Functional Characterization of Carboxylesterases in Pyrethroid Resistant House Fly,

Musca domestica

by

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Abstract

House fly, *Musca domestica*, is a major sanitary pest which can carry and transmit more than 100 human and animal intestinal diseases. Currently, the chemical control with insecticides is still the most efficient weapon to control its population. However, the intensive and inappropriate use of insecticides will lead to resistance issue. House fly can quickly develop resistance and cross-resistance to multiple insecticide classes. The easily development of resistance, large offspring population, and availability of genome and transcriptome database, all of which made house fly become a model insect for insecticide resistance study.

As one of the major detoxification enzymes, carboxylesterases play vital roles in metabolizing insecticides and thereby conferring resistance in insects. Up-regulation of carboxylesterase genes is thought to be a major component of resistance development. In our study, a total of 39 carboxylesterase genes have been identified in house fly, eleven of which were significantly overexpressed in resistant ALHF strain compared with susceptible aabys and wild-type CS strains. Eight up-regulated carboxylesterase genes with their expressions were further induced to higher levels in response to permethrin treatments, indicating that both of the constitutive and inductive overexpression of carboxylesterases is co-responsible for the enhanced detoxification of permethrin. Further spatial expression studies revealed that these carboxylesterases were abundantly

distributed in detoxification tissues and genetically mapped on autosome 2 and 3 of house flies, and their expressions could be regulated by factors on autosome 1, 2 and 5. The functions of up-regulated carboxylesterases were further explored through *in vitro* metabolism studies. Here, the baculovirus-mediated insect cell expression system was employed to large-scale produce interested carboxylesterase proteins. Our results indicated that these carboxylesterase proteins efficiently hydrolyzed esterase substrate α-naphthyl acetate. A cell-based MTT cytotoxicity assay revealed that Sf9 cells expressing targeted carboxylesterases enhanced the tolerance to permethrin, suggesting the important roles of carboxylesterases in metabolizing permethrin. The metabolic functions of carboxylesterases were further characterized by conducting *in vitro* metabolism toward permethrin, and our results suggested that these carboxylesterases showed significant efficiencies in metabolizing permethrin *in vitro*. Homology modelling and docking analysis were constructed to illustrate the interaction between carboxylesterases and permethrin, thus confirming the metabolic roles of carboxylesterases against insecticides in house flies.

Besides the quantitative overexpressions of carboxylesterases, the qualitative changes of carboxylesterases are also responsible for their enhanced hydrolytic activities toward permethrin and thereby conferring pyrethroid resistance in insects. For carboxylesterase MdαE7 gene, eight mutations have been identified in resistant ALHF strain and four homozygous offspring line A1234, A1245, A1235 and A2345, which not only confirming that the MdαE7 gene is located on autosome 2 of house flies, but also indicating that these mutations have correlated with pyrethroid resistance in house flies. We then introduced these mutations individually into the MdαE7 gene extracted from

aabys strain and investigated their functions through cell-based MTT assay and *in vitro* metabolism studies. Our results showed that three mutations could significantly enhance the hydrolytic activities of MdαE7 to permethrin at the expense of decreasing their carboxylic activities to generic esterase substrate α-NA, indicating that these mutations have similar effects with "mutant ali-esterase hypothesis" and play important roles in conferring pyrethroid resistance in house flies.

Taken together, this study firstly comprehensive investigated the carboxylesterases in house flies and emphasized their important roles in conferring pyrethroid resistance in insects, which may facilitate the better understanding of carboxylesterase-mediated resistance and thereby providing novel strategies to efficiently prevent or impede the development of insecticide resistance in insects.

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List of Abbreviations

ANOVA	analysis of variance
ASPCR	allele apecific PCR
BC1	back-cross generation 1
Bp	base pair
COE	carboxylesterase
cDNA	complmentary DNA
CBDP	2-(o-cresyl)-4H-1,2,3-benzodioxa-phosphorin-2-oxide
DDT	dichloro-diphenyl-trichloroethane
DEF	S, S, S-tributylphosphorotrithioate
DFP	fluorophosphorous derivative diisopropylphosphorofluoridate
DNA	deoxyribose nucleic acid
dsRNA	double stranded RNA
GABA	gamma aminobutyric acid
GST	glutathione S-transferase
kdr	knockdown resistance
LD_{50}	lethal dosage killing 50% of a tested population
Iso-OMPA	tetraisopropylpyrophosphoramide
mRNA	messenger RNA
MOI	multiplicity of infection

OP	organophosphate
ORF	open reading frame
P450	cytochrome P450 monooxygenase
PCR	polymerase chain reaction
qRT-PCR	quantitative real-time polymerase chain reaction
RNA	ribose nucleic acid
SNP	single nucleotide polymorphism
Sf9 cells	spodoptera frugiperd

Chapter 1: Literature Review

1.1 House flies

The house fly, *Musca domestica* L. (Diptera: Muscidae), is an important sanitary pest which can carry and transmit more than 100 human and animal intestinal pathogens, including bacteria (salmonellosis, shigellosis, typhoid fever and cholera); protozoan (amebic dysentery); helminth (roundworms, hookworms and tapeworms); and virus (palio, coxsackievirus, paramyxoviridae, and enterovirus) (Scott et al. 2014; Barin et al. 2010). Recent studies have found that house flies are also the potential carriers of lethal *Escherichia. coli* O157: H7 and pathogenic avian influenza virus H5N1 (Ripoll et al. 2015; Tyasasmaya et al. 2016). House flies picked up pathogens through their mouthparts and body surface, and transmitted to human and animals when making contacts (Malik et al. 2007). Besides that, high density of house fly population also causes stress to poultry farmers and affects the economic values of their products, and the annual cost used to control house fly in poultry farms in the USA has been estimated to be over 1.6 millions (Acevedo et al. 2009).

Current efforts to control house flies still heavily relied on the insecticide application. Multiple insecticides from organophosphates, pyrethroids, carbamates, to some relative new insecticides, such as abamectin, imidacloprid, indoxacarb, fipronil and spinosad, have been used to control fly populations (Memmi 2010; Khan et al. 2013;

Abbas et al. 2015; Naqqash et al. 2016). Some bioinsecticides, such as the essential oils extracted from plants, also been applied to control house flies (Palacios et al. 2009; Urzua et al. 2010; Kumar et al. 2012; Morey et al. 2012). Among them, pyrethroids constitude the majority of household insecticides widely used for fly control, owning to their high efficiency, low mammalian toxicology and environmental friendly (Kaufman et al. 2010; Abbas et al. 2014; Scott et al. 2013). However, house fly can rapidly develop resistance and cross-resistance to multiple insecticides (Liu and Yue, 2000), which has now become the global barrier to efficiently control the vector-borne infections (Nauen, 2007; Liu, 2015). Therefore, it is urgent to elucidate the mechanisms governing the insecticide resistance and therefore designing novel strategies to prevent the resistance development and ultimately reduce the prevalence of fly-carrier diseases.

1.2 The insecticide resistance

According to the World Health Organization (WHO), resistance refers to "the development of an ability in a strain of some organisms to tolerate doses of a toxicant, which would prove lethal to the majority of individuals in a normal population of the same species" (WHO, 1957). The insecticide resistance is a pre-adaptive phenomenon, where prior to insecticide exposure, rare individual carrying one or more resistant alleles can survive under stresses and make this ability quickly developed in their populations (Li et al. 2013). Up to date, more than 600 arthropod insects are assumed to be resistant to one or more insecticides and this number continuous arise with injudicious use of insecticides (Whalon et al. 2008). In recent decades, relative researches mainly focused on the development and mechanisms of insecticide resistance (Roush and Tabashnik, 2012), and insects can develop not only resistance to a single insecticide used to control

them, but also cross-resistance to multiple insecticides with same target sites and modes of action (Casida, 2009). Our previous studies have also pointed out that house flies can develop high resistant level not only to pyrethroids, but also to organophosphates and some relative new insecticide classes (Liu and Yue, 2000). Deeper exploration at molecular level has indicated that multiple mechanisms are indeed involved in insecticide resistance. Among them, the decreased target site sensitivity caused by the mutations on the target sites, such as the voltage-gated sodium channels, acetylcholinesterases and GABA receptor genes as well as the increased metabolic detoxification resulted from the transcriptional overexpressions of detoxifying enzymes are major mechanisms responsible for the resistance development (Ranson et al. 2010: Liu et al. 2015). Besides that, some other mechanisms, such as the insect behavioral changes (e.g. reducing the proximicity to insecticides) and physiological changes (e.g. reducing the penetration of insecticides via the evolvement of thickened or altered cuticles) have also involved in the insecticide resistance (Sokhna et al. 2013; Koganemaru et al. 2013). All these mechanisms work alone or together to confer high level of resistance in insects. The elucidation of mechanisms underlying the insecticide resistance will play a vital role in preserving insecticide efficacy, developing novel insecticides and finally implementing pest control efficiently.

1.2.1 The decreased target sites sensitivity

The target site insensitivity in insect nervous system caused by structural modifications of target proteins resulted from mutations on coding regions is recognized as one of the major mechanisms underlying insecticide resistance (Casida and Durkin, 2013). Three main target sites are well explored, including the insect voltage gated

sodium channel which is the major target of dichlorodiphenyltrichloroethane (DDT) and pyrethroids (Davies et al. 2008; Dong et al. 2014); Acetylcholinesterases which are key targets of organophosphates (OPs) and carbamates (Kwon et al. 2010; Walsh et al. 2001; Kim et al. 2011); and γ-animobutyric acid (GABA) receptors which are the target sites of cyclodiene and fipronil insecticides (Nakao et al. 2010; Wondji et al. 2011; Wang et al. 2013).

1.2.1.1 The insect voltage-gated sodium channel

The voltage-gated sodium channel is an integral transmembrane protein essential for the initiation and propagation of action potentials in the central nervous system. It is composed of a pore-forming α-subunit and several β subunits which modulate the sodium channel expression and gating properties (Brackenbury and Isom, 2011). The α-subunit is consisted of four homologous domains, each containing 6 transmembrane segments. The Segment 1-4 (S1-S4) form the voltage-sensing module, and S5, S6 and a connecting membrane loop together form the pore module (Catterall, 2014). Insecticides DDT and pyrethroids target on the insect sodium channel, exert their insecticidal activities by altering channel gating properties and prolonging its opening state for unusual time, which in turn lead to the repetitive discharges and depolarization of membrane potentials and later cause insects exhausted to die (Dong, 2007; Davies et al. 2008). Modifications of insect sodium channel structure via polymorphisms occurring on the coding region will result in a reduction or elimination of binding affinity of insecticides to channels, thereby decreasing the toxic effects of insecticides and leading to resistance development in insects (Burton et al. 2011). This kind of resistance was also known as the knockdown resistance (kdr), which was firstly reported in house flies and now has been widely

identified in a number of medically or agriculturally important insect species (Busvine, 1951; Dong, 2007). Among the multiple kdr mutations, Leu to Phe (the substitution of leucine by phenylalanine), located in the segment 6 of domain II (IIS6), was firstly identified in pyrethroid resistant house fly *Musca domestica*, with its roles in conferring pyrethroid resistance have been confirmed via *in vitro Xenopus* oocyte expression experiments (Knipple et al. 1994). This substitution is currently the most common kdr mutation widely identified in multiple pyrethroid resistant insects, including mosquitoes, cockroaches, moths, aphids, ticks and beetles (Ranson et al. 2000; Park and Taylor et al. 1997; Martinez-Torres et al. 1999; He et al. 1999; Nauen et al. 2012). Another point mutation, Met to Thr, known as super-kdr mutation, has also been identified in sodium channel gene which can confer higher pyrethroid resistance when combined with the Leu to Phe mutation (Lee et al. 1999). Up to date, more than 30 sodium channel mutations have been detected in more than one resistant insect species, multiple of which with their roles in conferring insecticide resistance have been examined through *Xenopus oocyte* expression systems (Rinkevich et al. 2013). These mutations can confer knockdown resistance either alone or in combination. Our lab has reported the strong correlation between the frequency of polymorphism distribution and insecticide resistance levels in several insect species, including *Culex quinquefasciatus*, *Musca domestica* and *Blattella germanica*, further suggesting the involvement and inheritance traits of these sodium channel mutations in insect resistance evolution (Xu et al. 2006; Tian et al. 2011; Li et al. 2012).

1.2.1.2 Acetylcholinesterases

As the primary target of organophosphates and carbamate insecticides, acetylcholinesterases can hydrolyze the neuron transmitter acetylcholine to acetate and choline and terminate the signal transduction at cholinergic synapse of insects. Organophosphates (OPs) and carbamates can inhibit the AChE activity and make insects over-exhausted to die. Therefore, the insensitivity of AChE to insecticides resulted from point mutations is another important mechanism underlying the insecticide resistance. Most insects and ticks have at least two acetylcholinesterases, AchE1 and AchE2 encoded by Ace-1 and Ace-2 genes, respectively, while only AchE1 has synaptic functions. Since the first report that AChE insensitivity caused by point mutations of Ace gene in *Drosophila melanogaster* (Mutero et al. 1994), various point mutations of Ace gene responsible for insecticide resistance have been continuously described in a variety of insect species (Fournier, 2005; Lee et al. 2015). Among these mutations, G119S has been frequently identified in multiple resistant mosquito species and then used as a diagnostic tool for carbamate and OP resistance (Djogbenou et al. 2010; Alou et al. 2010; Essandoh et al. 2013). Another mutation, F455W substitution has also been widely identified in *Tetranychus urticae*, *Musca domestica*, *Spodoptera frugiperda* and *Bemisia tabaci* with its association with carbamate and OP resistance (Khajehali et al. 2010; Yuan et al. 2012; Zhang et al. 2013; Kwon et al. 2010; Carvalho et al. 2013). Other Ace-1 mutations, such as G265A, S431F, A201S, G368A, A391T, and etc., were also identified in various insect species with their involvement in conferring insecticide insensitivity either individually or in combination (Walsh et al. 2001; Menozzi et al. 2004; Carletto et al. 2010; Khajehali et al. 2010; Kwon et al. 2010).

1.2.1.3 The GABA receptor

Gamma-aminobutyric acid (GABA) is the principal inhibitory neurotransmitter in vertebrates and invertebrates. The GABA receptor is a heteromultimeric gated chloride-ion channel in the insect's central nervous system and implicated as the target site for cyclodiene and fipronil insecticides. The GABA receptor is made up of five subunits, each of which containing four transmembrane domains (M1-M4) and one extracellular N-terminal domain. The transmembrane M2 provides the key residues lined the ion channel for insecticides binding. Once the insecticides bind within the ion channel, the flow of chloride ions through the receptor channel complex will be inhibited and which in turn cause the insects exhausted to die (Le Goff et al. 2005). Several substitutions in coding region of GABA receptor gene are closely related with fipronil and cyclodiene resistance in a variety of insects. Among them, A to S (or G) is the most common one which has already been identified in multiple resistant species, including *Musca domestica* (Thompson et al. 1993a), *Hypothenemus hampei* (ffrench-Constant et al. 1994), *Myzus persicae* (Anthony et al. 1998), *Tribolium castaneum* (Andreev et al. 1999), *Ctenocephalides felis* (Daborn et al. 2004), *Anopheles Arabiensis* (Du et al. 2005), *Drosophila simulans* (Le Goff et al. 2005), *Plutella xylostella* (Yuan et al. 2010), *Anopheles gambiae* (Kwiatkowska et al. 2013), and *Diabrotica virgifera* (Wang et al. 2013). Besides that, another V to I mutation can confer high resistant level to dieldrin in *Anopheles funestus* when co-existed with A to S substitution (Wondji et al. 2011). A point mutation R340Q in the GABA receptor was associated with fipronil resistance in *Sogatella furcifera* (Nakao et al. 2010).

1.2.2 Increased metabolic detoxification

As one of the predominant mechanisms underlying insecticide resistance, the increased metabolic detoxification primarily relied on three major detoxification enzyme families: cytochrome P450 monooxygenases (CYP450s), carboxylesterases (COEs) and glutathione-S-transferases (GSTs). The overexpression of these detoxifying enzymes resulted from either transcriptional up-regulation or gene amplification will lead to the enhanced enzymatic activities toward insecticides and thereby contributing to the development of insecticide resistance.

1.2.2.1 Cytochrome P450 monooxygenases

As the largest supergene family, cytochrome P450-dependent monooxygenases are hydrophobic, heme-containing enzymes involved in metabolism of numerous endogenous and exogenous compounds. The activities of P450s in eukaryotic organisms require the coexistence of flavoprotein NADPH and cytochrome P450 reductase (CPR), which is now considered as the novel target for the development of insecticide synergists (Lycett et al. 2006; Lian et al. 2011). The silence of CPR will lead to the reduced insecticide resistance in *Cimex lectularius* (Zhu et al. 2012) and *N.lugens* (Liu et al. 2013). Up to date, more than 2000 P450 genes have been identified in various insect species, and the number of cytochrome P450s extracted from whole genome databases of different organisms ranged from 37 in *Pediculus Humanus* (Lee et al. 2010) to 204 in *Culex quinquefasciatus* (Yang and Liu, 2011). The first report of CYP450s involved in insecticide resistance was in early 1960's that the sensitivity of house flies to carbaryl was increased by the P450 inhibitor sesame (Eldefrawi et al. 1960). From that on, the P450-mediated resistance has became one of the most recognized mechanisms involved

in insecticide resistance in various insects. Multiple P450s with their overexpression levels were closely related with resistance development, especially for the P450 genes belong to the CYP6, CYP4 and CYP9 families. The first insect P450 gene, CYP6A1, was cloned from a diazinon resistant house fly strain (Feyereisen et al. 1989). Since then, an increasing number of overexpressed P450s were identified in various insecticide resistant species, including *Drosophila melanogaster* (Daborn et al. 2002), *Drosophila simulans* (Le Goff et al. 2003), *Myzus persicae* (Puinean et al. 2010; Bass et al. 2014), *Tribolium castaneum* (Zhu et al. 2010), *Aedes Aegypti* (Stevenson et al. 2012; Riaz et al. 2013), *Aedes albopictus* (Ishak et al. 2016); *Anophels fruestus* (Riveron et al. 2013), *Anopheles gambiae* (Stevenson et al. 2011; Mitchell et al. 2012), *Culex quinquefasciatus* (Komagata et al. 2010), *Nilaparvata lugens* (Bass et al. 2011; Ding et al. 2013), *Bemisia tabaci* (Nauen et al. 2013), *Frankliniella occidentalis* (Cifuentes et al. 2012), and etc. In house flies, multiple P450s genes were also identified with their expression levels were higher in resistant strain compared with those in susceptible one (Liu and Scott, 1998; Zhu et al. 2008a; Li et al. 2013). Some P450s with their expressions can not only be constitutively up-regulated in resistant insect strains, but also can be further induced to high levels in response to insecticide exposures (Markussen et al. 2010; Liu et al. 2011; Gong et al. 2013). By using the GAL4/UAS system to express P450s in transgenic drosophila flies and further conducting transgenic fly bioassay, an increased tolerance to insecticides was observed, indicating the functions of P450s in metabolizing insecticides and thereby conferring resistance in insects (Daborn et al. 2007). The further heterologous expression studies as well as the homology modelling and docking analysis of multiple mosquito P450 genes have suggested that P450s can not only metabolize pyrethroids, but also

metabolize the potential pyrethroid metabolites, phenoxylbenzyl acohol (PBAlc) and phenoxybenzaldehyde (PBAld) *in vitro* (Chandor-Proust et al. 2013; Gong et al. 2017). In recent years, with the rapid development of cutting-edge genome-editing techniques, such as RNA interferences (RNAi) and CRISPR-Cas9 technique, some researchers have successfully characterize the roles of P450s in metabolizing insecticides by inhibiting or eliminating their expression in insects (Mao et al. 2007; Chung et al. 2009; Itokawa et al. 2016).

1.2.2.2 Esterases

The esterase family is another major enzyme family which can hydrolyze a wide range of ester-containing substrates, including insecticides. In insects, esterase-mediated metabolism is another important mechanism underlying resistance development of multiple ester-containing insecticides, including organophosphates (OPs), carbamates, and pyrethroids (Li et al. 2007; Hotelier et al. 2010; Bass and Field, 2011). Most esterases involved in resistance belong to the carboxylesterase gene family (Hotelier et al. 2010). Carboxylesterases mediated the resistance development in insects either through the quantitative changes (gene amplification/ transcriptional up-regulation) which result in the enhanced carboxylesterase activity toward insecticides or through the qualitative changes (gene coding sequence mutations) which can alter the binding affinity of carboxylesterases to insecticides. For the quantitative changes, multiple overexpressed carboxylesterases genes resulted from gene amplification and up-regulation have been documented in various insecticide resistant species, such as *Helicoverpa armigera* (Wu et al. 2011), *Rhipicephalus microplus* (Nandi et al. 2015), *Boophilus microplus* (Guerrero et al. 2012), *Plutella xyloestella* (Moharil et al. 2008), *Frankliniella occidentalis* (Maymo et

al. 2006), *Tetranychus urticae Koch* (Yorulmaz, 2010), and etc. In house flies, multiple up-regulated carboxylesterases were also identified in highly resistant strains (Zhang et al. 2007). For the qualitative changes, the point mutations occurring on the coding region of carboxylesterases can significantly shift the substrate preference from generic substrate (e.g. α-naphthyl acetate) to ester-containing insecticides. This mechanism, also known as "mutant ali-esterase hypothesis", has been identified in various OP resistant insects (Newcomb et al. 1997; Campbella et al. 1998; Cui et al. 2011; Gong et al. 2017; Gacar and Vatan, 2009). Two mutations, G137D and W251L found in the LcαE7 gene of *Lucilia cuprina* and its orthologous MdαE7 gene of *Musca domestica* were closely related with "mutant ali-esterase hypothesis" and were responsible for OPs resistance (Campbell et al. 1998; Claudianos et al.1999). In some cases, both of the qualitative changes and quantitative changes of carboxylesterases are co-responsible for the high level of resistance development (Pan et al. 2009; Zhang et al. 2010).

1.2.2.3 Gluathione S-transferases

The glutathione S-transferases (GSTs) belong to a diverse family of enzymes found ubiquitously in aerobic organisms. They play important roles in detoxifying a wide range of xenobiotics including insecticides. GSTs can metabolize insecticides by catalyzing their reductive dehydrochlorination or by conjugation reactions with reduced glutathione (GSH), producing water-soluble metabolites that are more easily excreted (Enayati et al. 2005). Elevated GSTs activity as a result of gene amplification or transcriptional up-regulation has been associated with resistance to nearly all the major insecticide classes. For organochlorine insecticides, dehydrochlorination catalyzed by GSTs is considered as a major mechanism for DDT resistance in various insect species,

such as *Aedes aegypti* (Lumjuan et al. 2011), *Anopheles gambiae* (David et al. 2005; Wang et al. 2008), and *Anopheles arabiensis* (Jones et al. 2012). The metabolic roles of overexpressed GSTs toward DDT were confirmed through crystallographic and NMR structure analysis (Low et al. 2010). Further genetic mapping of the major loci conferring DDT resistance indicated that both cis- and trans-factors were contributed to the overexpression of GSTs related with DDT resistance (Ranson et al. 2001; Ding et al. 2005). Besides the quantitative changes, the qualitative changes of GSTs associated with DDT resistance were also reported in insects. For instance, a single amino acid substitution L119F in an upregulated GST gene was responsible for the high level resistance to DDT in *Anopholes funestus* (Riveron et al. 2014). In addition to organochloride insecticide, the GSTs were also involved in conferring organophosphate resistance in various insects, such as *Plutella xylostella* (Sonoda and Tsumuki, 2005), *Locusta migratoria* (Qin et al. 2013), *Bombyx mori* (Yamamoto et al. 2009) and etc. In addition, GSTs were also reported to be implicated in pyrethroid resistance with its enhanced activity resulted from gene overexpression in various insects, such as *Sitophilus zeamais* (Fragoso et al. 2007), *Sarcoptes scabiei* (Mounsey et al. 2010), *Bombyx mori* (Yamamoto et al. 2009), *Aedes aegypti* (Lumjuan et al. 2011), *Helicoverpa armigera* (Ugurlu et al. 2007), *Bactrocera doesalis* (Hu et al. 2014), and etc. For carbamate insecticides, there were studies reported that GSTs may be involved in detoxification of carbaryl in *Locusta migratoria* (Qin et al. 2012; Qin et al. 2013).

1.2.3 Other mechanisms

1.2.3.1 The decreased penetration

Decreased penetration associated with insecticide resistance was firstly reported in early 1960. Even though this physiological modification plays minor roles in conferring resistance in insects, however, it can significantly reduce the insect sensitivity to insecticides by working together with other mechanisms, such as the enhanced metabolism and target site insensitivity (Scott, 1990; Wu et al. 1998; Wen and Scott, 1999; Valles et al. 2000; Ahmad et al. 2006). The thickening or remodeling of cuticle resulted from the increased steady-state transcript levels of multiple cuticle proteins may contribute to the decreased insecticide penetration, which in turn lead to resistance development in insects (Wood et al. 2010; Koganemaru et al. 2013; Lin et al. 2012; Zhang et al. 2008; Pan et al. 2009; Vannini et al. 2014; Strycharz et al. 2013; Lilly et al. 2016).

1.2.3.2 The accelerated excretion

The accelerated excretion is another mechanism implicated in insecticide resistance which has been reported in several insect species. For example, in *Leptinotarsa decemlineata*, the increased excretion has been regarded as a major mechanism to rapidly remove imidacloprid (Mota-Sanchez, 2003) and glycoalkaloids (Krishnan et al. 2007); In *Frankliniella occidentalis,* the excretion rate was higher in diazinon-resistant strain compared with that in susceptible one (Zhao et al. 1994); In *Heliothis virescens*, the excretion level of cypermethrin was higher in larvae of some field collected strain compared with that in susceptible reference strain (Ottea et al. 1995); *In vivo* distribution

studies have also revealed the increased excretion rate of carbaryl in resistant *Diabrotica virgifera virgifera* populations (Scharf et al. 1999).

1.2.3.3 The behavioral resistance

The behavioral resistance means that the insects have behavior to reduce their exposure to toxic compounds (e.g. insecticides) and thereby allowing them to survive in a harmful or fatal environment. Two different types underlying the behavioral resistance: the stimulus-dependent and stimulus-independent mechanism. Direct insecticide contact is required for the stimulus-dependent mechanism, such as the increased repellency and irritancy. While no insecticide contact is required for the stimulus-independent mechanism, such as exophily (Sparks et al. 1989). The behavioral resistance has been reported in several insect species. For example, in *Stomoxys calcitrans*, the repellency and irritancy of pyrethroids was greater in resistant strain (Quinenberry et al. 1984), and later dose-response studies further revealed the unique behavioral response patterns of resistant horn flies to insecticides, demonstrating the importance of behavioral resistance in insects (Lockwood et al. 1985). The *Blattella germanica* can exhibit a high level of behavioral resistance to Avert and Maxforce FC gel baits (Wang et al. 2004); In *Sitophilus zeamais*, the irritability were observed among different populations, while no significant correlation was discovered between this behavioral mechanisms and fenitrothion resistance (Braga et al. 2011).

1.2.4 The interactions of multiple resistant mechanisms

Given that a limited number of mechanisms conferring insecticide resistance, it is possible to establish general rules for interactions between multiple mechanisms. The interactions between different mechanisms can be synergistic, antagonistic or addictive,

which may play important roles in the evolution and/or maintenance of multigenic resistance in the field (Hardstone et al. 2009). Usually, the interaction is synergistic when multiple resistance loci are homozygous, while addictive when resistance loci are heterozygous. Multiple studies have investigated the interactions between different resistance factors. For example, Sawicki found that a penetration decaying factor and desethylation caused by gene alpha can synergistically increase the resistance level house flies to organophospates up to 5-10 times (Sawicki, 1970). Multiplicative interactions between target site insensitivity and enhanced detoxification by cytochrome P450 monooxygenases were observed in mosquito *Culex pipiens* and *Culex quinquefasciatus* (Hardstone et al. 2009).

1.3 Carboxylesterases

1.3.1 The description, classification, mechanism & structure

Carboxylesterase (COE) (EC 3.1.1.1, Pfam PF00135 domain) represents an important hydrolase enzyme widely distributed in nearly all living organisms, including mammals (Satoh and Hosokawa, 1998), plants (Marshall et al. 2003), insects (Montella et al. 2012) and microbes (Bornscheuer et al. 2002).

The systematic nomenclature of carboxylesterases remained to be established and multiple classification methods were employed currently. According to Aldridge (Aldridge, 1993), esterases can be classified as three different types (A, B and C) based on their interaction with organophosphate insecticides. Esterases which can hydrolyze OPs were termed as A-esterases, while those inhibited by OPs were named as B-esterases, and C-esterases were resistant to OPs but cannot degrade them. Carboxylesterases belong to B-esterases (Montella et al. 2012). Another nomenclature commonly classified

carboxylesterases as α-esterase and β-esterase based on the preference of hydrolyzing α-naphthyl acetate and β-naphthyl acetate substrate (Vaughan and Hemingway, 1995). Besides that, carboxylesterases can also be classified based on their electrophoretic mobility (Prabhakaran and Kamble, 1993). Presently, the phylogenetic criterion appears to be the best one for esterase classification, especially for unravelling esterases with different functions (Montella et al. 2012).

As the most important and largest hydrolase supergene family, carboxylesterases can hydrolyze a wide range of endogenous and exogenous ester-containing substrates via addiction of water (as shown in Figure 1.1). Current biochemical characterization of COEs mainly relied on the measurement of enzymatic activities toward generic esterase substrates, such as α-naphthyl acetate (α-NA), β-naphthyl acetate(β-NA) and p-nitrophenyl acetate (PNPA), with their absorbance values can be colorimetric detected in enzymatic assays (Wheelock et al. 2005). Several COE inhibitors, such as S,S,S-tributyl phosphorotrithioate (DEF), 2-(o-cresyl)-4H-1,2,3-benzodioxa-phosphorin-2-oxide (CBDP), tetraisopropylpyrophosphoramide (iso-OMPA) and fluorophosphorous derivative diisopropylphosphorofluoridate (DFP) were commonly used to study the biochemical properties of COEs and can be further served as synergists for insecticide application in the field.

The determination of carboxylesterase crystal structure can better facilitate the elucidation of carboxylesterase-mediated hydrolysis. The overall structure of carboxylesterase contains three functional domains, including αβ domain, regulatory domain and catalytic domain. Three key amino acid residues, Serine (Ser), Histidine (His) and Glutamic acid (Glu), form a catalytic triad involved in the hydrolytic process of

carboxylesterases (Jackson et al. 2013). COEs cleave the ester-bond of substrates via a two-step process including the formation and degradation of an acyl-enzyme intermediate with the involvement of three key amino acids (as shown in Figure 1.2).

