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Research

# Variation in Mitochondria-Derived Transcript Levels Associated With DDT Resistance in the *91-R* Strain of *Drosophila melanogaster* (Diptera: Drosophilidae)

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## Abstract

The organochloride insecticide dichlorodiphenyltrichloroethane (DDT) and its metabolites can increase cellular levels of reactive oxygen species (ROS), cause mitochondrial dysfunction, and induce apoptosis. The highly DDT-resistant *Drosophila melanogaster* Meigen 1830 (*Drosophila*) strain, *91-R*, and its susceptible control, *91-C*, were used to investigate functional and structural changes among mitochondrial-derived pathways. Resequencing of mitochondrial genomes (mitogenomes) detected no structural differences between *91-R* and *91-C*, whereas RNA-seq suggested the differential expression of 221 mitochondrial-associated genes. Reverse transcriptase-quantitative PCR validation of 33 candidates confirmed that transcripts for six genes (*Cyp12d1-p*, *Cyp12a4*, *cyt-c-d*, *COX5BL*, *COX7AL*, *CG17140*) were significantly upregulated and two genes (*Dif*, *Rel*) were significantly downregulated in *91-R*. Among the upregulated genes, four genes are duplicated within the reference genome (*cyt-c-d*, *CG17140*, *COX5BL*, and *COX7AL*). The predicted functions of the differentially expressed genes, or known functions of closely related genes, suggest that *91-R* utilizes existing ROS regulation pathways of the mitochondria to combat increased ROS levels from exposure to DDT. This study represents, to our knowledge, the initial investigation of mitochondrial genome sequence variants and functional adaptations in responses to intense DDT selection and provides insights into potential adaptations of ROS management associated with DDT selection in *Drosophila*.

**Key words:** dichlorodiphenyltrichloroethane, resistance, *Drosophila melanogaster*, mitochondria, reactive oxygen species

Mitochondria are double-membrane organelles with a highly permeable outer membrane and an impermeable inner membrane, which house the electron transport chain (ETC) that is responsible for oxidative phosphorylation and ATP generation (Zapico and Ubelaker 2013). Mitochondria function within metabolic, intracellular signaling, apoptotic, and oxidative phosphorylation pathways (Green and Reed 1998, Ryan and Hoogenraad 2007, Zapico and Ubelaker 2013). The highly reduced circular mitochondrial genome (mitogenome) contains 13 protein-coding genes (PCGs; *atp6*, *atp8*, *cox1*, *cox2*, *cox3*, *cytb*, *nad1*, *nad2*, *nad3*, *nad4*, *nad5*, *nad6*, *nad4L*), 22 transfer RNA (tRNA) genes, and 2 ribosomal RNA genes, small subunit rRNA (*rrnS*) and large subunit rRNA (*rrnL*), which use a genetic code unique from that of the nuclear genome (Lewis et al. 1995). According to the endosymbiotic theory, mitochondria are derived from free-living prokaryotes, which once engulfed, have undergone genome size reduction by gene translocation to the eukaryotic nucleus (Leister 2005). This has resulted in the incorporation of nearly 98% of mitochondrial genes into the nuclear genome, with subsequent translation via cytoplasmic ribosomal machinery and importation into the mitochondria. There are over 1,442 nuclear-encoded genes of mitochondrial origin or associated with mitochondrial function (D'Elia et al. 2006, Lotz et al. 2014, Smith and Robinson 2016). The function of several of the mitochondrial proteins is affected by exposures to the organochloride insecticide dichlorodiphenyltrichloroethane (DDT) in mouse (Payne et al. 1960, Chefurka et al. 1980) and also insect species, such as the cockroach [*Periplaneta americana* Linnaeus 1758 (Blattodea: Blattidae); Khan and Cutkomp 1982] and the housefly [*Musca domestica* Linnaeus 1758 (Diptera: Muscidae); Gregg et al. 1964, Chefurka 1983].

Earlier studies concluded that DDT is involved in the disruption of mitochondrial functions, primarily of ATPase, yet results were occasionally contradictory (Payne et al. 1960, Gregg et al. 1964, Byczkowski et al. 1973, Byczkowski 1976, Khan and Cutkomp 1982, Chefurka 1983). Subsequent studies show that DDT inhibits multiple components of the ETC including succinate dehydrogenase (respiratory complex II; Moffett and Yarbrough 1972, Nishihara and Utsumi 1985), ubiquinone-cytochrome *c* oxidoreductase (respiratory complex III; Moreno and Madeira 1991), and ATP synthase (Chefurka 1983, Nishihara and Utsumi 1985, Moreno and Madeira 1991, Donato et al. 1997, Younis et al. 2002, Mota et al. 2011). Thus, DDT and/or its metabolites have been identified to disrupt oxidative phosphorylation (Gregg et al. 1964, Byczkowski et al. 1973, Khan and Cutkomp 1982, Moreno and Madeira 1991). In addition, exposure to DDT resulted in an increased H+ concentration in the mitochondrial intermembrane space (hyperpolarization), which led investigators to suggest the voltage-dependent anion channel (VDAC) as a potential candidate causing the hyperpolarization (van Tonder et al. 2014). Studies also indicated that DDT could induce cell apoptosis, leading to cytochrome *c* upregulation that could cause mitochondrial dysfunction, and in turn caspase activation, ultimately leading to apoptosis (Song et al. 2011, Shi et al. 2013).

Three main mechanisms of DDT resistance have been identified among insects: 1) increased expression of detoxification enzymes (cytochrome P450s, esterases, glutathione-S-transferases), 2) increased direction excretion of DDT, and 3) reduced cuticular penetration (Boyer et al. 2012, Strycharz et al. 2013). Insect cytochrome P450 monooxygenases play key roles in the detoxification of xenobiotic compounds, including chemical insecticides (Ranson et al. 2002, McDonnell et al. 2012), and are subdivided into the CYP2, CYP3, CYP4, and mitochondrial clades (Feyereisen 2006). Mitochondrial P450s genes in vertebrates primarily serve essential physiological functions, including steroid metabolism (Feyereisen 2006). Cytochrome P450-mediated degradation or biosynthetic products can modulate intracellular signaling cascades that influence cell fate (Nebert and Dalton 2006). For instance, cytochrome P450 monooxygenases catalyze reactions that produce harmful reactive oxygen species (ROS), which cause lipid peroxidation and protein oxidation that, respectively, damage membranes and impair enzymatic function (Lewis 2002). ROS also act as secondary messengers that triggers signaling cascades that can either lead to cell death (apoptosis; Redza-Dutordoir and Averill-Bates 2016) or regeneration (Fogarty et al. 2016, Diwanji and Bergmann 2017).

