Differentially expressed microRNAs associated with changes of transcript levels in detoxification pathways and DDT-resistance in the *Drosophila melanogaster* strain 91-R

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Abstract

Dichloro-diphenyl-trichloroethane (DDT) resistance among arthropod species is a model for understanding the molecular adaptations in response to insecticide exposures. Previous studies reported that DDT resistance may involve one or multiple detoxification genes, such as cytochrome P450 monooxygenases (P450s), glutathione S-transferases (GSTs), esterases, and ATP binding cassette (ABC) transporters, or changes in the voltage-sensitive sodium channel. However, the possible involvement of microRNAs (miRNAs) in the post-transcriptional regulation of genes associated with DDT resistance in the *Drosophila melanogaster* strain 91-R remains poorly understood. In this study, the majority of the resulting miRNAs discovered in small RNA libraries from 91-R and the susceptible control strain, 91-C, ranged from 16–25 nt, and contained 163 precursors and 256 mature forms of previously-known miRNAs along with 17 putative novel miRNAs. Quantitative analyses predicted the differential expression of ten miRNAs between 91-R and 91-C, and, based on Gene Ontology and pathway analysis, these ten miRNAs putatively target transcripts encoding proteins involved in detoxification mechanisms. RT-qPCR validated an inverse correlation between levels of differentially-expressed miRNAs and their putatively targeted transcripts, which implies a role of these miRNAs in the differential regulation of detoxification pathways in 91-R compared to 91-C. This study provides evidence associating the differential expression of miRNAs in response to multigenerational DDT selection in *Drosophila melanogaster* and provides important clues for understanding the possible roles of miRNAs in mediating insecticide resistance traits.

Introduction

While chemical insecticidal agents have been developed and widely applied to suppress pest arthropod populations in ongoing efforts to enhance the efficiency of agricultural production...
and protect human health [1], this intensive use of chemical insecticides has also led to the development of resistance to one or more classes of insecticides [2–4]. The neurotoxic organochlorine insecticide, dichlorodiphenyltrichloroethane (DDT), has been extensively used to control many agricultural insect pests and insects that vector human diseases, but was banned in most countries in the 1980s due to environmental concerns [5]. The genetic basis of DDT resistance in the *Drosophila melanogaster* (*D. melanogaster*) provides a model system for studying the evolution of insecticide resistance. Indeed, the low-level DDT resistance phenotype in *D. melanogaster* is thought to be associated with a single cytochrome P450, *Cyp6g1* [6,7]. However, moderate to high-level DDT resistance is polygenic [8] involving modulation in DDT penetrance and excretion and multiple differentially-regulated Phase I, II, and III detoxification genes/enzymes, respectively, including P450s, glutathione S transferases (GSTs), and ATP binding cassette (ABC) transporters. Regarding the P450s, members of the *Cyp6* (*Cyp6g1, Cyp6g2, Cyp6a2*, and *Cyp6w1*) and *Cyp12* (*Cyp12d1*) subfamilies have been implicated in polygenic DDT resistance [9–13]. Furthermore, the overexpression and structural changes in membrane-spanning ABC transporters were shown to be involved in DDT efflux and contribute to the overall DDT resistance phenotype in *D. melanogaster* [14]. Full genome re-sequencing identified 13 different regions showing evidence of high nucleotide diversity, directional selection (selective sweeps), and thus putatively associated with the evolution of DDT resistance in the DDT-resistant strain, 91-R, when compared with a susceptible isolate, 91-C [15]. Analyses of this same whole genome re-sequencing data also identified a large panel of fixed amino acid changing mutations between 91-R and 91-C, many of which showed evidence of directional selection [16]. Most recently, a multigenic response to DDT selection was demonstrated within 91-R where transcripts with constitutive differential-expression compared to 91-C were enriched in cell survival, stress response, and neurological functions [17]. Additionally, reduced expression of the putative calcium/lipid binding domain-containing protein from gene model CG10737 is associated with short-term (3–5 hrs) DDT knockdown resistance within the *Drosophila* Genetic Reference Panel, suggesting a role for ameliorated effects of muscle overstimulation in DDT resistance phenotypes [13]. Despite these lines of evidence, the systemic basis and underlying genetic control of polygenic DDT resistance remains enigmatic.

Accumulating evidence demonstrates that the post-transcriptional regulation (RNA editing and alternative splicing) may contribute to the evolution of insecticide resistant phenotypes [18,19]. The miRNAs are a class of endogenous 18–25 nt non-coding small RNAs that, since their discovery in *Caenorhabditis elegans* over two decades ago [20], have been identified across several arthropod species including twelve *Drosophila* species [21]. Biogenesis of miRNAs occurs from transcript-derived stem-loop structures with canonical CNNC downstream elements [22] that are processed into a ~70 bp double-stranded precursor RNAs (pre-RNAs) within the nucleus by the ribonuclease type III enzyme Drosha [23]. After translocation to the cytoplasm, miRNA precursors exhibit canonical stem-loop secondary structures, which usually have two arms called the miRNA-5p arm or -3p arm [24]. The pre-RNAs are further processed into mature 21–22 nt miRNAs by Dicer [25,26]. Following the degradation of the passenger strand [27], the resulting single-stranded guide sequence is loaded into the RNA-induced silencing complex (RISC) [28]. Subsequent base pairing of the miRNA guide “seed sequence” (nucleotides 2–7 at the 5’ end of guide RNAs) [29] with the 3’-untranslated region (3’-UTR) of target cellular mRNA transcripts most often targets the mRNA for degradation by RISC [30], either blocking translation initiation factor binding or inhibiting elongation factor progression that leads to premature termination [31–33]. Alternatively, a miRNA binding of a target transcript can result in translational up-regulation [34] within their mRNA resulting in inhibition of translation or mRNA degradation [35]. Overall, miRNAs are recognized as
potent regulators of eukaryotic gene expression at the post-transcriptional level [36,37] that regulate cell differentiation [38], migration [39], and neuronal development [40]. Approximately 256 *D. melanogaster* miRNA precursors have been identified and deposited in the mirBase (Release 21) [24] and have been shown to be involved in regulating biological processes such as development [41], immune response [42], and metabolism [43].

