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# Interaction between Acetylcholinesterase and Choline Acetyltransferase: an Hypothesis to Explain Unusual Toxicological Responses*

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**Abstract:** Organophosphorus and carbamate insecticides are thought to have only one target site, acetylcholinesterase (EC 3.1.1.7). When this enzyme is inhibited, the neurotransmitter acetylcholine is not metabolized and polarization of the post-synaptic membrane does not take place. But, what happens when the cholinesterase becomes resistant or when neurotransmitter levels are diminished? Here, we report results suggesting that choline acetyltransferase (EC 2.3.1.6), the enzyme responsible for the acetylcholine production, may be involved either as an alternative pesticide target site or as a factor enhancing survival during insecticide exposure. This underlines the concept that the pivotal step for insecticide toxicology is not the acetylcholinesterase activity but the amount of acetylcholine present. This latter can only fluctuate between an upper and a lower threshold, and crossing one of these two thresholds leads to the death of the insect. The interaction between acetylcholinesterase and choline acetyltransferase activities would explain the astonishing toxicological phenomenon that, in some conditions, mortality decreases when insecticide concentration increases.

Key words: acetylcholinesterase, choline acetyltransferase, insecticide resistance, *Culex pipiens*

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## 1 INTRODUCTION

Acetylcholinesterase (AChE; acetylcholine acetylhydrolase, EC 3.1.1.7) and choline acetyltransferase (ChAT, acetyl-CoA-choline-O-acetyltransferase, EC 2.3.1.6) are two key enzymes of the cholinergic system because they regulate the level of acetylcholine (ACh), the primary sensory neurotransmitter in arthropods.[1,2] ChAT is implicated as a regulatory step for ACh production[3] whereas AChE terminates nerve impulses by catalysing the hydrolysis of ACh. Of the two enzymes, only AChE has been considered for the development of inhibitors as insecticides. Thus, organophosphorus and carbamate insecticides have properties analogous to ACh but are hemisubstrates: they quasi-irreversibly inhibit AChE by phosphorylating or carbamylating the active-site serine.[4] AChE inhibition leads to an accumulation of ACh in the synapses which, in turn, leaves the ACh receptors permanently open, resulting in the death of the insect.[5,6]

Insecticide resistance would arise through the selection of any mechanisms which balance ACh accumulation. One of them is an increase in ACh degradation. It corresponds either to an overproduction of the enzyme or to a decrease of AChE inhibition due to the appearance and selection of altered AChEs less sensitive to the insecticide.[7] Overproduction has not been studied in resistant populations due to the difficulty in estimating the AChE content in insects. However, modification of AChE amounts in *Drosophila* has been correlated to insecticide sensitivity.[8] Concerning AChE modification, some point mutations have been reported in the fruit fly,[9,10] housefly (Williamson and Devonshire, pers. comm.) and Colorado potato beetle.[11]

A second resistance mechanism would be a decrease in ACh production because of a reduced ChAT activity. Surprisingly, this second resistance mechanism has not yet been reported.

In this paper we report results suggesting the influence of modifications of ChAT activity on pesticide resistance, showing how ChAT modification may provide insecticide resistance, and that ChAT could be a pesticide target-site or a modifier of pesticide resistance.

## 2 EXPERIMENTAL METHODS

### 2.1 Mosquito strains

Three strains of the mosquito *Culex pipiens* L. were used as follows: *S-Lab*, a susceptible homozygous reference strain,[12] and two strains resistant to OP and carbamate insecticides, homozygous for an insensitive acetylcholinesterase: *MSE*, collected in 1979 from southern France[13,14] and *Ace-R*, collected in 1993 from Cyprus.[15]

*C. pipiens* possesses two acetylcholinesterases, AChE1 and AChE2, which are thought to be produced by distinct genes, *Ace.1* and *Ace.2*.[16,17] Only *Ace.1* is involved in insecticide resistance and two types of allele can be distinguished: $Ace.1^S$ and $Ace.1^R$, coding for sensitive and insensitive AChE1s, respectively.[18] The susceptible strain *S-Lab* possesses only sensitive AChE1 enzyme (and is thus homozygous $Ace.1^{SS}$) and the resistant strains (*MSE* and *Ace-R*) possess only insensitive AChE1 (and are homozygous $Ace.1^{RR}$). To obtain heterozygous individuals, resistant males of each strain were crossed with *S-Lab* females. Offspring were designated as *MSE-F1* or *AceR-F1* depending on the resistant strain used as the male parent.

