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Transcriptome modulation during host shift is driven by secondary metabolites in desert *Drosophila*

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Abstract

High-throughput transcriptome studies are breaking new ground to investigate the responses that organisms deploy in alternative environments. Nevertheless, much remains to be understood about the genetic basis of host plant adaptation. Here, we investigate genome-wide expression in the fly *Drosophila buzzatii* raised in different conditions. This species uses decaying tissues of cactus of the genus *Opuntia* as primary rearing substrate and secondarily, the necrotic tissues of the columnar cactus *Trichocereus terscheckii*. The latter constitutes a harmful host, rich in mescaline and other related phenylethylamine alkaloids. We assessed the transcriptomic responses of larvae reared in *Opuntia sulphurea* and *T. terscheckii*, with and without the addition of alkaloids extracted from the latter. Whole-genome expression profiles were massively modulated by the rearing environment, mainly by the presence of *T. terscheckii* alkaloids. Differentially expressed genes were mainly related to detoxification, oxidation–reduction and stress response; however, we also found genes involved in development and neurobiological processes. In conclusion, our study contributes new data onto the role of transcriptional plasticity in response to alternative rearing environments.

Keywords: alkaloids, environment adaptation, mescaline, plasticity, RNA-Seq

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Introduction

The remarkable diversity of plant-eating insects is related to the diversification of Angiosperms that started in the Cretaceous (Grimaldi & Engel 2005). Plants are capable of synthesizing a vast array of secondary metabolites that serve as defence against the assault of herbivores, while in turn insects can respond with counteradaptations to circumvent plant chemical defences. The enormous diversity of plant secondary compounds suggests that an insect experiencing a host plant switch may be confronted to dramatically different chemical environments (Vogel *et al.* 2014). Consequently, host plant shifts may impose new selective regimes driving phenotypic and genetic change (reviewed in Tilmon 2008).

Phytophagous insects offer excellent opportunities to investigate adaptation to novel environments, because host plants are the most immediate environmental factor affecting early life cycle stages (Schoonhoven *et al.* 2005). Although this issue has been intensively addressed, our understanding of how insects adapt to new environments is still insufficient.

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The genus *Drosophila* has been instrumental in a broad spectrum of disciplines, from genetics and development to ecology and evolution (Markow & O'Grady 2007). In the last 10 years, several sequencing projects led the phylogeny of *Drosophila* to the avant-garde of the postgenomic era, providing powerful genetic tools for studying adaptive processes (Clark *et al.* 2007; Roy *et al.* 2010; Guillén *et al.* 2015). In addition, many important aspects of the biology of *Drosophila melanogaster*, one of the most meticulously studied organisms in the history of modern science, can be transferred to other species (Adams 2000; Clark *et al.* 2007). However, *D. melanogaster* has the caveat of a poorly understood ecology, hindering extrapolation of findings in this model to other species (Goldman-Huertas *et al.* 2015). On the other hand, cactophilic *Drosophila* of the *repleta* group have proved to be valuable models in evolutionary ecology due to their tractable ecologies (Throckmorton 1975; Barker & Starmer 1982; Fogleman & Danielson 2001; Oliveira *et al.* 2012). These flies have the ability to utilize fermenting cactus tissues as breeding substrates, a feature that allowed them to successfully colonize the American deserts (Wasserman 1982; Ruiz & Heed 1988; Markow & O'Grady 2008). In turn, cactus diversification has been accompanied by the evolution of a wide variety of allelochemicals (Nobel 2002). Therefore, adaptation to new cactus hosts entailed the acquisition of mechanisms aimed to face such chemical challenges (Nobel 2002). Thus, patterns of host utilization have been largely explained by the chemical profile of each host in the *Drosophila* cactus model of the American deserts (Fogleman & Danielson 2001).

The South American *Drosophila buzzatii* is a cactophilic model species of the *repleta* group (mulleri subgroup, buzzatii complex) that has been studied for over 40 years and whose recent genome sequencing boosted its role in adaptation genomics (Manfrin & Sene 2006; Guillén *et al.* 2015). Originally from the arid lands of northwestern Argentina and southern Bolivia, *D. buzzatii* later became the only subcosmopolitan cactophilic fly following the expansion and transport of its main host, prickly pears of the cactus genus *Opuntia* (Manfrin & Sene 2006). Although *D. buzzatii* emerges primarily from several *Opuntia* species, it can also be recovered from chemically more complex columnar cacti of the genera *Trichocereus* and *Cereus*, which constitute secondary hosts (Hasson *et al.* 1992; Oliveira *et al.* 2012). In the sampling area for this study, the most common species used by *D. buzzatii* are *Opuntia sulphurea* and *Trichocereus terscheckii* (Hasson *et al.* 1992).

Unlike the plant species involved in the North American *Drosophila* cactus model, *T. terscheckii* is rich in the alkaloids mescaline and trichocereine, hallucinogenic compounds derived from phenylethylamine (Reti & Castrillón 1951; Ogunbodede *et al.* 2010). Besides a preference for oviposition in *Opuntia* cacti, previous studies have shown that breeding in *T. terscheckii* affects fitness in *D. buzzatii*, decreasing viability, body size and starvation resistance, and extending development (Fanara *et al.* 1999, 2006; Fanara & Hasson 2001; Soto *et al.* 2012). More recent studies showed that the addition of an alkaloid fraction extracted from *T. terscheckii* impairs viability and extends developmental time (DT), suggesting that the stressful conditions imposed by this cactus can be accounted for, at least in part, by the presence of alkaloids (Corio *et al.* 2013; Padró *et al.* 2014; Soto *et al.* 2014).

These results are consistent with studies in North American cactophilic *Drosophila*, which additionally identified mainly genes of the families cytochrome P450 and glutathione S-transferase involved in response to particular hosts and detoxification of cactus-derived isoquinoline alkaloids (Fogleman & Danielson 2001; Matzkin *et al.* 2006; Bono *et al.* 2008; Matzkin 2008, 2012).

The use of new technologies enables to achieve a broader panorama of the different elements involved in the genetic responses of organisms, with new levels of resolution and depth (Feder & Walser 2005; Roelofs *et al.* 2010). So far, transcriptomic studies (microarrays and recently RNA-Seq) on host plants in flies of this model system were restricted to the North American *Drosophila mojavensis*, a species with available genomic resources and main hosts without toxic alkaloids (Matzkin *et al.* 2006; Smith *et al.* 2013; Etges *et al.* 2015). Recently, a moderate-coverage RNA-Seq study based on *de novo* transcriptome assembly in isolated populations of *Drosophila mettleri* revealed that host shifts induce differences in expression profiles in thousands of genes (Hoang *et al.* 2015). *D. mettleri* has also the peculiarity of using soaked soils of cactus exudates as breeding substrate, where concentrations of plant secondary metabolites (in some cases isoquinoline alkaloids) may be tens of times more concentrated than in native tissues (Hoang *et al.* 2015).

Although alkaloids are among the most significant allelochemicals present in the host cactus of several desert *Drosophila*, to date, gene expression profile of model species feeding on its alkaloid-rich host plant has not yet been addressed. Notably, the transcriptome expression pattern of flies reared in *Opuntia*, the cactus proposed as the ancestral host of the repleta group and whose exploitation is shared by several species of the genus, also remains unknown.

The comparison of gene expression profiles across environments will help to understand the specific mechanisms that enable an organism to cope with environmental change and the associated stress. In addition,

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the study of gene expression profiles in flies reared in the presence of mescaline and phenylethylamine alkaloids in cactophilic flies may provide new insights into the genetics and metabolic routes associated with psychotomimetic amines. To address these issues, we assessed global gene expression in the desert fly *D. buzzatii* reared in primary and secondary natural hosts in different alkaloidiferous conditions by means of high-coverage transcriptome sequencing.