Recent publications implicate a role of specific miRNAs in the regulation of insecticide resistance mechanisms among insect species. Several comparative analyses have estimated significant differences in miRNA expression between chemical insecticide-resistant and -susceptible strains [44,45]. Additionally, miRNAs differentially expressed between insecticidal *Bacillus thuringiensis* (Bt) toxin-resistant and -susceptible strains of *Ostrinia furnacalis* were predicted to target potential receptor genes [46]. In *Culex pipiens*, miRNAs differentially expressed between deltamethrin-sensitive and -resistant strains were proposed to mediate the expression of putative cytochrome P450 target genes [47]. Specifically, *Culex pipiens* miR-285 and miR-278 were implicated in pyrethroid resistance through the transcriptional regulation of *Cyp6n23* and *Cyp6ag1* [48,49], as well as a miRNA cluster involved in regulation of *Cyp9j35* and *Cyp325bg3* [50] and upregulation of miR-932 that regulates transcript levels of the cuticular gene *CpCPR5* [51,52].

To date, the authors have no knowledge of any investigation of the contributions of post-transcriptional gene expression regulation on DDT resistance phenotypes. In order to partially address this knowledge gap, the present study estimated significant quantitative miRNA differences between the highly DDT-resistant *D. melanogaster* strain 91-R compared to the DDT-susceptible strain 91-C. Additionally, correlation between levels of differentially expressed miRNAs and corresponding putative computationally predicted P450 target transcripts were made within 91-R. This study provides insights into the role of miRNAs for the regulation of metabolic resistance to DDT as well as the effects of multigenerational DDT selection on the evolution of miRNA-mediated post-transcriptional regulation in a polygenic *D. melanogaster* DDT resistance phenotype.

**Materials and methods**

**miRNA library preparation, sequencing, and annotation**

The DDT-resistant 91-R and -susceptible 91-C strains were obtained from Dr. Ranjan Ganguly (University of Tennessee-Knoxville) and developed as described previously [53]. Both strains were reared on a commercially available medium (Jazz-Mix *Drosophila* Food, Fischer Scientific, Cat. No. AS153) under the conditions of 25 ± 1°C, 55–70% relative humidity and a 14 h light/10 h dark cycle. 91-R has been continually selected by maintaining the flies in colony bottles with the presence of a 150 mg DDT impregnated filter paper disk, while 91-C was maintained without any exposure to DDT. Recent topical bioassays estimated that 91-R is ~107-fold more resistant to DDT compared to 91-C [17].

Three biological replicates of one hundred 3–5 day-old virgin female adults were collected from both 91-R and 91-C (*n* = 6). In order to compare the constitutive expression of miRNAs in subsequent analyses (see below), neither population was exposed to DDT within that generation. The small RNAs (sRNAs) were immediately extracted from live collected flies from each replicate using the Qiagen miRNeasy Mini Kit according to the manufacturer’s instructions (Qiagen, Valencia, CA). RNA degradation and contamination were assessed for all samples using an Agilent 2100 Bioanalyzer (Agilent Technologies, Germany), and RNA concentrations were estimated using a NanoDrop One (Thermo, Wilmington, USA). Illumina sRNA libraries were constructed from each pool, and 50-bp single-end (SE50) sequence read data were
generated on an Illumina HiSeq 4000 at the Research Technology Support Facility (Michigan State University, East Lansing, MI).

All raw Illumina sequence data were imported into CLC Genomics Workbench v.9.5 (Qiagen) and all reads were processed to remove low-quality reads, poly A sequences, adapters, reads without 3’ adapter, and sequences shorter than 15 nt. Using the “small RNA analysis” tool in CLC Genomics Workbench, annotation of the trimmed read data from each library (n = 6) was made by comparing against the known miRNA precursors of *D. melanogaster* in the miRBase R.21 (http://www.mirbase.org/; file hairpin.fa); subsequent generation of relative miRNA counts were made within each library. Only tags matching exactly with the mature 5’ or 3’ regions of previously annotated *D. melanogaster* miRNAs (miRBase R.21; file mature.fa) were accepted and retained for the further analysis. The sRNA sequencing data with annotated tags were deposited to NCBI Short Read Archive (SRA) with the accession number SRP136631. Additionally, miRDeep2 v.0.0.8 software was used in order to predict novel miRNA candidates [54]. Illumina adapters of sRNA raw sequence reads were trimmed using cutadapt v.1.15 [55]. The trimmed reads were quality-checked and curated using personal perl script. The results were aligned to the *D. melanogaster* reference genome (Release 6; dmel-all-chromosome-r6.19. fasta.gz at http://flybase.org/) using bowtie v.1.2.2 [56] and also were mapped to *D. melanogaster* non-coding RNA database (dmel-all-trRNA-6.1/8.fasta and dmel-all-miscRNA-6.1/8.fasta file at flybase.org) in order to filter the small conditional RNAs (scRNAs), small nuclear RNAs (snRNAs), small nuclear RNAs (snRNAs) and transfer RNAs (tRNAs). Novel miRNAs were then identified against known miRNA precursors (hairpin.fa) and previously annotated miRNAs (mature.fa) using default parameters suggested by the developers [57]. The structure and minimal free energy (MFE) of all potential novel miRNAs were predicted using a RNAfold [58] with algorithms described previously [59]. The MFE ≤ -25 kcal mol⁻¹, the randomization test of secondary structure MFE, called randfold P-value ≤ 0.05, and miRDeep2 score ≥ 3 were used as the cutoff level to declare them as potential novel miRNAs [60].

