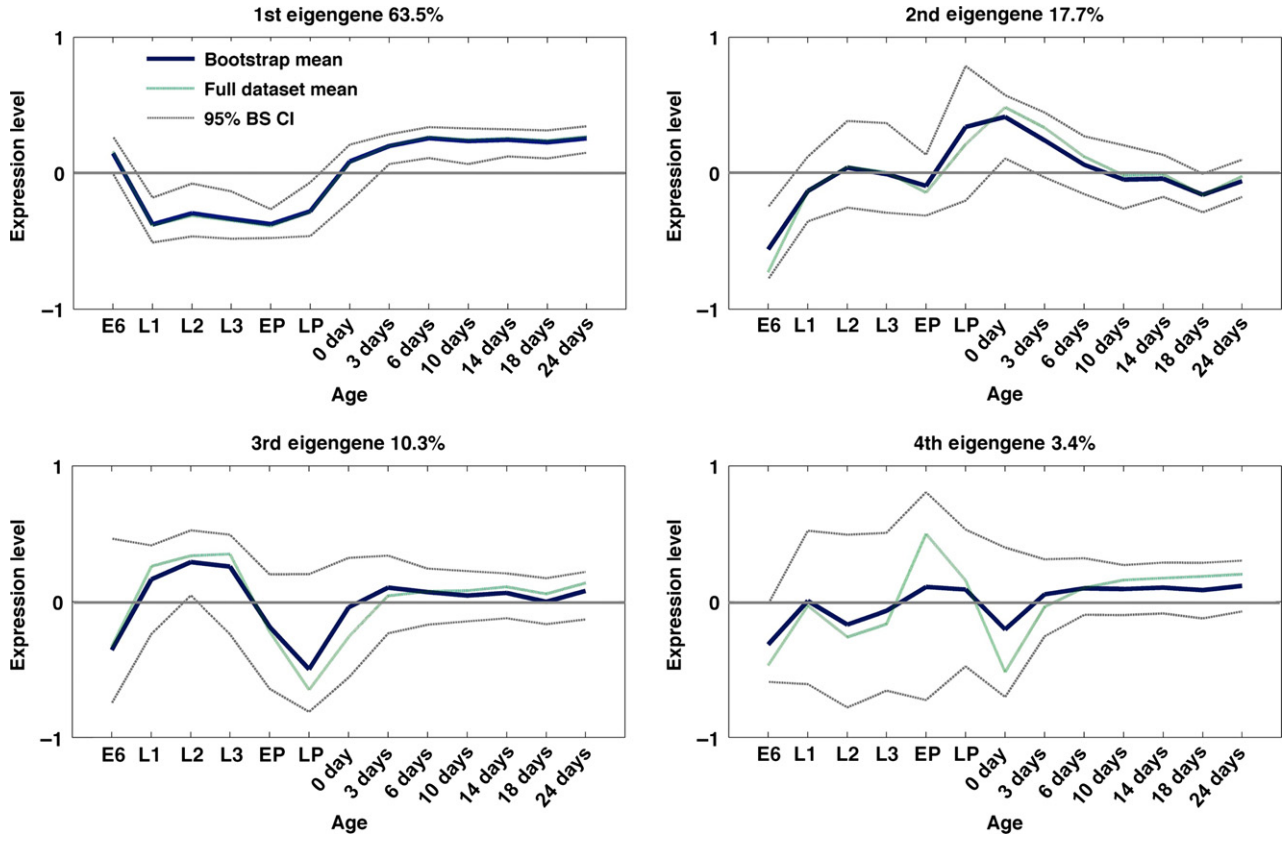

Fig. 2 The first four eigengenes plotted across the life cycle in *D. mojavensis*. The proportion of the total variation explained by each eigengene is listed. Plus/minus correlations with each eigengene are plotted for the overall data set means, bootstrap means and 95% bootstrap mean confidence intervals. The *X*-axis represents the six pre-adult stages: embryo, larval and pupal plus adults defined in Fig. 1 and the text.

cific variance of all predicted genes in their expression levels. Different numbers of individuals were sampled at different pre-adult stages that might affect genome-wide variance estimates, but only gene expression variance increases in second instar larvae and late pupae (384 h) were observed (see Results).

Pairwise stage and age comparisons

We also assessed a set of specific pairwise comparisons using data sets pooled in a different way, for example, comparing expression at two ages, or comparing expression under two environments with the primary aim of mapping gene expression levels into functional domains as in previous studies (Pletcher *et al.* 2002; Kim *et al.* 2005; Koutsos *et al.* 2007) and to search for shared components of gene co-expression underlying development and ageing (McCarroll *et al.* 2004). We chose to analyse targeted pairwise interactions rather than use a traditional linear model approach, since a fully parameterized model of our data would involve $2 \times 2 \times 13$ possible comparisons which, in the end, would have needed to be assessed with the same set of pairwise tests. A full linear model for all genes across the life cycle produced stage/age differences in gene expression that were >99% similar to our pairwise comparisons (results not shown). We identified transcripts that significantly increased or decreased in expression between each pair of consecutive life stages using *t*-tests corrected for false discovery rate (FDR) $P < 0.05$ (Benjamini & Hochberg 1995) and that had absolute fold changes >1.5 (the absolute value of the ratio of normalized intensities between two samples). These comparisons helped to tease out individual gene expression changes potentially responsible for, or caused by, important age–stage transitions.

We also identified transcripts that significantly increased or decreased in expression between 3-day-old (young adult) and >18-day-old (senescent) adults because many ageing studies have focused on such pairwise comparisions between 'young' and 'old' age classes (e.g. Landis *et al.* 2004; de Magalhães *et al.* 2009; Southworth *et al.* 2009). We pooled samples from ages 18+ days to increase sample sizes, since at older ages only enough flies remained to produce one or two replicate samples per treatment. We also assessed numbers

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of genes differentially expressed due to host cacti in pre-adult and adult stages using a data set with all pre-adult stages pooled together and all adult stages pooled together.

Host cactus and population effects

For each pre-adult stage and adults on day of eclosion, we assessed all gene expression differences due to cactus with t-tests with FDR $P < 0.05$ and absolute fold changes >1.5. The remaining adult data were assessed by ANOVA with cactus, population and cactus by population interaction included with ages pooled (Etges 2014).

Orthologue search and functional annotation clustering

Submission of the 14 528 *D. mojavensis* transcripts to Flybase (Tweedie *et al.* 2009) produced 9117 *D. melanogaster* orthologues, that is only ~63% of predicted *D. mojavensis* genes could be functionally analysed. Reciprocal BLAST searches with the other 10 available *Drosophila* genomes did not increase this number (results not shown). These 9117 orthologues were used in gene ontology analyses using DAVID Bioinformatics Resources 6.7 (Huang *et al.* 2009). Thus, for a given list of *D. mojavensis* transcripts of interest, we first determined the subset of those transcripts that had *D. melanogaster* orthologues and used the corresponding *D. melanogaster* genes in our gene ontology analysis.

Gene annotation clusters were determined by DAVID's clustering algorithm with initial classification stringencies set to 'Moderate'. We also used GO-Module (Yang *et al.* 2011) to reduce redundancy in numbers of annotated clusters when there were several overlapping functional clusters produced by DAVID. Further inspection of annotated gene function was enabled by identifying KEGG pathways (Kanehisa & Goto 2000).

Due to limited annotation of the *D. mojavensis* genome, our gene ontology analysis has two main potential sources of error. First, we could only include genes that have known *D. melanogaster* orthologues. Thus, the gene lists used in our analyses are missing ~37% of the original transcripts of interest. The addition of this missing data could change the significance of the clusters reported here and could also contain enriched clusters undetectable in our current data set. Second, our enrichment analyses compared gene lists of interest with the list of 9117 orthologues as background, not with the entire *D. melanogaster* genome. An 'enriched' cluster of GO-terms, then, means that terms within that cluster were proportionately over-represented in the subset of the original transcript list of interest that had known *D. melanogaster* orthologues, as compared to the total set of orthologues. Given these limitations, we interpret our gene ontology results with caution and focus primarily on broader trends. We performed the same annotation cluster analysis with the top 5% of genes corresponding to each eigengene, genes with maximal expression over the life cycle and genes differing in expression between consecutive life cycle stages/ages.

Results

Singular value decomposition (SVD) analysis

Singular value decomposition analysis revealed four eigengenes that explained 95% of the variation in the normalized data set (Fig. 2). All four eigengenes showed life cycle shifts in gene co-expression associated with transitions from egg to larval stages and pupae to day of eclosion, with relatively little change from eclosion to adults of older ages (Fig. 2). We pooled replicates from population and cactus diet treatments because there were no significant differences observed in eigengene structure between these groups, as revealed by overlap in their 95% bootstrap confidence intervals at all life stages (results not shown). SVD analysis of overall life cycle variation in gene expression was thus insensitive to differences due to rearing substrates or population origin, likely in part because the number of replicates for each age-population-diet combination was limited to four.