Materials and methods

Samples collection

The progeny of wild inseminated females collected in northwestern Argentina in summer 2010 (see Appendix S1, Supporting information) was used to establish inbred lines homozygous for the three most frequent second chromosome inversions (*standard*, *j* and jz^3) (Hasson *et al.* 1995) by sib-mating for eight generations. Crosses in which the progeny had the desired karyotype were selected for further inbreeding. Using this procedure, we derived three lines (from now on genotypes), fixed for the *standard* (line a), *j* (line b) and jz^3 (line c) inversions. Hereafter, strains were maintained in standard rearing medium under controlled conditions with frequent inspections to verify homozygosity.

During summer 2012, fresh pieces of *Opuntia sulphurea* and *Trichocereus terscheckii*, the main cactus hosts in the sampling area, were collected in Valle Fértil Natural Park (see Appendix S1, Supporting information) and stored *in situ* at −20 °C. Once in the laboratory, cacti were stored at −80 °C until the onset of the experiments (see Appendix S1, Supporting information).

Alkaloid extraction

Fresh tissues of each cactus (the same as used for experimental treatments) were ground and blended with absolute ethanol in a 1:1 proportion and left in agitation overnight at room temperature. The mixture was then filtered to remove the solid materials. The resulting alcoholic fraction was concentrated in a rotary evaporator at 40 °C to obtain an aqueous solution that then was acidified to pH 3 using diluted HCl 4%. From the latter phase, 1 mL was extracted and added to a Mayer's reagent aqueous solution (5% KI and 1.35% $HgCl_2$), resulting in a white precipitate that confirmed the presence of alkaloids in *T. terscheckii* and *O. sulphurea*. The aqueous acidic solution was partitioned with CH_2Cl_2 three times, retaining only the aqueous phase, which was further alkalized to pH 12 with NaOH 2N (the pKa of the protonated amino group of mescaline was 9.5). The resultant aqueous alkaline solution was partitioned again with CH_2Cl_2. Finally, the organic fraction was dried on a rotary evaporator and weighed. This protocol is based on the methodology described in Reti & Castrillón (1951) and Ogunbodede *et al.* (2010) and yields an alkaloid fraction enriched in phenylethylamines with mescaline-related compounds (Corio *et al.* 2013). Estimated alkaloid concentration in *T. terscheckii* was 0.4 mg/g, while *O. sulphurea* yielded 0.05 mg/g.

Alkaloid identification was accomplished by means of gas chromatography–mass spectrometry (GC-MS; Thermo Scientific EM/DSQ II—Trace GC Ultra AI3000). Running conditions were as follows: injector 200 °C; temperature programme 60 °C (1 min), increasing by 8 °C/min to 290 °C (10 min); splitless mode (1 min) and carrier gas He (108 935.5 Pa; 1.2 mL/min).

Experimental design

To investigate the effect of cactus host and phenylethylamine alkaloids on profiles of gene expression during *Drosophila buzzatii* development, the genotypes a, b and c were exposed to four treatments. The first two treatments aimed to assess the host plant effect. These treatments consisted in rearing batches of 50 individuals (from first to third larval stage) in seminatural media prepared with fresh tissues of *O. sulphurea* or *T. terscheckii*, supplemented with a killed yeast extract (see Appendix S1, Supporting information). The yeast extract was added to emulate the proteic contribution of cactophilic yeasts in their natural diet, thus avoiding a possible nutritional deficiency by the lack of this macronutrient.

The remaining treatments aimed to evaluate the effects of *T. terscheckii* alkaloids. To this end, a proper amount of *T. terscheckii* alkaloid extract was added to both cactus media in order to reach a final concentration of two-folds the estimated in *T. terscheckii* fresh tissues. Thus, alkaloid treatments were set up by the addition of 2.4 mg of the alkaloid extract in 0.1 mL of ethanol in the vials containing *T. terscheckii*, and 4.8 mg of the alkaloid extract in 0.1 mL of ethanol in the vials containing *O. sulphurea*. The same volume of ethanol was added to the vials not supplemented with the alkaloid fraction. All treatments are summarized in Table 1.

First-instar larvae of each genotype were obtained by releasing batches of sexually mature flies in egg-collecting chambers with a Petri dish containing egg-laying medium (2% agar). Petri dishes were removed after 12 h, inspected for the presence of eggs and incubated for 24 h to allow egg hatching.

For each treatment, groups of 50 first-instar larvae were randomly sampled from Petri dishes and

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Table 1 Experimental design. Summary of the composition of the four treatments

	Treatments			
	Native *Opuntia sulphurea*	*O. sulphurea* + alkaloids	Native *Trichocereus terscheckii*	*T. terscheckii* + alkaloids
Host	Primary	Primary	Secondary	Secondary
Cactus	*O. sulphurea*	*O. sulphurea*	*T. terscheckii*	*T. terscheckii*
Yeast extract	40 g/L	40 g/L	40 g/L	40 g/L
Alkaloids added	No	Yes	No	Yes
Alkaloids total	0× (native)	2×	1× (native)	2×

transferred to vials with the corresponding rearing medium. From a total of 15 vials per treatment (replicates), 10 were employed to obtain batches of third-instar larvae for the RNA-Seq study, while the remaining five were assigned to evaluate DT as a measure of a fitness-related trait. Vials were incubated at 25 ± 1 °C, 12:12-h light/dark photoperiod and mean relative humidity of 60 ± 10%. Replicates intended for larval collection were incubated until larvae reached the third-instar (start of the wandering phase). Larvae were gently picked out of the vials, rapidly washed three times in sterile PBS, snap-frozen in liquid nitrogen and then stored at −80 °C until RNA extraction. Vials assigned for DT measurement were incubated until adult emergence.

RNA extraction and transcriptome sequencing

Total RNA was extracted from batches of third-instar larvae using a combined TRIzol®/RNeasy® protocol optimized for *Drosophila* (Bogart & Andrews 2006). RNA concentration and quality was initially assessed by measuring A260/280 nm and A260/230 nm absorbance ratios using a NanoDrop® spectrophotometer. RNA integrity was checked in 1% agarose bleach gel (Aranda *et al.* 2012) and before library preparation in a 2100 Bioanalyzer system (Agilent Technologies, Palo Alto, CA, USA).

We used Illumina paired-end libraries with an insert size of 150–450 bp (maximal peak at 150 bp), and the Illumina HiSeq 2000 platform with 101 cycles for sequencing to generate 12 transcriptomes (one for each combination of *D. buzzatii* genotype and treatment). Both libraries preparation and sequencing were performed at Centre Nacional d'Anàlisi Genòmica (Barcelona, Spain).

Raw reads were quality-controlled using FASTQC v0.10.1 (Andrews 2010), filtered and trimmed for a quality score ≥26 and length ≥25 bases using FASTX-TOOLKIT v0.0.13 (http://hannonlab.cshl.edu/fastx_toolkit); only paired reads were retained.

Functional annotation of the reference genome and differential gene expression analysis

For a reference-based analysis, we used the sequence and the structural annotation consisting of 13 567 protein-coding genes (release 1) of the *D. buzzatii* genome (Guillén *et al.* 2015). Functional annotation of protein-coding genes was performed using BLAST2GO (Conesa *et al.* 2005) (see Appendix S1, Supporting information).

