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Functional genomic and phenotypic responses to desiccation in natural populations of a desert drosophilid

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Abstract

We used whole-transcriptome microarrays to assess changes in gene expression and monitored mortality rates and epicuticular hydrocarbons (CHCs) in response to desiccation stress in four natural populations of *Drosophila mojavensis* from Baja California and mainland Mexico. Desiccation had the greatest effect on gene expression, followed by biogeographical variation at regional and population levels. Genes involved in environmental sensing and cuticular structure were up-regulated in dry conditions, while genes involved in transcription itself were down-regulated. Flies from Baja California had higher expression of reproductive and mitochondrial genes, suggesting that these populations have greater fecundity and higher metabolic rates. Host plant differences had a surprisingly minor effect on the transcriptome. In most cases, desiccation-caused mortality was greater in flies reared on fermenting cactus tissues than that on laboratory media. Water content of adult females and males was significantly different and was lower in Baja California males. Different groups of CHCs simultaneously increased and decreased in amounts due to desiccation exposure of 9 and 18 h and were population-specific and dependent on larval rearing substrates. Overall, we observed that changes in gene expression involved a coordinated response of behavioural, cuticular and metabolic genes. Together with differential expression of cuticular hydrocarbons, this study revealed some of the mechanisms that have allowed *D. mojavensis* to exploit its harsh desert conditions. Certainly, for *D. mojavensis* that uses different host plants, population-level understanding of responses to stressors associated with future climate change in desert regions must be evaluated across geographical and local ecological scales.

Keywords: cuticular hydrocarbons, desert, desiccation, gene expression, genome, host plant, microarray, transcriptome

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Introduction

More than 30% of the earth's landmass consists of arid or semi-arid areas (Okin *et al.* 2006). Desertification is expected to increase as a result of global climate change and anthropogenic influence (Karl & Trenberth 2003). There is therefore a critical need to understand how organisms will respond to these environmental changes, and the study of desert species constitutes an important first step towards that goal. Many studies have examined the physiology and ecology of desert organisms, but our understanding of genetic and genome-level adaptation to deserts is minimal. The only desert organism whose genome has been sequenced so far is *Drosophila mojavensis* (Drosophila 12 Genomes Consortium 2007), a

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well-studied cactophilic species that presents a unique opportunity to obtain an integrated understanding of genomic responses to the harsh abiotic conditions that occur in arid lands. It is also a member of the well-studied cactus–yeast–*Drosophila* model system, providing a rich background for ecological genomic studies (Barker & Starmer 1982; Barker *et al.* 1990). *D. mojavensis* has diverged into ecologically diverse desert habitats (Heed 1978; Etges *et al.* 1999) and has undergone adaptation to different host plants (Etges 1989b, 1990, 1993), and some geographically isolated populations are now considered incipient species (Etges 1998; Etges & Ahrens 2001; Etges *et al.* 2007). Thus, *D. mojavensis* is an excellent model for understanding the genomics of adaptation to arid environments and reproductive isolation.

In the New World, about half of the *c.* 100 species in the large *Drosophila repleta* group use fermenting cactus tissues to carry out their life cycles (Heed 1982; Wasserman 1992; Oliveira *et al.* 2012). Within the *mulleri* species complex, *D. mojavensis*, *Drosophila arizonae* and *Drosophila navojoa* form a monophyletic group that is endemic to Mexico and the southwestern United States (Ruiz *et al.* 1990). In the Sonoran and Mojave Deserts and adjacent arid lands, *D. mojavensis* was isolated from the ancestor of its closest relative, *D. arizonae*, on the mainland in association with tectonic drift of present-day peninsular Baja California (Gastil *et al.* 1975; Riddle *et al.* 2000; Garrick *et al.* 2009). Derived mainland Mexico and Arizona populations of *D. mojavensis* use organ pipe, *Stenocereus thurberi*, and sina cactus, *Stenocereus alamosensis*, and diverged *c.* 250 kya (Reed *et al.* 2007; Smith *et al.* 2012) from Baja California populations that primarily use pitaya agria cactus, *Stenocereus gummosus*, with occasional use of cochal cactus, *Myrtillocactus cochal* (Fellows & Heed 1972; Heed & Mangan 1986).

Mainland Mexico and southern Arizona populations have undergone genetic differentiation in allozyme and chromosome inversion frequencies (Zouros 1974; Etges *et al.* 1999), host plant–specific life histories (Etges & Heed 1987; Etges 1989b, 1990, 1993; Etges *et al.* 1999) and physiological adaptation to alternate cactus host species (Starmer *et al.* 1977; Etges & Klassen 1989). Further, Baja California, mainland Mexico and Arizona *D. mojavensis* exhibit low, but significant sexual isolation (Zouros & d'Entremont 1974; Markow 1991) and postmating, prezygotic isolation (Knowles & Markow 2001) among populations with no observed postmating hybrid inviability/sterility. These rearing substrate shifts have also influenced sex-specific epicuticular hydrocarbon (CHC) differences that mediate premating isolation (Stennett & Etges 1997; Etges & Ahrens 2001), in addition to courtship song differences (Etges *et al.* 2006, 2007).

Epicuticular hydrocarbons also serve as a barrier to cuticular water loss (Gibbs 2002; Gibbs & Rajpurohit 2010), because differences in the amount and composition of CHCs affect the permeability of the cuticle (Johnson & Gibbs 2004). In *D. mojavensis*, CHCs are composed of long-chain (C_{29}–C_{50}) alkanes, alkenes, branched alkenes, alkadienes, alkatrienes and alkatetraenes (Etges & Jackson 2001; Yew *et al.* 2011) that can change with age and environmental temperature (Toolson *et al.* 1990; Gibbs *et al.* 1998). Wild-caught adults and flies reared from cactus rots have significantly lower amounts of CHCs than flies reared on cactus in the laboratory (Etges 2002), suggesting that natural environmental conditions may significantly affect water balance. While the underlying genetic pathways for some hydrocarbon components are partially understood in some drosophilids (reviewed in Gleason *et al.* 2009), genetic and environmental influences on hydrocarbon production and deposition are poorly known for most species.

In comparison with other drosophilids, *D. mojavensis* is tolerant of desert conditions, including high temperatures (Stratman & Markow 1998; Krebs 1999) and low humidity (Gibbs & Matzkin 2001; Gibbs *et al.* 2003b; Matzkin & Markow 2009; Kellermann *et al.* 2012). It is adapted to feed on necrotic cactus tissues rich in secondary compounds, for example, phytosterols, fatty acids and triterpene glycosides toxic to other insects (Fogleman *et al.* 1986; Fogleman & Heed 1989; Fogleman & Danielson 2001). Furthermore, adult flies can metabolize volatile compounds like ethanol vapour and other cactus fermentation by-products (Starmer *et al.* 1977; Etges 1989a; Etges & Klassen 1989). Thus, *D. mojavensis* thrives under a wide variety of environmental stressors and represents an ideal species in which to identify genomic mechanisms of stress resistance.

Here, we take advantage of the sequenced genome of *D. mojavensis* to investigate transcriptional responses to desiccation stress, the first of several studies from our group using whole-genome microarrays to uncover functional genomic responses to ecological variation in desert conditions, host plant use and geographical variation. We show that exposure to low humidity conditions causes transcriptional changes in more than a thousand genes, in addition to significant geographical differences in adult mortality, water loss and shifts in cuticular hydrocarbon profiles. Overall, greater than 90% of all predicted genes in *D. mojavensis* were differentially expressed in response to one or more environmental or biogeographical factors or interactions between them.

Material and methods

Origin of stocks and husbandry

Populations of *Drosophila mojavensis* were collected from cactus rots and over baits in the field, returned to the

laboratory and mass-reared on banana food (Brazner & Etges 1993) in 35-mL shell vials at 20–22 °C. Two populations from Baja California, San Quintin (SQ) and Punta Prieta (PP) were collected in 2008. A mainland Sonora population from Punta Onah (PO) was collected in 2007, and 20 isofemale lines collected in 2007 from Organ Pipe National Monument (OPNM), Arizona, were donated by S. Castrezana (Table 1).

After several generations in the laboratory to increase population sizes, thousands of adult flies from each population were introduced into 12 720 cm^3 plexiglass population chambers and allowed to mate for 7–10 days. For each of the four populations, eggs were collected in food cups attached to the cages and distributed into six 250-mL milk bottles containing banana food. Bottle cultures were established at moderate larval densities to minimize nutritionally caused maternal/environmental effects from the vial cultures and maintained in an incubator programmed on a 14:10 LD photoperiod that cycled from 27 to 17 °C. All bottle-reared adults were separated by sex on the day of eclosion and aged to maturity on laboratory food in vials in the incubator.