The first eigengene accounted for 63.5% of the overall variation in gene expression and so represented the largest correlated "structure" of life cycle gene expression in the normalized data set. This trajectory was characterized by a negative relationship between pre-adult and adult gene expression patterns – transcripts that were downregulated in pre-adult stages were upregulated in adults and vice versa (Fig. 2). Interestingly, gene expression in 6-h embryos was concordant with expression in adult ages (Table S2, Supporting information). This was expected in part because adult females contained developing eggs (cf. Graveley *et al.* 2011). Of the top 5% of all genes with the highest positive correlation with eigengene 1, just 38.7% were annotated and were significantly enriched for general growth and metabolic function including protein synthesis, cell division and secretory functions (Table 1).

Of the genes with transcription levels negatively correlated with eigengene 1, 86.4% were annotated and were enriched for protease activity, G-protein-coupled receptor function, ion transport, sensory perception and transcriptional regulation (Table S2). These functional groups were expressed from first instar larvae to late pupae consistent with protein degradation, larval moulting, tissue remodelling in pupation and increased

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Table 1 Gene ontology terms identified with DAVID using the top 5% (750 genes) of all predicted *D. mojavensis* genes. Results for the top 4 significant eigengene clusters based on all life stages and ages are shown. Numbers in parentheses are the percentages of the top 750 genes with *D. melanogaster* orthologs

Eigengene 1 (63.5%)				Eigengene 2 (17.7%)			
Positive correlation 290 (38.7%)+		Negative correlation 648 (86.4)		Positive correlation 574 (76.5)		Negative correlation 531 (70.8)	
GO-Term	Enrich	GO-Term	Enrich	GO-Term	Enrich	GO-Term	Enrich
Protein transport, localization	5.82****	G-protein-coupled receptor protein sensory perception, taste, chemical stimulus	4.62****	Membrane structure	8.9****	RNA, mRNA, tRNA processing, splicing, transport	10.38****
Vesicle transport, endocytosis, secretion	3.35***	Cell morphogenesis	2.1**	Glycoproteins	4.79****	Zinc finger, metal ion binding	6.05****
Nucleotide binding	2.74*	Ion transport, sodium channel activity	1.96*	ATP transmembrane metabolism, ion transport	4.32***	Chromosomal structure	4.24****
Synaptic transmission	2.06*	Transcriptional regulation	1.64*	Plasma membrane	3.2*	Transcription, protein synthesis	4.1****
Translation in mitochondrion	1.99**	Peptidase activity	1.25*	Larval behavior	2.35*	Chromosome organization, modification	3.86***
				Cell-cell signaling and neurological function	2.33**	Protein folding	3.21**
				Oxidative phosphorylation	2.1***	Ribonuclease activity	2.92*
				G-protein coupled receptors	1.54*	Epigenetic regulation of gene expression	1.36*
				Detection of external stimulus	1.38**		

Eigengene 3 (10.3%)				Eigengene 4 (3.4%)			
Positive correlation 457 (60.9%)		Negative correlation 422 (56.3%)		Positive correlation 406 (54.1%)		Negative correlation 545 (72.7%)	
GO-Term	Enrich	GO-Term	Enrich	GO-Term	Enrich	GO-Term	Enrich
Peptidase activity	3.78****	Neuron differentiation & development, axonogenesis	5.4****	Ribosome	6.27****	HOX/homeobox, development protein, DNA binding, transcriptional regulation	7.98****

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Table 1 *Continued*

Eigengene 3 (10.3%)				Eigengene 4 (3.4%)			
Positive correlation 457 (60.9%)		Negative correlation 422 (56.3%)		Positive correlation 406 (54.1%)		Negative correlation 545 (72.7%)	
GO-Term	Enrich	GO-Term	Enrich	GO-Term	Enrich	GO-Term	Enrich
Endoplasmic reticulum	2.88***	Cell migration	5.32****	Structural constituent of ribosome	2.81****	Mitochondrion, mitochondrial ribosome	5.13****
		Organ formation	4.58*			Organ formation	4.52****
		Eye development	4.22*			Regionalization, pattern specification	4.24****
		Instar larva or pupal morphogenesis, metamorphosis	3.23*			Neuron development	3.8***
		Salivary gland development	2.22***			Transcription repressor activity, negative regulation of transcription	3.67***
						Head segmentation	3.57**
						Oxidative phosphorylation	3.49**
						Gut development	3.14**
						Respiratory system development	2.31*
						Wnt signaling	2.16**

[+]Number (percentage of top 750) of genes annotated.
FDR *$P < 0.05$, **$P < 0.01$, ***$P < 0.005$, ****$P < 0.0001$.

larval expression of sensory and gustatory genes (Vosshall & Stocker 2007). Many of these genes in this cluster were olfactory (Or) and gustatory receptor (Gr) orthologues that were upregulated in first instar larvae (Table 2). Thus, the largest sources of life cycle gene co-expression variation for orthologues with inferred functions were due to increased expression of ribosomal-associated translation capacity in embryo and adult stages with correspondingly increased expression of gene clusters with protein degradation and sensory perception function in larval through pupal stages.

The second significant eigengene accounted for 17.7% of the variation in lifetime gene expression. The 'positive' complement of this trajectory was characterized by downregulated expression in egg and early pupa stages, with close to mean expression levels during larval stages, strongly upregulated expression in late pupae and day of eclosion, then a slow monotonic decrease in expression with adult age (Fig. 2). The negative complement showed, conversely, upregulated expression in egg and early pupal stages, mean expression levels in larval stages, downregulation at late pupae and eclosion and monotonic increases in expression with adult age. Thus, increased transcription in 6-h embryos and in ageing, post-eclosion adults likely involved common gene clusters.

The largest positive loadings on this eigengene occurred from late pupae to eclosion and in young adults. Of the top 5% positively correlated genes, 574 genes were annotated and enriched for functional clusters involved with plasma membrane structure and ion transport, glycoprotein metabolism, neural development and function, sensory perception and oxidative phosphorylation (Table 1). This enrichment is consistent with expression of developmental genes in late pupae, as well as peak neural and metabolic function in young adults with decreases in neural and metabolic function with increasing adult age. Negative associations with eigengene 2 included RNA processing and transport, transcriptional regulation, protein folding, chromosomal organization and epigenetic control of gene regulation (Table S3, Supporting information). This enrichment pattern is consistent with protein synthesis in egg and pupal stages, and interestingly, again in late adult life. Thus, eigengene 2 included a significant component of lifetime gene co-expression associated with embryonic gene clusters and the pupa–eclosion transition that then shifted with adult age. This suggested that eigengene 2 structure was driven by post-eclosion shifts in gene cluster transcription associated with slowing of protein metabolism, reduction in neural function, detoxification activity and chromatin silencing associated with ageing, including *Sirt6*, a known determinant of adult lifespan (Kusama *et al.* 2006). Eigengene 2 is therefore an excellent genelet to pursue to understand expression of ageing genes.

While the third and fourth significant eigengenes accounted for far smaller proportions of the total variation in our data, eigengene 3 was associated with contrasting larval and pupal patterns of gene expression and an overall lack of deviation from mean gene expression levels after eclosion (Fig. 2). The 'positive' trajectory of this eigengene had peak expression in larval stages, with strong downregulation in egg and pupal stages. Transcripts correlated with this trajectory were enriched for peptidase activity and endoplasmic reticulum function. The increased expression of these genes in larvae, with decreasing expression in pupal stages, is consistent with decreases in metabolic rates from early to late pupal stages (Lebo *et al.* 2009; Merkey *et al.* 2011).

The negative trajectory of eigengene 3 was characterized by peak expression in egg and pupal stages, with downregulated expression in larvae. Transcripts with correlated with this trajectory were enriched for brain and organ development, and metamorphosis consistent with upregulation of developmental processes in the embryo and pupae (Table 1).

The 'positive' trajectory of the fourth eigengene was characterized by downregulated expression in 6 h embryos, weaker downregulation in larval stages, peak expression in pupal stages followed by strong downregulation of expression at eclosion and slowly increasing expression at adult ages (Fig. 2). Transcripts correlated with this trajectory were enriched for ribosomal function, consistent with the tissue remodelling during pupal stages.

Transcriptional variation correlated with the negative trajectory of this eigengene was associated with pattern formation and larval development, and enriched for *Hox* genes, organ system formation, segmentation and neuron development genes, as well as *wnt* signalling (Table 1). Enrichment for developmental genes is consistent with expression patterns in the embryonic stage and likely has little to do with the eigengene's expected peak expression in young adults. *wnt* signalling was also associated with embryogenesis, and this enrichment is likely driven by overexpression of *wnt* associated genes in 6 h embryos. All *wnt* signalling homologs showed peak expression early in embryogenesis, but some, for example *boca, WntD, pangolin and wingless* (Table S2), also showed increased expression in adults consistent with the positive expression in young adults for eigengene 4 (Fig. 2) similar to modENCODE expression levels in *D. melanogaster* (Tweedie *et al.* 2009).