**Differential expression of miRNAs**

Estimates of miRNA expression level analysis within each replicate library from 91-R (n = 3) and 91-C (n = 3) were performed using the “Annotate and Merge” command on the CLC Genomics Workbench v.9.5 (Qiagen). This procedure used the *D. melanogaster* miRBase (release 21, http://www.mirbase.org/) as the primary database and the non-coding RNA database (dmel-all-trRNA-6.1/8.fasta and dmel-all-miscRNA-6.1/8.fasta) as the secondary database for annotation. The read counts of miRNAs were first normalized using the tag per million reads (TPM) method: TPM = (number of mapped reads for each miRNA/total number of mapped reads) × 10⁶. Subsequently, the Empirical analysis of Differential Gene Expression (EDGE) algorithm [61] was used to estimate differences in read counts comparing 91-R to 91-C strains, with P-values adjusted for multiple testing calculated using the Benjamini–Hochberg false discovery rate (FDR) procedure [62]. The variance in miRNA levels between the 91-R and 91-C with a log₂ fold-change > 1.0 or < -1.0, and a FDR ≤ 0.05, were defined as significant [63].

**Target prediction and functional annotation of differentially expressed miRNAs**

The potential target transcripts of miRNAs predicted to be differentially expressed between 91-R and 91-C were predicted using three different types of software packages, RNAhybrid [64], DIANA [65], and ComiR [66], using the following criteria: (1) RNAhybrid: the target site prediction was restricted to the 3’-UTR region obtained from the 3’-UTR database of *D. melanogaster*. 
melanogaster (dmel-all-three_prime_UTR-r6.19.fasta at http://www.flybase.org) with MFE \leq -30 kcal mol^{-1}; (2) DIANA: miTG score \geq 0.8; and (3) ComiR: high threshold \geq 0.8. Additionally, target gene ontology (GO) and corresponding pathways for all putative target transcripts were retrieved from the FlyMine database (http://www.flymine.org), and the GO terms were classified with CateGOrizer (https://www.animalgenome.org).

Validation and correlated expression between miRNAs and target transcripts

Reverse transcriptase-quantitative PCR (RT-qPCR) was carried out on selected eight known miRNAs for validation of sRNA sequencing estimated differences between the 91-R and 91-C strains. Moreover, the correlation was assessed between the relative expression levels of eight differentially expressed miRNAs and their potential targeted detoxification genes: Cyp6a8, Cyp6g1, Cyp6w1, Cyp4s3, Cyp6g2, Cyp309a2, Cyp313a4, Cyp313b1, Cyp4ac1, Cyp4d2, Cyp4g15, Cyp4p3, Cyp6d5, Cyp6t3, Cyp6u1, Cyp49a1, Cyp18a1, Cyp303a1, Cyp4aa1, Cyp4e3, Cyp4g1, Cyp6a19, Cyp6a22, Cyp4d20, GstD1, GstS1, GstE6, GstE10, Esterase10, Esterase7, ABC-B7, GABA-R, mAChR-A, nAcRalpha-A, and Cpr65Ec. Three biological replicates of 15 adult female flies were sampled per strain (91-R and 91-C) and—identically with treatments used in Illumina sRNA sequencing—all samples were not exposed to DDT within the generation used. Both miRNAs and total RNAs were extracted from each biological replicate using a miRNeasy Mini Kit and a RNeasy Mini Kit, with resulting extracts treated with DNase I (Qiagen) to remove contaminating genomic DNA. The first-strand cDNA was synthesized from mature miRNA and total RNA respectively with a miScript II RT kit (Qiagen) and a SuperScript™ III Reverse Transcriptase kit (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. RT-qPCR reactions were performed with a miScript SYBR® Green PCR Kit (Biorad, Hercules, CA) according to the manufacturer’s instructions using miRNA-specific forward primers (S1 Table) with miScript II RT kit (Qiagen) products as a template. Analogously, RT-qPCR reactions for corresponding putative target mRNA transcripts were performed using SYBR® Green Master Mix (Biorad) according to the manufacturer’s instructions using target transcript-specific forward and reverse primers (S1 Table), with products from the SuperScript™ III Reverse Transcriptase kit used as a template. All amplification reactions were performed on a StepOnePlus Real-Time PCR system (Applied Biosystems Inc., Foster City, CA), with three technical replicates across all biological replicates. Normalized miRNA and target transcript expression levels were calculated using the 2^{-ΔΔC(t)} method [67] with the internal references U6 snRNA and SS rRNA for miRNA normalization and rp49 for mRNA normalization, respectively. A one-way ANOVA was used to examine the significance of expression differences between the two samples using XLSTAT software (Addinsoft, USA). The correlation coefficient was determined by Pearson’s correlation analysis between the transcript levels of the selected eight miRNAs and their corresponding putative target genes.