The software package RSEM v1.2.18 (Li & Dewey 2011) was used to estimate gene expression levels (in fragments per kilobase of transcript per million mapped reads, FPKM) in all transcriptomes using the protein-coding annotation of the *D. buzzatii* genome as reference (Guillén *et al.* 2015). Differential gene expression analysis among treatments was performed using the NOISeqBIO method from the NOISEQ R package (Tarazona *et al.* 2011, 2015). The statistical strategy in this package considers both the difference in mean expression and the fold-change to measure the change of expression between conditions, and therefore, the differentially expressed (DE) genes are computed based on these two measurements. Consequently, there was no need for applying a fold-change filtering on the results obtained by NOISEQ. Functional enrichment analyses of the DE genes were performed using the R package GOSEQ (Young *et al.* 2010) and REVIGO (Supek *et al.* 2011) for summary visualization, using Gene Ontology (GO) categories. In both cases, the three *D. buzzatii* genotypes were used as biological replicates.

The functions *boxplot*, *hclust* (clustering method: 'ward.D2') and *heatmap* of the R packages GRAPHICS and STATS, respectively (R Core Team 2015), were used for the visualization of the results of differential expression analysis.

Among the DE genes identified experiment-wide, we searched for those that based on the BLAST2GO designated name belong to five gene families [alcohol dehydrogenases (ADHs), esterases, glutathione S-transferases (GSTs), cytochromes P450 (P450s) and UDP-glycosyltransferases (UGTs)] known to be involved in

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detoxification processes related to host plant utilization, according to reports in other model systems (Aminetzach 2005; Dow & Davies 2006; Matzkin *et al.* 2006; Després *et al.* 2007; Low *et al.* 2007; Li *et al.* 2008; Matzkin 2012). Thus, using expression scores of each one of the selected genes, each combination of biological replicate and treatment may be conceived as a point in a multidimensional expression space that can then be reduced to three dimensions by means of multidimensional scaling (MDS). This method uses the structure of global similarity relations among cases of a matrix (i.e. genes) to construct a low-dimensional space in which the structure can be depicted, facilitating visualization and interpretation of transcriptomic similarity across treatments and biological replicates for these gene families. The R package SMACOF (De Leeuw & Mair 2008) was used for MDS along with the package RGL for the visualization of differential expression data.

Developmental time measurement

Developmental time is a widely used indicator of the degree of adaptation of an organism to a particular environment (Parsons *et al.* 1979; Soto *et al.* 2014). Thus, we measured DT as the time elapsed from the moment first-instar larvae were seeded in the vials until adult flies emergence from the puparium in each combination of treatment and biological replicate.

A linear mixed-effects model was fitted to test DT differences across treatments, using vials as experimental units (mean DT per replicate as dependent variable), cactus and alkaloid condition as fixed crossed factors and genotype as random factor. We employed the R packages LME4 (Bates *et al.* 2014) and CAR. Specific random terms included in the final model were selected comparing the Akaike Information Criterion (AIC) of several models and picking the one with the lowest (AIC = 371.447). These terms turned out to be random intercepts for each combination of genotype and cactus, as well as genotype and alkaloid condition. Statistical significance of these terms proved to be highly significant using likelihood ratio test (P value >0.001; d.f. = 2; $\chi^2 = 28.759$).

Results

Alkaloid identification

The analysis of the gas chromatograms of the alkaloid fractions obtained from each cactus showed conspicuously different profiles (Fig. 1). Two major peaks (retention times 25.00 and 25.72 min) can be clearly distinguished in the *Trichocereus terscheckii* fraction, which according to mass spectrometry analysis correspond to mescaline and trichocereine (*N*,*N*-dimethylmescaline), respectively (Figs 1A and S1, Supporting information). The other peaks correspond to minority phenylethylamine (mescaline-like) alkaloids that were not further characterized (Fig. 1A). The GC-MS performed for the *Opuntia sulphurea* fraction is the first reported for this species. According to mass spectral considerations, preliminary analysis of the major peaks suggests the presence of proline derivative alkaloids similar to 4-hydroxyproline.

Transcriptome sequencing and functional annotation of the reference genome

After quality filtering, we obtained an average of 78 million reads per treatment with a modal length of 101 bases per read (Table S1, Supporting information). About 85% of the reads could be correctly mapped to the reference genome (data not shown).

Using BLAST2GO, we were able to functionally annotate 10 188 genes, nearly 75% of the total number of protein-coding genes in the *Drosophila buzzatii* genome. Because each gene takes the annotation from the reference genes that are better annotated and with nearest identity, our functional annotation is composed of the best combination of available information from the phylogenetically closest species (*Drosophila mojavensis*, *D. virilis* and *D. grimshawi*) and more accurately annotated species (*Drosophila melanogaster*) (Fig. S2; See Appendix S1, Supporting information).

Differential gene expression analysis

A total of 28% of all protein-coding genes (3867 of 13 567) were DE across treatments experiment-wide (false discovery rate (FDR) < 0.05).

First, we compared expression profiles between larvae grown in media prepared with fresh tissues of each cactus, which represents the closest rearing condition in nature (native condition). Of the 3617 genes (26%) DE across cacti, 3556 were up-regulated and 61 were down-regulated in *T. terscheckii* vs. *O. sulphurea*, respectively (Fig. 2A, Table S2A, Supporting information).

Second, we evaluated the differences in gene expression between both cactus media supplemented with the *T. terscheckii* alkaloid fraction (*O. sulphurea* + alkaloids and *T. terscheckii* + alkaloids). We only detected 22 DE genes (0.1% of *D. buzzatii* protein-coding genes) in this comparison. Fifteen were up-regulated and seven were down-regulated in *T. terscheckii* + alkaloids vs. *O. sulphurea* + alkaloids, respectively (Fig. 2A, Table S2A, Supporting information).

The next comparison aimed to analyse the differences in gene expression between samples reared with and

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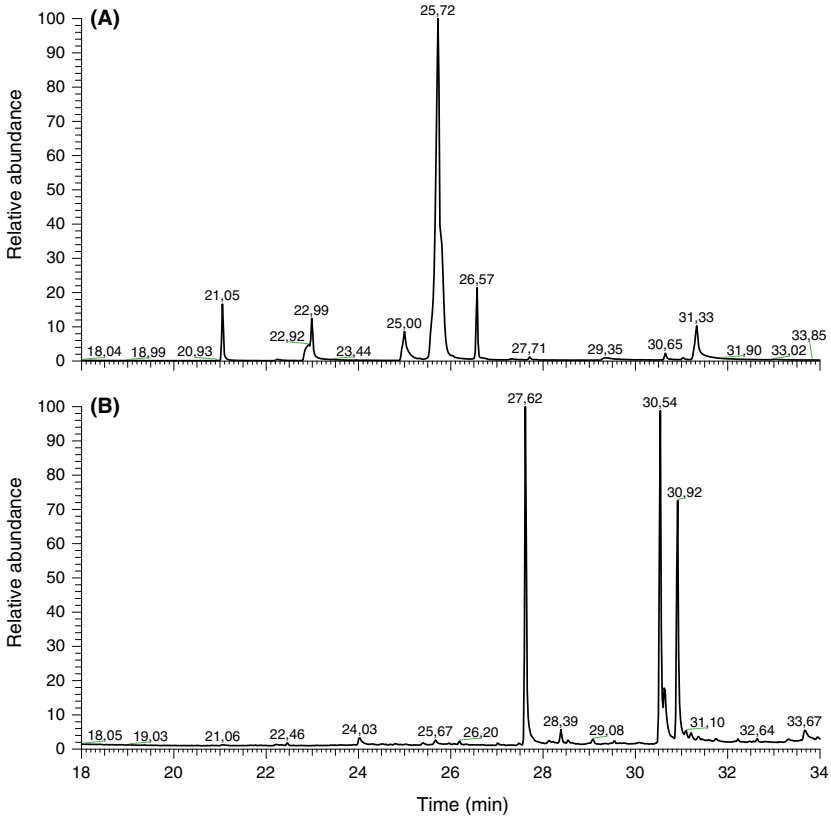

Fig. 1 Gas chromatograms of the alkaloid fractions extracted from fresh tissues of (A) *Trichocereus terscheckii* and (B) *Opuntia sulphurea*.

without the alkaloid supplement within each cactus. In *O. sulphurea*, we identified a total of 1039 genes: 1007 were up-regulated and 32 were down-regulated in *O. sulphurea* + alkaloids vs. native *O. sulphurea*, respectively (Fig. 2B, Table S2B, Supporting information). In contrast, only 36 genes were DE in *T. terscheckii*, 28 up-regulated and eight down-regulated in *T. terscheckii* + alkaloids vs. *T. terscheckii* in native condition, respectively (Fig. 2B, Table S2B, Supporting information).