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Table 2 Changes in expression (all 1.5 × fold change, FDR $P < 0.05$) of sensory genes from 6 h embryos to first instar larvae (Egg-L1), second to third instar (L2–L3), and late pupae to day of eclosion (LP-0D) in two populations of *D. mojavensis*. All significant changes were identified in enriched functional clusters of genes differing in expression between consecutive life stages identified by DAVID (Huang *et al.* 2009). Direction of arrows indicates increased or decreased transcript abundance

D. melanogaster ID	Gene	Egg-L1	L2–L3	LP-0D	GO annotation
FBgn0000120	*Arr1*	↑↑			Deactivation of rhodopsin-mediated signalling
FBgn0000206	*Boss*	↑↑			R7 cell fate commitment; GO:0007465 compound eye development; GO:0048749
FBgn0000313	*chp*	↑↑			Homophilic cell adhesion; GO:0007156 rhabdomere development; GO:0042052
FBgn0066293	*CheB42b*	↑↑			Detection of pheromone; GO:0043695
FBgn0040726	*dpr*	↑↑			Salt aversion; GO:0035199, sensory perception of salty taste
FBgn0004623	*Gbeta76C*	↑↑			Deactivation of rhodopsin-mediated signalling; GO:0016059, G-protein coupled receptor protein signaling pathway; GO:0007186
FBgn0028433	*Ggamma30A*	↑↑			G-protein coupled receptor protein signaling pathway; GO:0007186, phototransduction; GO:0007602
FBgn0004618	*gl*	↑↑			Response to red light; GO:0010114, entrainment of circadian clock by photoperiod
FBgn0045502	*Gr10a*	↑↑			Sensory perception of taste; GO:0050909
FBgn0041250	*Gr21a*	↑↑			Sensory perception of taste; GO:0050909
FBgn0041248	*Gr23a*	↑↑			Sensory perception of taste; GO:0050909
FBgn0041247	*Gr28a*	↑↑		↓↓	Sensory perception of taste; GO:0050909
FBgn0045495	*Gr28b*	↑↑			Sensory perception of taste; GO:0050909
FBgn0032416	*Gr33a*	↑↑		↓↓	Sensory perception of taste; GO:0050909
FBgn0041236	*Gr59d*	↑↑		↓↓	Sensory perception of taste; GO:0050909
FBgn0035468	*Gr63a*	↑↑		↓↓	Sensory perception of taste; GO:0050909, response to carbon dioxide; GO:0010037
FBgn0045479	*Gr64a*	↑↑		↓↓	Sensory perception of taste; GO:0050909, detection of glucose; GO:0051594
FBgn0045478	*Gr64b*	↑↑		↓↓	Sensory perception of taste; GO:0050909
FBgn0045477	*Gr64c*	↑↑		↓↓	Sensory perception of taste; GO:0050909
FBgn0045476	*Gr64e*	↑↑		↓↓	Sensory perception of taste; GO:0050909
FBgn0052255	*Gr64f*	↑↑		↓↓	Sensory perception of taste; GO:0050909
FBgn0035870	*Gr66a*			↓↓	Sensory perception of taste; GO:0050909
FBgn0046885	*Gr98d*	↑↑		↓↓	Sensory perception of taste; GO:0050909, sensory perception of taste; GO:0050909
FBgn0011672	*Mvl*			↓↓	Sensory perception of taste; GO:0050909, taste receptor activity; GO:0008527
FBgn0013972	*Gycalpha99B*	↑↑			Positive phototaxis; GO:0046956 rhodopsin-mediated phototransduction; GO:0009586, guanylate cyclase complex, soluble; GO:0008074
FBgn0004784	*inaC*	↑↑			Adaptation of rhodopsin-mediated signaling; GO:0016062 phototransduction; GO:0007602
FBgn0001263	*InaD*	↑↑			Deactivation of rhodopsin-mediated signaling; GO:0016059, phototransduction; GO:0007602
FBgn0053197	*mbl*	↑↑			Embryonic development; GO:0009790, muscle organ development; GO:0007517, compound eye photoreceptor cell differentiation; GO:0001751
FBgn0036414	*nan*	↑↑			Calcium ion transport; GO:0006816, sensory perception of sound; GO:0007605
FBgn0002936	*ninaA*	↑↑			Rhodopsin biosynthetic process; GO:0016063
FBgn0002938	*ninaC*	↑↑			Cytoskeleton organization; GO:0007010, phototransduction, visible light; GO: adaptation of rhodopsin-mediated signaling; GO:0016062
FBgn0002940	*ninaE*	↑↑			Phototransduction; GO:0007602, photoreceptor cell morphogenesis; GO:0008594
FBgn0037896	*ninaG*	↑↑			Retinoid metabolic process; GO:0001523

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Table 2 *Continued*

D. melanogaster ID	Gene	Egg-L1	L2–L3	LP-0D	GO annotation
FBgn0016047	*nompA*	↑↑			Dendrite morphogenesis; GO:0048813, detection of mechanical stimulus involved in sensory perception of sound; GO:0050910
FBgn0016919	*nompB*	↑↑			Flagellum assembly; GO:0009296, sensory cilium assembly; GO:0035058
FBgn0016920	*nompC*	↑↑			Calcium ion transport; GO:0006816, mechanosensory behavior; GO:0007638, sensory perception of sound; GO:0007605
FBgn0031110	*Obp19b*	↑↑			Sensory perception of chemical stimulus; GO:0007606
FBgn0033268	*Obp44a*	↑↑			Sensory perception of chemical stimulus; GO:0007606
FBgn0033508	*Obp46a*	↑↑		↓↓	Sensory perception of chemical stimulus; GO:0007606
FBgn0033573	*Obp47a*	↑↑			Sensory perception of chemical stimulus; GO:0007606, odorant binding; GO:0005549
FBgn0050067	*Obp50a*	↑↑			Sensory perception of chemical stimulus; GO:0007606
FBgn0033931	*Obp50e*	↑↑			Sensory perception of chemical stimulus; GO:0007606
FBgn0034468	*Obp56a*	↑↑			Sensory perception of smell; GO:0007608, response to pheromone; GO:0019236
FBgn0046879	*Obp56c*	↑↑		↓↓	Sensory perception of chemical stimulus; GO:0007606
FBgn0034471	*Obp56e*	↑↑			Sensory perception of chemical stimulus; GO:0007606
FBgn0034474	*Obp56 g*	↑↑			Sensory perception of chemical stimulus; GO:0007606
FBgn0034475	*Obp56 h*	↑↑			Sensory perception of smell; GO:0007608 response to pheromone; GO:0019236
FBgn0034768	*Obp58b*	↑↑		↓↓	Sensory perception of chemical stimulus; GO:0007606
FBgn0034769	*Obp58c*	↑↑		↓↓	Sensory perception of chemical stimulus; GO:0007606
FBgn0034770	*Obp58d*	↑↑		↓↓	Sensory perception of chemical stimulus; GO:0007606
FBgn0046876	*Obp83ef*			↓↓	Sensory perception of chemical stimulus; GO:0007606
FBgn0046875	*Obp83 g*	↑↑		↓↓	Olfactory behavior; GO:0042048, response to pheromone; GO:0019236
FBgn0038859	*Obp93a*	↑↑		↓↓	Sensory perception of chemical stimulus; GO:0007606
FBgn0039685	*Obp99b*	↑↑	↑↑	↓↓	Autophagic cell death; GO:0048102, salivary gland cell autophagic cell death; GO:0035071, olfactory behavior; GO:0042048, response to pheromone; GO:0019236
FBgn0039682	*Obp99c*	↑↑			Sensory perception of chemical stimulus; GO:0007606
FBgn0026396	*Or22c*			↓↓	Sensory perception of smell; GO:0007608
FBgn0026394	*Or24a*	↑↑			Sensory perception of smell; GO:0007608
FBgn0032096	*Or30a*	↑↑		↓↓	Sensory perception of smell; GO:0007608
FBgn0026390	*Or33c*	↑↑		↓↓	Olfactory behaviour; GO:0042048
FBgn0033041	*Or42a*	↑↑			Sensory perception of smell; GO:0007608
FBgn0026389	*Or43a*	↑↑		↓↓	Sensory perception of smell; GO:0007608
FBgn0033422	*Or45b*	↑↑			Sensory perception of smell; GO:0007608
FBgn0026388	*Or46a*	↑↑			Olfactory behaviour; GO:0042048, sensory perception of smell; GO:0007608
FBgn0033727	*Or49a*	↑↑		↓↓	Sensory perception of smell; GO:0007608
FBgn0028963	*Or49b*	↑↑		↓↓	Sensory perception of smell; GO:0007608
FBgn0034473	*Or56a*	↑↑		↓↓	Sensory perception of smell; GO:0007608
FBgn0026384	*Or59a*	↑↑		↓↓	Sensory perception of smell; GO:0007608
FBgn0035382	*Or63a*	↑↑		↓↓	Sensory perception of smell; GO:0007608
FBgn0036078	*Or67c*	↑↑		↓↓	Sensory perception of smell; GO:0007608
FBgn0036709	*Or74a*	↑↑		↓↓	Integral to membrane; GO:0016021, olfactory receptor activity; GO:0004984, odorant binding; GO:0005549
FBgn0037322	*Or83a*	↑↑		↓↓	Sensory perception of smell; GO:0007608
FBgn0037324	*Or83b*	↑↑		↓↓	Olfactory behavior; GO:0042048 sensory perception of smell; GO:0007608 response to pheromone; GO:0019236
FBgn0037576	*Or85a*	↑↑		↓↓	Sensory perception of smell; GO:0007608
FBgn0037594	*Or85d*	↑↑		↓↓	Sensory perception of smell; GO:0007608
FBgn0038798	*Or92a*	↑↑		↓↓	Sensory perception of smell; GO:0007608
FBgn0030204	*Or9a*	↑↑			Sensory perception of smell; GO:0007608
FBgn0060296	*Pain*	↑↑			

