MOLECULAR ECOLOGY

Molecular Ecology (2015) **24**, 2759–2776 doi: 10.1111/mec.13191

Differences in performance and transcriptome-wide gene expression associated with *Rhagoletis* (Diptera: Tephritidae) larvae feeding in alternate host fruit environments

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Abstract

Host race formation, the establishment of new populations using novel resources, is a major hypothesized mechanism of ecological speciation, especially in plant-feeding insects. The initial stages of host race formation will often involve phenotypic plasticity on the novel resource, with subsequent genetically based adaptations enhancing host-associated fitness differences. Several studies have explored the physiology of the plastic responses of insects to novel host environments. However, the mechanisms underlying evolved differences among host races and species remain poorly understood. Here, we demonstrate a reciprocal larval performance difference between two closely related species of *Rhagoletis* flies, *R. pomonella* and *R. zephyria*, specialized for feeding in apple and snowberry fruit, respectively. Microarray analysis of fly larvae feeding in apples versus snowberries revealed patterns of transcriptome-wide differential gene expression consistent with both plastic and evolved responses to the different fruit resources, most notably for detoxification-related genes such as cytochrome p450s. Transcripts exhibiting evolved expression differences between species tended to also demonstrate plastic responses to fruit environment. The observed pattern suggests that *Rhagoletis* larvae exhibit extensive plasticity in gene expression in response to novel fruit that may potentiate shifts to new hosts. Subsequent selection, particularly selection to suppress initially costly plastic responses, could account for the evolved expression differences observed between *R. pomonella* and *R. zephyria*, creating specialized races and new fly species. Thus, genetically based ecological adaptations generating new biodiversity may often evolve from initial plastic responses in gene expression to the challenges posed by novel environments.

Keywords: host adaptation, larval fitness, plant–insect interactions, *Rhagoletis*, speciation, transcriptome

Received 16 December 2014; revision received 30 March 2015; accepted 1 April 2015

Introduction

Under the biological species concept, speciation is defined as the evolution of barriers to gene flow between populations (Coyne & Orr 2004). If we are to resolve the ultimate cause(s) for speciation, then it is

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most important to discern reproductive isolation that arises earliest, initially causing populations to evolve along diverging trajectories. This is particularly true for ecological speciation, where divergent adaptation to different habitats is hypothesized to initially drive the evolution of reproductive isolation (Rundle & Nosil 2005). Systems of closely related host races or sibling species that are diverging in the face of gene flow can provide particularly valuable natural laboratories to test the role that ecological adaptation may play in initiating population divergence. In such cases, gene flow is predicted to homogenize phenotypic and genetic differences between populations not directly acted upon by divergent selection or indirectly affected by physical linkage (Berlocher & Feder 2002; Funk *et al.* 2002). Thus, reciprocal cross-rearing of diverging populations and sibling species in alternate parental habitats can reveal phenotypes under selection and help discern their genetic bases.

Here, we measure performance metrics and patterns of transcriptome-wide gene expression for larvae of two taxa of flies in the *Rhagoletis pomonella* (Diptera: Tephritidae) group feeding in native and non-native host fruits to examine the nature and relationship of plastic and evolved performance differences that may contribute to reproductive isolation between the taxa. The *R. pomonella* complex consists of a number of genetically and morphologically similar taxa (many are named species) that experience gene flow, yet inhabit discrete environments by infesting different sets of nonoverlapping host fruit. The complex has been hypothesized to radiate via sympatric shifting and ecological adaptation of flies to novel host plants (Bush 1969). For example, *R. pomonella* has recently shifted (in the last 160 years) from its ancestral host hawthorn (*Crataegus* spp.) to introduced domesticated apple in the eastern U.S. This is often cited as an example of host race formation in action, the hypothesized first stage of ecological speciation with gene flow (e.g. Wu 2001; Rundle & Nosil 2005; Feder *et al.* 2012). All taxa within the *R. pomonella* group are very closely genetically related, exhibiting only allele frequency variation and experiencing ongoing gene flow among taxa (Berlocher *et al.* 1993; Powell *et al.* 2013).

Differences in larval performance on alternative hosts have been hypothesized to be critical for the evolution of fitness trade-offs that present ecological barriers to gene flow between host specialist populations (Via & Hawthorne 2002; Bolnick & Fitzpatrick 2007; Calcagno *et al.* 2007; Laukkanen *et al.* 2013). Such trade-offs, wherein each taxon achieves superior fitness relative to other taxa in its native environment, may not be universal, however (Jaenike 1990; Fry 1996; Forister *et al.* 2012). Although *Rhagoletis* taxa often demonstrate marked reproductive isolation through differences in seasonal life history timing (Feder & Filchak 1999) and host fruit odour preference (Frey & Bush 1990; Linn *et al.* 2003; Dambroski *et al.* 2005), previous reciprocal transplant studies have not revealed true, host-specific trade-offs in larval performance (Diehl & Prokopy 1986; Prokopy *et al.* 1988; Bierbaum & Bush 1990). However, each of these studies revealed highly variable larval performance across host fruits, suggesting that selection is acting differentially on larval responses to the nutritional and chemical milieu of the different host fruits.

Larval performance may play a particularly critical role in maintaining barriers to gene flow between *R. zephyria*, which infests snowberry (*Symphoricarpos* spp.), and apple-infesting host races of *R. pomonella*. A mitochondrial phylogeny suggests that *R. pomonella* and *R. zephyria* are sister species (Smith & Bush 1997), although the two exchange genes at an appreciable rate. Up to 1% F1 hybrids have been detected in natural apple-infesting populations, and yet, the two taxa remain genetically and morphologically distinct (McPheron 1990; Feder *et al.* 1999; Yee *et al.* 2012; Green *et al.* 2013). *Rhagoletis zephyria*'s native distribution spans western North America from the Pacific Northwest to the Great Lakes, and both the fruit and fly are abundant (Gavrilovic *et al.* 2007). Apple-infesting *R. pomonella* flies have become abundant in the Pacific Northwest since initial detection in the 1970s (Yee *et al.* 2012), and infested apple trees are often microsympatric with infested snowberry plants (both taxa are caught on the same sticky traps) (Yee *et al.* 2009). The fruit environment experienced by larvae is quite distinct, as the apple and snowberry host plants are members of different families (*Rosaceae* and *Caprifoliaceae*, respectively) whose fruits contain different complements of phenolic and glycosidic secondary metabolites (Schieber *et al.* 2001; Szaufer-Hajdrych & Zgorka 2003; Makarevich *et al.* 2009), compounds with well-known toxic effects on herbivorous insects (Despres *et al.* 2007). In contrast to these pronounced differences in larval feeding environment, host plant differences in seasonal timing are limited because snowberry bushes fruit over a broad seasonal window (early summer to early fall) that overlaps the fruiting time of apples (Schwarz 2004). Ongoing behavioural trials suggest that host odour preference does differ between *R. zephyria* and *R. pomonella* (J. L. Feder, unpublished), although the magnitude of that difference relative to other studied taxa within the species group remains unresolved.

The rapidity with which new host races can form in the *Rhagoletis* species complex suggests that larvae are able to respond to different and potentially toxic host fruit environments with sufficient plasticity to exploit the new host, even if performance is initially poor.

Transplant and manipulative experiments examining insect larval responses to variation in toxins and plant secondary metabolites have illustrated physiological plasticity at the level of gene expression that is consistent with compensation for the novel chemical milieu. These studies reveal that a substantial proportion of the transcriptome plastically responds to the feeding environment and that candidate detoxification pathways are often well represented in these plastic responses (Matzkin *et al.* 2006; Govind *et al.* 2010; Oppert *et al.* 2010; Celorio-Mancera *et al.* 2013; Vogel *et al.* 2014). In addition to facilitating colonization of novel habitats, transcriptomic responses in the novel environment may also provide new, formerly covert targets of selection that influence evolutionary trajectories and adaptation (Moczek *et al.* 2011). Given that transcriptional plasticity is expected to mitigate the stresses of a novel host plant, evolved transcriptional responses across species will likely involve many genes displaying host-associated plasticity within species (Celorio-Mancera *et al.* 2013; Vogel *et al.* 2014). In general, study systems that allow comparisons across environments (e.g. plant hosts) and closely related populations or species experiencing different environments are best suited to investigate the impact of transcriptional plasticity on evolutionary responses (e.g. Xie *et al.* 2014; Etges *et al.* 2015).

Here, we use reciprocal transplants to test for a trade-off in larval performance between *R. zephyria* and *R. pomonella* and for underlying plastic and evolved differences in transcriptome-wide gene expression. In addition to standard functional enrichment analyses of expression data, we applied a specific test for the following simple conceptual model relating plastic and evolved differences in gene expression. Rearing in a novel environment produces transcriptional variation in many genes that is seldom exposed to natural selection in the native environment, and remains largely 'untested' in the novel environment (Rutherford & Lindquist 1998; Moczek *et al.* 2011). Thus, selection on expression of these genes should be disproportionately strong during the formation and differentiation of populations in discrete, novel environments such as different host fruits. In very shallowly diverged taxa still experiencing gene flow such as *Rhagoletis*, evolved genetic and phenotypic differences reflect the strength of divergent selection, whereas selectively neutral variation is rapidly homogenized (Rundle & Nosil 2005; Feder *et al.* 2012). Therefore, genes exhibiting evolved differences in expression between recently diverged host races or species should tend to be those that also demonstrate environment-dependent, plastic expression differences. Here, we test for this signature of selection on plastic expression by asking whether transcripts exhibiting plastic expression are overrepresented in the set of transcripts exhibiting evolved expression differences between the fly species. Treating the transcriptome of *R. zephyria* feeding in their native snowberry host fruit as a baseline, transcripts that show a signifi-