Results

miRNA library preparation, sequencing, and annotation

Six miRNA libraries (three biological replicates from each of 91-R and 91-C strains) were constructed, from which sequencing generated 216.2 million total raw reads (\geq 30.1 million per library; Table 1). After trimming reads (i.e., removal of reads without a 3’ adaptor and < 15 nt) 5.1 to 5.8 and 4.4 to 7.7 million reads were retained respectively among triplicates from 91-R and 91-C (Table 1). These were then used in subsequent analyses. Considering trimmed reads
across all six libraries, the majority of the sequences (55.5%) were distributed from 16–25 nt (S1 Fig), which is the standard size of described miRNAs [68]. A class of 26–31 nt long RNA sequences accounted for 25.4% of the total reads (34,598,261) and were classified as suspected piwi-interacting RNAs (piRNAs) [69]. Of the trimmed reads, 531,843 to 992,816 miRNA tags were identified across all replicate libraries of 91-R and 91-C (Table 1), and among these between 6,050 and 9,356 matched D. melanogaster miRNA entries in the miRBase R.21 database.

Annotation of known miRNAs in the 91-R and 91-C strains identified 163 precursor and 256 mature miRNAs following alignment of trimmed reads to the D. melanogaster precursor/mature miRNAs in the miRBase R.21 (S2 Table). None of the precursor or mature miRNAs were expressed uniquely in either strain. However, we failed to identify any of the 93 known D. melanogaster precursors that are recorded in the miRBase R.21 (S3 Table). In both strains, miR-184-3p miRNA was the most abundant among the means of non-normalized reads across triplicate libraries, with miR-8-3p, miR-276a-3p, bantam-3p, and miR-33-5p as the next most abundant miRNAs in both strains (Table 2). Algorithms in the miRDeep2 package identified 17 potential novel miRNAs in 91-R and 91-C, of which 15 were in common between libraries derived from both strains; putatively novel-miR-3L-18860981 and novel-miR-2R-20583765 were uniquely observed in 91-R and 91-C, respectively (S4 Table). The range of estimated MFE among potential novel miRNAs was between -31.7 and -25.5 kcal mol\(^{-1}\) and their mature lengths from 18–25 nt. These 17 novel miRNAs were named based on the chromosome and position on which the miRNA gene is located in the D. melanogaster genome.

### Differential expression analysis and RT-qPCR validation

Comparison of normalized estimates of miRNA quantity (log\(_2\) fold-changes; S5 Table) demonstrated an overall parity across replicated libraries derived from DDT resistance strain, 91-R, as compared to the susceptible control, 91-C (counts pooled across replicates within strain; S2 Fig). Exceptions were found among ten known miRNAs that showed significant levels of differential expression between 91-R and 91-C (log\(_2\) fold-change $\geq$ 1; FDR $\leq$ 0.05). Specifically, nine miRNAs were significantly down-regulated, and only one (miR-986-5p) was significantly up-regulated in 91-R (25.3-fold) when compared with the susceptible control 91-C (Table 3). Interestingly, four out of these nine down-regulated miRNAs [miR-310, miR-311, miR-312 and miR-313] are clustered miRNAs and belong to the miR-310 family. Overall, most of the differentially expressed miRNAs were down-regulated in the DDT resistant strain 91-R, suggesting that those down-regulated miRNAs may be potentially involved in DDT resistance.

#### Table 1. Small RNA sequences from 91-C and 91-R triplicate libraries read data.

| Category | Analyses of total reads data | Data Processing/Strains | Analyses of unique reads |
|----------|-------------------------------|-------------------------|--------------------------|
|          | 91-R-1 | 91-R-2 | 91-R-3 | 91-C-1 | 91-C-2 | 91-C-3 | 91-R-1 | 91-R-2 | 91-R-3 | 91-C-1 | 91-C-2 | 91-C-3 |
| Raw reads | 35,712,203 | 30,108,348 | 33,996,366 | 43,821,999 | 34,205,253 | 38,365,489 |
| Trimmed reads | 5,801,176 | 5,105,542 | 5,439,260 | 7,738,822 | 6,065,134 | 4,448,327 |
| Annotated with ncRNA (rRNA, tRNA, snRNA, snoRNA and others) | 4,399,524 | 4,072,155 | 4,196,227 | 5,961,313 | 4,553,746 | 3,182,916 |
| D. melanogaster miRNAs | 384,620 | 252,554 | 339,001 | 455,995 | 336,210 | 356,146 |
| Unannotated | 1,017,032 | 780,833 | 904,032 | 1,321,514 | 1,175,178 | 909,265 |

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The RT-qPCR validation of the predicted significant quantitative differences in miRNA levels among eight known and four novel miRNAs were amplified showing that the expression levels of miR-986-5p were highly up-regulated in 91-R, whereas miR-286-3p, miR-4919-3p, miR-311-3p, miR-312-3p, miR-995-3p, miR-311-3p, miR-310-3p, and miR-313-3p were down-regulated.

