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Acetylcholinesterase Activities in Adult Houseflies *Musca Domestica* L. of the Chlorfenapyr-Resistant Strain

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ABSTRACT

The study of changes that occurred in insect organisms in response to insecticidal exposure and their species-specific characteristics is important for a fuller understanding of the environmental and evolutionary patterns of pesticidal resistance. For chlorfenapyr from the pyrrole group of insecticides, the mechanism underlying the resistance in insects is not quite clearly described. This study evaluated the activity of acetylcholinesterase (AChE) in adults of the house fly *Musca domestica* of the chlorfenapyr-resistant (selected with chlorfenapyr) strain (ChlA). Also, we assessed the kinetic parameters of AChE in females and males of the ChlA strain compared to these of the unselected strain (Lab) of *M. domestica* for the first time. Specimens of Lab and ChlA strains had no statistically significant differences in specific AChE activity. The percentage remaining activities of propoxur-inhibited AChE was 3.81 times less ($p < 0.05$) and values of Vmax and Km were 43.3% and 46.9% ($p < 0.05$), respectively, less in females of the ChlA strain compared to these in females of the Lab strain. For both Lab and ChlA strains *M. domestica*, the catalytic efficacy of AChE based on Vmax/Km in males was more than that in females. In general, the results obtained suggest that the affinity of AChE to specific ligands (like a substrate acetylthiocholine and an inhibitor propoxur) increased without a rise of the catalytic activity in females of the ChlA strain *M. domestica* that was under selection with chlorfenapyr during 23-24 generations.

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

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INTRODUCTION

In the modern world, the protection of plants, animals, and humans against arthropod pests, ectoparasites, and vectors of vector-borne diseases is carried out mainly through the use of pesticides [1-4]. In response to insecticidal action, insects are capable of developing tolerance or resistance to insecticides [2, 4]. For example, more than 600 species of insects and mites are known to be resistant to at least one insecticide in their populations [5]. The study of chronic and sublethal effects of insecticidal exposure and their species-specific characteristics is important for a fuller understanding of the environmental and evolutionary patterns of pesticidal resistance. These patterns can shape modern approaches to pest and mite population control, prevention, and elimination of resistance [1, 6].

It is known that metabolic resistance is provided by three major groups of enzymes (P450 monooxygenases, glutathione-S transferases, and esterases), which are responsible for the biotransformation of insecticides. It is also provided by the ABC transporters, involved in the excretion of metabolites from the insect body, which were formed in the previous step [7]. Hydrolysis of insecticides by esterases is an important biochemical mechanism for the development of insecticide resistance that is common to several classes of chemical compounds [8-10]. Acetylcholinesterase (EC 3.1.1.7, AChE) is a serine esterase of the α-, β-hydrolase family. AChE acts as a regulator of acetylcholine levels in cholinergic synapses and thus partakes in nerve impulse transmission [11]. Therefore, AChE in insects is a specific molecular target of organophosphorus compounds (OPs) and carbamates. The resistance development to these compounds is often realized through the mechanism of decreasing the enzyme's sensitivity to them [12]. AChE can also contribute to the formation of insecticide resistance through detoxification, as evidenced by the ability of the enzyme with a high affinity for choline ethers to hydrolyze other ethers, including OPs [13]. In addition, it is assumed that solubilized AChE isoforms in insects can be involved in the sequestration of xenobiotics, including insecticides [14, 15].

Certain xenobiotics are pro-insecticides that are transformed into toxic metabolites in insects through a process of biotransformation (for example, mediated by monooxygenases). These compounds include chlorfenapyr (4-Bromo-2-(4-chlorophenyl)-1-(ethoxymethyl)-5-(trifluoromethyl)-1H-pyrrole-3-carbonitrile) from the pyrrole group [16]. According to the classification of the Insecticide Resistance Action Committee (IRAC), chlorfenapyr acts as a decoupler of oxidative phosphorylation [5]. Chlorfenapyr is effectively used as a non-repellent insecticide against various synanthropic insects (cockroaches, bedbugs, termites, ants, mosquitoes, etc.). In Russia, chlorfenapyr is commonly used to protect plants; insecticidal baits containing chlorfenapyr are also used to control Diptera insects in livestock facilities [17]. In countries where chlorfenapyr-containing means have been used in crop production long-term, the emergence of pest populations resistant to it has been noted [18, 19]. A possible mechanism of resistance development to chlorfenapyr has been described for the spider mite *Tetranychus urticae* Koch (Acari: Tetranychidae) [20] and the dusky cotton bug *Oxycarenus hyalinipennis* (Lygaeidae: Hemiptera) [18] and is associated with an increase in esterase and glutathione-S-transferase activity [18, 21], as well as with a decrease in cuticle permeability [19]. Meanwhile, the study on resistant diamondback moth, *Plutella xylostella* (Lepidoptera: Plutellidae), populations concluded that the aforementioned enzymes are not involved in the formation of resistance to chlorfenapyr [22].