Approximately 100 adults of each sex from each population were then introduced into separate oviposition chambers, and eggs were collected daily for 10 h in 5.5-cm-diameter Petri dishes containing agar-cactus media. Eggs from replicate chambers were washed in deionized water, 70% ethanol, again in sterile deionized water, counted into groups of 200, transferred to a 1-cm^2 piece of sterilized filter paper and placed on fermenting cactus in an incubator programmed as described above. Fermenting cactus cultures were set up in plugged half-pint bottles with 75 g of aquarium gravel at the bottom covered with a 5.5-cm-diameter piece of filter paper. Bottles were then autoclaved, 60 g of either agria or organ pipe tissues was placed in the bottles, and the bottles were autoclaved again for 8 min at low pressure. After cooling to room temperature, each culture was inoculated with 0.5 mL of a pectolytic bacterium, *Erwinia cacticida* (Alcorn *et al*. 1991), and 1.0 mL of a mixture of seven yeast species common in natural agria and organ pipe rots (Starmer 1982): *Dipodascus starmeri*, *Candida sonorensis*, *Candida valida*, *Starmera amethionina*, *Pichia cactophila*, *Pichia mexicana* and *Sporopachydermia cereana*. All unhatched eggs were counted to allow calculation of egg to adult viability in order to monitor culture conditions, and all eclosed adults from each replicate culture were counted daily under CO_2 anaesthesia, separated by sex and kept on banana food in vials in the incubator until flies were ready for the experiments. Females were aged until they were sexually mature, that is, 8 days, before the desiccation experiments began.

Desiccation treatments

Groups of 24 eight-day-old females were placed in 35-mL presterilized glass vials containing 10 g of Drierite desiccant in an incubator set at 25 °C with constant illumination. Flies were restricted to the bottom of a vial (1.5 cm) with a foam stopper, desiccant was added above the sponge, and the vial was sealed with Parafilm®. Preliminary experiments with cactus-reared flies exposed to low humidity conditions for 0 (control), 12 and 24 h after (Gibbs & Matzkin 2001) revealed that many flies were already dead by 24 h (see below), so we used exposure periods of 0, 9 and 18 h. Flies were removed after each time period, frozen in liquid nitrogen and stored at −80 °C prior to RNA extraction. A total of 24 treatments (4 populations × 2 host cacti × 3 desiccation times) were included with fourfold replication (in most cases; 3 or

Table 1 Origins of the four populations of *Drosophila mojavensis* in this study and estimates of the numbers of flies used to establish laboratory populations

Population	Stock number	Latitude and longitude	Number of founders
Punta Onah, Sonora	PO07	29°5′23.15″N 112°10′15.59″W	472*
Organ Pipe National Monument, AZ	OPNM08	31°58′4.88″N 112°46′5.75″W	20 isofemale lines†
San Quintin, Baja California	SQ08	30°30′41.88″N 115°53′34.51″W	372‡
Punta Prieta, Baja California	PP08	28°52′43.48″N 114°7′28.90″W	465

All flies were collected over banana baits in nature unless otherwise noted.
*Includes *c*. 80 adults aspirated from agria rots.
†Ten to 20 adults from each isofemale line collected in May 2007 were combined and mass-reared for at least six generations to form this stock. Lines were provided by S. Castrezana.
‡Includes 355 adults that emerged from three agria rots.

5 samples were collected for a few treatments), resulting in 95 groups of 24 females each for RNA extraction and microarray analysis. Additional replicates were included so that we could directly estimate mortality rates due to desiccation exposure, changes in mass and water content in both females and males, and assess cuticular hydrocarbon profiles.

Desiccation time and mortality rates

We directly assayed desiccation-caused mortality rates with male and female adults reared on laboratory food and males reared on fermenting agria or organ pipe cactus, to investigate the causes for increased mortality rates in cactus–reared flies. For the latter experiments, female flies were used for transcriptome experiments. Ten flies per vial were exposed to the desiccant as previously described or kept in empty (control) vials until all flies were dead at 25 °C with constant illumination. The number of dead flies was counted every 4 h, four times a day with a 12-h interval during the evening. The average time to death in hours per vial was calculated by linear interpolation of numbers of dead flies observed at each time point. To investigate possible bias in mortality due to fly age, we included fly age when exposure to low humidity began as a factor by starting experiments with flies aged 0, 3, 6, 9 and 12 days. Thus, a total of 80 treatment combinations were included for flies reared on laboratory food (4 populations × 2 sexes × 5 ages × 2 desiccation times) and 80 treatments for males reared on cacti (4 populations × 5 ages × 2 hosts × 2 desiccation times). Females reared on fermenting cactus were used for RNA extractions. In both cases, three replicates were performed for each treatment. All data were analysed by ANOVA in SAS (SAS Institute 2004).

Body mass and water content

We assessed body mass and water content differences as possible factors contributing to desiccation resistance. Adult fly water content was estimated as described by Gibbs *et al.* (2003a). Three groups of ten flies aged 8–12 days from the 16 treatments (4 populations × 2 cacti × 2 sexes) were frozen briefly at −80 °C, thawed and weighed on a Mettler microbalance. Flies were reweighed after drying overnight at 50 °C. Water content was calculated as the difference between wet and dry weight. Fly age ranged from 8 to 12 days.

Cuticular hydrocarbons

We sampled CHCs from five cactus-reared adult females from each of 24 treatments (4 populations × 2 hosts × 3 desiccation times) to reveal the dynamics of desiccation-caused CHC expression. Total CHCs were extracted by immersing single 8-day-old flies in hexane for 20 min in a 300-μL glass vial insert (Microliter Analytical Supplies, Suwanee, GA, USA), evaporating off all hexane in a 40 °C heating block and freezing each sample at −20 °C until analysis. Individual CHC extracts were redissolved in 5 μL of heptane containing a known amount of docosane (C_{22}) as an internal standard. One microliters of each sample was analysed by capillary gas-liquid chromatography using an automated Shimadzu GC-17A (Shimadzu Scientific Instruments, Columbia, MD, USA) fitted with a 15-m (ID = 0.22 mm) Rtx-5 fused-silica column (Restek Corporation, Bellefont, PA, USA). Injector and detector temperatures were set at 290 and 345 °C, respectively, with the injector port in split mode (ratio = 3:1), and the column was heated from 200 to 345 °C at 15 °C/min, holding at 345 °C for 4 min. Amounts of the CHCs were expressed as ng/fly and analysed by ANOVA, principal components analysis and canonical discriminant function (CDF) analysis in SAS (SAS Institute 2004).

RNA isolation, cDNA synthesis, microarray hybridization and visualization

RNA was isolated from groups of 24 female flies using RNeasy mini kits (Qiagen, Valencia, CA, USA). After extraction, RNA was stored at −80 °C until microarray hybridizations were performed. Invitrogen Superscript Double-Stranded cDNA Synthesis kits were used to synthesize double-stranded cDNA. Each cDNA sample was quantified using a NanoDrop spectrophotometer (NanoDrop Technologies) to verify that all samples met concentration and purity requirements (concentration $\geq$100 ng/uL; A_{260}/A_{280} $\geq$1.8; A_{260}/A_{230} $\geq$1.8). cDNA samples were labelled with Cy3 using a NimbleGen One-Color DNA Labeling kit (Roche Diagnostics). Hybridizations with custom NimbleGen 12-plex microarrays were performed with a NimbleGen Hybridization System 4. Probe selection for the microarrays was based on predicted transcripts from the genome assembly for *D. mojavensis* (http://flybase.net/genomes/Drosophila_mojavensis/current/fasta/dmoj-all-transcript-r1.3.fasta.gz), downloaded on April 14, 2009. Nine probes per transcript were generated for each of 14 528 predicted transcripts. Other spots in the design included negative (random probe) controls, controls for contamination from adjacent subarrays and blank (buffer) controls. Array scanning was performed with a GenePix 4000B scanner (Molecular Devices) and associated software at 532 nm, with photomultiplier gain settings that resulted in <1 in 10^5 normalized counts at saturation.

Processing of hybridization data

We normalized all gene expression data in a quantile manner as suggested by Bolstad *et al.* (2003) using NimbleScan v2.5. Gene '*.calls' files were generated using the Robust Multichip Average (RMA) algorithm (Irizarry *et al.* 2003), and analysis and visualization of fluorescence data were performed using ARRAYSTAR v3 software. Linear fluorescence intensities were used for ANOVA and false discovery rate (FDR) analyses in SAS (SAS Institute 2004). All microarray data were MIAME compliant and can be retrieved from the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo) under the series accession no. GSE43220.