1.3.2 The carboxylesterase-mediated resistance

In insects, carboxylesterases have been recognized as one of the most important enzymes involved in insecticide resistance to multiple ester-containing insecticides, including organophosphate, pyrethroid, carbamate and oxadiazine insecticides (Wheelock et al. 2005). Researches on carboxylesterase in insects have attracted a lot of attention due to their important roles in conferring insecticide resistance. The carboxylesterase-mediated resistance resulted from quantitative changes (overexpression of COE genes and enhanced COE activities) resulting from the constitutive gene amplification or transcriptional up-regulation or qualitative changes (mutations occurring on the COEs) are predominant mechanisms underlying insecticide resistance in insects.

1.3.2.1 Qualitative changes of COEs involved in insecticide resistance

The correlation between COE mutations and OP resistance was described as “mutant ali-esterase hypothesis”. It refers as the mutations occurring on the ali-esterase (carboxylesterase) can lead to the enhanced hydrolytic activities toward OPs at the expense of reducing its carboxylesterase activities against some generic esterase substrates. This hypothesis was firstly proposed in 1960s in house flies and later been discovered in several other insect species (Oppenoorth and Asperen, 1960; Cui et al. 2011). Two mutations, G137D and W251L, occurring on the carboxylesterase E3 (also classified as LcαE7) have been widely identified in resistant sheep blowfly *Lucilia cuprina*, are responsible for the mutant ali-esterase hypothesis with the loss of

carboxylesterase activity and the acquisition of a novel OP resistance (Newcomb et al. 1997; Campbell et al. 1998; Denvonshire et al. 2003; Heidari et al. 2004; Claudianos et al. 1999). These two mutations have also been identified in some other OP resistant insect species, such as *Musca domestica* (Cui et al. 2011; Gacar et al. 2009), *Helicoptera armigera* (Li et al. 2013), *Tribolium castaneum* (Haubruge et al. 2002), *Aphis gossypii* (Sun et al. 2005), *Cochliomyia hominicorax* (Carvalho et al. 2010). Except for the OPs, mutations of COEs were also involved in pyrethroid resistance in multiple insects, including *Lucilia cuprina* (Heidari et al. 2004; Coppin et al. 2012); *Drosophila melanogaster* (Heidari et al. 2005); *Musca domestica* (Wang et al. 2012); *Cochliomyia hominivorax* (daSilva et al. 2009).

1.3.2.2 Quantitative changes of COEs involved in insecticide resistance

The over-production of non-specific carboxylesterases caused by either transcriptional upregulation or amplification has been widely documented in OP, carbamate and pyrethroids resistant insect species, such as *Culex quinquefasciatus* (Paton et al. 2000), *Aedes aegypti* (Hemingway et al. 1998), *Aedes alnopictus* (Grigoraki et al. 2015), *Nilaparvata lugens* (Zhang et al. 2013; Wang et al. 2009), *Aphis gossypii* (Cao et al. 2008; Devonshire and Moores, 1982; Gong et al. 2014; Kwon et al. 2009), *Bemisia tabaci* (Alon et al. 2008), *Locusta migratoria manilensis* (Zhang et al. 2011), *Panonychus citri* (Zhang et al. 2013), *Helicoverpa armigera* (Wu et al. 2011; Wee et al. 2008), *Spodoptera frugiperda* (Carvalho et al. 2013), *Lygus lineolaris* (Zhu et al. 2004), and *Drosophila melanogaster* (Wang et al. 2015). Some studies also found that multiple carboxylesterases with their expression levels can be further enhanced in response to insecticide stimulis. For example, the expression of COEs can be induced by fipronil

which was used to control *Leptinotarsa decemlineata* (Lu et al. 2015). In acaricide resistant *rhipicephalus microplus*, the overexpression of COEs can be induced by acaricidae exposure (Bayugar et al. 2009). All these results suggested that not only constitutive overexpression, but also inductive overexpression of COEs are responsible for insecticide resistance in insects. Some studies also pointed out that rather than directly metabolize insecticides, the carboxylesterases can sequester insecticide firstly and later slowly hydrolyze insecticides to less- or non-toxic chemicals and therefore protecting the insects from toxins to some extent (Ketterman et al. 1992). Indeed, in some cases, both of the qualitative and quantitative changes of COEs are responsible for insecticide resistance in insects (Pan et al. 2009; Hernandez et al. 2002; Zhang et al. 2010; Cui et al. 2015).

1.3.3 Regulation of carboxylesterases expression

Up to date, multiple potential factors transcriptionally regulate the expression of COEs have been identified and characterized in human, rat and mouse (Satoh and Hosokawa, 2006; Zhang et al. 2012; Hosokawa et al. 2008). For example, Maruichi et al. reported that nuclear factor erythroid 2 related factor 2 (Nrf2) plays pivotal roles in regulation of human carboxylesterase 1A1 gene transcription (Maruichi et al. 2010). The hepatocyte nuclear factor 4 alpha is capable of regulating the expression of carboxylesterase in mice (Li et al. 2016; Xu et al. 2016). Some other nuclear receptors were also characterized in regulating the expression of carboxylesterases in human and mammals (Staudinger et al. 2010; Jones et al. 2013; Zhang et al. 2012). However, none relative studies were explored in insects. It still remained to be further explored on the regulatory factors and pathways controlling carboxylesterase expression in insects. With the availability of genome and transcriptome databased, as well as the application of

cutting edge genome editing tools, it is expected to better understand the regulatory pathway of the carboxylesterase-mediated resistance, thereby providing novel strategies to efficiently control some diseases vectors or pests in the future.

1.4 The baculovirus-mediated insect cell expression system

In recent decades, heterologous expression systems have been widely applied due to their high efficiencies in producing large amounts of target proteins, allowing the biochemically and functionally determination and characterization of functional proteins *in vitro*. Multiple model systems have been adopted for the recombinant protein expression, including *Escherichia coli*, *Pichia pastoris*, *Sacccharomyces cerevisiae*, and *Spodoptera frygiperda*, and the choice of suitable model is therefore crucial for large-scale generation of interested proteins (Bulter et al. 2003; Berger et al. 2004; Macauley-Patrick et al. 2005; Terpe, 2006). By comparing the expression of a rabbit liver carboxylesterase in different model systems, Morton et al. found that the baculovirus-mediated insect Sf9 cell expression system is the most favorable model to generate large amount of active carboxylesterases *in vitro* for subsequent functional studies (Morton et al. 2000). Since the first reported in early 1980s (Carbonell et al. 1985), the baculovirus-mediated foreign gene expression system has attracted a lot of attention for its high protein production level as well as eukaryotic protein processing capabilities (phosphorylation and glycosylation) (Javis et al. 2009). It is a binary system composed of two essential steps. The first is the construction of recombinant baculovirus used to deliver foreign gene encoding interested proteins into host cells, and the second is the transfection of host cells to large-scale express interested proteins *in vitro*. Over the past decades, the baculovirus-mediated insect cell expressing system has been widely used to

generate thousands of recombinant proteins, ranging from cytosolic enzymes to membrane-bound proteins in insect and mammal cells (Berger et al. 2004). Our previous studies have already successfully expressed multiple detoxifying mosquito CYP450 enzymes with this system, and these CYP450s showed strong *in vitro* metabolic activities to permethrin (Gong et al. 2017), suggesting that the baculovirus-mediated insect cell expression is no doubt a powerful and efficient tool to express our target carboxylesterase proteins *in vitro* for functional studies. The Figure 1.3 showed the basic procedures for the baculovirus-mediated insect cell expression system.To clearly observe the in *vitro* expression of proteins using baculovirus-mediated insect cell expression system, we chose a green fluorescent protein (GFP) as our target gene to conduct this system and visually observe the signs of cells after infected with GFP-recombinant bacuvirus at different stages (Figure 1.4).

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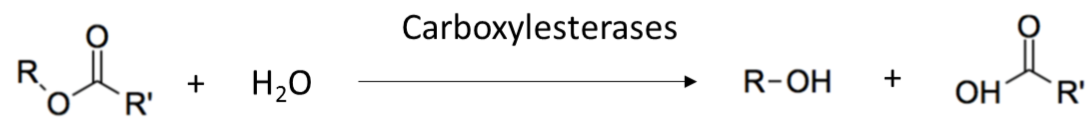

Figure 1.1.The basic mechanism of carboxylesterase-mediated hydrolysis. With the addition of water, ester-containing substrate can be hydrolyzed to form the corresponding alcohol and acid.

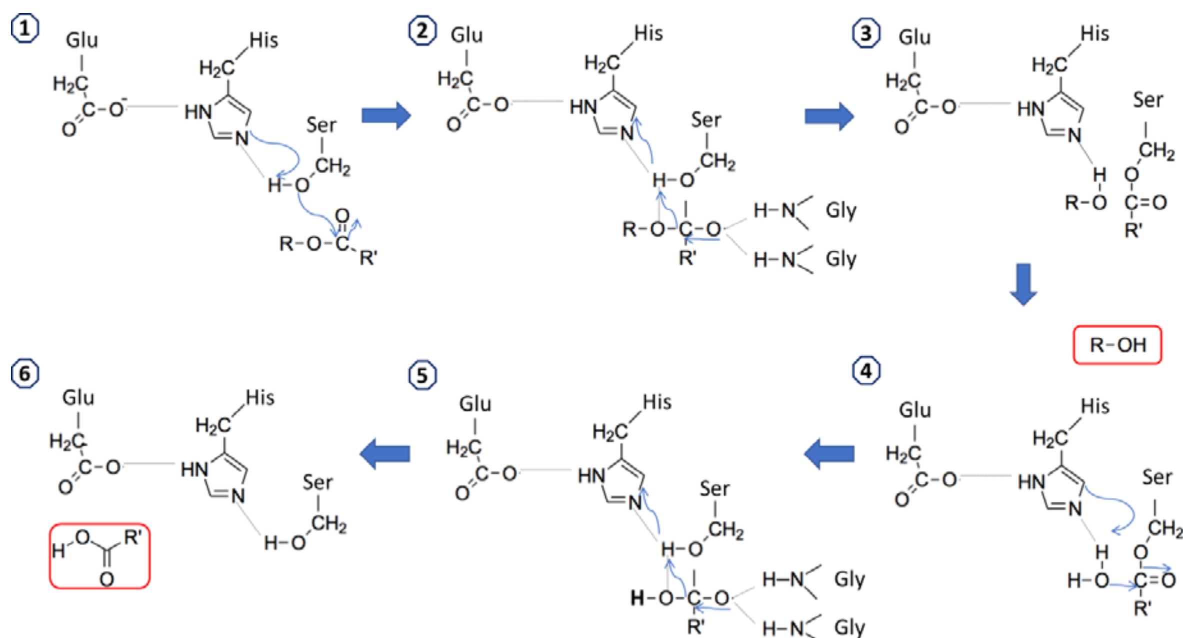

Figure 1.2.A proposed 2-step processes of COE-mediated ester bond cleavage. The first step is the formation of an acyl-enzyme intermediate, which includes: 1-2) A proton is transferred from Ser to His residue to increase the nucleophilicity of the Ser terminal hydroxyl group to attack the carbonyl moiety of ester substrate to form a tetrahedral intermediate which is further stabilized by two Gly residues in the oxyanion hole. A His residue is in turn stabilized by hydrogen bond formed by Glu (or Asp); 3-4) This intermediate collapses to form an acyl-enzyme intermediate, releasing Ser residue and the alcohol metabolite. The second step is degradation of an acyl-enzyme intermediate, which includes: 4-6) A His activated water attacks the acyl-enzyme intermediate, and release the acid metabolites. The conserved Ser later support and stabilize the structural of the catalytic triad formed by His, Glu and Ser residues.

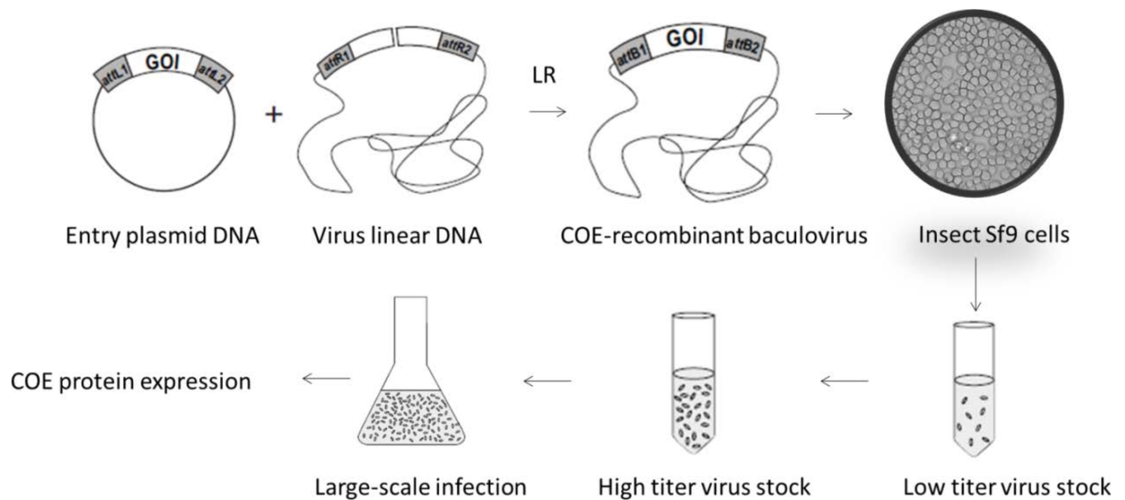

Figure 1.3.The basic procedures for the baculovirus-mediated insect cell expression system. The GOI indicated the gene of inserted, and it was ligated with the pENTR vector to construct the entry plasmid DNA with two att L arms. This entry plamid DNA was later ligated with virus linear DNA containing two att R arms by performing the Lambda Recombinantion reaction to build the COE-recombinant baculovirus. The recombinant baculovirus were initially used to infect insect Sf9 cells to produce and collect the low titer virus stock solution (P1), and later P1 was used to transfect Sf9 cells again to produce high titer virus stock (P2). The collected P2 was used to large-scale infect Sf9 cells to express our target carboxylesterase proteins *in vitro*.

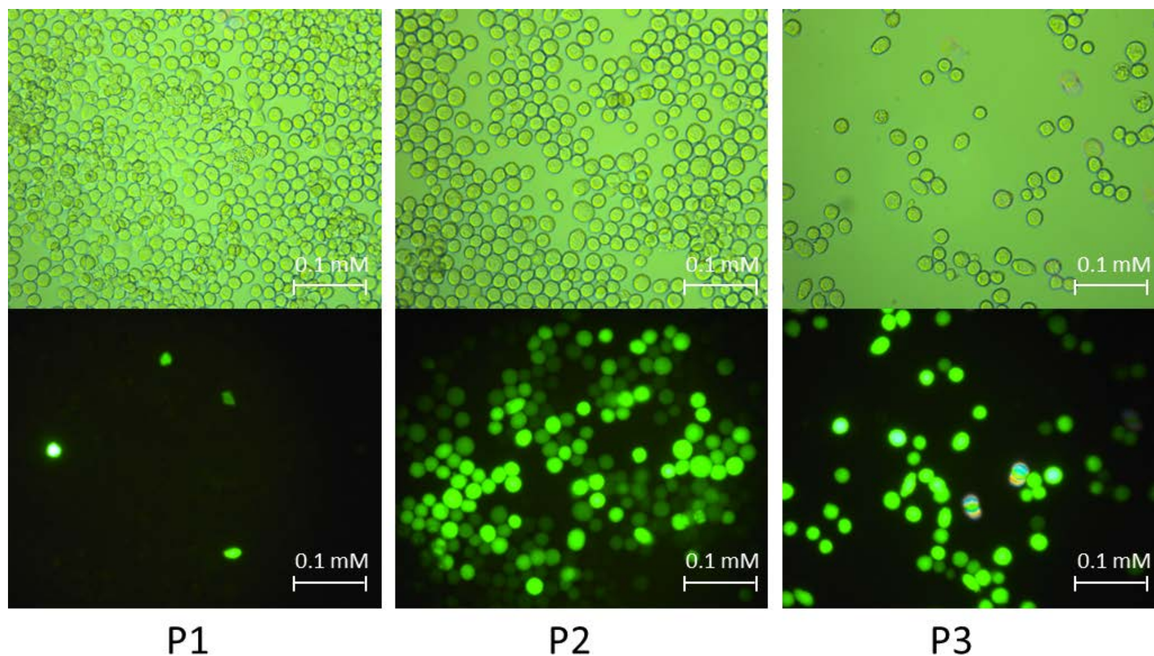

Figure 1.4: Example infected signs of Sf9 cells at different baculovirus amplification stages. The figure showed the signs of cells when infected by the GFP-recombinant baculovirus under natural light and fluorescent light. At P1 infection stage, the infection ratio is low. At P2 infection stage, the infection ratio was significantly enhanced. While at P3 infection stage, almost all the cells were infected with the symptoms of detachment from cell culture plate, increase of cell diameter as well as the cessation of cell growth.

Chapter 2: The Research Goal, Objectives and Significance

2.1 The long-term goal of the research and major objectives

In order to further explore the mechanisms underlying insecticide resistance at molecular level and thereby facilitating the better understanding of resistance development in insects, the long-term goal of my research is to characterize the roles of carboxylesterases in metabolizing insecticides and conferring insecticide resistance in house flies, *Musca domestica*. To achieve this goal, my research mainly addressed on these following objectives: 1) Comprehensive investigation of *M.domestica* carboxylesterases; 2) Profiling the constitutive and inductive overexpression patterns of carboxylesterases in resistant house flies; 3) Autosomal mapping of up-regulated carboxyleterases and their regulatory factors in house flies; 4) Functional characterization of up-regulated carboxylesterases in conferring pyrethroid resistance *in vitro*; 5) and exploration of carboxylesterase mutations associated with pyrethroid resistance in house flies.

2.1.1 Objective 1: Comprehensive investigation of *M.domestica* carboxylesterases

In my study, all the house fly carboxylesterase genes were extracted from its genome and transcriptome genome database (Li et al. 2013; Scott et al. 2014), and they were clustered together with all carboxylesterases genes of *Anopheles gambiae* and *Drosophila melanogaster* downloaded from their relative genome database ((https://www.ncbi.nlm.nih. gov/genome/?term=Drosophila+melanogaster) and

(https://www.ncbi.nlm. nih.gov/genome/?term =Anopheles+Gambiae)), respectively. All *Musca domestica* carboxylesterase genes were classified and annotated based on their amino acid sequence similarities with those in *Drosophila melanogaster* which have already been functional characterized and annotated. Based on this phylogenetic analysis, we are expected to estimate the potential functions of *Musca domestica* carboxylesterases and identify the candidate genes involved in detoxification and thereby conferring insecticide resistance in house flies. We have also investigated the tissue-specific distribution of carboxylesterases in house flies by dissecting different body parts and tissues (head, thorax, leg, midgut and fat body) from three house fly strains (ALHF, aabys and CS) and profiling their relative expression levels by conducting qRT-PCR analysis. In insects, midguts and fat bodies are major tissues involved in xenobiotics detoxification (Shen et al. 2013; Liu et al. 2011). Therefore, the abundantly distribution of our candidate carboxylesterases in midguts and fat bodies can further verify their potential relationship with insecticide detoxification in house flies.

2.1.2 Objective 2: Profiling the constitutive and inductive overexpression patterns of carboxylesterases in resistant house flies

Our lab has long-term reared different house fly strains and homozygous offspring lines. ALHF, is a highly insecticide resistant strain which develops resistance and cross-resistance to multiple insecticides (Liu and Yue, 2000). This strain was originally collected from a poultry farm in Alabama in 1998 after control failure with permethrin, and later consecutively selected with permethrin for 6 generations until it reached the high resistance ratio (≈2000 fold) to permethrin compared with the susceptible aabys strain. Its high resistant level was maintained under biannual selection

with permethrin (Liu and Yue, 2001; Tian et al. 2011). aabys, is an insecticide susceptible strain bearing five recessive morphological markers, ali-curve (*ac*), aristepedia (*ar*), brown body (*bwb*), yellow eyes (*ye*) and snipped wings (*snp*) on autosome 1, 2, 3, 4, and 5, respectively. CS, is a wild-type insecticide susceptible strain which has reared in our labs for more than 20 years. We analyzed and compared the relative expression levels of all *Musca domestica* carboxylesterases in resistant and susceptible strains and hypothesized that several genes with their expression levels were significantly up-regulated in resistant strain compared with those in both susceptible strains, indicating that these carboxylesterases play critical roles in conferring pyrethroid resistance in house flies. We further investigated the inductive overexpression patterns of these up-regulated genes in response to permethrin stimuli, and hypothesized that these up-regulated carboxylesterases with their expression levels can be further induced to higher levels in response to permethrin stimulus, suggesting that not only constitutive, but also inductive overexpression of carboxylesterases are responsible for the enhanced activitities to permethrin.

2.1.3 Objective 3: Autosomal mapping of up-regulated carboxylesterases and their regulatory factors in house flies

In this study, five backcrossed offspring lines (A2345, A1345, A1245, A1235 and A1234) were generated by the reciprocal cross of ALHF and aabys strains (Figure 2.1) (Li et al. 2013). These offspring lines contain different autosomal combination derived from ALHF and they were used together with two parental strains ALHF and aabys strain to autosomal mapping and genetical analysis of up-regulated genes identified in Objective 2. Allele-specific PCR was conducted using cDNA isolated from different

house fly strains and lines. We hypothesized that carboxylesterases were located on different house fly autosomes, mainly on autosome 2 and 3, which is consistent with our transcriptome analysis. We also expected that the overexpression of carboxylesterase is regulated by factors located on different house fly autosomes.

2.1.4 Objective 4: Functional characterization of up-regulated carboxylesterases in conferring pyrethroid resistance in house flies

To functional characterize the roles of carboxylesterases in metabolizing permethrin and conferring pyrethroid resistance in house flies, we use the heterologous expression system to large-scale produce carboxylesterase proteins and later investigate their metabolic capabilities to permethrin through cell-based MTT assay and *in vitro* metabolism studies. Here, several up-regulated genes identified in Objective 2 were selected as candidate genes to investigate their functions in pyrethroid resistance. We firstly constructed the COE recombinant baculovirus by ligating the COE-inserted pENTR vector with baculovirus linearized DNA through Lambda recombination (LR) reaction. After 2 rounds of amplifications of recombinant baculovirus, the obtained high-titer of recombinant baculovirus was used to infect *Spodoptera frugiperda* (Sf9) cells to large-scale produce our target carboxylesterase proteins. The *in vitro* metabolism study was operated by incubating obtained carboxylesterase proteins together with permethrin *in vitro* at certain conditions, and the depletion percentage of permethrin was detected and calculated based on HPLC analysis. Our study hypothesized that the obtained carboxylesterases not only have metabolic capabilities to general esterase substrates, such as α-naphthal acetate (α-NA), but also to permethrin insecticides, suggesting that these carboxylesterases play pivotal roles in metabolizing permethrin and conferring pyrethroid

resistance in house flies. Besides that, we also conducted the cell-based MTT assay to explore the roles of carboxylesterases in metabolizing permethrin in insect cells. We treated cultured cells expressing target proteins with permethrin at different concentrations, and examined the cell viabilities in response to permethrin exposure. We hypothesized that compared with two control groups (one with parental cells without baculovirus infection; another one with cells expressing chloramphenicol acetyltransferase (CAT) proteins), the cells with carboxylesterase expression can significantly enhanced their tolerance to permethrin, indicating the metabolic roles of carboxylesterases against permethrin and thereby protecting cells from chemical damages.

2.1.5 Objective 5: Exploration of carboxylesterase mutations associated with pyrethroid resistance in house flies

The "mutant ali-esterase hypothesis" is defined as the substrate preference shift from generic esterase substrate (e.g. α-napthyl acetate) to ester-containing insecticides (e.g. organophosphates, pyrethroids, and carbamates) caused by the mutations occurring on the coding regions of carboxylesterases. This qualitative mechanism underlying carboxylesterase-mediated resistance has been widely reported in multiple OP resistant insects (Cui et al. 2011; Gacar et al. 2009). However, none relative studies were investigated in pyrethroid resistant insects. The *Musca domestica* MdαE7 gene has been widely investigated with its overexpression responsible for the enhanced metabolic efficiencies toward permethrin *in vitro*. Also, its orthologous gene LcαE7 in *Lucilia cuprina* has been studies with its roles in conferring OP resistance resulted from mutations occurring on the coding region (Newcomb et al. 1997). However, the correlation between MdαE7 mutations and pyrethroid resistance remained to be further

explored. Based on this, I hypothesized that mutations within MdαE7 can enhance its hydrolytic activities to permethrin at the expense of decreasing its carboxylic activities against generic esterase substrates α-NA, thereby closely correlating with pyrethroid resistance in house flies. In this study, we try to identify multiple mutations occurring on MdαE7 gene by aligning the amino acid sequences extracted from resistant and susceptible house fly strains, and later conduct site-specific mutagenesis study to introduce mutations individually into the wild-type MdαE7 gene and express the mutant proteins through baculovirus-mediated expression system. The carboxylic activity toward α-NA and hydrolytic activity to permethrin of different mutants was investigated through *in vitro* functional studies. Through this study, we are expected to elucidate the roles of carboxylesterase mutations in conferring pyrethroid resistance in house flies.

2.2 Significances of the project

As one of the major detoxification enzymes, carboxylesterases play critical roles in detoxifying xenobiotics and endogenous compounds. Functional elucidating the roles of carboxylesterases in metabolizing insecticides can facilitate the better understanding of carboxylesterase-mediated resistance in insects and thereby providing novel ideas in impeding or minimizing the development of insecticide resistance, which in turn can provide us unique strategies to control agricultural pests or disease vectors in the future.

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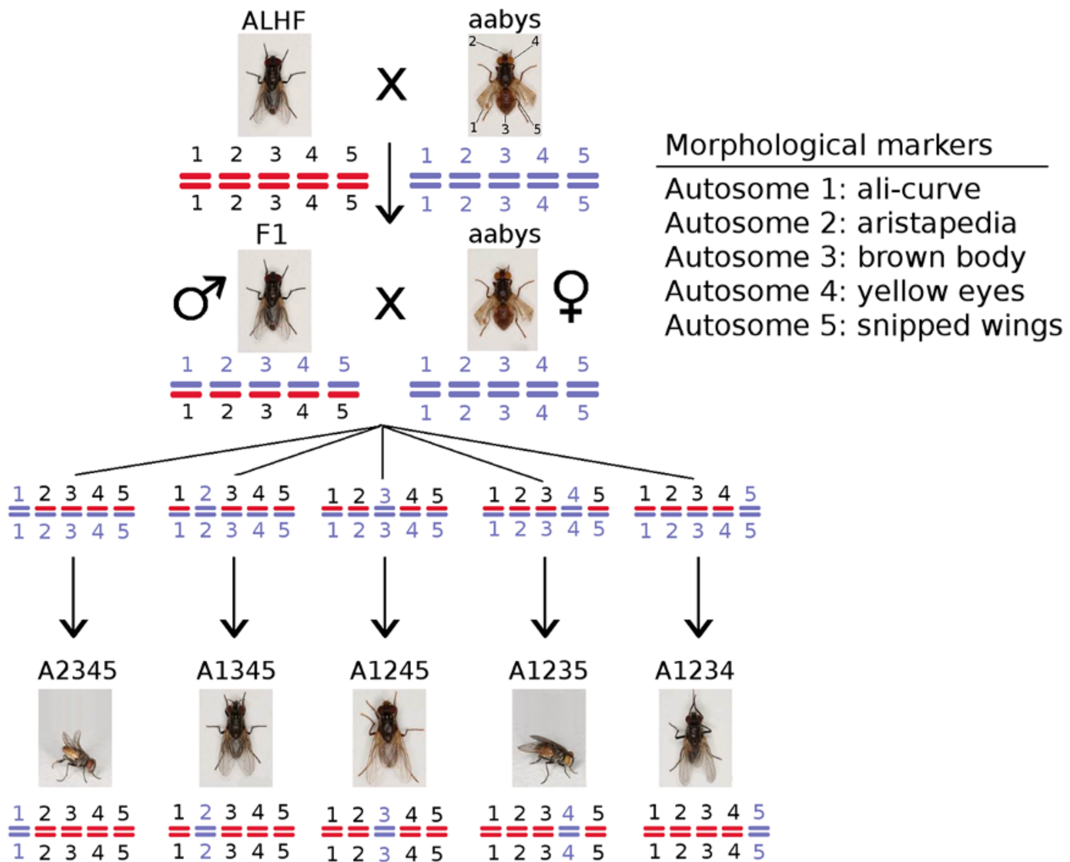

Figure 2.1 Schematic for the generation of the *M.domestica* combination lines used in our study. Strain ALHF is a highly insecticide resistant strain. Strain aabys is an insecticide susceptible strain with five morphological markers, ali-curve (*ali*), aristapedia (*ar*), brown body (*bwb*), yellow eyes (*ye*) and snipped wings (*snp*) on its five autosomes, respectively. Five homozygous offspring lines were generated by the reciprocal cross of ALHF and aabys strain, and later screened with single-pair crosses. Each line owns the unique morphological marker derived from aabys strain and was named based on the autosomes bearing wild-type markers from ALHF. (Li et al. 2013).

Chapter 3: Carboxylesterase genes in pyrethroid resistant house flies, *Musca domestica*

Abstract

Carboxylesterases are one of the major enzyme families involved in the detoxification of pyrethroids. Up-regulation of carboxylesterase genes is thought to be a major component of insecticide resistant mechanisms in insects. Based on the house fly transcriptome and genome database, a total of 39 carboxylesterase genes of different functional clades have been identified in house flies. In this study, eleven of these genes were found to be significantly overexpressed in the resistant ALHF house fly strain compared with susceptible aabys and wild-type CS strains. Eight up-regulated carboxylesterase genes with their expression levels were further induced to a higher level in response to permethrin treatments, indicating that constitutive and inductive overexpression of carboxylesterase are co-responsible for the enhanced detoxification of insecticides. Spatial expression studies revealed these up-regulated genes to be abundantly distributed in fat bodies and genetically mapped on autosome 2 or 3 of house flies, and their expression could be regulated by factors on autosome 1, 2 and 5. Taken together, these results demonstrate that multiple carboxylesterase genes are co-upregulated in resistant house flies, providing further evidence for their involvement in the detoxification of insecticides and development of insecticide resistance.