The housefly *Musca domestica* L. (Diptera: Muscidae), as a species of great health and economic importance in medicine and animal health [23, 24], is used as a model organism for testing insecticides and studying insecticide resistance [25]. In a controlled laboratory environment, *M. domestica* is capable of developing resistance rather quickly (within 5-7 generations) in response to exposure to certain insecticides [26, 27]. This study aims to evaluate the activity of acetylcholinesterase in adults of the chlorfenapyr-resistant *M. domestica* strain. The study included the identification of AChE activity in females and males of two strains (selected and not selected by chlorfenapyr), as well as the assessment of the main kinetic parameters of AChE, data on which can be a prerequisite to understanding the mutational effects in resistant insects [28].

MATERIALS AND METHODS

The following chemical compounds and reagents were used: Propoxur (100.0%, PESTANAL®), EDTA (≥99.0%, BioUltra), PTU (N-Phenylthiourea, ≥98.0%), PMSF (Phenylmethylsulfonyl fluoride, >98.5%), DTE (1,4-Dithioerythritol, ≥99.0%), Triton X-100 (t-Octylphenoxypolyoxyethethanol, ≥100.0%), DTNB (5,5′-Dithiobis(2-nitrobenzoic acid), ≥98.0%), Acetylthiocholine iodide (≥98.0%) were obtained from Sigma-Aldrich (Germany); Folin-Ciocalteu's Reagent (PanReac, AppliChem, Italy); BSA (bovine serum albumin) (ZAO Diakon-DC, Russia); mono- and disubstituted sodium and potassium phosphates, sulfurous copper, sodium carbonate of AR grade (OOO AO REACHIM, Russia).

The objects of the study were laboratory adult specimens, 3-5 days old, unexposed to chlorfenapyr (Lab, average weight of a female 13.43±4.24 mg, male 8.64±2.32 mg), and chlorfenapyr-resistant specimens (ChlA, resistance ratio 19.4; average weight of a female 17.26±3.03 mg, male 9.66±2.09 mg) of housefly *Musca domestica* L, provided by the Laboratory of Veterinary Problems in Animal Husbandry [17].

Both fly strains were kept in boxes with a constant temperature of 27±1℃ and relative humidity of 50±5%.

Homogenates were prepared from each specimen of *M. domestica* manually at low temperatures with the addition of 0.1 M of phosphate buffer pH=7.6, containing 1 mM EDTA, 1 mM PTU, 1 mM PMSF, 1 mM DTE, 20% Triton X-100. The supernatant obtained after centrifugation (2 min, 12500 rpm) was used to determine AChE activity and protein concentration. Protein content was determined photometrically by the Lowry protein assay, using bovine serum albumin solutions to construct a calibration curve [29].

AChE activity determination was performed on 96-well microtitration plates (MiniMed, Russia) on a Multiskan FC microplate photometer (Thermo Fisher Scientific Inc., Finland) according to the Ellman method with minor modifications [30]. To assess the specific activity of the enzyme, 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 (2 mM acetylthiocholine iodide and 0.23 mM DTNB mixed just before the measurement). To account for the non-enzymatic hydrolysis of acetylthiocholine, 10 µl of potassium phosphate buffer (pH=7.0) was added to the reaction mixture instead of a homogenate. The substrate content in the reaction mixture when determining AChE activity to analyze kinetic parameters (Michaelis constant, Km and maximal velocity, Vmax) was 0.0625 mM, 0.125 mM, 0.25 mM, 0.5 mM, 1 mM, and 2 mM. Propoxur (0.1 M) was used to determine the inhibition rate (or remaining activity) of AChE. A solution of acetylthiocholine iodide was mixed with propoxur solution in a ratio of 30:1, and the resulting mixture was used to prepare Ellman's reagent. To determine the specific activity of AChE, the optical density was measured at 405 nm in kinetic mode for 30 minutes at 30°C. The absorbance in the case of determination of kinetic parameters and remaining enzymatic activity was measured at 405 nm in kinetic mode for 5 minutes with 15-second intervals at 30ºC. AChE activity was represented as ΔOD/min/mg of protein (change in optical density per minute per mg of protein) [30].

Kinetic parameters were determined by non-linear regression using Excel Solver software [31, 32]. Statistical analysis of the enzyme activity results was performed by one-way ANOVA test and Tukey's test for multiple comparisons using Statistica 13.3 software package (StatSoft, Russia). The significance level of $p \leq 0.05$ was used to consider the identified differences as statistically significant.