Gene annotation

We performed reciprocal BLAST searches between the predicted transcriptomes of *D. melanogaster* and *D. mojavensis* to investigate the functions of genes exhibiting statistically significant changes in expression. A total of 9114 putative *D. melanogaster* orthologs were found after submission of 14 528 predicted *D. mojavensis* transcripts, that is, only *c.* 63% of *D. mojavensis* genes possessed substantial transcript similarity with *D. melanogaster*. We assumed that these genes had conserved functions across the *Drosophila* genomes so that annotations assigned to *D. melanogaster* genes were applicable to *D. mojavensis*. These annotations were used in further analyses, including Gene Ontology (GO) term enrichment, gene expression clustering and pathway analysis. Table S1 (Supporting information) provides a list of FBtr IDs for *D. mojavensis* transcripts and FBgn IDs for their putative orthologs in *D. melanogaster*.

Statistical analyses

Our microarray experimental design included 3–5 replicates for each combination of four populations (PO, OPNM, PP and SQ), two host diets (AG and OP) and three desiccation treatments (0, 9 and 18 h) for a total of 95 whole-transcriptome hybridizations. The normalized fluorescence for each microarray probe set was subjected to a replicated four-way mixed-model ANOVA in SAS using the model:

$$Y_{ijkl} = \mu + R_i + P_j + H_k + D_l + I_{R\times H} + I_{R\times D} + I_{P\times H} + I_{P\times D} + I_{H\times D} + I_{R\times H\times D} + I_{P\times H\times D} + E_{ijkl}$$

where μ is the grand mean, R_i is the effect of geographical region (Baja California vs. the mainland), P_j is the effect of population nested within regions, H_k is the effect of host cactus, D_l is the effect of desiccation, $I_{R\times H}$ is the interaction between region and cactus, $I_{R\times D}$ is the interaction between region and dry air treatment, $I_{P\times H}$ is the interaction between population and cactus nested within region, etc. and E_{ijk} is the error term. To correct for multiple comparisons, we calculated FDRs for the overall ANOVA data and for all pairwise comparisons between treatments (Benjamini & Hochberg 1995).

Bioinformatic analysis

We used DAVID v6.7 (Huang *et al.* 2009) for gene enrichment analyses. Because the annotation for our transcripts was derived from a subset of the transcriptome of *D. melanogaster*, the background gene list consisted of these 9114 putative orthologs instead of the entire *D. melanogaster* transcriptome. We used the medium stringency defaults of DAVID for analyses to minimize exclusion of potentially interesting GO terms. Gene Ontology GO_FAT and KEGG pathway terms were used to identify functionally related clusters of GO categories. Clusters with enrichment scores <1.3, corresponding to $P > 0.05$, were excluded from further analyses. We then submitted GO terms from these clusters to GO-Module, a web-based tool that identifies false positives included because of the hierarchical nature of the GO (Yang *et al.* 2011).

Because we were also interested in temporal patterns of gene expression as desiccation progressed, we performed expression profiling using the Short Time-series Expression Miner, STEM (Ernst & BarJoseph 2006). Based on STEM profiles, genes showing up- or down-regulation were grouped separately and submitted to DAVID and GO-Module for clustering and pathway analysis. We used CYTOSCAPE (www.cytoscape.org) to visualize heat maps and perform clustering analyses.

Results

Mean ± 1 SE per cent egg to adult viability and development time across the four populations reared on both cacti ($n = 24$ cultures) were 79.2 ± 1.7% and 15.9 ± 0.3 days, respectively. Thus, our cactus rearing conditions produced flies of consistent viability, development times and resulting adult body sizes as in previous studies (cf. Etges 1998).

Desiccation tolerance, mortality and water loss

Adult mortality varied significantly due to low humidity exposure, age and population for flies reared on laboratory food (Table S2, Figs S1 and S2, Supporting information). No mortality differences due to sex were observed, so the data were pooled; however, a significant sex × desiccation interaction ($P = 0.011$, Table S2, Supporting information) was caused by a greater increase in desiccation-caused mortality in males than

in females (results not shown). Mean age at death (h) ±1 SE of control adults, 66.52 ± 0.97 h, was significantly greater than that of adults exposed to zero humidity, 41.29 ± 0.93 h, but was not uniform across all populations (Fig. S1, Supporting information). Adults from both mainland populations survived longer than Baja California adults under control conditions, that is, they had greater starvation resistance in ambient humidity, consistent with previous studies (Starmer *et al.* 1977; Etges & Klassen 1989). However, there was no evidence for a mainland vs. Baja California difference in adult survivorship under low humidity conditions. Age at death declined almost monotonically with age in both control and desiccation treatments (Fig. S2, Supporting information) except for control 0-, 3- and 6-day-old and desiccated 9- and 12-day-old adults: these pairwise differences were not significantly different ($P > 0.05$). Thus, the starting age of flies exposed to low humidity had rather minor effects on subsequent survivorship under either control or experimental conditions.

We could only compare effects of pre-adult rearing substrates, cactus vs. laboratory food, on male desiccation tolerance, because cactus-reared females were used for microarray analyses. There were significant differences in desiccation tolerance due to population, food, desiccation and age as well as significant population × food, population × desiccation, population × age and food × age interactions (Fig. 1, Table 2). Mortality differences among populations were consistent with the first experiment performed on laboratory food (Fig. S1, Supporting information), but the population × food interaction demonstrated that desiccation effects on mortality of laboratory food-reared males differed significantly from that of flies reared on fermenting cactus (Fig. 1, Table 2).

Overall, rearing substrate effects were diminished under desiccation as compared to controls. Males reared on laboratory food tended to survive significantly longer than those reared on cactus substrates except for PP, Baja California males (Fig. 1), and a significant population × desiccation interaction was similar to that seen in the laboratory food-only study (Table S2, Fig. S1, Supporting information). Age effects were also similar to those in the first experiment with laboratory food-reared flies (results not shown). Therefore, cactus rearing substrates tended to increase adult mortality under desiccation compared with laboratory food. This may explain the higher survivorship of adults in previous laboratory food studies (Gibbs & Matzkin 2001; Matzkin *et al.* 2007; Matzkin & Markow 2009; Kellermann *et al.* 2012).

Wet and dry mass and adult body water content significantly differed among populations (Table 3, Fig. 2). Wet mass differences between males and females were population-specific as shown by a sex × population interaction ($F = 6.87$, $P = 0.001$).

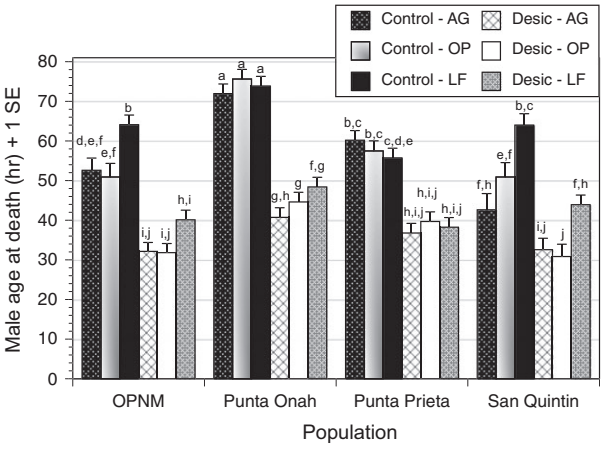

Fig. 1 Age at death of adult male *Drosophila mojavensis* reared on cactus or laboratory food from four populations that were of different ages when placed in control (ambient humidity) or zero humidity conditions to start the experiment. Ages were not graphed to emphasize rearing substrate and population differences. Females were not included in this analysis because they were used for microarray analysis. Letters above the error bars indicate post hoc groupings from Duncan's multiple range test ($P < 0.05$)—least-square means were unavailable for some treatment combinations due to unequal sample sizes.

Table 2 ANOVA results for survival time (h) between control and zero humidity for male *Drosophila mojavensis* reared on three substrates (food); agria cactus, organ pipe cactus and laboratory media

Source of variation	d.f.	Type III SS	*F*-value	Pr > *F*
Population	3	9837.657	36.94	<0.0001
Food	2	2218.595	12.50	<0.0001
Population × food	6	2509.596	4.71	0.0002
Desiccation	1	32163.290	362.33	<0.0001
Population × desiccation	3	1815.137	6.82	0.0002
Food × desiccation	2	20.138	0.11	0.893
Population × food × desiccation	6	837.621	1.57	0.156
Age	4	13758.417	38.75	<0.0001
Population × age	12	2465.595	2.31	0.009
Food × age	8	3625.428	5.11	<0.0001
Population × food × age	20	2036.042	1.15	0.304
Desiccation time × age	4	183.908	0.52	0.723
Population × desiccation × age	12	1071.280	1.01	0.445
Food × desiccation × age	8	2026.800	2.85	0.005
Population × food × desiccation × age	20	1321.758	0.74	0.777
Error	217	19262.75		

Table 3 ANOVA results for variation in body mass for groups of 8- to 12-day-old adult *Drosophila mojavensis* before (wet mass), after drying overnight at 50 °C (dry mass) and adult body water content (delta mass)

		Wet mass			Dry mass			Delta mass		
Source of variation	d.f.	Type III SS	*F*	*P*	Type III SS	*F*	*P*	Type III SS	*F*	*P*
Population	3	66.136	10.46	<0.0001	2.404	4.6	0.009	44.238	7.13	0.0009
Sex	1	26.223	12.44	0.001	18.601	106.9	<0.0001	0.653	0.32	0.578
Population × sex	3	43.449	6.87	0.001	0.711	1.36	0.273	33.826	5.45	0.004
Cactus	1	6.192	2.94	0.097	0.346	1.99	0.169	3.611	1.75	0.196
Population × cactus	3	11.167	1.77	0.175	0.336	0.64	0.594	8.758	1.41	0.259
Sex × cactus	1	0.150	0.07	0.791	0.346	1.99	0.169	0.953	0.46	0.503
Population × sex × cactus	3	1.817	0.29	0.834	0.313	0.6	0.620	2.829	0.46	0.715

Males and females from the four populations reared on both host cacti were included.