### 2.2 Drosophila strains

Three strains of *Drosophila melanogaster* Meig. were used, of which one was a standard susceptible strain (*Canton-S*). The other strains ($Cha^{tS1}$ and $Cha^{tS2}$) displayed temperature-sensitive ChAT alleles, expressing low choline acetyltransferase activities. They were obtained by chemical mutagenesis of the wild-type strain *Canton-S*.[19] All strains were reared on standard medium at 20°C; the restrictive temperature for these heat-sensitive strains is 25°C.

### 2.3 Insecticide bioassays

For mosquito strains (and their F1 progeny), resistance characteristics were analysed by bioassays performed on fourth instars as described by Raymond and Marquine[20] using the insecticide propoxur (95% pure), which is soluble in water at all concentrations employed (Bayer, Leverkusen, Germany). In all bioassays, larvae were exposed to the insecticide for 24 h, and the final concentration of alcohol was systematically adjusted to 10 ml litre$^{-1}$. Each bioassay cup held 20 larvae in aqueous propoxur (100 ml) and three replicates were done for each insecticide concentration tested. A control, where larvae experienced the same environmental conditions except for the presence of the insecticide, was run in each experiment. For *D. melanogaster* strains, $LD_{50}$ values were determined for the carbamate propoxur and the organophosphate parathion by tarsal contact at 20°C. Treatments were performed by putting 10 females in a glass bottle (30 ml capacity, 50 cm$^2$ internal surface) that had previously been soaked with an acetone solution of insecticide (100 $\mu$l). At least five concentrations of the insecticides were used, giving between 0 and 100% mortality. Mortality was recorded 24 h after treatment and $LD_{50}$ values were determined by fitting dose/mortality data to sigmoid curves by non-linear regression using the Prism program.

### 2.4 ChAT and AChE activity in mosquitoes

AChE activity (i.e. AChE1 plus AChE2 activity) was evaluated using acetylthiocholine iodide as a substrate according to Ellman *et al.*[21] Residual activities were recorded after 24 h exposure to propoxur. For the three mosquito strains, *S-Lab*, *MSE* and *AceR*, the three replicates of each dose (i.e. 60 larvae, dead or alive) were pooled, washed with water and rapidly mass-homogenized in sodium phosphate buffer (0·1 M, pH 7·0; 1·5 ml) containing 'Triton' *X*-100 (10 g litre$^{-1}$), using a glass pestle. Dead larvae did not appear until after 10 h treatment, so that no AChE degradation (by proteases) can occur before activity measurements since this enzyme is very stable over time.[22] Homogenates were centrifuged (10 000*g* for 5 min), and mosquito homogenate (100 $\mu$l) was added to the substrate-reagent solution (final concentrations: 5,5′-dithiobis-2-nitrobenzoic acid 1·7 mM; acetylthiocholine 3 mM). AChE activity was measured at 412 nm over a period of 1 min using a spectrophotometer (Kontron-Uvikon 930). Assay conditions were established so as to ensure that the rates of enzymatic reaction were linear during the recording period. ChAT activity was recorded as described by Chireux *et al.*[23] in the absence and presence (during 15 min) of three different propoxur concentrations: 20, 200 and 2000 mg litre$^{-1}$. Activity was measured respectively for 1, 2 and 10 adult mosquito heads of *MSE* strain homogenized in sodium chloride (0·2 M; 1 ml) containing 'Triton' X-100 (2 g litre$^{-1}$). Activity was measured after 15 min.