Strains of *Drosophila melanogaster* Meigen 1830, hereafter referred to as *Drosophila*, with varying resistance levels, including the highly DDT-resistant *91-R* strain and its DDT-susceptible counterpart *91-C*, serve as models of studying DDT resistance (Merrell 1959, 1965; Merrell and Underhill 1956). These resources were developed from a field-collected *Drosophila* population of common origin that was split, with *91-R* selected for DDT resistance through increasing DDT exposure >60 yr and *91-C* (not exposed to DDT) serving as a nonresistant control (Merrell 1959, 1965). DDT resistance in *91-R* has been attributed to a combination of mechanisms, including the differential expression of detoxification enzymes including P450s (Pedra et al. 2004) as well as ABC transporters (Strycharz et al. 2013, Seong et al. 2016), a reduction of cuticular penetration, and direct excretion of unmetabolized DDT (Strycharz et al. 2013). More recent studies further support the involvement of a polygenic mechanism of DDT resistance in *91-R*, including whole-genome resequencing that identified allelic fixation in *91-R* compared with variation being maintained within *91-C* (Steele et al. 2014), the identification of 13 genomic regions with reduced nucleotide diversity (selective sweeps) in *91-R* (Steele et al. 2015), and the differential expression of genes involved in stress response, cell survival, and neuronal development and function in *91-R* (Seong et al. 2017). Although DDT resistance within *91-R* has been studied in depth, the impact of DDT selection on mitochondrial structure and function has yet to be investigated between these two *Drosophila* strains. Therefore, the objectives in this study were to characterize any changes in mitochondrial genome structure within *91-R*, as well as the expression of mitochondrial-derived genes including those that are encoded in the nuclear genome. Results from this study provide novel insights into the potential mechanisms of ROS management triggered by DDT and thus identify candidate mitochondrial genes associated with DDT resistance in the *91-R* strain of *Drosophila*.

## Materials and Methods

### Fly Rearing Conditions and Collection

The high levels of DDT resistance in the *Drosophila* strain *91-R* were initially selected over six decades ago, for which a corresponding susceptible control strain, *91-C*, was maintained in parallel (Merrell and Underhill 1956). Both fly strains were provided by Dr. Ranjan Ganguly of the University of Tennessee–Knoxville, but have been reared in the Pittendrigh laboratory for over a decade. Flies have subsequently been maintained at ~25°C and 8:16 (L:D) h while reared in plastic bottles containing Jazz-Mix *Drosophila* Food brown diet (Fisher Scientific, Hanover Park, IL). The *91-R* strain has been continually selected by maintaining the flies in a colony bottle in the presence of a 150-mg DDT/filter paper disk, whereas *91-C* was maintained without any exposure to DDT. The *91-R* strain has been shown to be ~107-fold more resistant to DDT than susceptible *91-C* strain through the use of topical bioassays (Seong et al. 2017).

### Mitogenome Variant Prediction

Paired-end (PE) Illumina HiSeq read data from the whole genomes of *91-C* and *91-R* were previously generated (Steele et al. 2014) and were retrieved from the National Center for Biotechnology Information (NCBI) Short Read Archive database (accession number: SRP041176) previously. The reference *Drosophila* mitochondrial genome (version 6.06) was downloaded from flybase.org (Gramates et al. 2017) and imported into the CLC Genomics Workbench (Qiagen, Valencia, CA). The 'Map Reads to Reference' tool in CLC Genomics Workbench was used wherein trimmed reads for both *91-C* and *91-R* were mapped separately to the mitochondrial genome (parameters: mismatch cost 2, length fraction 0.5, and similarity fraction 0.8; PE read distances set to a minimum 200 bps and maximum of 430 bps). A consensus sequence was exported for both *91-C* and *91-R* and then used within a multiple sequence alignment to the reference *Drosophila* mitochondrial genome sequence (version 6.06) using Clustal Omega (Sievers et al. 2011) with default parameters.

### Estimation of Differential Mitochondrial Gene Expression

Four sets of candidate gene were developed for interrogation within RNA-seq data analyses. A literature search for potential mitochondrial-related genes affected by exposure to DDT and/or its metabolites yielded a list of 33 candidate genes (henceforth referred to as DDT-Lit). A list of nuclear genes associated with the mitochondria and/or DDT exposure affecting the mitochondria (henceforth referred to as Nuclear), containing 1,108 genes, was compiled from the online databases MitoDrome (D'Elia et al. 2006) and MitoMiner

(Smith and Robinson 2016), the Lotz et al. (2014) published list, a list of the ETC genes identified on flybase.org, and DDT-Lit gene set. The set of 13 PCGs and the two ribosomal RNA genes from the mitochondrial genome list (henceforth referred to as Mitogenome) was compiled from the reference *Drosophila* mitochondrial genome (version 6.06). A compilation list of all candidate gene sequences (henceforth referred to as Compiled) contained all 1,145 nonredundant genes across DDT-Lit, Nuclear, and Mitogenome gene sets. All data for these four gene sets were downloaded from flybase.org in fasta format (Supp Table 1 [online only]).

All RNA-seq read data sets (accession numbers: SRX2611754–SRX2611759) were previously generated from *91-C* and *91-R* in triplicate (Seong et al. 2017). Reads from replicate RNS-seq libraries from *91-R* ($n$ = 3) and *91-C* ($n$ = 3) were mapped to candidate genes to estimate the level of expression level. This was performed using the 'RNA-Seq Analysis' command on the CLC Genomics Workbench (Qiagen) using default setting and replicated for different reference sequences from 1) Compiled, 2) Nuclear, 3) DDT-Lit, and 4) Mitogenome gene sets. $Log_2$ transformation and quantile normalization of the data, as well as the Empirical Analysis of Differential Gene Expression (EDGE) statistical analysis (Robinson and Smyth 2007, 2008; Robinson et al. 2010), with a total count filter cutoff of 5.0 and a false discovery rate (FDR) ≤ 0.05 were implemented using CLC Genomic Workbench (Qiagen).