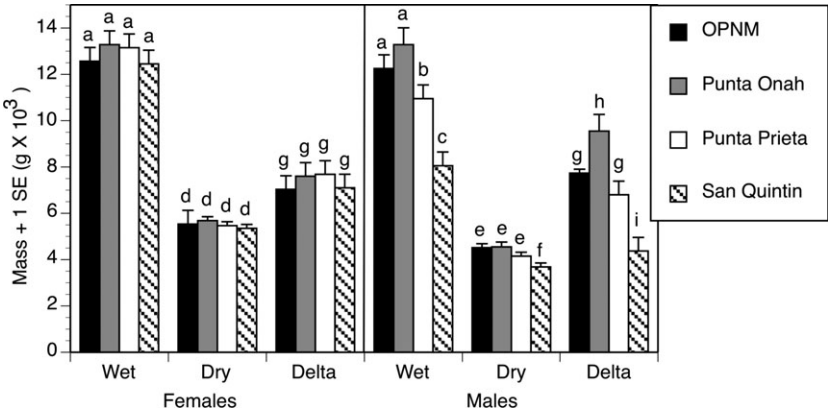

Fig. 2 Differences in wet and dry mass of male and female *Drosophila mojavensis* from the four populations in this study that were reared on agria and organ pipe cactus. Adult body water content, delta mass, is the difference between wet and dry mass after drying adults overnight at 50 °C. Different letters above the error bars indicate significant least-square mean differences between groups ($P < 0.05$).

Water content also showed a sex × population interaction (Table 3), where female water content tended to be much more uniform across populations than male water content. PO, Sonora, males had significantly higher water content than the other populations (Fig. 2) consistent with the greater survivorship of PO males in low humidity (Fig. 1). There were no significant rearing substrate effects, agria vs. organ pipe cactus, on body water content (Table 3), consistent with the general lack of cactus-induced differences in mortality in control or desiccation treatments (Fig. 1).

Cuticular hydrocarbon responses to desiccation

Female CHCs varied significantly between populations and cactus rearing substrates, as anticipated from previous studies (Etges & Ahrens 2001), as well as in response to desiccation exposure and its interaction with population and cactus substrates (Table S3, Supporting information). Thus, short-term CHC responses to low humidity were population-specific and depended on larval rearing substrates. Different groups of CHCs increased or decreased in amounts with increasing exposure to desiccation, even though total CHCs per fly did not change (Table 4). Among those that responded to low humidity, all but 31-methyldotricont-8-ene and 8,24-tritricontadiene (both C_{33}) are typically minor CHC components in *Drosophila mojavensis* (Etges & Jackson 2001), and these two CHCs increased in amounts with increasing exposure to low humidity. There was little evidence that specific kinds of CHCs increased or decreased in amount, that is, methylalkanes, alkenes, methylalkenes or alkadienes. The 12 CHCs that increased quantitatively included C_{31}, C_{32}, C_{34}, C_{35}, C_{36}, C_{38}, C_{39} and C_{40} components (Table 4).

These covarying CHCs that increased in quantity with exposure to low humidity showed negative correlations along the largest axis of variation from CDF analysis (Table 5). Inspection of the largest +/− scores for CV 1 revealed complete correspondence with the +/− changes in CHC amounts (Table 4): negative CV scores corresponded to those CHCs that increased in amounts and positive scores corresponded to those that decreased in amounts. Decreases in average CHC centroid scores from 0, 9 to 18 h of zero humidity

Table 4 Comparisons of least-square means of the 31 epicuticular hydrocarbon components in female *Drosophila mojavensis* included in this study in the three experimental desiccation treatments: 0 (control), 9 and 18 h in zero humidity

Hydrocarbon	ECL*	0 h vs 9 h vs 18 h	*P*
2-methyloctacosane	$C_{28.65}$	40.278, 42.241, 41.322	ns
2-methyltricontane	$C_{30.65}$	104.294, 106.515, 97.931	ns
7- and 9-hentricontene	$C_{30.78}$	7.171 > 5.984 < 7.565	0.03
Unknown	C_{32}	2.922, 3.529 < 4.578	0.009
Unknown alkene	C_{33br1}	0.533, 0.470, 0.581	ns
11- and 13-methyldotricontane	C_{33br2}	7.247, 6.391 > 5.761	0.03
Unknown alkene	C_{33br3}	5.553 > 4.739 > 3.928	<0.0001
31-methyldotricont-8-ene	$C_{32.47}$	36.560, 32.386 > 26.352	<0.0001
31-methyldotricont-6-ene	$C_{32.56}$	3.124, 3.271 > 2.771	0.007
8,24-tritricontadiene	$C_{32.63}$	32.574, 35.527 > 30.313	0.03
7,25-tritricontadiene	$C_{32.70}$	35.223, 37.0793, 35.5903	ns
10-, 12- and 14-tritricontene	$C_{32.79}$	9.767, 8.411 < 12.454	0.008
Unknown	$C_{32.86}$	1.318, 1.092, 1.011	ns
8,26-tetratricontadiene	$C_{34diene1}$	7.481, 6.099 < 7.528	0.007
6,24- and 6,26-tetracontadiene	$C_{34diene2}$	15.267, 16.282 < 17.176	0.03
10-, 12- and 14 tetretricontene	C_{34ene}	5.422, 6.802 < 8.420	<0.0001
33-methlytetratricont-10-ene	C_{35alk1}	10.417, 12.638, 12.649	ns
33-methlytetratricont-8-ene	C_{35alk2}	12.464 < 14.773 > 12.746	0.03
Unknown alkene	C_{35alk3}	25.122, 28.061 > 23.930	0.009
9,25-pentatricontadiene	$C_{34.59}$	88.664, 88.664, 89.458	ns
8,26-pentatricontadiene	$C_{34.66}$	306.191, 280.118, 295.936	ns
7,27-pentatricontadiene	$C_{34.73}$	32.792, 33.538 < 42.955	0.011
Unknown diene	C_{36a}	8.137, 6.923, <9.542	0.023
Unknown alkene	C_{36b}	10.649, 12.348 < 15.087	0.004
35-methylhexatricont-10-ene	C_{37br}	2.610, 2.755, 2.710	ns
9,27-heptatricontadiene	$C_{36.5}$	26.253, 29.090, 25.998	ns
8,28-heptatricontadiene	$C_{36.6}$	67.738, 66.200, 76.153	ns
14-, 16- and 12-hexatricontene	$C_{36.7}$	41.698, 37.238, 42.248	ns
Unknown alkene	C_{38}	5.084, 5.096 < 6.927	0.008
Unknown alkene	C_{39}	4.647 < 6.340, 6.790	0.04
Unknown alkene	C_{40}	3.447, 2.903 < 3.854	0.009
Total hydrocarbons per fly		960.646, 962.261, 970.177	ns

Mean hydrocarbon amounts are expressed in ng/fly. *P* values are from ANOVA.
*Equivalent chain length for each hydrocarbon component.

exposure were significantly different from each other (Fig. 3; all Euclidean distances, $P < 0.05$). CV 2 scores showed similar patterns of CHC covariation (Table 5), although mean CV 2 scores increased from controls at 9 h and then decreased at 18 h. Overall, significant increases in CHC amounts in response to 9 and 18 h of exposure to zero humidity involved mostly a variety of less abundant female *D. mojavensis* CHCs with fewer CHCs decreasing in amounts.

Transcriptome results: summary of differentially expressed transcripts

Our mixed-model nested ANOVA design included four main effects and seven interaction effects with populations nested within regions. At a FDR of $P < 0.01$, the ANOVA revealed a total of 768 808 statistically significant pairwise differences between treatments (Table 6). The magnitude of many effects was small (below 5% in some cases), so for some analyses, we set an arbitrary cut-off of 1.5-fold expression change for inclusion in further analyses, reducing the number of differences analysed by nearly two-thirds (Table 6). Our objective was to identify broad patterns of changes in gene expression, while also allowing us to identify specific gene categories that were especially affected by experimental variables.

