Genomics analysis of Drosophila sechellia response to Morinda citrifolia fruit diet

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Abstract

Drosophila sechellia is an island endemic host specialist that has evolved to consume the toxic fruit of Morinda citrifolia, also known as noni fruit. Recent studies by our group and others have examined genome-wide gene expression responses of fruit flies to individual highly abundant compounds found in noni responsible for the fruit’s unique chemistry and toxicity. In order to relate these reductionist experiments to the gene expression responses to feeding on noni fruit itself, we fed rotten noni fruit to adult female D. sechellia and performed RNA-sequencing. Combining the reductionist and more wholistic approaches, we have identified candidate genes that may contribute to each individual compound and those that play a more general role in response to the fruit as a whole. Using the compound specific and general responses, we used transcription factor prediction analyses to identify the regulatory networks and specific regulators involved in the responses to each compound and the fruit itself. The identified genes and regulators represent the possible genetic mechanisms and biochemical pathways that contribute to toxin resistance and noni specialization in D. sechellia.

Keywords: toxin resistance; Morinda citrifolia; noni; host specialization; RNA-seq

Introduction

Insects have intimate relationships with plants, ranging from pollination to parasitism, and mimicry to mutualism. One of the most common of these interactions is insect-host plant specialization. A well-studied example of this is the Seychelles Islands endemic fruit fly specialist Drosophila sechellia that feeds almost exclusively on the ripe fruit of the Morinda citrifolia or noni plant (Tsacas and Bachli 1981; Louis and David 1986; Matute and Ayroles 2014). Drosophila sechellia is considered to be a banner species for specialization because its closest relatives are not specialists, and because it relies heavily on M. citrifolia at all of its life stages (Louis and David 1986; R’Kha et al. 1991, 1997; Lavista-Llanos et al. 2014). Additionally, aside from the ease of cultivating fruit flies in a lab, the ability of D. sechellia to hybridize with close relatives facilitated early genetic studies (R’Kha et al. 1991). Much interest in D. sechellia arises from the observation that ripe M. citrifolia fruit is highly toxic to other species of fruit flies yet D. sechellia is resistant to this toxicity (R’Kha et al. 1991; Andrade Lopez et al. 2017).

The main toxins of M. citrifolia fruit are volatile fatty acids, to which D. sechellia has evolved both high resistance and preference (Legal et al. 1992, 1994; Farine et al. 1996; Dekker et al. 2006; Matute and Ayroles, 2014). A number of studies have centered around the mechanisms of this toxin resistance, most with a focus on the fatty acid volatile octanoic acid (OA; R’Kha et al. 1991; Jones 1998; Lavista-Llanos et al. 2014; Lanno et al. 2017, 2019; Peyser et al. 2017; Lanno and Coolon 2019). The other primary fatty acid volatile found in noni fruit, hexanoic acid (HA), is also toxic (Farine et al. 1996; Peyser et al. 2017; Lanno and Coolon 2019) but less so than OA and is responsible for attracting D. sechellia to its host (Amlou, Moreteau, and David 1998). In that vein, recent work has shown that D. sechellia prefers the fruit of M. citrifolia, it has adapted to it nutritionally and relies on it for normal reproduction (Lavista-Llanos et al. 2014; Watanabe et al. 2019).

Drosophila sechellia grows and reproduces better on M. citrifolia than other food sources in part because of adaptation to a lower carbohydrate to protein ratio (Watanabe et al. 2019), but also because of reliance on M. citrifolia for l-DOPA, a dopamine precursor (Lavista-Llanos et al. 2014). The results of Lavista-Llanos et al. (2014) explain the observation that maternal environment is more important for larval success in D. sechellia than genotype—it is the reliance on an external source of l-DOPA. Surprisingly, they also showed that dopamine confers toxin resistance in other Drosophilids, a result corroborated by Lanno et al. (2019). Additionally, the toxic environment in M. citrifolia fruit increases egg production in D. sechellia but decreases egg production in other Drosophilae (R’Kha et al. 1991). A gene expression study using microarrays found that D. sechellia fed M. citrifolia have increased expression of genes associated with egg production and fatty acid metabolism (Dworkin and Jones 2009).

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D. sechellia have low levels of 3,4-dihydroxyphenylalanine (L-DOPA), a dopamine precursor, relative to other Drosophila species, likely due a mutation in the *CatSpu* gene, which regulates the synthesis of L-DOPA from tyrosine. *Morinda citrifolia* contains high levels of L-DOPA. When the L-DOPA in *M. citrifolia* is removed, *D. sechellia* produce fewer eggs. Thus, *D. sechellia* supplement their own low levels of L-DOPA with the L-DOPA found in *M. citrifolia* to increase egg production. In addition, this process allows the eggs to survive in the fruit’s toxic environment (Lavista-Llanos et al. 2014). Furthermore, *Drosophila melanogaster* and *Drosophila simulans* adult flies fed L-DOPA have increased resistance to OA (Lavista-Llanos et al. 2014; Lanno et al. 2019).