### Reverse Transcriptase-Quantitative PCR Validation

Reverse transcriptase-quantitative PCR (RT-qPCR) validation was carried out on 33 mitochondrial-related genes, which were estimated to be differentially expressed between the *91-R* and *91-C* strains based on the described RNA-seq analyses. Total RNA was extracted from pools of flies (10 males and 10 females) for each *91-C* and *91-R* across three biological replicates. First-strand cDNA synthesis was completed using SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, Grand Island, NY), with either priming by $Oligo(dT)_{20}$ (for nuclear genes) and Random Hexamers (for mitogenome genes) as described by the manufacturer. Primers were designed using the Integrated DNA Technologies (IDT; Coralville, IA) PrimerQuest Tool (Supp Table 2 [online only]). The *rp49* gene was utilized as a reference gene to normalize all cDNA samples (Seong et al. 2018). RT-qPCR was run on a StepOne plus real-time PCR machine (Applied Biosystems, Carlsbad, CA), with parameters Holding Stage-Step 1 95°C 30 s, Cycling Stage-Step 1 95°C 15 s, Step 2 'Primer Temp' 1 min, Melt Curve-Step 1 95°C 15 s, Step 2 60°C 1 min, for a total of 40 cycles. Cycling Stage-Step 2 temperature settings for a specific primer are given in Supp Table 2 (online only). Three biological replicates of cDNA were run, as well as three technical replicates of each cDNA biological replicate. StepOne v2.0 Software was used to calculate the average threshold cycle ($C_T$) for each cDNA sample. Target genes delta CT values were normalized by the *rp49* $C_T$, and two-sample equal variance *t*-tests were done using Microsoft Excel. Fold difference was calculated from the average $2^{-\Delta\Delta CT}$ values (Livak and Schmittgen 2001).

### Functional Annotation of Differentially Regulated Mitochondrial Genes

Gene descriptions and gene symbols were obtained from flybase.org. Gene ontology (GO) annotation, including GO ID numbers, for Molecular Function, Biological Process, and Cellular Component were retrieved from flybase.org (last accessed 18 May 2018) for all differentially expressed genes. The GO enrichment analysis tool (release date 8 September 2017) at geneontology.org (PANTHER Overrepresentation Test, release date 12 May 2017) was used to assess the GO biological process annotations for both 1) the Compiled list of candidate genes and 2) the genes identified via RNA-seq as potentially differentially regulated (with a Fischer's exact with FDR multiple test correct $P$ value of <0.05; Mi et al. 2017). GO annotations for those genes verified as upregulated or downregulated via RT-qPCR were retrieved from flybase.org and the Gene Ontology Consortium (geneontology.org; Ashburner et al. 2000, The Gene Ontology Consortium 2017).

## Results

### Mitogenome Variant Prediction

The mapping of trimmed Illumina reads to the *Drosophila* mitogenome (v 6.06) resulted in the alignment of 1,165,938 (mean length of 86.61 bps) and 1,053,516 reads (mean length of 86.76 bps), respectively, for *91-C* and *91-R*. The subsequent alignment of consensus *91-R* and *91-C* mitogenomes to that of the *Drosophila* mitogenome predicted a total of five changes when the A+T-rich region was excluded. Three of these substitutions were located within gene coding regions (one each in *COX1* at position 2,190, *NAD5* at position 7,454, and the 16S ribosomal RNA at position 13,217; Supp Fig. 2 [online only]). All of the substitutions within PCGs were synonymous (silent: non-amino acid changing). A 2-bp deletion was predicted in the noncoding region upstream of tRNA-Ala in the *91-R* mitochondrial genome (positions 5,982 and 5,983; Supp Fig. 2 [online only]). Putative nucleotide variation was predicted within the A+T-rich region, but confidence in these predictions was not high due to the potential influence of short read misalignments along the highly repetitive sequences within.

### Estimation of Differential Mitochondrial Gene Expression

Box plots for each of the six replicated libraries were constructed and demonstrated the approximately equal distribution of variance for the original expression values, the transformed expression values, and the normalized expression values, and thus were deemed comparable (Suppl Fig. 1 [online only]). After $P$-value correction for multiple testing using EDGE and implementation of an FDR ≤ 0.05 cutoff for determining statistical significance, 175 candidate genes from the Compiled set were predicted to be differentially regulated; 105 and 70 were, respectively, upregulated and downregulated in *91-R* compared with *91-C* (Fig. 1; Supp Table 3 [online only]). These differentially regulated candidate genes belonged to nuclear-encoded mitochondrial protein ($n$ = 170; five of which were also components of the ETC), known DDT-response genes (DDT-Lit; $n$ = 4), and mitochondrial PCGs (Mitogenome; $n$ = 1). For the expression-level analysis of the individual gene lists yielded 197 candidate genes (102 upregulated and 95 downregulated in *91-R*; cutoff FDR ≤ 0.05) for the Nuclear list, 9 candidate genes (4 upregulated and 5 downregulated in *91-R*) of the Mitogenome list, and 8 candidate genes (4 upregulated and 4 downregulated in *91-R*) of the DDT-Lit gene set (Fig. 1; Supp Tables 4–6 [online only]).

Examination of all four lists of genes yielded a total of 221 unique candidate genes identified via RNA-seq analysis as potentially differentially regulated between *91-C* and *91-R*. In addition, there were 32 genes from the Nuclear list, 8 genes from the Mitogenome list, and 4 genes from the DDT-Lit list that were not identified in the compiled genes list analysis and, thus, would have been missed without this analysis methodology. Three of the DDT-Lit list only genes identified were subsequently verified as upregulated (*CG17140*) or downregulated (*Dif*, *Rel*) in *91-R*.

## Quantitative RT-qPCR Validation

In total, 33 unique transcripts (26 nuclear and 9 mitogenome) selected for RT-qPCR validation from among the 221 transcripts predicted to be differentially regulated between *91-C* and *91-R*. In total, six genes showed statistically significant ($P < 0.05$) for upregulation (*Cyp12d1-p*, *COX5BL*, *Cyp12a4*, *cyt-c-d*, *COX7AL*, and *CG17140*) and conversely two for downregulation in *91-R* (*Rel* and *Dif*; Table 1). [Note: *Cyp12d1-p* was initially misidentified as *Cyp12d1-d*, as *Cyp12d1-d* was included on the list from Lotz et al. (2014). McDonnell et al. (2012) had shown the two copies of the *Cyp12d1* gene to be 99.4% identical, making individual gene amplification impossible, and found that both *91-C* and *91-R* contain only the single *Cyp12d1-p* copy of the *Cyp12d1* gene.]