Table 2. The most abundant reads from 91-R and 91-C small RNA libraries corresponding to known Drosophila melanogaster miRNAs in miRBase R.21.

| miRNA      | Read counts<sup>a</sup> 91-R | Read counts<sup>a</sup> 91-C | Mature sequence                        |
|------------|-------------------------------|-------------------------------|----------------------------------------|
| miR-184-3p | 27,954                        | 27,402                        | UGGACGGAGAACUGAUAGGC                   |
| miR-8-3p   | 24,289                        | 25,956                        | UAAUAAUCUGACGGUGAAGAGUUC               |
| miR-276a-3p| 20,174                        | 17,849                        | UAGGAACUCUAUACGGUCCGUCU                |
| bantam-3p  | 7,498                         | 9,036                         | UUGGAGUCAUUUGAAGCGAGUUU                |
| miR-33-5p  | 4,208                         | 5,040                         | UGGCGAUUGAUCGUCCAUUGUC                 |
| miR-10-5p  | 3,464                         | 3,954                         | ACCCGUAAGUCCCCAUIUUGUU                |
| miR-317-3p | 2,908                         | 3,292                         | UGAAACACGCUGUGUUGAUCAGU               |
| miR-14-3p  | 2,603                         | 2,611                         | UCCAGUCUUUUCUCUCUCUCUU                |
| miR-31a-5p | 1,056                         | 2,318                         | UUGAGGACUGACGCGCUAGA                   |
| miR-11-3p  | 1,659                         | 2,275                         | CAUCAACACUGAGUXUUGC                   |
| miR-311-3p | 596                           | 2,170                         | UAIUGCACAIUACGCGCCUGA                 |
| miR-318-3p | 994                           | 1,977                         | UCACUGGCGUUGUUGUAAUUCUA               |
| miR-999-3p | 1,604                         | 1,660                         | UGGCAACGCUGACGGCGUCU                  |
| miR-957-3p | 1,744                         | 1,576                         | UGAAACCGUCCCCAIAACUGAGCC             |
| miR-276b-3p| 1,846                         | 1,553                         | UAGGAACUGUAAACGCGCUCU                 |
| miR-277-3p | 2,046                         | 1,489                         | UAAUGACUACUCUGUACAGACA               |
| miR-995-3p | 542                           | 1,275                         | UAGCACCAACAUUGUACGCUU                |
| miR-305-3p | 1,000                         | 1,235                         | CGGCACAXXUUGAAGACXACUCA              |
| let-7-5p   | 1,094                         | 1,208                         | UGAAGUGGUAUGUGUUGUAGU              |
| miR-986-5p | 3,069                         | 139                           | UCUGCAGAAAGCGUUGUACGGA             |
| miR-958-3p | 1,020                         | 899                           | UGAGAUUCUUCUUACUUCUU                |

<sup>a</sup> Non-normalized reads summed across triplicate libraries

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The RT-qPCR validation of the predicted significant quantitative differences in miRNA levels among eight known and four novel miRNAs were amplified showing that the expression levels of miR-986-5p were highly up-regulated in 91-R, whereas miR-286-3p, miR-4919-3p, miR-311-3p, miR-312-3p, miR-995-3p, miR-286-3p, miR-92a-3p, miR-4919-3p, miR-311-3p, miR-310-3p, and miR-313-3p were down-regulated.

Table 3. Differentially expressed miRNAs between 91-C and 91-R.

| miRNA      | p-value  | FDR<sup>a</sup> | Fold-change | Log₂ fold-change<sup>b</sup> | miRNA sequence                      |
|------------|----------|-----------------|-------------|-----------------------------|-------------------------------------|
| miR-986-5p | 3.0E-53  | 7.8E-51         | 25.3        | 4.66                        | TCTCGAATAGCGTGTTGACTGA               |
| miR-986-5p | 7.0E-04  | 1.3E-02         | -2.01       | -1.01                       | TATCCACAGCACGCTTTTGACTG             |
| miR-986-5p | 1.4E-07  | 5.3E-06         | -2.02       | -1.01                       | TATTGCCACTTGAGACGGCTG                |
| miR-995-3p | 2.8E-09  | 1.4E-07         | -2.17       | -1.12                       | TAGCCACCATGATTCGCTT                  |
| miR-286-3p | 2.1E-03  | 3.2E-02         | -2.29       | -1.19                       | TGACTGACGACAAACATGCTGCT             |
| miR-92a-3p | 2.9E-10  | 1.9E-08         | -2.34       | -1.23                       | CATTGCCACTTGTCCGCGCTAT              |
| miR-4919-3p| 1.4E-04  | 3.2E-03         | -2.57       | -1.36                       | TATCCCTGAAGAAGCATTTGCG              |
| miR-311-3p | 1.8E-15  | 1.5E-13         | -3.38       | -1.76                       | TATGGCAACATTACCGGCGTT               |
| miR-310-3p | 9.1E-16  | 1.2E-13         | -3.94       | -1.98                       | TATGGCAACATTCCGCGCTTT               |
| miR-313-3p | 4.4E-07  | 1.4E-05         | -4.89       | -2.29                       | TATGGCAACCTTTCAACGCGCA              |

<sup>a</sup> FDR: False discovery rate. Differentially expressed miRNAs were identified at the threshold [FDR < 0.05 and log₂(fold change) ≥1.0] of 91-C/91-R.

<sup>b</sup> Fold change was calculated as 91-C/91-R.

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miR-311-3p, miR-312-3p, and miR-313-3p were significantly down-regulated in 91-R (Fig 1). However, levels of miR-92a-3p and miR-310-3p showed no significant difference between strains (P-value > 0.05). Based on Pearson’s correlation coefficient test, the expression patterns of selected miRNAs showed a similar trend between the results of sRNA sequencing and RT-qPCR ($R^2 = 0.971$, $P$-value < 0.01), confirming the predicted differential expression between strains. Additionally, the expression level of one putative novel miRNA (novel-miR-3L-10365243) was significantly down-regulated in 91-R, whereas three other putative novel miRNAs showed no significant differences between two strains (Fig 1).