Many prior studies in other insects have focused on identifying genes important for insect specialization on specific host plants by using a genome-wide transcriptomic approach. This approach has been applied to investigations of insects in response to specific toxins found in their preferred host plant (Wang et al. 2015; Lanno et al. 2017, 2019; Li et al. 2019; Drum et al. 2022) while others investigated gene expression responses to the different host plant as a whole (Govind et al. 2010; Bansal et al. 2014; Hoang et al. 2015; Crava et al. 2016; De Panis et al. 2016; Birnbaum et al. 2017; Schweizer et al. 2017). Feeding on different food sources therefore differ in many ways (e.g. presence and concentration of many chemicals) but represent a more ecologically relevant condition. In this study, we compared transcriptomes of *D. sechellia* fed on a standard diet to a diet supplemented with rotten *M. citrifolia* fruit and compare to our prior work investigating gene expression responses to single chemicals found in *M. citrifolia* (Lanno et al. 2017, 2019; Drum et al. 2022). The rotten *M. citrifolia* represents a condition in which L-DOPA is preserved and toxicity to other Drosophilids due to OA and HA volatiles is not because microbial action reduces the volatile fatty acids in the fruit (David et al. 1989; Lavista-Llanos et al. 2014). We also analyze significantly differentially expressed genes (DEGs) from each treatment using software that examines shared regulatory motifs among DEGs to make predictions about which transcription factors (TFs) may be regulating the expression of DEGs in response to these different treatments. Therefore, we can make inferences about the role of each compound in altering gene expression in *D. sechellia* and compare the transcriptomic responses induced by each compound as well as identify regulatory networks that might be common to all chemical substances.

**Methods**

**Fly strains and culture**

*Drosophila sechellia* (14021-0428.25) flies were reared at low density on standard cornmeal medium under a 16:8 light:dark cycle maintained at 20°C.

**RNA-sequencing**

Adult female 0- to 3-day-old *D. sechellia* 14021-0428.25 flies were fed control food (Carolina Biological Supply) or control food mixed with 1 g rotten *M. citrifolia* fruit pulp for 24 h. The *Morinda* fruit used in this experiment was harvested when fully ripe (white/gray in color and soft) from plants grown on site. The fruit was then aged in 1 L plastic containers with holes in the lid to allow air movement for 7 days at 25°C and 70% relative humidity. Seeds were removed and pulp homogenized before incorporation in food media. After treatment, flies were snap frozen in liquid nitrogen and stored at −80°C until RNA extraction. Three replicates were analyzed per treatment, with 10 flies per replicate, generating 3 control and 3 noni fed samples. RNA was extracted using the Promega SV total RNA extraction system with modified protocol (Promega; Coolon et al. 2013). RNA quality was determined using gel electrophoresis (Thermo Fisher Scientific, USA) and NanoDrop spectrophotometer (Thermo Fisher Scientific USA). RNA was sent to the University of Michigan Sequencing Core Facility where mRNA selection was performed from total RNA using poly(A) selection. cDNA libraries were then sequenced using the Illumina HiSeq 4000 platform.

**BIOL310 genomics analysis**

The genomics analysis of RNA-seq data presented in this manuscript was performed by 2 high school, 20 undergraduate, and 3 graduate students as part of a semester-long course at Wesleyan University called Genomics Analysis (BIOL310). This is the fourth such manuscript (see Lanno et al. 2017, 2019; Drum et al. 2022) generated from this Course-Based Undergraduate Research Experience (CURE) where the aim is to provide early-stage undergraduate students an opportunity for hands-on research experience with active participation in the process of scientific discovery. Students in the course learn through engaging with newly generated genomics data and use cutting-edge genomics analysis and bioinformatics tools engaging in a discovery-based independent study. Every student in the course contributed to every aspect of the analysis including quality control, bioinformatics, statistical analyses, write-up, and interpretation of the findings, providing their own unique perspective of the results and the text written by each and every student was combined into this manuscript with little modification.

After sequencing output files were obtained from the University of Michigan Sequencing Core (Table 1), fastq files containing raw sequencing reads were uploaded to the Galaxy platform (Afgan et al. 2016) and an RNA-seq pipeline analysis was performed (Fig. 1) as previously described (Lanno et al. 2017, 2019; Drum et al. 2022). Briefly, reads were assessed for quality using FASTQC (Andrews 2010) and any overrepresented sequences were analyzed using NCBI Blast (Altschul et al. 1990). Bowtie2 was used for mapping reads to the appropriate reference genome for each species with default parameters (Langmead and Salzberg 2012), with the most recent genomes for each species available at the time of analysis acquired from Ensembl (www.ensembl.org, Yates et al. 2020; D. sechellia: Drosophila_sechellia_dsec_caf1.dna.toplevel.fa). The Bowtie2 output files were analyzed using Cuffdiff (Trapnell et al. 2013), for gene expression quantification and differential gene expression analysis using the aforementioned genome file along with the most recent annotated .gff3 file for each genome available at the time of analysis acquired from Ensembl (D. sechellia: Drosophila_sechellia_dsec_caf1.42.gff3). In Cuffdiff, geometric normalization and library size correction were performed, along with bias correction using the reference genome, giving an output of DEGs following false discovery rate multiple testing correction (Benjamini and Hochberg 1995, q < 0.05). Data were visualized using R. The list of DEGs was uploaded to geneontology.org for Gene Ontology (GO) term enrichment analysis (www.geneontology.org; Ashburner et al. 2000; Mi et al. 2019; Carbon et al. 2021). Drosophila melanogaster orthologs for each *D. sechellia* gene were downloaded using FlyBase (Thurmond et al. 2019). Data processing and visualization were performed in R (R Core Development Team 2020). The D. melanogaster orthologs for each DEG after feeding on noni food were analyzed through the cis-Target analysis software (https://med.kuleuven.be/icb/cisTarget) to identify putative cis-regulatory sequences shared among DEGs (Herrmann et al. 2012; Imrichová et al. 2015). The top 10 all non-TATA unique sequence elements representing predicted TF binding sites and their downstream targets.
were then visualized with Cytoscape (https://cytoscape.org; Shannon et al. 2003). DEGs following D. sechellia exposure to OA, HA, or L-DOPA were downloaded from the literature (Lanno et al. 2017, 2019; Drum et al. 2022). Gene overlap testing was performed using the “GeneOverlap” package in R (Shen 2021) with D. melanogaster orthologs.