## Functional Annotation of Differentially Regulated Mitochondrial Genes

GO annotation terms molecular function (F), biological process (P), and cellular component (C) were retrieved for each of the resulting four candidate gene list sets after expression-level analysis (Fig. 2). A merged list containing all unique candidate genes across the four gene sets revealed a majority of GO category M to be enzymatic activity, including oxidoreductase activity ($n = 43$), hydrolase activity ($n = 25$), and transferase activity ($n = 17$; Fig. 2a). GO category P had metabolic processes ($n = 52$) as the most prevalent, with cellular respiration ($n = 14$), chromatin organization ($n = 14$), and oxidation-reduction process ($n = 13$) also frequent (Fig. 2b). As expected, given the candidate gene selection, Gene category C showed a high number of genes as components of the mitochondrion ($n = 92$; Fig. 2c).

GO enrichment analysis for 1) the Compiled list of candidate genes and 2) the genes identified via RNA-seq as putatively differentially regulated showed that both are enriched for genes involved in metabolic processes (with a Fischer's exact with FDR multiple test correct $P$ value of <0.05). Specifically, the RNA-seq list had 63 metabolic process subcategories enriched out of the total 168 enriched categories (37.5%). The Compiled list of all candidate genes list had 123 metabolic process subcategories enriched out of a total of 496 enriched categories (24.8%).

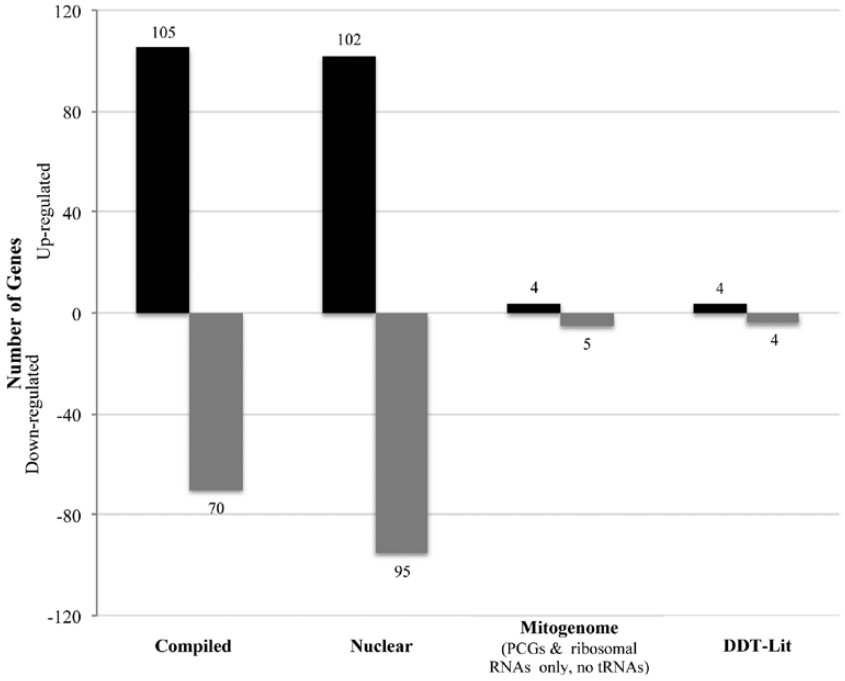


**Fig. 1.** Candidate genes identified via RNA-seq analysis, which was replicated for four different reference sequences from 1) Compiled, 2) Nuclear, 3) DDT-Lit, and 4) Mitogenome gene sets. Black bars represent upregulated genes, and gray bars represent downregulated genes in the *Drosophila melanogaster* DDT-resistant strain *91-R* compared to the DDT-susceptible strain *91-C*.

**Table 1.** Validation of the differentially regulated candidate genes in the DDT-resistant *91-R Drosophila melanogaster* (*Drosophila*) strain using relative RT-qPCR and their corresponding positions within the *Drosophila* genome. Differentially expressed genes were determined via a comparison of the *Drosophila melanogaster* DDT-resistant strain *91-R* and the DDT-susceptible strain *91-C*.

| Gene | Average ΔΔCτ | FC | *P* value | Expressed in *91-R* | Candidate list | Cytological position | Chromosome |
|---|---|---|---|---|---|---|---|
| *Cyp12d1-p* | −1.9652 | 3.9048 | 0.0002 | Over | C; N; L | 47D4 | 2R |
| *COX5BL* | −0.6709 | 1.5920 | 0.0148 | Over | C; N; MD | 26E3 | 2L |
| *Cyp12a4* | −0.7132 | 1.6395 | 0.0128 | Over | C; N; L | 91F3 | 3R |
| *cyt-c-d* | −0.6850 | 1.6078 | 0.0069 | Over | C; DDT-Lit | 36A11 | 2L |
| *COX7AL* | −0.6746 | 1.5961 | 0.0220 | Over | C; N; MD | 84F13 | 3R |
| *CG17140* | −0.4125 | 1.3310 | 0.0330 | Over | DDT-Lit | 32B1 | 2L |
| *Rel* | 0.9409 | 0.5209 | 0.0284 | Under | DDT-Lit | 85C3 | 3R |
| *Dif* | 0.6787 | 0.6247 | 0.0265 | Under | DDT-Lit | 36C7 | 2L |

Fold change (FC) and gene candidate source (C = compiled list of all candidate genes; N = gene list containing all candidate genes encoded in nuclear genome, excluding those encoded in mitochondrial genome; DDT-Lit = DDT literature search genes only) and corresponding references (L = Lotz et al. 2014; MD = MitoDrome [D'Elia et al. 2006]; MM = MitoMiner [Smith and Robinson 2016]) are provided.