For the last analysis, we pooled the transcriptomic profiles of larvae reared in both cactus media in each condition (native vs. alkaloids added) to assess the effect of alkaloids regardless of the possible influence of other factors that may differ between cacti (e.g. nutritional differences). A total of 34 DE genes were identified in this comparison, and all were up-regulated in the alkaloid-enriched condition vs. the native condition (Table S2C, Supporting information).

Functional enrichment analysis

To further characterize the genomic responses in the different treatments, we performed an enrichment analyses for GO terms related to biological processes and molecular functions on the set of DE genes in the comparisons outlined in the previous section.

Despite limitations of the annotation that may affect functional enrichment analysis (see Appendix S1, Supporting information), over-represented GO terms were related mainly to stress response and detoxification in larvae raised in the putatively stressful conditions (the secondary cactus host and in both cacti with alkaloids added) as compared to more benign conditions (FDR < 0.05, Table S3 and Fig. S3, Supporting information).

Detox-related terms such as 'glutathione transferase activity' and 'xenobiotic-transporting ATPase activity' were detected in the GO terms enrichment analysis in the *O. sulphurea* vs. *T. terscheckii* comparison. However, these terms did not appear from the set of enriched GO terms in this comparison after applying multiple testing correction.

Host adaptation and detoxification genes

Among the DE genes identified experiment-wide, 62 are members of five gene families known to be involved in detoxification processes and related to host plant utilization: 5 ADHs, 10 esterases, 15 GSTs, 25 P450s and 7 UGTs. Expression scores for each one of the 62 genes in each combination of biological replicate and treatment were reduced to three dimensions by means of MDS. The point of this methodology is to visualize similarity

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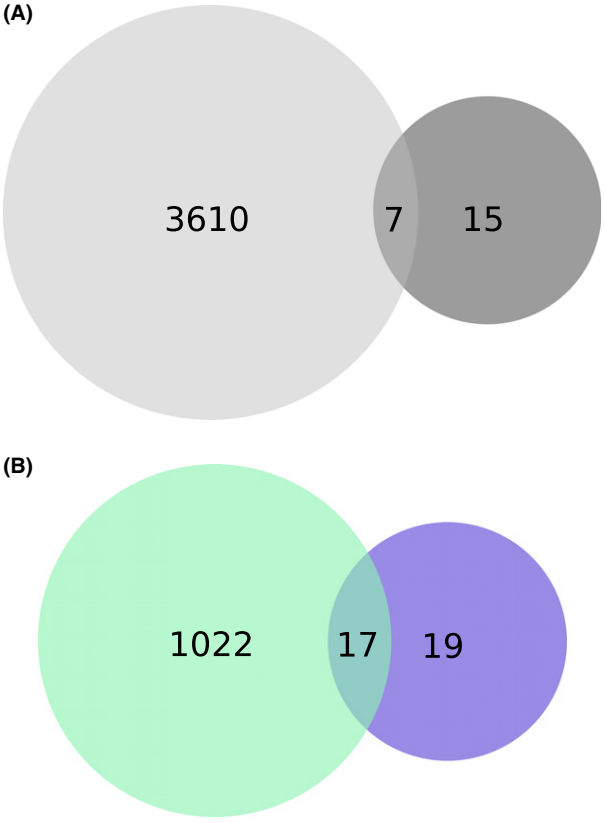

Fig. 2 The number of differentially expressed genes in pairwise comparisons between treatments. The intersection in the Venn diagrams discriminates the number of shared genes. (A) Between the two cactus in native conditions (large circle), and between the two cactus with alkaloids added (small circle). (B) Between the native and alkaloidiferous conditions in *Opuntia sulphurea* (large circle) and between the native and alkaloidiferous conditions in *Trichocereus terscheckii* (small circle).

relationships existing in the data sets. The results of the MDS analysis revealed that despite the large variation observed among genotypes (within treatments), treatments appear fairly distant in this particular gene expression space (Fig. 3A, stress = 0.023). Moreover, a common pattern across the five gene families can be observed in the box plots presented in Fig. 3. Noteworthy, 21 (~34%) of the 62 of these DE genes exhibited low expression scores (of a few FPKM) (Table S4, Supporting information). Despite the apparent heterogeneity among biological replicates and genes (within gene families), average expression scores tended to be higher in the more stressful treatments (Fig. S4, Supporting information), a trend that is particularly clear for ADHs (Fig. 3B), GSTs (Fig. 3D), P450s (Fig. 3E) and UGTs genes (Fig. 3F). Next, we explored in detail the functional annotation of the 62 genes related to detoxification and resource adaptation. In addition to the reactions in which the enzymes encoded by genes of these families are involved (typically solubilization and deactivation of metabolites), we found genes with possible roles in development, neurobiological processes and transcription regulation (Table S5, Supporting information).

Differential expression patterns

To narrow down the set of genes that are candidates to be involved in the shift from *O. sulphurea* to *T. terscheckii*, and in the response to phenylethylamine alkaloids, we raised the stringency of the FDR cut-off criterium to $q < 0.01$. This allowed us to identify with greater confidence a total of 99 genes that, beyond the families evaluated in the previous section, form part of the genetic response of the organism to the different rearing conditions (Table S6, Supporting information). The expression scores of the genes included in this reduced list were used to build a heatmap aimed to visualize expression profiles across biological replicates and treatments (Fig. 4). Prior to heatmap construction, genes for which functional annotation was null or scarce, or genes for which we could not discard that variation in expression profiles may possibly reflect noise, were eliminated from the data set. Thus, 45 genes were retained after filtering, because 40 were discarded due to the lack of informative annotation (Table S6, Supporting information) and 14 did not pass the noise criterion (see Appendix S1, Supporting information).

In general terms, the genes included in the heatmap can be classified into three categories according to expression patterns. On the one hand, we found a group of genes that exhibited higher expression in native *O. sulphurea* as compared to the other conditions (triangles in Fig. 4). Interestingly, this group includes four genes (of a total of 9) whose annotation is related to cuticular proteins (Table S6, Supporting information). Second, a group consists of 29 genes with higher expression scores at high alkaloid concentrations with a roughly dose-dependent pattern (squares in Fig. 4). Among these genes, nine (two ADHs, three GSTs and four P450s) belong to the gene families involved in host adaptation and detoxification evaluated in the previous section. Additionally, within this group, there are also other genes with annotations related to oxidation–reduction, development and neurobiological processes (Table S6, Supporting information). Finally, seven genes do not fit in the previous categories (circles in Fig. 4), which in some arguably cases appear to have increased expression in native *O. sulphurea* and *O. sulphurea* + alkaloids treatments.