**Target transcript predictions and correlated expression between miRNAs and target transcripts**

Considering only the ten miRNAs predicted to be differentially expressed between 91-R and 91-C, a total of 46,368 miRNA-target pairs were predicted by the algorithms applied by ComiR ($n = 5,577$), DIANA ($n = 2,548$), and RNAhybrid ($n = 38,243$; S6 Table). Overlap in output occurred among 664 transcript targets predicted by all three algorithms. Functional GO
annotations were received for 603 of these 664 transcript targets, with 64.3%, 20%, and 15.7% that respectively received terms in biological process (BP), molecular function (MF), and cellular component (CC) categories (GO level 2; S7 Table). Specifically, two putative functions (development and metabolism) were highly represented in the BP category, and the MF category showed the function of ‘binding’ and ‘catalytic activity’ was most prevalent among target genes. Moreover, cell, intracellular component, cytosol, cytoplasm, and cytoskeleton were largely overrepresented in CC category (Fig 2A). A total of 2,175 biological pathways were assigned to 258 of the 664 predicted transcript targets (38.9%; Fig 2B; S8 Table), and the target Ras85D (CG9375) is associated with regulation of tissue growth and development represented 93 biological pathways. The remaining 405 transcript targets (61.1%) received no pathway annotations. Among these 2,175 biological pathways, 58 (2.7%), 54 (2.5%), and 53 (2.4%) target genes of the known differentially expressed miRNAs, respectively, were assigned to metabolism, signal transduction, and metabolic pathway (Fig 2B).

Additional annotation focused on a subset of the 664 transcripts putatively targeted by differentially expressed miRNAs; specifically, those transcripts likely to be involved in xenobiotic metabolism. These putative transcripts were predicted to target phase I, II, and III detoxification pathways such as cytochrome P450s, GSTs, esterases, and ABC transporters (43 target transcripts; Fig 3). Additionally, differentially expressed miRNAs were predicted to regulate other genes associated with cuticular proteins, acetylcholine receptors, nicotinic acetylcholine receptors, and glutamate-gated chloride channels (13 target transcripts; Fig 3). The phase I, cytochrome P450 targets Cyp6a8, Cyp6g1, Cyp6g2, and Cyp6w1, were previously associated with DDT resistance as described in the Introduction. Also of note, the down-regulated miR-310-313 cluster in 91-R strain was predicted to share several P450s, sodium channel proteins, and cuticular proteins encoding target genes (Fig 3). Additionally, sixteen P450 genes (MFE -25; Cyp18a1, Cyp305a1, Cyp309a2, Cyp312a1, Cyp313b1, Cyp49a1, Cyp4ae1, Cyp4g1, Cyp4g15, Cyp4p2, Cyp4s3, Cyp6a19, Cyp6g1, Cyp6g2, Cyp6v1, and Cyp9h1) were among the predicted targets of 9 of the 17 putative novel miRNAs (S9 Table).

Moreover, the RT-qPCR-estimated quantities of 26 putatively targeted P450 transcripts showed an inverse relationship with the expression level of the corresponding miRNA(s) predicted to target them in 91-R. For example, the relative expression of three miRNAs (miR-311-3p, miR-312-3p, and miR-313-3p) were significantly down-regulated in 91-R strain (Fig 1), while the corresponding predicted targets (Cyp6a8, Cyp4s3, Cyp4ae1, Cyp6g1, Cyp6g2, Cyp6s3, Cyp6v1, Cyp18a1, Cyp49a1, Cyp303a1, Cyp309a2, Cyp313a1, and Cyp313b1) were significantly up-regulated (Fig 4). Furthermore, other corresponding predicted detoxification targets, Esterase8, Esterase10, GstD1, GstE10, GstS1, GABA-R, mAChR-A, nAcRalpha-A and GluR-IB were significantly up-regulated with the down-regulation of miR-286-3p, miR-2a-3p, miR-311-3p, miR-312-3p, and miR-313-3p in 91-R strain (Fig 4). The resulting coefficient of correlation showed that the strong negative correlation between miRNAs and putative target transcripts (R = -0.9558; P-value < 0.01) suggests the possibility that higher levels of detoxification transcripts may be influenced by the reduced levels of corresponding targeting miRNAs within the DDT resistant 91-R strain. The precise role of these detoxification genes in mediating DDT resistance remains unknown, but may include increasing cellular resistance to oxidative stress, cuticular penetration, or other systemic responses to exposure.

**Discussion**

Resistant phenotypes have evolved within arthropod populations that lead to survival when exposed to various chemical or biological insecticidal toxins and seemingly arise to each successively introduced novel mode of action [70]. Although direct correlations have been made
between single transposon-based mutations at single genetic loci (an *Accord* element associated with *Cyp6g1*) and corresponding insecticide resistance traits [71–73], in many instances the causal mutations and molecular mechanisms involved in resistance are yet to be fully understood. Moreover, definitive linkages between phenotypes and corresponding adaptive mutations remain difficult to define [74], especially in instances where phenotypes arise due to the contribution of multiple genes or gene interactions, or are complexed with variance due to
the environment [75]. For instance, DDT resistance was linked to the up-regulation of Cyp6g1 caused by the upstream integration of an Accord transposon at the DDT-R locus in D. melanogaster populations [71] but shown to be independent of DDT-R in the highly DDT resistant strain 91-R [76]. The prediction of thirteen selective sweeps that became fixed in the genome of 91-R during the course of DDT selection implicated a complex polygenic mode of evolution [15], which likely involves a combination of cis- and trans-regulatory mutations that modulate the function of stress response and neurogenic pathways [17]. Regardless of evidence that strongly implicates miRNAs as potent modulators of gene expression at the post-transcriptional level [77], the role of miRNAs in the evolution of differential gene expression in insecticide resistant phenotypes among arthropods remains suggestive or associative in many cases [44,45].

