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Polygenic Resistance In The Highly DDT-resistant 91-r Strain Of Drosophila Melanogaster Involves Decreased Penetration, Increased Metabolism And Direct Excretion Of Ddt

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POLYGENIC RESISTANCE IN THE HIGHLY DDT-RESISTANT 91-R STRAIN OF *DROSOPHILA MELANOGASTER* INVOLVES DECREASED PENETRATION, INCREASED METABOLISM AND DIRECT EXCRETION OF DDT

A Thesis Presented

by

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DEDICATION

To my family for their support during my graduate education.

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ABSTRACT

POLYGENIC RESISTANCE IN THE HIGHLY DDT-RESISTANT 91-R STRAIN OF *DROSOPHILA MELANOGASTER* INVOLVES DECREASED PENETRATION, INCREASED METABOLISM AND DIRECT EXCRETION OF DDT

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Resistance to dichlorodiphenyltrichloroethane (DDT) in the *91-R* strain of *Drosophila melanogaster* is extremely high compared to the susceptible *Canton-S* strain (>1500 times). Oxidative detoxification is involved in resistance but is not the only mechanism. Rates of DDT penetration, metabolism, and excretion were determined radiometrically between resistant *91-R* and susceptible *Canton-S* strains. Contact penetration was ~1.5-times slower with *91-R* flies compared to *Canton-S* flies. The *91-R* strain had 13-fold more cuticular hydrocarbons, possibly resulting in penetration differences. DDT was metabolized ~33-fold more extensively by *91-R* than *Canton-S* resulting in dichlorodiphenyldichloroethane (DDD), two unidentified metabolites and polar conjugates being formed in significantly greater amounts. *91-R* also excreted ~5.0 times more DDT and metabolites than *Canton-S*. Verapamil pretreatment reduced the LD_{50} value for *91-R* flies topically dosed with DDT by a factor of 10-fold. Thus, it is likely that the increased excretion by *91-R* flies is due to the increased expression of ATP-binding cassette (ABC) transporter genes, including *MDR50* (CG8525) that had a

36% higher transcript level by quantitative real time PCR than *Canton-S* flies. In summary, DDT resistance in *91-R* is polyfactorial and includes reduced penetration, increased detoxification and direct excretion.

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CHAPTER 1

INTRODUCTION

1.1 Pathways for DDT metabolism in animals

In vitro metabolism of p,p-dichlorodiphenyltrichloroethane (p,p-DDT) using rat liver microsomes results in the production of the dechlorinated metabolite, p,p-dichlorodiphenyldichloroethane (p,p-DDD), and the dehydrochlorinated metabolite, p, p-dichlorodiphenyldichloroethylene (p,p-DDE), under reducing conditions (Kitamura et al., 2002). The reductive dechlorination of p,p-DDT to p,p-DDD was induced by phenobarbitol and dexamethasone and was catalyzed enzymatically by cytochrome P450 monooxygenase proteins (P450) (Kitamura et al., 2002). The production of p,p-DDD also occurs non-enzymatically in the presence of reduced flavin and using the heme group of P450 as a cofactor (Kitamura et al., 2002) (Fig. 1). Following reductive dechlorination, the metabolism of p,p-DDD proceeds via a hydroxylated intermediate called α-hydroxyl-DDD (Fox et al., 1998). The α-hydroxyl-DDD metabolite can undergo subsequent enzymatic reactions to form dichlorodiphenyl acetic acid (DDA), which is the major excretable metabolite of DDT (Gold and Brunk, 1982) (Fig. 2). It has also been suggested that DDE is formed via DDD by rat liver microsomes but it has not been determined if insects form DDE via DDD or only through DDT-dehydrochlorinase (DDTase) (Kitamura et al., 2002; You, 2000; Matsumura, 1985).

The genetics of xenobiotic metabolism in *Drosophila melanogaster* has been extensively studied for the expression of P450 and glutathione S-transferase (GST) genes. P450 genes involved in insecticide resistance in *D. melanogaster* include *Cyp6a2*

Figure 1. Metabolic pathway for the detoxification of DDT to its primary metabolites (Kitmura et al., 2002).

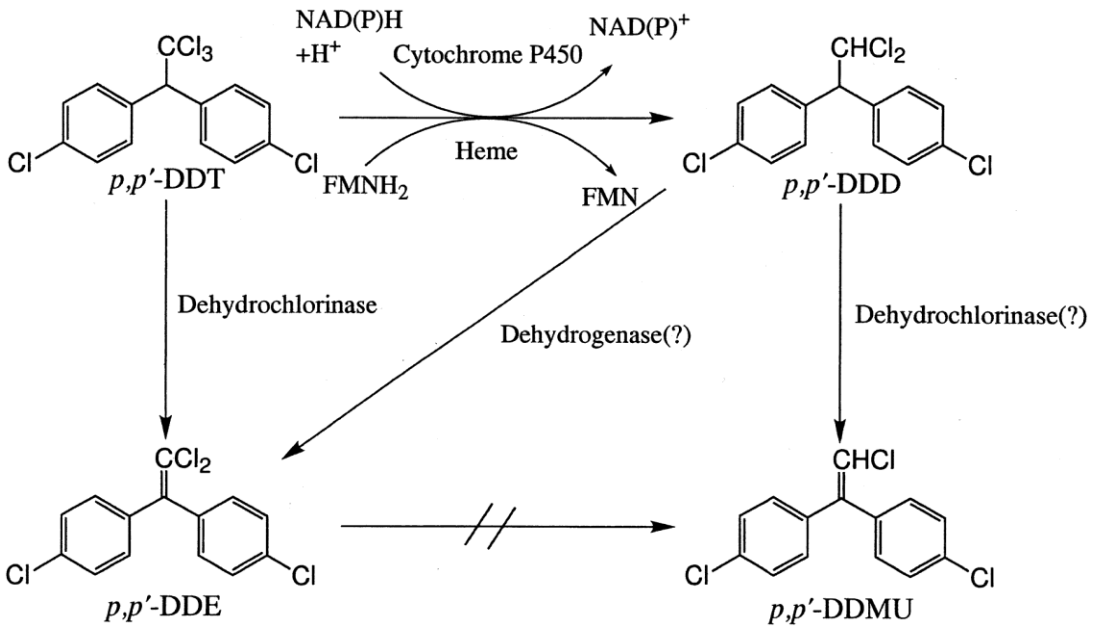
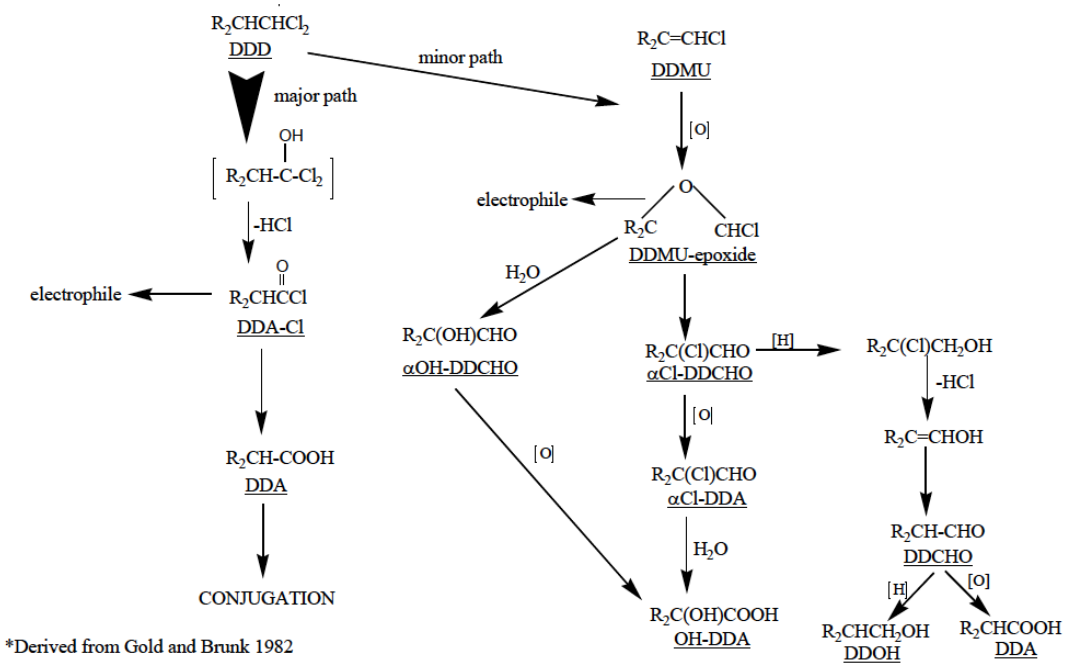

NAD(P)H
+H+
Cytochrome P450
NAD(P)+
CCl3
Cl
Cl
p,p′-DDT
FMNH2
Heme
FMN
CHCl2
Cl
Cl
p,p′-DDD
Dehydrochlorinase
Dehydrogenase(?)
Dehydrochlorinase(?)
CCl2
Cl
Cl
p,p′-DDE
CHCl
Cl
Cl
p,p′-DDMU

Figure 2. Metabolic pathway of DDT leading to the formation of DDA (Gold and Brunk, 1982).



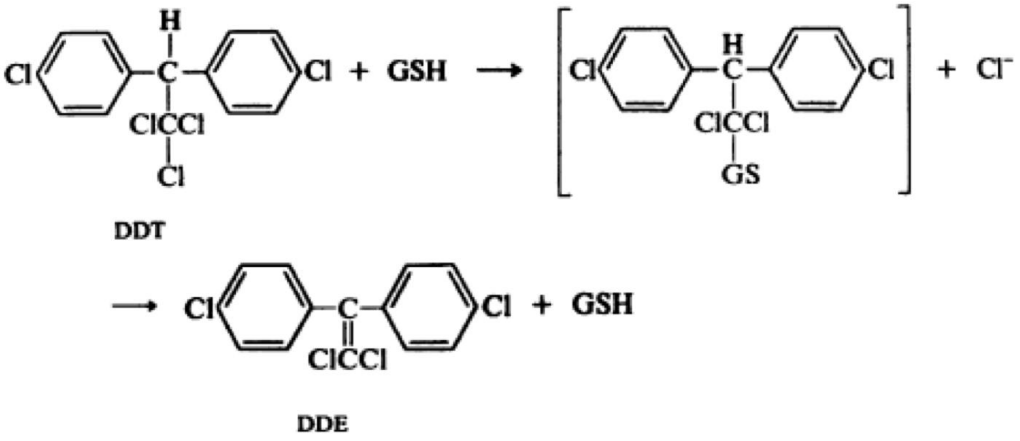
*Derived from Gold and Brunk 1982

(Waters et al., 1992; Brun et al., 1996; Amichot et al., 2004), *Cyp6g1* (Daborn et al., 2002), *Cyp6a8*, *Cyp12d1*, *Cyp4e2* and *Cyp12a4* (Amichot et al., 1994; Maitra et al., 2000; Le Goff et al., 2003; Pedra et al., 2004; Bogwitz et al., 2005) with some of these genes inducible by DDT (Brandt et al., 2002). Numerous laboratory-selected (e.g., *91-R*) and field-collected (e.g., *Hikone-R* and *Wisconsin*) DDT-resistant strains constitutively over-transcribe several P450 genes, including *Cyp6a2* and *Cyp6a8* (Brun et al., 1996; Pedra et al., 2004). There is also growing evidence that over-transcription of some P450 genes in resistant strains involves trans-regulatory elements but a link to these elements and induction levels remains to be determined (Brandt et al., 2002). Some P450 genes that are controlled by regulatory elements in *D. melanogaster* include *Cyp6a2* and *Cyp6a8* (Dombrowski, 1998; Maitra et al., 2000; Maitra et al., 2002) and appear to be induced with phenobarbitol (Dunkov et al., 1997; Dombrowski, 1998; Maitra et al., 2002). Phenobarbitol causes a 15-fold increase in the transcription of *Cyp6a2* in the DDT-susceptible *Canton-S* strain (Brun et al., 1996), increases P450 enzymatic activity, and caused these flies to become more resistant to DDT (Amichot et al., 1994; Amichot et al., 1998). Thus, evidence exists that phenobarbitol treatment of *D. melanogaster* results in increased expression of multiple P450s, some of which may be associated with the metabolism of DDT and resistance.

GSTs identified in the *D. melanogaster* genome are also thought to play a role in detoxifying insecticides, leading to resistance increased rate of transcription (Enyati et al., 2005; Ranson et al., 2001; Salinas and Wong, 1999). The mechanism of over-transcription that causes increased GST activity has not yet been established but increased activity has been shown to be associated with the over-expression of multiple

GST enzymes (Hemingway et al., 2000; Grant and Hammock, 1992). High levels of GST activity have been found in the fat body and midgut, which are the primary locations of insecticide detoxification in insects (Singh et al., 2001). *D. melanogaster* have class I type GSTs that are comprised of 6 intronless genes located on a single stretch of chromosome (Toung et al., 1993). It is probable that there are trans-acting regulatory elements upstream of GST genes producing resistance but none have been fully characterized (Hemingway et al., 2000). GSTs can metabolize insecticides by catalyzing their reductive dehydrochlorination or by conjugation reactions with reduced glutathione (GSH) to produce water-soluble metabolites that are readily excretable (Enyati et al., 2005, Matsumura, 1985) (Fig. 3). Conjugation driven by GSTs occurs when electrophilic compounds attack the thiol group on reduced glutathione making the adduct more water-soluble and excretable than the non-conjugated compound (Habig et al., 1974). Some GSTs in the *D. melanogaster* genome perform a dehydrochlorination reaction using reduced GSH as a catalytic cofactor rather than as an activated conjugative agent (Clark and Shamaan, 1984). GSH has also been shown to be a critical cofactor in the dehydrochlorination of DDT conferring resistance in insects such as housefly and mosquitoes (Enyati et al., 2005; Clark and Shamaan, 1984, Prapanthadara et al., 1993; Prapanthadara et al., 1995; Grant et al., 1991). A glutathione conjugate of DDT has never been isolated but another organochlorine insecticide, lindane, has been shown to form a glutathione conjugate (Tanaka et al., 1981). Conjugation of primary DDT metabolites (e.g. DDD and DDE) to GSH does occur in certain resistant mosquito strains, which acts as a Phase II xenobiotic metabolism mechanism in disequilibrium with a Phase I monooxygenase system (Hemingway et al., 1991).