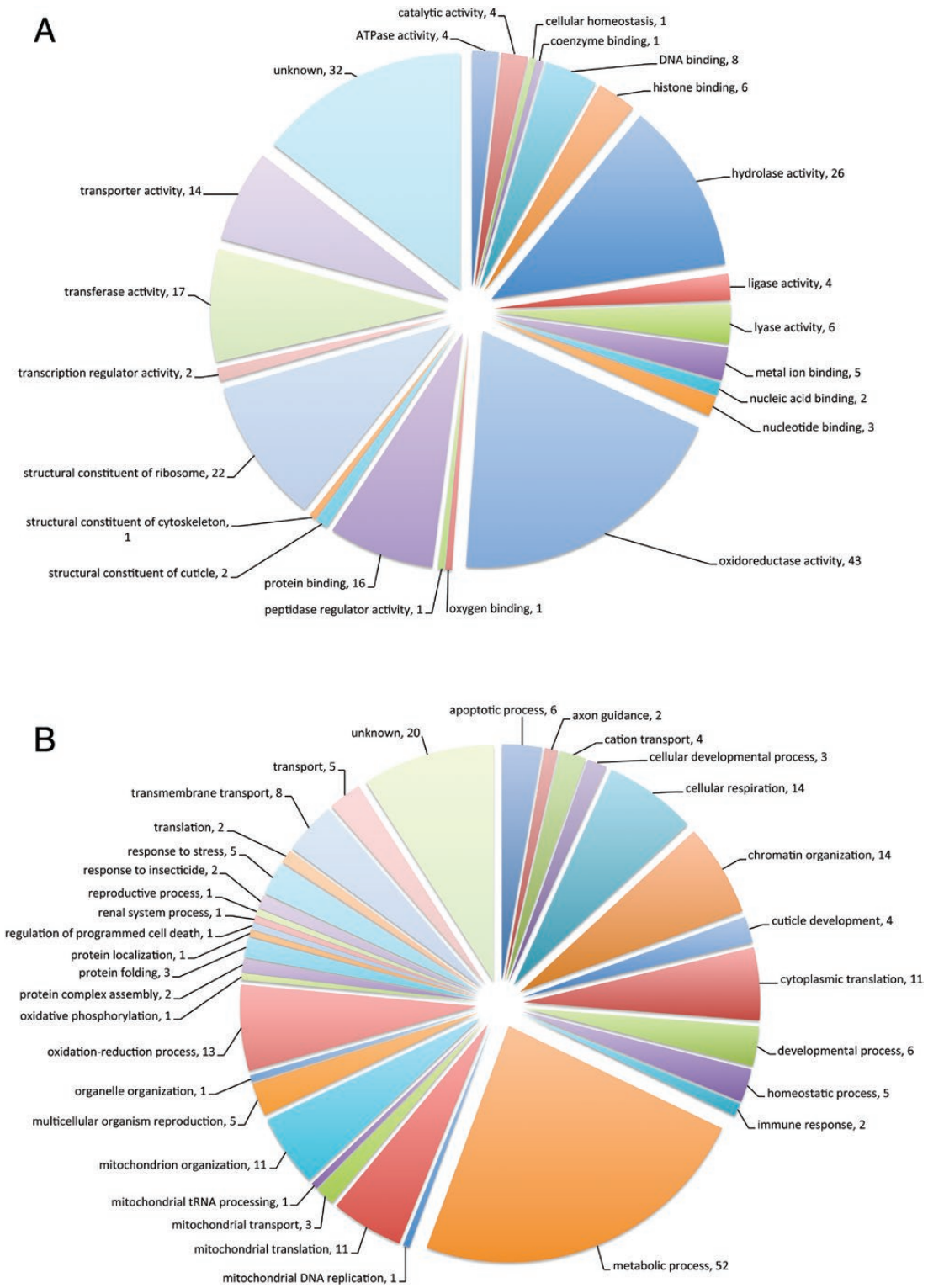


**Fig. 2.** Gene ontology (GO) annotations for a merged list of all 221 unique differentially expressed candidate genes for (a) molecular function, (b) biological process, and (c) cellular component, with the number of genes indicated after each GO annotation term. GO terms annotations obtained from flybase.org, and GO categories defined by the Gene Ontology Consortium (geneontology.org). Differentially expressed genes were determined via a comparison of the *Drosophila melanogaster* DDT-resistant strain *91-R* and the DDT-susceptible strain *91-C*.

Retrieval of gene information from flybase.org indicated that all but *Cyp12d1-p* are located on chromosome 2L or 3R (Table 1). Data from the Gene Ontology Consortium (geneontology.org) and records from flybase.org showed molecular functions of heme binding for three genes (*Cyp12d1-p*, *Cyp12a4*, *cyt-c-d*), cytochrome *c* oxidase activity for two genes (*COX5BL*, *COX7AL*), transcriptional activator activity for two genes (*Rel*, *Dif*), and channel activity for a single gene (*CG17140*; Table 2).