Additionally, using information available in the reference genome, we evaluated the genomic and contextual location of the 62 DE genes that belong to five gene families in the previous section (Table S5, Supporting

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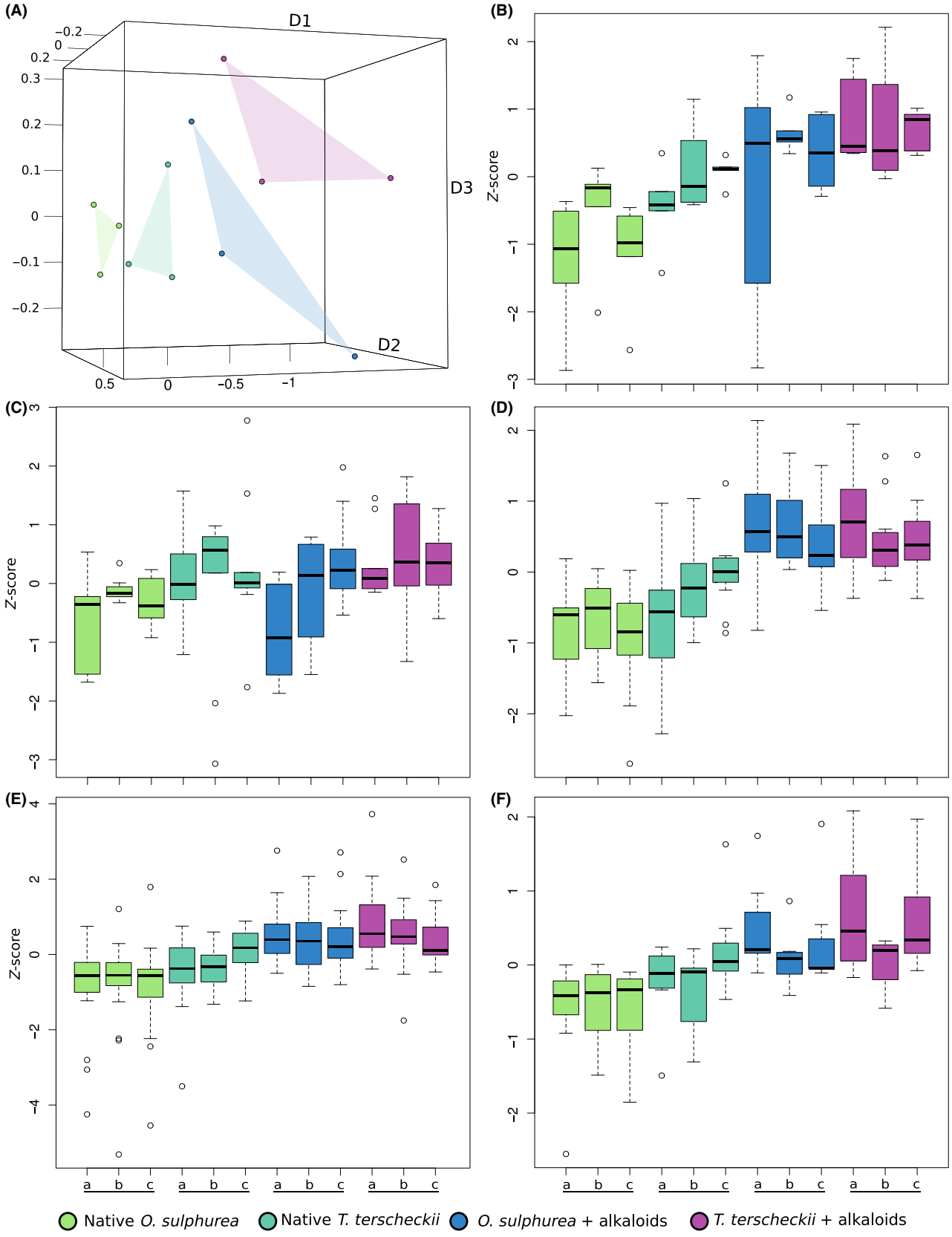

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Fig. 3 Expression analysis of 62 differentially expressed genes ($q < 0.05$), belonging to families previously reported as related to host adaptation and detoxification, among treatments and genotypes (a, b and c). (A) Multidimensional scaling for the expression scores of the evaluated genes. The triangular plane formed between the three points representing the biological replicates is intended only for better visualization. (B–F) Boxplots for the 5 ADHs (B), 10 esterases (C), 15 GSTs (D), 25 P450s (E), 7 UGTs (F).

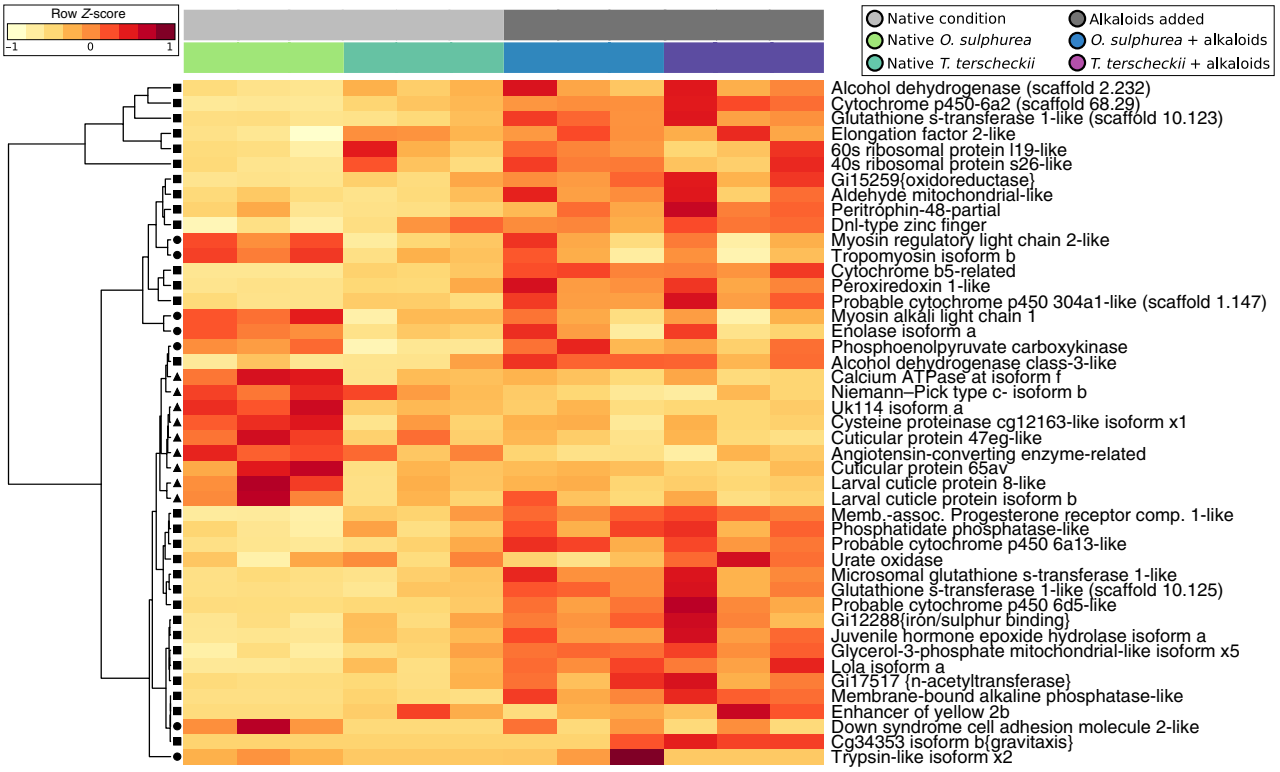

Fig. 4 Heatmap based on gene expression profiles across treatments and genotypes for differentially expressed genes significant at a FDR of 0.01 and after filtering due to scarce functional annotation or possible noise. For each treatment, the order of genotypes is as follows: a, b, c. Black triangles, squares and circles in each row refer to categories according to expression profiles (see text for further explanation).

information) and the 45 DE genes included in the heatmap (Fig. 4, Table S6, Supporting information). We identified 24 genomic neighbourhoods (2-Mb windows, see Appendix S1, Supporting information for detailed neighbourhood definition): seven in the second chromosome, six in the third, two in the fourth, six in the fifth, two in the chromosome X and one placed in a scaffold not yet anchored to chromosomes in the reference genome (Table S7, Supporting information).