Figure 3. Model of DDT dehydrochlorinase (DDTase) activity using reduced glutathione (GSH) (Enyati et al., 2005).


Cl
H
C
Cl
ClCCl
Cl
+ GSH
Cl
H
C
Cl
ClCCl
GS
+ Cl⁻
DDT
Cl
C
Cl
ClCCl
+ GSH
DDE

Detoxification of DDT and the formation of water-soluble conjugates of DDT metabolites has been observed *in vitro* using rat liver microsomes containing P450s (Pinto et al., 1965; Langen et al., 1989). DDT is partially detoxified to DDD through reductive dechlorination via P450-mediated reactions in rat liver microsomes (Zaidi, 1987). DDT is also reductively dehydrochlorinated to DDE through a DDD intermediate, which is then further oxidized to DDA (Kitamura et al., 2002).

1.2 Insecticidal resistance mechanisms in *Drosophila melaogaster*

Resistance to insecticides is an important and relatively common form of rapid natural selection and hence an efficient model to understand the evolution of drug resistance in general. Resistance mechanisms include; reduced cuticular penetration, reduced target site sensitivities, enhanced xenobiotic metabolism, including increased excretion and sequestration. DDT, a chlorinated aromatic hydrocarbon neurotoxin, has been widely and extensively used for over 60 years to control plant insect pests and for the control of insects that vector human diseases such as malaria, dengue and epidemic typhus. The use of DDT for the control of vectors continues and will almost assuredly increase as insect borne diseases expand (Edman, 1994).

Continuation of the use of DDT will allow for further selections of resistance mechanisms, both quantitatively and qualitatively. Resistance to DDT in *D. melanogaster* has been extensively studied and two types of resistance have been observed: (i) target-site insensitivity and (ii) enhanced xenobiotic metabolism. The *D. melanogaster para* gene encodes the α-subunit of the voltage-sensitive sodium channel and amino acid changes in the α-subunit due to point mutations in *para* results in channel

insensitivity to DDT and the pyrethroids (Pittendrigh et al., 1997). These resistance-associated mutations in *para* are analogous, in some cases identical, to mutations in other insect species and have been termed *knockdown resistance* (*kdr*) mutations.

1.3 Role of P450s in *Drosophila melanogaster* insecticidal resistance

Much of the observed metabolic resistance to DDT in *D. melanogaster* has been attributed to increased oxidative metabolism associated with P450 activity with two mechanisms responsible: (1) structural changes in specific P450s (Amichot, M. 2004) or (2) increased expression of P450s (Daborn et al., 2001; Daborn et al., 2002; Brandt et al., 2002; Pedra et al., 2004).

The *D. melanogaster* genome contains 85 P450 genes and some of them have been implicated in DDT resistance. Initial genetic mapping of DDT resistance supported the hypothesis that this form of resistance is polygenetic (Crow, 1954) and associated with each of the three large chromosomes (Dapkus and Merrell, 1977). Subsequently, lower levels of DDT resistance were mapped to a 64.5 centiMorgans loci (*Rst(2)DDT*) on the right arm of chromosome II. Within or near this mapped region, six P450 genes have been identified, including *Cyp6g1*, *Cyp6a2*, *Cyp12d1*, which have been shown to be differentially expressed in some DDT resistant-strains. *DDT-R* is a dominant allele, putatively involved in the increased metabolism of DDT, which maps to this location on chromosome II (Wilson, 1988). It has been shown to be associated with the over-transcription of *Cyp6g1* in three strains of *D. melanogaster*, although the expression levels did not correlate to the levels of DDT resistance (Kuruganti et al., 2007; Festucci-Buselli et al., 2005).

Independent microarray analyses of all the *D. melanogaster* P450 genes confirmed that *Cyp6g1* is over-transcribed in the limited set of DDT-resistant strains examined (Daborn et al., 2002; Pedra et al., 2004). Over-expression of *Cyp6g1* using the GAL4 system produced transgenic flies that were more tolerant to DDT than the non-transgenic flies (Daborn et al., 2002; Le Goff et al., 2003) and tissue-directed (midgut, Malpighian tubules and fat body) over-expression of eight P450s genes using the GAL4/UAS system produced DDT treatment survivors only when *Cyp6g1* and *Cyp12d1*were expressed (Daborn et al., 2007). Over-expression of *Cyp6a2* did not result in survivors as expected, as only the *Cyp6a2vSVL* variant has been shown to actually metabolize DDT, producing dicofol, DDD and DDA (Amichot et al., 2004).

Increased expression of *Cyp6g1* in *D. melanogaster* populations collected worldwide has been associated, in some cases, with an insertion of a 491-bp long terminal repeat (LTR) of an *Accord* retrotransposon 291 bp upstream of the *Cyp6g1* transcription start site (Daborn et al., 2002; Catania et al., 2004). Cis-regulatory elements in the *Accord* retrotransposon, all located within 1.2 kb of the promoter, resulted in increased expression of *Cyp6g1* in midgut, Malpighian tubules, and fat body (Chung et al., 2007). Expression of *Cyp6g1*, however, does not necessarily confer high levels of DDT resistance in all strains of *D. melanogaster* (Kuruganti et al., 2007). Using the DDT-resistant *91-R* and *Wisconsin* versus the DDT-susceptible ry^{506}, *91-C*, *Canton-S* and *Hikone-R* strains, Kuruganti et al., (2007) observed that although the *91-R* and *Wisconsin* are highly resistant to DDT compared to the susceptible strains (~600- to 10,000-times more resistant), they only had ~16 to 33-fold more *Cyp6g1* mRNA. Additionally, the *Cyp6g1* mRNA and protein levels in *Canton-S* and *Hikone-R* flies were as high as the

two resistant strains, yet they were as susceptible as the *91-C* strain. The susceptible phenotypes of the *Canton-S* and *Hikone-R* strains were not due to mutations in the *Cyp6g1* gene nor to the absence of the *Accord/Ninja* elements in the 5' regulatory region of this gene. Lastly, when the *Cyp6g1* allele of the *91-R* strain was replaced with the allele from the DDT susceptible *91-C* strain, recombinants all lacked the *Accord* insertion and expressed *Cyp6g1* at a low level as in the *91-C* strain, yet were still highly resistant like the *91-R* strain.

1.4 Reduced penetration as an insecticidal resistance mechanism

Reduced penetration of various insecticides through the cuticle of insects is a known resistance mechanism in tobacco budworm (*Heliothis virescens*) (Lanning et al., 1996; Ottea et al., 2000; Abd-Elghafar et al., 1994), cotton bollworm (*Heliocoverpa armigera*) (Ahmad et al., 2006; Gunning et al., 1991), corn earworm (*Heliocoverpa zea*) (Abd-Elghafar et al., 1996), beet armyworm (*Spodoptera exigua*) (Liu and Shen, 2003), diamondback moth (*Plutella xylostella*) (Noppun et al., 1989), housefly (*Musca domestica*) (Golenda et al., 1989; Sawicki et al., 1968; Plapp and Hoyer, 1968; Farnham et al., 1973; DeVries and Georghiou 1981, Liu and Pridgeon, 2002), cockroach (*Blattella germanica*) (Wu et al., 1998) and mosquito (*Aedes aegypti* and *Culex pipiens*) (Pan et al., 2009; Matsumura and Brown, 1961; Shrivastava, et al., 1970). In some cases, reduced penetration has little influence on the level of resistance on its own, but when combined with xenobiotic metabolism, it has been shown to increase resistance 5-10 fold when compared to that caused by metabolism alone (Oppennoorth, 1971). Reduced cuticular penetration can increase the effectiveness of detoxification mechanisms by prolonging

the time in which the insecticide can be detoxified by slowing the accumulation of insecticide into target cells. It has been acknowledged that reduced penetration of DDT can increase resistance due to *kdr* in housefly (O'Brien and Yamamoto, 1970). The actual mechanism that causes reduced cuticular penetration of insecticides is still unknown but is postulated to be related to increases in lipid or chitin content that constitute the cuticle. The gene, *pen*, has been implicated to be responsible for reduced penetration to fenvalerate in cotton bollworm and housefly (Gunning et al., 1996; Plapp and Hoyer, 1968). Additionally, overexpression of the laccase gene, a gene shown to be involved in cuticle scelerotization or tanning, in fenvalerate-resistant strains of mosquito has been suggested as a mechanism for reduced cuticular penetration (Pan et al., 2009).

Reduced cuticular penetration of (1R/1S)-trans-permethrin has been shown in the pyrethroid-resistant and DDT cross-resistant *147-R* strain of housefly compared to the susceptible *NAIDM-S* strain (DeVries and Georghiou, 1981).

Delayed cuticular penetration to deltamethrin has been observed in pyrethroid-resistant strains of cotton bollworm from Australia, Thialand, China and Pakistan (Ahmad et al., 2006) causing a 6 hr delay in the time it took 50% of the applied dose to accumulate in resistant compared to susceptible bollworms.

Reduced cuticular penetration has been determined as a resistance mechanism in fenvalerate-resistant strains of mosquitoes (Pan et al., 2009). Real-time PCR experiments of the laccase gene, *CpLac2*, in fenvalerate-resistant *Culex pipiens pallens* showed significantly higher transcript levels than a susceptible strain and suggests that the mechanism of reduced penetration is derived from reinforcement of the cuticle, which delays penetration into nerve cells (Pan et al., 2009).

Reduced cuticular penetration has also been identified as a resistance mechanism to the DDT and to the carbamate insecticide, thiodicarb, in tobacco budworms (Lanning et al., 1996). Lanning et al., (1996) suggested that reduced cuticular penetration is actually due to an overexpression and localization of a p-glycoprotein (ABCB-type of ATP-binding cassette protein transporters involved in phase III xenobiotic metabolism, see section 1.5) in the cuticle of resistant insects (Lanning et al., 1996). A 3-fold increase in thiocarb accumulation was achieved in resistant insects when synergized with the p-glycoprotein inhibitor, quinidine. Synergism with quinidine also reduced the LD_{50} by 12.5-fold in resistant tobacco budworms in contact exposure bioassay (Lanning et al., 1996).

1.5 Increased excretion as an insecticidal resistance mechanism

Increased excretion of insecticides is an established resistance mechanism in several insects. It is known that xenobiotics are actively effluxed out of cells by membrane-spanning proteins known as ATP-binding cassette (ABC) transporters. *D. melanogaster* have 56 genes encoding these transporters. ABCB-type transporters expressed by multiple drug resistance (MDR) genes, such as p-glycoprotein, are non-substrate specific and efflux a vast array of hydrophobic substrates, including insecticides (Buss and Callaghan, 2008). There are three p-glycoprotein genes in *D. melanogaster* including *MDR49*, *MDR50*, and *MDR65* (Wu et al., 1991; Gerrard et al., 1993; Buss and Callaghan, 2008). The ability of these transporters to efflux insecticides from cells can contribute to DDT resistance by limiting the ability of DDT to partition into the nervous

system and prolonging the time for detoxification to occur. It is important to note that p-glycoprotein is already accepted as the major cause of drug resistance in human cancer chemotherapy (Buss and Callaghan, 2008). Of note, a functional transport mechanism that is disrupted by p-glycoprotein inhibitors has been identified in the Malpighian tubules of *D. melanogaster* (Leader and O'Donnell, 2005).

The contribution of p-glycoprotein as a resistance mechanism has been demonstrated in several insects through the use of p-glycoprotein inhibitors in conjunction with bioassay. Using verapamil as a synergist for ivermectin in midge (*Chironomus riparius*) bioassays reduced the LD_{50} by 2.5-fold (Podsiadlowski et al., 1998). Verapamil also enhanced toxicity by reducing the LD_{50} of ivermectin (2.0-fold), endosulfan (1.6-fold) and cypermethrin (2.2-fold) in mosquitoes (*Culex pipiens*) (Buss et al., 2002). Thiocarb-resistant larvae of tobacco budworm (*H. virescens*) have a 6-fold increase of p-glycoprotein transporters using real-time PCR (Lanning et al., 1996). The p-glycoprotein inhibitor, quinidine, reduced the LD_{50} by 12.5-fold in the thiocarb-resistant strain and 1.8-fold in the susceptible strain. Additionally, field populations of insecticide-resistant cotton bollworm (*H. armigera*) have elevated levels of p-glycoproteins (Srinivas et al., 2004). Recently, it has been shown that *Bacillus thuringiensis* require ABC transporters to efflux β-exotoxin I. If insects possess homologous ABC transporter genes, they could use this as an ABC transporter-dependent resistance mechanism (Buss and Callaghan, 2008).

In addition to MDR transporters, *D. melanogaster* also have genes for multidrug resistance proteins (*dMRP*), which are members of the ABCC-type transporters (Tarnay et al., 2004; Buss and Callaghan, 2008). MRPs show different substrate specificity from

p-glycoprotein and actively transport organic anions, such as sugar, sulfate and glutathione conjugates (Zaman et al., 1995; Ishikawa et al., 1992).

1.6 Regulation of DDT for vector control

The *91-R* strain is an ideal model to study additional mechanisms of DDT resistance that clearly could be selected for in DDT-resistant flies currently in field populations if reselected with DDT. This possibility is highly likely given the emerging threat of various vector-borne diseases worldwide and the current status that WHO has taken on the increased use of DDT, particularly in Africa.

The WHO's position on using DDT for indoor residual spraying states, "Countries can use DDT for as long as necessary, in the quantity needed, provided that the guidelines and recommendations of WHO and Stockholm Convention are all met, and until locally appropriate and cost-effective alternatives are available for a sustainable transition from DDT" (WHO, 2007). The WHO and the Stockholm Convention on persistent organic pollutants agreed that indoor applications of DDT for control of vectors of diseases was appropriate as there is currently no alternative as equally effective and operationally feasible, especially for high-transmission areas. Both groups, however, shared a common commitment of reducing and eventually eliminating the use of DDT, while minimizing the burden in endemic countries (WHO, 2006; Stockholm, 2001).

In this study, we examine polygenetic resistance mechanisms in highly DDT-resistant *91-R* females compared to susceptible *Canton-S* females. Resistance mechanisms hypothesized to be involved in polygenetic resistance include reduced

penetration through the cuticle, increased metabolism via phase I oxidative enzymes and phase II conjugations, and increased excretion via phase III efflux transporters.