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Table 2 *Continued*

D. melanogaster ID	Gene	Egg-L1	L2–L3	LP-0D	GO annotation
					Calcium ion transport; GO:0006816, sensory perception of pain; GO:0019233, response to mechanical stimulus; GO:0009612, feeding behavior
FBgn0011283	*Pbprp5*	↑↑			Sensory perception of chemical stimulus; GO:0007606
FBgn0065109	*ppk11*	↑↑			Sodium ion transport; GO:0006814, liquid clearance, open tracheal system; GO:0035002, sensory perception of salty taste; GO:0050914
FBgn0085373	*rdgA*	↑↑			Diacylglycerol kinase activity
FBgn0004366	*rdgC*	↑↑			Phototransduction; GO:0007602, photoreceptor cell maintenance; GO:0045494, visual perception; GO:0007601
FBgn0003248	*Rh2*	↑↑			Phototransduction; GO:0007602
FBgn0003249	*Rh3*	↑↑			Phototransduction, UV; GO:0007604
FBgn0014019	*Rh5*	↑↑			Phototransduction; GO:0007602
FBgn0019940	*Rh6*	↑↑			Phototransduction; GO:0007602
FBgn0036260	*Rh7*	↑↑			G-protein coupled receptor protein signaling pathway; GO:0007186, phototransduction; GO:0007602
FBgn0003380	*Sh*	↑↑			Flight behavior; GO:0007629, potassium ion transport; GO:0006813, courtship behaviour; GO:0007619
FBgn0086367	*t*	↑↑			Flight behaviour; GO:0007629, cuticle pigmentation; GO:0048067, visual perception; GO:0007601, dopamine biosynthetic process; GO:0042416
FBgn0014395	*tilB*	↑↑			Sensory perception of sound; GO:0007605, male courtship behavior, veined wing generated song production; GO:0045433
FBgn0005614	*trpl*	↑↑			Calcium ion transport; GO:0006816, response to abiotic stimulus; GO:0009628, response to light stimulus; GO:0009416
FBgn0004514	*TyrR*	↑↑			Sensory perception of smell; GO:0007608, octopamine/tyramine signaling pathway; GO:0007211
FBgn0039482	CG14258		↑↑		Pheromone/odorant binding GO:0005549
FBgn0051345	CG31345	↑↑			Detection of calcium ion; GO:0005513, phagocytosis, engulfment; GO:0006911
FBgn0147028 (Dmoj)	Dmoj_GI24305		↑↑		IPR004272: Odorant binding protein, IPR013053: Hormone binding, (no *D. melanogaster* ortholog)

Peak expression and transitions in gene expression levels over the life cycle

Both maximum expression data (per cent of all genes at maximum lifetime expression levels, Fig. 3, Table S4, Supporting information) and expression change data (per cent of all genes with significant (FDR $P < 0.05$ and >1.5 × fold changes, Table S5, Supporting information) between successive life stages/ages showed the same three distinct peaks over the life cycle of *D. mojavensis* (Fig. 4). There was a clear burst of genome-wide levels of expression in 6-h embryos that declined throughout larval stages, an increase in pupae to day of eclosion, and an almost monotonic decline until adults were 14 days old (Table 3; Fig. 4). A slight late-life peak in gene expression levels was apparent from 14 to 18 and 24 days, a peak also seen for genes at their maximum lifetime expression levels (Fig. 4).

Genome-wide variance in expression levels peaked at second larval instar and late pupal stages and remained relatively unchanged over adulthood (Fig. 4). Since sexes were pooled until day of eclosion, and only female adults were analysed here, we could not separate variation due to sex-specific expression in pre-adult

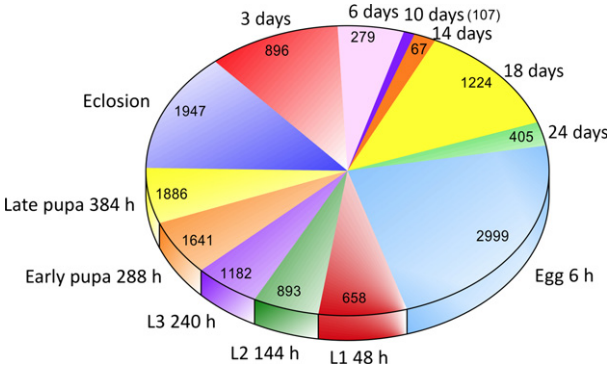

Fig. 3 Pie chart showing the numbers of genes at their maximum lifetime expression levels at each stage and age in this study. Stages and ages are defined in Fig. 1 and in the text.

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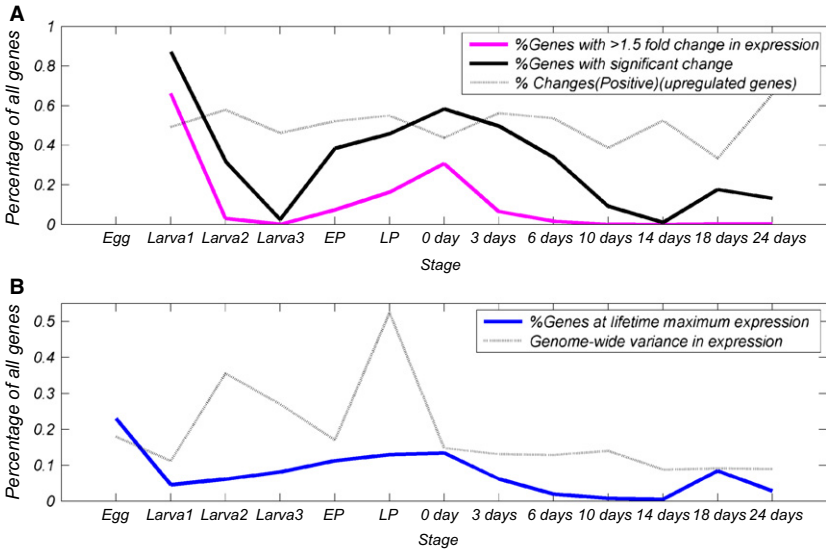

Fig. 4 (A) Plots of the changes in gene expression compared with previous stages/ages for the proportion of all 14 528 genes with 1.5 × fold changes, significant changes ($P < 0.05$), per cent of all genes that were upregulated from the previous stage/age. (B) Plot showing the percentage of all genes at maximum lifetime expression levels and changes in the variance in gene expression for all genes at each stage/age.

stages from other causes as a contributing factor to these variance increases. However, sex-specific differences in expression, particularly in pupae, were likely greater in germline than somatic tissues (Lebo *et al.* 2009).

Almost 21% of all predicted genes were at their maximum transcription levels in 6-h embryos ($n = 2999$, Fig. 3) and were significantly enriched for 26 different functional gene clusters (Table 3). The second transcriptional peak in late pupae to day of emergence involved 1886 and 1947 genes, respectively, and a third peak in 18-day-old females revealed 1224 genes at maximum lifetime expression (Fig. 3). This lifetime pattern was quite similar to that of eigengene 1 (Fig. 2). Numbers of functionally annotated clusters identified in DAVID (Huang *et al.* 2009) in each life stage were strongly correlated with numbers of *D. mojavensis* genes with *D. melanogaster* orthologues (Pearson $r = 0.95$, $t = 10.09$, $P < 0.0001$). Here, the average proportion ± 1 SD of annotated genes was 0.64 ± 0.18, with a range from 0.37 in early pupae to 0.80 on the day of eclosion (Table 3).

Annotation clustering of genes with maximal lifetime expression levels uncovered the largest number of functional terms in the 6-h embryo stage (Table 3), in part because 79% of these early developmental genes were annotated. A diverse set of gene clusters involved with development, segmentation, nucleic acid metabolism, oogenesis, cellular metabolism, negative and positive regulation of biosynthesis and transcription, mitosis, morphogenesis and imaginal disc development were significantly enriched. That meiotic gene expression enriched in embryos has been previously observed (Mukai *et al.* 2006) and was due to genes associated with meiotic chromosome segregation, microtubule binding and cell cycle dynamics (Table 3).

The transition from 6-h embryo to first instar revealed a precipitous decline in the numbers of genes with maximal expression, the proportion of genes with significant changes in expression from the embryo stage (Figs 3 and 4) and numbers of enriched gene clusters of diverse function (Table 3). The most enriched gene cluster in the first instar stage was associated with formation of the peritrophic membrane, a lining of a specialized extracellular matrix in the gut, indicating significantly increased expression of genes associated with feeding and digestion. Other enriched clusters included those annotated for ribosome assembly, increased metabolism and development (Table 3). Genes that increased in expression from embryo to first instar stages were significantly enriched for functional clusters with membrane, chitin, cuticle and a number of other metabolic pathways and sensory systems associated with larval development (Table S6A, Supporting information). This transition was also characterized by significant decreases in expression of many of the embryonic gene clusters with maximal gene expression (Table 3). Thus, the embryo to larval transition involved the largest downregulation of genome-wide expression across the life cycle in *D. mojavensis*.