## 3 RESULTS

### 3.1 Reduced ChAT activity: an insecticide resistance mechanism

Each of the two *Drosophila* strains $Cha^{tS1}$ and $Cha^{tS2}$ possesses a distinct ChAT structural gene mutant, resulting in a low level of acetylcholine in the central nervous system.[3] Dose–mortality curves for propoxur and parathion are shown in Fig. 1 along with the curve obtained for the wild-type *Canton-S*. Temperature-sensitive strains have a two-fold resistance level compared to the reference strain. The resistance ratio was identical for propoxur (a carbamate) and parathion (an organophosphate), which have the same target. Although we cannot exclude the involvement of another gene(s) present in $Cha^{tS}$ mutants in producing the same resistance ratio, this suggests that the resistance observed originates from the decrease of neurotransmitter in the central nervous system which renders the inhibition of AChE by the insecticide less drastic. The two ChAT mutations do not significantly affect neurotransmission at permissive temperature when AChE is not inhibited,[19] but when neurotransmission is affected by a partial AChE inhibition, a decrease in neurotransmitter synthesis may result in restoring an efficient neurotransmission.

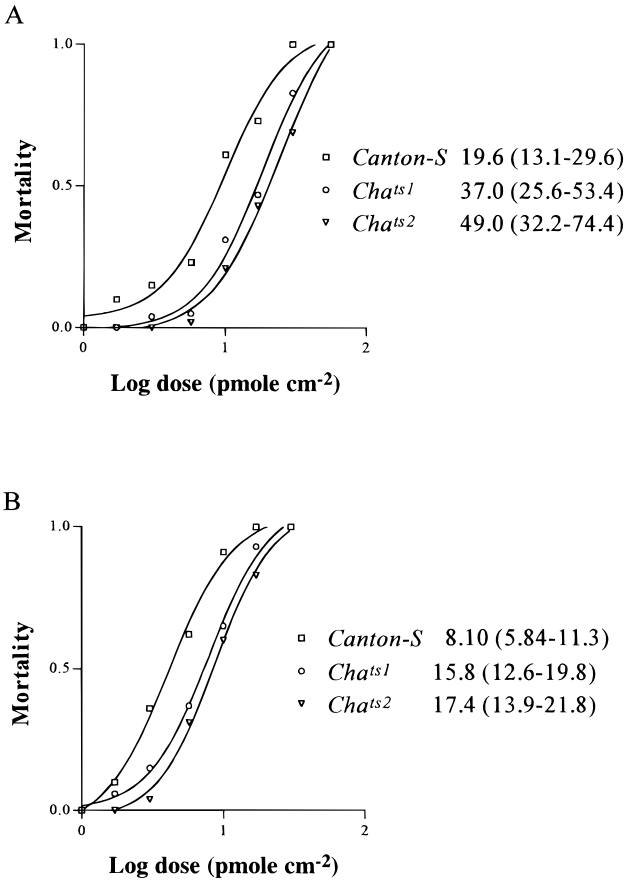



**Fig. 1.** Dose mortality curves obtained for the wild type *Drosophila melanogaster Canton-S* and for the two mutants $Cha^{tS1}$ and $Cha^{tS2}$ using (A) propoxur or (B) parathion as insecticide. $LD_{50}$ (95% confidence level) is indicated for each strain.

### 3.2 ChAT: an insecticide target

In the mosquito *C. pipiens*, several insensitive AChE1 have been described.[13,14,24,25] Using strains with different AChE sensitivities to propoxur, we studied the remaining enzymatic activity following in-vivo insecticide treatment at concentrations giving from 0 to 100% mortality. For *S-Lab* (a susceptible strain) and *AceR* (with a $\approx$200-fold less sensitive AChE1)[25] mortality levels were consistent with AChE inhibition (Figs 2A and 2B). By contrast, for *MSE* (with a $\approx$300 000-fold less sensitive AChE1),[25] AChE activity was unaffected by the pre-treatment, even at doses of insecticide giving 100% mortality (Fig. 2C). We cannot exclude that AChE activity recovered after extraction. However, the non-inhibition of the AChE enzyme is consistent with the fact that propoxur concentrations giving 100% mortality are far below the concentration needed to inhibit this insensitive AChE1. Thus this result suggests that mortality of *MSE* larvae is not due to AChE1 inhibition but to the interaction with another target-site.