CHAPTER 2

MATERIALS AND METHODS

2.1 *Drosophila melanogaster* strains

D. melanogaster stocks were raised on instant fruit fly media (Ed's Fly Meat, Port Orchard, WA) at ~25 °C. DDT-resistant *91-R* and DDT-susceptible *Canton-S* strains were obtained from Dr. Barry Pittendrigh at University of Illinois, Urbana-Champaign and used in all experiments. The *91-R* strain is ~1000 times more resistant to DDT than the *Canton-S* strain using a contact bioassay (Dapkus and Merrell, 1977; Kuruganti et al., 2007; Merrell and Underhill, 1956; Dapkus et al., 1992).

2.2 DDT and DDT metabolites

[^{14}C] p,p'-dichlorodiphenyltrichloroethane ([^{14}C]-DDT) was custom made by American Radiolabeled Chemicals, Inc., St. Louis, MO, with a specific activity of 12.8 mCi/mmole. Non-radioactive standards of DDT, dichlorodiphenyldichloroethylene (DDE), dichlorodiphenyldichloroethane (DDD), 4,4'-dichlorobenzophenone (DBP) and 2,2,2-trichloro-1,1-bis(4-chlorophenyl)ethanol (dicofol) were purchased from Chem. Service Inc. (West Chester, PA). All other chemicals, including verapamil, were purchased from Sigma Chemicals (St. Louis, MO) at the highest purity available (see Appendix A for structures).

2.3 Mortality bioassays

Three groups of 30 female flies from the *91-R* and *Canton-S* strains were used in all bioassays. For the topical bioassay experiments, DDT was dissolved in acetone at various concentrations and 1 µl of solution topically applied to the pronotum using a microapplicator (Houston Atlas, Houston, TX) equipped with a 10 µl glass syringe (Hamilton Co, Reno, NV). Females were knocked down prior to treatment by placing them in a 4 °C refrigerator for 30 min and then transferring them to an ice-chilled glass Petri dish. After treatment, groups of flies were transferred to 20 ml glass vials, each capped with cotton, and evaluated at room temperature for 24 hr. The cotton was initially moistened with 1-2 ml of a 5.0 % sucrose solution in distilled water. Females were considered dead if unable to right themselves and lacked leg movement when probed.

For contact bioassays, females (n = 10) were placed into 20 ml glass vials, which were coated with various concentrations of DDT. The vials were coated by applying 100 µl of an acetone solution containing DDT and rolling the vials on their sides until all the acetone had evaporated. The vials were capped with moistened cotton as described above. Exposure to DDT was confirmed by visually determining that flies spent little time on the cotton plug and rotated off onto the wall of the vial intermittently.

Log dose/concentration versus logit percent mortality regression lines were generated to determine the dose of topically applied DDT that killed 50% of the treated flies (LD_{50} topical) and the concentration of DDT on coated vials that killed 50% of the treated flies (LC_{50} contact), respectively. Maximum log-likelihood ratio tests were performed to test the equality (slope and intercept) of the regression lines ($p<0.05$, Polo PC, LeOra Software, 1987).

The p-glycoprotein ($ABCB_1$) blocking agent, verapamil, which functions as a competitive substrate, was used with DDT in bioassay to determine possible synergistic effects by inhibiting ATP-binding cassette protein transporters (ABC transporters), such as the multidrug resistance proteins, dMDR49, dMDR50 and dMDR65. Flies were treated with 1 µl of a verapamil solution (10 µg/µl acetone) using the same methods used for the topical application of DDT described above. A verapamil pretreatment was applied 6 hr prior to application of various concentrations of DDT. Synergistic effects of verapamil were determined using a synergist ratio ($SR = LD_{50}/sLD_{50}$, where *s* = synergized), relative percent synergism of the susceptible strain ($R\%S\ (S) = 100[\log LD_{50}(S) - \log sLD_{50}(S)]/[\log LD_{50}(R) - \log sLD_{50}(S)]$, and relative percent synergism of the resistant strain $R\%S(R) = 100[\log LD_{50}(R) - \log sLD_{50}(R)]/[\log LD_{50}(R) - \log sLD_{50}(S)]$). Verapamil applied at 10 µg/µl did not result in fly mortality over the 24 hr exposure period.

2.4 Penetration of DDT

[^{14}C]-DDT (0.03 µg/µl acetone) was applied topically to the pronotum of each fly. Ninety females were dosed for each of three replicate treatments. After exposure times of 0, 6, 12 and 24 hrs, batches of 30 flies were sequentially immersed three times into 5 ml aliquots of acetone for 30 sec/rinse. Rinses were combined, concentrated under nitrogen, and resuspended in a final volume of 0.8 ml acetone. Excrement (feces plus urine) remaining in the exposure vials was extracted sequentially using three 5 ml aliquots of acetone, followed by an additional three 5 ml aliquots of methanol. The acetone and methanol excrement extracts were combined separately, concentrated under

nitrogen, and resuspended in a final volume of 0.8 ml acetone or methanol, respectively. A 200 µl aliquot was taken from both the acetone and methanol extracts for liquid scintillation spectrophotometry and the remaining 600 µl aliquot was analyzed by thin layer chromatography (TLC) with the radioactivity (counts per minute, CPM) of parent DDT and its metabolites determined by TLC-linear analyzer for radiometric detection (see below).

Acetone-rinsed flies were homogenized (whole body extracts) using a 7 ml glass-glass tissue homogenizer in 5 ml acetone (~30 strokes for 1 min) and centrifuged at 7000 g for 10 min. Each supernatant was collected, the homogenization process repeated two additional times, and all supernatants combined. The remaining pellet was resuspended by homogenization and extracted using three sequential 5 ml aliquots of methanol, which were combined as above. The acetone and methanol whole body extracts were concentrated separately under nitrogen, and each resuspended into a final volume of 0.8 ml acetone or methanol, respectively. Then, 200 µl aliquots were taken from either the acetone or methanol extracts for liquid scintillation spectrophotometry and the remaining 600 µl aliquots analyzed separately by TLC-linear analyzer (See below).

The amount of [^{14}C]-DDT that penetrated the cuticle following topical application in acetone (% topical penetration) was determined from the amount of radioactivity (CPM) extracted from the external surface of the flies (rinse extracts) compared to the total amount of extracted radioactivity (rinse + whole body + excrement extracts = total radioactivity) as determined by liquid scintillation spectrophotometry using the following equation.

Equation 1: % Topical Penetration = [(CPM in whole body + excrement) / (CPM in rinse + whole body + excrement)] X 100.

Penetration of [^{14}C]-DDT was also determined using a contact exposure method where [^{14}C]-DDT was coated on the inside of the 20 ml glass exposure vials. This exposure scenario eliminated acetone used in the topical application, which is likely to disrupt the hydrocarbon constituents found on the waxy surface of the cuticle and allows the direct penetration of DDT. For this experiment, exposure vials were coated with 100 µl of acetone solution containing 3.0 µg of [^{14}C]-DDT by rolling the vials until all the acetone evaporated. Three groups of 30 females were used in each of three replicate experiments (n = 270). The amount of excreted radioactivity cannot be used to determine penetration in this experimental design due to the amount of [^{14}C]-DDT used to coat the vials. Thus, percent penetration for contact (% contact penetration) exposure was determined using the following equation.

Equation 2: % Contact Penetration = [(CPM in whole body homogenate) / (CPM in rinse + whole body)] X 100.

The amount of DDT administered either topically or by contact did not result in any mortality over the 24 hr assessment of penetration.

2.5 Excretion of DDT and metabolites

The amount of excreted radioactivity (% excretion) was determined from the amount of radioactivity extracted from the excrement (urine plus feces) left in the exposure vials compared to the total amount of extracted radioactivity (rinse + whole body + excrement extracts = total radioactivity) as determined by liquid scintillation spectrophotometry using the following equation.

Equation 3: % Excretion = [(CPM in excrement) / (CPM in rinse + whole body + excrement)] X 100.

2.6 Metabolite identification and quantification

Rinse, whole body and excrement extracts were analyzed for radiolabeled [^{14}C]-DDT and its metabolites by TLC. Extracts were applied to the TLC plates (silica gel GF, 250 µm) and developed using a hexane: acetone solvent system (9:1) (Kitmura et al., 2002). Plates were scanned for radioactivity (CPM) using a LB282 Berthold TLC-Linear Analyzer (Berthold Analytical Inc., Nashua, NH). The areas of radioactivity peaks detected during scanning were auto-integrated using the system's software program. DDT and its metabolites were identified based on the retention factor (Rf) values determined directly from the TLC plates of non-radioactive standards visualized by fluorescence under UV light (254 nm). Rf values were calculated using the distance traveled (cm) of the standard divided by the total distance that the solvent front traveled (18 cm). The Rf values for DDT, DDD, DDE and dicofol were determined to be 0.50, 0.35, 0.64 and 0.29, respectively, and were not statistically different from published values (Kitamura et al., 2002).

2.7 Cuticular hydrocarbon analysis

Groups of 90 females from the *91-R* or *Canton-S* strains were hexane-extracted and the cuticular hydrocarbon differences between the two strains determined for three replicate experiments (n = 270). Ninety females from *91-R* or *Canton-S* were placed separately into 20 ml glass scintillation vials and 1 ml of hexane added. The flies were extracted for 5 min and the hexane extract transferred to 2 ml amber glass autosampler vials (Desena et al., 1999). Extracts were reduced to dryness using a Speedvac concentrator, resuspended in 0.5 ml of hexane and 10 µl of an internal standard added to each extract (1mg/ml octadecane). Extracts were analyzed using a Hewlett-Packard 6890 gas-liquid chromatograph equipped with a flame ionization detector and a DB-5 column. Helium was used as a carrier gas at a flow rate of 3.8 ml/min. The column-containing oven was temperature programmed from 100 to 280 °C at a rate of 7.5 °C/min, with a final hold at 280 °C for 22 min. Integrations of chromatographic hydrocarbon peak areas were performed using Chemstation (revision A.07.01; Hewlett-Packard). Integrated areas of chromatographic peaks were normalized to octadecane and compared between *91-R* and *Canton-S* females to determine significant differences using Student's t-test ($p<0.05$).

2.8 RNA extraction and quantitative real-time PCR

Three-day old female flies from *91-R* and *Canton-S* strains were collected separately to extract total RNA for each biological replicate. For each strain, three biological replicates (females from different generations) were collected. Thirty females were used for each biological replicate. Each biological replicate was analyzed four times by quantitative (qPCR). The collected flies were flash frozen in liquid nitrogen and stored at -80 ^{o}C until total RNA extraction.

For each strain and replicate, total RNA was extracted using the Qiagen RNeasy kit (Qiagen, Valencia, CA) with an on column DNase digestion procedure. The first stranded cDNA for each biological replicate was synthesized by using 1 µg of total RNA with iScript cDNA kit from Bio-Rad (Hercules, CA) in a 20 µl reaction volume. A 25-fold dilution was made to each first stranded cDNA for qPCR. For each cDNA, four qPCR reactions were conducted using iQ SYBR Green Supermix from Bio-Rad (Hercules, CA) with SYBR Green dye on an iCycler Thermal Cycler from Bio-Rad (Hercules, CA). Ribosomal protein 49 (*rp49*) was used as the reference gene. A pre qPCR was done with *rp49* alone to further equalize the expression level of the reference gene among all cDNAs. The threshold cycle value (C_t) was calculated by the iCycler IQ software. The relative expression levels were calculated using the method of Pfaffl et al., (2002) and the statistical analysis of the relative gene expression level were performed using SAS (SAS Institute Inc., Cary, NC). The difference in C_t values (ΔC_t), is determined by subtracting the C_t value of *91-R* from *Canton-S* indicating an increase in transcription in *91-R* for genes with negative ΔC_t values. The primers used for qPCR and gene bank accession numbers are given in Table 1.

Table 1. Primer sequences used for qPCR experiments.

Gene name	Accession number	Primer sequence (5'-3')
rp49	U92431	CGGTTACGGATCGAACAAGCG TTGGCGCGCTCGACAATCT
CG6214	AY069827	GGACGCGGAACGCCAGAAGA GCATTCCGTCCGCGAAGCAG
CG10181	NM_057483	GGTGGAGAAAAATCAGAGGTGCAGC TACCAAAATGCTCCGGCGCA
CG10226	NM_139783	CGAGAGGGAATACACACCGGCG ATGGCCGAAGCCGATCCTCG
CG3879	NM_079000	CCAGATGCGAGCGCTTCCTG GATGTGCCCACGCCGACTGT
CG8523	NM_079016	ACTCCCAGATCCTCACCATCCGC TTCGGCCAAGCCATCCTCCA

CHAPTER 3

VALIDATION OF DDT RESISTANCE IN *91-R* FEMALE FLIES

3.1 Validation of DDT resistance in *91-R* female flies using contact exposure mortality bioassay

There was a significant difference in the mortality response of *91-R* females to a contact exposure of DDT compared to *Canton-S* females ($\chi^2 = 420$, d.f. = 2, $p < 0.001$) (Fig. 4). The LC_{50} values of *91-R* and *Canton-S* females to contact exposure of DDT were 1513 (808-4203, 95% C.L.) µg/vial and 0.99 (0.41-1.90, 95% C.L.) µg/vial, respectively (Table 2). The resistance ratio using the contact LC_{50} values of the *91-R* females versus *Canton-S* females was determined to be 1,526 (Table 2).

Figure 4: Log dose versus logit percent mortality for *91-R* and *Canton-S* females using topical application of DDT in acetone (µg/µl) and a contact application using DDT-coated glass vials (µg/vial).