Maximum expression of second and third instar larval genes was enriched for similar functional clusters associated with growth and development (Table 3). Membrane receptor function, HOX gene regulation and cuticle formation gene clusters were at maximum expression levels in second instar larvae accompanied by significantly increased transcription of cellular respiration, energy production and fatty acid metabolism

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Table 3 Functional annotation clustering of genes with maximum lifetime expression levels in two populations of *D. mojavensis* using DAVID. Numbers of transcripts having their maximum expression at each life stage are indicated with the number of *D. melanogaster* orthologs (in parentheses) used for GO clustering. FDR *P* values associated with top GOterms are indicated. L1 = first instar, L2 = second instar, L3 = third instar, EP = early pupae, LP = late pupae, 0 D = adult day of emergence, 3 D = 3 day old adults, etc. See text for details

Life stage									
Embryo 6 h *n* = 2999 (2363)		L1 *n* = 658 (432)		L2 *n* = 893 (345)		L3 *n* = 1182 (451)		EP *n* = 1641 (602)	
GOTerm	Enrich score	GOTerm	Enrich score	GOTerm	Enrich score	GOTerm	Enrich score	GOTerm	Enrich score
1. RNA splicing, processing	22.6****	1. peritrophic membrane structure, chitin binding	4.4****	1. G-protein coupled peptide receptor activity	4.2****	1. G-protein coupled receptor, sensory perception	12.3****	1. cuticle structure	4.2****
2. nuclear lumen, nucleoplasm	19.6****	2. signal peptide	3.1****	2. HOX, DNA binding	3.0****	2. detection of chemical stimulus	6.5****	2. salivary gland histolysis	3.6***
3. DNA binding, transcription regulation	19.3****	3. cell adhesion	2.6*	3. membrane receptor	2.7****	3. sodium channel activity	5.6****	3. chitin binding	2.7**
4. Chromosome	16.1****	4. proton transport ATPase	2.6****	4. carbohydrate binding	2.1**	4. peptide receptor activity	2.5*	4. neuron development	2.6*
5. Down regulation of gene expression	13.5****	5. transmembrane function	2.6**	5. cuticle structure	1.7*	5. ligand-gated channel activity	2.5****	5. Serine protease	1.8**
6. Mitosis, cell cycle	12.5****	6. ribosome structure	2.5****	6. serine-type peptidase activity	1.7*	6. chitin binding	2.3**	6. dynein complex	1.7
7. Embryonic morphogenesis	10.5****	7. procollagen-proline dioxygenase activity	2.0**					7. cell adhesion	1.7*
8. Chromatin organization/ modification	8.8****	8. neurotransmitter, gated channel activity	1.9**						
9. Up regulation of gene expression	8.1****								
10. Oogenesis	7.9****								
11. Transcription factor binding	7.9****								
12. DNA replication	6.9****								
13. DNA repair	6.2****								

Table 3 *Continued*

Life stage									
Embryo 6 h *n* = 2999 (2363)		L1 *n* = 658 (432)		L2 *n* = 893 (345)		L3 *n* = 1182 (451)		EP *n* = 1641 (602)	
GOTerm	Enrich score	GOTerm	Enrich score	GOTerm	Enrich score	GOTerm	Enrich score	GOTerm	Enrich score
14. Neuron differentiation, morphogenesis	5.8****								
15. Ribosome biogenesis	5.5****								
16. Nucleocytoplasmic transport	5.3****								
17. RNA helicase activity	4.9****								
18. DNA packaging, nucleosome assembly	4.8****								
19. Meiosis	4.8****								
20. cell migration, motion	4.7****								
21. compound eye development	4.7****								
22. Nucleotidyl-transferase activity	4.5****								
23. Stem cell maintenance	4.4****								
24. Imaginal disc development	4.1****								
25. Gene silencing	3.5****								
26. Embryonic segmentation	3.4****								

Life stage									
LP *n* = 1886 (818)		0 day *n* = 1947 (1562)		3 day *n* = 896 (613)		6 day *n* = 279 (213)		10 day *n* = 107 (79)	
GOTerm	Enrich score	GOTerm	Enrich score	GOTerm	Enrich score	GOTerm	Enrich score	GOTerm	Enrich score
1. cuticle structure	3.1***	1. cellular respiration	20.6****	1. fatty acid biosynthesis	5.8****	1. cofactor binding	2.5****	1. ribosome, translation	2.8****
2. microtubule cytoskeleton	2.6****	2. transit peptide: mitochondrion	6.6****	2. iron ion binding	5.0****	2. vitamin binding	2.3****		
3. ion channel activity	2.2***	3. TCA cycle	6.2****	3. cofactor binding, acyl-CoA DH activity	4.3****	3. steroid metabolism	2.3****		

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Table 3 *Continued*

Life stage									
LP *n* = 1886 (818)		0 day *n* = 1947 (1562)		3 day *n* = 896 (613)		6 day *n* = 279 (213)		10 day *n* = 107 (79)	
GOTerm	Enrich score	GOTerm	Enrich score	GOTerm	Enrich score	GOTerm	Enrich score	GOTerm	Enrich score
4. muscle development	1.8****	4. photo-transduction, light detection	5.5****	4. hexose metabolism	4.0****	4. Golgi vesicle membrane	1.9***		
5. membrane transport	1.8**	5. ion transport	4.9**	5. transaminase activity	3.8***	5. membrane, endocytosis	1.8*		
6. mitochondrial membrane	1.5	6. ATP metabolic process	4.6****	6. glycosidase, lysosome	3.8****	6. oogenesis	1.8		
7. neuropeptide binding	1.5*	7. mitochondrial electron transport	4.4****	7. signal peptide secretion	3.1***				
		8. adult locomotory behavior	4.3****	8. carboxylic acid catabolism	3.0***				
		9. membrane transport	4.2****	9. glutathione transferase activity, P450	2.9***				
		10. proteasome accessory complex	3.7****	10. galactose metabolism	2.9**				
		11. glycolysis, alcohol metabolism	3.7****	11. unsaturated FA synthesis	2.9***				
		12. synaptic transmission	3.3****	12. oxidoreductase activity, AA metabolism	2.3***				
		13. glycogen biosynthetic process	2.6***						
		14. ion transport	2.5****						
		15. axon projection	2.2**						

14 day *n* = 67 (50)		18 day *n* = 1224 (997)		24 day *n* = 405 (316)	
GOTerm	Enrich score	GOTerm	Enrich score	GOTerm	Enrich score
1. ribosome, translation	1.2	1. DNA repair	7.8****	1. peroxisomal membrane	3.0****
2. oogenesis	1.1	2. zinc finger, ion binding	7.1****	2. translation, ribosome	3.0****
		3. ATP binding	4.3****	3. protein transport	2.1*
		4. chaperonin, protein folding	3.6***		
		5. mitosis	2.5**		
		6. Golgi apparatus	2.2***		

Table 3 *Continued*

14 day n = 67 (50)		18 day n = 1224 (997)		24 day n = 405 (316)	
GOTerm	Enrich score	GOTerm	Enrich score	GOTerm	Enrich score
		7. phosphoinositide binding	2.1***		
		8. apoptosis regulation	2.1*		
		9. protein transport	2.1**		
		10. DNA helicase activity	2.0**		
		11. meiosis	2.0*		
		12. recombination	1.8		
		13. deoxyribonuclease activity	1.7*		
		14. oocyte development	1.7**		

FDR *P < 0.05, **P < 0.01, ***P < 0.005, ****P < 0.0001.

genes as in first instar larvae (Table S6A), while expression of cell division and DNA repair genes significantly declined. Third, instar larvae showed enrichment for genes at maximum expression for sensory perception of chemical stimuli and increased membrane receptor activity (Table 3) with decreased expression of metabolic pathways including energy, sugar, amino acid, lipid and P450 metabolism (Table S6A). Decreased transcription of endoplasmic reticulum genes and increased expression of cuticle structure, fat body-associated ADH and odorant-binding genes were consistent with the continued trajectory of increasing larval growth and size prior to pupation.

From first instar to late pupae, the numbers of genes at maximum expression increased (Fig. 3) while the fractions of annotated genes at maximum expression levels decreased from 0.66 (432/658) in first instars to as low as 0.37 (602/1641) in early pupae (Table 3), suggesting increases in expression of lineage-specific *D. mojavensis* genes, that is those with no *D. melanogaster* orthologues, during late preadult development. The third instar to early pupa transition revealed a drastic reduction in metabolic rates where mitochondrial, oxidative phosphorylation, citric acid cycle, and sugar, lipid and amino acid metabolism genes showed significant decreases in expression (Table S6A). DAVID also identified a gene cluster with 54 annotated *D. melanogaster* orthologues enriched for spermatogenesis that was significantly upregulated from L3 to EP (Tables S6A and S7, Supporting information) consistent with the known timing of testis development in *D. melanogaster* (Cooper 1950).