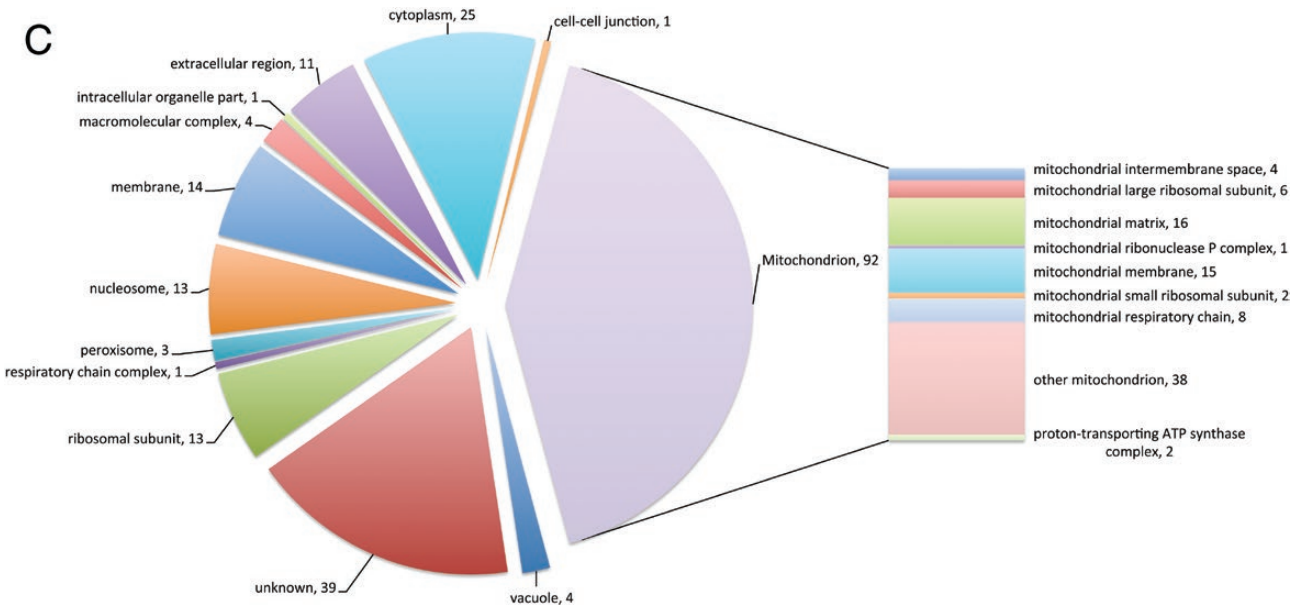


**Fig. 2.** *Continued*

**Table 2.** Gene ontology (GO) of genes identified as differentially expressed via reverse transcriptase-quantitative PCR (RT-qPCR). Differentially expressed genes were determined via a comparison of the *Drosophila melanogaster* DDT-resistant strain *91-R* and the DDT-susceptible strain *91-C*.

| Molecular function[a] | GO code | Gene | Biological process[b] | Cellular component[b] |
|---|---|---|---|---|
| Heme binding | GO:0020037 | *Cyp12d1-p* | Response to insecticide | Mitochondrion |
| | | *Cyp12a4* | Response to insecticide | |
| | | *cyt-c-d* | Oxidative phosphorylation; activity involved in apoptotic process | Mitochondrial inner membrane |
| Cytochrome *c* oxidase activity | GO:0001077 | *COX5BL* | Mitochondrial electron transport, cytochrome *c* to oxygen; oxidative phosphorylation | Mitochondrial respiratory chain complex IV; mitochondrial inner membrane; cytochrome complex |
| | | *COX7AL* | Mitochondrial electron transport, cytochrome *c* to oxygen; oxidative phosphorylation | Mitochondrial respiratory chain complex IV; mitochondrial inner membrane; cytochrome complex; |
| Transcriptional activator activity | GO:0001077 | *Rel* | Innate immune response; regulation of defense response to fungus; defense response to Gram-negative bacterium; peripheral nervous system neuron development and differentiation | Cytoplasm; nucleus |
| | | *Dif* | Innate immune response; regulation of defense response to fungus; defense response to Gram-negative bacterium; peripheral nervous system neuron development and differentiation | Cytoplasm; nucleus |
| Channel activity[c] | | *CG17140* | Transmembrane transport | Mitochondrial outer membrane |

[a]Geneontology.org.
[b]Flybase.org Gene Ontology records.
[c]Inferred from direct assay (Komarov et al. 2004).

## Discussion

The metabolism of DDT and other xenobiotics has been implicated as a source of oxidative stress and mitochondrial dysfunction (Hassoun et al. 1993, Barros et al. 1994, Abdollahi et al. 2004, Pérez-Maldonado et al. 2005). This study utilized the DDT-resistant strain *91-R* and its susceptible counterpart *91-C* to predict the impact of multigenerational DDT selection on the mitogenome and nuclear-encoded genes involved in mitochondrial pathways. In this study, no amino acid changes were predicted between the mitogenomes of *91-C* and *91-R*. In contrast, our results predicted the differential expression of 221 mitochondrial-associated genes between *91-R* and *91-C*, which agrees with previous studies implicating mitochondrial gene expression differences or inhibition of components of the ETC in response to DDT exposures (Byczkowski et al. 1973, Byczkowski 1976, Song et al. 2008, Jin et al. 2014).

The present study focused on the constitutive changes in expression of transcripts encoding proteins within mitochondrial pathways, and our results might suggest adaptions through the upregulation of mitochondrial components involved in ROS management. Oxidative stress is primarily a result of the accumulation of ROS from high rates of mitochondrial oxidative phosphorylation by the ETC (Velayutham et al. 2011) and detoxification of xenobiotics

by uncoupling of electron transfer during reduction reactions carried out by cytochrome P450 monooxygenases (Bast 1986, Bondy and Naderi 1994, Strolin-Benedetti et al. 1999, Zangar et al. 2004), which leads to damage of cellular constituents and components including membrane lipids, enzymes and nucleic acids (Kannan and Jain 2000, Stark 2005). The endoplasmic reticulum (ER) and mitochondrion are involved in the management of cellular stress responses and regulation of apoptosis during conditions when normal antioxidant capabilities are overwhelmed (Xu et al. 2005, Manoli et al. 2007). DDT exposure has previously been shown to increase oxidative stress (Hassoun et al. 1993, Barros et al. 1994), suggesting a possible role of modified mitochondrial or ER stress response as mechanism in adaptation. Furthermore, the significant enrichment of mitochondrial genes with metabolic process GO annotations could suggest a linkage with DDT resistance. More likely explanation may lie in the bias incurred via use of restricted candidate gene lists, and the resulting significant enrichment could be artifactual.

### Transcripts Upregulated in *91-R*

Out of 33 genes tested by real-time PCR for differential transcript level, eight were validated as differentially expressed, with four from the Nuclear set (*Cyp12d1-p*, *COX5BL*, *Cyp12a4*, *COX7ALI*) and four from the DDT-Lit set (*cyt-c-d*, *CG17140*, *Rel*, *Dif*), with their cytological positions distributed across both the second and third chromosomes (Table 1). These results corroborate with prior studies that determined the cytological positions of genes associated with DDT resistance in *Drosophila* (Dapkus and Merrell 1977, Shepanski et al. 1977, Dapkus 1992). In addition, previous studies identified a region on the second chromosome between the genes *cinnabar* (*cn*) and *vestigial* (*vg*), known as *Rst(2)DDT*, as being implicated in DDT resistance in some strains of *Drosophila* (Hällström 1985, Hällström and Blanck 1985, Daborn et al. 2002, Brandt et al. 2002).