3.1 Introduction

Carboxylesterases (COEs) represents a multigene superfamily that is widely distributed in insects (Montella et al. 2012), mammals (Satoh and Hosokawa, 1998), plants (Marshall et al. 2003) and microbes (Bornscheuer, 2002), playing a major role in hydrolyzing a broad range of ester-containing xenobiotics, including drugs, environmental toxicants, insecticides and carcinogens. In insects, COEs are of particular interest due to their roles in metabolizing insecticides (Wheelock et al. 2005; Zhang et al. 2010; Ranson et al. 2002; Sogorb and Vilanova, 2002; Hemingway and Karunaratne, 1998; Devonshire and Moores, 1982; Farnsworth et al. 2010). Qualitative changes (mutations occurring in the active sites of COEs) and quantitative changes (overexpressed COE genes and enhanced COE activities) resulting from constitutive gene amplification or transcriptional up-regulation of COEs are the predominant mechanisms implicated in the development of insecticide resistance in insects (Hemingway et al. 2004; Devonshire et al. 1986; Li et al. 2007; Zhang et al. 2010). The overexpression of carboxylesterases has been detected in many resistant insect species, including *Aphis gossypii*, *Culex quinquefasciatus*, *Bemisia tabaci*, *Myzus persicae*, *Musca domestica, Boophilus microplus*, *Aedes aegypti* and *Helicoverpa armigera* (Cao et al. 2008; Vaughan and Hemingway, 1995; Alon et al. 2008; Foster et al. 2003; Zhang et al. 2010; Hernandez et al. 2002; Poupardin et al. 2014; Wu et al. 2011), indicating that the COE-mediated metabolism due to constitutive gene overexpression plays a key role in governing increased levels of detoxification in insecticides, thus conferring insecticide resistance. In addition, the induction of carboxylesterases by insecticides is of considerable importance in the increased metabolic detoxification of insecticides in insect

species such as *Anopheles gambiae* (Vontas et al. 2005), *Aedes aegypti* (Poupardin et al. 2008), *Tetranychus cinnabarinus* (Wei et al. 2016) and *Leptinotarsa decemlineate* (Lü et al. 2015). Both constitutive and inductive overexpressions of COEs are thought to be responsible for the increased levels of detoxification of insecticides.

The house fly, *M. domestica*, is a major cosmopolitan pest that is capable of transmitting more than 100 human and animal intestinal diseases, including major illnesses such as cholera, typhoid fever, salmonellosis and polio (Hewwit, 2011; Abbas, et al. 2014; Scott et al. 2014). Although insecticides from the pyrethroid family, especially permethrin, are currently widely applied to control house flies, their extensive application is known to lead to resistance issues in insects (Liu and Yue, 2001; Kaufman et al. 2010; Scott et al. 2013; Scott et al. 2017). In a previous study, our group reported that house flies can and do develop resistance and cross-resistance to pyrethroids, organophosphates as well as some relatively new insecticides such as fipronil, imidacloprid and spinosad (Liu and Yue, 2000). Now that a transcriptome and genome database for the house fly *M. domestica* has become available (Li et al. 2013; Scott et al. 2014), a total of 39 COE genes have been identified and their constitutive and inductive expression profiles compared in different resistant and susceptible populations to demonstrate their involvement in detoxifying insecticides and conferring insecticide resistance in house flies. In the current study, which was designed to decipher and understand the importance of COE genes in insecticide resistance, we classified and annotated COE genes from the house fly genome by constructing a phylogenic tree with those genes from other insect species, characterizing the expression profiles of COEs in resistant and susceptible house flies, investigating the spatial expression patterns of the

COE genes, and examining COE gene expression in response to a permethrin challenge, as well as genetically mapping the COE genes in house flies

3.2 Material and Methods

3.2.1 House flies

Three house fly strains were used in this study. ALHF, a highly insecticide resistant strain, was originally collected from a poultry farm in Alabama. This strain exhibited a high level of resistance after subsequent selection with permethrin for six generations and has been annually selected with permethrin to maintain its highly resistant status (Liu and Yue, 2000; Tian et al. 2011). The first of the insecticide susceptible strains used in this study, aabys, bears five recessive morphological markers: ali-curve (ac), aristapedia (ar), brown body (bwb), yellow eyes (ye) and snipped wings (snp) located on autosomes 1, 2, 3, 4 and 5, respectively. The second, CS, is a wild type susceptible strain bearing the same phenotype as ALHF. The aabys and CS strains were originally obtained from Dr. J. G. Scott (Cornell University). All house flies were reared at 25±2°C under a photoperiod of 12:12 h (L: D), and fed with sugar and water.

A genetic cross of ALHF females with aabys males was performed. The F1 males (~400 flies) were then backcrossed with aabys females. Five back-cross (BC_1) lines with the following genotypes were isolated: *ac/ac*, +/*ar*, +/*bwb*, +/*ye*, +/*snp*; +/*ac*, *ar/ar*, +/*bwb*, +/*ye*, +/*snp*; +/*ac*, +/*ar*, *bwb/bwb*, +/*ye*, +/*snp*; +/*ac*, +/*ar*, +/*bwb*, *ye/ye*, +/*snp*; and +/*ac*, +/*ar*, +/*bwb*, +/*ye*, *snp/snp* (Li et al. 2013). Homozygous house fly lines *ac*/*ac*, +/+, +/+, +/+, +/+ (A2345); +/+, *ar* /*ar*, +/+, +/+,+/+ (A1345); (+/+, +/+, *bwb*/*bwb*, +/+, +/+ (A1245); +/+, + /+, +/+, *ye*/*ye*, +/+ (A1235) and +/+, +/+, +/+, +/+, *snp*/*snp* (A1234) were generated by sorting for appropriate phenotypic markers and selecting with permethrin at

corresponding doses causing ~70% mortality for each of the lines for three generations. One hundred single-pair crossings of each of the lines for the desired phenotype and genotype were then set up for each line (Liu and Yue 2000; Tian et al. 2011). The name of each line indicates which of its autosomes bear wild-type markers from ALHF. For instance, the A2345 strain has wild-type markers on autosomes 2, 3, 4 and 5 from ALHF, with a mutant marker on autosome 1 from aabys.

3.2.2 Phylogenetic analysis of *M. domestica* carboxylesterase genes

To comprehensively classify and annotate the carboxylesterase in *M. domestica*, a phylogeny tree was created based on the COEs of *M. domestica* extracted from the first adult whole transcriptome database and genome database (https://www.ncbi.nlm.nih.gov/assembly/GCF_000371365.1/) (Li et al. 2013; Scott et al. 2014), and the COEs of *An. gambiae* and *D. melanogaster*, downloaded from their respective genome databases (https://www.ncbi.nlm.nih. gov/genome/?term=Drosophila+melanogaster) and (https://www.ncbi.nlm. nih.gov/genome/?term =Anopheles+Gambiae). The repertoires of COEs of all the species used in the present project were aligned using Muscle (http://www.ebi.ac.uk/Tools/msa/muscle/) before constructing the phylogeny tree using FastTree utilizing the default settings (Price et al. 2009). An approximate-maximum-likelihood phylogeny tree was constructed based on the JTT model of amino acid evolution and adopting a Bayesian approach (Drummond and Rambaut, 2007) to compute the local support values. The phylogeny trees were run on MEGA6.0 for visualization (Tamura et al. 2013).

3.2.3 Bioassay

An insecticide bioassay was conducted on each of the strains by dropping 0.5ul of permethrin (dissolved in acetone) at a range of concentrations on the thoracic notum of 2-day old female house flies (Liu and Yue, 2000; Tian et al. 2011). Twenty flies were tested per dose with a total of 5-6 doses designed to produce >0% and <100% mortality. Control groups received acetone alone. Treated flies were reared in a paper box and fed with 15% sugar water. Mortality was assessed after 24 hr and any flies that did not move were scored as dead. All tests were performed at room temperature (25±2˚C). Three replications were prepared with house flies emerging on different days for each of three consecutive generations. Bioassay data were pooled and analyzed by PROBIT analysis in SPSS. Statistical analyses of LD_{50} values were based on non-overlapping 95% confidence intervals.

3.2.3 Permethrin treatment

In this study, two different permethrin treatment experiments were conducted: 1) Hundreds of female house flies of each strain were treated with permethrin at their corresponding LD_{10}, LD_{50} and LD_{90} doses and the surviving flies (20 flies/treatment) were collected for RNA extraction after 24 hr treatment with permethrin; and 2) Hundreds of female house flies of each strain were treated with permethrin at their corresponding LD_{50} dose and the surviving flies (20 flies/treatment) were collected 12 hr, 24 hr, 48 hr and 72 hr after permethrin treatment for RNA extraction. Control groups treated with acetone only (with no exposure to permethrin) were collected at the same time points as their permethrin treated counterparts. Three replications with different preparations were conducted.

3.2.4 RNA extraction and cDNA preparation

Twenty 2-day old virgin female house flies of each strain (ALHF, aabys and CS) and 20 surviving flies with permethrin treatment at different permethrin doses (LD_{10}, LD_{50} and LD_{90}) and time points (12 hr, 24 hr, 48 hr and 72 hr) and their respective counterparts (acetone treated only) were collected and total RNA was extracted using the acidic guanidine thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987). The DNA was removed from total RNA using DNase (TURBO DNA-free, Ambion), after which the DNA-free RNA (500ng per sample) was reverse-transcribed to cDNA with SuperScript II reverse transcriptase (Invitrogen) with a pair of random hexamer primers in a total volume of 20ul. The quantity of cDNA was measured by a spectrophotometer prior to qRT-PCR analysis. Each experiment was repeated three times with different RNA preparations and cDNA syntheses.

3.2.5 Quantitative Real-time PCR (qRT-PCR)

The expression patterns of all *M. domestica* COEs were examined by Quantitative Real-time PCR (qRT-PCR) (Bustin, 2002; Huggett et al., 2005). The gene expression levels were detected with SsoAdvanced™ Universal SYBR Green supermix (Bio-Rad) and CFX96™ Real Time PCR system (Bio-Rad). Each qRT-PCR reaction (15 ul final volume) consisted of 1×SYBR Green master mix, 1ul of cDNA, and 1.5uM of specific COE primer pair designed based on their nucleotide sequences (Table S1). The qRT-PCR reaction was heated to 95°C for 30s, followed by 40 cycles of 95°C for 15 s, 60°C for 30 s, and then a melting curve step (95°C for 0.05s, 65°C for 0.05s and 95 °C for 0.5s). The specificity of each PCR reaction was assessed by a melting curve analysis using Dissociation Curves Software. Relative expression levels were calculated by the 2^{-}

$^{\Delta\Delta Ct}$ method using SDS RQ software (Livak and Schmittgen, 2001). The RPS3 (ribosomal protein S3) gene and β-actin gene were used as reference genes to internally normalize the expression of the target genes (Gao et al. 2006; Zhang et al. 2010; Gao et al. 2012; Kita et al. 2014). All samples, including the "no template" negative control, were analyzed twice. Each experiment was repeated three times with different RNA samples. The statistical significance of the gene expression values was calculated using one-way analysis of variance (ANOVA) (SPSS 18.0 Software). A value with $P \leq 0.05\%$ was considered to be statistically significant. Significant overexpression was determined using a cut-off value of ≥2 fold change in expression (Strode et al. 2008).

3.2.6 Autosomal mapping of selected COE genes in *M. domestica*

Five house fly BC_1 lines were used to determine the genetic linkages of the selected COE genes. Allele specific PCR was conducted using the cDNA from 5 BC_1 lines (Liu et al. 1995). The ALHF allele specific primer pair (Table S3.1) was designed based on the target sequence of genes from ALHF by placing a nucleotide polymorphism at the 3' end of each primer to permit preferential amplification of those specific alleles from ALHF. Two rounds of PCR were conducted. For the first PCR reaction, the allele-independent primer pairs (Table S3.1) were used to generate COE cDNA fragments. The first PCR solution with cDNA template and a primer pair were heated to 95°C for 3 min, followed by 35 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 1 min, then 72°C for 10 min. The second PCR employed 0.5 μl of the first round PCR reaction solution and the allele specific primer pair (Table S3.1). The second PCR reaction was heated to 95°C for 3 min, followed by 35 cycles of 95°C for 30 s, 62°C for 30 s, and 72°C for 30 s, then

72°C for 10 min. Each experiment was repeated three times with different mRNA samples, and the PCR products were sequenced at least once for each gene.

3.3 Results

3.3.1 Classification and annotation of carboxylesterase genes in house flies

Through *M. domestica* transcriptome and genome analysis (Li et al. 2013; Scott et al. 2014), a total of 39 putative COEs were identified from the genome data of *M. domestica*. These genes were clustered together with the corresponding COEs from *D. melanogaster* and *An. gambiae* based on their sequence similarities. The constructed phylogeny tree (Figure 3.1) showed that all the COEs fall into 9 clades within the three main phylogenetic classes of dietary/detoxification, hormone/semiochemical processing and neuro/developmental functions. Among these, seventeen COE genes belong to the α-esterase clade, which is the only clade in the dietary/detoxification class that has been linked to lipid metabolism and xenobiotic detoxification (Flores et al. 2005; Jackson et al. 2013; Strode et al. 2008). The considerable expansion in the number of COEs in this class indicates their potentially important roles in detoxifying insecticides and thus conferring insecticide resistance in house flies. Ten COE genes were involved in the hormone/semiochemical processes, with 1 juvenile hormone esterase, 2 β-esterases and 7 integument esterases. The COEs within this class were catalytic enzymes with crucial roles in hormone/pheromone olfactory processes, reproductive behaviors and xenobiotics metabolism (Durand et al. 2010; Robin et al. 2009; Oakeshott et al. 2010). β-esterases within this class have also been reported to be associated with insecticide metabolism in insects (Flores et al. 2005; Hawks and Hemingway, 2002). The remaining 12 COE genes were located in the neuro/developmental processes class, with 1 acetylcholinesterase, 2

gliotactins, 3 neuroligins, 5 glutactins and 1 uncharacterized neuro-receptor. Within this class, except for catalytic acetylcholinesterase, which is known to be involved in OP resistance in several insects (Singh et al. 2016; Zhang et al. 2013), all the clades were non-catalytically adhesive proteins implicated in cell-to-cell interactions and signal transductions in the nervous system (Oakeshott et al. 2010). Neurotactin-like esterases were not detected in house flies (Figure 3.1).

3.3.2 Expression profiles of COEs in resistant and susceptible house flies

Qualitative real-time PCR (qRT-PCR) analysis was performed to examine and compare the expression levels of all the *M. domestica* COEs in three house fly strains bearing different resistant levels to permethrin, including a highly resistant strain (ALHF), a susceptible strain (aabys) and a wild-type susceptible strain (CS). To eliminate any differences in the genetic background, only those genes with expression levels that were significantly overexpressed in the resistant ALHF strain compared with that in both the susceptible aabys and CS strains were regarded as being up-regulated. Of the COE genes tested, a total of 11 *M. domestica* COEs were significantly up-regulated in resistant ALHF, ranging from ~2.1 fold to ~22.4 fold compared to those in the susceptible aabys strain (Figure 3.2 A) and the CS strain (Figure 3.2 B). The correlation between these up-regulated COEs with resistant phenotypes suggests their importance in permethrin tolerance in house flies. Six of these up-regulated COEs were found to belong to the dietary/detoxification class (α-esterase clade), three to the hormone/semiochemical processes class (with 1 β-esterase and 2 integument esterase), and the remaining 2 were located in the neuro/developmental functions group (with 1 acetylcholinesterase and 1 glutactin). In terms of the potential roles of these clades in detoxifying xenobiotics in

house flies, the overexpression of COEs may facilitate insecticide metabolism and thus confer insecticide resistance. These 11 up-regulated genes have been named based on their clustered clades and relative positions within that clade (Table S3.1). The expression levels of the remaining 28 *M. domestica* COE genes showed either down-regulated (eg. MdαE14) or no significant differences in resistant ALHF strain compared with that in either susceptible strain aabys or CS strain (Table S3.2).

3.3.3 Tissue-specific overexpression of COEs in resistant and susceptible house flies

The expression of COEs may vary somewhat in different tissues in response to environmental stimuli. In insects, their midguts and fat bodies are the primary detoxification tissues, containing most of the overproduced detoxification enzymes responsible for insecticide resistance (Shen et al. 2013; Liu et al. 2011). To determine whether the up-regulated COEs in resistant house flies are indeed tissue-specific, we further extracted the total RNAs from heads, thoraxes, midguts, fat bodies and legs of 2-3 day old female adults of the ALHF, aabys and CS strains and examined their gene expression levels using qRT-PCR analysis. Comparing the expression levels of these COEs in different tissues indicated that all these 11 genes were abundantly distributed in fat bodies (ranging from ~7.6 fold to ~565.7 fold) compared with in non-detoxification tissue thorax in resistant ALHF strain (Figure 3.3 A), suggesting that COEs are associated with the chemical storage metabolism in fat bodies, which agrees with the findings of previous studies that showed fat body COEs play important roles in lipid storage and metabolism in insects (Beller et al. 2006; Birner et al. 2012). Except for MdIntE3 and MdGluE3, all other carboxylesterase genes were also highly overexpressed in another detoxification tissue midguts (ranging from ~2.6 fold to ~253.6 fold),

indicating these carboxylesterases, especially those α-esterases, indeed play fundamental roles in detoxifying insecticides in house flies. Another interesting finding is the overexpression of MdAChE1, MdGluE3, MdIntE3 and MdIntE7 (ranging from ~2.8 fold to ~8.5 fold) in heads compared with in thoraxes in ALHF strain. As brain is the major component of central nervous system, the overexpression of MdAChE1, MdGluE3 in head may be closely related with their potential roles in signal transductions and other neuro/developmental processes (Oakeshott et al. 2010). For integument esterase genes, MdIntE3 and MdIntE7, their overexpression in heads can be explained by their potential roles in hormone/semiochemical processing, such as the recognizing, inactivating and degrading hormone, pheromone or other odorant released into the environments (Younus et al. 2017; Ishida and Leal, 2005). Besides that, none of these carboxylesterases were overexpressed in legs compared with in thoraxes, indicating that leg and thorax were not involved in detoxification insecticides in house flies. Similar tissue-specific expression patterns were also observed in susceptible aabys strain (Figure 3.3 B) and CS strain (Figure 3.3 C)

3.3.4 Responses of COEs expression with permethrin challenge at different doses in resistant and susceptible house flies

It has long been suspected that insects can over-produce detoxifying enzymes when they are exploring insecticides. To investigate the tolerance of house flies to a permethrin challenge, we examined the inductive profiles of all the constitutively up-regulated COEs in response to permethrin at different dosages of LD_{10}, LD_{50} and LD_{90} specifically tailored to each of the house fly strains (Table 3.1). Our results showed that after 24 hr permethrin treatment, eight genes with their expression can be induced with

varying levels in a dose-dependent manner, while no significant induction of MdαE5, MdGluE3 and MdAchE1 were detected in any of three house fly strains. Compared with their non-permethrin treated counterparts, although no significant induction of MdαE7 was detected in the susceptible aabys house flies, an elevated expression level of MdαE7 (~1.5 fold) was detected in both the resistant ALHF strain and wild-type CS strain when they were treated with permethrin at their corresponding LD_{50}s (Figure 3.4 A). A relative lower induction of MdαE9 was found in all three strains, with the inductive peak (~1.6 fold) achieved by LD_{50} permethrin treatment in ALHF strain (Figure 3.4 B). For gene MdαE13, although no significant induction was identified in the resistant ALHF strain, an initial induction (~1.8 fold) was detected in the CS strain when treated with permethrin at an LD_{90} dose, and induction in the susceptible aabys strain was even more evident, with an induction peak (~9.3 fold) appeared at the LD_{50} dose level (Figure 3.4 C). The inductive pattern of MdαE16 was similar in aabys and CS strains, initiating (~1.5 fold for aabys and ~3.2 fold for CS) at LD_{10} doses, arrived at a maximal (~2.0 fold for aabys and ~3.8 fold for CS) at LD_{50} doses and then declined (~0.8 fold for aabys and ~3.2 fold for CS) at LD_{90} doses. In ALHF strain, an initial induction (~3.4 fold) occurred at LD_{50}, but then significantly reduced (~1.9 fold) at LD_{90} (Fig. 4D). For gene MdαE17, a readily induced level (~1.5 fold, ~1.9 fold and ~3.5 fold for ALHF, CS and aabys, respectively) was achieved at their LD_{10}s, rose to a maximum (~2.3 fold, ~2.5 fold and ~4.1 fold for ALHF, CS and aabys, respectively) at LD_{50}s, but later kept stable at LD_{90s} (Figure 3.4 E). No significant induction of MdβE2 was detected in CS and aabys strain, while a slightly induction (~1.7 fold) was identified in ALHF when treated with permethrin at LD_{50} and LD_{90} doses (Fig. 4F). The MdIntE3 followed the same inductive

patterns in different house fly strains, with an initial induction (~1.5 fold, ~2.6 fold and ~6.2 fold for ALHF, CS and aabys, respectively) at LD_{10s}, reaching a peak (~1.9 fold, ~3.1 fold and ~7.0 fold for ALHF, CS and aabys, respectively) at LD_{50}s, and then no further elevation at LD_{90}s (Figure 3.4 G). In ALHF, MdIntE7 was initially induced (~2.3 fold) at LD_{50} dose, then slightly reduced (~1.9 fold) at LD_{90}. In aabys and CS strains, the inductive patterns were similar, with an initial induction (~3.8 fold) at LD_{10s}, reaching a peak (~3.9 fold) at LD_{50s}, and then slightly declined (~3.4 fold) at LD_{90}s (Figure 3.4 H). All these COEs can thus be induced at variable levels by different doses of permethrin, with a permethrin dose at LD_{50} producing the maximum inductive ability in all three house fly strains.

3.3.5 Responses of COE expression with permethrin challenge at different time points in resistant and susceptible house flies

In terms of the dose-dependent results, the LD_{50} for each house fly strains was chosen as the optimal permethrin dose to further explore the time-dependent inductive patterns of the *M. domestica* COEs. Our results indicated that nearly all these COEs can be induced with variable levels at different time points after exposure to permethrin at LD_{50} doses in all three house fly strains (Figure 3.5). No significant induction of MdαE7 was detected in susceptible aabys and CS strains, while an lower induction was discovered in ALHF (~1.7 fold) 48 hr after treatment (Figure 3.5 A). A similar induction pattern of MdαE9 was found in all three strains, with significant inductions (~2.3 fold, ~2.5 fold and ~2.7 fold for ALHF, aabys and CS, respectively) appeared 12 hr after treatment, declined 24 hr after treatment (Figure 3.5 B). For MdαE13, an evident time-dependent inductive pattern (ranging from ~8.5 fold to ~13.5 fold) was observed in aabys

at different time point, and an induction (~1.9 fold) was also observed in ALHF strain after 48 hr permethrin treatment. No time-dependent induction was identified in CS strain (Figure 3.5 C). A similar inductive pattern of MdαE16 was observed in ALHF and aabys strains, with an initiation (~6.7 fold for ALHF and ~4.7 fold for aabys) after 12 hr treatment, followed by a significant decline (~3.8 fold for ALHF and ~2.0 fold for aabys) after 24 hr, then enhanced (~8.3 fold for ALHF and ~5.1 fold for aabys) after 48 hr, and a final decrease, just as their untreated counterparts. In CS strain, the induction of MdαE16 started (~4.0 fold) after 12 hr treatment, maintained stable (~3.9 fold) after 24 hr, and finally reduced (~2.1 fold) after 48 hr (Figure 3.5 D). For MdαE17, a slightly inductive level (≤2.0 fold) was observed for CS strain at different time points, while in aabys strain an evident inductive peak (~9.5 fold) was appeared after 12 hr, and then dramatically reduced (~4.5 fold) after 24 hr permethrin treatment. The induction was initiated (~3.1 fold) after 12 hr, climbing to a peak (~5.8 fold) after 48 hr, and declined (~3 fold) after 72 hr in ALHF strain (Figure 3.5 E). For MdβE2, lower inductions occurred at 24 hr point (~1.7 fold) in ALHF and 12 hr (~2.0 fold) in aabys strain (Figure 3.5 F). The expression of MdIntE3 can be induced in different house fly strains at different time points after permethrin exposure, with the induction peak (~2.1 fold for ALHF , ~8.5 fold for aabys and ~3.5 fold for CS) occurring after 48 hr, 12 hr and 24hr permethrin treatment in ALHF, aabys and CS strain, respectively (Figure 3.5 G). The expression of MdIntE7 was only induced (~2.5 fold) 48 hr after permethrin treatment in the CS strain, while in the ALHF and aabys strain, it can be initiated (~3.8 fold for aabys and ~3.5 fold for ALHF) after 12 hr, reaching a peak (~4.3 fold for aabys and ~8.6 fold for ALHF) after 24 hr and 48 hr permethrin exposure in aabys and ALHF, respectively (Figure 3.5 H). The time-

dependent inductive patterns for all these COEs further suggest their close correlation with permethrin tolerance in house flies.

3.3.6 Autosomal mapping of COEs genes in *M. domestica*

Based on our transcriptome analysis of house flies, we went on to investigate the autosomal locations of the COEs that were up-regulated in the resistant house flies, *Musca domestica*. Our results indicated that these 11 up-regulated carboxylesterase genes were all either located on autosome 2 or autosome 3 in house flies (Table S3.3). To further confirm their autosomal location, five COE genes were chosen to conduct allele-specific PCR using ALHF allele specific primer pairs (Table S3.3). The autosome mapping results showed that the ALHF allele-specific primer sets for these five COE genes amplified specific DNA fragments only in flies where autosome 2 carried the wild-type marker from ALHF (Figure 3.6), demonstrating that these five COE genes were located on autosome 2 of the house flies, which is consistent with our results from transcriptome analysis (Table S3.3). Two carboxylesterase genes from different clades, MdαE7 and MdβE2, were then selected in order to examine their autosomal linkage of factors from different autosomes and thus determine the effects of the co-regulation on the expression of the up-regulated MdαE7 and MdβE2 genes among five house fly homozygous lines, A2345, A1345, A1245, A1235 and A1234. Analyzing the gene expression in these house fly lines with different autosomal compositions enabled us to evaluate the role of genes or factors on each of the autosomes involved in COE gene overexpression in the ALHF house flies. The results showed that when autosomes 2 and 5 in the ALHF house flies were replaced by the corresponding autosomes from aabys (i.e., lines A1345 and A1234), the expression of MdαE7 was down-regulated compared to that

observed in the ALHF house flies (Figure 3.7 A). When autosomes 1, 2 or 5 in the ALHF house flies were replaced by the corresponding autosomes from aabys (i.e., lines A2345, A1345 and A1234), the expression of MdβE2 was again down-regulated compared to that in ALHF house flies (Figure 3.7 B), suggesting that factors on autosomes 2 and 5 are indeed involved in regulating the expression of MdαE7 genes and that factors on autosomes 1, 2 and 5 are involved in regulating the expression of MdβE2 genes in ALHF house flies.

3.4 Discussion

Alterations in the esterase activities caused by mutant alleles or overexpression are two of the major mechanisms underpinning the COE hydrolytic detoxification-mediated resistance to pyrethroids in several insects (Wu et al. 2011; Devonshire and Moores, 1982; Heidari et al. 2005; Devonshire et al. 2007; Zhang et al. 2007; Coppin et al. 2012). However, the precise nature of COE-mediated resistance needs to be further understood. Both constitutively increased expression (overexpression) and induction of COEs are thought to be responsible for increased levels of detoxification in insecticides (Zhang et al. 2010; Wei et al. 2016).

The induction of gene expression may reflect a good compromise between energy saving (i.e. enhancing the activity of the detoxifying system only when a chemical stimulus occurs) and adjustment to a rapidly changing environment (Brattsten, 1979). In this study, we characterized the constitutive and inductive expression patterns of COEs in response to a permethrin challenge in house flies. Our results indicated that 8 *M.domestica* COEs have their expression level not only constitutively overexpressed in resistant house flies, but can be further elevated to higher levels with permethrin exposure.

Among these COEs, five belong to the α-esterase clade and one to the β-esterase clade, both of which are recognized as major clades involved in lipid and xenobiotics metabolism (Birner-Gruenberger et al. 2012; Jackson et al. 2013; Campbell et al. 2003; Claudianos, 1999). Their location in these clades point to their potentially important roles in metabolizing insecticides in house flies. Two other genes, MdIntE3 and MdIntE7, belong to the integument esterase clade that has been strongly linked to the inactivation and degradation of pheromones or detoxifying xenobiotics penetrating the integument (Oakeshott et al. 2010; Ishida and Leal, 2005; Yu et al. 2009). Previous studies have reported that the integument esterase E4 and FE4 can metabolize OPs and thus confers resistance in insects (Li et al. 2007). Therefore, it is possible that the overexpression of these two integument carboxylesterases contributes to the metabolism of permethrin in house flies.

Insecticide resistance is generally assumed to be a pre-adaptive phenomenon, where prior to insecticide exposure rare individuals carrying an altered genome already existed in the population, thus allowing their survival after permethrin selection. We expected that the individuals carrying this genetic variation would be quickly passed on and developed among house fly populations under permethrin selection and become dominant in house flies. This, to some extent, can better explain the constitutive and inductive overexpression patterns of these carboxylesterases in different house fly strains. Our previous inductive studies of multiple P450s in house flies and mosquitoes had revealed that the gene inductive levels were correlated with resistant levels of insects, with a much higher induction occurring in the resistant rather than the susceptible insects (Zhu et al. 2008; Liu et al. 2011). Unlike this finding, our current study found no specific

correlation between the inductive levels of multiple COEs genes and resistant levels in house flies. In fact, the inductive levels of several COEs, such as MdαE13, MdαE17, MdIntE3 and MdIntE7, were actually much higher in susceptible aabys and CS strains than in resistant ALHF strain. This result is, however, consistent with those of previous studies that indicated that phenobarbital can induce the expression of P450s in the susceptible house fly CS strain but not in the resistant LPR strain (Scott and Lee, 1993; Liu and Scott, 1997).

Our results also strongly support clear dose- and time-dependent induction in house fly COEs. For the majority of constitutively up-regulated COEs, expression levels can be significantly raised in response to permethrin at different doses and time points. For some COEs, such as MdαE16, the weak induction by permethrin at a relatively low dose (LD_{10}) in the resistant ALHF strain may be the consequence of the rapid metabolism of permethrin at lower doses, meaning that it never reaches the threshold needed for induction (Islam et al. 2006). Conversely, the low or nonexistent levels of induction at higher (LD_{90}) doses (e.g. MdαE7) may indicate a dysfunction of the induction system in insects that have been highly poisoned (Willoughby et al. 2006). The time-dependent inductive patterns observed also suggests that multiple genes, including MdαE9, MdαE16 and MdαE17, can only be induced by permethrin at certain time intervals, which is supported by the hypothesis that induction is a temporary event that can only occur at short time intervals or during some specific developmental stages of insects (Terriere, 1984).

Taken together, all these studies indicate that both the constitutive overexpression and inductive expression of COEs are co-responsible for insecticides detoxification,

evolutionary insecticide selection, and the ability of insects to adapt to changing environments. In further work, we plan to functional characterize the roles of these COEs in metabolizing insecticides and therefore conferring insecticide resistance in house flies via *in vitro* metabolism studies.