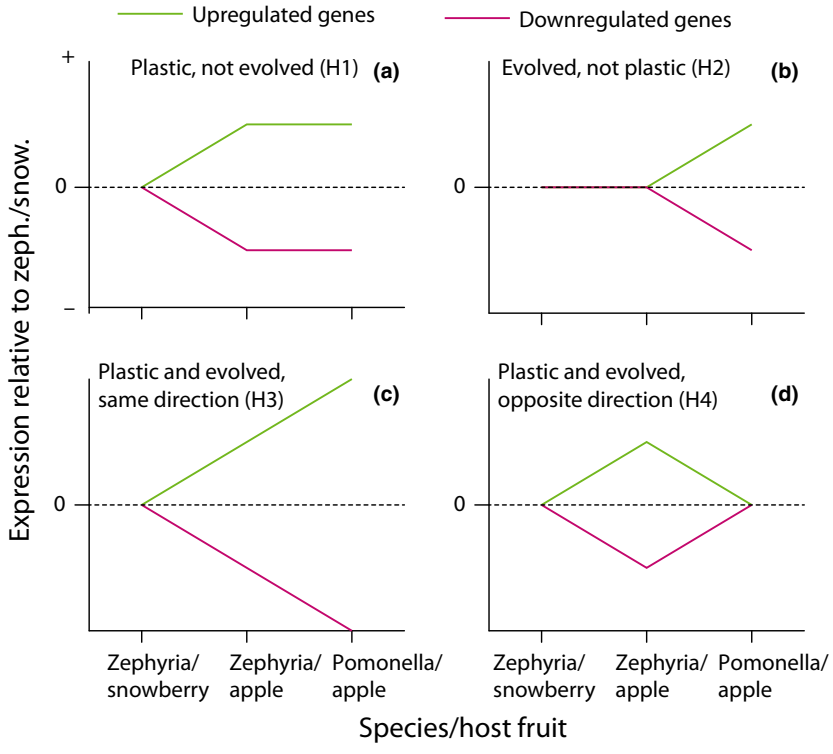

Fig. 1 Hypothetical patterns of gene expression comparing *R. zephyria* larvae feeding in snowberry, *R. zephyria* feeding in apple and *R. pomonella* feeding in apple. Differences between *R. zephyria* in apple vs. snowberry represent plastic responses (same species, different environment), while differences between *R. zephyria* and *R. pomonella* feeding on apple (different species, common environment) are evolved differences. The *y*-axis is scaled to represent log fold change in expression relative to *R. zephyria* in snowberry (e.g. log[*R. zephyria* in apple expression] – log[*R. zephyria* in snowberry expression]). Green and purple lines represent genes up- and down-regulated relative to *R. zephyria* in snowberry, respectively.

cant response in any treatment combination may follow four possible patterns of expression: (i) a plastic response within a taxon across environments with no evolved difference between taxa within an environment (Fig. 1a), (ii) an evolved difference with no plastic response (Fig. 1b), (iii) a plastic response and an evolved response in the same direction (Fig. 1c), or (iv) a plastic response and an evolved response in the opposite direction (Fig. 1d). Consistent with our model that initial plastic responses experience strong, divergent selection, the subset of transcripts demonstrating evolved and plastic responses (Fig. 1c,d) are overrepresented in the comprehensive set of transcripts demonstrating evolved responses (Fig. 1b–d). We discuss the potential selective patterns and candidate physiological mechanisms that may explain this observation in the context of host plant switching and ecological speciation in *Rhagoletis*.

Materials and methods

Sample collection, rearing and breeding

Rhagoletis zephyria and *R. pomonella* flies used in the study were initially collected as larvae in infested fruit in 2008 from three separate locations (no two more than 4 km apart) in Bellingham, Washington, United States. We note that each taxon has a much broader geographic distribution and thus that population pairs from other regions have almost surely diverged along somewhat independent evolutionary trajectories. Nevertheless, the two taxa are found only in their respective host's apple and snowberry throughout their entire range. While differences in the chemical profile between populations and cultivars of snowberry and apple may exist, they are expected to be minor compared to the differences between species from different plant families. The discrete difference between larval habitats is therefore consistent, and inferences from these samples relate to the general processes of differential adaptation occurring across populations.

Infested fruits were held at 21 °C in trays containing moist sand, and larvae egressing from fruit were allowed to pupate in the sand. Under these conditions, pupa will enter a diapause state. Diapausing pupae were overwintered for at least 5 months at 4 °C. We initiated adult development by transferring pupae from the cold to a constant temperature room held at 21 °C with a 16-/8-hour (L/D) photoperiod. Upon emergence from the puparium, adult flies were separately placed into one of four (per species) different intraspecific mating cages, with each cage containing 10 females and five males. Each cage was supplied with two parafilm-covered green agar semispheres acting as artificial oviposition substrates (30 g *Drosophila* agar, Genessee Scientific, and 3 mL green food dye per litre of water). Due to differences in preferred fruit diameter between *R. zephyria* and *R. pomonella* (Prokopy & Bush 1973), we used small (20 mm diameter) and large (42 mm diameter) agar semispheres for *R. zephyria* and *R. pomonella* mating cages, respectively. Oviposition substrates were changed at regular 2-day intervals to harvest eggs, and approximately equivalent numbers of eggs from each cage were randomly allotted to both apple and snowberry transplants.

Reciprocal transplants

Commercially available Gala apples (August 2009, harvested from organic growers in Chelan, Washington, USA) and wild snowberry fruit (2009, harvested from September 7–18 from Bellingham, Washington, USA) were used in the transplant experiments. To prevent fruit infestation with wild flies, ripening snowberry fruits in the field were covered with mesh bags. Apples were stored in a cold room, and snowberries were picked on the day of the experiment. Straight-tipped dissecting needles (Bioquip, Rancho Dominguez, CA, USA) were used to transfer fly eggs from the agar semispheres to petri dishes containing filter paper kept moist with autoclaved tap water. Eggs were kept at 21 °C and 16/8 (L/D) photoperiod until L1 larvae hatched. L1 larvae were collected daily and transplanted into snowberry or apple fruit in the laboratory. A short incision was made in the skin of each fruit with an alcohol-sterilized razor blade. An L1 was transferred into each incision using a fine brush. Before sealing the implant site with scotch tape, we checked whether larvae survived the transfer by visually inspecting the implant site for L1 movement. Dead larvae were replaced with live new individuals when necessary. We transferred fruit with implanted larvae into individual containers with moist sand that served as a pupariation substrate for egressing larvae. Transplants were stored under the same standardized conditions in the laboratory as described above for field-collected fruit.

Our experimental design for reciprocal transplants included two treatments – *R. zephyria* in apple fruit and *R. pomonella* in snowberry fruit – and the two corresponding controls – *R. zephyria* in snowberry fruit and *R. pomonella* in apple fruit. Within each treatment combination, larvae were split approximately equally into two groups, one group for phenotyping and the other group for RNA extraction at the L3 stage. Numbers of transplanted larvae per treatment were as follows (first number for phenotyping, second number for RNA extraction at the feeding L3 stage): (i) *R. pomonella* in apple, $n = 49$, 51; (ii) *R. zephyria* in apple, $n = 51$, 49; (iii) *R. pomonella* in snowberry, $n = 28$, 19; and (iv) *R. zephyria* in snow-

berry, n = 45, 43. Initial sample sizes for (iii) were lower because we discovered that early collections of snowberries used for *R. pomonella* -to-snowberry implants were improperly screened in the field, and thus were excluded from the analysis.

Genetic confirmation of species identity

While the commercial apples used in the experiment were insect free, snowberries were collected from wild plants that were protected from fly infestation at an early stage of fruit development using net screens. To test whether the screens effectively prevented oviposition by wild flies, the species identities of surviving implanted larvae were determined by SNP genotyping at the highly informative P1700 locus (Green *et al.* 2013). For the survivorship component of the transplant study, genomic DNA was extracted from a dissected portion of every surviving fly (in all transplant treatments) using Qiaquick blood and tissue extraction kits (Quiagen, Hilden, Germany). For the microarray component of the transplant study, genomic DNA was isolated from third instar stage larvae (L3) by first co-extracting and then separating DNA from RNA, as specified in the Ribopure kit (Life Technologies). These genetic tests confirmed that all pupae obtained from the transplants of *R. pomonella* larvae into snowberries had *R. pomonella* genotypes.

Performance measures

Three different aspects of larval performance were recorded as metrics of fly fitness in the transplant experiment: larval survivorship, larval development time and pupal mass. We note that all three of these measures of fitness can be regarded as general indicators of larval feeding efficiency in fruit, although they do not capture any element of adult performance. Our goal here was to test for the most immediate host-related performance differences occurring for larvae and newly pupariating flies, but not to conduct an exhaustive study for fitness effects across all life stages.

Larval survival. We measured survivorship as the proportion of transplanted larvae that completed development to form a puparium. Pupae were regularly sifted from the sand in collecting trays for 60 days (starting when feeding damage was visible), allowing enough time for successful development to pupariation. We tested for differences in survival between *R. pomonella* and *R. zephyria* within each transplant treatment using Fisher's exact tests.

Development time. Egressing larvae leave characteristic holes in the skin of infested apples. This allowed for non-destructive visual screening of apple fruit every 2 days to assess the larval development times of transplanted flies. Upon dissecting apples several days after egression holes had appeared, we discovered that a small proportion of larvae had died in the fruit after making an exit hole. These individuals were removed from calculating larval development times in apple. Egressing larvae from infested snowberry fruit do not leave a reliable exit mark, and older snowberry fruits were very delicate. Thus, we avoided handling snowberry fruit and were not able to measure development time, as we did for apples. Rather, we present a conservative estimate of maximum development time in snowberry based on the timing that the last puparium was found in the sand substrate.

Pupal mass. Individuals that successfully formed pupae were held at 85% RH and 21 °C for 1 week, the approximate time required for the completion of pupal development within the puparium (Ragland *et al.* 2009). Each pupa was then weighed to the nearest 10^{-5} g.

RNA extraction and microarrays

Because of high mortality of *R. pomonella* reared through snowberry (see Results and discussion), we were only able to obtain adequate samples for microarray analysis for three of the four treatments. No result was obtained for *R. pomonella* larvae gene expression in snowberries. Total RNA was extracted from L3 larvae >5 mm in length that were dissected out of fruit while actively feeding. Each larva was collected between 10:00 AM and 12:00 PM, briefly rinsed in DI water to remove decaying fruit material, measured, snap-frozen on dry ice and immediately homogenized in Tri Reagent (Life Technologies). We stored the homogenates at −80 °C for 2–4 months before extracting RNA using a Ribopure kit (Life Technologies). In addition to total RNA, we also isolated genomic DNA by processing the organic and interphase layers of the supernatant of the extraction and genotyped these extracts to determine species identities of flies, as described above.

cDNA preparation, labelling and hybridization. Gene expression levels were quantified based on a two-colour array experiment using custom Agilent 4x44k microarrays. The experimental design balanced equal numbers of replicates labelled with Cy3 and Cy5, with six biological replicate arrays (sample from one individual hybridized per dye per array) and 2–3 technical replicate arrays per biological replicate for each possible pairwise comparison of the three treatments (*R. pomonella* in apple vs. *R. zephyria* in apple, *R. zephyria* in apple vs. *R. zephyria* in snowberry and *R. zephyria* in

Table 1 Experimental design for two-colour microarrays, noting the Cy3- and Cy5-labelled samples hybridized to each array on the five Agilent 4x44k slides. Numbers are biological replicate (individual larva) codes, and letters indicate treatment/species combinations as follows: PA = *R. pomonella* in apple, ZA = *R. zephyria* in apple, ZS = *R. zephyria* in snowberry

Slide no	Cy3	Cy5
1	PA-1	ZA-4
1	PA-2	ZS-4
1	ZS-1	ZA-4
1	ZS-2	PA-4
2	ZS-1	PA-5
2	PA-3	ZA-4
2	ZS-2	ZA-4
2	ZA-1	PA-6
3	ZS-3	PA-6
3	ZA-2	ZS-5
3	PA-2	ZA-5
3	ZA-3	ZS-6
4	PA-1	ZS-5
4	ZS-3	ZA-5
4	ZA-2	PA-4
4	ZS-3	ZA-5
5	ZA-3	PA-5
5	PA-3	ZS-6
5	ZA-1	ZS-4
5	ZA-3	ZS-6

snowberry vs. *R. pomonella* in apple; Table 1). Smaller sample size per population/treatment combination introduces potential sampling error. On the other hand, using individuals rather than pools (as is commonly performed in microarray studies) produces more realistic estimates of interindividual variation, and we chose this strategy to produce more conservative statistical inferences (Zhang & Gant 2005).