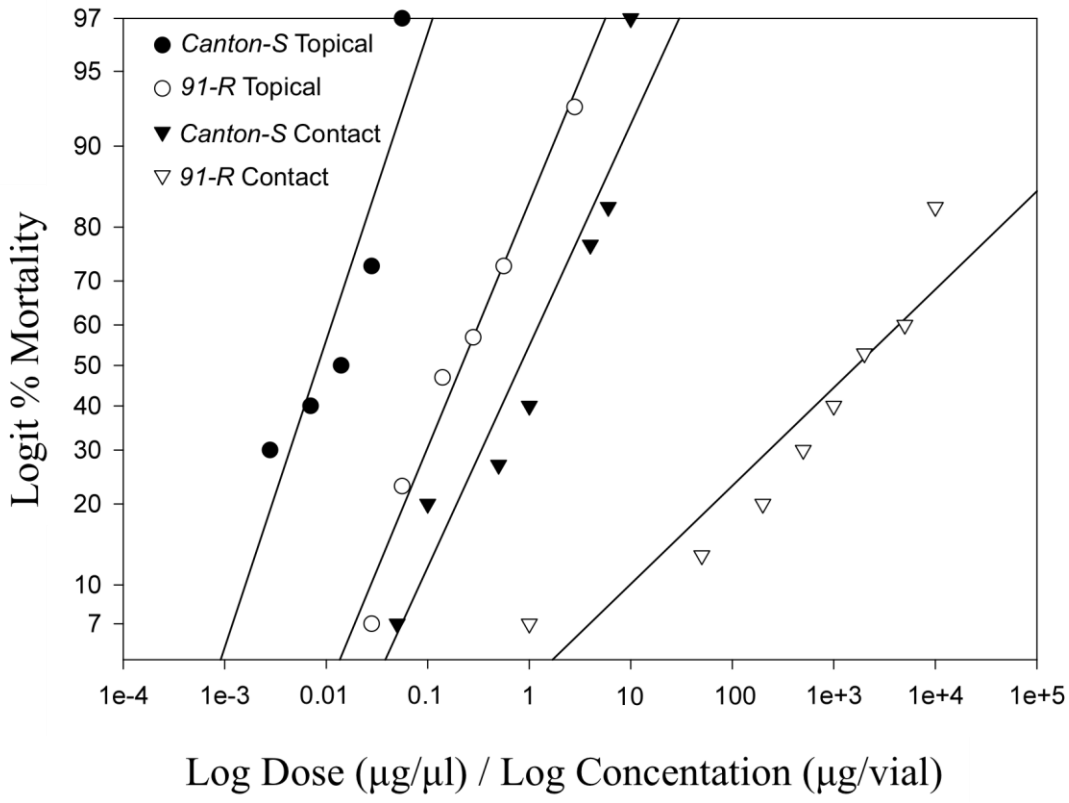

Logit % Mortality
97
95
90
80
70
60
50
40
30
20
10
7
Canton-S Topical
91-R Topical
Canton-S Contact
91-R Contact
1e-4
1e-3
0.01
0.1
1
10
100
1e+3
1e+4
1e+5
Log Dose (µg/µl) / Log Concentation (µg/vial)

Table 2. Lethal contact mortality bioassay of *91-R* versus *Canton-S* female flies.

Strain	LC_{50}[a] (95% C L)[c]	RR[b]	Slope ± S.D.
91-R	1,513 (808.1- 4,203.0)	1,526	2.1 ± 0.2
Canton-S	0.99 (0.41 – 1.90)		1.2 ± 0.1

[a] Lethal concentration (µg/vial) that killed 50% of the flies.
[b] Resistance ratio = *91-R* LC_{50} / *Canton-S* LC_{50}.
[c] 95% Confidence Limit.

3.2 Differential mortality response of *91-R* and *Canton-S* female flies to DDT administered by topical versus contact exposure

There was a significant difference in the mortality response of *91-R* females to a topical application of DDT in acetone compared to contact exposure (χ^2 = 386, d.f. = 2, $p < 0.001$) (Fig. 4). The LD_{50} value of *91-R* females to topically-applied DDT in acetone was 0.20 (0.14-0.29, 95% C.L.) µg/µl. The LD_{50} value of *Canton-S* females to topically-applied DDT in acetone was 0.009 (0.006-0.013, 95% C.L.) µg/µl. The resistance ratio of the LD_{50} values of the *91-R* females versus *Canton-S* females was determined to be 22.2. There is a 68-fold difference in the resistance ration of topically-applied DDT in acetone versus contact exposed females.

CHAPTER 4

TOXICOKINETICS OF [^{14}C]-DDT IN *91-R* AND *CANTON-S* FEMALE FLIES

4.1 Distribution of [^{14}C]-DDT and its metabolites determined on the cuticular surface, within the whole body, and in the excrement of *91-R* versus *Canton-S* females following topical exposures in acetone

The level of radioactivity of [^{14}C]-DDT and its metabolites (CPM) were determined in external rinses, whole body homogenates and excrements for *91-R* and *Canton-S* females at 24 hr post-topical exposure to DDT in acetone. There was no significant difference in the external rinses ($p = 0.87$) but there were significant differences in the whole body homogenate and excrement extracts for *91-R* versus *Canton-S* females ($p < 0.05$) (Table 3). There was 1.8-fold less radioactivity in the whole body homogenate extracts and 3.8-fold more radioactivity in the excrement extracts of *91-R* compared to *Canton-S* females (Table 3). Interestingly, the excrement extract contains the greatest amount of radioactivity for *91-R* females (51%), whereas, the whole body homogenates of *Canton-S* contained the greatest amount of radioactivity (67%) (Table 3).

Table 3. Distribution of radioactivity at 24 hr following topical application of [^{14}C]-DDT in acetone to female flies of the *91-R* and *Canton-S* strains of *D. melanogaster*.

	Percent Radioactivity Present (% of total applied CPM)[a]		
Fly Strain	External Rinse	Whole Body Homogenate	Excrement
91-R	15.4 ± 5.3	36.6 ± 2.7[b]	51.2 ± 2.5[b]
Canton-S	16.0 ± 3.6	67.2 ± 4.3	13.6 ± 2.3

[a] The total radioactivity applied was ~35,500 ± 5,000 CPM and the extraction efficiencies were greater than 90%.
[b] Indicates a statistical difference between *91-R* and *Canton-S* extracts using Student's t-test ($p < 0.05$).

4.2 Penetration differences determined by topical application of [^{14}C]-DDT in acetone to cuticle or by contact exposure to [^{14}C]-DDT-coated vials of *91-R* versus *Canton-S* female flies

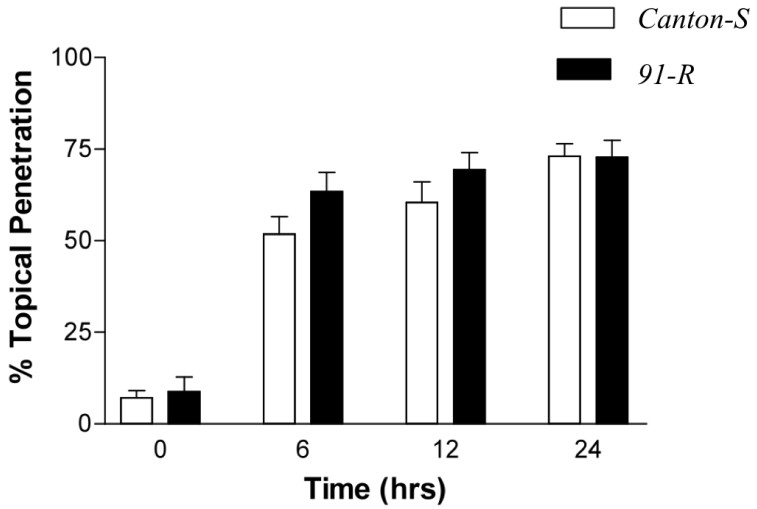
The percent of total [^{14}C]-DDT topically applied that penetrated the cuticle was determined from the amount of radioactivity found in the excrement and the whole body of *91-R* and *Canton-S* female flies. There were no significant differences between the amount of [^{14}C]-DDT that penetrated the cuticle of either strain following topical exposures in acetone at 0, 6, 12, and 24 hr post-application (Fig. 5A). Penetration of [^{14}C]-DDT was rapid and accounted for ~10% of the total applied dose for both *91-R* and *Canton-S* females at the 0 hr time point (Fig. 5A). Penetration of [^{14}C]-DDT significantly increased at the 6 hr time point and accounted for ~60% of the total applied dose for both *91-R* and *Canton-S* females (Fig. 5A). The amount of [^{14}C]-DDT penetration did not

significantly increase beyond the 6 hr time point and reached a maximal penetration level of ~75% of the total applied dose at 24 hr (Fig. 5A).

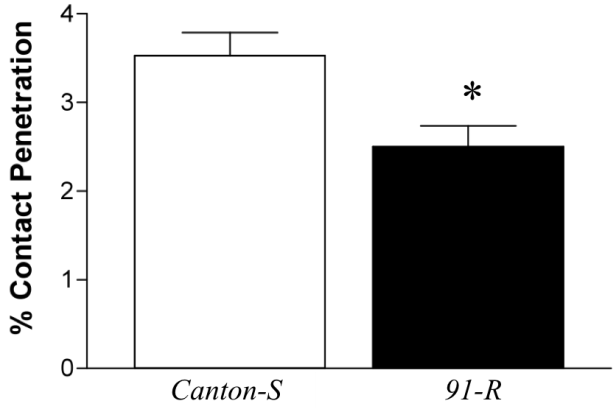
With contact exposure, there was a significant decrease in the percent of total available [^{14}C]-DDT that penetrated the surface of *91-R* compared to *Canton-S* females ($p < 0.05$) (Fig. 5B). Overall, there was a 1.5-fold reduction in the cuticular penetration of [^{14}C]-DDT with *91-R* versus *Canton-S* females at 24 hr following contact exposure. Only 2.5% of the total applied [^{14}C]-DDT used to coat the vial was found in whole body extracts of *91-R* females versus 3.5% found in *Canton-S* females (Fig. 5B).

Figure 5. Percent topical penetration versus time (A) and percent contact penetration at 24 hr (B) of [^{14}C]-DDT on *91-R* and *Canton-S* females determined by liquid scintillation spectrophotometry. An asterisk (*) indicates means are significantly different using Student's *t*-test ($p < 0.05$).

A


Canton-S
91-R
% Topical Penetration
100
75
50
25
0
0
6
12
24
Time (hrs)

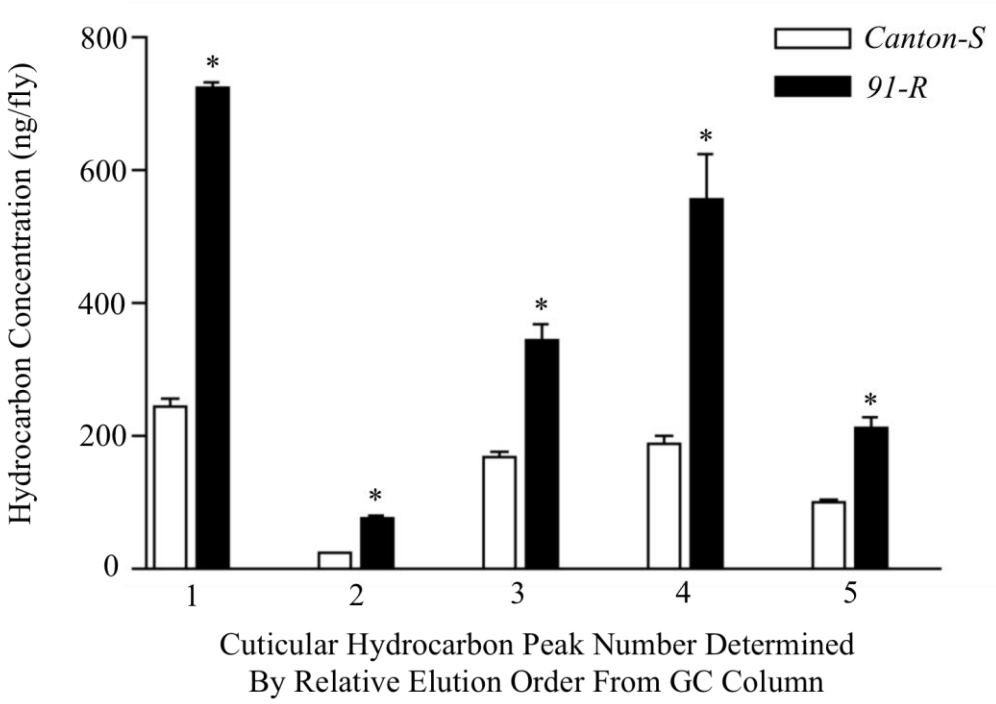
B


% Contact Penetration
4
3
2
1
0
*
Canton-S
91-R

4.3 Identification and quantification of hydrocarbons associated with the cuticle of *91-R* versus *Canton-S* female flies

Overall, there were no significant differences in the qualitative composition of the cuticular hydrocarbons present in *91-R* versus *Canton-S* females (data not shown). There were five hydrocarbon peaks, however, that were found in significantly larger amounts in the *91-R* females compared to *Canton-S* females (Fig. 6). Hydrocarbons peaks 1 to 5 were identified at retention times of 12.4, 14.1, 14.4, 14.7 and 16.5 min, respectively, in the chromatograms from the cuticular hydrocarbon extracts from both *91-R* and *Canton-S* females. The area under each hydrocarbon peak was integrated using Chemstation (revision A.07.01; Hewlett-Packard). Integrated areas of chromatographic peaks were normalized to octadecane (1 µg/µl). Hydrocarbon(s) in peak 1 was the most abundant hydrocarbon quantified for both *91-R* and *Canton-S* cuticular extracts and was ~3-fold more abundant in the cuticular hydrocarbon extract of *91-R* compared to *Canton-S* females (Fig. 6). Hydrocarbon(s) associated with peaks 2, 3, 4 and 5 were all more abundant in *91-R* compared to *Canton-S* females with ~3-, 2-, 3-, and 2-fold more hydrocarbon(s), respectively (Fig. 6).

Figure 6. Cuticular hydrocarbon analyses of females of the DDT-resistant *91-R* versus susceptible *Canton-S* strains of *D. melanogaster* using GC-FID. Integrations of chromatographic hydrocarbon peak areas were performed using Chemstation (revision A.07.01; Hewlett-Packard). Integrated areas of chromatographic peaks were normalized to octadecane (1µg/µl). Asterisks (*) indicate statistical differences between strains using Student's *t*-test ($p < 0.05$).