The differentially expressed *Cyp12d1-p* is positioned within the *Rst(2)DDT* locus, and *Cyp12d1-p* and *Cyp12a4* were upregulated in *91-R* compared with *91-C* within previous studies (Pedra et al. 2004, Festucci-Buselli et al. 2005, Seong et al. 2018), suggesting P450s may be involved in genetic responses to DDT. Specifically, DDT exposure was shown to induce *Cyp12d1* in the DDT-resistant field-selected fly strains Wisconsin and Hikone-R, as well as the laboratory-selected *91-R* strain (Festucci-Buselli et al. 2005). Moreover, upregulation of *Cyp12a4* was previously documented in *Drosophila* as a mechanism of resistance to lufenuron, an insect growth regulator that disrupts chitin synthesis and is typically used as a control method for the cat flea, *Ctenocephalides felis* Bouché 1835 (Siphonaptera: Pulicidae) (Bogwitz et al. 2005). Interestingly, a mutation in the *Cyp12d1-p* gene of *91-R* was predicted to produce a nonfunctional protein, which was not present in *91-C* (McDonnell et al. 2012, Seong et al. 2018) that could potentially aid in lowering ROS accumulation in the mitochondria. Alternatively, because some P450s are implicated in cuticle formation, possibly through their regulation of ecdysone levels (Sztal et al. 2012), this mutation could modify cuticular hydrocarbon composition that results in decreased insecticide absorption (Balabanidou et al. 2016). Undoubtedly, additional investigation is required to determine functions of these cytochrome P450s in the DDT resistance mechanism.

*Drosophila* contains two distinct cytochrome *c* genes, cytochrome *c* distal (*cyt-c-d*) and cytochrome *c* proximal (*cyt-c-p*; Limbach and Wu 1985, McClelland et al. 2014). Annotations indicate that *cyt-c-p* functions directly in mitochondrial respiration, whereas the upregulated *cyt-c-d* in *91-R* is involved with apoptosis via caspase activation as well as sperm differentiation and does not regulate cellular respiration (Arama et al. 2006, Mendes et al. 2006). *cyt-c* has also been implicated in caspase activation, apoptosis, and antioxidant and peroxidase activity in mammalian systems (Korshunov et al. 1999, Kagan et al. 2004, Velayutham et al. 2015).

The gene *cyt-c* was previously shown to initiate DDT-induced apoptosis and the concomitant upregulation of cytochrome *c* (Song et al. 2011, Shi et al. 2013). DDT and its metabolites, DDE and DDD, have been implicated in ROS production accompanied by apoptosis in human blood mononuclear cells (Pérez-Maldonado et al. 2005). Exposure to DDT can induce other indicators of oxidative stress including lipid peroxidation (Barros et al. 1994) and DNA single-strand breaks (Hassoun et al. 1993), suggesting that cytochrome *c* release into the cytosol, and subsequent triggering of apoptosis, probably occurs following DDT exposure. Regardless, the constitutive upregulation of the apoptosis-inducing *cyt-c-d* in *91-R* remains a quandary. It could be hypothesized that an increase in levels of apoptosis-inducing *cyt-c-d* in *91-R* could be advantageous as the death of apoptosis-sensitive cell types can lead to the activation of intercellular signals that promote repair and growth among the surrounding cells (Vriz et al. 2014). Alternatively, *cyt-c* in mammalian mitochondria has some antioxidative properties via regeneration of $O_2$ from superoxide, resulting in regeneration of the oxidized *cyt-c* within the ETC (Korshunov et al. 1999, Pereverzev et al. 2003, Arama et al. 2006). The upregulation of *cyp-c-d* in this study might suggest that *91-R* is utilizing this preexisting ROS elimination/control system in mitochondria as a method to manage DDT-induced increases in ROS levels. Regardless, the role of *cyt-c-d* in mediating DDT resistance in *91-R* will require further studies into the impact of *cyt-c-d* upregulation on DDT resistance phenotypes.

Cytochrome *c* oxidase activity is suppressed among susceptible insects when exposed to chemical insecticides (Price 1980), including *Drosophila* (Song and Scharf 2009). Two cytochrome *c* oxidase-like genes, *COX5BL* and *COX7AL*, are upregulated in DDT-resistant *91-R* and are thought to be gene duplications of *COX5B* (*CG11015*) and *COX7A* (*CG9603*), respectively (Tripoli et al. 2005). Interestingly, *COX5BL* and *COX7AL*, along with almost all other duplicated genes in the oxidative phosphorylation pathway in *Drosophila*, have testis biased in expression (Parisi et al. 2004, Tripoli et al. 2005). The observed upregulation of *COX5BL* and *COX7AL* in the present study may corroborate earlier studies that demonstrated higher cytochrome *c* oxidase activity in DDT-resistant strains of the housefly, *M. domestica* (Sactor 1950, Perry and Sactor 1954, Sacktor 1975). Specifically, Sactor (1950) found that both male and female houseflies from a DDT-resistant strain had higher cytochrome *c* oxidase activity compared with a susceptible strain. More recently, transcripts for *COXI* were shown to be upregulated in a pyrethroid-resistant German cockroach, *Blattella germanica* Linnaeus 1767 (Blattodae: Ectobiidae) (Pridgeon and Liu 2003), and analogously, *COX3* was significantly upregulated in pyrethroid-resistant strains of the mosquito, *Aedes aegypti* Linnaeus 1762 (Diptera: Culicidae) (Pridgeon et al. 2009).

The role of increased *COX5BL* and *COX7AL* transcript levels in facilitating DDT resistance remains unknown, but could be related to cellular resistance to stress. Specifically, it has been demonstrated that cells with chemically inhibited cytochrome *c* oxidase activity are more susceptible to oxidative stress-induced apoptosis (Schüll et al. 2015). These lines of evidence suggest that the putative increased activity may be compensatory for DDT-induced inhibition of cytochrome *c* oxidases or be involved in increased cellular resistance to apoptosis.

The gene *CG17140* (previously identified as *CG31722*, *CG31722*-B, and *CG17139* in the literature) was observed to be upregulated in *91-R* (Table 1), which encodes one of four VDAC

isoform genes identified in *Drosophila*, along with *porin* (*CG6647*), *porin2* (*CG17137*), and CG17139 (Park et al. 2010). The VDAC is a small pore-forming integral membrane protein located within the mitochondrial outer membrane that mediates movement of metabolites and small ions between the cytosol and mitochondrial intermembrane space (Oliva et al. 2002, Craigen and Graham 2008, Graham et al. 2010). *Drosophila* VDAC isoforms show different spatiotemporal expression patterns, as well as function, with *CG17140* exhibiting voltage-dependent anion selectivity typical of other VDACs but comparatively having only 40% conductance to normal VDACs and atypical voltage dependency (Komarov et al. 2004, Graham and Craigen 2005, Craigen and Graham 2008).