Labelled (Cy3 or Cy5) aRNA (amplified RNA) was produced from 250 ng of extracted RNA using the Quick-Amp labeling Kit (Agilent Technologies, Inc.) following the manufacturer's recommendations. We fragmented and hybridized 825 ng of each of the labelled samples to the arrays, using manufacturer-recommended incubation and wash steps. Arrays were scanned using a dual-laser DNA microarray scanner (Model G2505C; Agilent Technologies, Inc.), and the red and green foreground and background fluorescence intensities were extracted using FEATURE EXTRACTION 10.1.1.1 software (Agilent Technologies, Inc.).

The array platform was a modified version of the *R. pomonella* microarray designed from an *R. pomonella* EST database (Schwarz *et al.* 2009) as described in Ragland *et al.* (2011) and deposited in the Gene Expression Omnibus (GEO; platform GPL13946). The current version (GPL19895) used in this study had three probe sets: (i) two forward probes for all 14 805 ESTs annotated to the National Center for Biotechnology Information NR database; (ii) one forward probe for 8191 un-annotated ESTs; and (iii) one reverse probe for a subset (5803) of the un-annotated ESTs. All annotations are based on BLASTX searches against the *Drosophila melanogaster* translation database (Schwarz *et al.* 2009).

Data analysis of microarrays. Array data were loess-normalized and analysed using linear models in the Limma R package (R Core Team 2013) (Smyth & Speed 2003; Smyth 2004). After within-array normalization but prior to downstream analysis, we used the presence/absence calls from the Agilent FEATURE EXTRACTION software to exclude all probes called as 'absent' in four or more arrays per treatment. We estimated parameters for a standard ANOVA model including parameters for host fruit, fly species and technical replicate (nested within biological replicate) and then applied linear contrasts to estimate $\log_2$ fold changes in expression between treatments/taxa. Nominal *P*-values were then estimated using an empirical Bayes method (Smyth 2004). We corrected for multiple comparisons by calculating the Benjamini and Hochberg false discovery rate (FDR) statistic for each probe from the nominal *P*-values. Finally, we estimated the average log fold change and the combined FDR values for multiple probes for the same gene using Fisher's method for combined probability (Hess & Iyer 2007).

Several tests were performed to show that DNA sequence differences between *R. pomonella* and *R. zephyria* did not bias our results due to differential array hybridization efficiency. We first performed an analysis of sequence similarity, focusing on cytochrome P450 genes, many of which were strongly differentially expressed between our treatments (see Results). We randomly selected 20 cytochrome P450 loci that each annotated to a different *D. melanogaster* locus. These 20 loci were represented by 125 different probes (half of which only differed by a single base pair) that we used to search unassembled *R. pomonella* and *R. zephyria* Illumina reads of genomic DNA (H. M. Roberston, unpublished data) using BLASTN. The average Illumina read length of 180 bp is three times as long as our 60-bp probes. Thus, we obtained multiple blast hits of sequence reads covering the entire length of each probe. We then used the per cent sequence identity of the top blast hit with our probe in both genomes to calculate the following sequence identity metric: Log_2 (% identity of top *R. zephyria* genome sequence match/% identity of top *R. pomonella* genome sequence match). In the majority of cases, *R. pomonella* and *R. zephyria* had identical Illumina sequences to each other and to the probe,

resulting in a Log_2 score of 0. In addition, log2 proportions of per cent identities were not correlated with estimated average log fold changes in gene expression levels in the microarray analysis over all possible pairwise comparisons (Pearson's $r = -0.062$, $P = 0.352$; Fig. S1, Supporting information). Furthermore, after statistically removing the effects of dye (red or green), raw average intensities across all probes did not differ significantly between species.

After classifying significantly differentially expressed genes into the categories described in Fig. 1 (see Results and discussion), we submitted member gene lists to the DAVID web-based functional annotation tool (Huang *et al.* 2008) to test for enrichment of catalogued functional categories. This tool applies a Fisher exact test (columns, 'in submitted gene set', 'in genome'; rows, 'in functional category', 'not in functional category') to test for enrichment of a broad array of Gene Ontology and other pathway databases. We submitted only sets of transcripts with annotations to *Drosophila melanogaster* and tested them for enrichment against the *D. melanogaster* genome.

Results and discussion

Host-related performance differences

Rhagoletis pomonella and *R. zephyria* flies survived significantly better when transplanted into their respective native fruit (the control treatments) compared to larvae transplanted into non-native fruit (Fig. 2a). Overall survivorship of *R. pomonella* larvae in apples was 63.3%, while it was 29.4% for *R. zephyria* larvae transplanted into apples ($P = 0.027$, Fisher's exact test). In the reciprocal comparison, 35.6% of *R. zephyria* larvae survived in snowberries, while only 10.7% of transplanted *R. pomonella* larvae survived ($P = 0.0012$, Fisher's exact test). Survivorship estimates for *R. pomonella* and *R. zephyria* displayed a crossing pattern in the transplant experiment, which is generally taken as an indicator of the existence of a host-related fitness trade-off between populations (Fig. 2a). However, while survival significantly declined in non-native snowberry vs. native apple fruits for *R. pomonella* (10.7% vs. 63.3%; $P < 1 \times 10^{-5}$, Fisher's exact test), the slight decrease in survival in non-native apple vs. native snowberry for *R. zephyria* was nonsignificant (29.4% vs. 35.6%; $P = 0.68$, Fisher's exact test). Thus, the survivorship data alone do not definitively demonstrate the existence of a host-related fitness trade-off for *R. zephyria*.

When the survivorship data were considered in combination with development time, however, a clearer picture of potential performance trade-offs emerged. Specifically, developmental time differences among

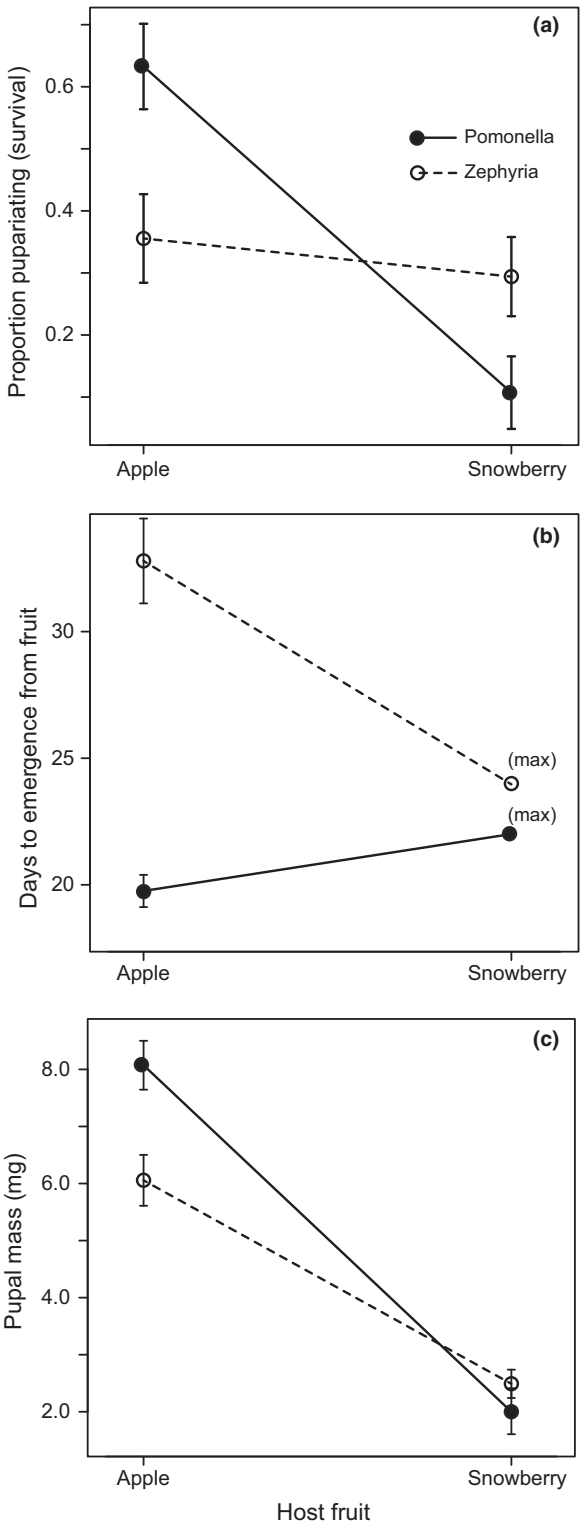

Fig. 2 (a) Proportion of transplanted larvae surviving to pupariation (±standard error) for *R. pomonella* (solid circle, solid line) and *R. zephyria* (open circle, dashed line) in native vs. non-native host fruit; (b) larval development times (only maximum estimates available in snowberry, see Materials and methods); and (c) pupal mass.

transplant treatments suggested that larval feeding efficiency of *R. zephyria* declined in non-native apple compared to native snowberry fruit. *Rhagoletis zephyria* larvae transplanted into apples took on average 31.71 ± 1.33 SE days to egress. In contrast, the maximal number of days for snowberry fly larval development in snowberries was 24 days, and the mean was probably considerably less but could not be measured due to the deterioration of snowberry fruit as the study progressed. In a natural ecological context, the increase in development time likely has substantial, negative fitness consequences. Previous studies have shown that abscised fruits tend to rot at a much greater rate in the field compared to more benign, sheltered conditions, with corresponding selection pressures on feeding larvae to develop faster in nature than in the laboratory (Filchak *et al.* 1999). For example, *R. pomonella* had a mean larval period duration of about 14 days in apples in the field compared to about 17 days in a sheltered garage (Filchak *et al.* 1999), and selection strongly favoured allozyme alleles associated with rapid development in the field. Given these results, it is extremely unlikely that *R. zephyria* requiring 30 or more days to develop in apples would have sufficient time to egress from fruit in the field before declining resource quality and/or rot-related ethanol build-up caused them to die. Moreover, parasitism rates are much higher for later developing *R. pomonella* (Feder 1995), a trend that has also been documented in other insect species (Bernays & Woods 2000; Blanckenhorn 2000). Thus, overall fitness, accounting for development time and survival, likely does decline in *R. zephyria* when feeding on apple compared to native snowberry, producing a reciprocal larval fitness trade-off between the two species. For *R. pomonella*, the maximum estimate of development time in snowberry did not substantially differ from the mean estimate in apple. Thus, increased survivorship of *R. pomonella* larvae in apple compared to snowberry fruit in the transplant experiment was not likely caused by the slower rate of decay of apples relative to snowberry fruits in the laboratory, providing more time for larval development for larvae feeding in apples. Rather, the increased survival of *R. pomonella* in apple reflects differences in fruit quality or nutrition for the larvae.