Canton-S
91-R
Hydrocarbon Concentration (ng/fly)
800
600
400
200
0
*
1
2
3
4
5
Cuticular Hydrocarbon Peak Number Determined
By Relative Elution Order From GC Column

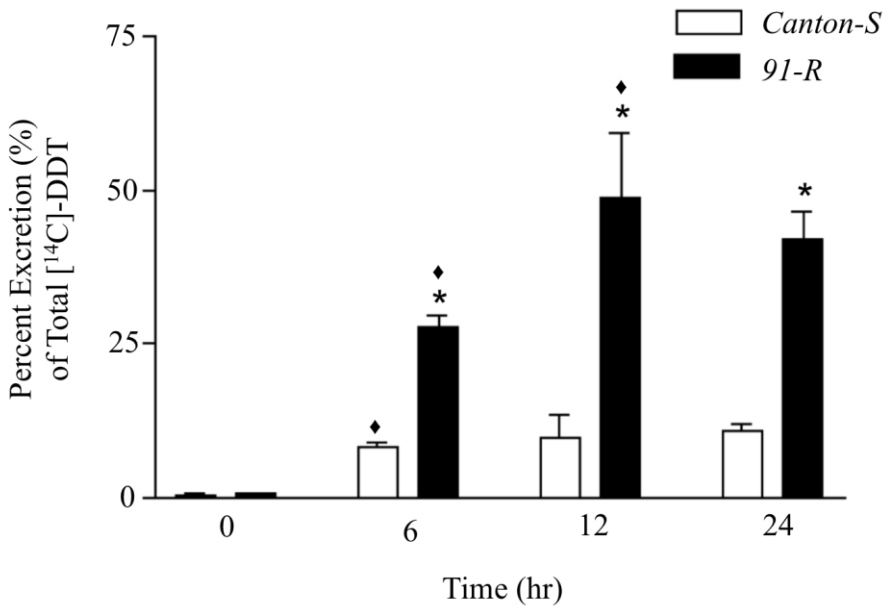
4.4 Excretion of [^{14}C]-DDT and metabolite determined by topical application of [^{14}C]-DDT to the cuticle of *91-R* and *Canton-S* female flies

There was a significant increase in the percent of excreted radioactivity with *91-R* females at 6, 12 and 24 hr following topical application of [^{14}C]-DDT with the largest difference at 12 hr compared to *Canton-S* females (Fig. 7). *91-R* females excreted 3.4-, 5.1- and 3.8-fold more radioactivity compared to *Canton-S* females at 6, 12 and 24 hr, respectively (Fig. 7). At the 0 hr time point, there was a small but detectable amount of radioactivity in the excrement (<5% of the total applied dose).

Radioactivity increased significantly at 6 hr with ~30% of the total applied dose excreted by *91-R* females and again at 12 hr when excretion was maximal (~50% of the total applied dose excreted by *91-R* females). The amount of excreted radioactivity remained constant at 24 hr compared to the amount of radioactivity excreted at 12 hr by *91-R* females (Fig. 7).

There was also a significant increase in the excretion of radioactivity at 6 hr with ~10% of the total applied dose excreted by *Canton-S* females (Fig. 7). Excretion of radioactivity did not significantly increase beyond 6 hr and reached maximal excretion at 12 hr with ~15% of the total applied dose excreted in *Canton-S* females.

Figure 7. Percent excretion of [^{14}C]-DDT and its metabolites from DDT-resistant *91-R* versus susceptible *Canton-S* female flies as determined by liquid scintillation spectrophotometry. Diamonds (♦) indicate significant increases in excretion for either *91-R* or *Canton-S* females flies compared to the excretion determined in the previous time point using Student's *t*-test ($p < 0.05$). Asterisks (*) indicate statistical differences between *91-R* and *Canton-S* female flies at indicated time point using Student's *t*-test ($p < 0.05$).


Canton-S
91-R
75
50
25
0
Percent Excretion (%)
of Total [14C]-DDT
0
6
12
24
Time (hr)

4.5 Increased DDT sensitivity of *91-R* female flies following pretreatment with a p-glycoprotein inhibitor verapamil

There was a significant difference in the mortality response of *91-R* females topically dosed with DDT in acetone compared to *Canton-S* females (χ^2 = 74.1, d.f. = 2, p< 0.001) (Fig. 8). The LD_{50} values of *91-R* and *Canton-S* females to topically-applied DDT were 0.20 µg/µl (0.14-0.29) and 0.009 µg/µl (0.006-0.013), respectively. The resistance ratio was calculated based on the LD_{50} values of the *91-R* females divided by the LD_{50} values of the *Canton-S* females and was determined to be 22.2 (Table 4).

A topical treatment of the p-glycoprotein inhibitor, verapamil, 6 hrs prior to treatment with DDT significantly decreased the LD_{50} values of *91-R* females to 0.02 µg/µl (0.015-0.027). Verapamil pretreatment did not significantly reduce the LD_{50} value of *Canton-S* females. A second resistance ratio was calculated based on the LD_{50} values obtained from the verapamil pretreatments of *91-R* versus *Canton-S* females and was determined to be 2.5.

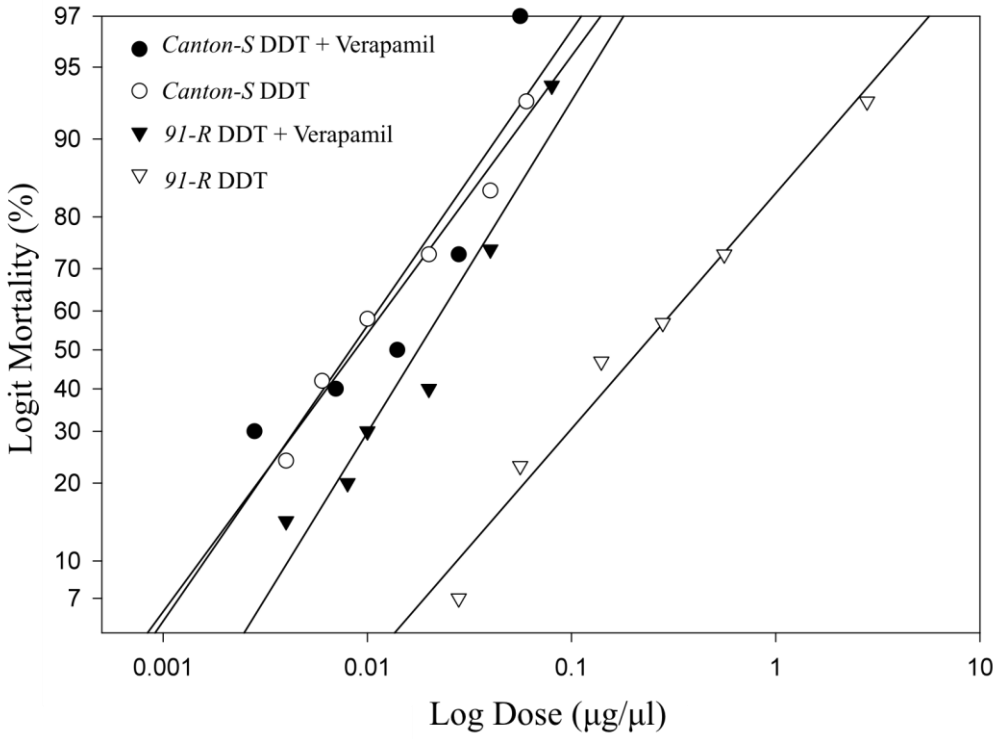
The verapamil synergist ratio calculated based on the above LD_{50} values of *91-R* females divided by the LD_{50} values of the verapamil pretreated *91-R* females were determined to be 10.0 (Table 4). The verapamil synergist ratio was calculated based on the above LD_{50} values of *Canton-S* females divided by the LD_{50} values of the verapamil pretreated *Canton-S* females and determined to be 1.1 (Table 4). The relative percent synergism of *91-R* was calculated as 71.4% (Table 4). The relative percent synergism of *Canton-S* was 3.7% (Table 4).

Table 4. Effects of verapamil pretreatment on resistance ratios, synergistic ratios and relative percent synergism of *91-R* and *Canton-S* females topically dosed with DDT in acetone.

Strain	LD_{50}[a] (95% C. L.)[e]	Slope ± S.D.	RR[b]	SR[c]	R%S[d]
91-R	0.20 (0.14 - 0.29)	2.4 ± 0.4	22.2	10.0	71.4%
91-R + verapamil[f]	0.02 (0.015 – 0.027)	3.3 ± 0.3	2.5		
Canton-S	0.009 (0.006 – 0.013)	2.5 ± 0.5		1.1	3.7%
Canton-S + verapamil[f]	0.008 (0.006 – 0.011)	2.9 ± 0.4			

[a] Lethal Dose (µg/ µl) that killed 50% of the flies.
[b] Resistance ratio = *91-R* LD_{50} / *Canton-S* LD_{50} or *91-R* + verapamil LD_{50} / *Canton-S* + verapamil LD_{50}.
[c] Synergistic ratio = *91-R* LD_{50} / *91-R* + verapamil LD_{50} or *Canton-S* LD_{50} / *Canton-S* + verapamil LD_{50}.
[d] Relative percent synergism. R%S for *91-R* = 100[log LD_{50}(R)-log sLD_{50}(R)]/[log LD_{50}(R) –log sLD_{50}(S)]). R%S for *Canton-S* = 100[log LD_{50}(S) –log sLD_{50}(S)]/[log LD_{50}(R)-log sLD_{50}(S)].
[e] 95% Confidence Limit.
[f] Verapamil was applied 6 hr prior to DDT treatment.

Figure 8: Log dose versus logit percent mortality of females from the DDT-resistant *91-R* and susceptible *Canton-S* strains of *D. melanogaster* using DDT alone or DDT+ verapamil. Verapamil (10 µg/µl) pretreatment occurred 6 hr prior to dosing with DDT.


97
95
90
80
70
60
50
40
30
20
10
7
Logit Mortality (%)
Canton-S DDT + Verapamil
Canton-S DDT
91-R DDT + Verapamil
91-R DDT
0.001
0.01
0.1
1
10
Log Dose (µg/µl)

4.6 Identification of over-expressed ABC transporter genes in *91-R* female flies

The transcript level of CG6214 (MRP1) was 35% higher (1.35 ratio) in *91-R* females compared to *Canton-S* females (ΔCT = -0.44; F=12.64; d.f. = 1; $p<0.001$) (Table 5). The transcript level of CG10181 (MDR65), was 39% higher in *91-R* females compared to *Canton-S* females (ΔCT = -0.48; F=10.79; d.f. = 1; $p<0.01$) (Table 5). The transcript level of CG8523 (MDR50), was 36% higher in *91-R* females compared to *Canton-S* females (ΔCT =-0.45; F=11.63; d.f. = 1; $p<0.01$) (Table 5). For CG3879 (MDR49), there were no differences in the transcript levels between *91-R* and *Canton-S* females (Table 5).

Table 5. Differential expression of *D. melanogaster* ABC transporter genes (MDR and MRP) in *91-R* versus *Canton-S* female flies by qPCR.

ABC Gene	Transcript ratio (ABC gene/rp49)	ΔCT* (S.E.)	p-value
P-glycoprotein			
MDR 49 (CG3879)	0.97	0.04 (0.12)	0.7172
MDR 50 (CG8523)	1.36	-0.45 (0.13)	0.0012*
MDR 65 (CG10181)	1.39	-0.48 (0.14)	0.0018*
Multidrug-resistance protein			
MRP1 (CG6214)	1.35	-0.44 (0.12)	0.0008*

Asterisk (*) indicates a significant difference using Bonferroni correction ($p<0.05$).

4.7 Discussion

P-glycoprotein is an ATP-dependant efflux pump protein of low substrate specificity in the family of proteins called ABC transporters that remove xenobiotics including insecticides from cells. They are critical for insecticide resistance produced by Phase III xenobiotic metabolism by effluxing intracellular insecticides out of target cells and excreting insecticides from the body. Very recently, the *D. melanogaster* multiple drug resistance associated protein (DmMRP) gene, which is orthologous to the human MRP1, 2, and 3 genes of the ABCC1 transporter super-family, has been shown to be associated with DDT resistance (Tarnay et al., 2004; Vache et al., 2006). qPCR analysis showed that *D. melanogaster* larval exposure to known vertebrate MRP substrates, chlorpromazine, phenobarbital and dexamethasone, causes DmMRP induction and increased DDT toxicity (Vache et al., 2006).

Using a competitive assay based on the efflux of the fluorescent dye rhodamine123 from intact mammalian HepG2 cells, Shabbir et al., (2004) found that DDT decreases rhodamine efflux, suggesting a competitive interaction between DDT and p-glycoprotein. Based on their results, they suggested that DDT was either a substrate or inhibitor of p-glycoprotein. Using verapamil as a competitive substrate of p-glycoprotein, they did not see an increase in the intracellular concentration of DDT suggesting that DDT is not a substrate for p-glycoprotein but an inhibitor. This finding is consistent with the work done by Bain and LeBlanc, (1996) where no efflux of DDT or DDE was observed in mouse cells.

The above findings are not consistent with our data that suggest that verapamil is a synergist of DDT toxicity by acting as a surrogate substrate of *D. melanogaster* MDR transporters. It may be possible that mammalian cells do not have the same substrate specificity for DDT as p-glycoprotein isolated from *D. melanogaster,* which have been continuously exposed to DDT for hundreds of generations. Considering there is only 50% sequence homology between human p-glycoprotein and *D. melanogaster* p-glycoprotein, their substrate specificity are likely different. Interestingly, Shabbir et al., (2005) also showed that both DDT and p,p'-DDE induce the p-glycoprotein gene MDR1 in HepG2 cells by 2-3 fold, which is also consistent with work shown by Eufemia et al., (2000) and Tampal et al., (2003). In addition to the induction of MDR1 gene expression, the endoplasmic reticulum molecular chaperone, Bip, was also induced suggesting the induction of an endoplasmic reticulum stress response to DDT and DDE.

CHAPTER 5

XENOBIOTIC METABOLISM OF [^{14}C]-DDT BY *91-R* AND *CANTON-S* FEMALE FLIES FOLLOWING TOPICAL APPLICATION IN ACETONE

5.1 Identification and quantification of [^{14}C]-DDT and its metabolites

The technical grade [^{14}C]-DDT standard used in these experiments had several contaminants present that were first determined via an un-metabolized radiogram of [^{14}C]-DDT applied directly to TLC plates and not extracted from flies (Table 6). The principle components present in the un-metabolized [^{14}C]-DDT standard included p,p-DDT, o,p-DDT, and two unknown contaminants, which are likely DDT isomers Unknown 3 (o,o-DDT) and Unknown 4 (o,m-DDT) with Rf values of 0.50, 0.51, 0.48 and 0.52, respectively, and comprised ~95% of the total CPMs detected (Table 6). Additional components identified were p,p-DDD, o,p-DDD, dicofol, DBP, o,p-DDE, p,p-DDE and DDMU with Rf values of 0.35, 0.39, 0.29, 0.45, 0.53, 0.64 and 0.60, respectively, and comprised ~5% of the total CPMs detected in the un-metabolized [^{14}C]-DDT standard.