3.5 References

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Table 3.1 Toxicity of permethrin to ALHF, aabys and CS strains of house flies, *Musca domestica*

Stain	df	n[a]	X^{2a}	LD_{10} (95%CI)[b]	LD_{50} (95%CI)[b]	LD_{90} (95%CI)[c]	Slope (SE)	RR*
ALHF	15	1200	70.5	975.0 (368.0-1565)	5239.0 (4454.0-6662.0)	13234.0 (10270.0-20442.0)	3.5 (0.2)	2095.0
aabys	23	1530	471.9	1.0 (0.5-1.5)	2.5 (2.0-3.5)	5.0 (3.5-12.0)	4.2 (0.2)	--
CS	25	1740	256.2	11.5 (5.0-16.5)	48.5 (35.9-93.0)	207.5 (103.5-323.0)	2.0 (0.1)	19.4

n[a]: Number of house flies tested.

[b]LD_{10}, LD_{50} and LD_{90} values in ug per house flies.

* Resistance Ratio (RR) was calculated as the ratio of LD_{50} of ALHF or CS to LD_{50} of aabys.

Table S3.1. List of primers used for qRT-PCR analysis

Gene	Accession No.	Forward primer	Reverse Primer	efficiency	R^2
MdαE1	XP_011291889	ACACAACATGGTCTCCGGTG	ATTGTTGCCGTTCCAGTTCG	91.35%	99.28
MdαE2	XP_005174776	TGGTGCTGTTGGTTGTACGT	GCCAGAATTCCATGCGTTCC	95.36%	97.25
MdαE3	XP_005178691	TGATAGACCGGCGACAATGG	AGCGATACAGGTAGGTGGGT	98.38%	99.15
MdαE4	XP_005178701	TGTATGTCGGGCACAATGTT	CTCCTCGTCGTTGTCTCCTC	96.32%	98.23
MdαE5	XP_005178692	TAACAGCCACCGAACAGACC	TGTTTGGCCAGGCGATAGG	96.84%	98.60
MdαE6	XP_011290557	CGTCCCTTAATGTTCCCGGT	AATGAACCGAAGCAGCACCA	100.13%	98.25
MdαE7	XP_005178694	GGGAGTTGGCTGACAGTGAA	CGATGCATGGGGAAGAGGAA	93.80%	99.87
MdαE8	XP_005178696	TAATGGCTCTGCGATGGGTG	ATGTAATGGACCGAGGCACC	89.69%	99.89
MdαE9	XP_011290558	ATGACGTTGCCAGTGACAGT	GTTCATGTCGTTGGCTGCTG	105.35%	99.46
MdαE10	XP_005182287	GCATACCTTACGCCTTGCCT	ATTTCGTTGCATGGCCTTCG	92.38%	97.19
MdαE11	XP_005178697	GGTGAGGTTAAGGGTGCCAA	TCCAAAACACCATCCCAGGG	91.34%	99.81
MdαE12	XP_005175162	GCCGCCACATATTGCTACTT	TTGGGATCGGAACATAGGAA	92.65%	98.61
MdαE13	XP_005175161	GCACGCTCGAAATATGCTCC	ATGACACACCCCTCGAATCG	95.30%	99.39
MdαE14	XP_011295261	GGATGATCCGGAGCTGAATA	CGGCACTTTCACCAAAAACT	90.68%	98.65
MdαE15	XP_005178699	CAGACCAAATATGGCCGAGT	CCTCACACCACAGGGTCTTT	95.65%	99.25
MdαE16	XP_011295279	GTCAAAGTGCTGGAGCATCA	TTTTTGATGCCCTGATAGCC	97.24%	98.27
MdαE17	XP_005175160	CCCCATGCCAAGGAGACATT	ATAGGTGGGTGTTGGTGCAG	98.44%	99.31
MdGluE1	XP_011296425	ACCCAGGCTAGACAGCAGAA	CCCGTGGCATATGGAATATC	88.79%	98.17
MdGluE2	XP_011296426	TTGCCTGACCATGACCATTA	AGTTGCTGCCCTCAAAGAAA	96.37%	96.69
MdGluE3	XP_005177159	GATCCTGTACCGTGCTCCTG	CGTAAGTGGCCGAAACGATG	93.07%	99.95
MdGluE4	XP_011290463	TGGAGCAAGCTGAACGGATT	GCCACATTACGCAAACACACA	103.27%	98.47
MdGluE5	XP_005185445	GCCCCTTAACTTCGACCTCC	GCCCAAAACACCCTCCAAAC	96.32%	99.15
MdNeuE1	XP_011293138	TAAATTCGGCGGCATTTTAC	TGATGGAATGTTTGCTGCAT	101.23%	98.57
MdNeuE2	XP_011295266	TGCTCAAACTGTGGATTTGC	CCATGTATGCAACCTTGACG	86.59%	97.56
MdNeuE3	XP_011295269	ATGGATGCGGTGAGAAAATC	CCACGGTGGAGCAACTTAAT	99.74%	98.65
MdGliE1	XP_011296159	TCTCATCAGGCAGCCCTACT	GCCCATTATGCTCAAACGAT	96.23%	99.14
MdGliE2	XP_005178235	CCCCGCAGACTAGACACATT	GTTGGCCAAAAACACGACTT	100.26%	96.13
MdAChE1	XP_005183285	TTCAGGGTCGCGATGTACAC	GCATCTAGGACACCATGCCA	94.36%	99.77
MdUnE1	XP_005185078	TAATGGCCAGTACAGCGACG	GAACCGCAACAAGTCCCAAC	93.28%	99.78
MdIntE1	XP_005180750	GTTGGGGAGTTGCGTTTCAA	TAGCCATGTCCGATTCACCG	90.64%	98.21

MdIntE2	XP_005180749	AGGGTCTGTTTCATCGTGCC	GGGATGCACAGGACACTTCA	89.79%	99.21
MdIntE3	XP_005180748	TGCCGGAAGTCTAAGTGTGG	CCCAATGGCGATGCACTTTG	107.71%	98.42
MdIntE4	XP_005180752	AGGGCGTGGAAATGGAAACT	TCACTGGCTGGGGATTTTGA	97.31%	99.46
MdIntE5	XP_005177449	GCCCTCCGAAAAGAATCCCA	TCACCAATACCACACTGCGA	84.59%	98.98
MdIntE6	XP_005180039	CCACTGGGTCCACTGAGATT	GTTGGTGTGGCTGGAGATTT	99.26%	97.56
MdIntE7	XP_005177448	CCAATGGCCATATACGGAAC	GGAAACAGCAACACCACCTT	101.35%	98.66
MdβE1	XP_005181018	TTCGAATGTGAGGCCCGTAC	ATCAGATGACGGCCCACAAG	98.79%	99.31
MdβE2	XP_005183940	GGCTTTTAGTACATCTGCTCGG	GCACTGGGTAGTGGAAGATTAG	91.91%	98.61
MdJhE1	XP_005181511	AAAGTGCTGGTGGGGTATCG	CAAAGGGAACATTGGCGGTG	96.37%	97.64
β-actin	XP_005183264	ATGAGGCTCAGAGCAAACGTGG	AGTCATCTTCTCGCGATTGGCCT	96.45%	99.09
RPS3	XP_005183698	GTGCGTCGTGCCTGCTAT	ATGGGTCACCAGAGTGGATC	99.25%	99.51

Table S3.2. The relative expression ratios of remaining 28 carboxylesterase genes in *M. domestica*

Gene	Accession Number	The relative expression level (mean±SEM) in different strains		
		ALHF	aabys	CS
MdαE1	XP_011291889	1.98±0.15	1.00	2.43±0.20
MdαE2	XP_005174776	1.23±0.12	1.00	0.81±0.08
MdαE3	XP_005178691	2.12±0.16	1.00	2.24±0.28
MdαE4	XP_005178701	1.07±0.28	1.00	1.57±0.41
MdαE6	XP_011290557	0.83±0.09	1.00	1.12±0.10
MdαE8	XP_005178696	1.85±0.25	1.00	1.75±0.24
MdαE10	XP_005182287	1.56±0.22	1.00	1.62±0.49
MdαE11	XP_005178697	1.65±0.17	1.00	1.15±0.14
MdαE12	XP_005175162	2.45±0.61	1.00	2.08±0.45
MdαE14	XP_011295261[a]	0.02±0.01*	1.00	1.28±0.21
MdαE15	XP_005178699	2.02±0.21	1.00	2.43±0.29
MdGluE1	XP_011296425	1.31±0.29	1.00	1.87±0.35
MdGluE2	XP_011296426	1.10±0.22	1.00	1.90±0.52
MdGluE4	XP_011290463	2.09±0.89	1.00	2.74±0.83
MdGluE5	XP_005185445	1.60±0.30	1.00	1.99±0.15
MdNeuE1	XP_011293138	1.67±0.96	1.00	1.23±0.69
MdNeuE2	XP_011295266	1.68±1.02	1.00	2.15±1.30
MdNeuE3	XP_011295269	2.84±0.36	1.00	3.49±0.62
MdGliE1	XP_011296159	0.51±0.10	1.00	0.74±0.13
MdGliE2	XP_005178235	2.11±0.41	1.00	3.72±0.45
MdUnE1	XP_005185078	1.25±0.21	1.00	1.43±0.19
MdIntE1	XP_005180750	0.65±0.08	1.00	0.83±0.15
MdIntE2	XP_005180749	1.21±0.21	1.00	2.75±0.46
MdIntE4	XP_005180752	0.36±0.05	1.00	0.56±0.08
MdIntE5	XP_005177449	0.78±0.15	1.00	1.24±0.17
MdIntE6	XP_005180039	0.52±0.11	1.00	1.64±0.33
MdβE1	XP_005181018	1.43±0.15	1.00	1.51±0.10
MdJhE1	XP_005181511	0.55±0.09	1.00	1.76±0.25

XP_011295261[a]: This gene is significantly down-regulated in resistant ALHF strain compared to susceptible aabys and CS strains.

Table S3.3 The autosomal location of 11 up-regulated carboxylesterases genes and allele-specific primers used for autosomal mapping

Gene	Clade	Accession No.	Autosome #	ALHF allele-specific primer pairs	
				Forward(5'-3')	Reverse(5'-3')
MdαE5	α-esterase	XP_005178692	2	F1:CCCGGCAATGCTGGTATCAAAGA F2:AATGGGTTAAGCAATACATC	AACCTCGACATCCTTATTTGC
MdαE7	α-esterase	XP_005178694	2	F1:GTTTGGGTGTGTTGGGTTTC F2:CGGTAATTCCATGTGCTCATT	TTCACTATGGCAGCCCTTTC
MdαE9	α-esterase	XP_011290558	2	F1:AAACATCTTCTCCGGTCTGTG F2:CTGACCGGTCGGTCACAT	TTGACGGCAATTCGCATTTGAT
MdαE17	α-esterase	XP_005175160	2	F1:ACTATTCGGAGAGAGTGCCGG F2:AAGAAGAACAATTCAATCATCTA	AATAATAATAGGTGGGTGTT
MdβE2	β-esterase	XP_005183940	2	F1:TTGAAATGTCCCAATTTGGA F2:TTATGACTCGGCATCCAAGA	AGAGCATATCCCAAACTATAATC
MdαE13	α-esterase	XP_005175161	2		
MdαE16	α-esterase	XP_011295279	2		
MdGluE3	Glutactin	XP_005177159	3		
MdIntE3	Integument esterase	XP_005180748	3		
MdIntE7	Integument esterase	XP_005177448	3		
MdAchE1	Acetylcholin esterase	XP_005183285	2		

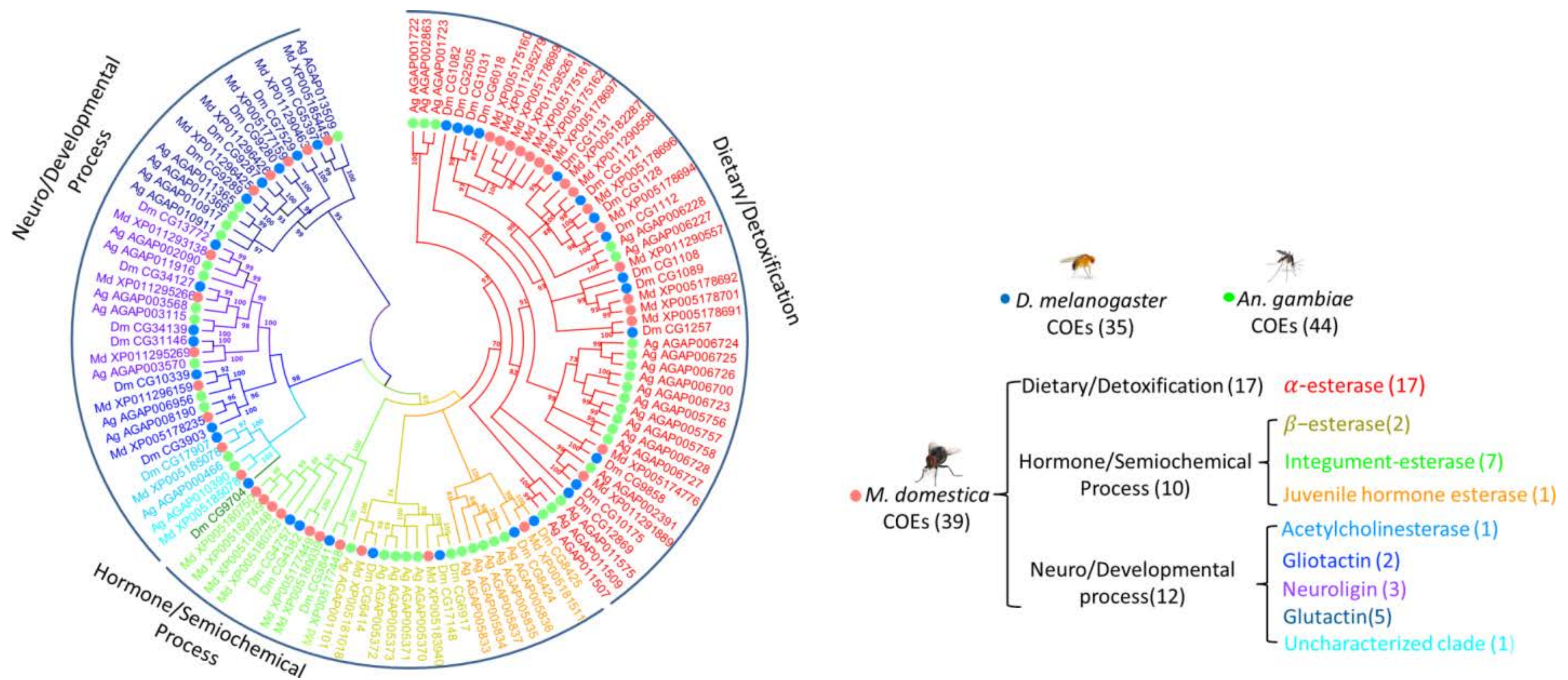

Figure 3.1. The classification of carboxylesterases. Muscle was used to perform the multiple sequence alignment. The phylogeny tree was constructed by FastTree with default settings using amino acid sequences from the *Musca domestica* (Md) database (https://www.ncbi.nlm.nih.gov/ assembly/GCF_000371365.1/), the *Drosophila melanogaster* (Dm) database (https://www.ncbi.nlm.

nih. gov/genome/?term=Drosophila+melanogaster) and the *Anopheles gambiae* (Ag) genome database (https://www.ncbi.nlm.
nih.gov/genome/?term=Anopheles+Gambiae). Mega 6.0 was utilized to visualize the constructed phylogeny tree. Different clades are labeled in different colors.

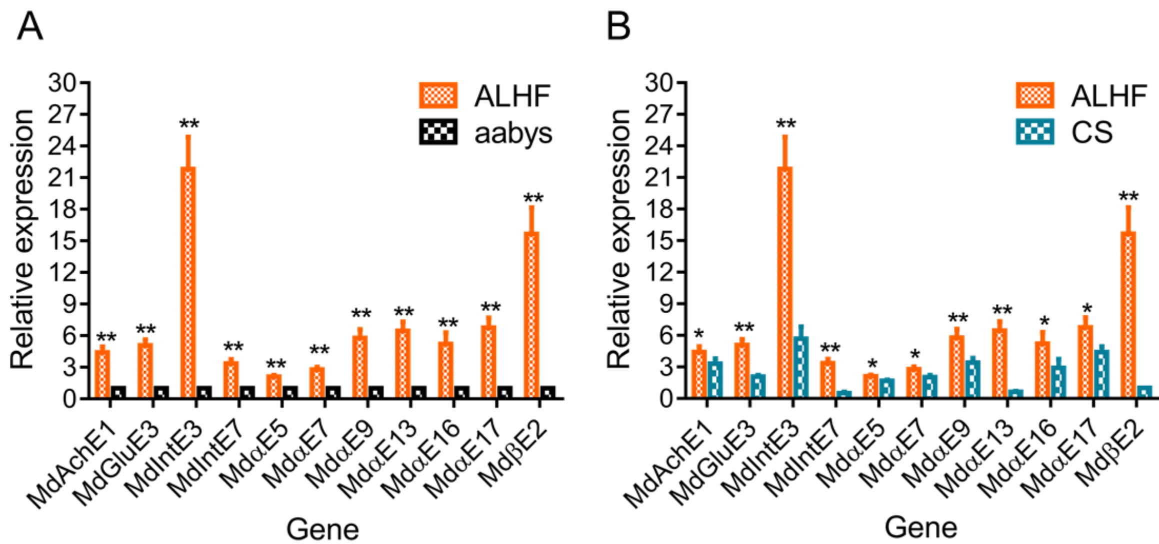

Figure 3.2 Relative expressions of carboxylesterase genes in different house fly strains. The relative gene expression ratios were calculated by comparing the expression of COEs in the aabys strain. In order to eliminate any differences due to the genetic background, only those genes whose expression levels were significantly increased more than 2.0-fold in the resistant ALHF strain compared with that in both the susceptible aabys and CS strains were considered to be up-regulated. A) The relative expression levels of up-regulated COEs in the resistant ALHF strain compared with those in the susceptible aabys strain. B) The relative expression levels of up-regulated COEs in the resistant ALHF strain compared with those in the susceptible wild-type CS strain. Data are shown as the mean±SEM (n=4). Asterisks above the bars indicate significant differences in the gene expression levels. (* indicates $P<0.05$, **indicates $P<0.01$).

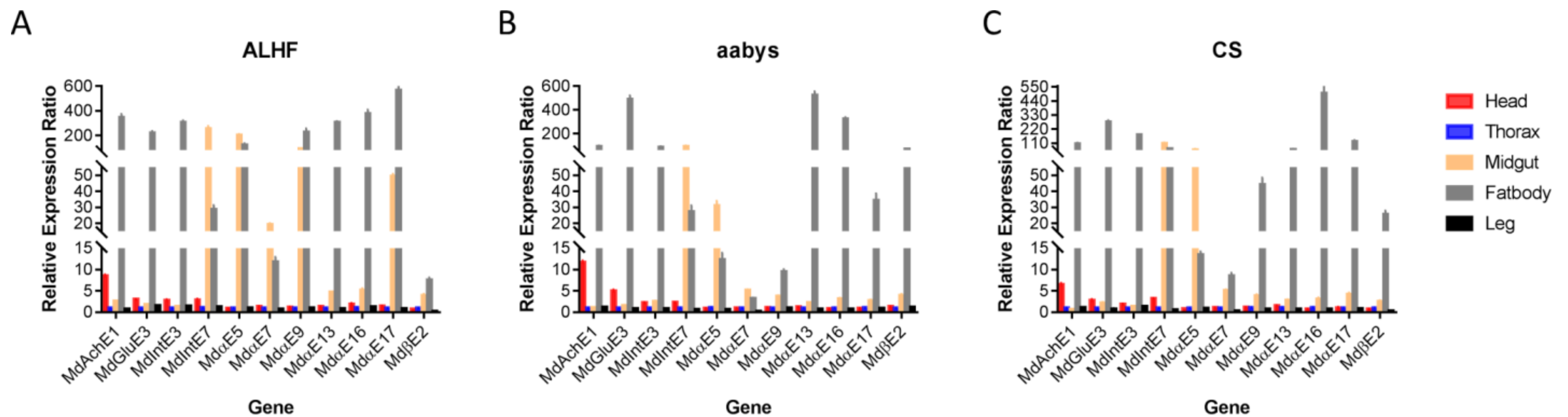

Figure 3.3 The tissue-specific expressions of carboxylesterase genes in different house fly strains. The gene expression values in different tissues were validated relative to that in thorax for each up-regulated COE among three different house fly strains. A) The relative expression ratios of COEs in different tissues of ALHF strain. B) The relative expression ratios of COEs in different tissues of aabys strain. C) The relative expression ratios of COEs in different tissues of CS strain. All data are shown as the mean±SEM (n=3).

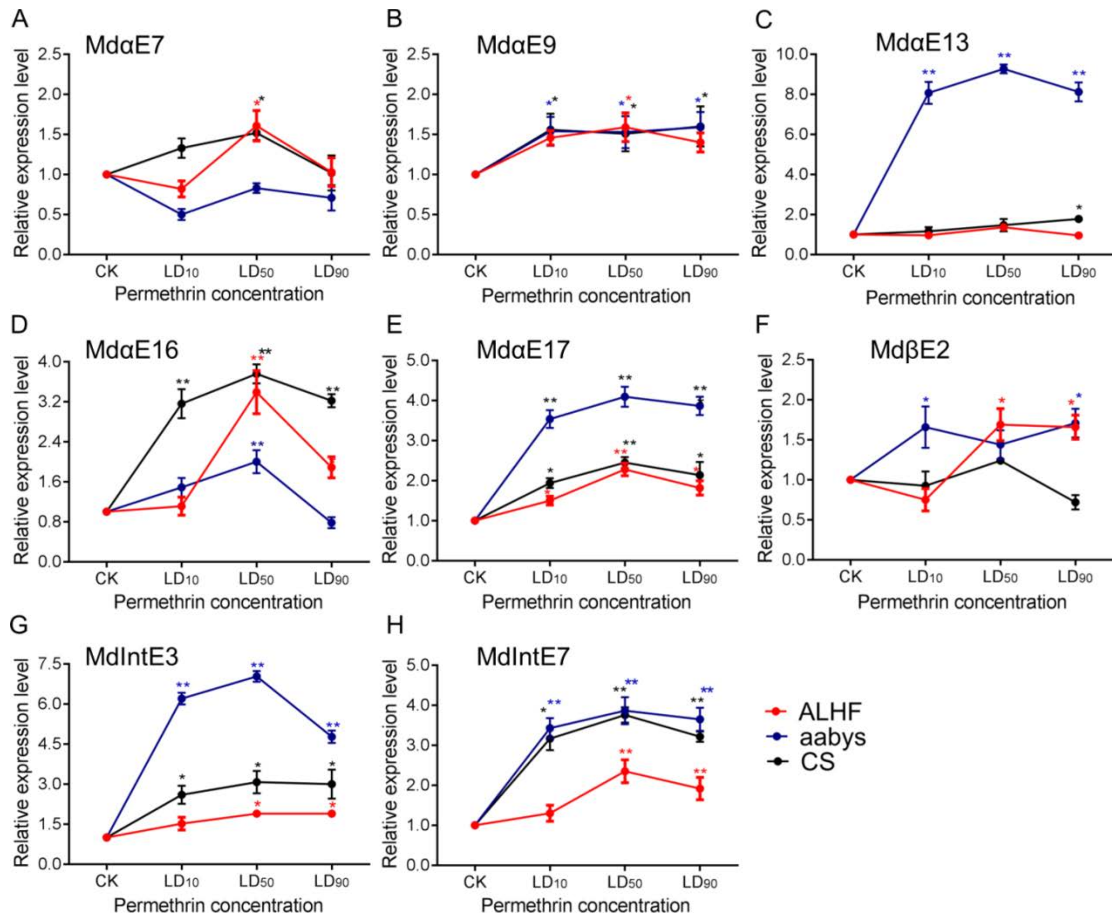

Figure 3.4 The dose-dependent inductive expression patterns of carboxylesterase genes in house flies. The up-regulated genes were chosen to investigate the inductive capabilities after 24 hr exposure to permethrin at different doses (LD_{10}, LD_{50} and LD_{90}). The Y axis represents the ratio of the gene expression in each treatment to that in acetone treated control house flies. The red lines represent the inductive profiles of COEs in the resistant ALHF strain, the blue lines represent the inductive profiles of COEs in the susceptible aabys strain, and the black lines represent the inductive profiles of COEs in the susceptible wild-type CS strain. The horizontal dashed line represents the 1.5-fold expression level. All data are shown as the mean±SEM (n=3). Significant differences are indicated by * ($P \leq 0.05$) and ** ($P \leq 0.01$).

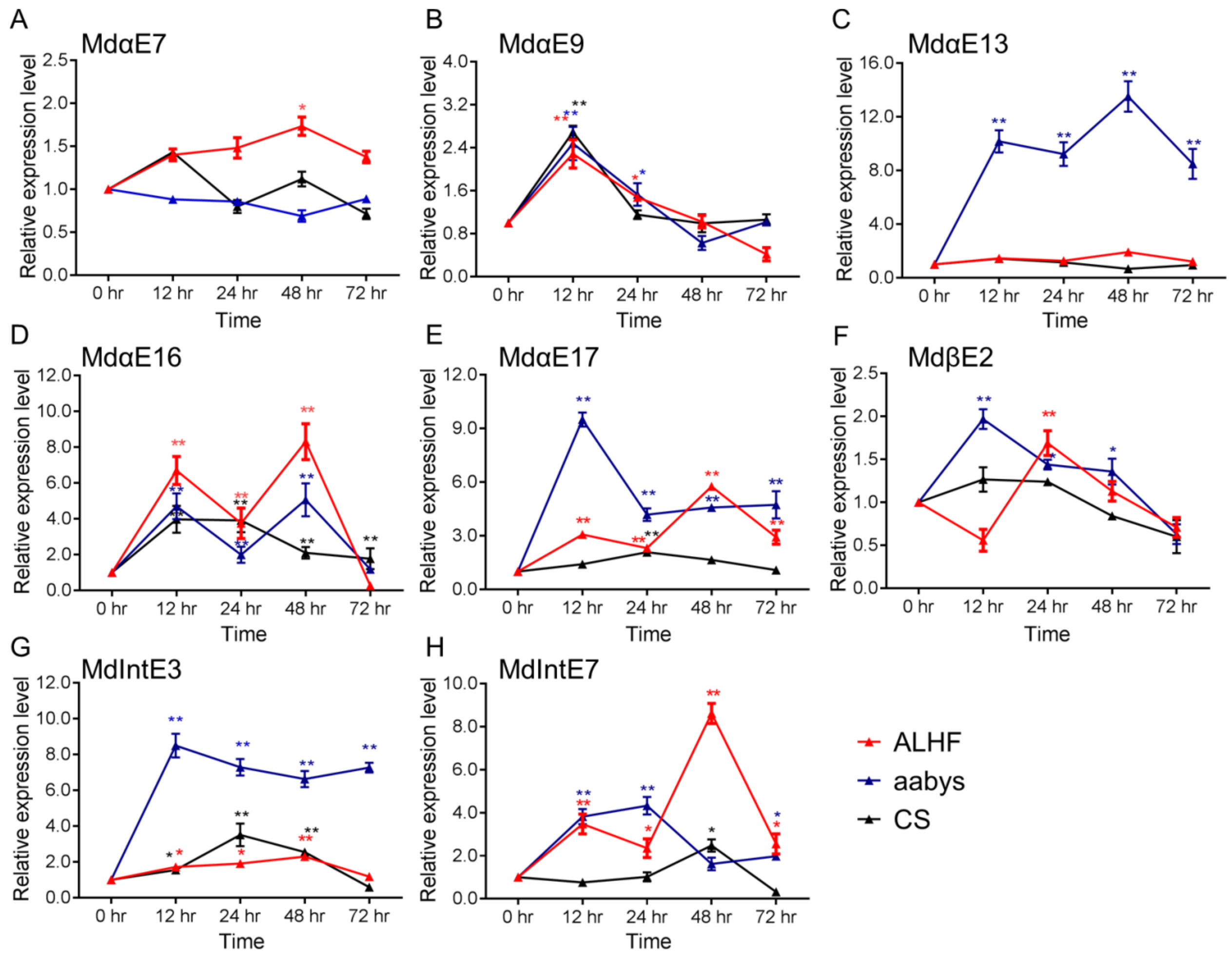

Figure 3.5 The time-dependent inductive expression patterns of carboxylesterase genes in house flies. The inductive expression patterns of COEs in different strains were tested in response to permethrin at an LD_{50} concentration after 12 hr, 24 hr, 48 hr and 72 hr exposure. The Y axis represents the ratio of the gene expression in each treatment to that in acetone treated control house flies (note that the Y axis scales vary among the sub-figures). The red lines represent the inductive profiles of COEs in the resistant ALHF strain, the blue lines represent the inductive profiles of COEs in the susceptible aabys strain, and the black lines represent the inductive profiles of COEs in the susceptible wild-type CS strain. The horizontal dashed line represents the 1.5-fold expression level.

All data are shown as the mean±SEM (n=3). Significant differences are indicated by *($P \leq 0.05$) and **($P \leq 0.01$).

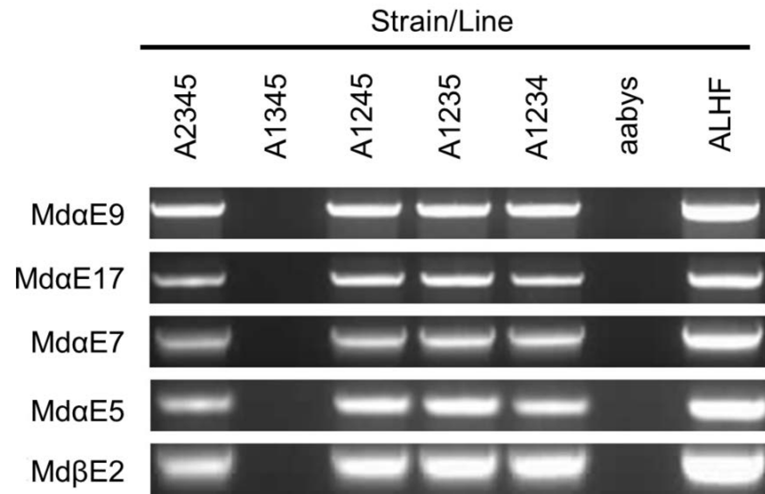

Figure 3.6 The allele-specific RT-PCR autosomal mapping of the *Musca domestica* carboxylesterase genes. The absence of a PCR product band in a house fly backcross line indicates that the gene is located on the corresponding autosome of house flies (i.e. the absence of a band in the A1345 line indicates that the gene was located on autosome 2).