Relatively little is known about the function of *CG17140* apart from a suggested role in the male reproductive tract due to its expression isolation to that specific tissue (Graham and Craigen 2005). Although not *CG17140* specific, there is some evidence that VDACs play a potential role in mitochondrial-mediated apoptosis by the release of cytochrome *c* (Shimizu et al. 1999, Harris and Thompson 2000, Cheng et al. 2003). Studies suggest that the interaction of Bcl-2 family proteins, particularly proapoptotic molecules Bak or Bax, with VDAC can induce a conformational change in VDAC that permits cytochrome *c* release across the outer mitochondrial membrane (Shimizu et al. 1999, Harris and Thompson 2000). In addition, the interaction of VDAC2, the mammalian VDAC isoform, with Bak forms an inactive form of Bak, inhibiting Bak-initiated mitochondrial apoptosis by blocking the release of cytochrome *c* (Harris and Thompson 2000).

Dorsal-related immunity factor (*Dif*) and Relish (*Rel*) are involved in the *Drosophila* innate immune response and are downregulated in *91-R* (Table 1). Both *Dif* and *Rel* are nuclear factor-kappa B (NF-κB) transcription factors that regulate expression of a large suite of innate immune-response genes (Goto et al. 2014). Interestingly, signaling pathways in immunity and apoptosis are intertwined (Ekert and Vaux 1997), with the crux of this association laying in immune response functions that determine survival or death of infected cells, wherein NF-κB plays a key role in both prosurvival and proapoptotic pathways (Gupta 2002, Karin and Lin 2002).

*Dif* plays a role in immune response to gram-positive bacteria on activation of the Toll pathway, and defects in this pathway are observed only when *Dif* is knocked out in conjunction with another NF-κB transcription factor, Dorsal (*dl*). Moreover, *Dif* and *dl* were shown to promote hemocyte cell survival in *Drosophila* (Matova and Anderson 2006). This prosurvival function is mediated via the *Dif* and *dl* stimulation of the *Drosophila* inhibitor of apoptosis 1 (*diap1*) gene (Matova and Anderson 2010), and repression of this NF-κB/*diap*1 pathways is required for progression of apoptosis (Tavignot et al. 2017). *Rel* functions in the response to gram-negative bacteria as part of the immune deficiency (IMD) pathway and controls the expression of antimicrobial peptides (Buchon et al. 2014), but also is involved in the cell type-dependent promotion or inhibition of apoptosis (Barkett and Gilmore 1999). *Rel* is proteolytically activated by proapoptotic *Drosophila* caspase, *Dredd* (Stöven et al. 2003) and promotes apoptosis in neuronal cells (Chinchore et al. 2012). Within the extrinsic apoptosis pathway the Fas ligand (FasL/CD95L) stimulates the cell surface death receptor Fas (CD95; Strasser et al. 2009), which subsequently recruits the Fas-associated death domain adapter protein (FADD) followed by caspase-8 to form the apoptome (Wang et al. 2010). Analogous to the immune response, NF-κB transcription factors are involved in the regulation of Fas and thus the progression of apoptosis (Liu et al. 2012, Jin et al. 2014).

Given that apoptosis induction has been implicated in *Drosophila* cellular responses following DDT exposure (Song et al. 2011, Shi et al. 2013), and the above evidence regarding the cellular control of apoptosis, the constitutive downregulation of *Dif* and *Rel* in *91-R* could be hypothesized to either suppress or induce the progression of cell death. Specifically, reduced levels of the transcription factor *Dif* may lead to reduced expression of *diap*1, which would in turn lead to a reduced capacity to suppress caspase function and leave cells with a greater susceptibility to enter into apoptosis. In contrast, reduced levels of *Rel*, and its translated product REL, could potentially lead to a suppression of the apoptotic signal. Jin et al. (2014) observed that DDT exposure leads to the elevation of NF-κB signaling via the mammalian ortholog p65 protein in HL-7702 cells, suggesting that repression of expression in NF-κB transcription factors may concomitantly repress downstream immune and/or apoptotic pathways. However, ROS signaling affects many cellular pathways, notably the mammalian immune response NF-κB pathway, and in *Drosophila* the Toll pathway and the IMD pathway (Finkel 1998, Buchon et al. 2014).

Indeed, mitochondrial ROS were shown in both mice cells and human cells to activate NF-κB transcription factors (Chandel et al. 2000, Nemoto et al. 2000, Sena and Chandel 2012), suggesting that the constitutively decreased expression of *Dif* and *Rel* in *91-R* could potentially be adaptive by priming cells for xenobiotic exposures, even in the absence of ROS. In addition, organochlorine insecticides, such as DDT, cause immune dysregulation (reviewed by Corsini et al. 2013), and DDT and DDE are immunosuppressive in rats, mice, and humans (Gabliks et al. 1975; Banerjee 1987a,b; Rehana and Rao 1992; Banerjee et al. 1997; Nuñez et al. 2002; Cooper et al. 2004). A state of readiness was predicted for cells with increased expression of transcripts involved in immune response among larvae resistant to *Bacillus thuringiensis* toxins (Dubovskiy et al. 2016), suggesting that the upregulation of immune response- or apoptosis-related genes may be adaptive with respect to survival when exposed to xenobiotics. Regardless of the enticing hypothesis that *Dif* and *Rel* may affect the expression of transcripts in immune-response pathways, additional investigation is required to elucidate the impact of these NF-κB transcription factors on apoptotic resistance among DDT exposed *Drosophila*.

## Conclusions

*Drosophila* strains *91-R* and *91-C* provide unique opportunities to examine potential long-term effects of intensive DDT selection on mitochondrial-encoded genes. The findings presented here suggest that constant exposure to DDT in *Drosophila* did not generate amino acid changes in the PCGs of the mitogenome. However, changes in expression level of genes involved in oxidative stress regulation and intracellular defects associated with mitochondrial dysfunction. Our results suggest *91-R* may have been selected for adaptations that combat increased ROS in the mitochondria following DDT exposure by 1) constitutively upregulating genes that remediate ROS created during oxidative phosphorylation in the mitochondria and 2) in instances when ROS cannot be lowered to normal physiological levels, affecting the initiation of apoptosis or regenerative cascades. Although the present study requires further investigations to determine the functional roles of the genes discussed, it provides a solid foundation of baseline information regarding the involvement of mitochondrial gene pathways in the evolution of the DDT resistance trait of *91-R*.

## Supplementary Data

Supplementary data are available at *Journal of Insect Science* online.

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