Results
Identifying genes that are regulated in response to noni fruit diet
To identify the genes expressed differently when adult D. sechellia flies are fed a diet of control food (instant Drosophila media) compared to flies fed control food supplemented with rotten noni fruit, we used RNA-seq. Statistical analyses of genome-wide gene expression in these 2 diets identified 503 significantly DEGs (Fig. 2). Of these DEGs, 179 were upregulated in response to noni fruit and 324 DEGs were downregulated (Table 2). Of these 503 DEGs, 421 had annotated D. melanogaster orthologs. Of the 82 DEGs without annotated D. melanogaster orthologs, 31 DEGs were 5.8S rRNA genes, all of which were downregulated (Table 1). Five of the DEGs had uncertain orthologs based on the presence of paralogs in one or more species were removed for further analysis. For the remainder of the analysis, only genes with D. melanogaster orthologs were used so annotation for the identified genes could be utilized.

Experimental Design

Fig. 1. Experimental design and bioinformatics pipeline. Female D. sechellia were exposed to either control food or food supplemented with rotten noni fruit. RNA was extracted, underwent polyA selection, library preparation, and sequencing. Raw-sequencing reads were checked for quality using FastQC, and then aligned to the D. sechellia reference genome with Bowtie2. Differential expression testing was performed using Cuffdiff, and expression data were analyzed using R, Gene ontology, and i-cis-Target.

Table 1. Samples, mapped reads, and read lengths for each sequencing library.

| Sample   | ID    | No. of reads | No. of mapped reads | % Mapped | Read length (nt) |
|----------|-------|--------------|---------------------|----------|-----------------|
| Control 1| 76332 | 19,222,060   | 18,496,450          | 96.23    | 65              |
| Control 2| 76333 | 20,704,811   | 19,440,620          | 93.89    | 65              |
| Control 3| 76334 | 17,696,868   | 17,123,579          | 96.76    | 65              |
| Noni 1   | 76350 | 18,937,596   | 17,939,488          | 94.73    | 65              |
| Noni 2   | 76351 | 16,870,429   | 15,801,506          | 93.66    | 65              |
| Noni 3   | 76352 | 14,595,340   | 14,181,949          | 97.17    | 65              |

Table 2. DEGs in D. sechellia in response to noni treatment.

| No. of DEGs | No. of upregulated | No. of downregulated | No. of D. melanogaster orthologs |
|-------------|-------------------|----------------------|---------------------------------|
| 503         | 179               | 324                  | 426                             |

were then visualized with Cytoscape (https://cytoscape.org; Shannon et al. 2003). DEGs following D. sechellia exposure to OA, HA, or L-DOPA were downloaded from the literature (Lanno et al. 2017, 2019; Drum et al. 2022). Gene overlap testing was performed using the “GeneOverlap” package in R (Shen 2021) with D. melanogaster orthologs.
Significantly DEGs are shown in red. Lavista-Llanos et al. (2014) investigated the regulatory network(s) of DEGs identified in D. sechellia that showed increased egg production in response to feeding on noni (R’Kha et al. 1991, 1997; Lavista-Llanos et al. 2014; Lanno and Coolon 2019). The most significantly enriched cellular component GO terms from upregulated DEGs included egg chorion (GO:0042600, \( P = 3.58e-08 \)), external encapsulating structure (GO:0030312, \( P = 4.43e-08 \)), chromosome (GO:0005694, \( P = 5.04e-06 \)), and nonmembrane-bounded organelle (GO:0043228, \( P = 1.15e-05 \), Fig. 3b). No significant enrichment of molecular function GO terms was found for upregulated DEGs. The most significantly enriched molecular function GO terms from downregulated genes were alkaline phosphatase activity (GO:0004035, \( P = 2.53e-03 \)), hydrolase activity, hydrolyzing O-glycosyl compounds (GO:0004553, \( P = 5.53e-03 \)), hydrolase activity, acting on glycosyl bonds (GO:0016798, \( P = 8.43e-03 \)), and hydrolase activity (GO:0016787, \( P = 4.66e-02 \), Fig. 3c). The most significantly enriched cellular component GO terms from downregulated DEGs were extracellular region (GO:0005576, \( P = 4.31e-06 \)), cell surface (GO:0009986, \( P = 4.57e-04 \)), plasma membrane (GO:0005920, \( P = 8.77e-03 \)), smooth septate junction (GO:0005920, \( P = 2.83e-02 \)), and nucleus (GO:0005634, \( P = 4.49e-02 \), Fig. 3d, Supplementary Tables 7 and 8). No significant enrichment of biological processes GO terms was found for downregulated DEGs.

Investigating the regulatory network(s) of DEGs responding to noni fruit diet

Identified DEGs were analyzed using i-cisTarget to determine which TFs may be involved in regulating gene expression upon feeding on rotten noni. Predicted TFs from DEGs that responded to noni treatment were Adj1, GATAd, GATAe, grn, ham, pnr, sd, single-minded (sim), srp, and zld (Supplementary Fig. 2). This analysis predicted all 5 GATA family TFs as regulators of DEG expression (GATAd, GATAe, grn, pnr, srp). GATA factors are important in dietary restriction (Dobson et al. 2018) and gut stem cell maintenance (Okumura et al. 2005), and a possible role in egg formation in insects (Liu et al. 2019) making them excellent candidates for roles in evolved responses to altered diet and downstream effects of diet on egg production. Of the predicted TFs responding to noni treatment, only sim, a transcriptional repressor involved in nervous system development was significantly upregulated in D. sechellia (Estes et al. 2001; Supplementary Fig. 1). Of the 31 DEGs predicted to be regulated by sim in noni treatment, 23 are downregulated (Supplementary Fig. 2).