Fitness-related trait

Flies reared in the presence of higher doses of alkaloids extended DT as compared to individuals reared in native cactus conditions ($P < 0.05$, Table S8, Supporting information). This result is in line with gene expression data, because the greatest variation occurs between treatments of added alkaloids and the natives. Moreover, we observed that in general larvae reared in the secondary host took longer time to reach adulthood than in the primary host (Fig. 5).

Discussion

Genome-wide expression studies based on RNA-Seq are revolutionizing the fields of ecological and evolutionary genomics, contributing new insights into the responses that organisms deploy in alternative environments. In effect, RNA-Seq allows the evaluation of the magnitude and nature of genetic changes, from a broad view and at the level of candidate genes or genetic pathways underlying ecologically relevant phenotypes. This approach has been employed in a few insect/host systems to investigate the responses to protease inhibitors and phenolic compounds in lepidopteran species (Vogel *et al.* 2014) and in response to different hosts in the cactophilic flies *D. mojavensis* (Smith *et al.* 2013) and *Drosophila mettleri* (Hoang *et al.* 2015). In the present study,

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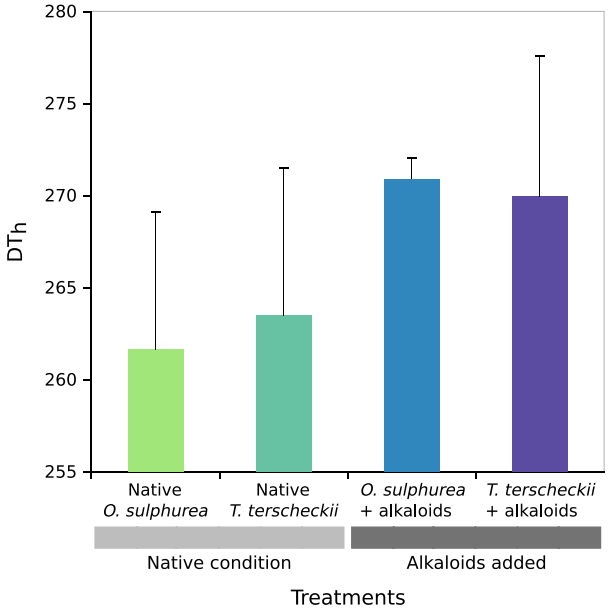

Fig. 5 Barplot for developmental time expressed in hours (DT_h). The analysis detected significant differences between native condition and alkaloids added.

we provide new data onto the role of transcriptional plasticity in response to alternative hosts and a novel insight into the study of genome-wide responses to mescaline-like alkaloids.

The cactus hosts are chemically different

A large number of field and laboratory studies have documented the host cactus effects on the life cycle of the cactophilic fly *Drosophila buzzatii* (Fanara *et al*. 1999; Fanara & Hasson 2001). Together, these results point out that the two cactus hosts represent unique ecological and developmental challenges during larval rearing. Moreover, the available evidence indicates that *Trichocereus terscheckii* imposes stressful conditions to the growing larvae as compared to the more benign environment offered by *Opuntia sulphurea* (Soto *et al*. 2008, 2014).

This has lead to the hypothesis that differences in cactus chemistry (both nutritional and allelochemical) could account for the observed variation in larval performance across cacti (Fogleman & Danielson 2001). However, the lack of nutritional profiles and a thorough characterization of secondary metabolites of both cacti precluded its verification until now.

Recent nutritional studies of both cacti revealed that *O. sulphurea* has slightly more free sugars (although lower than other *Opuntia*) and total fat, while *T. terscheckii* has a richer composition of fatty acids (Padró & Soto 2013; Carreira *et al.* 2014).

Concerning alkaloid content, the comparison between cacti revealed large differences. *T. terscheckii* not only has ten times more alkaloids than *O. sulphurea*, but also a completely different chemical profile. The compounds detected in *T. terscheckii* were phenylethylamine derivatives, mainly mescaline and trichocereine. These alkaloids were also present in extracts that were shown to affect survival, DT, body size and wing development in *D. buzzatii* (Corio *et al.* 2013; Padró *et al.* 2014; Soto *et al.* 2014). On the other hand, the GC-MS results of the *O. sulphurea* fraction showed the presence of compounds that did not match the phenylethylamine alkaloids profile. Interestingly, we found similarity with proline derivatives of unreported toxicity, similar to those reported for *Opuntia vulgaris* (Jiang *et al.* 2003).

Transcriptomic responses and functional enrichment

The results of performance assays in both cacti and in media supplemented with *T. terscheckii* alkaloids indicate that the stressful conditions imposed by the secondary host are related to the presence of mescaline and trichocereine. These differences are reflected in the numbers of DE genes detected in the comparison between the two hosts under native conditions (3617 genes), as well as between native *O. sulphurea* and *O. sulphurea* + alkaloids (1039 genes). Of the 1039 DE genes in the comparison *O. sulphurea* vs. *O. sulphurea* + alkaloids, 886 (85%) were also found among the 3617 DE genes detected in the comparison *O. sulphurea* vs. *T. terscheckii*. These results involving the only phenylethylamine alkaloid-free treatment (native *O. sulphurea*) strongly contrast with those obtained in the remaining comparisons, where the numbers of DE genes were two orders of magnitude lower. In fact, the numbers of DE genes detected in the comparison native *T. terscheckii* vs. *T. terscheckii* + alkaloids (36 genes), and between the transcriptomic profiles of the pooled samples native *O. sulphurea* plus native *T. terscheckii* vs. *O. sulphurea* + alkaloids plus *T. terscheckii* + alkaloids (34 genes), suggest that part of the transcriptional response is sensitive to the dose of mescaline-like alkaloids. Together, these results reveal that a very important proportion of the transcriptome is inducible by the rearing environment, mainly explained by the presence of *T. terscheckii* alkaloids.

Such transcriptional plasticity may help to cope with stressful rearing conditions, inherent to the use of *T. terscheckii*. However, it should be kept in mind that the influence of other factors, such as nutritional differences between host cacti, cannot be ruled out. In this sense, the comparison between the two hosts with added alkaloids indicates that a small fraction of the transcriptomic response (22 genes) may be responding to

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components different than alkaloids. Nevertheless, there is a common pattern to all comparisons: from the total number of DE genes, the great majority of the changes in expression profiles occurred in larvae reared under the more stressful conditions, that is high alkaloid content (Table S2, Supporting information).