Table 6. Total radioactivity (CPM) associated with DDT and its metabolites detected in external rinse, whole body homogenate and excrement extracts for *91-R* versus *Canton-S* female flies at 24 hr post topical application of [^{14}C]-DDT as determined by radiometric TLC-linear analysis.

Compounds (Rf of Standards)	CPM associated with un-metabolized [^{14}C]-DDT (% of Total CPM)	CPM associated with metabolized [^{14}C]-DDT following application to *91-R* females (% of Total CPM applied)		CPM associated with metabolized [^{14}C]-DDT following application to *Canton-S* females (% of Total CPM applied)
p,p-DDE (0.64)	350 ± 140 (1)	261 ± 27 (1)	2.4[b]	108 ± 11 (0.3)[a]
p,p-DDMU (0.60)	70 ± 11 (0.2)	252 ± 43 (1)[a]	2.6	97 ± 12 (0.3)
o,p-DDE (0.53)	1,050 ± 350 (3)	1,815 ± 109 (5)[a]	2.8	652 ± 54 (2)
Unknown 4 (0.52)	8,050 ± 700 (23)	8,397 ± 1,112 (23)		8,439 ± 2,163 (25)
o,p-DDT (0.51)	11,550 ± 2,100 (33)	10,819 ± 1,052 (29)		10,124 ± 1,061 (30)
p,p-DDT (0.50)	11,900 ± 1,050 (34)	9,309 ± 1,050 (25)[a]		10,070 ± 1,040 (30)
Unknown 3 (0.48)	2,100 ± 350 (6)	1,862 ± 292 (5)	1.7	3,111 ± 397 (9)[a]
DBP (0.45)	350 ± 70 (1)	291 ± 42 (1)	2.8	105 ± 17 (0.3)[a]
o,p-DDD (0.39)	14 ± 4 (0.04)	212 ± 31 (1)[a]		191 ± 26 (1)[a]
p,p-DDD (0.35)	14 ± 4 (0.04)	162 ± 23 (0.4)[a]		123 ± 19 (0.4)[a]
Dicofol (0.29)	35 ± 7 (0.1)	122 ± 41 (0.3)[a]	3	42 ± 20 (0.1)
Unknown 2 (0.19)	N.D.	119 ± 28 (0.3)[a]	5	23 ± 6 (0.1)[a]
Unknown 1 (0.14)	N.D.	1,610 ± 475 (4)[a]	10	158 ± 49 (1)[a]
p,p-DDA (0.04)	N.D.	90 ± 23 (0.2)[a]	3	27 ± 6 (0.1)[a]
Conjugates (0.0)	N.D.	1,997 ± 368 (5)[a]	6	330 ± 98 (1)[a]
	35,484 ± 4,750 (100)	37,318 ± 4,716[c]		33,600 ± 4,979[c]

[a]Indicates significant differences between the CPM of the un-metabolized [^{14}C]-DDT and the CPM from either *91-R* and *Canton-S* metabolized [^{14}C]-DDT using Student's t-test ($p < 0.05$).
[b]Indicates fold-increase in the CPM of *91-R* versus *Canton-S* females for significantly different metabolites.
[c]Total CPM associated with metabolized [^{14}C]-DDT was not significantly different from the un-metabolized [^{14}C]-DDT standard.

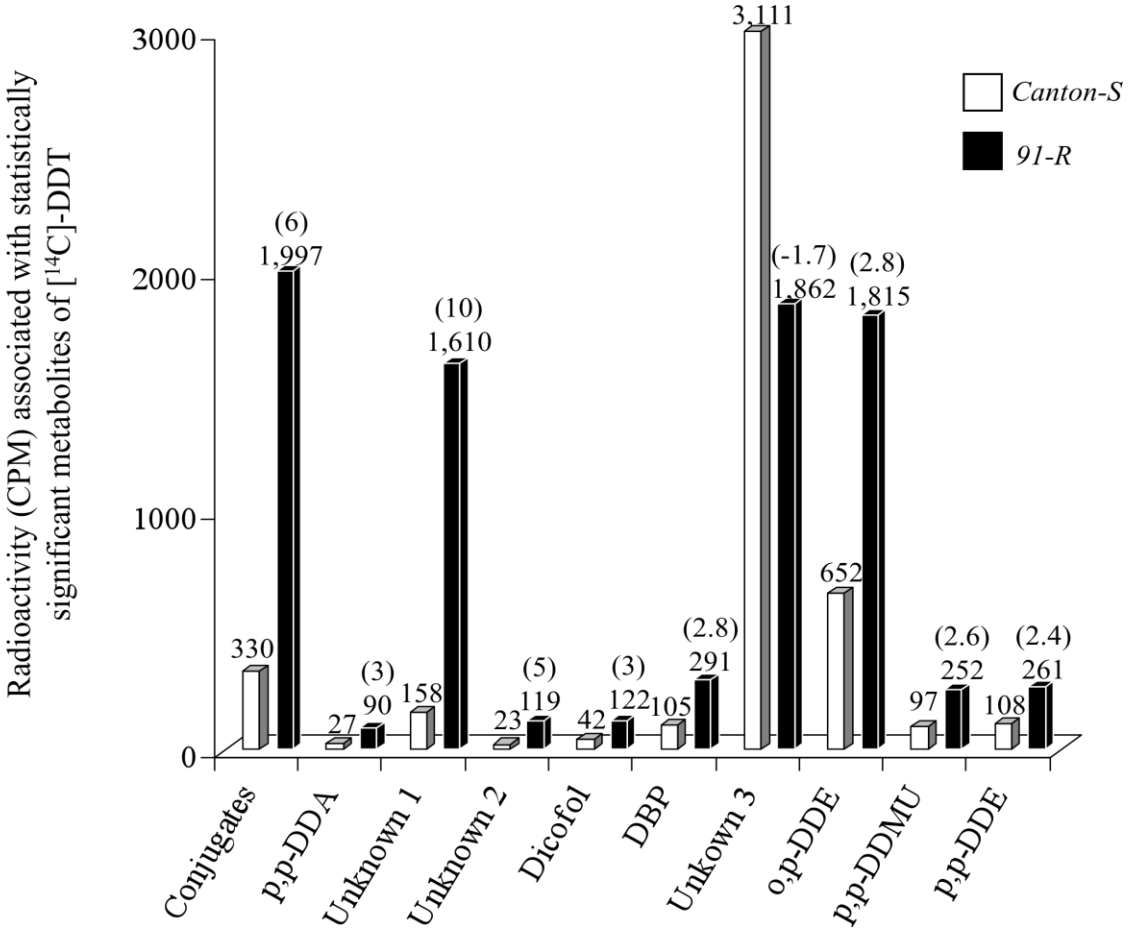
Radiometric TLC-linear analyzer results indicated that [^{14}C]-DDT was converted to numerous metabolic products following topical application to *D. melanogaster* females (Table 6). In addition to the DDT isomers and contaminants found in the un-metabolized [^{14}C]-DDT standard, extracts from flies treated with the standards contained three additional metabolites detected (Unknown 1, Unknown 2, p,p-DDA) and water-soluble CPMs at the origin of the TLC plates, likely representing water-soluble conjugates formed by phase I and II xenobiotic metabolism (Table 6). The presence of these additional compounds and the altered percent composition of the total radioactivity detected (% of total CPM applied) substantiate that *91-R* and *Canton-S* flies were capable of metabolizing [^{14}C]-DDT differently.

Specifically, the relative amounts of 10 DDT isomers and its metabolites detected in the extracts from *91-R* females were significantly different from the composition determined for the 15 compounds detected in the un-metabolized [^{14}C]-DDT standard. *91-R* females metabolized 13.8% of the total applied dose compared to the un-metabolized [^{14}C]-DDT (Table 6). *Canton-S* females metabolized 5.4% of the total applied dose compared to the un-metabolized [^{14}C]-DDT (Table 6). Overall, *91-R* females metabolized ~2.6-fold more of the total applied dose of [^{14}C]-DDT compared to *Canton-S* females.

Similarly, the relative abundance of 9 DDT isomers and metabolites that were extracted from *Canton-S* females were significantly different from the un-metabolized [^{14}C]-DDT standard. Seven metabolites were increased in abundance, 2 were reduced and DDT was unaltered.

Of the 15 DDT isomers and metabolites identified in extracts following [^{14}C]-DDT topical application, the relative abundance of 10 compounds (see boxed compounds, Table 6) were found to be significantly different between the *91-R* and *Canton-S* female flies. The radioactivity at the origin of the TLC plates, likely representing water-soluble conjugates, and Unknown 1 were the most abundant metabolites and were significantly more abundant in extracts from *91-R* females versus *Canton-S* females (Fig 9). Conjugates were 6-fold more abundant and Unknown 1 was 10-fold more abundant (Fig. 9). Unknown 2 and p,p-DDA were the next most abundant metabolites and were significantly more abundant in extracts from *91-R* versus *Canton-S* females. Unknown 2 was 5-fold more abundant and p,p-DDA was 3-fold more abundant. Present in relatively smaller amounts were dicofol (3-fold), DBP (2.8-fold), o,p-DDE (2.8-fold), p,p-DDMU (2.6-fold) and p,p-DDE (2.4-fold), which were all significantly more abundant in extracts from *91-R* versus *Canton-S* females. Only Unknown 3 (putatively identified as o,o-DDT) was found in higher amounts in extracts from *Canton-S* versus *91-R* females. In total, *91-R* females formed ~33-fold more metabolites from [^{14}C]-DDT standard compared to *Canton-S* females.

Figure 9. Radioactivity (CPM) associated with statistically significant metabolites of [^{14}C-]-DDT as determined in Table 6. Values in parenthesis above bars are the fold-difference between CPMs associated with the metabolism of *91-R* versus *Canton-S* female flies.


Radioactivity (CPM) associated with statistically significant metabolites of [14C]-DDT
3000
2000
1000
0
Canton-S
91-R
330
(6)
1,997
27
(3)
90
158
(10)
1,610
23
(5)
119
42
(3)
122
105
(2.8)
291
3,111
(-1.7)
1,862
652
(2.8)
1,815
97
(2.6)
252
108
(2.4)
261
Conjugates
p,p-DDA
Unknown 1
Unknown 2
Dicofol
DBP
Unkown 3
o,p-DDE
p,p-DDMU
p,p-DDE

Radiometric TLC-linear analyzer results were also analyzed separately for significantly different amounts (CPM) of DDT isomers and metabolites in external rinse, whole body, and excrement extracts following topical application of [^{14}C]-DDT to *D. melanogaster* females (Table 7). Of the 15 DDT isomers and metabolites identified in extracts following [^{14}C]-DDT topical application, the relative abundance of metabolites in each extract were found to be significantly different between the *91-R* and *Canton-S* female flies (see boxed compounds, Table 7).

Table 7. CPM of DDT and its metabolites determined separately in external rinse, whole body homogenate and excrement extracts from *91-R* versus *Canton-S* female flies at 24 hr post-topical application of [^{14}C]-DDT in acetone as determined by radiometric TLC-linear analysis.

	CPM (% of Total CPM)						
Compounds	Unmetabolized	External Rinse		Whole Body		Excrement	
(Rf of Standards)	[^{14}C]-DDT	91-R	Canton-S	91-R	Canton-S	91-R	Canton-S
p,p-DDE (0.64)	350 ± 140 (1)	32 ± 3 (0.1)	31 ± 3 (0.1)	54 ± 7 (0.2)	57 ± 6 (0.2)	175 ± 17 (1)*	20 ± 2 (0.1)
p,p-DDMU (0.60)	70 ± 11 (0.2)	33 ± 5 (0.1)	28 ± 3 (0.1)	36 ± 6 (0.1)	51 ± 7 (0.2)	183 ± 32 (1)*	18 ± 2 (0.1)
o,p-DDE (0.53)	1,050 ± 350 (3)	204 ± 8 (1)	190 ± 20 (1)	464 ± 6 (1)*	337 ± 21(1)	1,147 ± 41 (1)*	125 ± 13 (0.4)
Unknown 4 (0.52)	8,050 ± 700 (23)	1,343 ± 192 (4)	1,206 ± 105 (4)	2,583 ± 300 (6)*	6,186 ± 1,011 (18)	4,471 ± 620 (12)*	1,047 ± 81 (4)
o,p-DDT (0.51)	11,550 ± 2,100 (33)	1,714 ± 69 (5)	1,724 ± 164 (5)	3,151 ± 341 (8)*	6,861 ± 794 (21)	5,954 ± 642 (16)*	1,539 ± 103 (5)
p,p-DDT (0.50)	11,900 ± 1,050 (34)	1,573 ± 279 (5)	1,642 ± 157 (5)	2,891 ± 296 (8)*	6,962 ± 800 (20)	4,845 ± 475 (14)*	1,466 ± 83 (5)
Unknown 3 (0.48)	2,100 ± 350 (6)	322 ± 85 (1)	436 ± 43 (1)	739 ± 67 (3)*	2,242 ± 275 (6)	801 ± 140 (2)*	433 ± 79 (1)
DBP (0.45)	350 ± 70 (1)	35 ± 6 (0.1)	30 ± 5 (0.1)	65 ± 8 (0.2)	56 ± 9 (0.2)	191 ± 28 (1)*	19 ± 3 (0.1)
o,p-DDD (0.39)	14 ± 4 (0.04)	22 ± 3 (0.1)	11 ± 3 (0.03)	125 ± 16 (0.4)	174 ± 22 (1)	65 ± 12 (0.2)*	6 ± 1 (0.02)
p,p-DDD (0.35)	14 ± 4 (0.04)	18 ± 4 (0.1)	9 ± 2 (0.04)	90 ± 10 (0.3)	110 ± 16 (0.3)	53 ± 9 (0.2)*	4 ± 1 (0.01)
Dicofol (0.29)	35 ± 7 (0.1)	12 ± 4 (0.03)	12 ± 6 (0.04)	47 ± 15 (0.1)	22 ± 10 (0.1)	63 ± 22 (0.1)*	8 ± 4 (0.02)
Unknown 2 (0.19)	N.D.	21 ± 4(0.1)*	5 ± 2 (0.01)	51 ± 13(0.2)*	17 ± 4 (0.1)	47 ± 11 (0.1)*	1 ± 0.3 (0.01)
Unknown 1 (0.14)	N.D.	33 ± 6 (0.1)*	10 ± 1 (0.03)	649 ± 196 (3)*	142 ± 46 (0.4)	928 ± 273 (3)*	6 ± 2 (0.02)
p,p-DDA (0.04)	N.D.	7 ± 2 (0.02)	4 ± 1 (0.01)	48 ± 12 (0.1)*	20 ± 4 (0.1)	35 ± 9 (0.1)*	3 ± 1 (0.01)
Conjugates (0.0)	N.D.	31 ± 6 (0.1)	34 ± 7 (0.1)	1,012 ± 130 (4)*	229 ± 80 (1)	954 ± 232 (3)*	67 ± 11 (0.2)
	35,484 ± 4,750 (100)	5,400 ± 676 (15)	5,372 ± 522 (16)	12,005 ± 1,423 (37)	23,466 ± 3,105 (67)	19,912 ± 2,563 (51)	4,762 ± 386 (14)