Comparing DEGs responsive to noni fruit, OA, HA, and L-DOPA

The unique niche that D. sechellia utilizes by specializing to feed almost solely on noni fruit includes multiple highly abundant plant chemicals including OA, HA, and L-DOPA (Legal et al. 1994; Farine et al. 1996; Andrade Lopez et al. 2017). Genome-wide gene expression investigations of responses to each of these individual chemicals were published previously (Lanno et al. 2017, 2019; Drum et al. 2022). Comparing the separate transcriptional responses of D. sechellia to the individual chemicals with D. sechellia fed noni fruit may help elucidate how they evolved to specialize on this toxic fruit. Previous studies investigated the transcriptional response of D. sechellia to OA (Lanno et al. 2017), HA (Drum et al. 2022), and 3,4-dihydroxyphenylalanine (L-DOPA, Lanno et al. 2019). DEGs that do not have annotated D. melanogaster orthologs in FlyBase were found to be many members of different RNA classes. Upon OA, L-DOPA, and noni treatment, several 5.8SrRNAs, snorRNAs, and 18SrRNAs were all downregulated. In contrast, upon HA treatment several 5.8SrRNAs were upregulated (Supplementary Table 3). Analyzing DEGs in each treatment with annotated D. melanogaster orthologs yields 8 DEGs that were significantly differentially expressed in all 4 treatments and all 8 genes were downregulated (Fig. 4). The antimicrobial peptides Defensin, GNBP-like3, edn were significantly
downregulated in all 4 treatments, as was the TF Neu2, as well as other genes: Sry-alpha, CG14915, CG15876, and CG6885. Gene expression responses in *D. sechellia* exposed to rotten noni or L-DOPA treatment yielded 173 genes in common. Of these 173 genes, 149 genes were significantly differentially regulated in the same direction in both treatments. The TF sim was upregulated in both noni and L-DOPA treatments. Interestingly, only 25 of 127 genes differentially expressed in OA treatment (Lanno et al. 2017) were specific to only OA treatment and not to other compounds from noni fruit. The 2 medium chain fatty acids OA and HA shared only 2 DEGs [E(spl)gamma-HLH and AttA] between them that were specific to only fatty acid treatment and not L-DOPA or rotten noni (Supplementary Tables 1–4).

Overlap significance testing was performed to compare the number of significant DEGs found between each treatment with the “GeneOverlap” package in R (Shen 2021). *Drosophila sechellia* had 17,275 annotated genes in its genome and 13,095 genes with annotated *D. melanogaster* orthologs, so 17,275 was used for the genome size measurement. L-DOPA treatment resulted in 743 DEGs, noni treatment yielded 421 DEGs, OA caused 127 DEGs, and HA treatment resulted in 56 DEGs (Fig. 4). L-DOPA and noni treatments had 173 overlapping DEGs between these treatments (Fisher’s exact test, $P = 1.9\times10^{-127}$), noni and OA treatments had 62 overlapping DEGs between these treatments (Fisher’s exact test, $P = 2.9\times10^{-66}$), and noni and HA treatments had 29 overlapping DEGs between these treatments (Fisher’s exact test, $P = 2.6\times10^{-32}$). We compared between previously analyzed treatments: OA and HA, which had 18 overlapping DEGs (Fisher’s exact test, $P = 1.9\times10^{-25}$), OA and L-DOPA treatments had 82 overlapping DEGs (Fisher’s exact test, $P = 4.8\times10^{-28}$). The overlap for every pairwise comparison of DEGs between all 4 treatments was

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**Fig. 3.** GO analysis of DEGs in response to noni fruit. See Supplementary Tables 7 and 8 for complete lists. a and b) Upregulated DEGs analyzed for enriched GO processes. a) Upregulated DEGs are enriched for several cellular components, including nonmembrane-bound organelle, external encapsulating structure, egg chorion, and chromosome. b) Upregulated DEGs are enriched for several biological processes, including sexual reproductive processes, eggshell formation, and cell cycle processes. c and d) Downregulated DEGs analyzed for enriched GO processes. c) Downregulated DEGs are enriched for several cellular components, including smooth septate junction, plasma membrane, nucleus, extracellular region, and cell surface. d) Downregulated DEGs are enriched for several molecular functions, including hydrolase activity and alkaline phosphatase activity.
significant suggesting that there may be common regulatory changes that evolved in *D. sechellia* controlling similar gene expression responses to different aspects of their host food species.

**Comparing predicted TFs among treatments**

Significantly DEGs identified in previous studies that examined OA, L-DOPA, and HA exposure in adult female *D. sechellia* flies (Lanno et al. 2017, 2019; Drum et al. 2022) and were used for i-cisTarget analysis in addition to DEGs we identified here in response to noni and we predicted TFs that control the plasticity of these DEGs. For this analysis, all DEGs, both up- and downregulated were used for all 4 treatments (OA, L-DOPA, HA, and noni). The TF zelda (*zld*) was predicted to regulate the expression of genes in all 4 treatments (Fig. 5). All 5 GATA family of TFs (grn, pnr, GATAd, GATAe, and srp) were predicted to regulate expression in both noni and L-DOPA treatments, and srp was also predicted to regulate expression in HA treatment. sim (*single-minded*) was predicted to regulate expression in noni, OA, and HA treatments. The TFs Relish (*Rel*), Hsf, and Blimp-1 were predicted to regulate expression in both OA and HA treatments. Additionally, GATAd expression was significantly increased in L-DOPA treatment, sim expression was significantly increased in both L-DOPA and noni treatments, and dl expression was significantly increased in L-DOPA treatment (Supplementary Tables 1 and 2).

Predicted regulatory networks for each treatment can be found in Supplementary Fig. 3.