RESULTS AND DISCUSSION

According to the results of the statistical analysis of the obtained data, the specific AChE activities in homogenates, prepared from adult specimens of *M. domestica* of two strains (ChlA and Lab) were not significantly different **(Figure 1)**. The values of the enzyme activity in females and males of the ChlA strain were 20.9% ($p=0.879$) and 13.7% ($p=0.996$) lower respectively, compared to the specimens of the Lab strain. It was noticed **(Figure 2)** that the remaining AChE activity (in the reaction mixture with the inhibitor) was statistically significantly lower in Lab males than in Lab females by 3.61 times ($p=0.000021$, $p < 0.05$), whereas in ChlA specimens there was no statistically significant difference in this parameter depending on sex ($p=0.999$). The remaining enzyme activity of the ChlA females was 3.81 times lower ($p=0.000018$, $p < 0.05$) compared to the Lab females; the ChlA and Lab males showed no statistically significant difference in remaining AChE activity ($p=0.998$).

Table 1 represents the AChE kinetic parameters of the Lab and ChlA strains. The maximal velocity (or Vmax) during the initial reaction period in specimens of both strains had no statistically significant differences depending on sex. However, it should be noted that the Vmax value was 36.8% lower ($p=0.082$) in Lab males and 40.7% higher ($p=0.576$) in ChlA males than in females of the corresponding strain. At the same time, the Vmax value in females of the ChlA strain was 43.3% lower than in females of the Lab strain ($p=0.013$, $p < 0.05$).

The value of Michaelis constant (or Km) in males of Lab strain was 2.74 times lower ($p=0.014$, $p < 0.05$) than in females of the same strain, while in specimens of ChlA strain there were no statistically significant differences of Km value depending on sex. It can be noted that the Km value in females of the ChlA strain was 1.88 times lower ($p=0.132$) than in females of the Lab strain. The Vmax/Km ratio in males was 48.0%

(p=0.030) and 35.1% (p=0.105) higher than in females of Lab and ChlA strains, respectively **(Table 1)**.

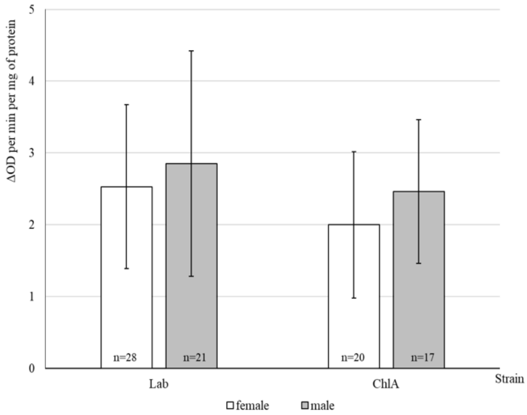

Figure 1. The specific activity of AChE in adults in the laboratory chlorfenapyr-selected (ChlA) and unselected (Lab) strains of *M. domestica* L. RR (resistance ratio) for the ChlA-strain is 19.4. Values are represented as M±SD.

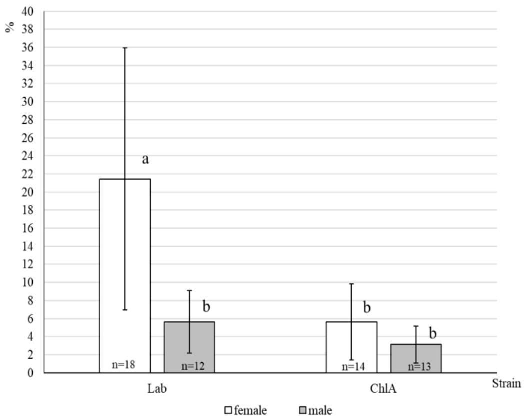

Figure 2. The remaining activity of AChE in adults in the laboratory chlorfenapyr-selected (ChlA) and unselected (Lab) strains of M. domestica L. RR (resistance ratio) for the ChlA-strain is 19.4. Values with the same letters do not differ significantly at p<0.05. Values are represented as M±SD.

Table 1. Kinetic parameters of AChE in adults of the laboratory chlorfenapyr-selected (ChlA) and unselected (Lab) strains of *M. domestica* (M±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 is targeted by insecticides of the OP and carbamate classes [5]. Changes in its activity, substrate specificity, and sensitivity to insecticides are important mechanisms of detoxification and resistance development [33]. Published studies show an increase in activity and a change in quality indicators of AChE in insects, resistant not only to OPs and carbamate classes [28] but also to pyrethroids [34, 35]. The possible involvement of this enzyme in the detoxification of insecticides through hydrolysis [13] and sequestration [14, 15] has been previously reported. This study focused on the activity and kinetic parameters of AChE in adults of *M. domestica* of Lab (chlorfenapyr-unselected) and ChlA (chlorfenapyr-selected) strains. Compared with the Lab strain, the ChlA strain was characterized by a resistance ratio (RR) to chlorfenapyr of 19.4. This RR value indicates a low [36] or medium [37] insect resistance to the insecticide.