Significantly increased transcription of gene clusters enriched for mitochondrial and aerobic respiration function, the TCA cycle and glycolysis (Table S6A) was consistent with increases in metabolic rates in late pupae (Merkey *et al.* 2011). Increased expression of flight muscle genes (Fernandes *et al.* 1991; DeSimone *et al.* 1995) and associated mitochondrial genes, as well as gene clusters enriched for glycolysis, were accompanied by significant decreases in transcripts associated with DNA replication and RNA processing, DNA repair, sensory perception and steroid synthesis.

Almost 2000 genes were at maximum expression levels on the day of eclosion that accounted for 15 significantly enriched gene clusters, second only to the diversity of genes expressed in 6-h embryos across the entire life cycle (Fig. 3, Table 3). Highly significant GO-terms included cellular respiration, mitochondrial and TCA cycle function, vision, adult movement and other metabolic functions (Table 3). Eighty per cent of genes showing increased expression from late pupa to day of emergence were annotated and were functionally enriched for a number of metabolic functions including

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membrane transport, ATP binding, protein transport and catabolism, mitochondrial function and biogenesis, growth and others (Table S6A). Upregulation of fatty acid metabolism was also apparent in KEGG pathway analysis (Kanehisa & Goto 2000). The transition from late pupae to emergence was characterized by decreases in cytoskeleton formation, ion transport, peptidase activity and KEGG pathways involving carbohydrate and glutathione metabolism, and oxidative phosphorylation. An annotated cluster of 21 taste and olfactory receptor genes showed significant decreases in expression from late pupa to eclosion (Table S6A). This was a subset of the 94 sensory, taste and olfactory orthologues that were significantly upregulated from egg to first instar (Table 2). Thus, the pupa–adult transition involved a large decrease in expression of sensory genes that were upregulated in early larval stages.

From eclosion into adulthood, far fewer genes were expressed at maximum lifetime levels, except in day 18 adults, and there was a corresponding decrease in the numbers of genes showing significant decreases/increase in expression between sampling points (Fig. 3, Table S6). In three-day-old adults, genes at maximum lifetime expression were functionally enriched for diverse metabolic functions including fatty acid metabolism, iron ion binding, sugar metabolism, carboxylic acid catabolism and P450 activity (Table 3), and there were significant increases in gene expression for DNA replication, cell division, ribosome manufacture, egg production and DNA repair (Table S6A). In six-day-old adults, fewer genes were at maximal expression (Fig. 3) and these were enriched for vitamin and cofactor binding, steroid hormone manufacture and oogenesis. Three to six days is approximately the age at first reproduction for *D. mojavensis* females depending on temperature and nutrition (Markow 1982; Etges & Klassen 1989). Also seen in the transition from three- to six-day-old adults were 621 genes associated with oogenesis, meiosis, cell division, DNA repair and downregulation of metabolism, as well as decreased expression of cuticle formation, immune response, melanin metabolism, sugar transport and muscle development genes (Table S6A), suggesting decreasing gene expression associated with somatic maintenance with the onset of female reproduction.

From the 6- to 10-day and 10- to 14-day intervals, there were continuing decreases in expression for cuticle gene expression, immune response, melanin metabolism and muscle formation, and few significant increases in gene expression. Sixty-seven genes were at maximal expression levels at day 14 (Fig. 3) that were enriched for translation and oogenesis (Table 3). A larger number of genes, 1224, were at maximal lifetime expression at day 18 that were enriched for genes associated with ageing including DNA repair, protein chaperones, DNase activity and apoptosis regulation, as well as control of gene regulation and oocyte development. Significant decreases in expression from 14 to 18 days involved gene clusters enriched for signal peptides, lipid synthesis, microsome-associated iron binding, immune response and a number of other cellular catalytic functions (Table S6A). Many of these same gene clusters were then upregulated from 18 to 24 days (Fig. 2), including antimicrobial peptides, immune response, lipid metabolism and microsome-associated iron binding, as well as amino acid metabolism, glutathione metabolism (KEGG), and P450 activity suggesting further regulatory changes associated with ageing, increased oxidative stress and immune response to microbes (Table 3, Table S6A). This "late life" transition in gene expression from 14 to 18 to 24 days was also observed in both eigengenes 2 and 3 (Fig. 2).

Assessing differential expression between 'young' and 'old' adults (3–6 day vs. 18+ day) revealed changes consistent with other studies of ageing (see de Magalhães *et al.* 2009 for a review). In 'old' samples, there was increased gene expression in DNA repair, DNA replication, stress response, mitosis and meiosis, and decreased expression of electron transport chain, muscle development, signalling and transport, hormone binding and locomotor genes (Table S6B). However, as seen above, this simple young–old comparison missed the nonmonotonic trajectories of expression through adulthood, particularly the 'late life' transitions observed between ages 14 and 18, and 18 and 24 (Fig. 2).

Host cactus effects on gene expression across the life cycle

Both host cactus and population effects influenced preadult stage-specific patterns of gene expression (fold change >1.5 × and FDR $P < 0.05$). From the embryo stage to eclosion, there were significant differences between these two populations in the timing of differentially expressed genes due to host cactus (Table 4). Variation in egg-to-adult development time and viability in this experiment (Tables S8 and S9; Fig. S1, Supporting information) was consistent with previous studies, so transcriptional variation here should help to identify causes of cactus-influenced shorter development times and higher viabilities of Baja populations vs. those on the mainland (Etges 1990; Etges *et al.* 2010). Cactus rearing substrates caused expression levels to differ in first and second instar stages in the mainland population, but in the Baja California population, most transcriptional differences due to cactus occurred in early and late pupal stages (Table 5; Table S10, Supporting information). There were no differences in the

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Table 4 Total numbers of genes differentially expressed (fold change >1.5 × and FDR < 0.05) due to cactus rearing substrates in preadult stages, from egg to eclosion, in two populations of *D. mojavensis* reared on two cactus hosts. Contrasts indicate numbers of genes overexpressed due to organ pipe (OP) vs. agria (AG) cactus, OP>AG, and vice versa. Life stages are eggs (6 h), L1 – first instar larvae (48 h), L2 – second instar larvae (144 h), L3 – third instar larvae (240 h), EP – early pupae (288 h), LP – late pupae (384 h) and 0D – adults at day of eclosion

Life stage														
	Egg		L1		L2		L3		EP		LP		0D	
Population Las Bocas*	OP>AG	AG>OP	OP>AG	AG>OP	OP>AG	AG>OP	OP>AG	AG>OP	OP>AG	AG>OP	OP>AG	AG>OP	OP>AG	AG>OP
D. mojavensis genes	—	—	570	631	61	15	—	3	3	2	—	—	1	—
D. melanogaster orthos	—	—	222	529	53	9	—	3	1	2	—	—	0	—
Proportion annotated	—	—	0.389	0.838	0.869	0.6	—	1	0.33	1	—	—	0	—
Average % annotated	0.637													
	Egg		L1		L2		L3		EP		LP		0D	
Population Punta Prieta#	OP>AG	AG>OP	OP>AG	AG>OP	OP>AG	AG>OP	OP>AG	AG>OP	OP>AG	AG>OP	OP>AG	AG>OP	OP>AG	AG>OP
D. mojavensis genes	4	4	1	4	—	—	—	—	15	21	213	132	—	4
D. melanogaster orthos	0	1	0	4	—	—	—	—	2	14	137	59	—	3
Proportion annotated	0	0.25	0	1	—	—	—	—	0.13	0.67	0.643	0.447	—	0.75
Average % annotated	0.553													

*mainland Sonora.
#Baja California.

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Table 5 Gene ontology results for predicted genes that were differentially expressed (>1.5 fold difference, FDR $P < 0.05$) from the egg stage to day of eclosion in two populations of *D. mojavensis* reared on agria and organ pipe cacti. Life stages are defined in Table 3 and individual genes and their *D. melanogaster* ortholog names are listed when only several genes differed in expression and no functional clustering was observed in DAVID (Huang *et al.* 2009)

Population								
	Las Bocas (mainland)				Punta Prieta (Baja California)			
	OP>AG		AG>OP		OP>AG		AG>OP	
	GOTerm (gene)	Enrich score	GOTerm (gene)	Enrich score	GOTerm (gene)	Enrich score	GOTerm (gene)	Enrich score
Egg	—	—	—	—	—	—	GI22080 – Cep78 protein	—
L1	1. cuticle, chitin structure	5.9****	1. transport	5.9****	—	—	GI24491 – Rheb	—
			2. cell metabolism	4.3****			GI24142 – Rab–protein 7 – GTPase mediated signal transduction	
	2. Odorant binding, olfactory reception	2.1***	3. protein transport	3.5****				
			4. OXPHOS process/ ion transport	3.4****				
			5. mitotic spindle/ ribosome protein	3.3****			GI10537 – alternative testis transcripts open reading frame A	
			6. cell morphogenesis	3.0***				
			7. RNA binding	3.0**				
			8. larval development	2.9**			GI17029 – split ends – postembryonic morphogenesis	
			9. actin filament processes	2.9**				
			10. protein synthesis	2.5**				
			11. RNA splicing	2.5*				
			12. axon development	2.2**				
			13.mitochondria transport peptide	2.1**				
			14. germ cell develop.	1.8**				
L2	1. cytoskeleton, ribosomal protein	1.1	1. metal ion binding	0.5	—	—	—	—
L3	—	—	GI16482 – ribosomal protein L40 GI12755 – Rh50 (membrane transport)	—	—	—	—	—