Analysing the functional annotation (biological processes and molecular functions) of the DE genes in each comparison, we found that the enriched GO terms in the sets of overexpressed genes in the alkaloid-rich treatments are mainly related to stress response and detoxification mechanisms (Fig. S3, Supporting information). According to the functional enrichment analyses, the cellular stress response is apparently mediated by the ubiquitin-proteasome pathway and other related processes (protein folding and transport, translational regulation and response to DNA damage). The ubiquitin-proteasome pathway is responsible for a large amount of intracellular proteolysis, including the selective degradation of oxidatively damaged proteins. It also plays a central role in the post-translational quality control, by removing unrepaired proteins that process-related chaperones failed to refold (Shang & Taylor 2011). Moreover, elements of this pathway affect other important cellular processes such as transcription and DNA repair (Muratani & Tansey 2003; Bergink & Jentsch 2009). This observation is very interesting because one of the causes of abnormal proteins is due to denaturation induced by various environmental stresses, such as oxidation. Thus, the ubiquitin-proteasome pathway seems to be key in coping with oxidative stress and, at the same time, is modulated by the cellular redox status (Shang & Taylor 2011). Notably, the ubiquitin-proteasome has been reported to be involved in detoxification and adaptation to different hosts in tephritid fruit flies and cactophilic flies (Smith *et al.* 2013; Hoang *et al.* 2015; Ragland *et al.* 2015). Precisely, another important part of the enriched GO terms in our study is related to detoxification processes (mainly oxidation–reduction, glutathione and alcohol metabolism). These well-known pathways that usually deactivate or increase hydrophilicity of toxic metabolites involving enzymes such as cytochrome P450s and GSTs can modify the oxidative state of the cell and produce different types of reactive oxygen species (Feyereisen 1999; Tu & Akgül 2005). Therefore, our functional enrichment analysis (Table S3, Fig. S3, Supporting information) revealed elements associated with detoxification and regulation of the enhanced redox state, probably related to the stressful conditions experienced by larvae raised in mescaline-/trichocereine-rich media. The observation of genes with no direct implication in alkaloid metabolism (i.e. detoxification) suggests a complex genetic basis underlying stress-buffering mechanisms in *D. buzzatii*. In fact, polygenic resistance is proposed to be involved in responses to mild stresses and/or in species with a generalist habit as an adaptation to cope with unpredictable plant defences (Hoffmann & Hercus 2000; Ali & Agrawal 2012).

Expression profiles are affected by the rearing environment

Differences in expression profiles across treatments were clear, despite the variation among genotypes, when we considered the set of 62 DE genes that belong to gene families known to be involved in detoxification and host adaptation (Fig. 3A). These results illustrate the sensitivity of the response orchestrated by these genes to cope with diverse environmental challenges. Likewise, this observation can be extended to the 45 genes included in the heatmap shown in Fig. 4, where three apparent categories according to expression patterns can be discerned. Interestingly, the observation of the location of these DE genes in the reference genome showed that more than half are found within any of 24 genomic neighbourhoods identified and that genes in the same neighbourhood seem to be functionally related (Table S7, Supporting information). Because the position effect is a phenomenon that can alter gene expression depending on its surroundings (De *et al.* 2009), a genomic neighbourhood influence on the regulation of the evaluated genes should be considered to fully account for the complexity of the transcriptomic assays.

Detox genes related to secondary host's xenobiotics

Using the available annotation of the set of DE genes mentioned in the previous section (Tables S5 and S6, Supporting information), we explored the possible functional links among genes that contribute to shape the spectrum of the differential genetic response to the experimental conditions. In our study, several genes were up-regulated in treatments with higher concentration of phenylethylamine alkaloids as compared to more benign environments for larval development. For instance, nine DE genes annotated as P450s are associated with the response to insecticide (Table S5, Supporting information). Among these genes, two annotated as *probable cytochrome p450 304a1-like* were reported as up-regulated in *D. mettleri* larvae reared in soil soaked with the exudate of a columnar cactus that contains isoquinoline alkaloids (Hoang *et al.* 2015). Moreover, these genes along with *cytochrome p450 6a9* were also detected in the response to different hosts in larvae of *Rhagoletis* fruit flies (Ragland *et al.* 2015). In addition, four of these P450s are also related to the metabolism of particular xenobiotics. These are the cases of *probable cytochrome*

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p450 6a14-like and two genes annotated as *cytochrome p450 6a2* that are associated with 'response to caffeine', a methylxanthine alkaloid with psychoactive properties (Fredholm *et al.* 1999). Interestingly, both *p450 6a2* genes were differentially regulated in comparisons between *Rhagoletis* larvae reared on different host fruits (Ragland *et al.* 2015). In addition, *probable cytochrome p450 6v1-like* and one of the genes annotated as *cytochrome p450 12a2-like* are associated with the biological process 'response to DDT', a formerly worldwide used insecticide. These genes may provide a link with the detox-related gene family of the GSTs, because four DE genes annotated as *glutathione S-transferase d1* share the molecular function 'DDT-dehydrochlorinase activity' (Willoughby *et al.* 2006).

Also among xenobiotic-responsive genes, *cytochrome p450 6d1-like*, which was overexpressed in alkaloid-rich conditions, is associated with 'response to camptothecin', a cytotoxic quinoline alkaloid (Ulukan & Swaan 2002). Aside from P450s and GSTs genes (although sharing the same expression pattern), the *membrane-bound alkaline phosphatase-like* gene is associated with 'response to nicotine', a pyridine alkaloid with insecticide properties due to its antiherbivore characteristics and also known by its psychoactive properties (Ujváry 1999).

All these genes related to the detoxification of xenobiotics showing higher expression in alkaloid-rich treatments are candidates to form part of the genetic response deployed by *D. buzzatii* when exposed to a resource that contains mescaline and trichocereine.

The response to challenging resources has a strong redox component

Many of the individually analysed DE genes have functional terms associated with processes related to oxidation and reduction (Tables S5 and S6, Supporting information). These associations are shared by all P450 genes up-regulated under the more stressful treatments (Fig. S4, Supporting information). Noteworthy, the *probable cytochrome p450 28a5-like*, *probable cytochrome p450 6d5-like* and two genes annotated as *cytochrome p450 4p1-like* were reported as up-regulated on *D. mettleri* larvae reared in cactus soil media (Hoang *et al.* 2015). Likewise, these P450s *6d5-like* and *4p1-like* along with *probable cytochrome p450 6a13-like*, *cytochrome p450 4g1-like* and *probable cytochrome p450 4s3-like* were also shown to be sensitive to rearing in alternative hosts in *Rhagoletis* fruit flies (Ragland *et al.* 2015).

Additionally, we found other genes up-regulated in alkaloid-rich media that are associated with redox processes, such as *cytochrome b5-related* and five ADHs. All these ADHs share the functional term 'ethanol oxidation', a biological process that is also associated with the DE gene *aldehyde mitochondrial-like*. Among these genes, *alcohol dehydrogenase class-3-like* may provide a link with GSTs, as it is associated with the oxidation of glutathione functions and has glutathione dependence (Danielsson & Jörnvall 1992).

Finally, some of the DE GSTs are associated with the biological process 'response to oxidative stress'. Among these genes, *glutathione S-transferase e1* and *glutathione S-transferase isoform b* are also associated with 'response to heat' and 'determination of adult lifespan', respectively. The latter is shared by the DE gene *peroxiredoxin 1-like*, which is also linked to the glutathione-mediated response by the associated molecular function 'glutathione peroxidase activity'. Interestingly, this redox state-regulated gene that encodes an antioxidant enzyme (Radyuk *et al.* 2001) is also associated with 'response to DNA damage stimulus'.

Altogether, these DE genes associated with oxidation–reduction processes may be part of the detox–redox regulating elements involved in the stress response inherent to the exploitation of *T. terscheckii*.

The host switch affects the expression of developmental genes

It is interesting to note that some of the DE P450 genes detected in our study are associated with the development of different body structures. This is not an unexpected observation because P450 enzymes, which are at the core of the response to environmental challenges, are found in the biosynthetic pathways of ecdysteroids and juvenile hormones, regulating growth and development of insects (Feyereisen 1999). In fact, the genes *probable cytochrome p450 9f2-like* and *probable cytochrome p450 6d4-like*, both overexpressed in treatments containing the secondary host, are associated with 'wing disc development'. Noteworthy, these two genes have also been detected in the response to alternative fruit hosts in larvae of *Rhagoletis* fruit flies (Ragland *et al.* 2015). Along with these genes (and sharing the same expression profile), *probable cytochrome p450 310a1-like* is associated with 'regulation of Wnt pathway', a highly conserved pathway in animal development involved in the patterning of adult body structures, including the wings of *Drosophila melanogaster* (Bejsovec 2006). These observations may help to explain the results of previous studies in *D. buzzatii*, showing that wing morphology, bilateral symmetry and venation patterning are severely affected in flies reared in *T. terscheckii* and in media with high alkaloid content (Soto *et al.* 2008; Padró *et al.* 2014).