Despite the longer time taken to develop in apple, *Rhagoletis zephyria* achieved significantly larger pupal size when reared through apple compared to snowberry (Mann–Whitney *U*-test, $P < 0.0001$; Fig. 2c). Similarly, *R. pomonella* pupae were also larger when reared through apple compared to snowberry (Mann–Whitney *U*-test, $P = 0.007$; Fig. 2c). The larger size of *R. zephyria* reared in apple may reflect the longer period of time these flies have to develop in this fruit versus snowberry. However, as discussed above, the higher rate at which apple fruits rot in the field likely limits the ecological realization of these body size differences in nature (Filchak *et al.* 1999). In comparison, the larger size of *R. pomonella* reared in apple compared to snowberries cannot be explained by developmental time differences and likely reflects adaptation to feeding in its native host fruit.

Given that host fruiting phenology and fly life cycle timing are not as distinct between *R. pomonella* and *R. zephyria* as they are for other *Rhagoletis* taxa, it is likely that the larval fitness differences detected in the transplant experiment represent an important selective force counteracting gene flow between *R. pomonella* and *R. zephyria* populations in nature. Very low survival of *R. pomonella* in snowberry compared to *R. zephyria* in apple may also contribute to the previously observed asymmetric gene flow, with *R. zephyria* alleles primarily introgressing into *R. pomonella* (Green *et al.* 2013).

Models of ecological speciation with gene flow predict that habitat choice and/or divergent natural selection typically must be strong to produce a level of reproductive isolation that maintains genetically distinct populations with moderate to high levels of gene flow (Rundle & Nosil 2005). Just like other taxa in the genus *Rhagoletis*, *R. pomonella* and *R. zephyria* show prezygotic isolation via host choice (Schwarz *et al.* 2007). Divergent selection driving adaptation of the univoltine life cycle to different hosts fruiting at different times (Feder & Filchak 1999) and host odour preference (Linn *et al.* 2003; Dambroski *et al.* 2005) has been demonstrated between the hawthorn and apple host races of *R. pomonella*. But, because of the extended phenology of snowberries, allochronic isolation may be less pronounced between *R. zephyria* and *R. pomonella* in the Pacific Northwest (Schwarz 2004). Here, we have established different larval host environments as an additional cause of host-specific divergent selection that may be of particular importance when temporal isolation in fruiting time between taxa is less distinct, as with *R. pomonella*/*R. zephyria*. In fact, our estimates of performance differences almost surely underestimate true fitness differences because we did not track adult emergence or adult reproductive performance. This may also explain why true reciprocal fitness trade-offs were not detected by measuring larval survival of reciprocal transplants between apple and hawthorn host races of *R. pomonella* (Prokopy *et al.* 1988) or between *R. pomonella* and the blueberry-infesting *R. mendax* (Diehl & Prokopy 1986; Bierbaum & Bush 1990). Insects with a dormant stage, such as the overwintering pupal diapause in *Rhagoletis* flies, may face severe survival challenges after larval feeding is complete that are highly dependent upon nutrient reserves acquired during larval feeding (Filchak *et al.* 2000; Hahn & Denlinger 2011; Ragland

et al. 2012). Moreover, larval host use can affect multiple metrics of adult mating and reproductive performance, and incorporating these added selective dimensions should clarify the true nature of fitness differences among populations exploiting different resource environments. (Grimaldi & Jaenike 1984; Brazner & Etges 1993; Stennett & Etges 1997; Jaureguy & Etges 2007).

Testing for plastic and evolved responses in gene expression

As detailed in Fig. 1, our experimental design tests for both plastic and evolved responses to differences in larval host fruit environments. Because we were unable to rear sufficient numbers of *R. pomonella* through snowberries, our analysis specifically explores the plastic response of *R. zephyria* reared in snowberry (ZS) vs. apple (ZA) and the evolved differences between *R. zephyria* and *R. pomonella* reared in apple (ZA and PA, respectively). If genes displaying plastic expression across host environments are more prone to adaptive evolution, we predict that plastically expressed transcripts should be overrepresented in the set of transcripts exhibiting evolved differences between the fly species. Below, we describe the observed patterns of differential expression and hypothesis tests for overrepresentation. We also discuss evidence for the functional basis of plastic responses and evolutionary differences in larval performance. Rather than relying on identification and confirmation of individual candidate genes, we leverage patterns of expression of multiple genes within functional categories through enrichment analyses.

Gene expression data indicated that plastic and evolved responses to host fruit differences were extensive and transcriptome-wide in the transplant experiment. Postfiltering, 20% (2614 of 13 357) of transcripts were significantly differentially expressed between *R. zephyria* feeding in native snowberry compared to *R. zephyria* feeding on non-native apple fruit (Fisher combined P-value ≤ 0.05), and 547 of the transcripts were $\geq$ twofold ($\geq$200%) differentially expressed (absolute value of $\log_2$ fold change ≥ 1; Table S1, Supporting information). These differences represent induced, plastic responses in gene expression levels in *R. zephyria* larvae associated with feeding in the novel apple fruit environment. An even greater proportion of transcripts (30%; 4002 of 13 357) were differentially expressed between *R. pomonella* and *R. zephyria* when both were reared through apple, with 1337 of the transcripts being $\geq$ twofold differentially expressed. These 4002 transcripts represent evolved differences between the two species. Although many expression differences probably reflect adaptation to host fruit environment (especially in detoxification responses, see below), some differences may reflect responses to other nonhost fruit-related selection pressures differentially acting between *R. pomonella* and *R. zephyria*. However, it is not likely that the expression differences are neutral or due to maternal or paternal effects. Genetic drift should not be responsible for the observed 'evolved' expression differences given that moderate rates of gene flow between the fly species are sufficient to homogenize nonadaptive variation (McPheron 1990; Green *et al.* 2013). Although parental effects from the previous generation have been shown to affect larval performance (Fox *et al.* 1997), maternal transcripts degrade early in fly larval development (Arbeitman *et al.* 2002), and parental effects account for a small percentage (~10–15%) of variation relative to genetic variation in sea urchin larvae (Garfield *et al.* 2013). Moreover, the influence of parental effects on transcriptional variation degraded quickly during larval development in the sea urchin study. Our measures of gene expression were taken relatively late in development, after the completion of the first and second larval instars, as well as part of the third instar, and so should not be greatly affected by maternal or paternal effects.

Grouping transcripts into the four combinations of plastic and evolved responses depicted in Fig. 1 and testing for overrepresentation indicated that plastically regulated transcripts were more likely to demonstrate evolved differences compared to nonplastic transcripts. Using an FDR cut-off of 0.05, we classified genes significantly differentially regulated between host fruits in *R. zephyria* (plastic response) and between species when reared in apple (evolved response) into four categories (i) plastic, not evolved, (ii) evolved, not plastic, (iii) plastic and evolved, same direction, and (iv) plastic and evolved, opposite direction. The results are summarized in Fig. 3, including average expression profiles and transcript numbers per category. There was a significant overrepresentation of transcripts showing significant plastic responses (Z/A comparison: categories H1, H3 and H4, Fig. 3) within the set of transcripts showing significant evolved expression differences (categories H2, H3 and H4 in the P/A comparison, Fig. 3). Overall, we observed 922 transcripts displaying both significant plastic and significant evolved expression differences, a significant overrepresentation (predicted transcripts = $[2614 \times 4002]/13\ 357 = 783.2$, $P \ll 0.001$, hypergeometric test). In other words, transcripts plastically expressed between snowberry and apple host fruits for *R. zephyria* were more likely than chance alone to exhibit differences in expression between the species when both were reared through apple.