For the four main effects, desiccation affected the largest number of transcripts (8917; Table 6), while the expression of only 18 genes was affected by host plant differences. In the latter case, the largest statistically significant difference was <25% so that no diet-related genes passed the 1.5-fold change cut-off. Region and population (nested within region) affected a total of 3098 and 5262 transcripts, respectively. There were 5 two-factor interactions and 2 three-factor interactions

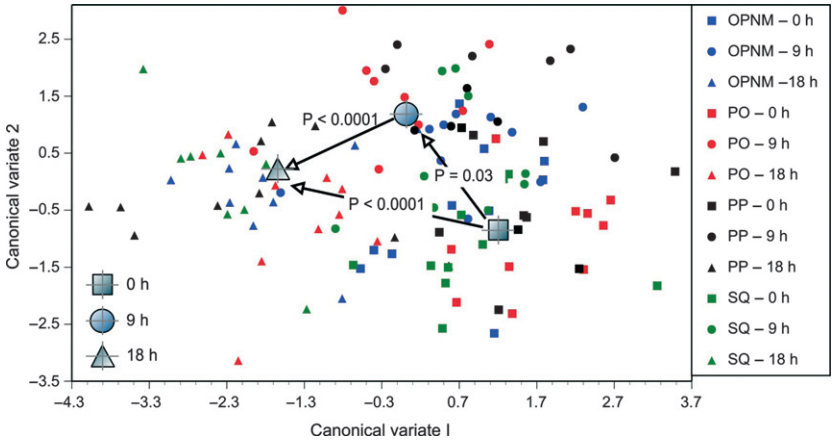

Fig. 3 Canonical discriminant function biplot showing shifts in cuticular hydrocarbon profiles of female *Drosophila mojavensis* from the four populations in this study exposed to zero humidity, 0, 9 and 18 h. mean centroids for CV 1 and 2 are shown, and *P* values refer to significantly different Euclidean distances between means. Cactus differences were not included. OPNM, Organ Pipe National Monument, Arizona; PO, Punta Onah, Sonora; PP, Punta Prieta, Baja California; SQ, San Quintin, Baja California.

Table 5 Total structure of the first 5 canonical variates (CV) for the 31 female cuticular hydrocarbons in this study

Cuticular hydrocarbon	ECL*	CV 1	CV 2	CV 3	CV 4	CV 5
2-methyloctacosane	$C_{28.65}$	−0.057	0.022	−0.011	0.001	0.013
2-methyltricontane	$C_{30.65}$	0.152	0.155	−0.008	0.043	0.049
7- and 9-hentricontene	$C_{30.78}$	−0.103	**−0.290**	0.020	−0.108	−0.033
Unknown	C_{32}	**−0.291**	0.034	0.074	−0.101	−0.002
Unknown alkene	C_{33br1}	0.065	0.059	−0.005	0.003	−0.018
11-and 13-methyldotricontane	C_{33br2}	**0.202**	**−0.109**	0.059	−0.082	−0.080
Unknown alkene	C_{33br3}	**0.318**	−0.105	−0.001	0.016	0.014
31-methyldotricont-8-ene	$C_{32.47}$	**0.429**	−0.078	0.020	0.029	0.031
31-methyldotricont-6-ene	$C_{32.56}$	**0.244**	**0.139**	−0.018	0.064	0.055
8,24-tritricontadiene	$C_{32.63}$	**0.099**	0.185	0.066	0.016	−0.066
7,25-tritricontadiene	$C_{32.70}$	−0.056	−0.033	0.020	−0.037	−0.026
10-, 12- and 14-tritricontene	$C_{32.79}$	**−0.240**	**−0.169**	−0.068	0.182	0.102
Unknown	$C_{32.86}$	0.047	0.103	−0.064	0.052	0.080
8,26-tetratricontadiene	$C_{34diene1}$	**−0.136**	**−0.168**	−0.012	−0.029	0.031
6,24- and 6,26-tetracontadiene	$C_{34diene2}$	**−0.181**	−0.070	0.091	−0.053	0.032
10-, 12- and 14 tetretricontene	C_{34ene}	**−0.343**	0.110	−0.045	−0.003	0.134
33-methlytetratricont-10-ene	C_{35alk1}	−0.050	**0.202**	−0.003	0.031	0.043
33-methlytetratricont-8-ene	C_{35alk2}	**0.099**	**0.210**	−0.025	0.131	−0.009
Unknown alkene	C_{35alk3}	**0.176**	**0.181**	0.023	0.080	0.086
9,25-pentatricontadiene	$C_{34.59}$	−0.018	0.023	0.033	0.010	−0.011
8,26-pentatricontadiene	$C_{34.66}$	0.052	−0.097	0.036	0.010	0.018
7,27-pentatricontadiene	$C_{34.73}$	**−0.222**	**0.112**	−0.008	0.156	−0.015
Unknown alkene	C_{36a}	**−0.181**	−0.059	0.069	0.196	0.042
Unknown alkene	C_{36b}	**−0.256**	−0.063	−0.014	−0.028	−0.069
35-methylhexatricont-10-ene	C_{37br}	0.056	**0.132**	−0.011	0.081	−0.005
9,27-heptatricontadiene	$C_{36.5}$	0.006	−0.015	−0.027	0.017	−0.070
8,28-heptatricontadiene	$C_{36.6}$	−0.043	−0.049	0.052	−0.037	−0.012
14-, 16- and 12-hexatricontene	$C_{36.7}$	−0.006	0.010	0.012	0.027	0.069
Unknown alkene	C_{38}	**−0.342**	−0.089	−0.129	0.007	0.047
Unknown alkene	C_{39}	**−0.210**	**0.147**	0.378	0.119	0.043
Unknown alkene	C_{40}	**−0.228**	**−0.247**	0.127	0.018	0.076

CV 1 and 2 loadings in bold correspond to the hydrocarbons that covaried (+/−) and showed significant shifts in amounts due to desiccation exposure. See Table 4.
*Equivalent chain length for each hydrocarbon component.

included in our statistical model. When interaction terms were included, more than 90% of predicted genes were differentially expressed under some combination of experimental variables (Table 6).

We used CYTOSCAPE v 2.8.2 (Smoot *et al.* 2011) to generate and visualize gene expression networks with the EXPRESSIONCORRELATION plugin (www.baderlab.org/Software/ExpressionCorrelation), which creates networks

Table 6 Summary of gene expression differences

Treatments	No. of pairwise differences; FDR < 0.01	No. of unique genes	No. of genes with *Dmel* orthologs	No. of pairwise differences; 1.5× fold change cut-off	No. of unique genes; 1.5× cut-off	No. of genes with *Dmel* orthologs
Main effects						
Region (*R*)	3098	3098	1963	184	184	64
Population (*P*)	11 926	5263	3417	1182	514	217
Host cactus (*H*)	18	18	15	0	0	0
Desiccation (*D*)	16 794	8917	6089	1152	828	507
Interaction effects						
5. $R \times H$	6896	2556	1594	790	249	96
6. $R \times D$	56 983	11 467	7401	8625	2825	1520
7. $P \times H$	25 097	5041	3198	4609	835	346
8. $P \times D$	152 055	12 351	7941	50 539	5731	3062
9. $H \times D$	43 037	8507	5865	5266	1413	813
10. $R \times H \times D$	131 779	11 148	7231	35 836	4454	2364
11. $P \times H \times D$	321 125	12 607	8011	161 234	8336	4572
Column totals	768 808	80 973	52 725	269 417	25 369	13 561
Number of unique genes		13 342	8481		8394	4587

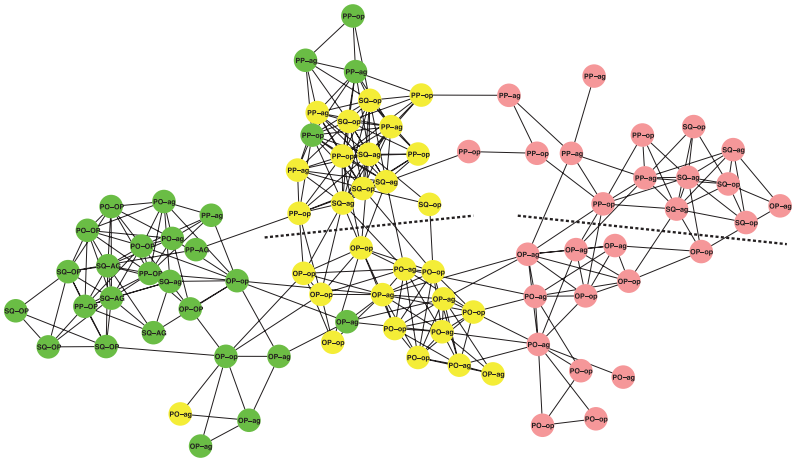

Fig. 4 Gene expression network generated using Cytoscape. All genes whose expression differed by at least 50% in at least one pairwise comparison of experimental treatments (Table 6) were included in the analysis. Dashed lines separate samples from the mainland and Baja California after 9 and 18 h of desiccation.

of gene expression similarities based on the Pearson correlation coefficient. For this analysis, we included all 8394 genes with at least a 50% fold change in expression for one or more main or interaction effects. Gene expression clustered into three main subgroups based on desiccation time (Fig. 4). Fly samples desiccated for 9 or 18 h clustered with each other; within these treatments, samples from Baja California and mainland Mexico and Arizona tended to cluster with each other. Thus, desiccation had the greatest effect on global gene expression, followed by geographical region, with little effect of host plant.