The current study identified a total of 163 precursors with 256 mature known miRNAs and 17 novel miRNAs. This study failed to identify 93 known precursor miRNAs, previously recorded in the miRBase R.21. The extraction of sRNAs from 3–5 day-old virgin adult female samples may likely have biased the number of miRNA types. Previously, miRNAs were shown to be sex-biased in D. melanogaster, where expression was preferentially associated with the reproductive functions [78]. More recent work that compared miRNA expression between mature male and female reproductive organs in Bactrocera dorsalis demonstrated that sex-biased miRNAs are likely involved in sexual differentiation [79]. Furthermore, the expression of miRNAs varied across developmental stages of Xenopus laevis [80] and across different human tissues [81]. This suggests that our use of virgin female adults might have narrowed the pool of potential miRNAs that could be encountered within the resultant sRNA libraries but is justified since adults are exposed to DDT selection within the 91-R laboratory colony. Another hypothesis may reside in the potential saturation of sRNA samples with 2S ribosomal RNA (rRNA). Specifically, D. melanogaster rRNA is composed of four individual rRNAs, 28S, 18S, 5.8S, and 2S, with the 2S rRNA being 30-nt in length [82]. Analysis of our sRNA read data revealed that 32% of non-miRNA sequences matched the D. melanogaster 2S rRNA, suggesting the possibility that the 2S rRNA component of our libraries might have affected the subsequent read depths and the inability to identify 93 known miRNAs from 91-R and 91-C strains if they
Fig 4. Expression level analysis of potential target detoxification genes of differentially expressed miRNAs between 91-R and 91-C strains. The expression levels were normalized by rp49. Statistical significance was analyzed using one-way ANOVA. The asterisks represent significance, with a single asterisk indicating $P < 0.05$.

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would have been at low copy number. Neither of the above explanations can be ruled out but require additional investigation. The current study nonetheless predicted the significant quantitative difference in ten miRNA levels between 91-R and 91-C at the adult virgin female stage.

Ten differentially expressed miRNAs and their corresponding putative target transcripts were predicted and received GO and pathway database annotations in this study. Among these, escargot regulates D. melanogaster [86], and beta-catenin that in turn regulates cell adhesion and outgrowth [87]. Furthermore, melanogaster D. titative difference in ten miRNA levels between require additional investigation. The current study nonetheless predicted the significant quan

Findings from this study show that the down-regulation of miR-311-3p, miR-312-3p, and miR-313-3p is correlated with the up-regulation of their respective in silico predicted cytochrome P450 target transcripts, Cyp6a8, Cyp4f3, Cyp4ae1, Cyp4g1, Cyp6g2, Cyp6f3, Cyp6v1, Cyp18a1, Cyp49a1, Cyp303a1, Cyp309a2, Cyp313a4, and Cyp313b1 in 91-R. This up-regulation of Cyp6g1 target is predicted to occur via a decreased miR-310-313 expression in 91-R and also corresponds to the cytochrome P450 initially implicated in conferring DDT resistance at the DDT-R locus via integration of the Accord transposon among D. melanogaster populations [71]. Our computational and empirical data suggests that the miR-310 family members may be involved in the posttranscriptional regulation of these cytochrome P450s, which in turn might be directly involved in DDT detoxification or stress response [17]. Thus, the involvement of miR-310-313 could explain prior evidence that even though DDT resistance in 91-R is independent of the Accord insertion [76], significant up-regulation of Cyp6g1, as well as Cyp6a8, Cyp4f3, Cyp4ae1, Cyp6g2, Cyp6f3, Cyp6v1, Cyp18a1, Cyp49a1, Cyp303a1, Cyp309a2, Cyp313a4, and Cyp313b1 occurs constitutively in the strain. Regardless of these strong correlations, additional functional analyses are required to confirm these predicted impacts of miRNA-based posttranscriptional regulation.

The function of the most highly up-regulated miRNA in 91-R strain, miR-986-5p, is currently unknown, but levels in the hemolymph of adult virgin males are known to significantly decrease over time [90]. Interestingly, the miR-986 precursor is located on the second
Differentially expressed microRNAs are associated with DDT detoxification pathways

chromosome within the third intron of the Cyp4e2 gene, which is involved in the metabolism of endogenous and exogenous compounds [91]. Our transcript target site predictions suggest that miR-986-5p could interact with transcripts of cytochrome P450s, GSTs, esterases, and superoxide dismutases (SODs). Specifically, the GSTs and SODs are a group of a multifunctional antioxidant enzymes that play an important role in mediating oxidative stress caused by reactive oxygen species (ROS) in insects[92–94]. Therefore, the miR-986 may be involved in the posttranslational regulation of genes that alleviate oxidative stress induced by DDT insecticide exposures. The putative targeted transcripts of miR-986-5p include Cyp6g1 and Cyp6g2. However, the RT-qPCR performed here, as well as prior RNA-seq results [17], indicate that the expression levels of putative targets Cyp6g1 and Cyp6g2 were not decreased. In contrast, both Cyp6g1 and Cyp6g2 were significantly up-regulated in 91-R, suggesting miR-986-5p may have a transcript stabilizing or enhancing effect, as was demonstrated previously [95,96]. Alternatively, algorithms used for in silico prediction of miRNA-transcript target interactions can produce variable results depending on the input database and models that are applied [97,98]. Regardless, in vivo or in vitro validation of these assumptions will be required.