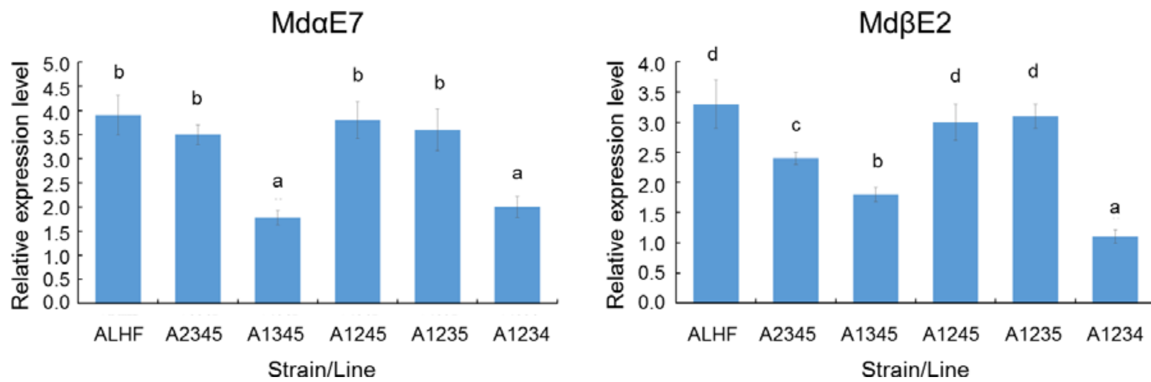

Figure 3.7 The relative expressions of MdαE7 and MdβE2 in ALHF and five house fly homozygous lines. The relative expression levels of COEs are shown as a ratio in comparison with that in the aabys strain. The results are shown as the mean ± SEM (n = 3). Different letters above the bars indicate significant differences of gene expression in different strains/lines ($P \leq 0.05$). (A) MdαE7. (B) MdβE2

Chapter 4: Characterization of carboxylesterases in permethrin resistant house flies, *Musca domestica*

Abstract

Carboxylesterase-mediated metabolism is thought to play a major role in insecticide resistance mechanisms in insects. Several carboxylesterase genes were not only up-regulated in a pyrethroid resistant house fly strain ALHF, but also induced to a higher expression level by permethrin in different house fly strains. These genes were expressed in insect *Spodoptera frugiperda* (Sf9) cells and their hydrolytic activities toward different esterase substrates and permethrin were characterized. These carboxylesterases efficiently hydrolyzed α-naphthyl acetate rather than β-naphthyl acetate. A cell-based MTT cytotoxicity assay revealed that Sf9 cells expressing carboxylesterases increased the tolerance to permethrin, suggesting the important roles of these carboxylesterases in metabolizing permethrin. The functions of these carboxylesterases were further characterized by conducting *in vitro* metabolism studies toward permethrin and its potential metabolites 3-phenoxybenzyl alcohol and 3-phenoxybenzaldehyde. All carboxylesterases showed significant efficiencies in metabolizing permethrin compared with controls. This finding indicated not only a potential route for permethrin metabolism in insects, but also an important role of these carboxylesterases in metabolizing permethrin and conferring resistance in house flies. Homology modeling and docking analysis were used to explore the interaction between permethrin and carboxylesterase

protein, thereby confirming the metabolic roles of these carboxylesterases toward insecticides in house flies.

4.1 Introduction

House flies, *Musca domestica*, are ubiquitous agricultural and sanitary pests that can mechanically transmit more than 100 human and animal disease pathogens, including bacterial, protozoan, helminthic and viral pathogens (Barin et al. 2010; Abbas et al. 2014; Scott et al. 2014; Sasaki et al. 2000; Hewitt et al. 2011). Pyrethroids are currently the most widely used insecticides for the management of many different insects including house flies due to their high insecticidal potency, low mammal toxicity and environmental friendliness (Casida et al. 1983; Soderlund et al. 2003). However, house flies can rapidly develop resistance and cross-resistance to insecticides, which is a major concern for house fly control strategies worldwide (Abbas et al. 2014; Kaufman et al. 2010; Scott et al. 2013; Liu and Yue, 2000).

Previous work to characterize the underlying molecular basis for the development of insecticide resistance has already laid the foundation for a better understanding of this important issue and facilitated efforts to design novel strategies to efficiently prevent or minimize the spread and evolution of resistance development and, therefore, control many insect pests (Roush et al. 2012; Hemingway et al. 2000). The interactions of multiple mechanisms (i.e., increased detoxification and decreased target site sensitivity) or genes (i.e., cytochrome P450s and carboxylesterases) responsible for insecticide resistance have been extensively studied in recent years (Liu, 2015; Corbel et al. 2007; Bass et al. 2014). In particular, carboxylesterases, as one of the major detoxifying enzymes in insects, have attracted attention for their potential role in sequestering and

metabolizing insecticides (Grigoraki et al. 2015; Grigoraki et al. 2017; Wheelock et al. 2005). Multiple carboxylesterase genes have been shown to be transcriptionally up-regulated in various resistant insects, including house flies (Cao et al. 2008; Bao et al. 2010; Bass and Field, 2011; Adelman et al. 2011; Zhang et al. 2010; Demkovich et al. 2015; Fuentes et al. 2013). These overexpressed carboxylesterases are thought to sequester the insecticides and hydrolyze them into less harmful substances, thus facilitating excretion outside the insect bodies (Wheelock et al. 2005; Field and Blackman, 2003). In both *Aedes aegypti* and *Anopheles gambiae* mosquitoes, pyrethroids can be metabolized by carboxylesterases to form PBOH (phenoxybenzoic alcohol) and PBCHO (phenoxybenzaldehyde), which can be further metabolized by cytochrome P450 monooxygenases to PBCOOH (phenoxybenzoic acid) (Somwang et al. 2011; Chandor-Proust et al. 2013).

Previous studies have revealed that the expression levels of four carboxylesterase genes, MdαE7, MdβE2, MdαE17 and MdIntE7, are not only up-regulated in the pyrethroid resistant house fly strain ALHF, but can also be induced to much higher levels in response to permethrin treatments, demonstrating their close relationship with insecticide resistance (Feng et al. 2018). For gene MdαE7, there is evidence to suggest that both qualitative and quantitative changes may contribute to insecticide resistance in resistant house fly strains based on measurements of its hydrolytic activity toward insecticide-like substrates (Zhang et al. 2010; Wang et al. 2012; Cui et al. 2015). However, as yet the functions of these carboxylesterase genes in metabolizing insecticides have not been well-studied. In this study, the tolerance of insect Sf9 cells expressing carboxylesterases toward permethrin at different concentrations was tested in

the presence or absence of S, S, S-tributylphosphorotrithioate (DEF), a common inhibitor of carboxylesterases, suggesting the important roles of these carboxylesterases in metabolizing permethrin. Moreover, an *in vitro* metabolism assay with a baculovirus-mediated insect Sf9 cell expression system that was performed to characterize the functions of these carboxylesterases in metabolizing insecticides *in vitro* found direct evidence that these carboxylesterase are indeed involved in pyrethroid resistance by metabolizing permethrin insecticides, adding to our understanding of the carboxylesterase-mediated mechanism governing the development of insecticide resistance. Finally, based on the crystal structure of *Lucilia cuprina* αE7 structure (LcαE7) which was constructed to reflect the interaction between carboxylesterase and OP insecticides (Jackson et al. 2013), the homology modellings of *Musca domestica* carboxylesterases were built to display the interactions of pyrethroids within protein active site and thereby demonstrating their metabolic roles toward pyrethroids and thus conferring insecticide resistance in house flies.

4.2 Materials and Methods

4.2.1 House fly strains

ALHF, the multi-insecticide resistant house fly strain used in this study, was collected from a poultry farm in Alabama after a control failure with permethrin. Its high resistance level (~2000 fold to permethrin) was achieved by selection with permethrin for 6 consecutive generations and maintained under biannual selection with permethrin (Liu and Yue, 2000; Feng et al. 2018; Tian et al. 2011). The house flies were reared at 25±2 ˚C under a photoperiod of 12:12 (L: D) hr and fed with sugar and water.

4.2.2 Construction of pENTR™ expression plasmids of carboxylesterase genes

Total RNA was extracted from 20 3-day old adult female ALHF house flies using the acidic guanidine thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987). The DNA was removed from the total RNA using DNase (TURBO DNA-free, Ambion). The first-strand cDNA was synthesized with DNA-free total RNA using a Transcriptor First Strand cDNA Synthesis Kit (Roche) and oligo-dT primer following the manufacture's instructions. The pENTR™ expression plasmids of carboxylesterases were constructed with gene-specific primers designed based on their full-length nucleotide sequences (https://www.ncbi.nlm.nih.gov/genome/?term=Musca+domestica) with four nucleotide bases CACC added to the 5'end of forward primer (immediately upstream of the ATG transcription start codon) (shown in Table 2), which enables the carboxylesterase genes to be directly cloned into the pENTR™ TOPO® vector (Invitrogen) by annealing the CACC sequence in the PCR products with the overhang tag GTGG in the vector. The recombinant vector was then transformed into One Shot® competent *E.coli*. pENTR™ plasmids with target carboxylesterase genes were purified using the PureLink HQ Mini plasmid purification Kit (Invitrogen). The orientation of the inserted genes was detected by using the forward primer of each of the specific genes and the reverse primer of M13. Expression plasmids were further verified by sequencing.

4.2.3 Recombinant baculovirus expression of carboxylesterases in Sf9 cells

The pENTR™ plasmid of each carboxylesterase gene was ligated with BaculoDirect Linear DNA using the LR clonase™ II enzyme mix through the BaculoDirect™ Baculovirus Expression System. The constructed recombinant baculovirus was then transfected into *Spodoptera frugiperda* (Sf9) cells using CellfectinR

II Reagent (Invitrogen) to produce recombinant baculovirus stock solutions. The large-scale expression of carboxylesterase proteins in the Sf9 cells was performed according to the manufacture's instructions (Invitrogen). The titer of the baculovirus was measured by plaque forming assay and a titer of ~2×10^8 pfu/mL was used to infect Sf9 cells for the large-scale expression of carboxylesterase proteins. The parental Sf9 cells and pENTR™ CAT (plasmid producing baculovirus expressing chloramphenicol acetyltransferase (CAT) protein [Invitrogen]) infected cells served as controls. The cell lysate protein was harvested after 72 hrs infection, and centrifuged at 1000 rmp for 10 min at 4 ºC. The cell pellets were washed twice using ice-cold PBS buffer (pH 7.4) and then re-suspended in insect cell PE LB™ buffer. Subsequently, the dissolved cell lysate was centrifuged at 9800 rmp for 15 min, and then the supernatant was collected and stored at -80 °C.

4.2.4 Carboxylesterase activity assays

The activities of the carboxylesterases were determined by measuring their hydrolysis toward α-naphthyl acetate (α-NA) and β-naphthyl acetate (β-NA), both of which are ester substrates commonly used for esterase activities. The 25 µL of 10-fold diluted carboxylesterase protein solution (diluted with 0.1 M PBS buffer, pH 7.5) and 90 µL of 3×10^{-4} M substrate solution (either α-NA or β-NA dissolved in 0.1 M PBS buffer) were added to a 96-well microplate and incubated at 30 °C for 30 min. The reaction was stopped by the addition of 45 µL of freshly prepared diazoblue-sodium lacrysulphate solution (containing 2 parts of 1% fast blue B salt and 5 parts of 5% sodium dodecyl sulfate solution) in each well. After 15 min incubation at room temperature, the absorbance value of hydrolysis product α-naphthol or β-naphthol was measured at 600 nm or 550 nm respectively with a 96-well microplate reader (Cytation 3 imagine reader,

BioTekUSA) and then converted to product formation rate (pmol/min/mg protein) based on the standard curves for α-naphthol or β-naphthol. The protein concentration was measured with Bradford method (Bradford, 1976). The kinetic parameters, including Michaelis constant (Km) and maximal velocities (Vmax) for each carboxylesterase were measured using a series of substrate (α-NA or β-NA) concentrations ranging from 0.1 to 1.2 mM (add 3.8, 7.7, 11.5, 15.3, 19.2, 23.0, 26.8, 30.7, 34.5, 38.3, 42.2 and 46.0 μL of 3×10^{-3} M substrate solution respectively into each well). The reactions with proteins extracted from the parental Sf9 cells or CAT-recombinant baculovirus infected cells served as controls.

4.2.5 MTT cytotoxicity assay

The cytotoxicity assay of the carboxylesterases was conducted according to Gong et al. with modifications (Gong et al., 2017). The cells were infected by carboxylesterase-recombinant baculovirus (with a titer of $\sim2\times10^{8}$ pfu/mL) and cultured in 25 cm^2 flasks at 27 °C. Controls were parental Sf9 cells and pENTR^{TM} CAT expressing cells cultured under the same conditions. After 48 hrs cultivation, cells expressing different carboxylesterases were seeded onto 24 well plates with a density of 2×10^{5} cells/well, and later treated with permethrin standard solutions (the mixture of cis- and trans-isomers dissolved in acetonitrile) (analytical standard, Sigma-Aldrich), with final concentrations ranging from 50 μM to 400 μM. The cytotoxic effects of the permethrin standards were evaluated by MTT assays using a MTT cell viability assay kit (Sigma). After 48 hrs treatment, the cell culture medium was removed and the cells were washed with PBS buffer (0.1 M, pH 7.4). Later, 200 μL of triazolyl blue tetrazolium bromide solution (Sigma-Aldrich) (5 mg/mL) was added to each well and the plate was incubated at 37 °C

for 4 hrs, after which the absorbance values were measured at 540 nm using the Cytation 3 imagine reader (BioTek, USA). Three replications were conducted with independent protein preparations. The cell viability was calculated in comparison with acetonitrile-treated cells. For the inhibition assay, the inhibitor S, S, S-tributylphosphorotrithioate (DEF) (Sigma-Aldrich) (with final concentrations of 0.1, 1 and 10 µM) was added, together with 200 µM permethrin in each well, and cell viability was calculated in comparison with treatments with no DEF added.

4.2.6 *In vitro* metabolism of permethrin and its metabolites by carboxylesterases

Each substrate standard (Permethrin, PBOH or PBCHO) was initially dissolved in acetonitrile to make 1 mM stock solution. Serial dilutions of stock solution were then prepared in acetonitrile to create the standard curve for each. The 700 µL samples of metabolism reaction contained 20 uM substrate standard and 1 mg carboxylesterase protein (MdαE7, MdαE17, MdβE2 or MdIntE7) in 0.2 M Tris-HCl buffer. After 2 hrs incubation at 30 °C with orbital shaking, the reaction was quenched by adding 700 µL ice-cold acetonitrile and incubated with shaking for an additional 15 min, after which it was centrifuged at 10000 rmp for 2min. The supernatant was collected after filtering through 0.45 µm membranes and transferred to ultraclean glass vials for HPLC analysis. The HPLC analysis was monitored by a reverse-phase HPLC system (Alliance Waters 2695) equipped with a Nova-Pak C18 column (60 Å, 4 µm, 3.9 mm×150 mm, 1/pkg [WAT086344]) and a Waters 2487 Dual λ absorbance detector. Two mobile phases (mobile phase A: 90% acetonitrile and 10% water; mobile phase B: 5% acetonitrile adjusted to pH 2.3 with 85% phosphoric acid) were used for the gradient elution with a flow rate of 1 ml/min and measured at a wavelength of 232 nm. The gradient system was

initiated with 50% of solvent A and 50% of solvent B rising to 75% of mobile phase A at 6 min and finishing at 100% of solvent A at 8 min. The flow of 100% mobile phase A was maintained for 4 min and then reduced to 50% at 13 min and continued for a further 4 min to return the column to the initial conditions for the next run. Reactions containing no enzymes were used to calculate the substrate depletion percentage. Three replications were performed and a paired t-test was used to analyze the results. Reactions with proteins extracted from parental Sf9 cells and CAT expressing cells served as controls.

4.2.7 *In silico* modeling and docking analysis

In silico 3D structure modeling of each carboxylesterase protein was performed by the I-TASSER server utilizing the combined methods of threading and *ab initio* modeling (http://zhanglab.ccmb.med.umich.edu/I-TASSER/) (Roy et al. 2010; Zhang et al. 2008). Five models were predicted for each carboxylesterase gene and the top scoring model submitted to the FG-MD server for fragment guided molecular dynamics structure refinement (Zhang et al. 2011). Model quality was controlled by Ramachandran plots generated with Procheck (Laskowski et al., 1993) (http://services.mbi.ucla.edu/SAVES/) and ProSA-web (http://prosa.services.came.sbg.ac.at/prosa.php) (Sippl, 1993; Wiederstein et al. 2007). Proteins and ligands were prepared for docking with Autodock Tools V1.5.6 (http://mgltools.scripps.edu/downloads). Molecular docking was performed by Autodock 4.2 (Morris et al. 2009). Ligand permethrin structures were retrieved from the ZINC database (http://zinc.docking.org/) (Irwin et al. 2005). For all dockings, a search space with a grid box of 60 x 60 x 60 Å centered on the serine of the catalytic triad of the carboxylesterase was used. All protein structure images were produced by Pymol (http://www.pymol.org/) (Delano et al. 2002). The binding cavity and its constitutive

amino acids were predicted by LigPlot (Wallace et al. 1995). Protein structure diagrams were produced using TopDraw (Bond et al. 2003).

4.3 Results

4.3.1 Carboxylesterase activity

The expression of carboxylesterase protein was accomplished by infecting insect Sf9 cells with constructed carboxylesterase-recombinant baculovirus. The carboxylesterase proteins obtained were isolated from Sf9 cells and further used for biochemical characterization. Our results indicated that these carboxylesterase proteins from insect Sf9 cells were capable of hydrolyzing α-naphthyl acetate (α-NA) to produce α-naphthol at different efficiencies, with a hydrolytic activity of 11396.6±484.8 pmol min^{-1} mg^{-1} for MdαE7, 13810.1±635.6 pmol min^{-1} mg^{-1} for MdαE17, 8610.8±335.2 pmol min^{-1} mg^{-1} for MdβE2 and 6083.5±326.6 pmol min^{-1} mg^{-1} for MdIntE7, all of which were considerably higher than that measured in either the parental Sf9 cells (3458.2±168.2 pmol min^{-1} mg^{-1}) or the pENTR™ CAT (plasmid producing baculovirus expressing chloramphenicol acetyltransferase (CAT) protein [Invitrogen]) infected cells (3645.0±173.5 pmol min^{-1} mg^{-1}) used as controls (Table 1), indicating the strong hydrolytic capabilities of these carboxylesterase in metabolizing esterase substrate α-NA. The kinetic parameters for the hydrolytic reactions of each carboxylesterase were also measured with α-NA at final concentrations ranging from 0.1 mM to 1.2 mM. All the hydrolytic reactions of these carboxylesterases followed the Michaelis-Menten equation; their corresponding kinetic parameters are listed in Table 1. The maximum velocity (Vmax) for MdαE7 was 53555.5±1649.7 pmol min^{-1} $mg^{-1}$and the Michaelis constant (Km) was 568.0±22.3 μM; for MdαE17, the Vmax was 71586.7±2585.8 pmol min^{-1} $mg^{-1}$and

Km was 651.9±33.8 μM; for MdβE2, the Vmax was 46414.3±1401.9 pmol min^{-1} $mg^{-1}$and Km was 523.3 ±19.5 μM; and for MdIntE7, the Vmax was 41048.9 ±1748.2 pmol min^{-1} $mg^{-1}$and Km was 521.7±15.4 μM. The Km and Vmax were 186.4±7.6 μM and 9503.9±151.0 pmol min^{-1} mg^{-1}, respectively, in the parental Sf9 cells, and 213.2±10.7 μM and 10343.6±186.8 pmol min^{-1} mg^{-1}, respectively, in the pENTR™ CAT infected cells, significantly below the values obtained in the four carboxylesterase expressed cells, thus suggesting the strong hydrolytic capabilities of these four carboxylesterases toward substrate α-NA. The hydrolytic activities of these carboxylesterases were also examined toward another substrate, β-naphthyl acetate (β-NA), and the results revealed that all four carboxylesterases exhibited extremely low hydrolytic abilities, with 12.9±0.6 pmol min^{-1} mg^{-1} for MdαE7, 18.4±1.7 pmol min^{-1} mg^{-1} for MdαE17, 13.8±0.9 pmol min^{-1} mg^{-1} for MdβE2 and 11.5±0.6 pmol min^{-1} mg^{-1} for MdIntE7, although all were slightly higher than that of the parental Sf9 cells (8.4±0.5 pmol min^{-1} mg^{-1}) and pENTR™ CAT infected cells (8.8±0.6 pmol min^{-1} mg^{-1}) (Table 1). The kinetic parameters of these carboxylesterases toward β-NA were also determined following the increased β-NA concentrations, with Km being 146.8±4.1 μM for MdαE7, 190.1±4.3 μM for MdαE17, 188.3±3.5 μM for MdβE2 and 146.1±4.8 μM for MdIntE7; $Vmax_s$ were 15.7±0.3 pmol min^{-1} mg^{-1} for MdαE7, 21.9±0.7 pmol min^{-1} mg^{-1} for MdαE17, 16.0±0.3 pmol min^{-1} mg^{-1} for MdβE2 and 13.9±0.1 pmol min^{-1} mg^{-1} for MdIntE7, again slightly above the values obtained in either parental Sf9 cells (where Vmax was 9.9±0.2 pmol min^{-1} mg^{-1} and Km was 56.4±3.0 μM) or pENTR™ CAT infected cells (with a Vmax of 9.8±0.1 pmol min^{-1} mg^{-1} and a Km of 58.1±3.0 μM) (Table 4.1). Taking all the hydrolytic data together, we found that α-esterases (MdαE17 and MdαE7) had relative stronger hydrolytic efficiency toward either

α-NA or β-NA substrate compared with MdβE2 and MdIntE7, which belong to the β-esterase and integument esterase, respectively. This may be due to their various protein structures, which could lead to different substrate specificities (Younus et al. 2017; Hosokawa, 2008). This finding also provides support for the involvement of the α-esterase clade in xenobiotic metabolism and detoxification reported in previous studies (Flores et al. 2005; Campbell et al. 2003; Wang et al. 2015).

4.3.2 Cytotoxicity of permethrin in carboxylesterase-expressing Sf9 cells (MTT assays)

The cytotoxicity of permethrin was examined for MdαE7, MdαE17, MdβE2 and MdIntE7-recombinant virus-infected Sf9 cells; pENTR^{TM} CAT infected Sf9 cells and parental Sf9 cells served as controls. The cell viability against different permethrin concentrations (50, 100, 200 and 400 μM) was calculated in comparison with cells treated with acetonitrile alone. We found that the viability of MdαE7 expressing cells was significantly higher (ranging from 86.0% to 101.9%) (Figure 4.1 A-1) than that of the parental Sf9 cells (which ranged from 64.7% to 79.4%) (Figure 4.1 E-1) and the pENTR^{TM} CAT expressing cells (which ranged from 57.7% - 88.9%) (Figure 4.1 F-1), when exposed to permethrin at different concentrations. Similarly, the tolerance of MdαE17 expressing cells against the cytotoxic effects of permethrin at different concentrations also increased (ranging from 78.8% to 94.1%) compared to the control cells (Figure 4.1 B-1). The cell viability of MdIntE7 expressing cells ranged from 89.4% to 92.4% when exposed to different permethrin concentrations, again more tolerant than the control cells (Figure 4.1D-1); the cell viability of the MdβE2 expressing cells, though weaker than the other three carboxylesterase expressing cells, was still significantly

higher than the controls, ranging from 73.3% to 85.5% (Figure 4.1C-1). Taken together, these results show that all four carboxylesterase expressing cells have a greater tolerance to permethrin treatments compared with the two controls, with MdαE7 having the highest ability to detoxify permethrin when expressed in insect cells.

To confirm the roles of these carboxylesterases in detoxifying permethrin in insect cells, we then explored the permethrin cytotoxicity to insect Sf9 cells in the presence of S, S, S-tributylphosphorotrithioate (DEF), a carboxylesterase inhibitor. The cell viability against permethrin cytotoxicity in MdαE7 expressing cells decreased significantly, from 93.2% (control only treated with 200 μM permethrin) to 76.8%, 75.8% and 69.3% when co-treated with 200 μM permethrin and 0.1 μM, 1 μM or 10 μM DEF, respectively (Figure 4.1 A-2). Similar results were also observed in the MdαE17 expressing cells, whose viability decreased from 84.7% (control) to 77.8%, 64.4% and 53.8% when co-treated with 200 μM permethrin and 0.1 μM, 1 μM or 10 μM DEF, respectively (Figure 4.1 B-2). For the MdβE2 expressing cells, a slight decrease in cell viability was detected between the control (79.1%) and the cells subjected to co-treatment with 200 μM permethrin and 0.1 μM, 1 μM or 10 μM DEF (70.5%, 70.2% and 67.4%, respectively) (Figure 4.1 C-2). For the viability of MdIntE7 expressing cells, although no significant difference was detected between the control (89.4%) and cells co-treated with 200 μM permethrin and 0.1 μM DEF (91.0%), significant decreases to 76.8% and 78.9% were observed when co-treated with 200 μM permethrin and 1 μM or 10 μM DEF, respectively (Figure 4.1 D-2). No significant differences in the cell viability against permethrin cytotoxicity were found in either the parental Sf9 cells or pENTR™ CAT infected cells when co-treated with 200 μM permethrin and DEF at different

concentrations (Figure 4.1 E-2 and Figure 4.1 F-2). The significant decrease in cell viability against permethrin cytotoxicity in the four carboxylesterase expressing cells when co-treated with DEF at different concentrations strongly supports the involvement of these carboxylesterases in metabolizing permethrin in insect cells.

4.3.3 *In vitro* metabolism of permethrin by carboxylesterases

Permethrin metabolism was assayed by incubating a 20 µM permethrin standard together with different carboxylesterase proteins extracted from infected insect Sf9 cells. The reactions of 20 µM permethrin incubated with proteins from either parental Sf9 cells or pENTR^{TM} CAT infected cells served as controls. The depletion percentage for permethrin was calculated in comparison with reactions where only 20 µM permethrin was added. Reactions were monitored by reverse-phase HPLC after a 120 min incubation period. Since the permethrin standard is actually a mixture of cis- and trans- isomers, two peaks were observed in the HPLC chromatographic profiles, with elution times of 10.7 min and 10.9 min for trans-permethrin and cis-permethrin, respectively (Figure 4.2 A). The highest depletion percentage of permethrin was achieved by MdαE7, at 39.2±3.8% (Figure 4.2 B), followed by 29.4±2.3% for MdαE17 (Figure 4.2 B). Relative lower depletion percentages of permethrin were observed for MdβE2 and MdIntE7, which achieved 16.2±0.7% and 16.4±0.7%, respectively (Figure 4.2 B). All depletion percentages for these four carboxylesterases were significantly higher than those of either the parental Sf9 cells (7.9±0.8%) or pENTR^{TM} CAT genes (7.3±0.8%) used as controls, which was not only consistent with the MTT results presented above, but also directly reflects the capabilities of these four carboxylesterases in metabolizing permethrin *in vitro*.

4.3.4 *In vitro* metabolism of two permethrin metabolites, PBOH and PBCHO

The depletion percentages of two permethrin metabolites, PBOH and PBCHO, were also measured by incubating 20 µM substrate together with different carboxylesterase proteins extracted from insect Sf9 cells. The reactions of 20 µM substrates incubated with proteins extracted from parental Sf9 cells or pENTR™ CAT infected cells again served as controls. The depletion percentage of the substrate was again calculated in comparison with reactions in which only 20 µM substrates were added. Reactions were monitored by reverse-phase HPLC after a 120 min incubation period. The retention time of PBOH was 3.3 min (Figure 4.3 A). The depletion percentages of PBOH by MdαE7, MdαE17 and MdβE2 were 8.0±0.8 %, 7.5±0.5% and 7.2±0.6%, respectively, none of which were significantly different from those of either the parental Sf9 cells (6.8±0.5%) or the pENTR™ CAT infected cells (8.9±0.5%) (Figure 4.3 B). For MdIntE7, the depletion percentage of PBOH was 2.2±0.5%, lower than that achieved by the control groups (Figure 4.3 B). The retention time of the other substrate, PBCHO, was 5.4 min (Figure 4.4 A). Again, no significant differences were found in the depletion percentages of PBCHO achieved by MdαE7 (12.0±0.7%), MdαE17 (12.1±1.00%), and MdβE2 (13.0 ±1.2%) compared with those for the parental Sf9 cells (10.5±0.7%) and pENTR™ CAT infected cells (9.9±0.8%) (Figure 4.4 B). The minimal significant metabolic effects of these carboxylesterases toward the two main permethrin metabolites, PBOH or PBCHO, further supports the potential metabolic route of permethrin that has been proposed in mosquitoes, where permethrin can be metabolized by carboxylesterases to form PBOH or PBCHO, which are then further metabolized by other enzyme systems (Somwang et al. 2011; Chandor-Proust, 2013).

4.3.5 Homology modeling and permethrin docking analysis

Homology modeling and permethrin docking analysis were conducted to investigate the interactions between carboxylesterases and permethrin. Several missing or inserted motifs were found in certain carboxylesterases, such as the missing of start antiparallel β-sheets, β6 and αD in MdαE17 (Figure 4.5 B-1 and B-2); the missing β1, β2, β5 and β6 and the insertion of a short helix following αB in MdβE2 (Figure 4.5 C-1 and C-2); and a missing β4 and the insertion of a short sheet after the start antiparallel β-sheets in MdIntE7 (Figure 4.5 D-1 and D-2). The overall structures of these carboxylesterases were similar with other α/β hydrolases, containing an eight-stranded β-sheet (β1-β8) surrounded by six α-helices (αA-αF), together with two pairs of antiparallel β-strands at the start and end of the protein structure, all of which were comprised of the catalytic domain (shown as the magenta area in Figure 4.5). Two bundles of α-helices at the top of the catalytic domain formed the αβ domain and the regulatory domain (shown as orange and green areas, respectively, in Figure 4.5). A catalytic triad made up of Ser, His and Glu/Asp (labeled as black dots and sticks in Figure 4.5) was highly conserved among these four carboxylesterases. Two subdomains on either side of the active site cleft on the upper face of the protein formed the substrate binding cavity (white surface area in Figure 4.5). Subdomain I consisted of two short antiparallel α-helices inserted after β1, two short α-helices inserted after β3 and four α-helices following β6 (boxed orange in Figure 4.5). Subdomain II was composed of four α-helices inserted after β7 with the last two α-helices near the C-terminals (boxed green in Figure 4.5). A detailed comparison revealed that two small regions were divergent in the four carboxylesterase proteins, which could have a major impact on the rearrangement of the two subdomains

that form the binding cavity (Jackson et al. 2013). The first of these is the antiparallel β-sheet after β1 (shown as a red helix in Figure 4.5.A-1 and B-1) which is present in MdαE7 and MdαE17, but not in MdβE2 and MdIntE7. This antiparallel β-sheet can create a groove against the N-terminal α-helices when packing, thereby preventing the partial closure of the active site. The second subdomain affected is the short helix before the αD (shown as red sheets in Figure 4.5 A-1 and B-1), which is again present in MdαE7 and MdαE17 but absent in MdβE2 and MdIntE7. This helix is thought to hold apart two subdomains that comprise the binding cavity, leaving a much more open space for substrate binding. From this analysis, we can clearly see that even a small divergence in homology modelling can significantly impact the overall topology of the substrate binding sites. Compared with MdβE2 and MdIntE7, MdαE7 and MdαE17 have much more open active sites, allowing more space for the substrate binding and explaining the higher metabolic capabilities of MdαE7 and MdαE17 toward permethrin.