Our study also unveiled several DE genes related to the development and structure of chitin-based cuticle

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(Tables S5 and S6, Supporting information). In particular, chitin-related genes have recently gained prominence in studies of plant-feeding insects, introducing a new factor in the interaction between insects and the environment (Matzkin 2012; Celorio-Mancera *et al.* 2013; Vogel *et al.* 2014; Hoang *et al.* 2015). Among these genes, *cytochrome p450 6d4-like*, up-regulated in treatments with higher alkaloid concentrations as compared to less harsh conditions, is associated with chaeta development, a structure mainly composed of chitin. Similarly, the *cytochrome p450 4g1-like* gene (up-regulated in *T. terscheckii* media with respect to *O. sulphurea*) is involved in the biosynthesis of cuticular hydrocarbons (Qiu *et al.* 2012). This gene, which is proposed as a key acquisition that enabled insects to colonize dry environments endowing increased desiccation resistance (Qiu *et al.* 2012), has been also reported as overexpressed in silkmoths entering diapause, a mechanism that helps to overcome unfavourable environmental conditions (Yang *et al.* 2008).

Interestingly, we also detected chitin-related developmental genes up-regulated in native *O. sulphurea* relative to alkaloid-rich treatments (Figs 4 and S4, Supporting information). It is noteworthy among these, *probable cytochrome p450 4e1-like* gene (the only DE P450 that exhibited an inverse expression pattern), which is associated with 'structural constituent of cuticle' and has a proposed function related to metabolism of hormones and breakdown of synthetic insecticides in *D. melanogaster* (UniProt Consortium 2011). This functional link between metabolism, hormonal regulation and detox–redox response is explicit in the DE genes *cytochrome p450 306a1-like* and *membrane-associated progesterone receptor component 1-like*. The former, which corresponds to one of the halloween genes (*phantom*, *phm*) in *D. melanogaster* (Rewitz *et al.* 2006), exhibited low expression in native *O. sulphurea*, while the latter was overexpressed in alkaloid-rich treatments. Both genes are associated with redox processes and metabolism of xenobiotics as well as ecdysone, as steroid hormone that is directly related to the timing of developmental programmes in *Drosophila* (Yamanaka *et al.* 2013). Sharing the same expression pattern is the gene annotated as *juvenile hormone epoxide hydrolase isoform a*, involved in the degradation of the juvenile hormone, a major regulator of insect physiology, from development and reproduction to diapause and polyphenisms (De Kort & Granger 1996). Hence, this gene may have an important role in adaptation to varying environmental conditions due to its possible involvement in phenotypic plasticity (Merilä & Hendry 2014). In addition, *juvenile hormone epoxide hydrolase* is proposed as an enzyme that evolved from an original form involved in detoxification, reinforcing the link between hormones, xenobiotics metabolism and development (Wojtasek & Prestwich 1996). These results are consistent with a transcriptomic study in the predaceous pentatomid insect *Arma chinensis* fed with alternative diets, which showed a relationship between DT and the expression of a P450 gene related to moulting hormone metabolism, among other juvenile hormone metabolism genes (Zou *et al.* 2013). Moreover, the same study related *venom carboxylesterase-6-like* to DT (Zou *et al.* 2013), a gene of the esterase family that we found up-regulated in native *T. terscheckii* (as compared to native *O. sulphurea*).

Therefore, these DE genes may help to explain DT variation observed in *D. buzzatii* larvae reared in their secondary host and to explain other reported effects on its life cycle associated with the presence of phenylethylamine alkaloids (Fanara *et al.* 1999, 2006; Soto *et al.* 2008, 2014).

Final conclusions and future prospects

Our study uncovers considerable transcriptional plasticity upon the host plant switch from the primary host *O. sulphurea* to the secondary *T. terscheckii*. Even though we acknowledge that the present study may be considered as an approximation to the natural breeding sites of *D. buzzatii*, we were more interested in the effect of the native plants themselves and *T. terscheckii* alkaloids on gene expression, avoiding to add less controlled factors (e.g. the community of microorganisms associated with the decaying cacti). Such differences in expression profiles may be representative of the genetic changes that facilitate the colonization of novel habitats and help to mitigate the stresses imposed by a novel host which, additionally, contains particular xenobiotics.

In the repleta group, the ecological transition from the use of fermenting fruits of noncactus plants in moist forests to arid-adapted desert plants like *Opuntia* and other cacti is one of the most extensive and successful events in *Drosophila* (Oliveira *et al.* 2012). Two main habits in terms of host plant use can be recognized in the repleta group, *Opuntia* and columnar breeders. Phylogenetic studies suggest that the acquisition of *Opuntia* is ancestral and that the shift to columnar cacti (the derived state) occurred several times in the evolution of the repleta group (Oliveira *et al.* 2012).

Drosophila buzzatii is a representative of the ancestral condition because it is mainly an *Opuntia* breeder, but also has the ability to use several genera of columnar cacti, highlighting its generalist features within the group. Our gene expression survey reveals a massive response involving genes related to detoxification, oxidation–reduction and stress response. Moreover, more than a few DE genes also affect development, behaviour

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and perception of different types of stimuli. However, more studies are needed to confirm the link between many of these genes and rearing in alternative host plants. Thus, we may conclude that niche width in *D. buzzatii* may be related to a complex genetic architecture underlying buffering stress mechanisms in order to cope with unpredictable chemical cactus defences.

Interestingly, *Drosophila koepferae* (*D. buzzatii*'s sibling species) offers the chance to examine expression profiles in an organism that may be considered the opposite complement of *D. buzzatii* because it is a columnar cactus specialist that can secondarily use *Opuntia*. Such comparative transcriptomic approach would help to deepen our understanding on the role of host plant shifts and natural chemical stress driving ecological specialization.

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D.N.D.P., J.P., I.M.S. and E.H. designed the experiments. D.N.D.P. and J.P. performed the experiments. D.N.D.P., P.F., S.T. and P.M.C. performed bioinformatics and statistical analysis of the results. A.C. supervised bioinformatics analysis. D.N.D.P., J.P. and E.H. drafted the article. I.M.S., S.T., P.M.C., H.D. and A.C. provided useful comments and contributed to revisions. All authors have read and approved the submitted version.

Data accessibility

The RNA-Seq data discussed in this publication have been deposited in NCBI's Short Read Archive with accession no. SRP078503. The R scripts and data produced in the developmental time and differential expression analyses are available through Dryad: doi:10.5061/dryad.55c8q.

Supporting information

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

Fig. S1 Mass spectra of mescaline and trichocereine found in *T. terscheckii* alkaloids extract.

Fig. S2 Descriptive charts of the functional annotation with BLAST2GO.

Fig. S3 Semantic similarity-based scatterplots for summarize GO terms resulting from functional enrichment of the DE genes in the comparisons.

Fig. S4 Heatmap of 62 DE genes members of five gene families related with host adaptation and detox pathways.

Table S1 Sequencing data of the transcriptomes.

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Table S2 Numbers of differentially expressed genes.

Table S3 Functional enrichment of the DE genes in the comparisons.

Table S4 Gene expression scores of 62 DE genes members of five gene families related with host adaptation and detox pathways.

Table S5 Functional annotation of 62 DE genes members of five gene families related with host adaptation and detox pathways ($q < 0.05$).

Table S6 Functional annotation of 99 DE genes ($q < 0.01$).

Table S7 Genomic and contextual location of the analysed DE genes.

Table S8 Linear mixed model testing differences in DT.

Appendix S1 Additional information about samples collection, experimental design, functional annotation and differential expression patterns.

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