According to the obtained results **(Figure 1)**, specimens of Lab and ChlA strains had no statistically significant differences in specific AChE activity, although a study by Nazar *et al*. (2020) demonstrated an increase in AChE activity in the highly chlorfenapyr-resistant mealybug strain *Phenacoccus solenopsis* Tinsley (Hemiptera: Pseudococcidae) [38]. Shabbir *et al*. (2021) reported that AChE genes were highly expressed in chlorantraniliprole-treated larvae of diamondback moth Plutella xylostella L. (Lepidoptera: Plutellidae) [8]. It is worth noting that high enzymatic activity does not always associate with resistance to individual insecticides. For example, in a study by Li *et al*. (2018), houseflies of *M. domestica* of the field population displayed an increased AChE activity, when compared to the specimens of the sensitive strain, and a high level of resistance to propoxur and cypermethrin, but were not resistant to chlorfenapyr [34]. In addition, it is evident from the published articles that in *M. domestica*, the development of insecticidal resistance can be accompanied by both activation [27] and inhibition of esterase systems [9].

In our study no statistically significant differences in remaining AChE activity between the groups of males of Lab and ChlA strains were found **(Figure 2)**, although the ChlA strain had a 47.3% decrease in this parameter relative to the Lab strain specimens. The low values of remaining activity of this enzyme in the presence of propoxur inhibitor in the incubation mixture, observed in females of the ChlA strain, may indicate an increase in its sensitivity to the inhibitor when compared with the enzyme of Lab females. The opposite change, which is a high residual activity due to decreased sensitivity of the enzyme to inhibitors in "in vitro" experiments, can be observed in insects with resistance to insecticides, that target AChE. For example, this was observed in the housefly *M. domestica* resistant to propoxur [28], and in the psyllidae *Agonoscena pistaciae* Burckhardt and Lauterer (Hemiptera: Psyllidae) resistant to fosalone (OP) [39]. High levels of remaining AChE activity (30-70%) were observed in field populations of the whitefly *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) resistant to OPs and neonicotinoids [40].

In addition to a decrease or an increase in the degree of inhibition, the qualitative changes in the enzyme molecule in insecticide-resistant insects can also be manifested through changes in its activity and affinity for specific substrates, which can be observed through kinetic parameters. Previously, the kinetic parameters of AChE were usually determined for insects, resistant to OPs and carbamates, the development of resistance to which is based on a mechanism of the decreased sensitivity of the target (i.e. AChE) to insecticides. For instance, Shi *et al*. (2002) reported changes in the affinity and rate of hydrolysis of three substrates by acetylcholinesterase of propoxur-resistant specimens of *M. domestica* strain: Km and Vmax values of AChE for acetylthiocholine (ATC) in adults of the resistant strain were higher than in specimens of the insecticide sensitive strain [28]. The above-mentioned study has concluded that in the propoxur-resistant strain there was a decrease in the affinity of the enzyme to insecticides and substrate, as well as a decrease in the catalytic efficiency of AChE against the specific substrate (ATC). Similar results were obtained in other studies, where authors investigated AChE in OPs- and carbamate-resistant insects [41-43]. We evaluated the kinetic parameters of AChE in the chlorfenapyr-resistant *M. domestica* strain for the first time. There was a 43.3% decrease in Vmax in females of the ChlA strain compared to this parameter in specimens of the Lab strain ($p < 0.05$), which may indicate a decrease in the enzyme catalytic activity against a specific substrate (ATC) in chlorfenapyr-resistant insects. Considering the decrease in value of Km (1.88-fold) and remaining AChE activity (3.81-fold) in females of the ChlA strain, compared with the parameters in females of the Lab strain, we can assume that AChE of the chlorfenapyr-resistant strain had a greater affinity of the enzyme for specific ligands (in this case, the ATC substrate and propoxur inhibitor) with no increase in catalytic activity against ATC. It is known that mutational changes in the genes that encode the enzyme, can change such enzyme characteristics as substrate specificity, affinity to the substrate, and kinetic parameters [44]; therefore, in our opinion, it would be useful to analyze the ACE gene sequence in specimens of the chlorfenapyr-resistant *M. domestica* strain in the future.

CONCLUSION

Overall, the results suggest that AChE in *M. domestica* does not contribute to the development of resistance to chlorfenapyr, since no increase in its activity was detected in specimens of the resistant ChlA strain. At the same time, in *M. domestica* females of the ChlA strain, where adults were exposed to sublethal exposure to chlorfenapyr for 23-24 generations, qualitative changes of AChE occurred in adults of the 24-25 generations, affecting the affinity of the enzyme for ligands. Further molecular studies of AChE in insects of this strain are necessary to analyze possible mutational changes and to provide a fuller characterization of the enzyme.

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