Of those transcripts exhibiting significant plastic expression in *R. zephyria* and evolved differences

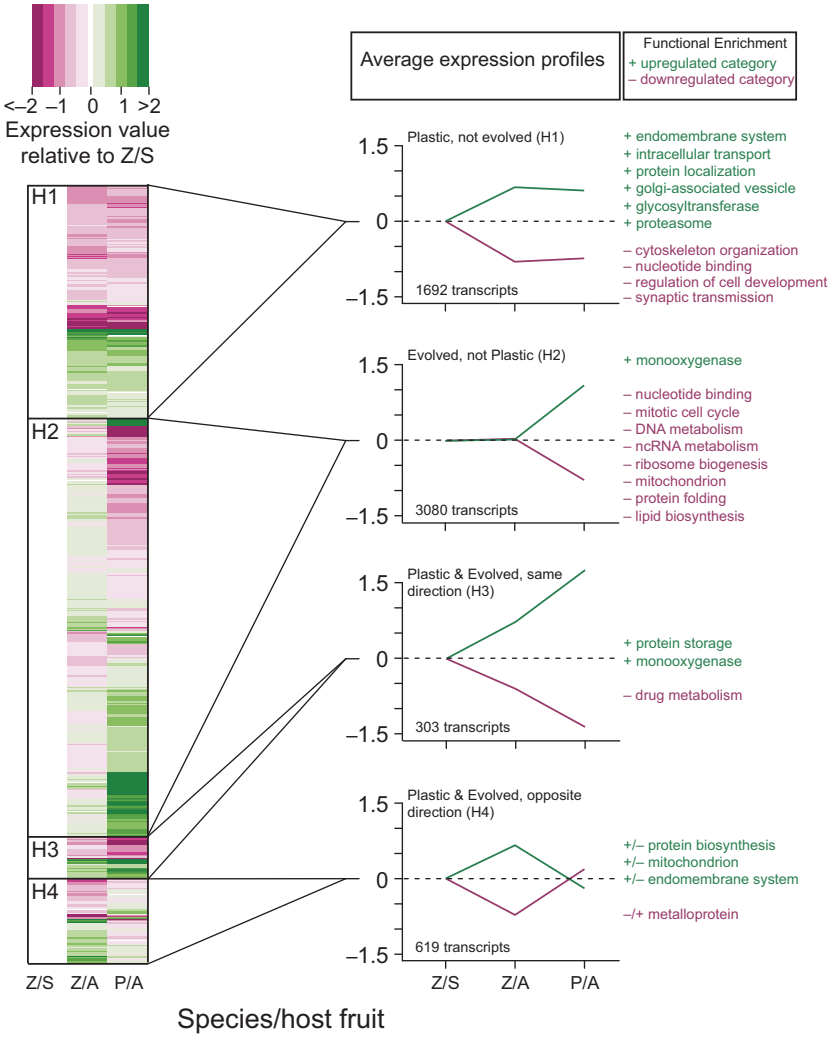

Fig. 3 Heatmap (left panel) and average expression profiles (right panel) for significantly differentially expressed genes displaying the four different patterns of plastic and evolved responses (H1–H4) presented in Fig. 1. Z/S and Z/A represent *R. zephyria* in snowberry and apple, respectively, and P/A represents *R. pomonella* in apple. Expression values are $\log_2$ fold changes relative to the expression value of Z/S. Green and purple represent up- and down-regulated genes relative to Z/S in one or both comparisons. Note that enriched functional categories represent a subset of all enriched categories reported in Table S2 (Supporting information).

between the species, the null expectation is that equal proportions of transcripts will show evolved and plastic responses in the same direction (Fig. 1c) and in the opposite direction (counteracting plasticity; Fig. 1d). Our results suggest that a significantly greater proportion of transcripts exhibiting plastic and evolved responses follow the 'opposite', or 'counteracting' pattern of Fig. 1d compared to those with plastic and evolved responses in the same direction (619 of 922 transcripts, 67%; $P \ll 0.001$, binomial test; see Fig. 3 for relative transcript numbers). Another study comparing plastic vs. evolved responses to discrete environmental variation in *Drosophila melanogaster* yielded a very similar result. Yampolsky *et al.* (2012) report results of a gene expression analysis comparing plastic responses of larva to ethanol exposure vs. evolved differences between control *D. melanogaster* lines and lines selected for increased ethanol tolerance for 320 generations. We categorized their significantly differentially expressed transcripts into categories of plastic and evolved responses as above (values taken from Table 1; Yampolsky *et al.* 2012) and applied the same tests as above. The results show an overrepresentation of plastic genes in the set of transcripts showing evolved responses among selected and control lines ($P \ll 0.001$, hypergeometric test). Moreover, most transcripts displaying plastic and evolved responses in this *Drosophila* study showed opposite patterns of regulation (80%, $P \ll 0.001$, binomial test), for example transcripts down-regulated in the plastic response to ethanol were up-regulated in the ethanol-selected vs. the control lines. The *Drospophila* study is analogous to our current study because both studies manipulated a discrete environmental variable (host fruit type or ethanol presence/absence) and compared lines or species with an evolutionary history of exposure to only one of the two environments. The results of both studies suggest that for plastically expressed genes, evolved differences between species or lines largely suppress the initial plastic responses (the 'opposite'

pattern of Fig. 1d) of gene expression to a foreign, non-native environment.

For taxa relatively early in the divergence process like *Rhagoletis* species, there are several reasons to expect that evolved differences in gene expression may occur most frequently in plastically regulated transcripts. First, many plastic responses like those occurring with exposure to a novel host fruit are likely pathological, reflecting a disruption of normal metabolic functions caused by changes in nutrition and toxin exposure. Such plastic expression patterns would tend to be suppressed by natural selection during host race formation, over evolutionary time yielding a pattern wherein evolved differences in expression counter plastic expression differences. Transcripts following the pattern of Fig. 3, H4, fit this 'opposite' pattern. This group includes, for example, a number of mitochondrion-associated transcripts whose functions may be disrupted when feeding on a foreign host fruit. Second, even plastic responses that initially increase fitness in stressful, foreign environments may later be selected against because they are energetically costly. Induced chaperonin responses, for example are a common response to stressful or foreign environments with major energetic costs (Feder & Hofmann 1999). Thus, selection pressures may be particularly strong on genes that are misexpressed or whose plastic, differential expression is costly compared to genes whose expression patterns are largely invariant across environments. Indeed, our data demonstrate an overrepresentation of expression patterns consistent with an evolved suppression of the plastic response. In the face of gene flow, evolved differences in expression reflect the strength of selection, and the majority of evolved differences in plastically expressed genes were consistent with selection countering costly plastic responses. The importance of plasticity for adaptive evolution during colonization events and via processes such as genetic accommodation has been well established (Rutherford & Lindquist 1998; Suzuki & Nijhout 2006; Moczek *et al.* 2011; Green & Extavour 2014; Morris *et al.* 2014), but studies in this area generally focus on plastic phenotypes with positive (i.e. adaptive) fitness effects in novel environments. Our results highlight that selection acting to suppress potentially maladaptive plastic responses may also be critical during the establishment of populations in novel environments such as foreign host fruits.

Although the predominate expression patterns suggest evolved differences countering plastic responses, we also identified hundreds of transcripts whose expression patterns are consistent with selection to maintain, or even enhance the plastic response of snowberry larvae to apple fruits. Transcripts that were plastically regulated but showed no between-species differences (Fig. 3, H1) represent cases where transcripts differentially regulated in *R. zephyria* in response to the novel apple environment occur at comparable levels between *R. pomonella* and *R. zephyria* larvae feeding in their respective native fruit. These include candidates functioning in detoxification and oxidative stress responses in the 'glycosyltransferase', 'proteasome' and various categories associated with endomembrane systems where detoxification processes (and cytochrome p450 enzymes) are often localized (Feyereisen 1999). Although less frequent, there were also many transcripts that were plastically up-regulated in response to apple and up-regulated in the apple specialist *R. pomonella* compared to *R. zephyria* (or down-regulated in the same direction; Fig. 3, H3). These also include candidates for detoxification responses (in the 'monooxygenase' and 'drug metabolism' functional categories) that are likely targets of selection imposed by plant secondary metabolites. Detoxification-related genes are strong candidates likely to underlie the ability of the flies to initially colonize (through plastic expression) and subsequently adapt (through evolution of expression levels) to host fruits with different suites of chemical defences.

Mechanisms such as expression plasticity that allow feeding and development in novel host fruit are important for host race establishment, but the current and previous studies suggest that fitness on a new host can initially be lower than fitness on the native host. For new host races to persist in nature, low larval survival may often be offset by other fitness benefits (Bush 1975). Indeed, evidence for escape from competition and parasitism in the newly established apple host race of *R. pomonella* suggests that field conditions can be permissive for host race formation despite initially lower feeding performance of the fly in the novel apple vs. ancestral hawthorn host fruit (Feder 1995). Nevertheless, larvae must acquire enough nutrition and adequately metabolize toxins in the novel host environment to successfully pupate, and this requires a plastic physiological adjustment, whether to actively respond to stressors or to suppress physiological 'noise' and maintain homoeostasis. Given the prevalence and rapid rate of host race formation in the *Rhagoletis* species complex (Bush 1969; Feder *et al.* 1988; Berlocher & Feder 2002; Schwarz *et al.* 2005), standing variation may frequently include plastic responses that allow successful feeding in novel host fruits, perhaps maintained by variation in host fruit quality within a host species.

Specific responses to toxins and nutrition

Plant secondary metabolites are widely considered to be one of the primary stressors imposed by novel larval host plant environments (Awmack & Leather 2002).

Accordingly, we observed major plastic and evolved changes in expression of candidate genes likely to affect fitness through responses to plant defences. Cytochrome p450s (Cyps) are proteins with well-documented roles in metabolism of plant secondary compounds (Feyereisen 1999). We found that Cyps drove functional enrichment in three of the four major expression categories of plastic and evolved responses depicted in Fig. 3. The member transcripts of the 'monooxygenase' functional category, largely composed of Cyps, were enriched in sets of transcripts showing both evolved differences (Fig. 3, H2) and plastic and evolved differences (Fig. 3, H3). In both cases, expression levels were higher in *R. pomonella* compared to *R. zephyria*, and the plastic response increases expression levels in *R. zephyria* reared in apple vs. snowberry. However, the 'metalloprotein' class of genes, also primarily composed of Cyps, was enriched in transcripts whose evolved differences in expression counter the plastic response (Fig. 3, H4). There are several possible explanations, alone or in combination, that could account for the different result between monooxgenases and metalloproteins. Many Cyps have highly specialized functions, each binding very specific classes of molecules (De Montellano 2005). Moreover, even in model organisms such as *D. melanogaster*, the specific function of each Cyp protein has not been characterized, and some may also play important roles in other processes such as insect hormone metabolism (Rewitz *et al.* 2006). Cyps may also catalyze reactions that actually increase the toxicity of a compound, as in bioactivation of xenobiotics (Feyereisen 1999). Given this diversity of function, it is perhaps unsurprising that we find Cyps showing all possible patterns of plastic, evolved and up-/down-regulation (Fig. 4). Moreover, even within Cyp subfamilies (designated by 4, 6, 12, etc.), there appears to be no conservation of expression pattern. Although the specific patterns of expression were diverse, Cyps were among the most highly differentially expressed transcripts in our data set, reaching greater than 16-fold changes ($\log_2$ fold change > 4) in expression between the species. In addition, we found plastic and evolved differential regulation of a number of glutathione S-transferases and glycosyltransferaes (Fig. 5a,b), major constituents of the 'drug metabolism' class of genes enriched in category H3 (Fig. 3). Both glutathione S-transferases and glycosyltransferaes are gene families with well-known functions in insect detoxification responses (Despres *et al.* 2007; Ahn *et al.* 2012; Fang 2012; Heckel 2014). Finally, we also found enrichment of proteasome-related pro-