Desiccation effects

Analyses using DAVID are restricted to lists of 3000 or fewer genes, but >6000 genes affected by desiccation stress had orthologs in *D. melanogaster*. We therefore used STEM (Ernst & BarJoseph 2006) to investigate desiccation-related patterns of expression in these genes. STEM classified our data into 16 temporal patterns, six of which were highly over-represented relative to chance (chi-squared test, $P < 10^{-14}$ for each pattern). These included two patterns each in which expression increased or decreased consistently over time. In the other two patterns, gene expression increased or decreased early in desiccation then did not change between 9 and 18 h.

We performed separate DAVID and GO-Module analyses for the up-regulated and down-regulated gene lists. For up-regulated genes, eight clusters of GO terms were uncovered with enrichment scores >3, corresponding to $P < 0.001$. Summaries of these categories are listed in Table 7(A), and more detailed information is provided in Table S4 (Supporting information). Two of these groups, including that with the second greatest

enrichment, contained genes related to sensing of the environment. The third and fourth most enriched clusters included genes encoding cuticular components and two others included genes involved in ion transport and neuropeptides. Ten down-regulated GO clusters included four genes related to transcription, chromatin organization and development (Table 7(B), Table S5, Supporting information).

To further characterize desiccation-related changes in gene expression, we separately analysed sets of genes that were up- or down-regulated, during early (0–9 h) and late (9–18 h) desiccation stress. In the first 9 h, four up-regulated GO clusters had enrichment scores >3, corresponding to $P < 0.001$, and included genes related to mitochondrial respiration, ribosome components, fatty acid biosynthesis and photoreception (Table S6, Supporting information). Lower enrichment clusters included several clusters composed of genes related to mitochondria, chitin metabolism and intermediary metabolism. Between 9 and 18 h, broadly similar categories of genes continued to show increased expression, except for mitochondrial respiration.

Clusters of genes that were down-regulated during the first 9 h of desiccation stress included a wide array of cellular processes, many related to chromosome structure and gene transcription (Table S7, Supporting information). Note that the third most highly enriched cluster (EASE score >12), which initially included several GO terms related to cation binding, was reduced to a single term (GO:0008270; zinc ion binding) after elimination of false positives with GO-Module. Several clusters were related to oogenesis, suggesting reduced reproductive effort. Broadly similar responses were seen in the subsequent 9 h of desiccation.

In analysing these data, we noticed that 19 genes were included on lists of up- and down-regulated genes. Expression of 10 genes decreased from 0 to 9 h of desiccation and then increased from 9 to 18 h. Six had putative orthologs in *D. melanogaster*, and interestingly, four of these six had been annotated as structural components of the chorion. Of the nine genes (3 with *D. melanogaster* orthologs) whose expression increased and then decreased, two are annotated as structural components of the vitelline membrane. The other annotated gene was *phantom* (*phm*), which encodes a cytochrome P450 catalysing an early step in ecdysone biosynthesis (Warren *et al.* 2004).

Geographical variation

Of 3098 genes (1963 with putative orthologs in *D. melanogaster*) differed in expression between populations from Baja California and mainland Mexico and Arizona, but only 187 differed by at least 50%. Of these, only 64 had putative orthologs in *D. melanogaster*. One cluster of functionally related genes with enrichment scores >1.3, that is, a type 1 error of 0.1, was recovered with DAVID. This cluster (11 GO categories, enrichment score 1.54)

Table 7 Summary of functional information for Gene Ontology categories up-regulated or down-regulated during desiccation stress

Cluster no.	General GO categories	Enrichment	Total no. of genes
(A) Clusters of GO categories that were up-regulated during desiccation stress			
1	Membrane proteins	14.60	273
2	Perception of smell	10.65	119
3	Structural constituent of chitin-based cuticle	8.37	42
4	Chitin metabolism	7.52	93
5	Cation transport	4.99	90
6	Plasma membrane	4.65	77
7	Detection of chemical stimulus	3.73	23
8	Neuropeptide hormone, signalling	3.44	15
(B) Clusters of GO categories that were down-regulated during desiccation stress			
1	Zinc ion binding	12.04	216
2	Regulation of transcription	5.57	240
3	Chromatin organization and modification	4.86	62
4	Positive regulation of transcription	4.42	62
5	Cell migration	3.92	64
6	Neuron differentiation and morphogenesis	3.82	121
7	Nucleoplasm, transcription	3.58	75
8	Metamorphosis, development	3.56	82
9	Tracheal development	3.29	43
10	DNA repair	3.02	56

Only categories with enrichment scores >3.0 (corresponding to $P < 0.001$) are included. More information on genes included in these categories and GO clusters with lower enrichment scores is included in Tables S2 and S3 (Supporting information).

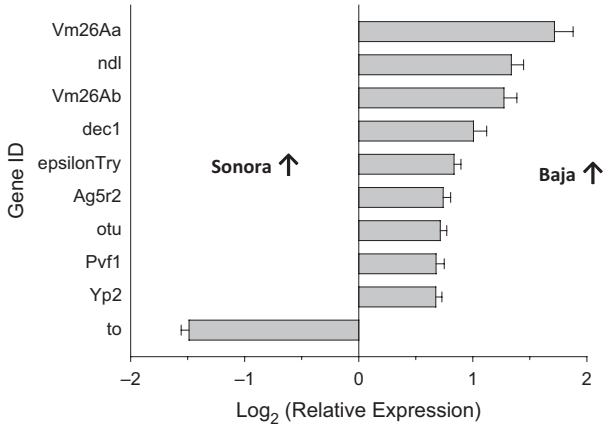

Fig. 5 Relative expression of genes associated with reproduction in flies from the mainland and Baja California. These genes were identified by DAVID from a list of genes with at least a 50% difference in expression between these geographical regions.

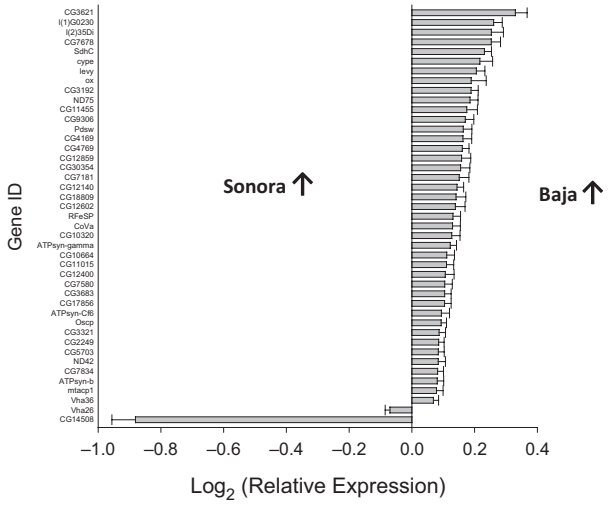

Fig. 6 Relative expression of genes associated with oxidative phosphorylation (GO:0006119) in flies from the mainland and Baja California.

contained several GO terms related to female reproduction (e.g. formation of gametes, ovarian follicular cells and the eggshell). Because of overlap among these categories, this cluster represented only 10 genes, 9 of which were more highly expressed in flies from Baja California (Fig. 5; two-tailed sign test, $P < 0.022$).

To investigate broader transcriptome differences between Baja California and mainland flies, we relaxed the 1.5-fold change cut-off restriction and re-analysed the larger data set containing all 1963 genes with orthologs in *D. melanogaster* and FDR $P < 0.01$. Four of the six most enriched GO term clusters (enrichment scores 1.97–4.18) included categories associated with mitochondria and oxidative phosphorylation (Table S8, Supporting information). Inspection of pairwise differences for individual genes indicated that mitochondrial protein genes, especially those related to electron transport and oxidative phosphorylation, were more highly expressed in flies from Baja California. The most highly over-represented GO term cluster included 129 genes, 86 of which were expressed at higher levels in flies from Baja California (two-tailed sign test, $P < 0.002$). Figure 6 shows the relative expression of 43 differentially expressed genes in the category GO:0006119, oxidative phosphorylation. Forty-one of these were expressed at higher levels in flies from Baja California (two-tailed sign test, $P < 0.0001$). Thus, Baja California females exhibited higher expression of transcripts associated with mitochondrial energy production under low humidity conditions.

