Eurasia Specialized Veterinary Publication

Entomology Letters

ISSN:3062-3588

2023, Volume 3, Issue 1, Page No: 28-35

Available online at: www.esvpub.com/

Chlorfenapyr Resistance and Acetylcholinesterase Enzyme Activity in *Musca domestica*

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ABSTRACT

Understanding the changes in insect organisms due to insecticide exposure and their species-specific responses is crucial for comprehending the evolutionary and environmental aspects of pesticide resistance. While the mechanism behind resistance to chlorfenapyr, a pyrrole group insecticide, remains unclear, this study investigates the acetylcholinesterase (AChE) activity in adult houseflies (*Musca domestica*) of a chlorfenapyr-resistant strain (ChlA) selected with chlorfenapyr. This research also compares the kinetic parameters of AChE in female and male ChlA strains to those in an unselected strain (Lab) of *M. domestica* for the first time. No significant differences were observed in the specific AChE activity between the ChlA and Lab strains. However, the remaining activity of propoxur-inhibited AChE in ChlA females was significantly lower (3.81 times less, $P < 0.05$), and the Vmax and Km values were reduced by 43.3% and 46.9% ($P < 0.05$), respectively, when compared to Lab females. Furthermore, the catalytic efficiency of AChE expressed as Vmax/Km, was higher in males than females in both strains. Overall, the results indicate that in ChlA females, the affinity of AChE for specific ligands, such as acetylthiocholine (substrate) and propoxur (inhibitor), increased without a corresponding increase in catalytic activity, which may be attributed to the selection of chlorfenapyr during 23-24 generations.

Keywords: Enzyme, Insecticide resistance, Chlorfenapyr, Kinetic parameters, Diptera

Received: 17 March 2023
Revised: 23 May 2023
Accepted: 25 May 2023

How to Cite This Article: Kinareikina AG, Silivanova EA. Chlorfenapyr Resistance and Acetylcholinesterase Enzyme Activity in *Musca domestica*. Entomol Lett. 2023;3(1):28-35. https://doi.org/10.51847/TnXtgnRRyx

Introduction

In modern agriculture, public health, and pest control, pesticides play a crucial role in safeguarding animals, plants, and humans from arthropod pests, ectoparasites, and vectors of diseases. However, over time, insects have developed mechanisms to tolerate or resist these insecticides, a phenomenon that poses significant challenges to pest management efforts. It is well-documented that more than 600 insect and mite species have evolved resistance to at least 1 insecticide, affecting pest control strategies [1-4]. The study of chronic and sublethal insecticidal effects, along with understanding species-specific responses, is essential for a comprehensive grasp of the environmental and evolutionary patterns of pesticide resistance. These insights are key in shaping modern strategies for pest control, including prevention and elimination of resistance [5-7].

Resistance to insecticides is primarily driven by metabolic mechanisms, involving major enzymes such as P450 monooxygenases, glutathione-S transferases, and esterases, which play pivotal roles in the breakdown of insecticides. In addition, ABC transporters help in the excretion of metabolites formed in the process, contributing

to resistance. Esterases, in particular, are crucial in the hydrolysis of insecticides and are involved in resistance against various classes of insecticides [8, 9]. Acetylcholinesterase (AChE), a serine esterase from the α-, β-hydrolase family, is a key enzyme in the regulation of acetylcholine at cholinergic synapses and is essential for nerve impulse transmission. AChE is a primary molecular target for organophosphates (OPs) and carbamates, and resistance to these chemicals often results from reduced enzyme sensitivity. Additionally, AChE may contribute to resistance by detoxifying insecticides, as its high affinity for choline ethers enables it to hydrolyze OPs and other chemicals. Some isoforms of AChE in insects are also thought to play a role in sequestering insecticides and other xenobiotics, further contributing to resistance mechanisms [10-14].

Certain insecticides, including chlorfenapyr (a pyrrole group compound), function as pro-insecticides. These chemicals are converted into toxic metabolites through biotransformation processes, such as those mediated by monooxygenases [15, 16]. Chlorfenapyr, classified by the Insecticide Resistance Action Committee (IRAC) as a decoupler of oxidative phosphorylation, is a widely used insecticide for controlling various pests, including bedbugs, cockroaches, termites, ants, and mosquitoes. It is especially prevalent in agricultural settings in Russia, where it is used to protect plants, and insecticidal baits containing chlorfenapyr are employed in livestock facilities to control Diptera pests [17-19].