Further docking analysis revealed the interactions between carboxylesterase proteins and permethrin. Four different permethrin isomers were individually docked into the different carboxylesterase structures. Among the different permethrin isomer binding modes, 1S-trans-permethrin was found to fit most snugly into the binding pockets of all the carboxylesterase proteins, with the lowest binding energies (Table 4.3). We therefore chose the 1S-trans-permethrin isomer for this analysis to examine its interactions with the carboxylesterases. Figure 6 shows the binding cavities of each of the carboxylesterases, as well as their constitutive amino acids. The majority of the amino acids were hydrophobic, including Gly, Phe, Ala, Val, Leu, Ile, Met and Trp, which facilitated efforts to provide a hydrophobic environment for permethrin binding. The conserved

catalytic triad composed of Ser, His and Glu/Asp (shown in Figure 4.5) is involved in the catalytic process, with the nucleophile Ser residue first attacking the electron deficient carbonyl group of permethrin to form a tetrahedral intermediate, which then collapses to form the acyl-enzyme complex, releasing both Ser and the alcohol portion of the substrate. This opens the way for the His-active water soluble component to attack the acyl-enzyme complex and release the acid portion of the substrate (Wheelock et al. 2005; Satoh and Hosokawa, 2006). Based on this process, the distance between the oxygen atom in the OH side chain of the catalytic Ser residue and the carbon atom in the carbonyl group of the substrate can be used to determine the distance between permethrin and the carboxylesterases. The results suggest that MdαE7 has the shortest distance (=2.95 Å) to permethrin along with the lowest binding energy (= -8.74 Kcal/mol) (Figure 4.6 A), indicating that MdαE7 will have the highest binding affinity toward 1S-trans-permethrin, which is consistent with the finding that MdαE7 has the highest metabolism ability. The distance between MdαE17 and permethrin was found to be 3.01 Å with a binding energy of 8.02 Kcal/mol (Figure 4.6 B); both MdβE2 and MdIntE7 were even further from permethrin, at 7.70 Å and 6.77 Å and with relatively higher binding energies of -7.35 Kcal/mol and -6.26 Kcal/mol (Figure 4.6 C and Figure 4.6 D), respectively. This explains the relative lower metabolism capabilities of MdβE2 and MdIntE7 to permethrin.

4.4 Discussion

In insects, carboxylesterase is one of the major metabolic enzymes that detoxify insecticides in the first phase of metabolism (Somwang et al. 2011; Chandor-Proust et al. 2013). Overexpressed carboxylesterases lead to increased activities, which further results in the enhanced metabolism of xenobiotics or endogenous compounds (Grigoraki et al.

2015; Bass and Field, 2011; Zhang et al. 2013). In previous studies we identified four carboxylesterase genes, MdαE7, MdαE17, MdβE2 and MdIntE7, whose expressions were not only constitutively up-regulated in the resistant house fly ALHF strain, but can also be induced to much higher levels in response to permethrin, indicating the important role they play in metabolizing permethrin in house flies (Feng et al. 2018). However, a functional characterization of these carboxylesterases *in vitro* is still lacking. Here, a baculovirus-mediated insect Sf9 expression system was used to investigate the heterozygous expression of these carboxylesterases *in vitro*; their hydrolytic activities toward different esterase substrates and permethrin insecticides were also measured in this study. All four carboxylesterases showed strong activities to α-NA (~6083 pmol min^{-1} mg^{-1} to ~13810 pmol min^{-1} mg^{-1}), while none of these carboxylesterases revealed obvious activities to β-NA (~20 pmol min^{-1} mg^{-1}), suggesting that β-NA may not be the most favorable substrate for measuring the activities of these carboxylesterases. The choice of a substrate with which to monitor carboxylesterase activity remains a major obstacle hampering efforts to accurately characterize carboxylesterase activities, especially for multiple isozymes (Wheelock et al. 2005). Measuring activities toward insecticides is thus still the best strategy to directly reflect the capabilities of these carboxylesterases in metabolizing insecticides and consequently conferring insecticide resistance in insects. In this study, by incubating different carboxylesterases together with insecticides *in vitro*, we have successfully characterized the roles of these carboxylesterases in metabolizing permethrin. After one hour's incubation, the calculated depletion percentage of permethrin by the different carboxylesterases ranged from ~16% to ~40%, less efficient than those achieved when multiple cytochrome P450s were

incubate with permethrin at same conditions in mosquitoes (~40%-~45%)(Gong et al. 2017), which may be explained by the hypothesis that the high titer of carboxylesterases serves as an "insecticide sink" that delays or prevents the interactions of insecticides and target sites rather than directly metabolizing them (Oakeshott et al. 2005; Li et al. 2007). We also found that MdαE7 and MdαE17, which belong to the α-esterase clade, have higher metabolic abilities to permethrin compared with MdβE2 and MdIntE7, which is consistent with the metabolism and detoxification roles of the α-esterase clade reported in classification studies (Flores et al. 2005; Campbell et al. 2003; Wang et al. 2015). The metabolic efficiencies of these carboxylesterases toward the two main permethrin metabolites, PBOH and PBCHO, were also investigated *in vitro* and no significant metabolic effects were detected for any of the four carboxylesterases, suggesting that the carboxylesterases may only play roles in the first phase of permethrin metabolism, with other enzymes such as cytochrome P450s or glutathione S-transferases becoming involved in the further metabolism of the permethrin metabolites. This finding supports the proposed metabolic route of permethrin in other insects (Somwang et al. 2011; Chandor-Proust et al. 2013). Except for *in vitro* metabolic studies, the functions of these carboxylesterases in metabolizing permethrin were also confirmed by cell based MTT assays in the presence and absence of the DEF inhibitor. Finally, homology modelling and docking analysis of carboxylesterases were built based on the crystal structure of LcαE7, an α-esterase isolated from the Australian sheep blowfly *Lucilia cuprina* with its roles in OP resistance has already been reported in blow flies (Jackson et al. 2013) . By comparing structures of these four carboxylesterases, we found that even small divergences within their structures can lead to variable substrate accommodation. The

house fly MdαE7 is orthologous to blow fly LcαE7 and also contribute to insecticides resistance either through qualitative or quantitative changes (Zhang et al. 2010). However, few studies have explored the interactions between MdαE7 and insecticides, either OP or pyrethroids, through docking analysis. In this study, we firstly investigated the binding modes of permethrin within binding cavities of carboxylesterases, which were constructed based on the asymmetric and hydrophobic binding cavities of LcαE7. The binding energy together with hydrogen bond distance further analyzed to reveal the interactions between permethrin and the carboxylesterases proteins. Besides that, the stereochemistry is another important factor in esterase-mediated metabolism, and our docking analysis found that 1S-trans-permethrin had the best fit with the binding cavities of the four carboxylesterases with the lowest binding energy compared with other permethrin isomers. Similar findings have also been reported in carboxylesterase E4 of the aphid *Myzus persicae*, which exhibits absolute specificity for hydrolyzed 1S-trans-permethrin rather than other isomers (Devonshire et al. 1982). In conclusion, the successful heterozygous expression and characterization of four different carboxylesterases *in vitro* reported here provides functional evidence of esterase-mediated resistance in house flies that sheds fresh light on the mechanisms governing the development of insecticide resistance and could lead to the development of innovative new pest management strategy.

4.5 References

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Table 4.1: Hydrolytic activities and kinetic parameters of *Musca domestica* carboxylesterases

Enzyme	α-naphthyl acetate			β-naphthyl acetate		
	Activity[a]	Km[b]	Vmax[c]	Activity[a]	Km[b]	Vmax[c]
Sf9 cells	3458.2± 168.2	186.4± 7.6	9503.9± 151.0	8.4± 0.5	56.4± 3.0	9.9± 0.2
CAT gene	3645.0± 173.5	213.2± 10.7	10343.6± 186.8	8.8± 0.6	58.1± 3.0	9.8± 0.1
MdαE7	11396.6± 484.8	568.0± 22.3	53555.5± 1649.7	12.9± 0.6	146.8± 4.1	15.7± 0.3
MdαE17	13810.1± 635.6	651.9± 33.8	71586.7± 2585.8	18.4± 1.7	190.1± 4.3	21.9± 0.7
MdβE2	8610.8± 335.2	523.3± 19.5	46414.3± 1401.9	13.8± 1.0	188.3± 3.5	16.0± 0.3
MdIntE7	6083.5± 326.6	521.7± 15.4	41048.9± 1748.2	11.5± 0.6	146.1± 4.8	13.9± 0.1

All data were listed as mean±STE

a: COE activity=pmol·min^{-1}·mg^{-1}
b: Km (Michaelis constant)= μM
c: Vmax (Maxinum velocity)= pmol·min^{-1}·mg^{-1}

Table 4.2: The primer list for the Musca domestica carboxylesterase gene

Gene	Accession No.	Clade	Primers for Sf9 expression	
			Forward (5'-3')	Reverse (5'-3')
MdαE7	XP_005178694	α-esterase	CACCATGAAATTTA AACTTTTCGTATT	TTAGACAACAATG GGTTTACAATCAT
MdαE17	XP_005175160	α-esterase	CACCATGGATTTAA ATATTGG	TTAACACAATGGCT CTTTG
MdβE2	XP_005183940	β-esterase	CACCATGAATTTCA AAGTTAG	TTAAAACAATTCCT TCTTTTTA
MdIntE7	XP_005177448	Integument esterase	CACCATGAAAGCA TTGTGGTTC	TTAACTTAATTTCC AAATGCTTAACACT

Table 4.3: The binding energies of different permethrin isomers in four carboxylesterases

protein	Binding Energy (Kcal/mol)			
	1R-cis-permethrin	1R-trans-permethrin	1S-cis-permethrin	1S-trans-permethrin
MdαE7	-7.83	-8.18	-7.98	-8.74
MdIntE7	-5.69	-5.71	-5.54	-6.26
MdαE17	-7.46	-7.89	-7.2	-8.02
MdβE2	-6.89	-7.03	-6.32	-7.35

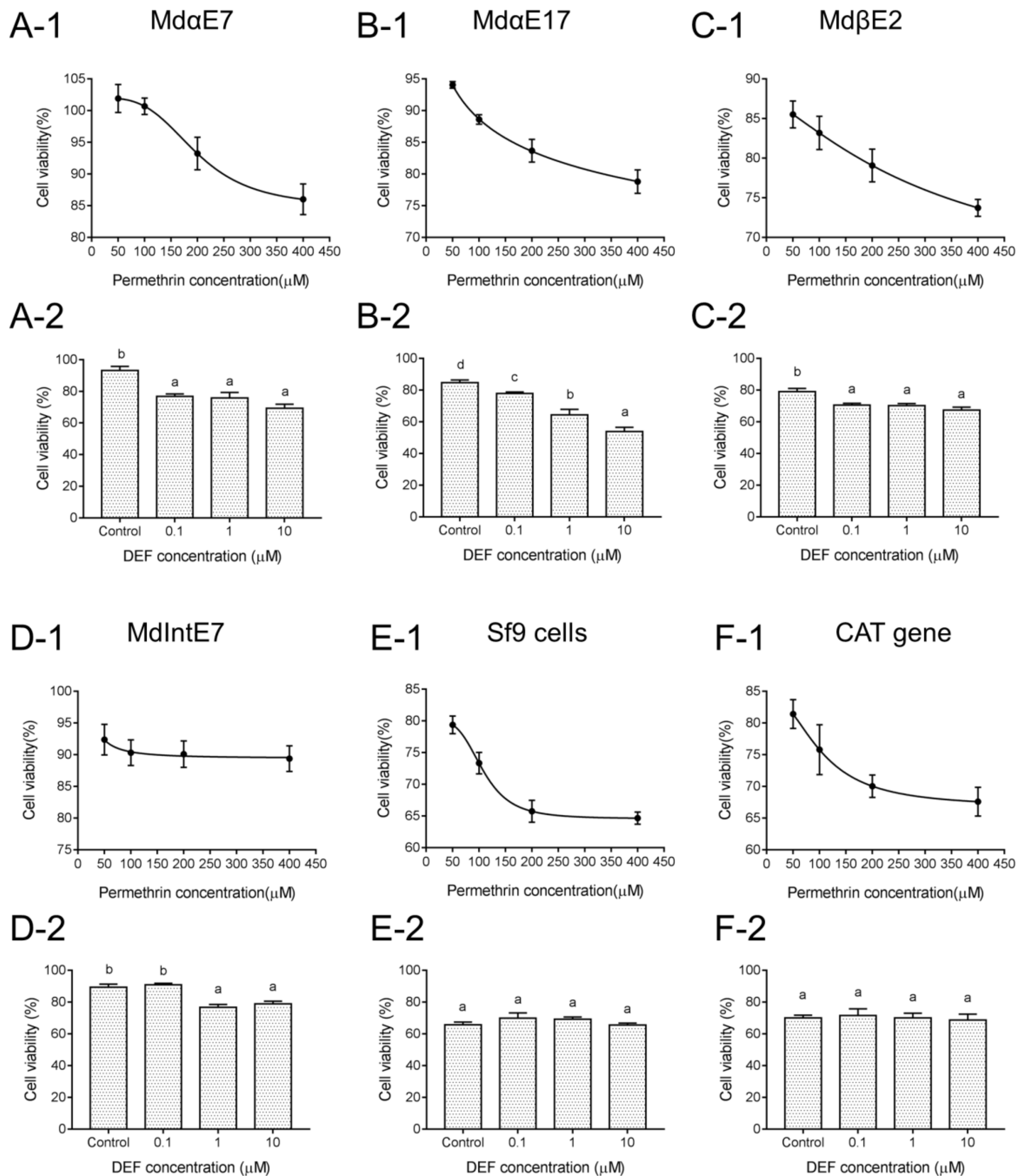

Figure 4.1 Roles of carboxylesterases in detoxification of permethrin in insect Sf9 cells. A-1), B-1), C-1), D-1), E-1) and F-1) Viability of Sf9 cells expressing MdαE7, MdαE17, MdβE2, MdIntE7 gene, parental Sf9 cells alone and CAT gene, respectively, treated with 50, 100, 200 and 400 μM of permethrin. A-2), B-2), C-2), D-2), E-2) and F-2) Viability of Sf9 cells expressing MdαE7, MdαE17, MdβE2, MdIntE7, parental Sf9 cells

alone and CAT gene, respectively, co-treated with 200 μM permethrin and 0.1, 1, or 10 μM of DEF. Student's t-test was used for the statistical significance analysis. Different letters above the bars indicate the significant differences of cell viabilities under different treatments ($P \leq 0.05$).

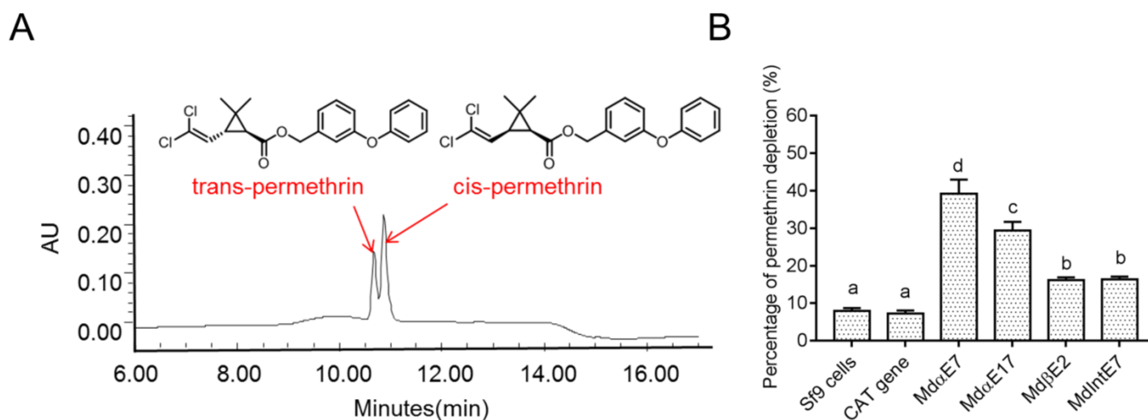

Figure 4.2 Permethrin metabolism by carboxylesterase proteins. A) HPLC profile of permethrin standard (a mixture of trans-permethrin and cis-permethrin isomers). The red arrows indicate the peaks for the trans-permethrin and cis-permethrin isomers. B) The depletion percentage of permethrin by different carboxylesterase proteins, parental Sf9 cells alone and CAT-gene expressing cells. Different letters above the bars indicate the significant differences for the depletion percentage of permethrin under different treatments ($P \leq 0.05$).

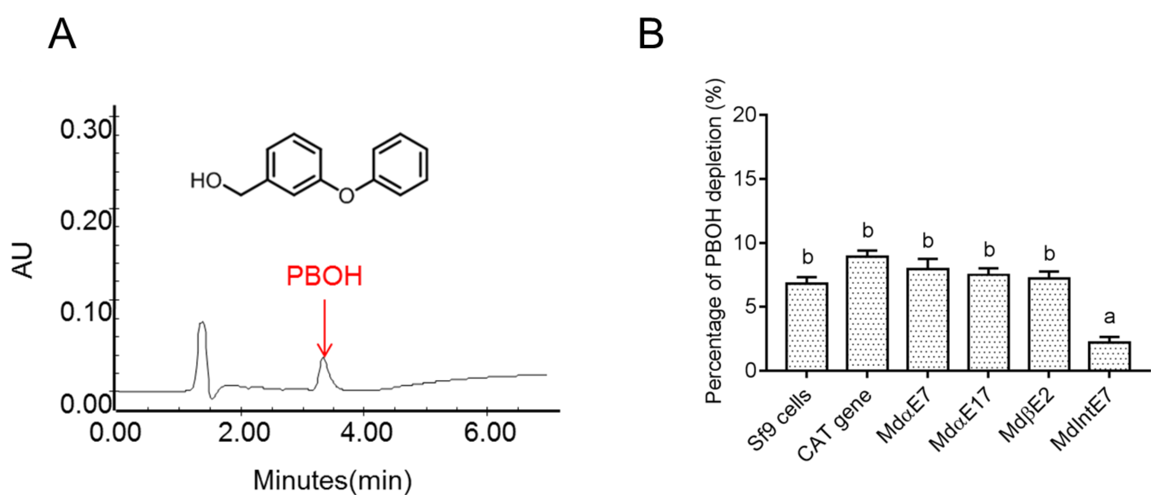

Figure 4.3 PBOH metabolism by carboxylesterase proteins. A) HPLC profile of PBOH standard. The red arrow indicates the peak for PBOH. B) The depletion percentage of PBOH by different carboxylesterase proteins, parental Sf9 cells alone and CAT-gene expressing cells. Different letters above the bars indicate the significant differences for the depletion percentage of PBOH under different treatments ($P \leq 0.05$).

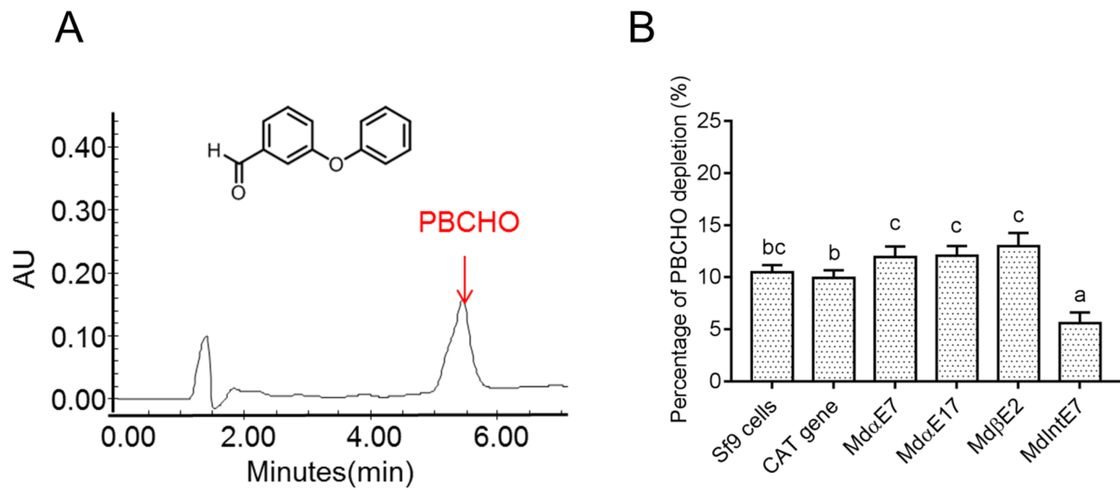

Figure 4.4 PBCHO metabolism by carboxylesterase proteins. A) HPLC profile of PBCHO standard. The red arrow indicates the peak for PBCHO. B) The depletion percentage of PBCHO by different carboxylesterase proteins, parental Sf9 cells alone and CAT-gene expressing cells. Different letters above the bars indicate the significant differences for the depletion percentage of PBCHO under different treatments ($P \leq 0.05$).

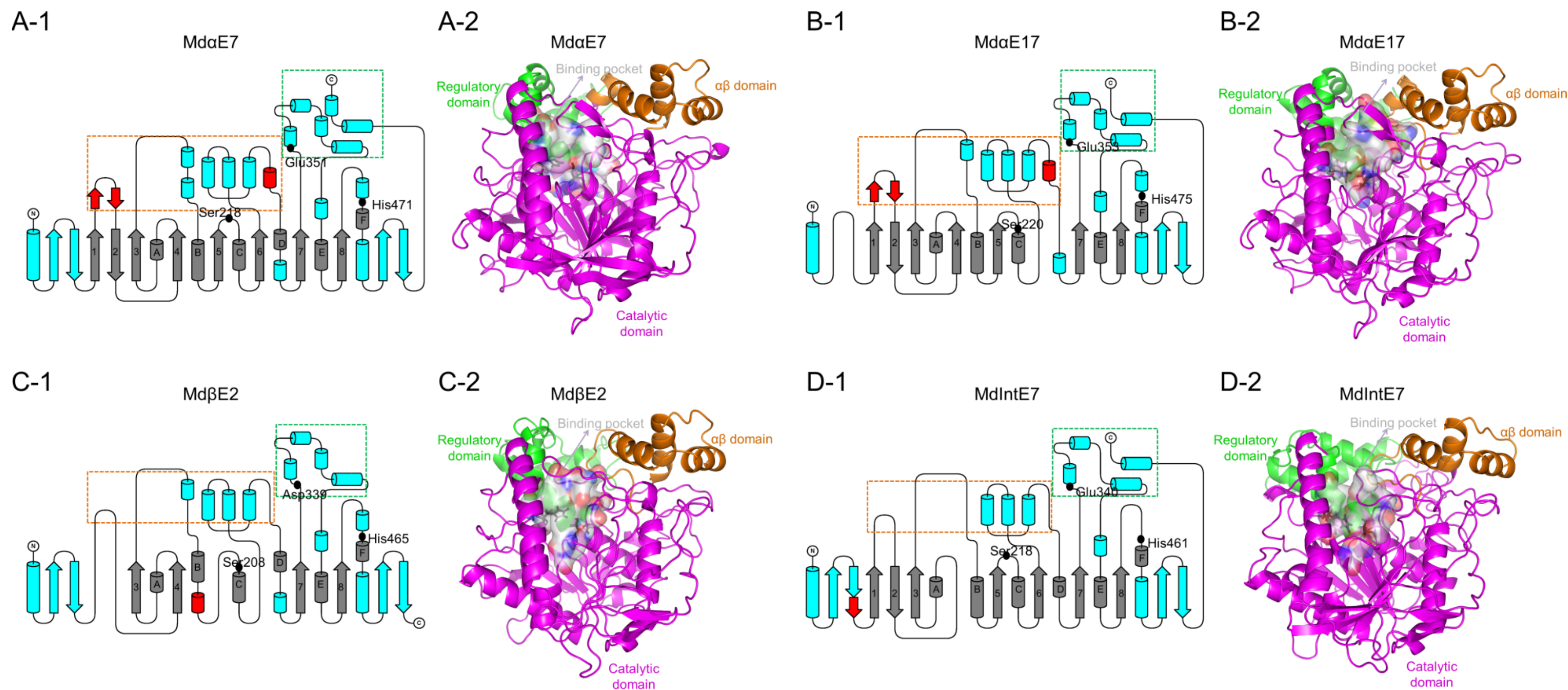

Figure 4.5 The structures of carboxylesterases in *M.domestica*. A-1), B-1), C-1) and D-1) Topological representations of the secondary structures of MdαE7, MdαE17, MdβE2 and MdIntE7, respectively, displaying the conserved α/β hydrolase fold (labeled as gray), conserved motifs among four carboxylesterase proteins (labeled as blue) and unique motifs belong to certain carboxylesterase

structure (labeled as red). Two subdomains made up of bundles of α-helices (framed by orange and green boxes) form the substrate binding cavity. Three conserved amino acids (Serine, Histidine and Glutamine/Aspartic acid) consisting of a catalytic triad are indicated as black dots. A-2), B-2), C-2) and D-2) Cartoon representations of the structures of MdαE7, MdαE17, MdβE2 and MdIntE7, respectively, highlighting the regulatory domain (green area), αβ domain (orange area), catalytic domain (magenta area) and binding pocket (white surface area). The conserved catalytic residues are labeled as black sticks.

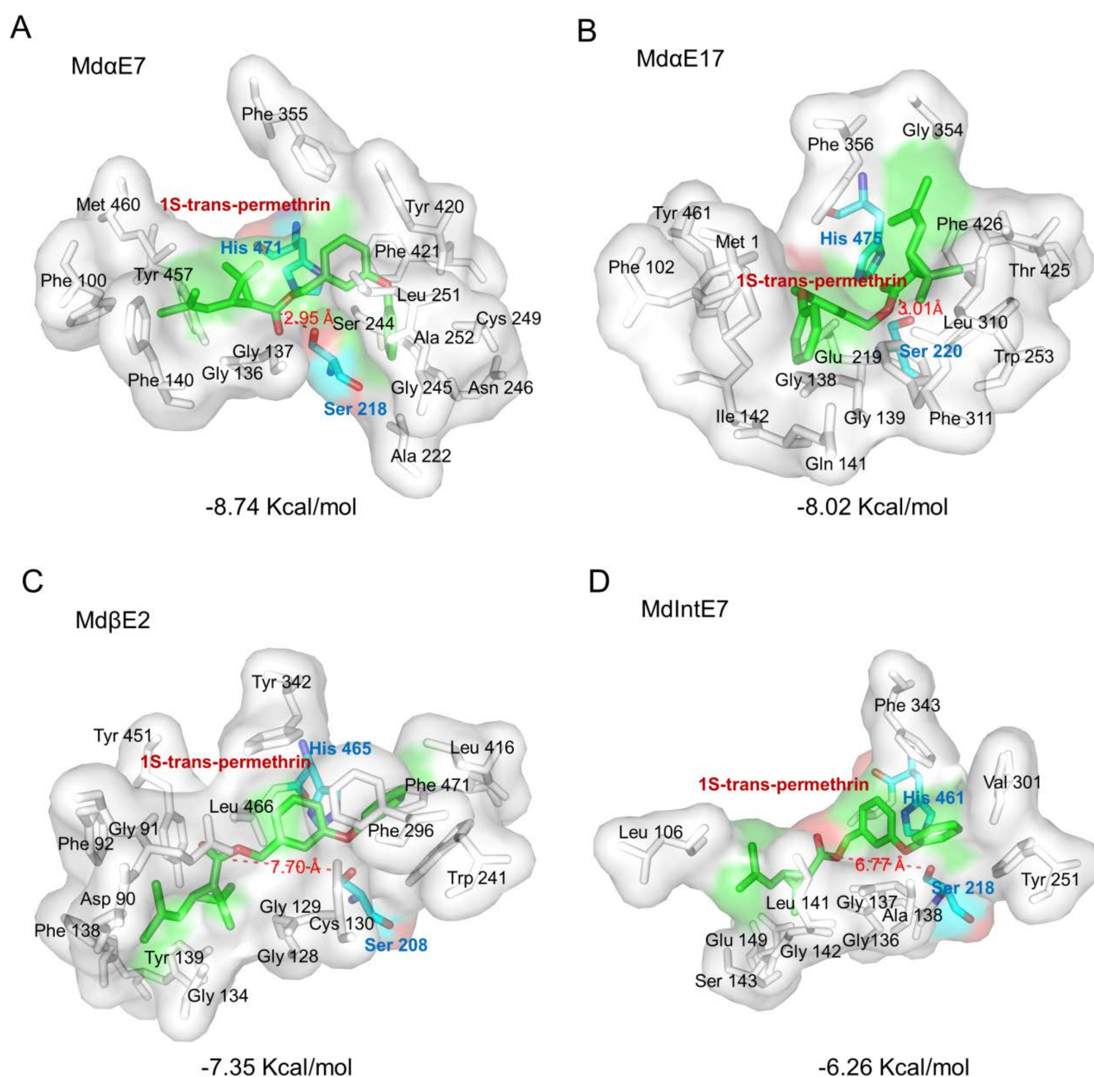

Figure 4.6 Stereo view of permethrin bound within the active site cavity. Permethrin bound within the active site cavity of: A) MdαE7; B) MdαE17; C) MdβE2; and D) MdIntE7. The Serine (Ser) and Histidine (His) residues are labeled as green sticks and the 1S-trans-permethrin isomer as cyan sticks. The distance between permethrin and the Ser residue of the carboxylesterase is indicated by a red dashed line. The binding energy is also shown. All amino acid active site cavities are labeled in each carboxylesterase structure.

Chapter 5: Qualitative changes of MdαE7 gene associated with pyrethroid resistance in house fly, *Musca domestica*

Abstract

The qualitative changes of carboxylesterases responsible for insecticide resistance have been widely investigated in multiple OP resistant insects. However, the correlation between carboxylesterase mutations and pyrethroid resistance remained to be further explored. In this study, with the alignment of MdαE7 amino acid sequences isolated from different house fly strains and lines, eight mutations have been identified in resistant ALHF strain and four offspring line A1234, A1245, A1235 and A2345, not only suggesting that these mutations occurring on MdαE7 own potential capabilities of conferring pyrethroid resistance in house flies, but also confirming that the MdαE7 gene was indeed located on the autosome 2 of house flies, which was consistent with our previous conclusions. These eight single mutations were then respectively introduced into the MdαE7 gene extracted from aabys strain and their roles were later investigated through *in vitro* functional studies. Our results showed that seven MdαE7 mutants with their kinetic efficiencies to generic esterase substrate α-naphthyl acetate (α-NA) were decreased compared with that of MdαE7 isolated from aabys. Moreover, three of these mutations can significantly enhance the cell tolerance to permethrin and simultaneously efficiently metabolize permethrin *in vitro*, suggesting that these mutations have similar effects with "mutant ali-esterase hypothesis", which can convert the substrate preference

of MdαE7 from generic esterase substrate a-NA to ester-containing pyrethroids, thereby correlating with pyrethroid resistance in house flies. The correlation between MdαE7 mutants and permethrin ligand were finally confirmed via the homology modeling and docking analysis.