Table 5 *Continued*

Population								
	Las Bocas (mainland)				Punta Prieta (Baja California)			
	OP>AG		AG>OP		OP>AG		AG>OP	
	GOTerm (gene)	Enrich score	GOTerm (gene)	Enrich score	GOTerm (gene)	Enrich score	GOTerm (gene)	Enrich score
			GI11050 – yu (ptn and RNA binding)					
EP	GI11311 – GNBP2 (Gram-negative bacteria binding protein 2) GI24782+ GI12221+	—	GI18191 – fbp (fructose–1,6–bisphosphatase) GI15376 – mesoderm development	—	GI24313 – Cpr30F (pupal chitin structure) GI19286		1. ubl conjugation pathway, proteolysis	2.0 *
LP	—	—	—	—	1. mitochondrial membrane	1.6	1. cuticle structure	1.2
0D	GI23541+	—	—	—	—	—	GI16633 – heat shock ptn/metal ion binding GI24251 – nucleotide binding GI16531 – Dpy-30 (adenylate kinase) GI19042	—

False Discovery rate *P* values, **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001.
[+]gene of unknown function.

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numbers of genes showing significant up/down transcription differences due to agria or organ pipe substrates (paired $t = 0.66$, $P > 0.05$), and the average proportion of predicted *D. mojavensis* genes with *D. melanogaster* orthologues that were influenced by rearing substrates for these two populations ranged from 55% to 64%.

While just eight genes in 6-h embryos showed significant expression differences due to cactus (Table 5), just GI22080 was annotated, a Cep78 homolog, a centrosomal protein (The-UniProt-Consortium 2011) that showed increased transcription due to agria cactus (Table 5). A larger number of orthologues in first ($n = 1201$) and second ($n = 76$) instar larvae were differentially expressed in this mainland population than in the Baja population due to cactus substrates. Organ pipe cactus caused increased transcription of cuticle and odorant binding-related orthologues in first instar larvae, and moderate increases in expression in genes associated with cytoskeleton and ribosome function in second star larvae compared with agria-reared larvae. Agria cactus caused greater expression of a broad range of significantly enriched genes in first instar larvae associated with growth and development including protein transport, cell division and ion transport than organ pipe cactus (Table 5). Just four genes of diverse function in Baja California first instar larvae were significantly overexpressed due to agria cactus, and few third instar genes showed any effect of cactus on expression levels. Of the 381 early and late pupal genes showing expression differences due to cactus, significant enrichment for ubiquitin conjugation function (proteolysis) genes was observed, as well as genes responsible for cuticle structure and mitochondrial membrane function. Thus, agria cactus caused increased expression of a broader spectrum of genes in different parts of the preadult life cycle than organ pipe cactus, particularly those associated with early larval development and metabolism, but the overall number of genes with significantly different levels of expression influenced by cactus was small.

For adults, samples were pooled across ages and variation in expression levels was assessed with a mixed model ANOVA with population, cactus and population X cactus effects where cactus was a fixed effect. Organ pipe cactus caused increased expression of genes enriched for neurotransmitter binding, circadian rhythm, and courtship and mating behavioural functions. Mainland females showed significantly increased expression of genes enriched for fatty acid metabolism genes and iron binding functions, such as P450 genes associated with xenobiotic detoxification. Baja females had higher overall expression of genes associated with transcription than mainland females (Etges 2014). Overall, these patterns of differential gene expression in populations of *D. mojavensis* were influenced by cactus rearing substrates in both pre-adult and adult stages, where different cactus substrates influenced expression of a greater number of orthologues in preadult stages than in adults.

Discussion

The holometabolous life cycle of *D. mojavensis* is marked by major transitions in the expression of functional clusters of genes similar to those in *D. melanogaster* (Arbeitman *et al.* 2002; Graveley *et al.* 2011; Tennessen & Thummel 2011). SVD captured a portrait of gene expression throughout pre-adult development and adult ageing in *D. mojavensis* expressed in environments designed to simulate natural conditions, yet these eigengenes were not overly sensitive to population or host cactus differences. This suggests that population origin and cactus substrates influenced expression for relatively small numbers of genes in relation to the major developmental transitions in gene expression, that is the life cycle transcriptome of *D. mojavensis* is relatively well buffered from differences in its host plants and has yet to become strongly geographically differentiated.

Population and cactus differences have previously been shown to influence both egg-to-adult development time and average longevity in adults in *D. mojavensis* (Etges 1990; Jaureguy & Etges 2007). Our sampling from egg to eclosion was stage based, not age based, and so would not reveal expression differences related to rate of development. Our sampling of adults was necessarily destructive, so it was not possible to infer whether gene expression levels of adults at different ages were related to their ultimate lifespans.

However, our experimental design included just four replicate samples at each age/stage with often surprisingly high within-age variance, so perhaps some influences of these environmental factors may be distinguishable with increased replication. The top four eigengenes revealed three major transitions, that is, from embryos to larvae, larvae to pupae and pupae to adults (Fig. 2). Thus, the latent patterns of biological organization and function revealed in eigengene analysis (Alter 2006; Ponnapalli *et al.* 2011) of the variation in lifetime gene expression occurred between, rather than within life stage types, and were uncovered through functional gene ontology clustering.

All four eigengenes were significantly influenced by variation in ribosome function and protein production (Table 1), suggesting that life cycle SVD analyses, when used to compare patterns of life gene expression in this and other organisms, may reveal fundamental insights

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into the general processes of development and senescence. Eigengene 1 encompassed most of overall transcriptional variation (63.5%) due to increased expression of larval and pupal endopeptidases, embryonic and adult protein production and transport, as well as sensory perception (Fig. 2, Tables 1 and 3, Table S6). While biased due to the lack of annotation for ca. a third of predicted genes, life cycle shifts in protein metabolism and tissue remodelling were not surprisingly major sources of variation in lifetime gene expression.

In addition, a large number of gustatory, odorant binding, olfactory receptor, ion transport and photoreceptor gene orthologues that increased in expression from embryo to first instar larvae and then were downregulated in adults were also highly correlated with eigengene 1 and revealed in comparisons of consecutive life cycle stages (Table 2). While adult sensory perception has been intensively studied because of its roles in chemical, host plant attraction/repulsion, and adult mating behaviour (Carlson 1996; Amrein 2004; Olsson *et al.* 2006; Stokl *et al.* 2010; Thistle *et al.* 2012), sensory perception in pre-adult stages has been less well studied, but is known to be a determinant of successful larval feeding behaviour, growth and attainment of body critical mass prior to pupation (Beadle *et al.* 1938; Tennessen & Thummel 2011). The functional consequences of sensory genes across the life cycle in *Drosophila* species have been documented (Cobb 1999; c.f. de Belle *et al.* 1989; Gerber & Stocker 2007; Kent *et al.* 2009; Matsuo *et al.* 2007), but rarely in flies reared under natural conditions. In larvae, foraging behaviours are facilitated by chemical perception (Fishilevich *et al.* 2005) and thus resource acquisition during larval development. While *D. mojavensis* larval behaviour in the wild has not been well studied (but see Fogleman *et al.* 1981), our results suggest that further study of the expression and evolution of these sensory gene families may help to unravel sensory behaviour variation in nature and how it is related to resource exploitation, that is the cactus-influenced preadult life history differences between Baja California and mainland populations (Etges 1990, 1993; Etges *et al.* 2010). These patterns were far more subtle in the stage-specific GO clustering analyses (Tables 1 and 2) exemplifying the utility of SVD, and also emerged in analyses of host cactus effects (Tables 4 and 5).

Most emphasis on understanding host cactus preferences and subsequent larval and adult performance in desert *Drosophila* has been on production of and attraction to cactus fermentation by-products (Starmer *et al.* 1977; Starmer 1982; Etges & Klassen 1989; Newby & Etges 1998; Fanara *et al.* 1999), xenobiotic metabolism of cactus secondary compounds (Fogleman & Heed 1989; Fogleman *et al.* 1998; Matzkin 2008) and host cactus resource availability (Heed & Mangan 1986; Etges 1990; Etges *et al.* 2010). For *D. mojavensis*, use of agria and organ pipe cacti in the Sonoran Desert is due largely to its tolerance of medium sized fatty acids [C_6–C_{18}, but most are C_{10}–C_{12} (Fogleman & Kircher 1986)], sterol diols and high levels of triterpene glycosides. It can also tolerate the isoquinoline alkaloids present in the rarely used alternate hosts saguaro, *Carnegiea gigantea*, and cardón, *Pachycereus pringlei*, cacti (Fogleman & Danielson 2001), but secondary compounds of other alternate hosts, for example California barrel cactus, *Ferocactus cylindraceus*, sina cactus, *S. alamosensis*, cochal cactus, *Myrtillocactus cochal* in Baja California, and *Opuntia* species on Santa Catalina Island have not been as intensively studied. While differences between agria and organ pipe cacti on overall gene expression were sometimes small, there were significant pre-adult stage-specific differences in gene expression between populations (Table 5) and population and cactus effects on adult gene expression (Etges 2014). There was little evidence of cactus-induced differences in expression of detoxification genes in pre-adult stages (Table 5), but there was significantly greater enrichment of P-450 genes in adult mainland females reared on organ pipe cactus. Thus, larvae were less sensitive to differences in cactus secondary compounds than adults, perhaps helping to explain genetic evidence for host plant generalism in larval performance in *D. mojavensis* (Etges 1993).