In regions where chlorfenapyr has been extensively applied in agricultural production, cases of resistance among pest populations have been documented. The development of resistance to chlorfenapyr has been explored in certain insect species, including the spider mite *Tetranychus urticae* (Acari: Tetranychidae) and the dusky cotton bug *Oxycarenus hyalinipennis* (Lygaeidae: Hemiptera) [20, 21]. Research suggests that resistance in these species is linked to elevated esterase and glutathione-S-transferase activity, as well as reduced cuticle permeability. However, studies on *Plutella xylostella* (Lepidoptera: Plutellidae) resistant populations indicate that these enzymes do not contribute to chlorfenapyr resistance [22-25].

The housefly *Musca domestica* L. (Diptera: Muscidae) is an insect of both medical and economic significance, particularly in the fields of public health and veterinary medicine. Due to its adaptability and rapid reproduction, *M. domestica* is frequently used as a model species for evaluating insecticidal efficacy and investigating resistance mechanisms. Under laboratory conditions, housefly populations can develop resistance within a short period (approximately 5–7 generations) when exposed to specific insecticides [26-29].

This study focused on assessing acetylcholinesterase (AChE) activity in adult *M. domestica* specimens from a chlorfenapyr-resistant strain. The research involved comparing AChE activity in both male and female flies from resistant and non-resistant strains. Additionally, key kinetic parameters of AChE were analyzed, providing insights into potential mutational adaptations in resistant individuals. These findings contribute to a broader understanding of resistance mechanisms in *M. domestica* and their implications for insecticide resistance management.

Materials and Methods

This study utilized various chemical compounds and reagents, including propoxur (100.0%, PESTANAL®), EDTA (≥ 99.0%, BioUltra), N-Phenylthiourea (PTU, ≥ 98.0%), phenylmethylsulfonyl fluoride (PMSF, > 98.5%), 1,4-Dithioerythritol (DTE, ≥ 99.0%), and Triton X-100 (t-Octylphenoxypolyoxyethethanol, ≥ 100.0%). Additionally, 5,5′-Dithiobis (2-nitrobenzoic acid) (DTNB, ≥ 98.0%) and acetylthiocholine iodide (≥ 98.0%) were obtained from Sigma-Aldrich (Germany). Other reagents included Folin-Ciocalteu's Reagent (PanReac, AppliChem, Italy), bovine serum albumin (BSA) (ZAO Diakon-DC, Russia), as well as mono- and disubstituted sodium and sulfurous copper, potassium phosphates and analytical-grade sodium carbonate (OOO AO REACHIM, Russia).

Two groups of adult *M. domestica* flies, aged between 3 and 5 days, were used in the experiments. One group consisted of laboratory-reared flies with no prior exposure to chlorfenapyr (Lab), with an average female weight of 13.43 ± 4.24 mg and a male weight of 8.64 ± 2.32 mg. The second group comprised chlorfenapyr-resistant specimens (ChlA), exhibiting a resistance ratio of 19.4, with females averaging 17.26 ± 3.03 mg and males 9.66 ± 2.09 mg. These houseflies were obtained from the Laboratory of Veterinary Problems in Animal Husbandry [17]. Both fly strains were maintained in controlled conditions, with a stable temperature of 27 ± 1 °C and relative humidity at 50 ± 5%.

To prepare homogenates, individual flies were manually processed at low temperatures. A 0.1 M phosphate buffer (pH = 7.6) containing 1 mM EDTA, 1 mM PTU, 1 mM PMSF, 1 mM DTE, and 20% Triton X-100 was used for

homogenization. The mixture was then centrifuged at 12,500 rpm for 2 minutes, and the resulting supernatant was collected for the assessment of AChE activity and protein concentration. The protein content was quantified using the Lowry protein assay, with BSA solutions serving as calibration standards [29].

AChE activity was measured using a Multiskan FC microplate photometer (Thermo Fisher Scientific Inc., Finland) with 96-well microtitration plates (MiniMed, Russia), following a modified version of Ellman's method [30]. The reaction mixture contained 10 µl of homogenate, 90 µl of 50 mM potassium phosphate buffer (pH = 7.0), and 100 µl of Ellman's reagent, freshly prepared by combining 2 mM acetylthiocholine iodide with 0.23 mM DTNB. To account for non-enzymatic hydrolysis of the substrate, control wells contained 10 µl of potassium phosphate buffer (pH = 7.0) instead of the homogenate.