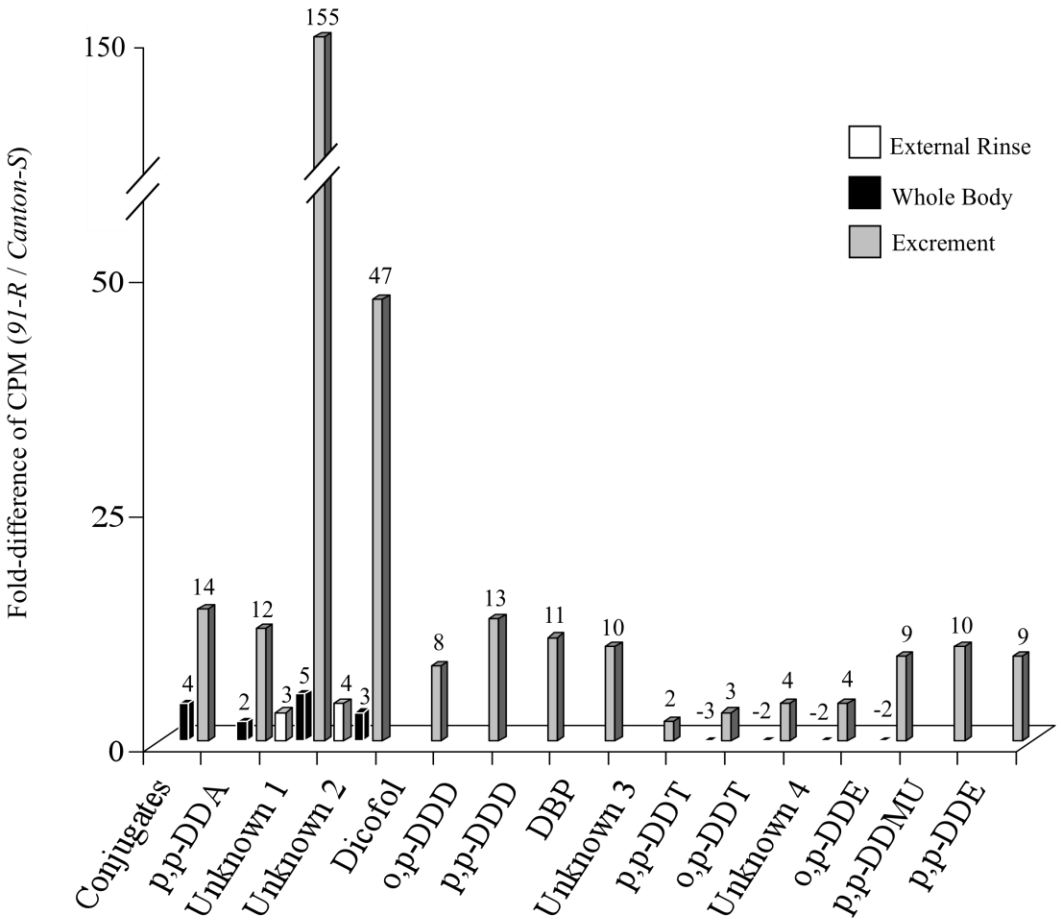
Significant differences between *91-R* versus *Canton-S* females were determined for external rinse, whole body homogenate and excrement extracts using three replicate experiments of 90 female flies from each strain by comparing the mean CPM and standard deviations of each DDT isomer and metabolite. Asterisks (*) indicate a statistical difference between *91-R* and *Canton-S* using Student's t-test ($p < 0.05$).

External rinse extracts of *91-R* females contained significantly greater amounts of Unknown 1 (3-fold more, $p = 0.02$) and Unknown 2 (4-fold more, $p = 0.02$) compared to *Canton-S* females (Fig. 10).

Whole body homogenate extracts of *91-R* females contained significantly greater amounts of water-soluble conjugates (4-fold more, $p = 0.007$), p, p-DDA (2-fold more, $p = 0.04$) Unknown 1 (5-fold more, $p = 0.04$) and Unknown 2 (3-fold more, $p = 0.04$) compared to *Canton-S* females (Fig. 10). Whole body homogenate extracts of *91-R* contained significantly less amounts of p, p-DDT (3-fold less, $p = 0.01$) o, p-DDT (3-fold less, $p = 0.01$) Unknown 3 (2-fold less, $p = 0.01$) and Unknown 4 (3-fold less, $p = 0.03$) compared to *Canton-S* females (Fig. 10).

Excrement extracts of *91-R* females contained significantly greater amounts of all metabolites and DDT isomers, including water-soluble conjugates (14-fold more, $p = 0.02$), p, p-DDA (12-fold more, $p = 0.03$), Unknown 1 (155-fold more, $p = 0.03$), Unknown 2 (47-fold more, $p = 0.01$), dicofol (8-fold more, $p = 0.04$), DBP (10-fold more, $p = 0.004$), p, p-DDD (11-fold more, $p = 0.006$), o, p-DDD (13-fold more, $p = 0.008$), p, p-DDE (9-fold more, $p < 0.001$) o, p-DDE (9-fold more, $p < 0.001$), DDMU (10-fold more, $p = 0.007$), p, p-DDT (3-fold more, $p = 0.002$), o, p-DDT (4-fold more, $p = 0.002$), Unknown 3 (2-fold more, $p = 0.04$) and Unknown 4 (4-fold more, $p = 0.005$) than *Canton-S* females indicating an increased rate of excretion of parent DDT along with its metabolites (Fig. 10).

Figure 10. Fold-differences of CPM of DDT and its metabolites that were found to be significantly different (Student's t-test, $p < 0.05$) for external rinse, whole body homogenate and excrement extracts from *91-R* versus *Canton-S* females determined in Table 7.


Fold-difference of CPM (91-R / Canton-S)
150
50
25
0
External Rinse
Whole Body
Excrement
155
47
14
12
4
2
3
5
4
3
8
13
11
10
2
-3
3
-2
4
-2
4
-2
9
10
9
Conjugates
p,p-DDA
Unknown 1
Unknown 2
Dicofol
o,p-DDD
p,p-DDD
DBP
Unknown 3
p,p-DDT
o,p-DDT
Unknown 4
o,p-DDE
p,p-DDMU
p,p-DDE

5.2 Hydrolysis of water-soluble metabolites of [^{14}C]-DDT

The water-soluble metabolites from the origin of the TLC plate for *91-R* extracts were hydrolyzed to determine what metabolites were conjugated (Table 8). Following an acid hydrolysis, enzyme hydrolysis, and finally base hydrolysis, ~100% of the radioactivity moved from the origin of the TLC plate to higher Rf values. The total amount of identifiable metabolites liberated by hydrolysis was determined to be 39% of the un-hydrolyzed metabolites originally at the origin. The metabolites that were liberated by hydrolysis were p,p-DDA, dicofol, DBP, p,p-DDD, o,p-DDD, Unknown 1, Unknown 2, p,p-DDE, o,p-DDE and DDMU (Table 8). The metabolites liberated from the water-soluble conjugates in the greatest amounts were Unknown 1 (5.5% of total CPM), Unknown 2 (3.6% of total CPM) and dicofol (5.9% of total CPM).

Table 8. Putative metabolite identification of the radioactivity (CPM) at the origins of previously analyzed TLC plates from combined *91-R* extracts following acid, enzyme (glycosidase) and base hydrolysis.

Compounds (Rf of Standards)	CPM associated with acid hydrolysis of [^{14}C]-DDT water soluble metabolites (% of Total CPM)[a]	CPM associated with glycosidase hydrolysis of [^{14}C]-DDT water soluble metabolites (% of Total CPM)[a]	CPM associated with base hydrolysis of [^{14}C] following acid or glycosidase hydrolysis (% of Total CPM)[a]
p,p-DDE (0.64)	7 (0.2)	9 (0.3)	22 (0.7)
p,p-DDMU (0.60)	6 (0.2)	9 (0.3)	21 (0.7)
o,p-DDE (0.53)	8 (0.3)	9 (0.3)	25 (0.8)
Unknown 4 (0.52)	N.D.	N.D.	N.D.
o,p-DDT (0.51)	8 (0.3)	8 (0.3)	23 (0.7)
p,p-DDT (0.50)	7 (0.2)	8 (0.3)	22 (0.7)
Unknown 3 (0.48)	N.D.	N.D.	N.D.
DBP (0.45)	10 (0.3)	15 (0.5)	39 (1.2)
o,p-DDD (0.39)	13 (0.4)	12 (0.4)	35 (1.1)
p,p-DDD (0.35)	14 (0.4)	13 (0.4)	37 (1.2)
Dicofol (0.29)	38 (1.2)	50 (1.6)	98 (3.1)
Unknown 2 (0.19)	14 (0.4)	24 (0.8)	78 (2.4)
Unknown 1 (0.14)	49 (1.5)	26 (0.8)	101 (3.2)
p,p-DDA (0.04)	11 (0.3)	15 (0.5)	42 (1.3)
	185 (5.8)	198 (6.2)	543 (17)

[a] The total number of CPM (~3,200) was split in half and used for acid and glycosidase hydrolysis. The remaining CPM at the origin following acid and glycosidase hydrolysis was used for base hydrolysis.

5.3 Discussion

The results from this study suggest that metabolic resistance to DDT in the *91-R* strain of *D. melanogaster* occurs through an increased ability to detoxify DDT via dechlorination to DDD, dehydrochlorination to DDE, and oxidation to dicofol/DBP. *91-R* also has an enhanced ability to conjugate a number of DDT metabolites, including, dicofol resulting in increased excretion of non-toxic, water-soluble conjugates. The contribution of phase I and phase II metabolism of DDT, however, appears to be secondary to the contributions of reduced penetration and enhanced excretion via phase III metabolism (ABC transporters).

Metabolite analysis of external rinse, whole body homogenate and excrement extracts revealed that [^{14}C]-DDT was converted to several metabolic products that were more polar than DDT (Table 6). DDT metabolites were formed ~30-fold more by *91-R* compared to *Canton–S* females include p, p-DDA, p, p-DDD, o, p-DDD, dicofol, DBP, p, p-DDE, o, p-DDE, DDMU, Unknown 1, and Unknown 2, and water-soluble conjugates. Although 30-fold more metabolites were formed by *91-R*, this only accounts for ~8% of the total applied dose. The minimal amount of metabolism observed by biochemical analysis may still provide substantial resistance to *91-R* females if the enzymes responsible are localized around glial tissue and nerve cells.

The hypothesis tested in this present study suggested that DDT would be directly detoxified by oxidation to dicofol based on previous genetic analysis of the *91-R* strain that indicated overexpression of P450s as the main cause for resistance (Sun et al., 2006). We examined the metabolic products of DDT produced by *91-R* with the intention of

identifying dicofol and/or dichlorobenzphenone (DBP), which is the major excretory product following oxidative conversion of DDT to dicofol. We did detect a significantly greater amount of dicofol and DBP (8- and 10-fold more, respectively) in the excrement extract of *91-R* versus *Canton-S* females. Although there is a significant increase in dicofol and DBP, it is not being formed in amounts as substantial as DDD and Unknown 1. It is possible that dicofol and DBP are being rapidly conjugated as they form but it has never been reported that dicofol or DBP are excreted as conjugates. Our hydrolysis data indicates a proportion of the conjugated metabolites at the origin of the TLC plate were dicofol or DBP. Nevertheless, the oxidative conversion of DDT to dicofol and then to DBP is relatively small. It may be significant, however, if the P450s responsible are highly expressed in glial or nerve tissues, allowing the small increase in oxidative metabolism to significantly affect overall DDT resistance.

DDT is dechlorinated/dehydrochlorinated to DDD and DDE, respectively, with formation of DDD occurring at a faster rate than DDE in most organisms as the conversion to DDE is dependent on DDT dehydrochlorinase activity (Fig. 1, Kitamura et al., 2002). In the present study, we found that the detoxification of DDT in *91-R* occurs via the dechlorination/dehydrochlorination of DDT to DDD or DDE. DDD is the major reductive lipophilic metabolite of DDT and is ultimately converted to the water-soluble metabolite DDA, an organic anion acid, through two proposed mechanisms (Fig. 2, Gold and Brunk, 1983). Additionally, P450s could be involved in detoxification of DDT via reductive metabolism by the mechanism proposed by Kitmura et al., (2003) (Fig 1.). If the over-expressed P450s in *91-R* are reducing DDT under anaerobic conditions, there would be an increase in DDD formation followed by subsequent phase I and phase II

reactions. Previously, the overexpression of P450s in *91-R* was thought to lead to oxidative detoxification and the formation of dicofol but under reductive conditions P450s will form DDD which is more consistent with the results observed in this study.