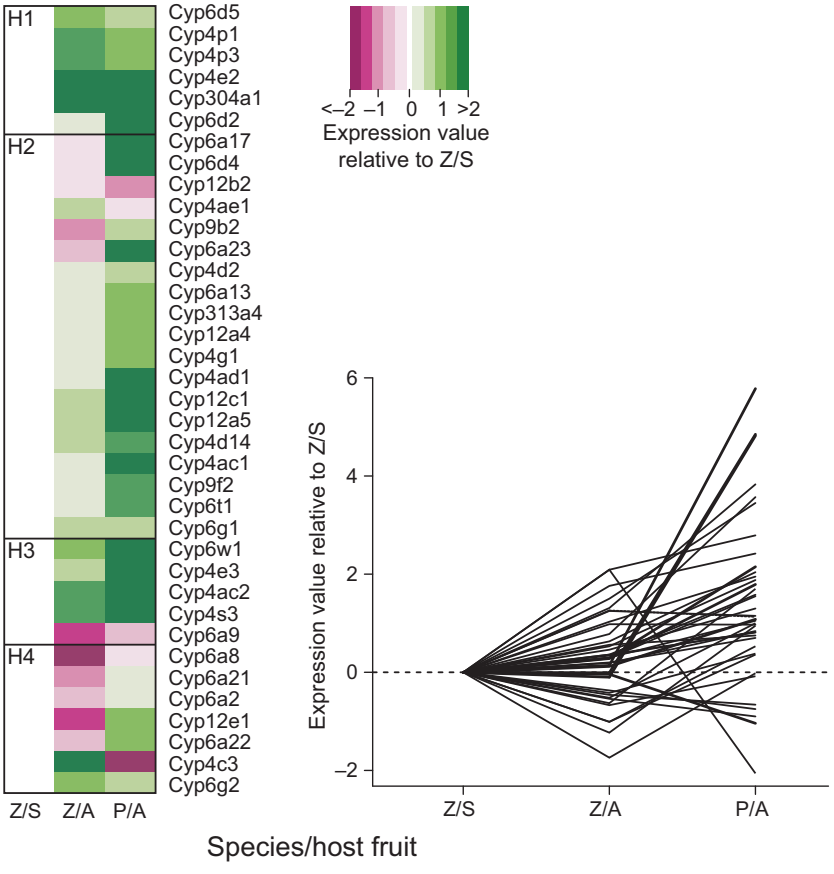

Fig. 4 'Heatmap (left panel) and average expression profiles (right panel) for representatives of the cytochrome p450 gene family. Expression values are $\log_2$ fold changes relative to the expression value of Z/S.

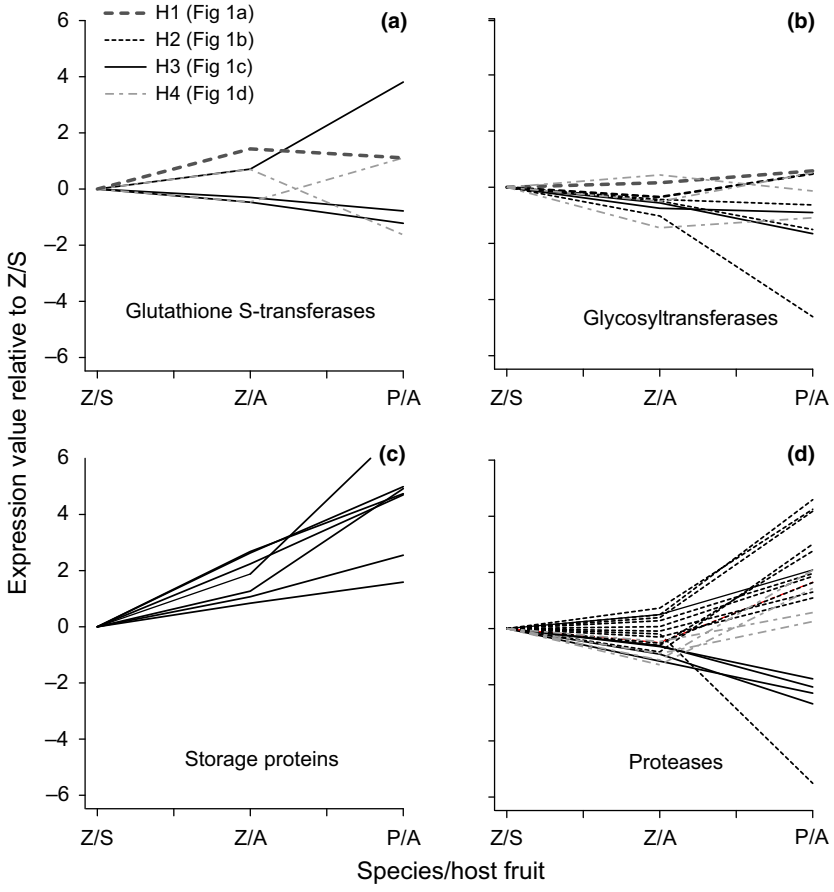

Fig. 5 Average expression profiles of genes from four candidate functional categories. Line patterns designate the membership of each transcript in one of the hypothesized plastic/evolved expression profiles from Fig. 1 (H1–H4). Expression values are $\log_2$ fold changes relative to the expression value of Z/S.

teins in plastic transcripts (Fig. 3, H1), and ubiquitin-targeted protein degradation mediated by the proteasome is commonly associated with response to oxidative stressors such as those related to toxin metabolism (Halliwell 2006).

The many detoxification-related genes that were differentially regulated in our study suggest that plastic responses to secondary metabolites are critical for successful infestation of novel host plants. Moreover, we propose that natural selection acts on these genes to refine their expression levels as fly species adapt to new host plant environments across generations. In our experiment, differences in chemical defences between apple and snowberry may have a large effect on expression patterns. Both fruits contain numerous phenolic compounds and glycosides. However, the lists of compounds found in extracts of apples and snowberries are largely distinct (Schieber *et al*. 2001; Szaufer-Hajdrych & Zgorka 2003; Makarevich *et al*. 2009), implying that the two fruits impose very different chemical challenges on feeding fly larvae. Overall, the plastic changes in transcription that we observed in *R. zephyria* would seem to allow relatively high survival to pupation on apple, albeit with a cost of slower development rate. Although survival in the reciprocal transplant, *R. pomonella* into snowberry, was very low, similar processes (that we did not measure, due to inadequate sample sizes) very likely underlie the successful infestation of at least a few *R. pomonella* larvae in the novel snowberry fruit.

The enrichment analysis also provides evidence for plastic and evolved transcriptional differences in nutrient provisioning and growth. Protein storage-related genes were enriched in transcripts increasing in abundance in the plastic response of *R. zephyria* to apple and in *R. pomonella* compared to *R. zephyria* (Fig. 3, H3, Fig. 5c). This category contains hexamerins, haemocyanin-derived proteins that store amino acids, and fat body proteins, receptor proteins that allow the fat body to take up storage proteins at the completion of larval development (Burmester & Scheller 1999; Burmester 2001). Genes involved in anabolism of lipids ('lipid biosynthesis' category) were enriched in the set of transcripts that were not plastically regulated but demonstrated down-regulation in *R. pomonella* compared to *R. zephyria* (Fig. 3, H2). Moreover, many functional categories associated with rate of cellular turnover and growth ('nucleotide binding', 'noncoding RNA metabolism', 'mitotic cell cycle', etc.) were enriched in transcripts showing only plastic or only evolved responses (Fig. 3, H1, H2). These patterns may

initially appear inconsistent with the observed differences in development rates, for example *R. pomonella* develops much faster than *R. zephyria* in apple, but transcripts related to cell turnover are also up-regulated in *R. zephyria* compared to *R. pomonella*. The clear signal of differential detoxification and survival responses among the species suggests that *R. zephyria* are incurring substantial cellular damage when feeding on apple, and responding to and repairing such damage (e.g. through degradation of oxidized proteins by the proteasome and greater turnover of cellular components) likely slows whole-organism developmental processes.

Response to novel hosts in other systems

Mechanisms of plastic responses to foreign host plant environment in phytophagous insects are relatively well documented, and our study demonstrates many elements of what seems to be a general response to host plant environment. This response includes detoxification proteins such as cytochrome p450's and glutathione S-transferases (Govind *et al.* 2010; Matzkin 2012; Celorio-Mancera *et al.* 2013; Vogel *et al.* 2014). We did not observe overt enrichment of other categories such as carboxylesterases that seem to be important in other systems (e.g. Celorio-Mancera *et al.* 2011), despite relatively thorough representation on our array (24 genes associated with GO:0004091, 'carboxylesterase activity'). Other transcriptomic studies have also identified strong plastic responses in ribosomal proteins, a somewhat surprising result hypothesized to potentially play an adaptive role in, for example, responses to plant-produced ribosome-inactivating proteins (Celorio-Mancera *et al.* 2013). Our data indicate significant enrichment for mitochondrial ribosomal proteins (the 'mitochondrion' functional category contains many ribosomal proteins) in the expression cluster demonstrating plastic responses, but an evolved response in the opposite direction, a pattern consistent with an energetically expensive stress response or mis-regulation (Fig. 3, H4).

In contrast to plastic responses, adaptive differentiation driven by host plant environment has received less attention, being primarily limited to detoxification responses that diverge between species well past the initial stages of ecological speciation (Bass *et al.* 2013). Our comparisons between *R. pomonella* and *R. zephyria* in the common apple environment clearly also identify detoxification responses as a major target of selection with several cytochrome p450 genes >16-fold differentially regulated between species (Fig. 4), and many of the detoxification-related genes demonstrating plastic responses (discussed above) also exhibiting evolved differences. Studies of plastic responses often identify digestive proteases as a highly differentially expressed category, hypothesized to be a response to plant protease inhibitors (Vogel *et al.* 2014). Although our enrichment analysis did not identify proteases as enriched in any plastic/evolved pattern, examination of representative protease transcripts on our array suggests that many demonstrate substantial evolved differences among the species, but most demonstrated no plastic response (Fig. 5d). Overall, our data provide a signal of evolved differences related to feeding in different milieus of potential toxins, supported by reciprocal fitness differences wherein each fly species performs relatively better on its native host fruit.