**Discussion**

Examining an organism’s genome-wide gene expression response to different environmental conditions can inform how organism physiology is modified to increase fitness in an environment-specific manner (Coolon et al. 2009; De Nadal et al. 2011). Comparing the physiology that allows *Drosophila sechellia* to feed on toxic *M. citrifolia* fruit to generalist susceptible sister species is an excellent model to study how insects evolve to specialize on toxic resources (Vogel et al. 2014). The chemicals found in noni fruit have driven the specialization of *D. sechellia* to its host, as *D. sechellia* has become resistant to the toxic volatile OA, is attracted to the fruit by toxic volatile HA, and utilizes consumed L-DOPA found in noni fruit to facilitate dopamine biosynthesis (Lavista-Llanos et al. 2014). Previous studies examined the whole organism transcriptional response to these separate components of noni fruit that has driven specialization (Lanno et al. 2017, 2019; Drum et al. 2022), and by comparing these transcriptional responses to the transcriptional response to noni fruit alone, we can better understand which responses are specific and which

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**Fig. 4.** Overlap of significantly DEGs in response to components of noni fruit. DEGs changing expression in response to OA (white), HA (gray), noni (blue), and L-DOPA (red). Overlaps of shared DEGs are shown, as are DEGs specific to each treatment.
are more general in the evolution of D. sechellia noni specialization.

Genes involved in reproductive processes are upregulated in response to noni, L-DOPA, and HA, and genes involved in egg chorion formation are significantly enriched in noni treatment, but significantly downregulated in OA treatment. To better understand how D. sechellia has specialized on ripe noni fruit, which contains OA and HA volatiles, understanding the transcriptional response to all components in noni fruit together is necessary. Drosophila sechellia selectively oviposits on noni fruit and this increase in proteins involved in egg formation and development is consistent with the evolved reproductive traits for this species when it feeds on noni fruit (R’Kha et al. 1997; Lavista-Llanos et al. 2014).

The expression of many rRNAs is significantly downregulated in response to both OA and noni treatment, whereas several rRNAs are upregulated in response to HA treatment (Lanno et al. 2017, Drum et al. 2022). rRNA synthesis is induced by Ras/Erk signaling in Drosophila (Sriskanthadevan-Pirahas et al. 2018), and Myc and Max are predicted to regulate DEG expression in HA and OA treatments, respectively (Fig. 5). Future work examining how rRNA synthesis and protein translation are involved in the specialization of D. sechellia to noni fruit may elucidate a role for these genes in specialization on noni.

Previous studies examining gene expression responses in D. sechellia to the volatile fatty acids in noni fruit have focused on examining all of the DEGs in response to either OA or HA treatment to identify genes involved in evolved toxin resistance (Lanno et al. 2017, 2019; Drum et al. 2022). Interestingly, only 19.7% and 28.9% of DEGs found in these studies are responding specifically to OA or HA treatment, respectively, and only 2 genes are found that respond to both OA and HA treatment but no other treatments. In order to better understand how insects evolve gene expression responses to plant secondary defenses, it is helpful in laboratory studies to not only examine the response to the toxic chemical but to examine the wider context of response that more accurately portrays how this interaction would happen in nature where all compounds are experienced simultaneously, similar to that previously described in other species (Govind et al. 2010; Bansal et al. 2014; Hoang et al. 2015; Crava et al. 2016; De Panis et al. 2016; Birnbaum et al. 2017; Schweizer et al. 2017). The genetic basis of the resistance of D. sechellia to OA is polygenic, with the locus conferring the most resistance to OA residing on chromosome 3R (Hungate et al. 2013). Previous work has shown that the knockdown of Osi6, Osi7, and Osi8 in adulthood, which reside on this locus, drastically decreases survival to OA (Andrade Lopez et al. 2017). Expression of Osi6 and several other Osiris genes are significantly increased in D. sechellia in response to OA (Lanno et al. 2017), and Osi6 is one of the 25 DEGs found only in OA and not in response to any of the other noni components. However, esterase 6 (Est-6) RNAi has been previously shown to alter survival in D. melanogaster exposed to OA and the activity of esterase enzymes has previously been shown to be involved in OA resistance (Lanno et al. 2017, 2019; Lanno and Coolon 2019).

Interestingly, Est-6 expression is significantly enriched in response to L-DOPA and noni treatments, but not upon OA or HA exposure. This result may suggest that gene expression responses to the nontoxic chemicals found in the plant may be

![Fig. 5. Several TFs are predicted to be involved in the response to multiple components of noni fruit. TFs predicted by i-cisTarget analysis to regulate DEG expression in noni, L-DOPA, HA, and OA treatments in D. sechellia are shown. The GATA family of TFs (pnr, grn, GATAe, GATAd, and srp) is predicted to regulate DEG expression in both noni and L-DOPA treatment, with srp also being predicted in HA treatment. Zelda is predicted to regulate expression of DEGs in all 4 treatments. Single-minded is predicted to regulate DEG expression in noni, L-DOPA, and OA treatment. Rel, Hsf, and Blimp-1 are predicted to regulate DEG expression in both OA and HA treatments. Predicted regulatory networks for each treatment are found in Supplementary Fig. 3.](image)
utilized as an indicator of toxin presence and therefore eliciting a gene expression response to help overcome the toxic chemicals that would also be found in the plant. As these genes expression response to each of these chemicals evolved together in response to exposure to all components simultaneously, it may be that responses to one chemical confer trait differences important for life in the presence of different components found in noni fruit.

Most TFs in our data set are expressed lowly and change little but small changes in TF abundance can alter gene expression in response to external stressor or chemical and may be observable by looking at changes in expression of downstream targets of TFs by RNA-seq. In order to predict which TFs are regulating the transcriptional response to noni in D. sechellia, we utilized i-cisTarget to analyze DEGs to find shared TF binding motifs between DEGs. Expression of each predicted TF was analyzed in response to each treatment, and only a handful are significantly differently expressed between control and treatment measured by RNA-seq (Supplementary Tables 1–4).