Additional over-represented GO clusters were related to ageing (23 genes) and immune function (42 genes). Because of their potential relevance to life history and survival differences between mainland and Baja California populations, we investigated these for potential geographical patterns. No consistent geographical differences were detected for ageing-related genes (two-tailed sign test, $P > 0.6$). Immune function genes were not differentially expressed between regions overall ($P > 0.8$), but there was a tendency for higher expression of innate immune response genes (GO:0045087) in mainland flies and humoral immune response genes (GO:0006959) in flies from Baja California. Because of overlap between these categories, however, overall expression did not differ significantly between regions (two-tailed sign tests, $P > 0.3$).

Nearly 5262 genes differed in expression among populations (nested within geographical region). This group included all of the 3098 genes differentially expressed between mainland and Baja California regions, and >85% of significant pairwise differences were between one of the mainland populations and one of the Baja Californian populations. Because >3000 of these genes had orthologs in *D. melanogaster*, we used DAVID to identify over-represented GO terms in the subset of genes whose expression differed by at least 50%. Our DAVID analysis revealed four clusters with enrichment scores >2 ($P < 0.01$). The most over-represented cluster included GO terms related to female reproduction.

Host plant and population-by-host plant interactions

As noted above, only 18 genes (15 with orthologs in *D. melanogaster*) differed in expression at FDR <0.01. Interestingly, 16 of these were expressed at higher levels in flies reared on organ pipe cactus than on agria.

Clustering analysis using DAVID found one poorly enriched cluster (enrichment score = 0.54) consisting of genes annotated as binding cations. When we further relaxed our criteria for inclusion to allow FDR <0.05, 106 genes (62 with orthologs in *D. melanogaster*) were differentially expressed. Analysis using DAVID uncovered a single over-represented cluster (enrichment score = 2.08), including five genes annotated as having anion transport activity.

Host plant and population have been shown to interact in affecting traits such as CHC and reproductive isolation between populations (Stennett & Etges 1997; Etges & Ahrens 2001), so we were particularly interested in identifying genes that might contribute to these effects. About 835 genes with significant population × cactus interactions differed in expression by >50% for at least one pairwise comparison. Thus, there were significant differences among populations (nested within region) in gene expression when reared on agria or organ pipe cactus. This set included all 247 genes with significant region × cactus interactions. Inspection of functional clusters revealed that these were similar to the categories revealed by analysis of regional or population effects alone. Only one region × cactus cluster was identified at $P < 0.05$ (enrichment score >1.3), including GO terms associated with female reproduction (Table S9, Supporting information). For population × cactus interactions, eight clusters were identified (Table S10, Supporting information), including GO terms related to female reproduction, immune function and lipid metabolism.

More than 4000 genes exhibited significant region × desiccation × host plant effects, but only 2354 had putative orthologs in *D. melanogaster*. DAVID analyses of these genes produced 17 clusters of enriched GO terms (Table S11, Supporting information). In general, the categories represented overlapped with those returned in main effect and two-way interaction analyses. This was probably because all of the genes included in this analysis also appeared as differentially regulated in region and desiccation analyses or in pairwise interactions between each other or with host plant.

In another attempt to identify GO categories associated with host plant-by-population interactions, we considered all genes exhibiting statistically significant host-by-region interactions. We then removed all genes exhibiting host or region effects, to leave a list of genes specific to host plant × region interactions, and analysed the remaining list using DAVID. We performed a similar analysis for population × host plant interactions. The goal was to identify GO terms specifically associated with interactions between host plants and geographical location. In both cases, no over-represented GO term clusters were identified.

Discussion

Desert insects, like *Drosophila mojavensis*, are often exposed to extremes in heat, cold and desiccation and are especially vulnerable to desiccation because of their large surface area to volume ratio. Among drosophilids, *D. mojavensis* is the most desiccation resistant species studied to date (Gibbs & Matzkin 2001; Matzkin & Markow 2009; Kellermann *et al.* 2012). Desiccation exposure in *D. mojavensis* resulted in manifold effects on adult mortality, directional increases and decreases in CHC quantities, and region-, population-, host plant- and desiccation-dependent variation in gene expression. The relatively large number of observed differentially expressed genes caused by treatment interactions vs. main effects (Table 6), made possible by a completely replicated experimental design, suggests that population-level understanding of genomic responses to relevant environmental stressors like desiccation must be evaluated across geographical and local ecological scales.

Effects of desiccation

An important aspect of survival during desiccation stress is water conservation, and two highly enriched clusters of up-regulated genes included chitin metabolism and cuticle constituents (Table 7). Similar results have been obtained in desiccation-stressed mosquitoes (Wang *et al.* 2011). The cuticular constituent cluster included more than 30 genes with orthologs in *D. melanogaster* coding for known cuticular proteins. This suggests that *D. mojavensis* can improve the water-retaining properties of the cuticle during desiccation stress by increasing production of cuticular proteins (Gibbs & Rajpurohit 2010). It is important to note that this occurs even as overall protein synthesis is being reduced, as several highly enriched clusters of down-regulated genes involved transcription (Table 7). Our data indicate that *D. mojavensis* generally decreases protein synthesis and overall biosynthesis when desiccated, but increases the relative expression of genes involved in processes that protect against water stress.

Cuticular hydrocarbons (CHCs) provide a hydrophobic layer that is the main barrier to water loss (Gibbs 2002). We found that total CHC content was not affected by desiccation stress, but CHC composition did change. Gleason *et al.* (2009) identified 30 potential elongases and desaturases thought to be involved in CHC biosynthesis in *D. melanogaster*, eighteen of which have orthologs in *D. mojavensis*. Ten of these were differentially expressed as a result of desiccation, with six consistently up-regulated from 0 to 18 h [*Cyt-b5-r*, *desat2*, CG2781 (fatty acid elongase), CG9743 (stearoyl-CoA 9-desaturase), CG5278 and CG33110 (both elongases)] and two

consistently down-regulated, CG17928 (Δ5 desaturase) and CG5326 (elongase). A particularly interesting candidate gene was *desat2* (FBtr0161209), whose expression increased 65% in the first 9 h and then declined by 28% at 18 h. As there were significant shifts in groups of CHCs with increasing desiccation exposure (Table S3, Supporting information, Table 4), particularly significant increases in 12 CHCs comprising monoenes and dienes from C_{31} to C_{40}, changes in desaturase and elongase expression may be responsible. We note that these changes in CHC amounts and profiles occurred rapidly, suggesting that CHC pools are dynamic and respond to environmental conditions. Increases in expression of genes associated with fatty acid biosynthesis and desaturation during the first 9 h of desiccation (Table S6, Supporting information) support this interpretation.

Our data also suggest an important behavioural component to desiccation responses. Highly enriched up-regulated gene clusters identified by STEM analysis include sensory perception (chemical and visual), neuropeptides and ion transporters that may act in signal processing by the nervous system. When *D. mojavensis* is first exposed to low humidity (in the absence of chemical or visual cues), it becomes immobile for *c.* 12 h and then becomes active (Gibbs *et al.* 2003a). This behaviour may be very different if flies are provided with a potential water source during desiccation stress. Increases in ion transporter expression (Table S4, Supporting information) could also reflect increased water retention by the Malpighian tubules and cells throughout the body. Conversely, reduced expression of tracheal morphogenesis genes (Table S5, Supporting information) may help to reduce respiratory water loss by reducing turnover and remodelling of the tracheal system. Our data are consistent with findings in desiccation-selected *D. melanogaster*, in which genes associated with respiratory system development were highly over-represented in single-nucleotide polymorphisms under selection (Telonis-Scott *et al.* 2012).

Given the behavioural changes described above, it is surprising that the GO cluster with the greatest over-representation during short-term desiccation was enriched in terms related to mitochondrial respiration (Table S6, Supporting information), suggesting an increase in metabolism. Previous work suggested that *D. mojavensis* primarily uses carbohydrates when desiccated, but also metabolizes proteins (Marron *et al.* 2003). Lipids are not metabolized to a significant extent, although we did observe increased expression of lipid biosynthetic enzymes in the first 9 h, followed by increased expression of triacylglycerol lipases between 9 and 18 h. A potential explanation for these observations is that flies utilize glycolysis to metabolize carbohydrates during early desiccation stress and release bound water of hydration, while synthesizing fatty acids from the resulting acetyl-CoA, some of which may be used in CHC turnover (Figs 1 and 2). As desiccation stress progresses, flies may switch fuels to metabolize lipids. Lipid metabolism is obligately aerobic, so up-regulation of mitochondrial genes during the early stages of desiccation may serve to allow later fatty acid oxidation.