Conclusion

Although reciprocal fitness differences are not a critical component of all models of ecological speciation/specialization (e.g. Holt 2003) (reviewed by Forister *et al.* 2012), when detected they can provide strong evidence for ecologically driven reproductive isolation (Futuyma & Moreno 1988; Agrawal 2000; Via & Hawthorne 2005; Egan & Funk 2009). Our results demonstrate the existence of such a trade-off in larval feeding performance of *R. pomonella* and *R. zephyria* between apple and snowberry fruit. Nevertheless, we also found evidence that even specialist insects such as *Rhagoletis* fruit flies possess a high degree of plasticity, particularly in detoxification responses, that may help to partly ameliorate fitness differences observed as a result of larval feeding. The general pattern of highly plastic transcriptomic responses in the *R. pomonella* system likely underlies past and recent successful colonization of diverse host fruits across the *Rhagoletis* species complex. Specifically, we hypothesize that plastic responses allow populations to persist on novel hosts long enough for adaptive radiation and species formation to eventually occur. This plastic variation is then exposed to strong selection, leaving a signature of adaptive differentiation in transcript expression levels among taxa that have recently diverged in different, discrete environments. These evolutionary changes contribute to the earliest stages of host-feeding specialization and sustain the long-term survival of colonizing populations on novel hosts.

Acknowledgements

We wish to thank Lex Burgin for assistance with data analysis; Tom Powell, Scott Egan and Glen Hood for formative discussions; and three anonymous reviewers for constructive comments on the manuscript. This work was supported by Western Washington University, the University of Notre Dame Environmental Change Initiative and grants from the National

Science Foundation (IOS 1451274 to GJR and JLF; IOS 1257298 to DAH; DEB 1145573 to JLF).

References

Agrawal AA (2000) Host-range evolution: adaptation and trade-offs in fitness of mites on alternative hosts. *Ecology*, **81**, 500–508.

Ahn S-J, Vogel H, Heckel DG (2012) Comparative analysis of the UDP-glycosyltransferase multigene family in insects. *Insect Biochemistry and Molecular Biology*, **42**, 133–147.

Arbeitman MN, Furlong EEM, Imam F *et al.* (2002) Gene expression during the life cycle of *Drosophila melanogaster*. *Science*, **297**, 2270–2275.

Awmack CS, Leather SR (2002) Host plant quality and fecundity in herbivorous insects. *Annual Review of Entomology*, **47**, 817–844.

Bass C, Zimmer CT, Riveron JM *et al.* (2013) Gene amplification and microsatellite polymorphism underlie a recent insect host shift. *Proceedings of the National Academy of Sciences of the United States of America*, **110**, 19460–19465.

Berlocher SH, Feder JL (2002) Sympatric speciation in phytophagous insects: moving beyond controversy? *Annual Review of Entomology*, **47**, 773–815.

Berlocher SH, McPheron BA, Feder JL, Bush GL (1993) Genetic differentiation at allozyme loci in the *Rhagoletis pomonella* (Diptera: Tephritidae) species complex. *Annals of the Entomological Society of America*, **86**, 716–727.

Bernays EA, Woods HA (2000) Foraging in nature by larvae of *Manduca sexta* influenced by an endogenous oscillation. *Journal of Insect Physiology*, **46**, 825–836.

Bierbaum TJ, Bush GL (1990) Genetic differentiation in the viability of sibling species of *Rhagoletis* fruit flies on host plants, and the influence of reduced hybrid viability on reproductive isolation. *Entomologia Experimentalis Et Applicata*, **55**, 105–118.

Blanckenhorn WU (2000) The evolution of body size: what keeps organisms small? *Quarterly Review of Biology*, **75**, 385–407.

Bolnick DI, Fitzpatrick BM (2007) Sympatric speciation: models and empirical evidence. *Annual Review of Ecology Evolution and Systematics*, **38**, 459–487.

Brazner JC, Etges WJ (1993) Pre-mating isolation is determined by larval rearing substrates in cactophilic *Drosophila mojavensis*. 2. Effects of larval substrates on time to copulation, mate choice and mating propensity. *Evolutionary Ecology*, **7**, 605–624.

Burmester T (2001) Molecular evolution of the arthropod hemocyanin superfamily. *Molecular Biology and Evolution*, **18**, 184–195.

Burmester T, Scheller K (1999) Ligands and receptors: common theme in insect storage protein transport. *Naturwissenschaften*, **86**, 468–474.

Bush GL (1969) Sympatric host race formation and speciation in frugivorous flies of genus *rhagoletis* (Diptera: Tephritidae). *Evolution*, **23**, 237–251.

Bush GL (1975) Modes of animal speciation. *Annual Review of Ecology and Systematics*, **6**, 339–364.

Calcagno V, Thomas Y, Bourguet D (2007) Sympatric host races of the European corn borer: adaptation to host plants and hybrid performance. *Journal of Evolutionary Biology*, **20**, 1720–1729.

Celorio-Mancera MD, Ahn SJ, Vogel H, Heckel DG (2011) Transcriptional responses underlying the hormetic and detrimental effects of the plant secondary metabolite gossypol on the generalist herbivore *Helicoverpa armigera*. *BMC Genomics*, **12**, 575, doi: 10.1186/1471-2164-12-575.

Celorio-Mancera MD, Wheat CW, Vogel H *et al.* (2013) Mechanisms of macroevolution: polyphagous plasticity in butterfly larvae revealed by RNA-Seq. *Molecular Ecology*, **22**, 4884–4895.

Coyne JA, Orr HA (2004) *Speciation*. Sinauer Associates, Incorporated Publishers, Sunderland, Massachusetts.

Dambroski HR, Linn C, Berlocher SH *et al.* (2005) The genetic basis for fruit odor discrimination in *Rhagoletis* flies and its significance for sympatric host shifts. *Evolution*, **59**, 1953–1964.

De Montellano PRO (2005) *Cytochrome P450: Structure, Mechanism, and Biochemistry*. Kluwer Academic/Plenum Publishers, New York.

Despres L, David JP, Gallet C (2007) The evolutionary ecology of insect resistance to plant chemicals. *Trends in Ecology & Evolution*, **22**, 298–307.

Diehl SR, Prokopy RJ (1986) Host-selection behavior differences between the fruit fly sibling species *Rhagoletis pomonella* and *Rhagoletis mendax* (Diptera: Tephritidae). *Annals of the Entomological Society of America*, **79**, 266–271.

Egan SP, Funk DJ (2009) Ecologically dependent postmating isolation between sympatric host forms of *Neochlamisus bebbianae* leaf beetles. *Proceedings of the National Academy of Sciences of the United States of America*, **106**, 19426–19431.

Etges WJ, Trotter MV, Oliveira CC *et al.* (2015) Deciphering life history transcriptomes in different environments. *Molecular Ecology*, **24**, 151–179.

Fang SM (2012) Insect glutathione S-transferase: a review of comparative genomic studies and response to xenobiotics. *Bulletin of Insectology*, **65**, 265–271.

Feder JL (1995) The effects of parasitoids on sympatric host races of *Rhagoletis pomonella* (Diptera: Tephritidae). *Ecology*, **76**, 801–813.

Feder JL, Filchak KE (1999) It's about time: the evidence for host plant-mediated selection in the apple maggot fly, *Rhagoletis pomonella*, and its implications for fitness trade-offs in phytophagous insects. *Entomologia Experimentalis Et Applicata*, **91**, 211–225.

Feder ME, Hofmann GE (1999) Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. *Annual Review of Physiology*, **61**, 243–282.

Feder JL, Chilcote CA, Bush GL (1988) Genetic differentiation between sympatric host races of the apple maggot fly *Rhagoletis pomonella*. *Nature*, **336**, 61–64.

Feder JL, Williams SM, Berlocher SH, McPheron BA, Bush GL (1999) The population genetics of the apple maggot fly, *Rhagoletis pomonella* and the snowberry maggot, *R. zephyria*: implications for models of sympatric speciation. *Entomologia Experimentalis Et Applicata*, **90**, 9–24.

Feder JL, Egan SP, Nosil P (2012) The genomics of speciation-with-gene-flow. *Trends in Genetics*, **28**, 342–350.

Feyereisen R (1999) Insect P450 enzymes. *Annual Review of Entomology*, **44**, 507–533.

Filchak KE, Feder JL, Roethel JB, Stolz U (1999) A field test for host-plant dependent selection on larvae of the apple maggot fly, *Rhagoletis pomonella*. *Evolution*, **91**, 187–200.

Filchak KE, Roethele JB, Feder JL (2000) Natural selection and sympatric divergence in the apple maggot *Rhagoletis pomonella*. *Nature*, **407**, 739–742.

Forister ML, Dyer LA, Singer MS, Stireman JO III, Lill JT (2012) Revisiting the evolution of ecological specialization, with emphasis on insect-plant interactions. *Ecology*, **93**, 981–991.

Fox CW, Nilsson JA, Mousseau TA (1997) The ecology of diet expansion in a seed-feeding beetle: pre-existing variation, rapid adaptation and maternal effects? *Evolutionary Ecology*, **11**, 183–194.

Frey JE, Bush GL (1990) *Rhagoletis* sibling species and host races differ in host odor recognition. *Entomologia Experimentalis Et Applicata*, **57**, 123–131.

Fry JD (1996) The evolution of host specialization: are trade-offs overrated? *American Naturalist*, **148**, S84–S107.

Funk DJ, Filchak KE, Feder JL (2002) Herbivorous insects: model systems for the comparative study of speciation ecology. *Genetica*, **116**, 251–267.

Futuyma DJ, Moreno G (1988) The evolution of ecological specialization. *Annual Review of Ecology and Systematics*, **19**, 207–233.

Garfield DA, Runcie DE, Babbitt CC *et al.* (2013) The impact of gene expression variation on the robustness and evolvability of a developmental gene regulatory network. *PLos Biology*, **11**, e1001696.

Gavrilovic V, Bush GL, Schwarz D, Crossno JE, Smith JJ (2007) Rhagoletis zephyria (Diptera: Tephritidae) in the Great Lakes Basin: a native insect on native hosts? *Annals of the Entomological Society of America*, **100**, 474–482.

Govind G, Mittapalli O, Griebel T *et al.* (2010) Unbiased transcriptional comparisons of generalist and specialist herbivores feeding on progressively defenseless *Nicotiana attenuata* plants. *PLoS One*, **5**, e8735.

Green DA, Extavour CG (2014) Insulin signalling underlies both plasticity and divergence of a reproductive trait in *Drosophila*. *Proceedings of the Royal Society B: Biological Sciences*, **281**, 20132673.

Green E, Almskaar K, Sim S *et al.* (2013) Molecular species identification of cryptic apple and snowberry maggots (Diptera: Tephritidae) in Western and Central Washington. *Environmental Entomology*, **42**, 1100–1109.

Grimaldi D, Jaenike J (1984) Competition in natural populations of mycophagous Drosophila. *Ecology*, **65**, 1113–1120.

Hahn DA, Denlinger DL (2011) Energetics of insect diapause. *Annual Review of Entomology*, **56**, 103–121.