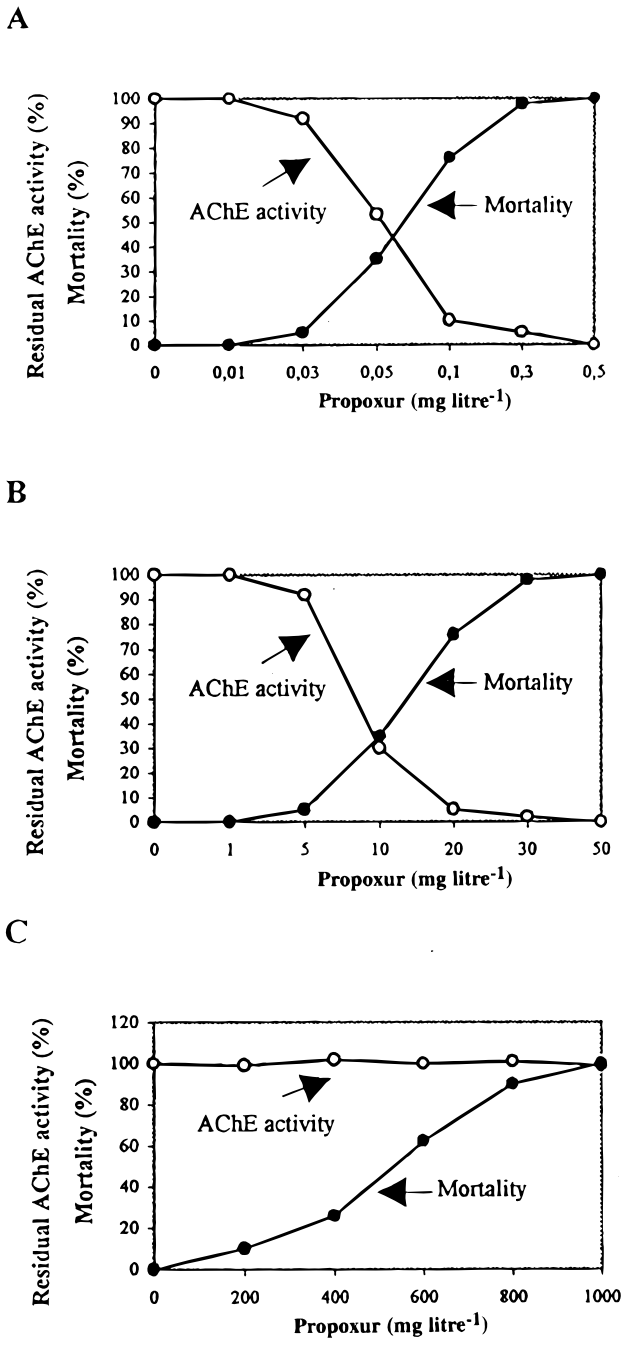



**Fig. 2.** Residual AChE activities and mortality rate of the three mosquito strains. (A) *S-lab*. (B) *AceR* and (C) *MSE*.

Mortality characteristics provide helpful insights on the second target, as the mortality pattern of *MSE* larvae is different from those of *S-Lab* and *AceR* larvae. These two latter undergo violent convulsions, become tetanized and die ($ACh^+$ mortality). To verify this hypothesis, we used nicotine, an ACh agonist which 'mimics' ACh accumulation. In the presence of lethal doses of nicotine, all mosquitoes (i.e. from *S-Lab*, *AceR* and *MSE*) showed an $ACh^+$ mortality. Conversely, when propoxur was used as insecticide, *MSE*-resistant mosquitoes did not display convulsion or tetanization: instead they stayed at the surface without moving and progressively shrivelled up and died. We conclude that the inhibition of the second target induced mortality through a physiological process different from ACh accumulation. These symptoms are tentatively assigned to a defect in neurotransmission arising from a lack of acetylcholine in the synapse ($ACh^-$ mortality), because they resemble the behavioural abnormality of paralysis shown by *D. melanogaster* strains with reduced ChAT activity.[19]