Numerous genes involved in amino acid transport and metabolism were expressed at higher levels during desiccation stress ($P < 0.01$; Table S4, Supporting information). In addition, genes involved in pyridoxal phosphate (vitamin B_6) increased in expression from 9 to 18 h of desiccation. Pyridoxal phosphate is an essential cofactor for transaminases that interconvert amino acids and related compounds, so this finding supports an overall increase in amino acid metabolism. Inspection of the up-regulated genes directly associated with amino acid metabolism suggests that catabolism of amino acids increases during desiccation. Amino acid catabolism generates small carbohydrates such as oxaloacetate and fumarate, which can be metabolized by the Krebs cycle to generate ATP. We also note that several amino acids are used as compatible osmolytes to protect cellular function during water stress in many organisms (Yancey 2005). Changes in amino acid metabolism and transport are consistent with the idea that *D. mojavensis* uses some amino acids to protect its tissues from dehydration.

Expression patterns of genes related to reproduction showed several interesting features. During both early and late desiccation stress, GO categories related to reproduction were significantly over-represented among down-regulated genes. An additional 19 genes exhibited complicated expression patterns. Nine were expressed at higher levels after 9 h, followed by significantly lower expression after 18 h. One of these was *phm*, two encoded vitelline membrane proteins, and six were unannotated. Ten genes decreased in expression and then increased, including four chorion structural proteins. These results suggest that desiccation causes dynamic changes in ovarian function. Increasing egg production seems counterintuitive during a period of resource stress, but late increases in egg structural components suggest that water-stressed female *D. mojavensis* may try to lay eggs while they still can.

We compared our desiccation-responsive genes to a database of 263 potential desiccation-related genes (in *D. melanogaster*) available from the Center for Environmental and Stress and Adaptation Research (CESAR; cesar.org.au). One hundred and sixty of these had putative orthologs in *D. mojavensis*, but only eight of these (including *desat2*) were differentially expressed in response to desiccation in our study. Our low success rate may reflect the fact that the majority of the genes in

this database were identified in desiccation-selected populations, rather than flies that been directly exposed to desiccation (Sørensen *et al.* 2007). Thus, laboratory selection may have acted primarily on the constitutive expression of genes that do not respond directly to desiccation. Other genes identified as differentially expressed in *D. melanogaster* either do not have a putative ortholog in *D. mojavensis* (*Smp-30*; Sinclair *et al.* 2007) or did not respond to desiccation in our study (*desiccate*; Kawano *et al.* 2010; FBtr0163396 in *D. mojavensis*).

Matzkin & Markow (2009) concluded that desiccation stress in *D. mojavensis* was associated with a decrease in metabolic rates, based largely on changes in expression of several metabolic enzymes. For comparison, we specifically investigated four central metabolism genes these authors identified as being differentially expressed during desiccation stress: *Adh-2*, *GAPDH*, *Tal* and *PEPCK* (Matzkin & Markow 2009). Our results confirmed a decrease in *Adh*-2 expression of *c.* 30%, but desiccation had no effect on expression of *GAPDH* or *Tal* in our experiments. Phosphoenolpyruvate carboxykinase (*PEPCK*) decreased in expression by *c.* 30% from 0 to 9 h and then doubled from 9 to 18 h. There are several reasons why our results may differ from previous work. We exposed our populations to a maximum of 18 h of desiccation stress, while Matzkin & Markow (2009) used a 40-h desiccation treatment. Their flies suffered significant mortality, while ours did not. Flies in our experiments were, however, significantly stressed, as preliminary experiments revealed that many flies were dead by 24 h, causing us to alter our experimental design to exposure times of 9 and 18 h (see Methods). These differences in desiccation resistance could reflect geographical differences in source populations (Matzkin *et al.* 2007) or rearing conditions. We observed significant increases in desiccation-related adult mortality when flies were reared on natural host plants vs. laboratory food (Fig. 1, Table 2), whereas Matzkin & Markow (2009) reared their flies on banana–*Opuntia* medium only. Pre-adult rearing substrates, cactus vs. laboratory food, also influence adult desiccation responses, in addition to other adult phenotypes such as mating behaviour, CHCs and body size (Etges 1990, 1992; Etges & Ahrens 2001).

Population and host plant effects

Populations of *D. mojavensis* from Baja California and the mainland differed in expression of numerous genes related to reproduction, particularly chorion structural proteins. These and additional related genes also exhibited significant region-by-host and population-by-host interactions. These results are consistent with previous studies demonstrating inter- and intrapopulation variation in fecundity (Etges & Klassen 1989; Etges & Heed 1992) and host-mediated reproductive isolation among populations (Etges & Ahrens 2001). We also observed differential expression of reproduction genes during desiccation stress.

Expression of only 18 genes differed between host plant treatments, and none of the differences exceeded 25%. Our results contrast sharply with those obtained for third-instar larvae, in which >500 genes were found to be differentially expressed on agria and organ pipe (Matzkin *et al.* 2006; Matzkin 2012). This may not be surprising, because we assayed transcription in adult females that had been reared on fermenting cactus through eclosion and then matured on laboratory food. Although 15 of the differentially expressed genes we detected had putative orthologs in *D. melanogaster*, no experimental evidence was available relating to the functions of any of them. Electronic annotations indicated two potential odorant-binding proteins, which could be involved in host plant recognition.

Reproductive isolation between mainland and peninsular populations is affected by the host cactus on which flies are reared (Etges & Ahrens 2001). We therefore examined genes with significant region-by-host and population-by-host interactions to try to identify candidates that might be involved. Genes involved with reproduction were over-represented, as were haeme-binding proteins. A total of 19 differentially expressed genes included 7 cytochrome P450 genes, some of which may be involved in detoxifying host secondary compounds. One of the P450 genes was *phantom*, whose product catalyses an early step in ecdysone synthesis (Warren *et al.* 2004). Ecdysteroids act to regulate oogenesis in adult females, so *phm* may have a role in host-mediated differences in fecundity. In general, however, multiple approaches did not suggest any transcriptional component to host plant effects on flies from different populations. Post-transcriptional processes (e.g. post-translational modifications of gene products) may be more important.

A critical limitation of this study (and any functional 'omic' research) is the poor annotation of most genomes. Whether RNA sequencing or microarrays, the latter still accurate and useful tools (Malone & Oliver 2011), are used to assess variation in transcription, lack of functional, lineage-specific gene functional information will constrain interpretation of results. Approximately 75 of the 14 528 predicted genes in *D. mojavensis* have formal annotations entered into FlyBase, and these are primarily accessory gland proteins and odorant-binding proteins. We were therefore forced to rely on the annotation of putative orthologs from *D. melanogaster*. More than one-third (5414) of the predicted genes in *D. mojavensis* did not have a clear ortholog and so were

excluded from our analysis. Of those that did, frequently the functional annotation for *D. melanogaster* was based on sequence similarity to a gene of known function, rather than direct experimental evidence. For historical reasons, *Drosophila* geneticists have concentrated on developmentally regulated genes that yield a morphological phenotype (or death) when disrupted. The annotated fraction of the transcriptome is therefore highly biased towards genes expressed during pre-adult stages. Our findings that many such genes are affected by desiccation in adult flies clearly demonstrate that these genes are active in adults, but their adult functions are largely unknown. Genome annotation remains a very weak link in our understanding of how gene expression is integrated to affect organismal physiology.

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S.R. performed the microarray hybridizations and image processing. C.C.O. performed the desiccation experiments. S.R. and A.G.G. performed functional analyses of gene expression. W.J.E. and A.G.G. designed the experiments. All authors contributed to data analyses and writing the manuscript.

Data accessibility

Microarray data: Gene Expression Omnibus accession no. GSE43220.

Body mass, CHC, mortality and viability data: doi:10.5061/dryad.rp187.

Supporting information

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

Fig. S1 Age at death of lab food-reared adult *Drosophila mojavensis*, sexes pooled, from four populations in control (ambient humidity) or zero humidity conditions.

Fig. S2 Age at death of lab food-reared adult *Drosophila mojavensis* from four populations that were of different ages when placed in control (ambient humidity) or zero humidity conditions to start the experiment.

Table S1 *Drosophila mojavensis* Fbtr IDs and corresponding *D. melanogaster* orthologs used for functional analyses.

Table S2 ANOVA table for desiccation survival of flies reared on laboratory media.

Table S3 MANOVA table for female CHC levels.

Table S4 Functional analysis of genes upregulated during desiccation stress.

Table S5 Functional analysis of genes downregulated during desiccation stress.

Table S6 Functional analysis of genes upregulated during early (0-9 hr) desiccation stress.

Table S7 Functional analysis of genes downregulated during early (0-9 hr) desiccation stress.

Table S8 Functional analysis of population differences in gene expression.

Table S9 Functional analysis of region-by-host plant differences in gene expression.

Table S10 Functional analysis of population-by-host plant differences in gene expression.

Table S11 Functional analysis of region-by-host plant-by-desiccation differences in gene expression.