5.1 Introduction

Carboxylesterases, as one of major detoxifying enzymes, can hydrolyze ester-containing insecticides and conferring resistance in insects (Hemingway and Karunaratne, 1998). Carboxylesterase-mediated resistance is recognized as one of the major mechanisms involved in insecticide resistance (Kwon et al. 2014; Baffi et al. 2007; Alon et al. 2008; Wu et al. 2011). Both of the quantitative (overexpression caused by either gene amplification or transcriptional up-regulation) and qualitative (mutations occurring on the active site) changes of carboxylesterases are responsible for carboxylesterase-mediated resistance (Coppin et al. 2012; Cui et al. 2015; Zhang et al. 2010). Up to date, multiple carboxylesterases have been identified with their overexpression levels were closely associated with insecticide resistance in various insect species, including house flies (Wang et al. 2015; Cao et al. 2008; Zhang et al. 2013; Feng et al. 2018). The qualitative mechanism, resulted from the changes of enzymatic properties of carboxylesterase, can significantly shift the substrate preference from generic substrate (e.g. α-naphthyl acetate) to ester-containing insecticides. This mechanism, also known as “mutant aliesterase hypothesis”, has been identified in various organophosphate resistant insect species, such as *Lucilia cuprina*, *Cochliomyia hominivorax*, *Musca domestica*, *Anisopteromalus calandrae*, *Helicoverpa armigera*, *Aphis gossypii*, *Apis mellifera*, *Tribolium castaneum*, and etc. (Carvalho et al. 2010; Sun

et al. 2005; Silva et al. 2009; Haubruge et al. 2002; Cui et al. 2011; Li et al. 2013; Pan et al. 2009; Zhu et al.1999). Two mutations, G137D and W251L found in the LcαE7 of *L. cuprina* and in its orthologous MdαE7 of *M. domestica* were responsible for this "mutant ali-esterase" and thereby conferring OP resistance (Campbell et al. 1998; Claudianos et al. 1999; Cui et al. 2011; Devonshire et al. 2003; Gacar et al. 2009; Heidari et al. 2004; Newcomb et al. 1997). However, this qualitative mechanism has not been widely investigated in pyrethroid resistant insects. One study has modified the active site of *L. cuprina* and *Drosophila melanogaster* carboxylesterase E3 with *in vitro* mutagenesis study, and found that the W251L/F309L double mutant has the best overall effects in enhancing the enzyme's ability to hydrolyze pyrethroids (Heidari et al. 2005). However, relative studies are still lacking in pyrethroid resistant house flies.

The house fly, *Musca domestica*, is a sanitary pest which can carry and transmit more than 100 intestinal pathogens (Li et al. 2013; Scott et al. 2014). Our previous study of house flies has identified multiple carboxylesterases, with their expression levels not only constitutively overexpressed in pyrethroid resistant strain, but also can be induced to higher levels in response to permethrin stimulus, indicating the important roles of these carboxylesterases in conferring pyrethroid resistance in house flies (Feng et al. 2018). Among these carboxylesterases, MdαE7 achieved the highest metabolism abilities toward permethrin through *in vitro* metabolism studies. This gene has also been reported associated with deltamethrin resistance either in qualitative and quantitative-mediated mechanism (Zhang et al. 2010). However, functional characterization of mutations occurring on MdαE7 in altering hydrolysis activities to permethrin remained to be further explored. In this study, eight non-synonymous mutations were identified by comparing

amino acid sequences extracted from permethrin susceptible and resistant house fly strains and lines. To functional characterize these MdαE7 mutants, the opening reading frame of MdαE7 gene from the susceptible aabys house fly strain were cloned and later single mutations were introduced respectively using site-directed mutagenesis techniques. After heterologous expression of different MdαE7 mutant in insect cells with baculovirus mediated expression system, their carboxylesterase activities toward generic substrate α-naphthyl acetate (α-NA) and hydrolytic activities against permethrin were examined via *in vitro* functional studies. Homology modeling and docking analysis were also conducted based on the crystal structure of LcαE7 gene from *L.cuprina* to illustrate the interactions between carboxylesterase variants and permethrin ligand. Taken together, all these results contributed to a better understanding of qualitative mechanisms of carboxylesterase-mediated resistance and thus providing novel strategies for insecticide resistance manipulation and management in insects.

5.2 Materials and Methods

5.2.1 House fly strains and lines

Two parental house fly strains and lines were used in this study. ALHF, a highly insecticide resistant strain, was originally collected from a poultry farm in Alabama. This strain exhibited a high level of resistance after subsequent selection with permethrin for six generations and has been annually selected with permethrin to maintain its highly resistant status; aabys, an insecticide susceptible strain bearing five recessive morphological markers: ali-curve (*ac*), aristapedia (*ar*), brown body (*bwb*), yellow eyes (*ye*) and snipped wings (*snp*) located on autosomes 1, 2, 3, 4 and 5, respectively. A reciprocal cross of ALHF female and aabys male was performed to obtain F1 generaction,

and F1 males were then backcrossed with aabys female to produce five back-cross (BC_1) lines. Five homozygous house fly lines *ac*/*ac*, +/+, +/+, +/+, +/+ (A2345); +/+, *ar* /*ar*, +/+, +/+,+/+ (A1345); +/+, +/+, *bwb*/*bwb*, +/+, +/+ (A1245); +/+, + /+, +/+, *ye*/*ye*, +/+ (A1235) and +/+, +/+, +/+, +/+, *snp*/*snp* (A1234) were generated by sorting for appropriate phenotypic markers and selecting with permethrin at corresponding doses causing ~70% mortality for each of the lines for three generations. The name of each line indicates which of its autosomes bear wild-type markers from ALHF. For instance, the A2345 strain has wild-type markers on autosomes 2, 3, 4 and 5 from ALHF, with a mutant marker on autosome 1 from aabys. A1235 homozygous line (with a recessive morphological marker on autosome 4 from aabys strain) showed no significant differences in resistant level compared with resistant ALHF strain based on the overlapping 95% confidence intervals. A2345, A1345, A1245, and A1234 house fly lines with recessive morphological markers on autosome 1, 2, 3, and 5 derived from susceptible aabys strain respectively, had significantly decreased resistant level to permethrin compared with ALHF, implying that factors on autosome 1, 2, 3 and 5 play important roles in conferring pyrethroid resistance in house flies (Tian et al. 2011).

5.2.2 Identification of MdαE7 mutations associated with pyrethroid resistance in house flies

Total RNAs were extracted from 20 3-day old adult female ALHF and aabys house flies respectively, using the acidic guanidine thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987). The DNA was removed from the total RNA using DNase (TURBO DNA-free, Ambion). The first-strand cDNA was synthesized with DNA-free total RNA using a Transcriptor First Strand cDNA Synthesis Kit (Roche) and

oligo-dT primer following the manufacture's instructions. The pENTR™ expression plasmids of carboxylesterases were constructed with gene-specific primers designed based on their full-length nucleotide sequences of MdαE7 with four nucleotide bases CACC added to the 5'end of forward primer (immediately upstream of the ATG transcription start codon) (See Table 5.1), which enables the carboxylesterase genes to be directly cloned into the pENTR™ TOPO® vector (Invitrogen) by annealing the CACC sequence in the PCR products with the overhang tag GTGG in the vector. The recombinant vector was then transformed into One Shot® competent *E.coli*. pENTR™ plasmids with target carboxylesterase genes were purified using the PureLink HQ Mini plasmid purification Kit (Invitrogen). The orientation of the inserted genes was detected by conducting PCR using the forward primer of each of the specific genes and the reverse primer of M13. Expression plasmids were further verified by sequencing and ten independent clones were picked in order to ensure the accuracy of DNA sequences. The obtained nucleotide sequences and amino acid sequences of MdαE7 from permethrin-resistant ALHF strain, susceptible aabys strain, and five homozygous offspring lines were further aligned using T-Coffee server (http://tcoffee.vital-it.ch/apps/tcoffee/do:regular) and ESPript 3.0 (http://espript.ibcp.fr/ESPript/ESPript/) to identify the non-synonymous mutations associated with pyrethroid resistance in house flies.

5.2.3 Site-directed mutagenesis studies

Non-synonymous mutations identified in 5.2.2 were introduced into the MdαE7 allele via the site-directed mutagenesis using a QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies, USA) by following the manufacturer's protocols. One mutation was introduced at corresponding site each time. The mutagenesis primers

were designed based on the nucleotide polymorphisms occurring on the MdαE7 allele of resistant ALHF strain (listed in Table 1). The template DNA used for mutagenesis study was pENTR™ plasmids of MdαE7 isolated from aabys strain. Briefly, the PCR reaction contained 1.0 µL (200-400 ng/ µL) of template MdαE7 DNA, 5.0 µL of 10× QuickChange Lightning Buffer, 1.25 µL of forward primer, 1.25 µL of reverse primer, 1.0 µL of dNTPs, 1.5 µL of QuickSolution reagents, and add water to bring final reaction volume to 50 µL. The procedure for PCR was 95˚C for 2min, then 18 cycles of 95˚C for 20s, 60˚C for 10s, 68 ˚C for 1.5 min, and finally 68 ˚C for 5 min. The PCR product was later digested with DpnI, and transformed into MAX Efficiency® Stbl2™ chemically competent cells. The plasmid DNA for each MdαE7 mutant was sequenced to verify the successful mutagenesis.

5.2.4 Heterologous expression of MdαE7 mutants in Sf9 cells

The pENTR™ plasmids of different MdαE7 mutants were respectively ligated with BaculoDirect Linear DNA using the LR clonase™ II enzyme mix through the BaculoDirect™ Baculovirus Expression System (Invitrogen). The constructed recombinant baculovirus was then transfected into *Spodoptera frugiperda* (Sf9) cells using CellfectinR II Reagent (Invitrogen) to produce recombinant baculovirus stock solutions. The large-scale expression of carboxylesterase proteins in the Sf9 cells was performed according to the manufacture's instructions (Invitrogen). The titer of the baculovirus was measured by plaque forming assay and a titer of ~2×10^8 pfu/mL was used to infect Sf9 cells for the large-scale expression of carboxylesterase mutant proteins. The proteins extracted from MdαE7 allele of aabys-recombinant baculovirus infected cells was served as controls. The cell lysate protein was harvested after 72 h infection,

and centrifuged at 1000 rmp for 10 min at 4 ºC. The cell pellets were washed twice using ice-cold PBS buffer (pH 7.4) and then re-suspended in insect cell PE-LB™ buffer. Subsequently, the dissolved cell lysate was centrifuged at 9800 rmp for 15 min, and then the supernatant was collected and stored at -80 °C. The Bradford method was used to measure concentration of each MdαE7 mutant protein (Bradford et al. 1976).

5.2.5 Carboxylic activity assays

The carboxylic activities for different MdαE7 mutants were determined by measuring the kinetic efficiencies of MdαE7 mutant proteins toward α-naphthyl acetate (α-NA), a generic substrate commonly used for esterase activities. In our previous study, the α-NA was proven to be a favorable substrate used for the measurement of carboxylesterases activities. Here, a 25 µL of 10-fold diluted carboxylesterase protein solution (diluted with 0.1 M PBS buffer, pH 7.5) and a series of substrate (α-NA) concentrations ranging from 0.1 to 1.2 mM (by adding 3.8, 7.7, 11.5, 15.3, 19.2, 23, 26.8, 30.7, 34.5, 38.3, 42.2 and 46 µL of 3×10^{-3} M substrate solution, respectively) were added to each well of a 96-well microplate, then use 0.1M PBS buffer to make final volume 115.0 µL. The reaction was incubated at 30 °C for 30 min and terminated by adding 45 µL of freshly prepared diazoblue-sodium lacrysulphate solution (containing 2 parts of 1% fast blue B salt and 5 parts of 5% sodium dodecyl sulfate solution). After 15 min incubation at room temperature, the absorbance value of hydrolysis product α-naphthol was measured at 600 nm with a 96-well microplate reader (Cytation 3 imagine reader, BioTekUSA). The reactions with proteins extracted from MdαE7 allele of aabys-recombinant baculovirus infected cells was served as controls. The kinetic efficiencies were calculated by the Kcat and Km values.

5.2.6 Cell-based MTT assay

The cytotoxicity assay of permethrin by different MdαE7 mutant proteins was conducted based on the protocol of Gong et al. with modifications (Gong et al. 2017). The cells were infected by constructed MdαE7 mutant-recombinant baculovirus and cultured in 25 cm^2 flasks at 27 °C. Controls were MdαE7 allele of aabys strain expressing cells cultured under the same conditions. After 48 h cultivation, cells expressing MdαE7 mutant proteins were seeded onto 24-well cell culture plate with a density of 2×10^5 cells/well, and later treated with permethrin standard solutions (the mixture of cis- and trans- isomers dissolved in acetonitrile) (analytical standard, Sigma-Aldrich), with final concentrations ranging from 50 μM to 400 μM. The cytotoxicity of permethrin standards were measured by MTT assay. After 48h incubation with permethrin, the cell cultural medium was removed. Later, 200 μL of 5 mg/ml triazolyl blue tetrazolium bromide solution (Sigma-Aldrich) was added to each well and the plate was incubated at 37 °C for 4h, after which the absorbance value was detected at 540 nm using the Cytation 3 imagine reader. Three replications were conducted with independent cell and protein preparations. The cell viabilities were calculated in comparison with acetonitrile-treated cells.

5.2.7 *In vitro* hydrolytic activities against permethrin

Permethrin (99.99% purity, analytical standard) was initially dissolved in acetonitrile to make 1 mM stock solution and later diluted to a series of concentrations to create the standard curve. A700 μL metabolism reaction was prepared with 20 uM permethrin standard and 1 mg carboxylesterase mutant protein (isolated from insect cells) in 0.2 M Tris-HCl buffer. After 2 h incubation at 30 °C with orbital shaking, the reaction

was quenched by adding 700 μL ice-cold acetonitrile and incubated with shaking for an additional 15 min, after which it was centrifuged at 10000 rmp for 2min to pelletize the proteins. The supernatant was collected after filtering through 0.45 μm membranes and transferred to ultraclean glass vitals for HPLC analysis. The HPLC analysis was monitored by a reverse-phase HPLC system (Alliance Waters 2695) equipped with a Nova-Pak C18 column (60 Å, 4 μm, 3.9 mm×150 mm, 1/pkg [WAT086344]) and a Waters 2487 Dual λ absorbance detector. Two mobile phases (mobile phase A: 90% acetonitrile and 10% water; mobile phase B: 5% acetonitrile adjusted to pH 2.3 with 85% phosphoric acid) were used for the gradient elution with a flow rate of 1 ml/min and measured at a wavelength of 232 nm. The gradient system was initiated with 50% of solvent A and 50% of solvent B rising to 75% of mobile phase A at 6 min and finishing at 100% of solvent A at 8 min. The flow of 100% mobile phase A was maintained for 4 min and then reduced to 50% at 13 min and continued for a further 4 min to return the column to the initial conditions for the next run. Reactions containing no enzymes were used to calculate the substrate depletion percentage. Three replications were performed and a paired t-test was used to analyze the results. Reaction of MdαE7 proteins extracted from aabys was served as control.

5.2.8 *In silico* modeling and docking analysis

In silico 3D structure modeling was performed by the I-TASSER server utilizing the combined methods of threading and *ab initio* modeling (http://zhanglab.ccmb.med.umich.edu/I-TASSER/) (Roy et al. 2010; Zhang et al. 2008). Five models were predicted for MdαE7 isolated from aabys and different MdαE7 mutants and the top scoring model submitted to the FG-MD server for fragment guided molecular

dynamics structure refinement (Zhang et al. 2011). Model quality was controlled by Ramachandran plots generated with Procheck (Laskowski et al. 1993) (http://services.mbi.ucla.edu/SAVES/) and ProSA-web (http://prosa.services.came.sbg.ac.at/prosa.php) (Wiederstein and Sippl, 2007). Molecular docking was performed by Autodock 4.2 (Morris et al., 2009). Ligand permethrin structures were retrieved from the ZINC database (http://zinc.docking.org/) (Irwin and Shoichet, 2005). For all dockings, a search space with a grid box of 60 x 60 x 60 Å centered on the serine of the catalytic triad of the carboxylesterase was used. All protein structure images were produced by Pymol (http://www.pymol.org/) (Delano, 2002). The binding cavity and its constitutive amino acids were predicted by LigPlot (Wallace and Laskowski, 1995). The volume of binding cavity was characterized by KVF_{INDER} with a 0.14 nm probe (Oliveira, 2014).

5.3 Results

5.3.1 Identification of MdαE7 mutations associated with insecticide resistance in house flies

MdαE7 mutations were identified by aligning amino acid sequences of MdαE7 allele extracted from insecticide resistant ALHF strain, susceptible aabys strain and their five homozygous offspring lines A1234, A1235, A1245, A1345 and A2345 using T-Coffee Server (http://tcoffee.crg.cat/) and ESPript 3.0 Server (http://espript.ibcp.fr/ESPript/ESPript/). The nucleotide sequence of MdαE7 is 1713bp which encodes a 571 amino acid sequence. Eight non-synonymous mutations were identified in resistant ALHF strain and four homozygous lines A1234, A1235, A1245 and A2345 compared with susceptible aabys strain and A1345 line. They were, Tyr^{148}-Phe

(Y^{148}F) resulted from TAT-TTT; Lys^{176}-Ile (K^{176}I) resulted from AAA-ATA; Trp^{251}-Leu (W^{251}L) resulted from TGG-TTG; Glu^{273}-Asp (E^{273}D) resulted from GAG-GAC; Ile^{303}-Met (I^{303}M) resulted from ATA-ATG; Asp^{364}-Glu (D^{364}E) resulted from GAT-GAG; Ala^{365}-Val (A^{365}V) resulted from GCG to GTT; and Arg^{378}-Trp (R^{378}W) resulted from CGG-TGG (Table 5.1 and Figure 5.1). All these mutations identified in resistant ALHF strain and four homozygous offspring lines not only suggested their potential relationship with pyrethroid resistance in house flies, but also reflected the genetic location of MdαE7 on autosome 2 of house flies, which is consistent with our previous autosomal linkage studies of carboxylesterases (Feng et al., 2018).

5.3.2 Carboxylesterase activity of different MdαE7 variants toward artificial substrate α-NA

Eight mutations identified in 5.3.1 were respectively introduced into MdαE7 allele extracted from aabys strain with the site-specific mutagenesis studies. By constructing different MdαE7-recombinant baculovirus and large-scale infecting insect Sf9 cells, different MdαE7 variant proteins were produced in vitro. The carboxylic activities of MdαE7 variants were later examined by measuring their kinetic efficiencies [catalytic constant (Kcat)/Michaelis constant (Km)] toward generic esterase substrate α-NA. Compared with the kinetic efficiencies MdαE7 isolated from aabys strain, six mutants of MdαE7, including Y^{148}F, K^{176}I, W^{251}L, E^{273}D, D^{364}E, and R^{378}W, have significantly decreased kinetic efficiencies toward α-naphthyl acetate, ranging from ~1.5 fold to ~5.1 fold compared with that of aabys MdαE7 (Table 5.2). Among these mutations, the D^{364}E mutation has the highest capabilities to decrese the substrate preference of MdαE7 to α-NA, more than ~5.1 fold compared with that of control (Table

5.2). $W^{251}L$ mutation, with its involvement in mutant ali-esterase hypothesis has been reported in OP resistant *Lucilia cuprina*, also has decreased kinetic efficiencies to α-NA for ~4.4 fold compared with that of MdαE7 extracted from aabys strain (Table 5.2). $Y^{148}F$ mutation also has significant impact in decreasing the kinetic efficiency of MdαE7, reaching ~3.2 fold (Table 5.2). The kinetic efficiencies of MdαE7 to α-NA were decreased for ~2.4 fold, ~2.4 fold and ~1.5 fold when introduced the $E^{273}D$, $R^{378}W$ and $K^{176}I$ mutations into MdαE7 alleles of aabys strain, respectively (Table 5.2). No significant differences were observed for the kinetic efficiency of $A^{365}V$ mutant and control based on the 95% overlapping intervals, while the kinetic efficiency of MdαE7 was significantly enhanced for more than when introducing the $I^{303}M$ into the wild-type allele of MdαE7 (Table 5.2). All these results suggested that the mutations can alter the structures of MdαE7, which may in turn influence the carboxylic efficiencies of MdαE7 toward generic esterase substrate α-NA.

5.3.3 Cytotoxicity of permethrin in MdαE7 variant-expressing Sf9 cells

In different MdαE7 variant-expressing Sf9 cells, the cell viabilities against different permethrin concentrations (50, 100, 200 and 400 μM) was calculated in comparison with cells treated with acetonitrile only. The viabilities of cells expressing MdαE7 extracted from aabys strain were ranged from 91% to 79% under different permethrin concentration treatments (Figure 5.2). No significant differences were found in cell viabilities between MdαE7 extracted from aabys and several MdαE7 variants, including $Y^{148}F$ (87%-74%), $E^{273}D$ (92%-78%), $I^{303}M$ (90%-77%), $D^{364}E$ (88%-72%), $A^{365}V$ (90%-75%) (Figure 5.2). For $K^{176}I$, $W^{251}L$ and $R^{378}W$ variants, the tolerance of cells when expressing these three mutants were slightly enhanced by 5%-8% percentage,

indicating that the capabilities of MdαE7 in metabolizing permethrin in insect cells can be enhanced to certain degree when these three mutations were independently introduced into the wild-type MdαE7 allele.

5.3.4 Hydrolytic activities of different MdαE7 variants toward permethrin

Permethrin metabolism was assayed by incubating a 20 µM permethrin standard together with different carboxylesterase variant proteins extracted from infected insect Sf9 cells. The reactions of 20 µM permethrin incubated with MdαE7 proteins from aabys strain served as control. The depletion percentage for permethrin was calculated in comparison with reactions where only 20 µM permethrin was added. Reactions were monitored by reverse-phase HPLC after a 120 min incubation period. Since the permethrin standard is actually a mixture of cis- and trans- isomers, two peaks were observed in the HPLC chromatographic profiles, with elution times of 10.67 min and 10.87 min for trans-permethrin and cis-permethrin, respectively. No significant differences were observed in the depletion percentage of permethrin between MdαE7 protein extracted from aabys strain and several MdαE7 mutants, including $Y^{148}F$, $E^{273}D$, $I^{303}M$, $D^{364}E$, $A^{365}V$ mutants (Figure 5.3), while $K^{176}I$, $W^{251}L$ and $R^{378}W$ mutations occurring on MdαE7 can significantly enhance the metabolic efficiencies of MdαE7 toward permethrin by 6%-9% suggesting that these mutations can significantly alter the properties of MdαE7 proteins and thereby increasing the substrate preference of MdαE7 to permethrin (Figure 5.3), which is also consistent with the cytotoxicity results we obtained in MTT assay (Figure 5.2). Among these MdαE7 mutants, $K^{176}I$ has the highest capabilities to metabolize permethrin, with the depletion percentage of permethrin arriving at 30.22±3.78% (Figure 5.3).

5.3.5 Homology modeling and docking analysis

To better understand the roles of different mutations in altering structure of MdαE7 protein and thereby changing the metabolic capabilities toward permethrin, the homology modelling and permethrin docking analysis were conducted to investigate the metabolism differences caused by the introduction of different mutations occurring on the MdαE7 gene. By aligning the amino acid sequences of MdαE7 isolated from aabys and different mutants, several conserved COE characteristics, such as a catalytic triad composed of Ser218, Glu351 and His471 residue which is involved in substrate metabolism process, and an oxyanion hole composed of Ala219 and Gly136-137 residues were observed in the sequence of MdαE7 (Figure 5.4). The Autodock tool was later used to display the interaction of COE structure and permethrin ligand. The overall structure of COE is composed of the catalytic domain, ab domain and regulatory domain. A binding pocket buried in the structure with a catalytic triad lined at the bottom (Figure 5.5). The binding analysis showed that 1S-trans-permethrin can better fit snugly into the catalytic pocket of different MdαE7 mutant structures. Among all the MdαE7 mutants, only $W^{251}L$ and $Y^{148}F$ mutations are located around the binding pocket of MdαE7, while others are far away from the binding pockets. We later ligated the permethrin isomer into each mutants, and found that $W^{251}L$ mutation can significantly enhance the binding affinity of permethrin within MdαE7, which could be reflected by the decreased binding energy of $W^{251}L$ mutant compared with that of the MdαE7 isolated from aabys, suggesting that the replacement of large molecular Trp residue to small Leu can leave more open space for permethrin binding (Figure 5.6), which is consistent with our

previous metabolism studies. No significant difference in binding energy was oberserved in aabys MdαE7 and $Y^{148}F$ mutant (Figure 5.6).

5.4 Discussion

The qualitative changes of carboxylesterases have been widely identified and investigated in association with OP resistance in insects (Sun et al. 2005; Carvalho et al. 2010; Silva et al. 2009; Cui et al. 2011; Li et al. 2013), however, relative studies on pyrethroid resistance remained to be explored. Multiple mutations and overexpression of the MdαE7 carboxylesterase are responsible for the OP resistance in house flies (Zhang et al. 2018), and the transcriptional upregulation of MdαE7 has also been identified in pyrethroid resistance house flies (Feng et al. 2018).

In this study, by comparing the amino acid sequences extracted from different resistant and susceptible house fly strains and lines, eight mutations were identified in pyrethroid resistant house fly strains. These 8 mutations were individually introduced into the MdαE7 allele from aabys strain to obtain different MdαE7 mutants, with their *in vitro* metabolic functions were later characterized after constructing recombinant baculovirus and heterologous expressing in insect Sf9 cells. Among these 8 mutations, six of them will significantly decrease the kinetic efficiencies of MdαE7 to generic esterase substrate α-NA. At the same time, three mutations, $K^{176}I$, $W^{251}L$ and $R^{378}W$, will also enhance the hydrolytic activities of MdαE7 toward permethrin either in insect cells and *in vitro*. This has the similar effects with that of “mutant ali-esterase hypothesis” which proposed that the polymorphisms occurring on the MdαE7 enable the enzymes to hydrolyze ester-containing insecticides at the expense of their carboxylic activities toward generic esterase substrates (Oppenoorth et al. 1960; Jackson et al. 2013; Pan et al. 2009; Zhu et

al. 1999). Actually, the $W^{251}L(S)$ mutation either in MdαE7 or its orthologues genes, such as LcαE7 gene in *Lucilia cuprina*, has been studied with its crucial contributions in conferring malathion resistance in various insects (Cui et al. 2011; Campbell et al. 1998). It has been proposed that the replacement of a bulky hydrophobic Trp (W^{251}) residue with some smaller residues, like Ser or Leu, can create a more open space to accommodate substrates with bulkier acid moieties, and enhance the binding affinity and hydrolytic capabilities of carboxylesterase to OP insecticides (Devonshire et al. 2003; Heidari et al. 2005). Another mutation, $R^{378}W$ has also been identified in malathion resistant house flies (Zhang et al. 2018), suggesting that carboxylesterase evolution resulted from qualitative changes maybe closely related with cross-resistance development in insects. Notably, the $K^{176}I$ mutation was the first discovery in resistant insects. For another mutation, $I^{303}M$ mutation, has been detected in certain house fly strains with its association with high malathion resistance (Taskin et al. 2004), however, none correlations were discovered for this mutation with pyrethroid resistance based on the functional studies even though it was also identified in our pyrethroid resistant house flies.

Some studies have investigated the interactions of different carboxylesterase mutations in conferring insecticide resistance. They pointed out that rather than single mutations, it is the combination of different mutations will play significant roles in enhancing hydrolytic activities toward insecticides. For example, Gong et al found that the co-occurrence of carboxylesterase mutations, H104R/A128V or H104R/T333P, have considerably higher hydrolytic capabilities against parathion compared with the wild-type carboxylesterase enzyme (Gong et al. 2017). In contrast, some other antagonistic effects were also observed for some carboxylesterase mutations. For instance, in *Lucilia cuprina*,

the W251L mutation occurring on E3 gene owned more than 30-fold hydrolytic activity to OP insecticide compared with wild-type, and another mutation F309L also slightly improved the OP hydrolytic activities compared with wild-type, however, a decreased hydrolytic activities were observed for the combination of W251L and F309L mutations compared with that of W251L alone (Heidari et al. 2005).Therefore, it is necessary to investigate the effects of different mutation combinations in conferring pyrethroid resistance in house flies.

Besides the transcriptional upregulation of MdαE7, this study revealed that the polymorphisms occurring on the MdαE7 genes are also responsible for the enhanced metabolism efficiencies toward pyrethroids, thereby conferring resistance in house flies. This study will not only facilitate the better understanding of carboxylesterase-mediated resistance mechanism, but also provide novel strategies to efficiently monitor and manage resistance development in house fly populations.

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Table 5.1: Non-synonymous mutations identified in pyrethroid resistant house flies

	Amino Acid Substitution	Nucleotide Substitution	House Fly Strains and Homozygous Offspring Lines						
			ALHF	aabys	A2345	A1345	A1245	A1235	A1234
148	Y-F	TAT-TTT	✓	-	✓	-	✓	✓	✓
176	K-I	AAA-ATA	✓	-	✓	-	✓	✓	✓
251	W-L	TGG-TTG	✓	-	✓	-	✓	✓	✓
273	E-D	GAG-GAC	✓	-	✓	-	✓	✓	✓
303	I-M	ATA-ATG	✓	-	✓	-	✓	✓	✓
364	D-E	GAT-GAG	✓	-	✓	-	✓	✓	✓
365	A-V	GCG-GTT	✓	-	✓	-	✓	✓	✓
378	R-W	CGG-TGG	✓	-	✓	-	✓	✓	✓

Note: ✓ indicated that this mutation has been identified in this house fly strain and/or line; - indicated that this mutation has not been identified in this house fly strain and/or line.

Table 5.2: Catalytic efficiencies of carboxylesterase variants toward artificial substrate α-naphthyl acetate (a-NA)

Variants	Km	Vmax	Kcat	Kcat/Km
aabys	119.14±3.08	110.81±1.31	7.23±0.02	0.061±0.002
$Y^{148}F$	259.72±5.65***	76.36±0.81***	4.98±0.05***	0.019±0.0003***
$K^{176}I$	141.47±6.97*	89.72±0.94***	5.85±0.06***	0.042±0.002***
$W^{251}L$	539.43±32.46***	117.92±4.01	7.69±0.26	0.014±0.001***
$E^{273}D$	96.07±5.31**	37.28±0.18***	2.43±0.01***	0.026±0.002***
$I^{303}M$	416.67±48.11***	916.67±48.11***	59.84±3.14***	0.147±0.009***
$A^{365}V$	217.13±3.63***	262.61±2.23***	15.01±2.01***	0.062±0.004
$D^{364}E$	623.97±35.44***	118.07±1.73**	7.71±0.11**	0.012±0.001***
$R^{378}W$	362.11±3.70***	137.54±1.97***	8.98±0.13***	0.025±0.0001***

Note: Kcat: catalytic constant, unit: min^{-1}; Km: Michaelis constant, unit: uM; Vmax: Maximum velocity, unit: nmol/min/mg; Kcat/Km: catalytic constant/Michaelis constant, unit: min^{-1} uM^{-1}.