Other ecological aspects of cactus rots influencing larval growth and development involving sensory perception include selective foraging and predator/parasite avoidance. Larval *D. mojavensis* prefer particular yeast species over others in naturally occurring rots, so larval olfactory and gustatory receptors are likely to be directly involved with foraging preferences (Fogleman *et al.* 1981). In addition to bacteria and yeasts, cactus rots comprise a complex fermenting environment of degraded cactus tissues, secondary compounds, volatiles and other invertebrates as rots progress from early bacterial fermentation, but interactions between these organisms and drosophilids have only been partially assessed (Mangan 1979; Escalante & Benado 1990; Polak 1998; Kiontke *et al.* 2011). Thus, understanding patterns of gustatory, odorant binding, olfactory receptor and photoreceptor gene expression throughout the life cycle in *D. mojavensis* may contribute to our general understanding of patterns of resource use, life history variation and host plant adaptation in natural populations of *Drosophila*.

Expression of life histories in contrasting environments

Central to a general understanding of life history evolution are the consequences of lifetime differences in

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environmental variability on survivorship and reproduction, and uncovering the environment-dependent expression of genetic variation underlying these components of fitness. Genetic differences in life histories between Baja California and mainland populations of *D. mojavensis* are host plant dependent, and thought to be influenced by differences in resource predictability at different stages of the life cycle (Heed 1978, 1981; Etges 1990, 1993; Etges *et al.* 1999). Cactus substrate-influenced development time differences between populations (Fig. S1, Table S8) were accompanied by larval, stage-specific differences in gene expression (Tables 4 and 5). Consistent with the increased development times of organ pipe-reared Las Bocas (mainland) flies and a Population X Cactus interaction, organ pipe-reared first instar larvae were enriched for 14 downregulated gene clusters associated with larval development and metabolism. A handful of annotated orthologues were also downregulated in the Punta Prieta, Baja California population due to organ pipe cactus, including GI17029, a *D. melanogaster* orthologue of *split ends*, involved in nucleic acid binding and postembryonic development (Table 5). Thus, decreased expression of developmental genes due to organ pipe cactus and increased expression of larval cuticle and olfactory reception genes in first instar larvae (Tables 4 and 5), suggests longer mainland development times result in part from transcriptional events early in larval development. Just a few gene clusters were functionally enriched for proteolysis associated with metamorphosis including the ubl conjugation pathway, and cuticle structure, where organ pipe cactus again caused reduced transcription levels in Baja flies. Several of these functional clusters including genes responsible for nucleic acid binding, cuticle proteins, and larval growth and metabolism were correlated with a trade-off between larval mass and survival in *D. melanogaster* (Bochdanovits & de Jong 2004), suggesting there may be a shared genetic basis for pre-adult growth rates in these species.

In adults, co-expression of genes associated with ageing and age-specific reproduction was revealed by different eigengenes, patterns of maximum lifetime gene expression (Table 3) and in pairwise comparisons between adjacent ages (Table S6). From a positive eigengene 2 peak at eclosion through 18–24 days (Fig. 2), there was a monotonic shift from eclosion onwards reflecting shifts in neural functioning, cellular maintenance, metabolic rates and P450 activity through adulthood (Table S2). Also at day 18, there were 14 significantly enriched gene clusters based on genes at maximum lifetime expression levels (Table 3), most that were associated with ageing-related traits, patterns strikingly similar to those in *D. melanogaster* and *Caenorhabditis elegans* (McCarroll *et al.* 2004). After 18 days, there was significantly increased expression of five gene clusters enriched for antimicrobial peptides, immune response, lipid metabolism, membrane function and further P450 activity (Table S6) similar to replicate lines of *D. melanogaster* selected for late life reproduction (Remolina *et al.* 2012). The noticeable late life shift in eigengene 2 and 3 expression at 18 days was due in part to increased expression of genes responsible for DNA repair, protein chaperones, signal transduction, ATP production, apoptosis and others. Thus, *D. mojavensis* females at ~3 weeks of age reared on fermenting cactus exhibited transcriptional shifts associated with physiological signs of increased cellular maintenance and protection from microbes and harmful chemical compounds.

A classic life history trade-off between somatic maintenance and reproduction was evident in decreases in gene expression associated with somatic maintenance with the onset of female reproduction. At the onset of sexual maturity at 3–6 days (Table S6A), increased expression of 621 genes were functionally enriched for reproduction and DNA repair and showed decreased expression of cuticle formation, immune response, melanin metabolism, sugar transport and muscle development genes (Table S6A). However, there was little evidence for downregulation of genes associated with egg production as in *D. melanogaster* where decreases in transcript abundance of chorion formation genes with increasing age have been observed (Pletcher *et al.* 2002). Likely explanations for this are as follows: (i) *D. mojavensis* females cultured on fermenting cactus and ethanol vapour rarely live more than 30 days and so may not reach reproductive senescence vs. the 60+ day survivorship of *D. melanogaster* cultured on artificial media; and (ii) our adult female *D. mojavensis* were unmated, so it is unlikely that we would expect to observe realistic lifetime shifts in expression of gene clusters associated with mating and egg production because female longevity, fecundity and metabolism are significantly influenced by mating and remating (Markow *et al.* 1990; Etges & Heed 1992).

Conclusions

Comparative life cycle studies of genomic expression in different organisms are imperative for characterizing the genetic architecture and ontogeny of gene expression responsible for the life history variation we seek to understand. Only then can we evaluate the expression of genomic elements responsible for fitness trade-offs and senescence in relation to phenotypic variation in life histories. Despite the limitations of genome annotation for most nonmodel species, SVD analysis

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successfully resolved many of the major developmental and adult shifts in the expression of correlated groups of genes from embryogenesis through senescence in this model insect. Ideally, future whole-genome expression SVD studies should involve direct comparisons of the same life stages and ages under controlled environmental conditions. Although few whole-genome studies assessing such life cycle variation have been performed under natural conditions for comparison, the transcriptome of *D. mojavensis* reared on two of its major host cacti throughout its life cycle has revealed similar core developmental transitions to those in *D. melanogaster*. However, there remains a significant fraction of the genome that is still unknown due to limited gene annotation, much that is necessary for understanding subtle expression differences due to population or host plants (Table 5). This will limit future comparative studies whether microarrays or other transcriptome methods are used.

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W.J.E., A.G.G. and S.T. designed the study. C.C.O. and W.J.E. carried out the experiments, and S.R. and A.G.G. facilitated microarray processing and data acquisition. Final data analyses were performed by M.V.T., S.T., A.G.G. and W.J.E. W.J.E. wrote the manuscript with input from M.V.T. and S.T. All authors read and approved the final version of the manuscript.

Data accessibility

MIAME compliant microarray data have been deposited at Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) with Accession no. GSE54510.

Supporting information

Additional supporting information may be found in the online version of this article.

Fig. S1. A. Egg to adult development time of male and female *D. mojavensis* from Punta Prieta, Baja California, and Las Bocas, Sonora reared on agria and organ pipe cacti expressed as deviation form the overall grand mean development time. B. Egg to adult viability, *ibid*. Letters over each bar denote significant differences between groups, $P < 0.05$.

Table S1. Numbers of microarrays included in this study for each stage/age, population, and cactus. Stage/age designations are defined in the text.

Table S2. Gene ontology clustering results from DAVID (Huang *et al*. 2009) for positive and negative loadings of the top 750 genes associated with each of the four eigengenes identified in this study. See text for details.

Table S3. Functional enrichment of genes associated with epigenetic control of gene expression negatively associated with eigengene 2.

Table S4. Lists of genes with maximum levels of expression over the life cycle. Gene names are *D. melanogaster* orthologues.

Table S5. Lists of genes showing significant increases or decreases in expression levels across adjacent life cycle stages and ages. #N/A indicates there is no orthology with genes in *D. melanogaster*.

Table S6. A. Stage and age-specific up and down regulated functional gene clusters. B. Up and down regulated functional gene clusters in 'old' (age 18+ days) adults as compared to 'young' adults (ages 3 and 6 days).

Table S7. Identified gene orthologs up-regulated from L3 to EP in *D. mojavensis* with spermatogenesis function.

Table S8. ANOVA of egg to adult development time for two populations of *D. mojavensis* reared on agria and organ pipe cactus.

Table S9. ANOVA results for egg to adult viability, numbers of dead embryos (brown eggs), and unhatched eggs for two populations of *D. mojavensis* reared on agria and organ pipe cactus.

Table S10. Gene lists and functional GO clustering results for orthologues showing significant expression differences due to host cactus in pre-adult stages.

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