Thus, ChAT is a good candidate for the second target, taking in account that some anti-cholinesterase compounds have already been found to inhibit the ChAT enzyme. For example, the anti-cancer drug caracemide, *N*-acetyl-*N*,*O*-di(methylcarbamoyl)hydroxylamine, has been found to inhibit both AChE by carbamylation of the active site serine[26] and ChAT by competition with the substrate acetyl-CoA.[27] The ethylcholine mustard, aziridium (AF64A), is also a co-inhibitor of the two enzymes.[28] To verify this hypothesis, we tested *in vitro* the inhibition of ChAT activity by propoxur (Fig. 3). At doses which induced mortality in the *MSE* strain (Fig. 2C), we observed an inhibition of the ChAT activity by propoxur. Apparently, this inhibition cannot account solely for propoxur mortality in *MSE*-resistant individuals since in-vitro inhibition was not complete, and *D. melanogaster* mutants were still viable with 8% of wild type activity.[19] However, inhibition took place only during 15 min (see Section 2.4) so that the actual in-vivo inhibition may be greater than the observed in-vitro inhibition. More generally this result shows that insecticides analogous to the neurotransmitter acetylcholine may also interact with proteins responsible for the acetylcholine release in the synapse, and hence reduce the neurotransmitter concentration in the synapse. When acetylcholinesterase is not affected by the insecticide, reduction may become lethal.

Therefore, we tentatively concluded that for *S-Lab* and *AceR* mosquitoes, inhibition of their AChE1 induces an increase of ACh at the synapse leading to the $ACh^+$ mortality. Conversely, for *MSE* insects, the important doses of propoxur probably inhibit ChAT and thus ACh synthesis. The resulting lack of ACh release would induce $ACh^-$ mortality.

### 3.3 ChAT: an insecticide resistance modifier

In *MSE-F1* mosquitoes (i.e. heterozygous individuals possessing both sensitive and insensitive AChEs) a complex toxicological phenomenon is observed: within a concentration range of propoxur, mortality decreases when insecticide concentration increases (Fig. 4). This is not an artefact. First, the decrease in mortality is observed systematically within a wide range of insecticide concentrations (10 to 300 mg litre$^{-1}$), using distinct insecticide stock solutions and in different laboratories.

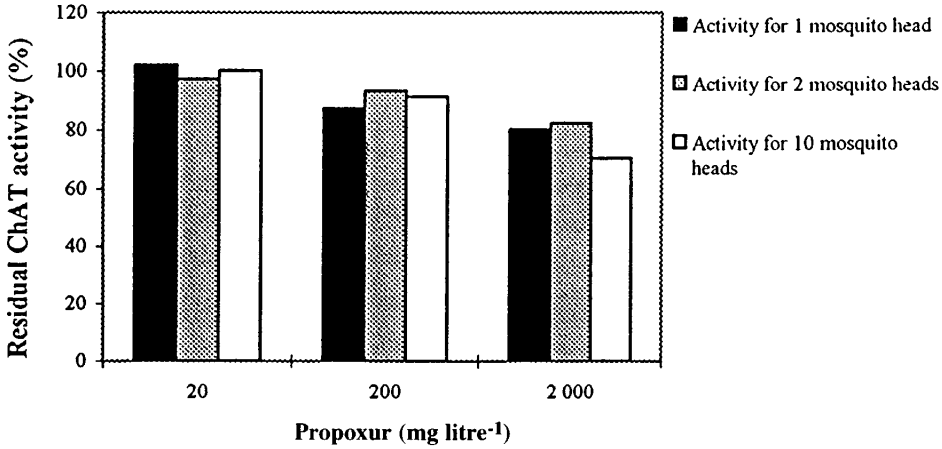



**Fig. 3.** In-vitro ChAT activity of the *MSE* mosquito strain in the presence of different propoxur concentrations.

Second, this puzzling phenomenon has been observed repeatedly since 1985 and was found for three resistant strains possessing the same insensitive allele but different genetic backgrounds (data not shown). Third, a decrease in mortality when propoxur doses increase has also been observed in field samples, where heterozygous mosquitoes are at relatively high frequencies.[20]