Halliwell B (2006) Oxidative stress and neurodegeneration: where are we now? *Journal of Neurochemistry*, **97**, 1634–1658.

Heckel DG (2014) Insect detoxification and sequestration strategies. In: *Annual Plant Reviews Volume 47: Insect-Plant Interactions*(eds Voelckel C, Jander G), pp. 77–114. John Wiley & Sons Ltd, Chichester.

Hess A, Iyer H (2007) Fisher's combined p-value for detecting differentially expressed genes using Affymetrix expression arrays. *BMC Genomics*, **8**, 96.

Holt RD (2003) On the evolutionary ecology of species' ranges. *Evolutionary Ecology Research*, **5**, 159–178.

Huang DW, Sherman BT, Lempicki RA (2008) Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nature Protocols*, **4**, 44–57.

Jaenike J (1990) Host specialization in phytophagous insects. *Annual Review of Ecology and Systematics*, **21**, 243–273.

Jaureguy LM, Etges WJ (2007) Assessing patterns of senescence in *Drosophila mojavensis* reared on different host cacti. *Evolutionary Ecology Research*, **9**, 91–107.

Laukkanen L, Leimu R, Muola A, Lilley M, Mutikainen P (2013) Genetic factors affecting food-plant specialization of an oligophagous seed predator. *Journal of Evolutionary Biology*, **26**, 141–149.

Linn C, Feder JL, Nojima S *et al.* (2003) Fruit odor discrimination and sympatric host race formation in *Rhagoletis*. *Proceedings of the National Academy of Sciences of the United States of America*, **100**, 11490–11493.

Makarevich I, Kovalenko S, Gusarova T, Gubin YI (2009) Iridoids from *Symphoricarpos albus*. *Chemistry of Natural Compounds*, **45**, 40–44.

Matzkin LM (2012) Population transcriptomics of cactus host shifts in *Drosophila mojavensis*. *Molecular Ecology*, **21**, 2428–2439.

Matzkin LM, Watts TD, Bitler BG, Machado CA, Markow TA (2006) Functional genomics of cactus host shifts in *Drosophila mojavensis*. *Molecular Ecology*, **15**, 4635–4643.

McPheron BA (1990) Genetic structure of apple maggot fly (Diptera: Tephritidae) populations. *Annals of the Entomological Society of America*, **83**, 568–577.

Moczek AP, Sultan S, Foster S *et al.* (2011) The role of developmental plasticity in evolutionary innovation. *Proceedings of the Royal Society B: Biological Sciences*. doi: 10.1098/rspb.2011.0971.

Morris MRJ, Richard R, Leder EH *et al.* (2014) Gene expression plasticity evolves in response to colonization of freshwater lakes in threespine stickleback. *Molecular Ecology*, **23**, 3226–3240.

Oppert B, Elpidina EN, Toutges M, Mazumdar-Leighton S (2010) Microarray analysis reveals strategies of *Tribolium castaneum* larvae to compensate for cysteine and serine protease inhibitors. *Comparative Biochemistry and Physiology D-Genomics & Proteomics*, **5**, 280–287.

Powell THQ, Hood GR, Murphy MO *et al.* (2013) Genetic divergence along the speciation continuum: the transition from host race to species in *Rhagoletis* (Diptera: Tephritidae). *Evolution*, **67**, 2561–2576.

Prokopy RJ, Bush GL (1973) Ovipositional responses to different sizes of artificial fruit by flies of *Rhagoletis pomonella* species group (Diptera: Tephritidae). *Annals of the Entomological Society of America*, **66**, 927–929.

Prokopy RJ, Diehl SR, Cooley SS (1988) Behavioral evidence for host races in *Rhagoletis pomonella* flies. *Oecologia*, **76**, 138–147.

R Core Team (2013) *R: A Language and Environment for Statistical Computing*. Available from http://www.R-project.org

Ragland GJ, Fuller J, Feder JL, Hahn DA (2009) Biphasic metabolic rate trajectory of pupal diapause termination and post-diapause development in a tephritid fly. *Journal of Insect Physiology*, **55**, 344–350.

Ragland GJ, Egan SP, Feder JL, Berlocher SH, Hahn DA (2011) Developmental trajectories of gene expression reveal candidates for diapause termination: a key life-history transition

in the apple maggot fly *Rhagoletis pomonella*. *Journal of Experimental Biology*, **214**, 3948–3959.
Ragland GJ, Sim SB, Goudarzi S, Feder JL, Hahn DA (2012) Environmental interactions during host race formation: host fruit environment moderates a seasonal shift in phenology in host races of *Rhagoletis pomonella*. *Functional Ecology*, **26**, 921–931.
Rewitz K, Rybczynski R, Warren JT, Gilbert LI (2006) The Halloween genes code for cytochrome P450 enzymes mediating synthesis of the insect moulting hormone. *Biochemical Society Transactions*, **34**, 1256–1260.
Rundle HD, Nosil P (2005) Ecological speciation. *Ecology Letters*, **8**, 336–352.
Rutherford SL, Lindquist S (1998) Hsp90 as a capacitor for morphological evolution. *Nature*, **396**, 336–342.
Schieber A, Keller P, Carle R (2001) Determination of phenolic acids and flavonoids of apple and pear by high-performance liquid chromatography. *Journal of Chromatography. A.*, **910**, 265–273.
Schwarz D (2004) Natural hybridization and speciation in Rhagoletis (Diptera: Tephritidae) Doctoral, University of Illinois.
Schwarz D, Matta BM, Shakir-Botteri NL, McPheron BA (2005) Host shift to an invasive plant triggers rapid animal hybrid speciation. *Nature*, **436**, 546–549.
Schwarz D, Shoemaker KD, Botteri NL, McPheron BA (2007) A novel preference for an invasive plant as a mechanism for animal hybrid speciation. *Evolution*, **61**, 245–256.
Schwarz D, Robertson HM, Feder JL *et al.* (2009) Sympatric ecological speciation meets pyrosequencing: sampling the transcriptome of the apple maggot *Rhagoletis pomonella*. *BMC Genomics*, **10**, 633.
Smith JJ, Bush GL (1997) Phylogeny of the genus *Rhagoletis* (Diptera: Tephritidae) inferred from DNA sequences of mitochondrial cytochrome oxidase II. *Molecular Phylogenetics and Evolution*, **7**, 33–43.
Smyth GK (2004) Linear models and empirical Bayes methods for assessing differential expression in microarray experiments. *Statistical Applications in Genetics and Molecular Biology*, **3**, 1–25.
Smyth GK, Speed TP (2003) Normalization of cDNA microarray data. *Methods*, **31**, 265–273.
Stennett MD, Etges WJ (1997) Premating isolation is determined by larval rearing substrates in cactophilic *Drosophila mojavensis*. III. Epicuticular hydrocarbon variation is determined by use of different host plants in *Drosophila mojavensis* and *Drosophila arizonae*. *Journal of Chemical Ecology*, **23**, 2803–2824.
Suzuki Y, Nijhout HF (2006) Evolution of a polyphenism by genetic accommodation. *Science*, **311**, 650–652.
Szaufer-Hajdrych M, Zgorka G (2003) Phenolic acids from *Symphoricarpos albus* (L.) Blake (Caprifoliaceae). *Acta Poloniae Pharmaceutica*, **60**, 91–96.
Via S, Hawthorne DJ (2002) The genetic architecture of ecological specialization: correlated gene effects on host use and habitat choice in pea aphids. *American Naturalist*, **159**, S76–S88.
Via S, Hawthorne DJ (2005) Back to the future: genetic correlations, adaptation and speciation. *Genetica*, **123**, 147–156.
Vogel H, Musser RO, Celorio-Mancera M (2014) Transcriptome responses in herbivorous insects towards host plant and toxin feeding. In: *Annual Plant Reviews Volume 47: Insect-Plant Interactions* (eds Voelckel C, Jander G), pp. 197–234. John Wiley & Sons, Ltd., Chichester.
Wu CI (2001) The genic view of the process of speciation. *Journal of Evolutionary Biology*, **14**, 851–865.
Xie W, Wu QJ, Wang SL *et al.* (2014) Transcriptome analysis of host-associated differentiation in *Bemisia tabaci* (Hemiptera: Aleyrodidae). *Frontiers in Physiology*, **5**, 487.
Yampolsky LY, Glazko GV, Fry JD (2012) Evolution of gene expression and expression plasticity in long-term experimental populations of *Drosophila melanogaster* maintained under constant and variable ethanol stress. *Molecular Ecology*, **21**, 4287–4299.
Yee WL, Chapman PS, Sheets HD, Unruh TR (2009) Analysis of body measurements and wing shape to discriminate *Rhagoletis pomonella* and *Rhagoletis zephyria* (Diptera: Tephritidae) in Washington State. *Annals of the Entomological Society of America*, **102**, 1013–1028.
Yee WL, Klaus MW, Cha DH *et al.* (2012) Abundance of apple maggot, *Rhagoletis pomonella*, across different areas in central Washington, with special reference to black-fruited hawthorns. *Journal of Insect Science*, **12**, 1013–1028.
Zhang S-D, Gant TW (2005) Effect of pooling samples on the efficiency of comparative studies using microarrays. *Bioinformatics*, **21**, 4378–4383.

G.J.R., D.A.H., J.L.F. and D.S. designed the study. K.A., K.L.V., H.M.G. and D.S. performed the manipulative experiments. G.J.R., H.M.G., D.A.H. and D.S. analyzed the data and G.J.R., D.S., D.A.H. and J.L.F. drafted the manuscript.

Data accessibility

Microarray gene expression data, metadata and array platform design deposited in the NCBI Gene Expression Omnibus (GSE66975). Supplemental information includes a full table of log fold changes between all comparisons for all nonredundant transcripts provided in supplementary information (S1), full results of David enrichment analysis (S2), raw data for pupal mass (S3), emergence timing (S4) and survival (S5).

Supporting information

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

Fig. S1 Average $\log_2$ fold change vs. the $\log_2$ ratio of % identity of *R. zephyria* and R.

Table S1 Average log fold changes, minimum *P*-values, and *P*-values estimated using Fisher's method for all transcripts after transcripts annotating to the same gene were collapsed (see methods).

Table S2 Enrichment of functional categories (DAVID analysis) within clusters of genes with membership in one of the following categories: plastic, not evolved (Fig. 1a); evolved, not plastic (Fig. 1b); plastic and evolved, same direiction (Fig. 1c); plastic and evolved, opposite direction (Fig. 1d).

Table S3 Pupal masses (mg).

Table S4 Egg to pupariation emergence data.

Table S5 Survival to pupariation.