All 5 members of the GATA family of TFs are predicted to regulate the expression of DEGs in noni and I-DOPA treatment, along with srp being predicted to also regulate DEG expression in HA treatment, but only GATA6 is significantly upregulated in I-DOPA treatment. U-shaped, the Friend of GATA protein that metabolize volatile fatty acids found in noni fruit that are concurrent with its toxic host. Sim, a TF that has been previously shown to be a repressor involved in nervous system development (Thomas et al. 1988; Estes et al. 2001), is significantly upregulated in both noni and I-DOPA treatments (Supplementary Fig. 1). In our network prediction, sim is predicted to regulate genes responding to OA, HA, and noni treatments, making it an excellent candidate for a master regulator that evolved to facilitate D. sechellia host specialization.

Examining the TFs that are predicted to regulate Osiris gene expression may help elucidate how they are regulated in response to OA in D. sechellia. From our network analysis of genes responding to OA exposure in D. sechellia, the TFs Ken and Barbie (Ken) and Blimp-1 are predicted to regulate the expression of Osiris, one of the Osiris genes that was upregulated upon OA exposure in D. sechellia and shown to be involved in resistance to OA toxicity (Andrade Lopez et al. 2017; Lanno et al. 2017). More closely examining possible interaction(s) between these TFs and Osiris genes may shed light on their role in OA resistance.

Separating out genes involved in fruit metabolism compared to genes involved in responding to toxic substances is important for understanding this interaction. Previous work has shown that specialist fruit flies D. sechellia and D. elegans live significantly longer on protein-rich foods than generalist sister species (Watada et al. 2020). Noni fruit has a low amount of sugar and is relatively nutrient poor compared to other fruits (Singh et al. 2012), so understanding how D. sechellia has specialized to use this resource for feeding and breeding and if their transcriptional response plays a role in the metabolism of noni may shed light on how animals alter metabolism to specialize on nutrient poor sources. Examining the potential role of predicted TFs and other DEGs may help us understand the transcriptional response of D. sechellia to noni fruit and shed light on the genetic basis of D. sechellia evolved specialization on its toxic host plant. Using network prediction tools to understand the regulatory environment of gene expression may elucidate how gene regulation is being altered that would be missed in the analysis of DEGs alone, especially when there are hundreds of DEGs to analyze. Using a combination of transcriptome sequencing with methods to predict which TFs are responsible for gene expression responses due to external compounds may elucidate how insects are able to adapt to harsh environments and evolve to specialize on new and frequently toxic host plant species.

**Data availability**

All RNA-seq data generated in this article have been submitted to the NCBI Gene Expression Omnibus under accession number GSE205467.

Supplemental material is available at G3 online.

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**Conflicts of interest**

None declared.

**Literature cited**

Afgan E, Baker D, van den Beek M, Blankenberg D, Bouvier D, et al. The Galaxy platform for accessible, reproducible and collaborative biomedical analyses: 2016 update. Nucleic Acids Res. 2016; 44:W3–W10. doi:10.1093/nar/gkw343.

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol. 1990;215:403–410. doi: 10.1016/S0022-2836(05)80360-2.

Andrews S. FastQC: a quality control tool for high throughput sequence data. 2010. Available at: http://www.bioinformatics.babraham.ac.uk/projects/fastqc.

Bansal R, Mian RAR, Mittapalli R, Michel AP. RNA-Seq reveals a xenobiotic stress response in the soybean aphid, aphis glycines, when fed aphid-resistant soybean. BMC Genomics. 2014;15(1):972. doi: 10.1186/1471-2164-15-972.
Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J R Stat Soc B Methodol. 1995;57(1):289–300.

Birnbaum SSL, Rinker DC, Gerardo NM, Abbot P. Transcriptional profile and differential fitness in a specialist milkweed insect across host plants varying in toxicity. Mol Ecol. 2017;26(23):6742–6761. doi:10.1111/mec.14401.

Carbon S, Douglass E, Good BM, Unni DR, Harris NL, Mungall CJ, Basu S, Chisholm RL, Dodson RJ, Hartline E, et al.; The Gene Ontology Consortium. The Gene Ontology resource: enriching on a Gold mine. Nucleic Acids Res. 2021;49(D1):D325–D334. doi:10.1093/nar/gkaa1113.

Coolon JD, Jones KL, Todd TC, Carr BC, Herman MA. Caenorhabditis elegans genomic response to soil bacteria predicts environment-specific genetic effects on life history traits. PLoS Genet. 2009;5(6):e1000503. doi:1371/journal.pgen.1000503.

Coolon JD, Webb W, Wittkopp PJ. Sex-specific effects of cis-regulatory variants in Drosophila melanogaster. Genetics. 2013;195(4):1419–1422. doi:10.1534/genetics.113.156331.

Crava CM, Bruetting C, Baldwin IT. Transcriptome profiling reveals differential gene expression of detoxification enzymes in a hemimetabolous tobacco pest after feeding on jasmonate-silenced Nicotiana attenuata plants. BMC Genomics. 2016;17(1):1–15. doi:10.1186/s12864-016-3348-0.

David JR, McEvey SF, Solignac M, Tzacasa L. Drosophila communities on Mauritius and the ecological niche of D. mauritiana (Diptera, Drosophilidae). J Afr Zool. 1989;103:107–116.

De Nadal E, Ammerger G, Posas F. Controlling gene expression in response to stress. Nat Rev Genet. 2011;12(12):833–845. doi:10.1038/nrg3055.