This phenomenon is unusual and has been described, as far as we know, only twice in the literature: in *Macrosporium sarcinaeforme* Cav. resistant to the fungicide tetramethylthiuram disulphide (Dimond *et al.*, 1941, in Finney[29]) and in *Venturia inequalis* (Cooke) Wint. resistant to thiuram sulfides.[30] For the first case, the decrease in resistance with increasing doses was explained by the dose-dependent dissociation of the fungicide into molecules of higher toxicity.[29,30] This explanation does not apply for the present observations, as dissociation of propoxur into more toxic compounds is unlikely (Fukuto, pers. comm.).

This unusual phenomenon could be explained by the inhibition of the ChAT as previously described for *MSE*. *MSE-F1* heterozygotes possess both sensitive and insensitive AChE1 enzymes. When propoxur doses increase, there is a progressive inhibition of the sensitive fraction until only the insensitive AChE1 remains. This insensitive fraction alone is not sufficient to decrease the

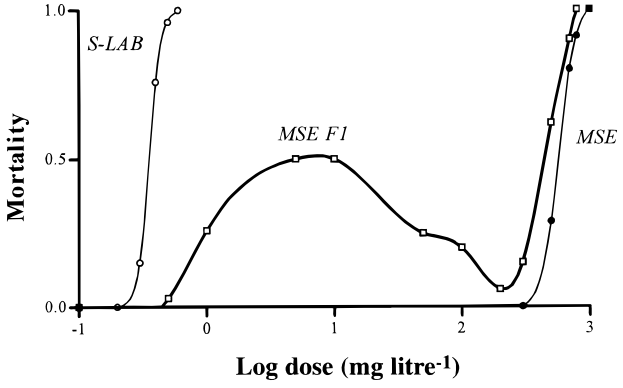



**Fig. 4.** Mortality curves of *MSE*, *S-lab* and *MSE-F1* mosquitoes, using propoxur.

ACh accumulation and $ACh^+$ mortality is observed. Indeed, resistant AChE1 is altered and has only 21% of wild type activity at 1 mM of substrate.[13] Thus, in heterozygotes, when the susceptible counterpart is inhibited, the remaining AChE1 activity is near the minimum for viability. At higher doses, propoxur starts to inhibit the ChAT (see above). The resulting decrease in ACh synthesis could balance the previous ACh accumulation and explain the observed decrease in mortality. When propoxur doses continue to increase, $ACh^-$ mortality appears to be due probably to the complete inhibition of the ChAT.

Conversely, *AceR-F1* heterozygote mortality curves do not show any decrease when propoxur concentrations increase. This is because both sensitive and insensitive AChE1 are inhibited at lower propoxur concentrations than the ChAT so that mortality is always the result of ACh accumulation. As expected, for *AceR-F1*, only $ACh^+$ mortality is observed.

## 4 DISCUSSION

We have taken advantage of mutant ChAT flies and of mosquitoes with a fully insensitive AChE1 to estimate the influence of ChAT and the interaction between AChE and ChAT on insecticide resistance. Our results suggest that a reduction of choline acetyltransferase is a potential resistance mechanism (as shown in mutant *D. melanogaster*), and that ChAT represents also a pesticide target-site (as for example in mosquitoes with insensitive AChE1) or a modifier of insecticide resistance (in heterozygotes, where it probably induces a mortality decrease when insecticide concentration increases).