The results are shown as mean±S.E. (n≥3).

The catalytic efficiency of each MdαE7 mutant was compared with that of MdαE7. One-way Analysis was used for the statistical significance analysis. * indicated the significant differences with P≤0.05; ** indicated the significant differences with P≤0.01; *** indicated the significant differences with P≤0.001.

Table S 5.1 The primers used in this study.

Full length amplification of MdαE7 gene

Gene	Forward(5'-3')	Reverse(5'-3')
MdαE7	CACCATGAATTTCAAAGTTAG	TTAAAACAATTCCTTCTTTTTA

Mutagenesis Studies

Mutant	Forward(5'-3')	Reverse(5'-3')	Detected Primer(5'-3')
$Y^{148}F$	GCTAATCGTAACTGGTTTGG GCCCGACTACTTC	GAAGTAGTCGGGCCCAAAC CAGTTACGATTAGC	TCGGAGGATTGTCTAT ACCTG
$K^{176}I$	GTTGGGTTTCCTTAGCCTGAT ATCGGAAAATCTCAATG	CATTGAGATTTTCCGATAT CAGGCTAAGGAAACCCAAC	TCGGAGGATTGTCTAT ACCTG
$W^{251}L$	GGTAATTCCATGTGCTCATTG GCCTCTACAGAATG	CATTCTGTAGAGGCCAATG AGCACATGGAATTACC	GTCTTCGGCGAAAGTG CTGGT
$E^{273}D$	CGTGTTGGCTATAAGGGAGA GGACAATGAAAAAGATATTT TGGAG	CTCCAAAATATCTTTTTCAT TGTCCTCTCCCTTATAGCCA ACACG	CGTGGTTTATTCCATC GTGGT
$I^{303}M$	GCCACAAGTTTTGACACCCG AAGAAATGCAAAATAAGGTC ATG	CATGACCTTATTTTGCATTT CTTCGGGTGTCAAAACTTG TGGC	CGTGGTTTATTCCATC GTGGT
$D^{364}E$	GCCAAACAATATCCGGAGGC GGTAAAAGAGTTGG	CCAACTCTTTTACCGCCTCC GGATATTGTTTGGC	GAAGAGCGCCTGGGG AAATTCG
$A^{365}V$	GCCAAACAATATCCGGATGT TGTAAAAGAGTTGGAATCCT G	CAGGATTCCAACTCTTTTA CAACATCCGGATATTGTTT GGC	GAAGAGCGCCTGGGG AAATTCG
$R^{378}W$	CCTGTGTTAATTATGTGCCTT GGGAGTTGGCTGACAGTGAA CGC	GCGTTCACTGTCAGCCAAC TCCCAAGGCACATAATTAA CACAGG	GAAGAGCGCCTGGGG AAATTCG

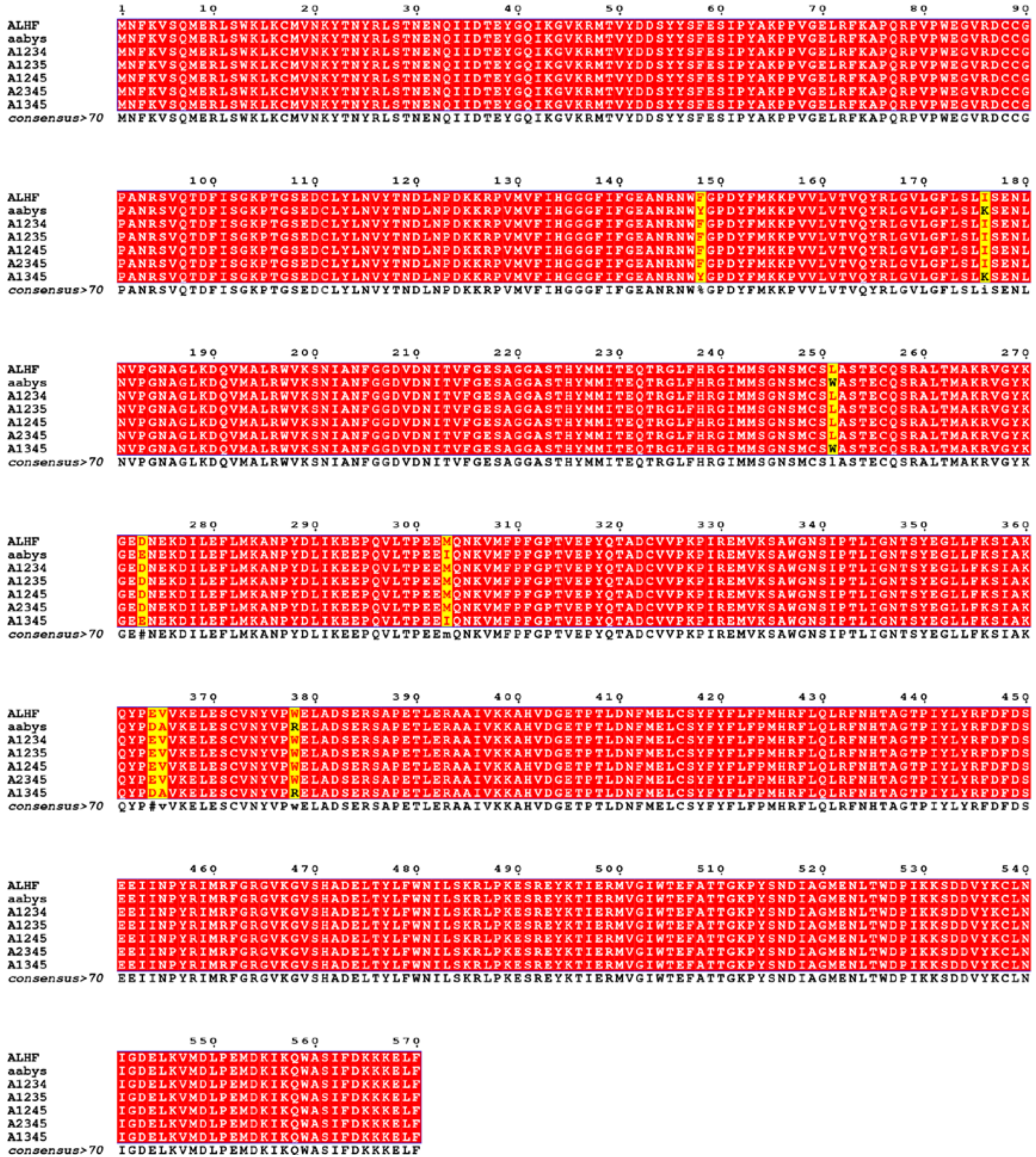

Figure 5.1 The sequence alignment of carboxylesterase MdαE7. Comparison of MdαE7 amino acid sequences from resistant ALHF strain, susceptible aabys strain and five homozygous offspring line A1234, A1345, A1245, A1235 and A2345. The amino acid mutations occurring on pyrethroid resistant strain/line were boxed with yellow color.

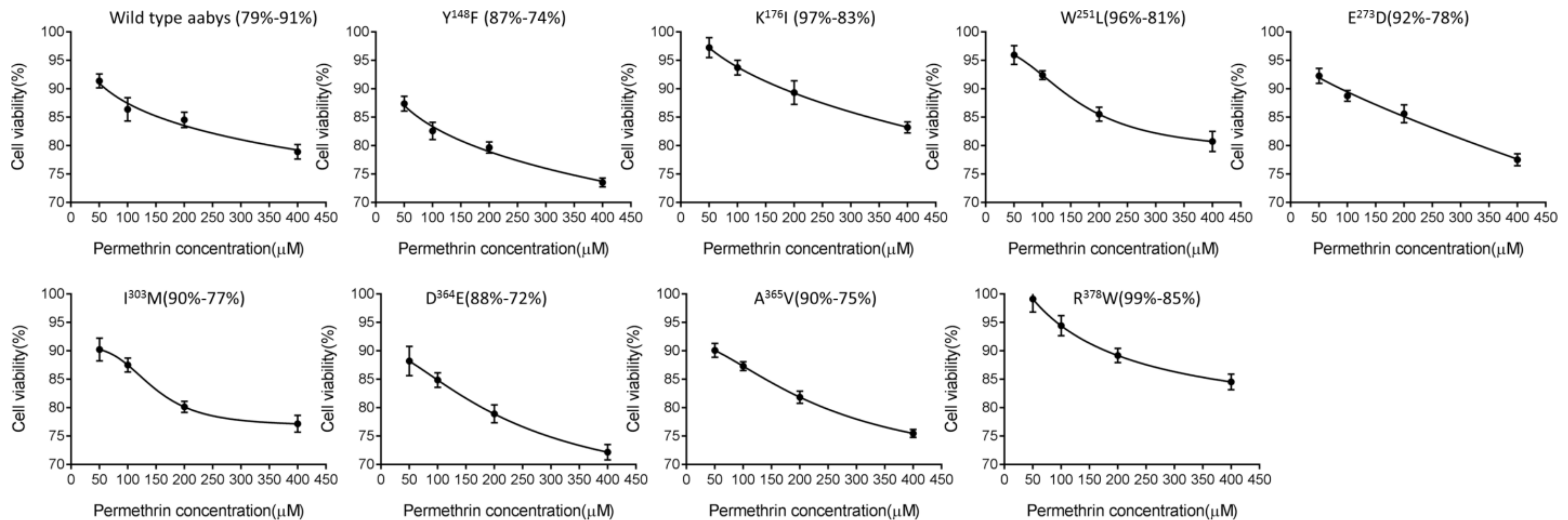

Figure 5.2 The cytotoxicity of permethrin in Sf9 cells. The viabilities of Sf9 cells against permethrin at concentration of 50, 100, 200 and 400 uM, respectively, when expressing different MdαE7 mutants. The viabilities were calculated by comparing with that of cells treated with acetonitrile only. The control group was cells expressing wild-type MdαE7 extracted from aabys strain.

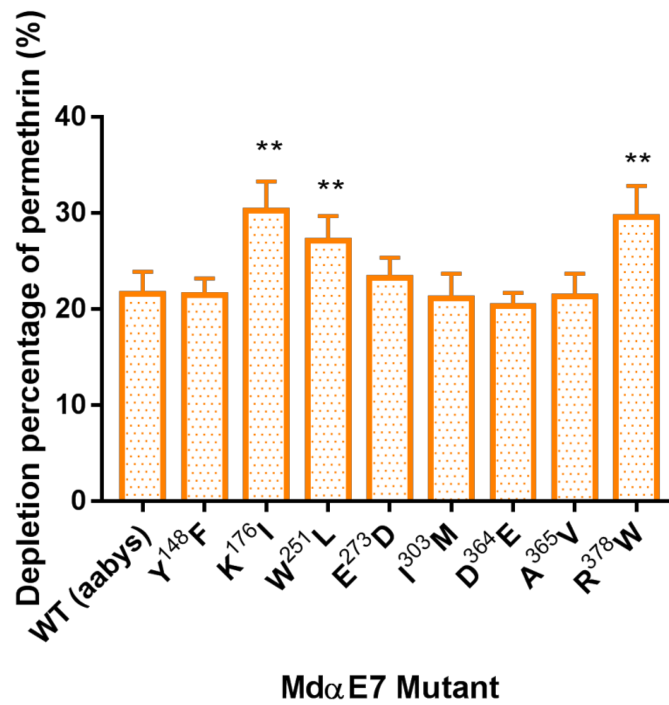

Figure 5.3 *In vitro* metabolism of permethrin by MdαE7. The depletion percentages of permethrin by wild-type MdαE7 extracted from aabys strain and different MdαE7 mutants were compared. ** indicated the significant differences with P≤0.01.

MdαE7

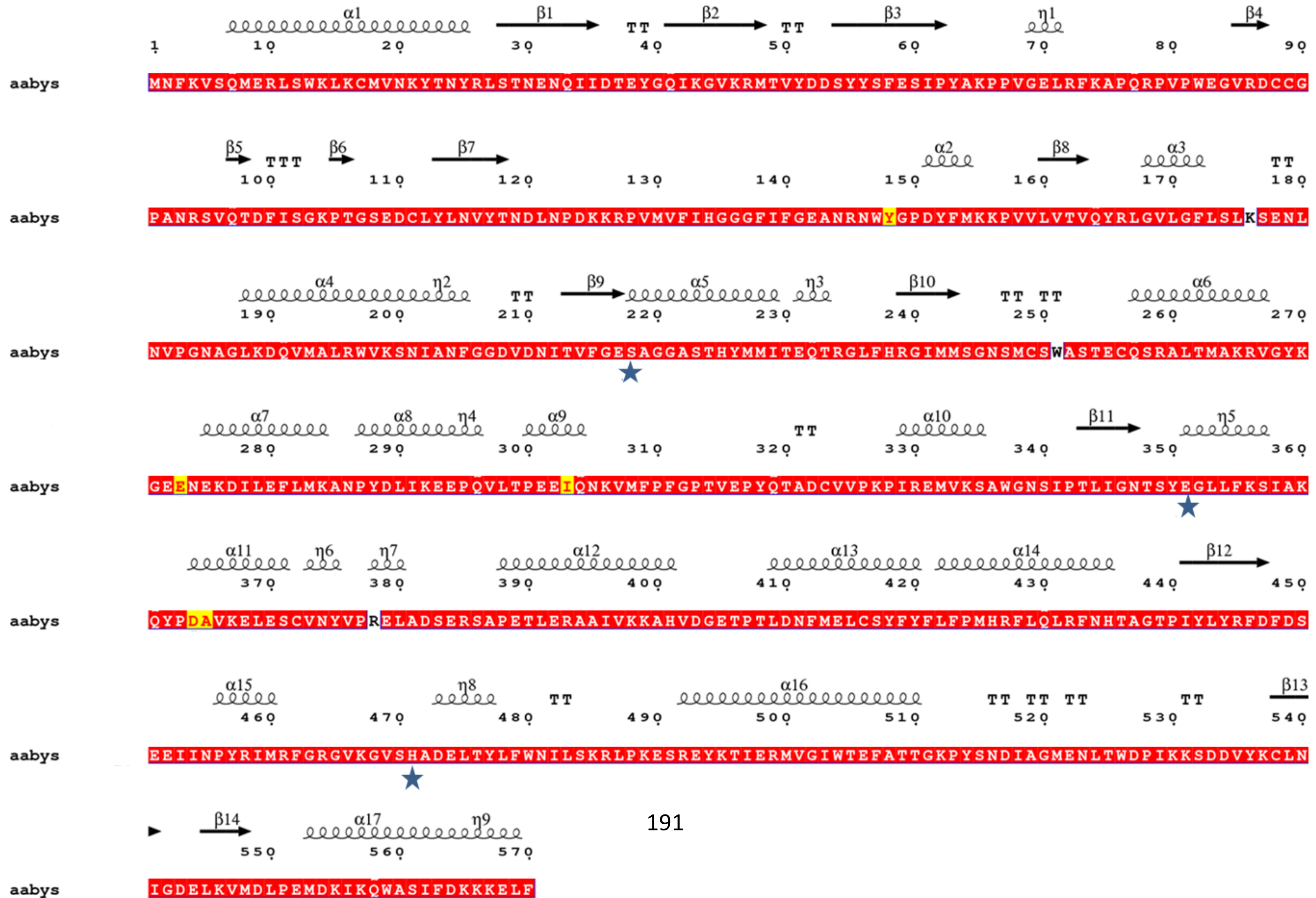

Figure 5.4 Deduced amino acid sequence of carboxylesterase MdαE7. The sequence analysis of MdαE7 was conducted with T-Coffee (http://tcoffee.crg.cat/) and ESPript 3.0 (http://espript.ibcp.fr/ESPript/ESPript/). Alpha-helices, eta-helices, beta sheets and beta turns were marked with α, η, β and TT respectively. The residue Ser218, Glu351 and His471 forming the catalytic triad was marked as blue star.

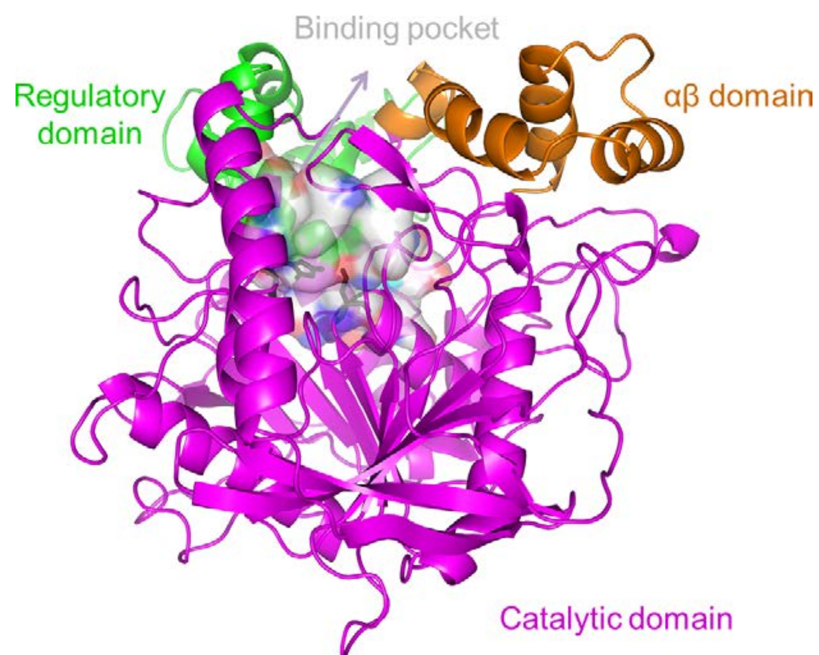

Figure 5.5 The overall structure of MdαE7. The regulatory domain, αβ domain, catalytic domain and binding pocket was colored as green, orange, purple and white, respectively. The residues of the catalytic triad (Ser218, Glu351 and His471) are marked as black sticks.

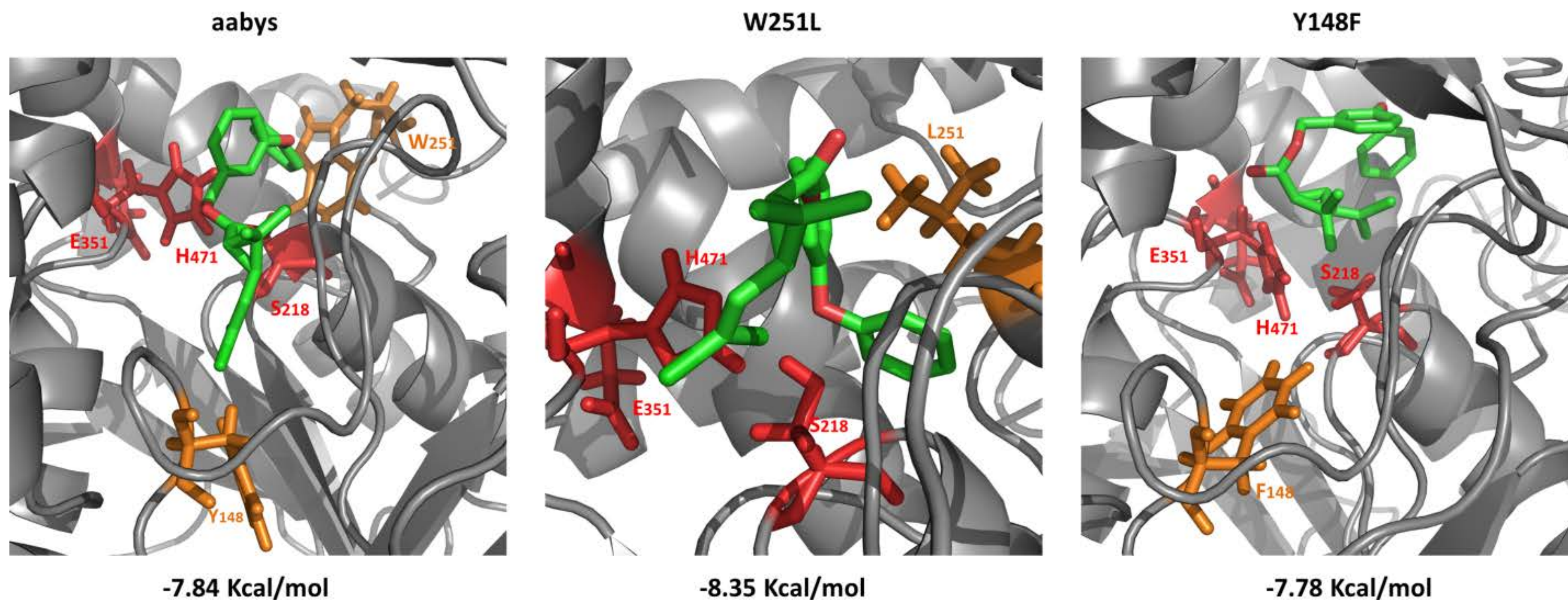

Figure 5.6 Binding modes of 1S-trans-permethirn ligand within the structure of MdαE7 mutants. The homology model of MdαE7 was represented as gray ribbon; The permethrin was presented as green sticks; The amino acid residue substitution was marked as yellow sticks; The amino acid resisue Ser218, His471 and Glu351 composed of catalytic triad were labeled as red sticks A) The MdαE7 gene from aabys strain; B) The $W^{251}L$ mutant; C) The $Y^{148}F$ mutant.

Chapter 6: Research Summary and Future Studies

6.1 Research Summary

My doctoral research project was mainly focused on the mechanisms underlying insecticide resistance in house fly, *Musca domestica*. Specifically, I have investigated the roles of carboxylesterases in conferring pyrethroid resistance in house flies. Based on the whole transcriptome and genome database of *Musca domestica*, a total of 39 carboxylesterase genes of different functional clades have been identified, which were involved in dietary/detoxification, hormone/semiochemical processing and neuro/developmental functions. Among these genes, eleven of them were significantly up-regulated in the resistant ALHF strain compared with susceptible aabys and CS strains. Eight up-regulated carboxylesterase genes with their expressions were further induced to higher levels in response to permethrin treatments, indicating that not only constative but also inductive overexpression of carboxylesterases are co-responsible for the enhanced detoxification of insecticides. Further spatial expression studies revealed that these up-regulated carboxylesterase genes were abundantly distributed in detoxification tissues (including midguts and fat bodies). Autosomal linkage analysis has mapped these carboxylesterases on autosome 2 and 3 of house flies, with their expression could be regulated by factors on autosome 1, 2, and 5. All these results demonstrate that multiple carboxylesterases genes are co-upregulated in resistant house flies, providing strong evidence for their involvement in degrading insecticides and thereby conferring

insecticide resistance in population. The roles of carboxylesterases in metabolizing permethrin were further explored by conducting *in vitro* metabolism studies. We expressed up-regulated carboxylesterase gene in insect *Spodoptera frugiperda* (Sf9) cells using baculovirus-mediated cell expression system, and examined their hydrolytic activities toward different esterase substrates and permethrin. Our results suggested that the targeted carboxylesterase proteins can efficiently hydrolyze substrate α-naphthyl acetate (α-NA), suggesting that the carboxylesterase proteins produced by this heterologous expression system own strong hydrolytic activities *in vitro*. Later, a cell-based MTT cytotoxicity assay was conducted to reveal the roles of carboxylesterase in insect cells, and we found that Sf9 cells expressing targeted carboxylesterase proteins significantly increased their tolerance to permethrin, suggesting the important characteristics of these carboxylesterases in metabolizing permethrin in insect cells. The functions of these carboxylesterases were further characterized by conducting *in vitro* metabolism studies against permethrin and its potential metabolites 3-phenoxybenzyl alcohol and 3-phenoxybenzaldehyde, and efficient metabolism capacities were observed toward permethrin rather that its metabolites. This finding indicated not only a potential route for permethrin metabolism in insects, but also an important role of these carboxylesterases in metabolizing permethrin and conferring resistance in house flies. Homology modeling and docking analysis were used to explore the interaction between permethrin and carboxylesterase protein, thereby confirming the metabolic roles of these carboxylesterases toward insecticides in house flies.

Besides the quantitative changes of carboxylesterase, the qualitative changes of carboxylesterases were also investigated in association with pyrethroid resistance in

house flies. One carboxylesterase gene, MdαE7, has been investigated in organophosphate resistant insects with its potential roles in conferring mutant ali-esterase hypothesis, which means that the mutations occurring on MdαE7 gene can significantly increase the hydrolytic activities toward ester-containing at the expense of its carboxylic activities against generic esterase substrates. However, the relationship between MdαE7 mutations and pyrethroid resistance remained to be explored. In this study, by aligning the amino acid sequences of MdαE7 extracted from different house fly strains and lines, eight mutations have been identified in resistant ALHF strain and homozygous offspring line A1234, A1235, A1245, and A2345, while none of them were identified in susceptible aabys and A1345 line, not only suggesting that these mutations occurring on MdαE7 gene are closely related with pyrethroid resistance in house flies, but also confirming that the MdαE7 gene was indeed located on autosome 2 of house flies. These eight mutations were then individually introduced into MdαE7 gene isolated from aabys strain and their roles were investigated through *in vitro* functional studies. Our results suggested that three mutations, $I^{176}K$, $W^{251}L$ and $R^{378}W$, can significantly increase the capabilities of wild-type MdαE7 in metabolizing permethrin at the cost of reducing its carboxylic efficiencies toward generic esterase substrate α-NA, which has the similar effects of "mutant ali-esterase hypothesis" reported in OP resistant insects. Taken together, our studies strongly supported the vital roles of carboxylesterases in conferring pyrethroid resistance in house flies through either quantitative or qualitative changes.

6.2 Future Studies

In my study, the roles of carboxylesterases in conferring pyrethroid resistance have been characterized in house flies. However, the regulatory factors or pathways

controlling the carboxylesterase-mediated resistance remained to be explored. With the availability of cutting-edge genome-editing techniques, such as CRISPR/Cas9 system, it is expected to elucidate the regulatory elements in controlling the up-regulation of carboxylesterases in pyrethroid resistant insects, thereby providing novel strategies to efficiently minimize and prevent the development of insecticide resistance in the populations.

6.2.1 Characterization of the regulatory elements of up-regulated carboxylesterases

Usually, the regulatory elements controlling the up-regulation of carboxylesterases were found at the upstream of the promotor regions and responsible for regulatory protein binding. Here, we are expected to use the electrophorathic mobility shift assay (EMSA), also known as the gel shift assay to identify the regulatory factors of carboxylesterase-mediated resistance.

Firstly, the sequences of promotor regions extracted from resistant and susceptible strain were aligned to identify the differences, which maybe the potential regulatory elements controlling the transcriptional expression of target genes. We design and synthesize the radioactive probe DNA and incubated with nuclear protein, and the DNA-protein complex will be separated by electrophoresis on a non-denaturing polyacrylamide gel and visualized by autoradiography by exposure to X-ray film with intensifying screens. By conducting these experiments, it is expected to identify the regulatory factors controlling the carboxylesterase-mediated resistance.

6.2.2 Functional characterize the roles of carboxylesterases and regulatory factors controlling the carboxylesterase-mediated resistance

We expected to use the CRISPR/Cas9 to knock-out the interested carboxylesterase genes or their potential regulatory factors, and later characterize their functions by investigating the effects caused by the gene disruption. We firstly knock-out the house fly gene MdY controlling the house fly brown body color (bwb) phenotype.

We designed the two sgRNA based on the MdY sequence using the CRISPR RGEN Tools (www.rgenome.net). The template DNA was synthesized based on the sgRNA primer and a common reverse primer. The sgRNA was later synthesized following the protocol of Megatranscript T7 kit (Ambion) using 300 ng target template with a 5' flanking T7 promotor as starting material. After sgRNA synthesis, the template was removed by incubating with TurboDNase (Ambion). For the embryonic microinjection, we prepared the injection mixture with 300 ng/uL Cas9 protein (PNABio), 300 ng/uL sgRNA-1 and 300 ng/uL sgRNA-1. The house fly embryos were collected within 30 min after egg lay. The embryos were aligned on a cover slip with posterior end pointing to the injection site, and covered with 1: 1 oil mix of Halocarbon oil 27: Halocarbon oil 700. Injection was performed by a micromanipulator connected to an Eppendorf Femtojet pumping device. After injection, the eggs were carefully removed from the cover slip and the covered oil was also cleaned with water. The eggs were later put on a plate filled with jazz-mix drosophila food overnight. After 24 h, the hatched eggs were transferred to a beaker containing house fly larva rearing medium (Figure 6.1 A). The phenotype of house fly with MdY gene knock-out was observed after eclosion (Figure 6.1 B).

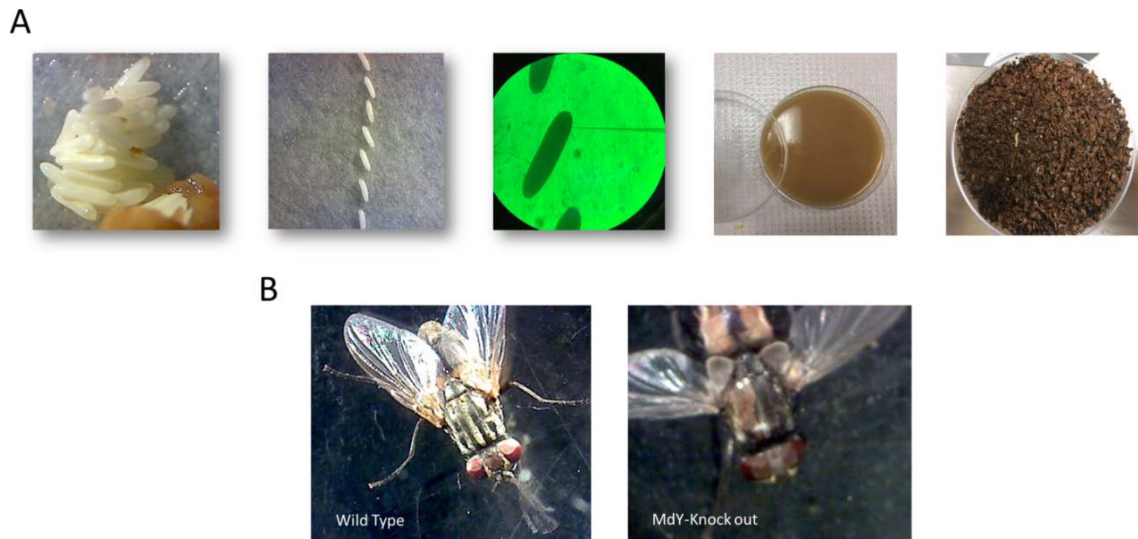

Figure 6.1 The scheme of CRISPR/Cas9 system in house flies. A) The basic procedures of the house fly embryonic microinjection. B) The phenotype of house flies after MdY gene knockout.