De Panis DN, Padró J, Furió-Taró P, Tarazona S, Milla Carmona PS, Soto IM, Dopazo H, Conesa A, Hassan E. Transcriptome modulation during host shift is driven by secondary metabolites in desert Drosophila. Mol Ecol. 2016;25(18):4534–4550. doi:10.1111/mec.13785.

Dekker T, Iba I, Sijou KP, Stensmyr MC, Hansson BS. Olfactory shifts parallel superspecialism for toxic fruit in Drosophila melanogaster sibling, D. sechellia. Curr Biol. 2006;16(1):101–109. doi:10.1016/j.cub.2005.11.075.

Dobson AJ, He X, Blanc E, Bolukbasi E, Feseha Y, Yang M, Piper MDW. Tissue-specific transcriptome profiling of Drosophila reveals roles for GATA transcription factors in longevity by dietary restriction. NPJ Aging Mech Dis. 2018;4(1):5. doi:10.1038/s41514-018-0024-4.

Drum ZA, Lanno SM, Gregory SM, Shimshak SJ, Ahamed M, Barr W, Bekele A, Castro C, Connelly L, DelGaudio N, et al. Genomics analysis of hexanoic acid exposure in Drosophila species. G3 (Bethesda). 2022;12(1):jkb354. doi:10.1093/g3journal/jkb354.

Dworkin J, Jones CD. Genetic changes accompanying the evolution of host specialization in Drosophila sechellia. Genetics. 2009;181(2):721–736. doi:10.1534/genetics.109.089319.

Estes P, Mosher J, Crews ST. Drosophila single-minded represses gene transcription by activating the expression of repressive factors. Dev Biol. 2001;232(1):157–175. doi:10.1006/dbio.2001.0174.

Farine J-P, Legal L, Moreau B, Le Quere J-L. Volatile components of ripe Drosophila Estes P, Mosher J, Crews ST. Drosophila melanogaster. Transcriptional variation associated with cactus host plant adaptation in Drosophila mettleri populations. Mol Ecol. 2015;24(20):5186–5199. doi:10.1111/mec.13388.

Hungate EA, Early RJ, Boussy IA, Turissini DA, Ting C-T, Moran JR, Wu M-L, Wu C-I, Jones CD. A locus in Drosophila sechellia affecting tolerance of a host plant toxin. Genetics. 2013;195(3):1063–1075. doi:10.1534/genetics.113.154773.

Irimiohává H, Hulselmans G, Atak ZK, Potier D, Aerts S. i-cisTarget 2015 update: generalized cis-regulatory enrichment analysis in human, mouse and fly. Nucleic Acids Res. 2015;43(W1):W57–W64. doi:10.1093/nar/gkv395.

Jones CD. The genetic basis of Drosophila sechellia resistance to a host plant toxin. Genetics. 1998;149(4):1899–1908.

Langmead B, Salzberg SL. Fast gapped-read alignment with Bowtie 2. Nat Methods. 2012;9(4):357–359. doi:10.1038/nmeth.1923.

Lanno SM, Coolon JD. Derived esterase activity in Drosophila sechellia contributes to evolved octanoic acid resistance. Insect Mol Biol. 2019;28(6):798–806. doi:10.1111/imb.12587.

Lanno SM, Gregory SM, Shimshak SJ, Alversen MK, Chiu K, Feil AL, Findley MG, Forman TE, Gordon JT, Ho J, et al. Transcriptomic analysis of octanoic acid response in Drosophila sechellia using RNA-sequencing. G3 (Bethesda). 2017;7(12):3867–3873. doi:10.1534/g3.117.300297.

Lanno SM, Lam I, Drum Z, Linde SC, Gregory SM, Shimshak SJ, Becker MV, Brew KE, Budhiraja A, Carter EA, et al. Genomics analysis of L-DOPA exposure in Drosophila sechellia. G3 (Bethesda). 2019;9(12):3973–3980. doi:10.1534/g3.19-1400552.

Lavista-Llanos S, Svatø A, Kai M, Riemensperger T, Birman S, Stensmyr MC, Hansson BS. Dopamine drives Drosophila sechellia adaptation to its toxic host. Elife. 2014;3:1–17. doi:10.7554/eLife.03785.

Legal L, Chappe B, Jallon JM. Molecular basis of Morinda citrifolia (L.): toxicity on drosophila. J Chem Ecol. 1994;20(8):1931–1943.

Lenz J, Liefke R, Funk J, Shoup S, Nist A, Schulz R, Tokusumi Y, Albert L, Raifer H, et al. Ush regulates hemocyte-specific gene expression, fatty acid metabolism and cell cycle progression and cooperates with dNurD to orchestrate hematopoiesis. PLoS Genet. 2021;17(2):e1009318. doi:10.1371/JOURNAL.PGEN.1009318.

Li Q, Sun Z, Shi Q, Wang R, Xu C, Wang H, Song Y, Zeng R. RNA-Seq analyses of midgut and fat body tissues reveal the molecular mechanism underlying Spodoptera litura resistance to tomatine. Front Physiol. 2019;10:8–12. doi:10.3389/fphys.2019.00008.

Liu H, Lin Y, Shen G, Gu J, Ruan Y, Wu J, Zhang Y, Li K, Long W, Jia L, et al. A novel GATA transcription factor GATA4 promotes vitellogenin transcription and egg formation in the silkworm Bombyx mori. Insect Biochem Mol Biol. 2019;107(January):10–18. doi:10.1016/j.ibmb.2019.01.004.

Louis L, David JR. Ecological specialization in the Drosophila melanogaster species subgroup: a case study of D. sechellia. Acta Oecol. Oecol. Gen. 1986;7:215–229.

Matute DR, Ayroles JF. Hybridization occurs between Drosophila simulans and D. sechellia in the Seychelles archipelago. J Ecol Biol. 2014;27(6):1057–1068. doi:10.1111/jeb.12391.