For kinetic analysis, acetylthiocholine iodide was used at final concentrations of 0.0625, 0.125, 0.25, 0.5, 1, and 2 mM to determine the Michaelis constant (Km) and maximal velocity (Vmax). AChE inhibition was evaluated using a 0.1 M propoxur solution. Acetylthiocholine iodide was mixed with propoxur in a 30:1 ratio before being incorporated into Ellman's reagent. Optical density at 405 nm was recorded at 30 °C for 30 minutes in kinetic mode when determining AChE activity, while kinetic parameters and residual enzymatic activity were assessed by measuring absorbance at 405 nm every 15 seconds over 5 minutes at 30 °C. The enzymatic activity was expressed as ΔOD/min/mg of protein, representing the change in optical density per minute per milligram of protein [30].

Kinetic parameters were analyzed using non-linear regression modeling with Excel Solver software [31, 32]. Statistical comparisons of enzyme activity were performed using one-way ANOVA, followed by Tukey's post hoc test for multiple comparisons, in the Statistica 13.3 software package (StatSoft, Russia). Statistical significance was set at $P \leq 0.05$.

Results and Discussion

Statistical analysis of the experimental data revealed no significant differences in specific AChE activity between the two strains of *M. domestica*, ChlA, and Lab, based on homogenates prepared from adult flies **(Figure 1)**. The enzymatic activity levels in females and males of the ChlA strain were observed to be lower by 20.9% ($P = 0.879$) and 13.7% ($P = 0.996$), respectively, in comparison to those of the Lab strain, though these variations were not statistically significant.

As depicted in **Figure 2**, the remaining AChE activity, measured in the presence of an inhibitor, was found to be significantly lower in Lab males compared to Lab females, exhibiting a 3.61-fold reduction ($P = 0.000021$, $P < 0.05$). In contrast, within the ChlA strain, no notable sex-related variation was observed in this parameter ($P = 0.999$). Furthermore, the remaining AChE activity in ChlA females was 3.81 times lower than that of Lab females ($P = 0.000018$, $P < 0.05$). However, no significant differences were found when comparing ChlA and Lab males in terms of remaining enzyme activity ($P = 0.998$).

The kinetic parameters of AChE for both strains are presented in **Table 1**. Analysis of maximal velocity (Vmax) during the first reaction phase indicated no statistically significant sex-based differences within either strain. Nonetheless, Lab males displayed a 36.8% lower Vmax value than Lab females ($P = 0.082$), while ChlA males exhibited a 40.7% higher Vmax compared to females of the same strain ($P = 0.576$). Additionally, a notable difference was detected between females of the two strains, with ChlA females showing a 43.3% lower Vmax than lab females ($P = 0.013$, $P < 0.05$).

Regarding the Michaelis constant (Km), the value in Lab males was significantly lower—by a factor of 2.74—compared to Lab females ($P = 0.014$, $P < 0.05$). However, within the ChlA strain, no statistically significant sex-dependent differences in Km were found. When comparing females across strains, the Km value in ChlA females was observed to be 1.88 times lower than in Lab females, though this difference was not statistically significant ($P = 0.132$). The Vmax/Km ratio in Lab males was found to be 48.0% higher than in Lab females ($P = 0.030$), while in ChlA males, this ratio was 35.1% greater than in ChlA females ($P = 0.105$) **(Table 1)**.

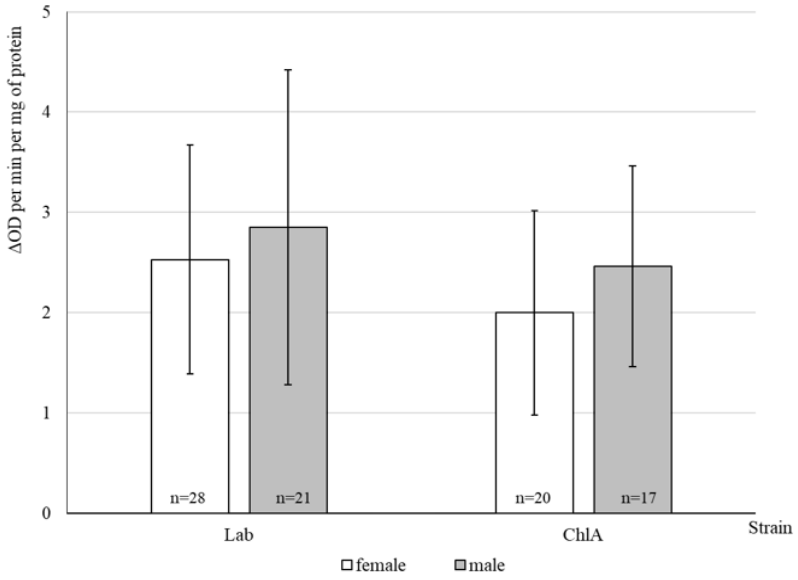

Figure 1. The specific activity of AChE in adults in the laboratory chlorfenapyr-selected (ChlA) and unselected (Lab) strains of *M. domestica* L. RR (resistance ratio) for the ChlA-strain is 19.4; values are represented as Mean ± SD.