Resistance to insecticides results from three main physiological mechanisms: reduced penetration, increased detoxification or reduced target sensitivity.[31] Here, we have found another potential mechanism which has not been described as yet, namely the underproduction of the target's substrate (here through a

reduced ChAT activity). This modification is a resistance mechanism when excess of the target's substrate is lethal, as it is the case for the AChE target. This situation confirms that the only relevant effect of organophosphate and carbamate toxicology is on the neurotransmitter level, which can be decreased either by over-production of acetylcholinesterase,[8] or by under-production of ChAT (this study), the two changes resulting in insecticide resistance. Apparently, this mechanism confers only a low resistance level in *D. melanogaster*, but in the presence of other resistance mechanisms, it may contribute additively or multiplicatively to a higher resistance level.[32] Since there is no direct binding between the insecticide and the ChAT, the resistance mechanism of reduced ChAT activity could potentially exist for all carbamates and organophosphates (i.e. to approximately half of insecticides used currently in the world).

Toxicological studies state that insecticide toxicity is due to the action on only one major target-site, either the GABA receptor, acetylcholinesterase or sodium channel for most common insecticides.[31] Thus OPs and carbamates act on acetylcholinesterase and owe their toxicity to their effects on this enzyme. This was indicated by the correlation between thoracic AChE inhibition and poisoning,[8] and the link between AChE modification and insecticide resistance.[33] However, *in vitro*, insecticides may affect several other interacting target-sites.[5,6] Since these targets are less sensitive to insecticides, they are normally not relevant to explain insect mortality. But when the main target becomes less sensitive, higher insecticide concentrations are required and secondary targets may be involved. Here, we show that, *in vivo*, ChAT may be an alternative insecticide target-site for at least a carbamate insecticide (propoxur).

Organophosphorus and carbamate insecticides are substrate analogues and modifications giving less sensitive AChE usually result in a partial decrease in substrate metabolization. This alteration in acetylcholine metabolization may be quite important. For example, the *MSE* strain used in this study presents only 21% of the AChE1 wild type activity at the apparent Vm.[13] This remaining AChE1 activity is sufficient for life under laboratory conditions and may even be decreased to much lower levels, as shown by the residual activity following insecticide treatments[34] or rescue experiments with an injected minigene in *D. melanogaster*.[35] However, in natural conditions, a fitness cost seems to be associated with the mosquito's insensitive allele.[36] It appears that ChAT under-production is a good candidate to decrease the deleterious effect on fitness produced by the AChE1 alteration. Such a balancing effect by mutations of an enzyme that restore the steady state level disturbed by a mutation in another enzyme has already been described. For example, in *Escherichia coli* Castell & Chalm., *top A* (the gene coding the topoisomerase I) mutants are viable only if they acquire compensating mutations that reduce the level of gyrase.[37] In the case of AChE and ChAT enzymes, each alteration compensates the physiological effect of the other. ChAT mutations may thus also be considered as a potential modifier that allows maintenance of altered mutations in untreated areas.

Under-production of an enzyme such as ChAT must be quite frequent; many mutations may result in a decreased activity, *via*, for example, under-transcription or inefficient folding. But we may expect insecticide treatments to select another kind of mutation: a modified ChAT with a higher sensitivity to insecticides. In insects harbouring a wild-type AChE, pest control would favour a ChAT more sensitive to insecticides because the co-inhibition of the two enzymes would confer insecticide resistance. On the other hand, when insects display an insensitive AChE, insecticide treatments would select for a less sensitive ChAT enzyme. These ChAT modifications may arise by point mutations modifying the active site, such as those previously described for AChE.[9] In AChE only a few amino-acid changes render the AChE less sensitive while retaining enough enzyme activity for an efficient neurotransmitter metabolization. The same constraint would apply for ChAT modifications: whatever the mutations, the enzyme activity should remain in sufficient amount to maintain the ACh above the lower threshold. We therefore predict that studying in detail the ChAT enzyme or gene will lead to better understanding of the evolution of insecticide resistance in natural populations.

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