The major pathway for the conversion of DDT to DDA involves the direct hydroxylation of the C_1 carbon of the ethane moiety forming DDD-OH, which is subsequently hydrolyzed to DDA (Gold and Brunk, 1982). A minor pathway involves the intermediate DDMU, which is formed from dechlorination of DDD. This pathway proceeds with the conversion of DDMU to DDMU-epoxide (Gold and Brunk, 1982). DDMU-epoxide is converted to αOH-DDCHO and excreted as αOH-DDA. In a more complex pathway, DDMU-epoxide is converted to αCl-DDCHO, which can be further metabolized to either αCl-DDA or to αOH-DDA for excretion. αCl-DDCHO can also be dechlorinated to DDCHO and excreted as DDA or DDOH (Gold and Brunk, 1982). Our results indicate that *91-R* females are forming DDA through one or more of these mechanisms. This assumption is reasonable based on the number of unidentifiable metabolite peaks found, and because base hydrolysis of the water-soluble conjugates found at the origin of the TLC plate resulted in the liberation of several products, including DDA.

DDA, an organic acid (anion) metabolite, is likely conjugated to amino acids which further increases its water solubility and the rate of excretion of DDA. DDA forms conjugates with glycine (Gingell, 1976; Reif and Sinsheimer, 1975), serine (Gingell, 1976; Pinto et al., 1965; Reif and Sinsheimer, 1975), aspartic acid (Pinto et al., 1965; Reif and Sinsheimer, 1975), alanine (Gingell, 1976) and glucuronic acid in bile of mammals (Gingell and Wallcave, 1974; Gingell, 1976). In the present study, the major

differences in the metabolites formed by *91-R* versus *Canton-S* females were the amounts of water-soluble metabolites that did not migrate from the origin of the TLC plate. *91-R* females produced ~ 19-fold more water-soluble conjugates versus *Canton-S* females. Base hydrolysis of the water-soluble metabolites resulted in the liberation of most of the radioactivity from the origin of the TLC plates with significant increases of p,p-DDA, Unknown 1, Unknown 2, dicofol, p,p-DDD, o,p-DDD, and DBP (3%, 6%, 5%, 6 %, 2%, 2% and 2% more respectively).

Based on the findings in this study along with previously reported work done on the metabolism of DDT in animals, we conclude that metabolic resistance to DDT is occurring through both direct oxidative detoxification and reductive dechlorination/dehydrochlorination. Dicofol/DBP and other metabolites that are formed by oxidative detoxification of DDT are produced in greater amounts (2.8-, 2.9-fold more) in *91-R* versus *Canton-S* females. DDD (1.2-fold more) and DDE (2.7-fold more) are the only identifiable metabolite that lead to reductive detoxification and are both present in enhanced levels in the *91-R* strain. Oxidation is likely a secondary process enhancing the rate of elimination of DDT, DDD and DDE via DDA. DDD/DDE formed in greater amounts than dicofol/DBP. DDD → DDA formed in greater amounts and as a water-soluble amino acid conjugates. Thus, reductive dechlorination of DDT→DDD→DDA→ conjugate is likely a more important detoxification pathway in *91-R* than is oxidative metabolism of DDT → dicofol/DBP.

CHAPTER 6

SUMMARY AND FUTURE DIRECTIONS

The findings in this study have implicated the importance of reduced penetration, increased metabolism and rapid excretion as DDT resistance mechanisms in the highly DDT-resistant *91-R* strain of *D. melanogaster*. Since the *91-R* females used in this study were shown by bioassay to be more resistant to DDT compared to the insecticide-susceptible *Canton-S* strain, we were able to compare the contribution of each mechanism to the overall level of resistance by measuring them directly by biochemical analysis and indirectly by mortality bioassay.

Examination of reduced penetration by biochemical analysis revealed a 1.5-fold decrease in DDT penetration in *91-R* females versus *Canton-S* females. When penetration was examined by mortality bioassay, there was a 68-fold reduction in resistance levels between *91-R* females and *Canton-S* females using a topical exposure bioassay (RR = 22) versus a contact exposure bioassay (RR = 1,500). Difference in penetration assessed by biochemical analysis versus by mortality bioassay was 45-fold greater in the bioassay analysis.

Examination of increased excretion by biochemical analysis revealed a 5.1-fold increased in DDT excretion by *91-R* females versus *Canton-S* females. When excretion was examined by mortality bioassay, there was a 10-fold difference in resistance levels between *91-R* females and *Canton-S* females using verapamil as a substrate to inhibit efflux of DDT (RR = 22 versus RR = 2.2 in the presence of verapamil). Differences in

excretion assessed by biochemical analysis versus bioassay were 2-fold greater in the bioassay analysis.

Examination of increased metabolism by biochemical analysis revealed a 33-fold increase in DDT metabolite formation by *91-R* females versus *Canton-S* females. The 33-fold difference in metabolite formation was only a small portion of the total applied dose of [^{14}C]-DDT. Of the total applied dose, *91-R* females metabolized 13.8% versus 5.4% for *Canton-S* females. This accounts for a 2.6-fold increase in metabolism by *91-R* versus *Canton-S* females when examining metabolism by biochemical analysis. The contribution of metabolism was 2.2-fold when examining *91-R* females versus *Canton-S* females by the remaining level of resistance in mortality bioassay. Differences in metabolism assessed by biochemical analysis versus bioassay were 1.2-fold greater in the biochemical analysis.

Genetic interactions that contribute to resistance have previously been determined as additive or multiplicative (Raymond et al., 1989). The justification for determining if resistance mechanisms should be combined additively or multiplicatively is unknown because the number of genes and transcript levels of those genes involved in each mechanisms is unknown. Raymond et al., (1989) presents a model based on experimental data to help determine how resistance mechanisms should be combined. A gene responsible for decreased penetration will contribute multiplicatively with other resistance genes. Detoxification genes will combine with an insensitive target site gene multiplicatively during the first moments of intoxication but will then combine additively after saturating the detoxification enzymes. The combination of resistance genes will also vary depending on the type and duration of insecticidal exposure (Raymond et al., 1989).

Using biochemical analysis, the level of resistance that is attributed to penetration, excretion and metabolism multiply (Raymond et al., 1989) to account for ~20-fold level of resistance in *91-R* versus *Canton-S* (1.5-fold X 5.1-fold X 2.6-fold) (Table 9). From the mortality bioassay results, the level of resistance is attributed to penetration, excretion and metabolism multiply to account for ~1,496-fold level of resistance in *91-R* versus *Canton-S* (68-fold X 10-fold X 2.2-fold) (Table 9). Based on these calculations, we attribute 68% of the resistance level to penetration, 10% of the resistance level to phase III excretion, and 2.2% of the resistance level to phase I and phase II metabolism.

Table 9. Summary of the contribution of separate resistance mechanisms to overall DDT resistance determined by biochemical analysis or mortality bioassay in *91-R* females.

Contribution To Level Of Resistance in *91-R*		
Resistance Mechanism	Biochemical Analysis	Mortality Bioassay
Reduced Penetration	1.5-fold	68-fold
Increased Excretion	5.1-fold	10-fold
Increased Metabolism	2.6-fold	2.2-fold

The contribution of each resistance mechanism in the overall level of resistance seen in the *91-R* strain is different when examined by biochemical analysis versus mortality bioassay. There are at least two possible explanations for the differences observed. First, it is possible that we have missed the existence of another resistance mechanism, such as target site insensitivity. Target site insensitivity occurs via *kdr* mutations through mutations in the sodium channel that typically provide an additional ~10-fold level of resistance. It has been shown that point mutation in *D. melanogaster*

sodium channel at sites analogous with those defined by *kdr* mutations in houseflies and other insects, but are located in different homologous units of the channel peptide (Pittendrigh et al., 1997). To date, a *kdr* mutation has not been identified in the 91-R strain and linkage studies would need to be completed to confirm if any sodium channel mutations impart increased levels of resistance. The second explanation for the discrepancies in biochemical analysis versus mortality bioassay resistance level measurements may include the way mechanisms interact. It is possible that the mechanisms are contributing to resistance synergistically. Penetration had a difference of 45-fold when measured by biochemical analysis versus bioassay and is, therefore, the most likely mechanism for synergistic interactions with phase I and phase II metabolism and with phase III excretion.

The mechanisms that produce reduced penetration of DDT, providing resistance to *91-R* females, are not yet known. Due to its lipophilicity, the ability of DDT to traverse the cuticle of flies is largely dependent on the lipid hydrocarbon content of the waxy layer of the cuticle in resistant flies. The findings in the present study establish that there are significantly greater amounts of at least 5 hydrocarbons in the cuticle of *91-R* versus *Canton-S* females. The increased quantity of hydrocarbon in the waxy layer of the cuticle could potentially produce variations in the cuticle slowing the penetration of DDT to the underlying cells. The 10-fold increase determined in the total amount of significantly different hydrocarbons in *91-R* versus *Canton-S* females caused a 1.5-fold reduction in the ability of DDT to penetrate the cuticle. The impact of this is observed substantially in that resistance ratios between *91-R* and *Canton-S* females went from 1,500 to 22.2 when

DDT was applied topically in acetone, resulting in 68-fold reduction in overall resistance determined for *91-R* females.

The mechanisms by which increased excretion of DDT is capable of providing resistance to *91-R* females involves effluxing DDT from cells and from the excretion of DDT from the body of flies via the Malphigian tubules. The findings in this study suggest that ABC-transporters are involved in the increased excretion of DDT from *91-R* females. The resistance ratio between *91-R* and *Canton-S* females was shifted from 22.2 to 2.2 when ABC-transporters were inhibited with verapamil, suggesting that p-glycoprotein provides 10-fold increase in resistance for *91-R* females. The increased transcript levels of p-glycoprotein in *91-R* females are consistent with such a mechanism, providing protection from DDT by lowering the concentration of DDT at the receptor site.

Overall, the implications from this research suggest that the observed resistance levels in *91-R* females involves more than just the contribution of xenobiotic metabolism and that the high resistance levels seen in *91-R* females is due to multiple mechanisms including reduced penetration and increased excretion of DDT. The contribution of reduced penetration is to delay the ability of DDT to partition into target cells and the contribution of ABC-transporters is to excrete DDT from the hemolymph, reducing the ability of DDT to bind nerve receptors. Slowing uptake and increasing excretion may allow *91-R* females enough time to detoxify a critical amount of DDT.

Addition studies to support the findings in this study could include RNA interference (RNAi) of P450 genes, linkage studies to confirm kdr, identification and induction of genes in cuticle formation and further examination of ABCB and ABCC-type efflux transporters. Studies in the examination of ABC-transporters is the next most

logical approach for understanding resistance in *91-R*. There are currently several research groups that are experts in P450 genetics and are taking on the task of further examination of their role in DDT-resistance in insects. Examining penetration as a resistance mechanism would also be a good approach but very little is known about genes involved in cuticular penetration and identification of those genes will provide a better interpretation of the role reduced penetration has in resistance.

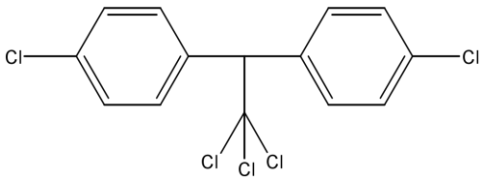
Our findings suggest that ABC-transporters are a major mechanism leading to DDT resistance in *91-R* females. To determine if ABC transporter genes, such as p-glycoprotein or MRPs over-expressed in *91-R*, are able to efflux DDT, the next line of investigation would entail heterologous expression of ABC-transporters from *91-R* females in *Xenopus laevis* oocytes and monitor efflux of DDT in the presence and absence of selected ABC-transporter inhibitors. Since ABC-transporters efflux insecticides out of cells, it would be expected that injecting DDT into the oocyte would cause an increased rate of efflux of DDT out of the cell compared to eggs not overexpressing these transporters. In the presence of transporter inhibitors such as verapamil, it would be expected that efflux activity would decrease and there would still be significant concentrations of DDT inside the oocytes.

To determine if one or multiple ABC transporter genes are over-expressed by *91-R*, the next line of investigation would also entail RNAi of multiple transporter genes from *91-R* females to monitor transcript levels of these genes by qPCR. Since injection of RNAi does not kill female flies it would be possible to bioassay the injected flies with DDT, after knockdown of ABC-transporter genes by RNAi. It would be expected that injected flies would become more sensitive to DDT in bioassay and the ABC-transporter

most responsible for DDT efflux could be identified. Once identified, ABC transporters could be functionally screened in oocytes and surrogate substrates identified that could be used as directed synergists in a resistance management scheme.

APPENDIX

STRUCTURE OF DDT, METABOLITES AND VERAPAMIL



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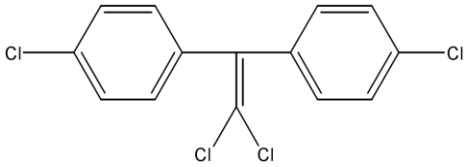

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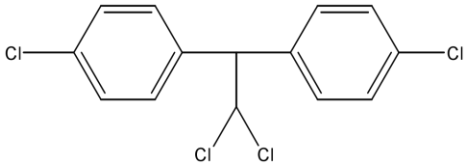
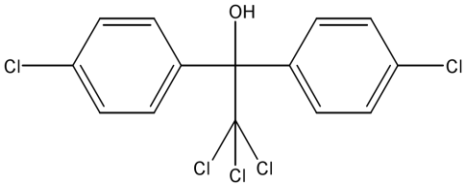
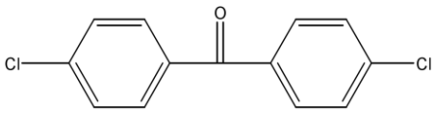


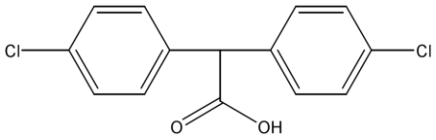
DDD



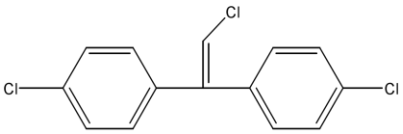
Dicofol



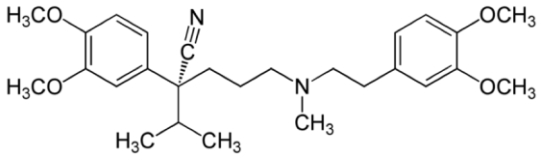
DBP



DDA



DDMU



Verapamil

Appendix: The structures of DDT, metabolites of DDT and verapamil examined in this study.

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