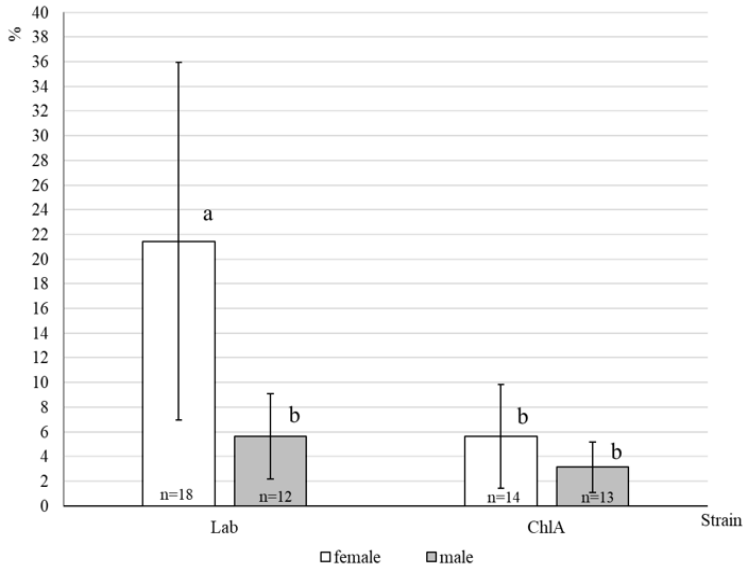

Figure 2. The remaining activity of AChE in adults in the laboratory chlorfenapyr-selected (ChlA) and unselected (Lab) strains of *M. domestica* RR (resistance ratio) for the ChlA-strain is 19.4; values with the same letters do not differ significantly at $P < 0.05$; values are represented as Mean ± SD.

Table 1. Kinetic parameters of AChE in adults of the laboratory chlorfenapyr-selected (ChlA) and unselected (Lab) strains of *M. domestica* (Mean ± SD).

Strain		Vmax, ΔOD/min/mg of protein	Km, mM of ATC	Vmax/Km
Lab	Females	3.93 ± 2.22^{a}	1.17 ± 0.88^{a}	3.73 ± 0.90^{a}
	Males	2.48 ± 0.67^{ab}	0.43 ± 0.15^{b}	5.52 ± 1.64^{b}
ChlA	Females	2.23 ± 0.52^{b}	0.62 ± 0.29^{ab}	4.15 ± 1.73^{ac}
	Males	3.20 ± 0.68^{ab}	0.69 ± 0.39^{ab}	5.61 ± 1.90^{bc}

Note: Vmax = the maximal velocity; Km = the Michaelis constant; OD = optical density; ATC = acetylthiocholine iodide; RR (resistance ratio) for the ChlA-strain is 19.4; values with the same letters in the same column do not differ significantly at $P < 0.05$.

Acetylcholinesterase (AChE) serves as a primary target for insecticides belonging to the organophosphate (OP) and carbamate groups [5]. Variations in substrate specificity, enzymatic activity, and sensitivity to these insecticides play a crucial role in both detoxification processes and resistance mechanisms [33]. Previous studies have demonstrated that insects resistant to OPs and carbamates [28], as well as pyrethroids [34, 35], often exhibit increased AChE activity along with modifications in their functional properties. The potential involvement of the enzyme in insecticide detoxification has been linked to mechanisms such as sequestration [14, 15] and hydrolysis [13].

This study examined the kinetic and activity characteristics of AChE in adult houseflies (*M. domestica*) from two strains: Lab (not subjected to chlorfenapyr selection) and ChlA (selected for chlorfenapyr resistance). The ChlA strain exhibited a resistance ratio (RR) of 19.4 to chlorfenapyr when compared to the Lab strain. Based on classification criteria, this level of resistance is considered either low [36] or moderate [37].