Mi H, Muruganujan A, Ebert D, Huang X, Thomas PD. PANTHER version 14: more genomes, a new PANTHER GO-slim and improvements in enrichment analysis tools. Nucleic Acids Res. 2019;47(D1):D419–D426. doi:10.1093/nar/gky1038.
Okumura T, Matsumoto A, Tanimura T, Murakami R. An endoderm-specific GATA factor gene, dGATAe, is required for the terminal differentiation of the Drosophila endoderm. Dev Biol. 2005;278(2):576–586. doi:10.1016/j.ydbio.2004.11.021.

Peyser RD, Lanno SM, Shimshak SJ, Coolon JD. Analysis of cytochrome P450 contribution to evolved plant toxin resistance in Drosophila sechellia. Insect Mol Biol. 2017;26(6):715–720. doi:10.1111/imn.12329.

R Core Development Team. R: A Language and Environment for Statistical Computing. RDC Team, editor. Vienna (Austria): R Foundation for Statistical Computing; 2020. ISBN:3-900051-07-0. https://www.R-project.org/

R’Kha S, Capy P, David JR. Host-plant specialization in the Drosophila melanogaster species complex: a physiological, behavioral, and genetic analysis. Proc Natl Acad Sci U S A. 1991;88(5):1835–1839. doi:10.1073/pnas.88.5.1835.

R’Kha S, Moreteau B, Coyne JA, David JR. Evolution of a lesser fitness trait: egg production in the specialist Drosophila sechellia. Genet Res. 1997;69(1):17–23. doi:10.1017/S0016672396002546.

Schweizer F, Heidel-Fischer H, Vogel H, Reymond P. Arabidopsis glucosinolates trigger a contrasting transcriptomic response in a generalist and a specialist herbivore. Insect Biochem Mol Biol. 2017;85:21–31. doi:10.1016/j.ibmb.2017.04.004.

Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D, Amin N, Schwikowski B, Ideker T. Cytoscape: a software environment for integrated models. Genome Res. 2003;13(11):2498–2504. doi:10.1101/gr.1239303.metabolite.

Shen L. GeneOverlap: An R Package to Test and Visualize Gene Overlaps. 2021. p. 1–10. http://shenlab-sinai.github.io/shenlab-sinai/ (accessed 2021 Nov 17).

Singh DR, Singh S, Salim KM, Srivastava RC. Estimation of phytochemicals and antioxidant activity of underutilized fruits of Andaman Islands (India). Int J Food Sci Nutr. 2012;63(4):446–452. doi:10.3109/09637486.2011.634788.

Stikanthadevan-Pirahas S, Lee J, Grewal SS. The EGF/Ras pathway controls growth in Drosophila via ribosomal RNA synthesis. Dev Biol. 2018;439(1):19–29. doi:10.1016/j.ydbio.2018.04.006.

Thomas JB, Crews ST, Goodman CS. Molecular genetics of the single-minded locus: a gene involved in the development of the Drosophila nervous system. Cell. 1988;52(1):133–141. doi:10.1016/0092-8674(88)90537-5.

Thurmond J, Goodman JL, Strelets VB, Atrill H, Gramates LS, Marygold SJ, Matthews BB, Millburn G, Antonazzo G, Trovisco V, et al.; FlyBase Consortium. FlyBase 2.0: the next generation. Nucleic Acids Res. 2019;47(D1):D759–D765. doi:10.1093/nar/gky1003.

Trapnell C, Hendrickson DG, Sauvageau M, Goff L, Rinn JL, Pachter L. Differential analysis of gene regulation at transcript resolution with RNA-seq. Nat Biotechnol. 2013;31(1):46–53. doi: 10.1038/nbt.2450.

Tsacas L, Bachli G. Drosophila sechellia, n. sp., huitième espèce du sous-groupe melanogaster des îles Seychelles (Diptera, Drosophilidae). Rev Fr Entomol. 1981;3:146–150.

Valanne S, Wang J-H, Rämet M. The Drosophila toll signaling pathway. J Immunol. 2011;186(2):649–656. doi:10.4049/jimmunol.1002302.

Vogel H, Musser RO, Celorio-Mancera MDLP. Transcriptome responses in herbivorous insects towards host plant and toxin feeding. In: Voelckel C, Jander G. editors. Annual Plant Reviews: Insect-Plant Interactions. Vol. 47. 2014. Hoboken, New Jersey: John Wiley & Sons, Ltd. doi:10.1002/9781118829783.ch6.

Wang LH, Chi YH, Guo FG, Li-Byarlay H, Balfé S, Fang JC, Pittendrigh BR, Zhu-Salzman K. Transcriptomic response of cowpea bruchids to N-acetylglucosamine-specific lectins. Insect Sci. 2015;22(1):83–94. doi:10.1111/1744–7917.12108.

Watanabe M, Hayashi Y, Watanabe K, Mizutani S, Mure A, Hattori Y, Uemura T. Divergence of Drosophila species: longevity and reproduction under different nutrient balances. Genes Cells. 2020;25(9):626–636. doi:10.1111/gtc.12798.

Watanabe K, Kanaoka Y, Mizutani S, Uchiyama H, Yajima S, Watanada M, Uemura T, Hattori Y. Interspecies comparative analyses reveal distinct carbohydrate-responsive systems among Drosophila species. Cell Rep. 2019;28(10):2594–2607.e7. doi:10.1016/j.celrep.2019.08.030.

Yates AD, Achuthan P, Akanni W, Allen J, Allen J, Alvarez-Jarreta J, Amode MR, Armean IM, Azov AG, Bennett R, et al. Ensembl 2020. Nucleic Acids Res. 2020;48(D1):D682–D688. doi:10.1093/nar/gkaz966.