**Conclusion**

Analyses conducted in this study focused on differentially expressed miRNAs that were predicted to regulate transcript levels of both phase I, II and III detoxification genes previously shown to be associated with the DDT resistance phenotype. Cytochrome P450s that are involved in many cellular processes, including xenobiotic detoxification, have been studied [99] for associations between miRNA levels and corresponding putatively targeted P450 transcripts. For example, a negative relationship was shown between up-regulated miRNAs miR-8534-5p and miR-375-5p and their respective predicted targeted cytochrome P450s, Cyp6b6 and Cyp4g15, in chlorantraniliprole-resistant strains of Plutella xylostella [44]. Several miRNAs down-regulated in deltamethrin-resistant mosquitoes played a role in pyrethroid resistance through the reduced targeting of Cyp325bg3, Cyp6n23, and Cyp9j35 [47,48,50], while the miRNAs (miR-155, miR-216b, miR-499) modulate the abundance of Cyp561d2 transcripts in response to fipronil exposure [100]. Additionally, miR-285 and miR-278 differentially regulate Cyp6n23 and Cyp6ag11 in pyrethroid resistant compared to susceptible Culex pipiens [48,49].

Thus, our implication of down-regulated miR-311-3p, miR-312-3p, and miR-313-3p with the corresponding constitutive up-regulation of in silico predicted targets Cyp6a8, Cyp4e1, Cyp4ae1, Cyp4g2, Cyp6d3, Cyp6v1, Cyp18a1, Cyp49a1, Cyp303a1, Cyp309a2, Cyp313a4 in 91-R may provide yet another example of a miRNA-mediated posttranscriptional modification that contributes to an insecticide resistance trait. Additionally, 4 out of 10 miRNAs predicted to be differentially expressed between 91-R and 91-C (miR-986-5p, miR-995-3p, miR-375-3p, and miR-2a-3p) were also predicted to interact with and impact the transcript level of multidrug resistance-associated protein B7 (ABC-B7). The ABC-B subfamily member, MDR49, had been previously been reported to show significant levels of differential expression between 91-R and the DDT-susceptible strain Canton-S [12] but not between 91-R and 91-C [12,17]. It was subsequently shown that the 91-R-derived 91-R-MDR49B allele provided increased DDT tolerance via transgenic expression in susceptible D. melanogaster, implicating structural, as opposed to dosage, effects on the DDT resistance trait [14]. Thus, one could hypothesize that ABC-B7 might be regulated by one or multiple miRNAs that are differentially expressed between 91-R and 91-C. In addition to further empirical study, however, it should also be kept in mind that multiple different mutational mechanisms can contribute to DDT resistance in 91-R.

In this study, we identified a set of differentially expressed known miRNAs and several novel miRNAs from DDT-resistant 91-R and -susceptible 91-C strains. Since 91-R and 91-C
strains have a common initial origin (genetic background), and 91-R has been selected for survival when exposed to chronic high levels of DDT for over six decades, changes between the strains are speculated to result from either the effects of random genetic drift or from selection. The experiments described here do not allow the differentiation of any impacts from drift versus selection, but the strong correlation between differential expression of miRNAs and their corresponding in silico predicted target transcripts suggests the potential for the involvement of posttranscriptional regulation of several detoxification genes.

Moreover, the experimental procedures do not allow the formulation that down-regulated miRNAs identified in the present study could lead directly to the overexpression of detoxification genes in the 91-R strain, thus requiring further functional experiments in order to elucidate the mechanisms of miRNAs involvement in DDT resistance in 91-R. As such, the overexpressed P450s putatively targeted by differentially-regulated miRNAs in 91-R strain need to be further evaluated to identify the ones directly involved in the DDT resistance mechanism. Due to the polygenic nature of DDT resistance in 91-R (see Introduction), however, this trait may be the result of interactions or additive/non-additive effects from several distinct genetic factors. This study, for the first time, suggests that these might include impacts mediated by miRNA posttranscriptional regulation. Our results provide valuable information for exploring the mechanisms of miRNAs involved in insecticide resistance and for understanding the evolution of post-transcriptional regulation in response to DDT pressure in D. melanogaster.

Supporting information

S1 Fig. Size distribution of small RNAs from the 91-R and 91-C libraries. (TIF)

S2 Fig. Scatter plot of differentially expressed miRNA in the 91-R and 91-C strains. Each plot represents a miRNA. The X- and Y-axis show the normalized read counts of miRNAs in the two strains respectively. (TIF)

S1 Table. The list of RT-qPCR primers. (XLSX)

S2 Table. Known miRNAs identified in this research. (XLSX)

S3 Table. Known miRNA precursors not identified in this research. (XLSX)

S4 Table. Novel miRNAs predicted in this research. (XLSX)

S5 Table. Differentially expressed miRNAs between 91-R and 91-C. (XLSX)

S6 Table. Target prediction of differentially expressed miRNAs between 91-R and 91-C with three algorithms. (XLSX)

S7 Table. Gene ontology analysis of target transcripts from differentially expressed miRNAs between 91-R and 91-C. (XLSX)
S8 Table. The pathway annotation of target transcripts from known differentially expressed miRNAs.
(XLSX)

S9 Table. Target genes for novel miRNAs predicted by RNAhybrid in this research.
(XLSX)

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