Experimental findings **(Figure 1)** indicate that there were no statistically significant differences in specific AChE activity between the Lab and ChlA strains. However, previous research by Nazar *et al*. [38] observed an increase in AChE activity in a highly chlorfenapyr-resistant strain of the mealybug *Phenacoccus solenopsis* (Hemiptera: Pseudococcidae). Similarly, a study by Shabbir *et al*. [8] found that AChE genes were highly expressed in larvae of *Plutella xylostella* (Lepidoptera: Plutellidae) after exposure to chlorantraniliprole. Despite these observations, elevated AChE activity does not necessarily correlate with resistance to specific insecticides. For instance, Li *et al*. [34] reported that field-collected *M. domestica* populations exhibited higher AChE activity than their insecticide-susceptible counterparts. While these flies demonstrated strong resistance to propoxur and cypermethrin, they remained susceptible to chlorfenapyr. Furthermore, research suggests that resistance development in *M. domestica* can be associated with both the activation [27] and suppression of esterase-based detoxification pathways [9].

Our findings revealed no statistically significant differences in residual AChE activity between male specimens from the Lab and ChlA strains **(Figure 2)**, despite a 47.3% reduction in this parameter in the ChlA strain compared to Lab specimens. Notably, in female flies of the ChlA strain, the remaining enzyme activity in the presence of propoxur was significantly lower than that of Lab females, suggesting an increased sensitivity to the inhibitor. In contrast, resistant insects often exhibit the opposite trend—higher residual enzyme activity due to reduced sensitivity to AChE-targeting insecticides under in vitro conditions. This phenomenon has been documented in propoxur-resistant *M. domestica* [28] and in *Agonoscena pistaciae* (Hemiptera: Psyllidae), which developed resistance to the organophosphate insecticide fosalone [39]. Additionally, field populations of *Bemisia tabaci* (Hemiptera: Aleyrodidae) resistant to OPs and neonicotinoids displayed remaining AChE activity levels between 30% and 70% [40].

Beyond alterations in inhibition levels, qualitative modifications in the enzyme molecule can occur in insecticide-resistant populations, affecting both activity and substrate affinity. These shifts are often reflected in changes to kinetic parameters. Research on AChE kinetics has traditionally focused on OP- and carbamate-resistant insects, where resistance mechanisms involve reduced target-site sensitivity to insecticides. For example, Shi *et al*. [28] reported significant modifications in AChE properties in a propoxur-resistant *M. domestica* strain, including altered affinity and hydrolysis rates for three different substrates. Their results showed that AChE in resistant houseflies had higher Km and Vmax values for acetylthiocholine (ATC) compared to specimens from a susceptible strain [28]. This study concluded that resistance in the propoxur-resistant strain was linked to a reduced affinity of AChE for both insecticides and substrates, as well as decreased catalytic efficiency for ATC. Similar observations have been reported in other research involving OP- and carbamate-resistant insect populations [41-43].

For the first time, we assessed AChE kinetic parameters in *M. domestica* resistant to chlorfenapyr. A significant 43.3% reduction in Vmax was observed in ChlA females compared to Lab females ($P < 0.05$), indicating a decline in catalytic efficiency against the ATC substrate in chlorfenapyr-resistant flies. Moreover, a 1.88-fold decrease in Km and a 3.81-fold reduction in remaining AChE activity in ChlA females, relative to Lab females, suggest that AChE in the resistant strain exhibited greater affinity for specific ligands (i.e., ATC substrate and propoxur inhibitor) without a corresponding increase in catalytic efficiency. Mutations in the genes encoding AChE are known to influence enzyme properties such as substrate specificity, affinity, and kinetic behavior [44]. Given this, future research should focus on sequencing the AChE gene in chlorfenapyr-resistant *M. domestica* to further explore these potential genetic alterations.

Conclusion

The findings of this study indicate that AChE does not play a significant role in the resistance of *M. domestica* to chlorfenapyr, as no increase in its enzymatic activity was observed in the resistant ChlA strain. However, in ChlA females, which underwent sublethal exposure to chlorfenapyr over 23–24 generations, qualitative modifications in AChE became evident in adults of the 24th–25th generations, influencing the enzyme's affinity for ligands. To gain a deeper understanding of these changes, further molecular investigations are needed to identify potential mutations and provide a more comprehensive characterization of AChE in this resistant strain.

Acknowledgments: None

Conflict of Interest: None

Financial Support: None

Ethics Statement: None

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