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Efficacy of insecticide mixtures against larvae of *Culex quinquefasciatus* (Say) (Diptera: Culicidae) resistant to pyrethroids and carbamates

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Abstract: The efficacy of insecticide mixtures of permethrin (pyrethroid) and propoxur (carbamate) was tested by larval bioassays on two strains of *Culex quinquefasciatus* (Say), one resistant to pyrethroids and the other resistant to carbamates. The method consisted in combining one insecticide at the highest concentration causing no mortality (LC_0) with increasing concentrations of the second one. The concentration–mortality regression lines were determined for permethrin and propoxur alone and in combination, and synergism ratios (SR) were calculated in order to determine the magnitude of an increase or decrease in efficacy with use of the mixtures. With the pyrethroid-resistant strain (BK-PER), the results showed that propoxur at LC_0 significantly enhanced the insecticidal activity of permethrin ($SR_{50} = 1.54$), especially on the upper range of the concentration–mortality regression. Conversely, when permethrin at LC_0 was tested with propoxur against the carbamate resistant strain (R-LAB), an antagonistic effect was observed ($SR_{50} = 0.67$). With the BK-PER strain, an increased oxidative detoxification (MFO) appeared to be the main mechanism responsible for the synergistic interaction. Nevertheless, antagonism in the R-LAB strain is probably due to a physiological perturbation implying different target sites for pyrethroid (ie sodium channel) and carbamate insecticides [ie acetylcholinesterase (EC 3.3.3.7) and choline acetyltransferase (EC 2.3.1.6)].

Keywords: *Culex quinquefasciatus* larvae; insecticide mixture; resistance mechanism; synergism; antagonism; acetylcholinesterase; choline acetyltransferase

1 INTRODUCTION

In 2001, resistance to insecticides concerned 540 species of arthropod, of which 198 were of medical and veterinary importance.[1] This was all the more worrying as insecticides have, for a long time, played a major role in the control of pests and insects, as well as of vectors of diseases. For example in West Africa, resistance to pyrethroids is widespread in *Anopheles gambiae* spp,[2] a major malaria vector in Sub-Saharan Africa and in *Culex quinquefasciatus* (Say),[3] the main nuisance mosquito in urban environments. Resistance to organophosphate compounds has developed in many species of mosquitoes of the genera *Culex*[4] and *Anopheles*.[5] Resistance to carbamates has been noted in *C quinquefasciatus*[6] and, more recently, in two populations of *A gambiae* from Côte d'Ivoire.[7]

Given that there are few alternative insecticides in public health coming on-stream, the main concern in resistance management strategies for vector species consists in making a judicious use of the compounds already available. The use of mixtures or recourse to a strategy of rotation over time of insecticides with different modes of action has already made it possible to prevent or to delay the appearance of resistance in the field.[8–10] However, mixtures of appropriate dosages of unrelated compounds may have better prospects for managing resistance effectively than rotations of the types of compounds.[11–13] This strategy is based on the fact that, if the probability for resistance

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to one of the two insecticides is a rare and independent event, then the probability that resistance will occur simultaneously to both insecticides of the mixture is extremely low.[14] The advantage of mixtures is that each insecticide eliminates most insects which are genetically susceptible to it.[13]

However, the toxicological risks for humans, as well as the cost involved in the use of several insecticides at operational dosage, are major concerns, unless the combined effect of the mixture is significantly stronger than the sum of the single effects (synergism effect). Such a phenomenon may increase the efficacy of treatment while reducing substantially cost and toxicity, because of a reduction of insecticide amounts. Many authors have already demonstrated the synergistic effect on insect pests of carbamates (or organophosphates) and pyrethroids.[15–17] With insects of medical importance, a synergistic effect between pyrethroids and carbamates was reported on larvae of *C quinquefasciatus*[18] and adults of *A gambiae*[19] susceptible to these insecticides. Given the development of resistance in most mosquito species, we investigated the interaction between a pyrethroid and a carbamate on larval stages of two *C quinquefasciatus* strains, one resistant to pyrethroids and the other resistant to carbamates.

2 MATERIAL AND METHODS

2.1 Insects

Pyrethroid- (BK-PER) and carbamate- (R-LAB) resistant strains of *C quinquefasciatus* were used for bioassays. The BK-PER strain originated from Côte d'Ivoire and was maintained under constant selection pressure of permethrin. This strain is homozygous for the *Kdr* mutation[20] and also exhibits an increased metabolic detoxification through the cytochrome P450-dependant monooxygenases.[3] The R-LAB strain is resistant to carbamates and organophosphates, although it remains fully susceptible to pyrethroids and DDT. The R-LAB strain is homozygous for an insensitive acetylcholinesterase with a genetic background identical to the susceptible reference strain S-LAB.[21] Mosquitoes were maintained by standard methods in an insectary at 27 (±2) °C and 80 (±10)% relative humidity.

2.2 Insecticides

The bioassays were carried out using technical-grade permethrin (pyrethroid insecticide) and propoxur (carbamate insecticide). Permethrin (*cis/trans* isomeric ratio 25/75: 94.4%) and propoxur (99.6%) were obtained from Agrevo (Berkhamsted, UK) and Bayer (Leverkusen, Germany), respectively. Each insecticide was prepared in absolute ethanol and stored at 4 °C throughout the experimentation.

2.3 Larval bioassay procedure

The larval bioassays were performed using a standard protocol described by the World Health Organization.[22] Each bioassay was repeated three times using late third- and early fourth-instar larvae of BK-PER and R-LAB *C quinquefasciatus*. For each bioassay, 20 larvae of each strain were transferred to cups containing 99 ml of distilled water. For each bioassay, we used five cups per concentration (100 larvae) and five to eight concentrations of each insecticide in a range that causes 0 to 100% mortality. One millilitre of each insecticide, at the desired concentration, was added to the cups. Control treatments of 1 ml of ethanol were performed for each test. Each bioassay was maintained at 27 (±1) °C throughout all tests. Larval mortality was recorded after 24 h of exposure, corrected by the formula of Abbott[23] if necessary, and data were analysed by the log-probit method of Finney,[24] using the Probit software (Praxème) programmed by Raymond *et al*.[25] This software uses the iterative method of maximum likelihood to fit a regression between the logarithm of concentration and the probit of mortality. The goodness-of-fit is estimated by a weighted chi-squared test. It also estimates the lethal concentrations and the slope of the regression lines with their confidence intervals ($P = 0.05$).

2.4 Synergism study

The effect of permethrin and propoxur in binary combination was evaluated using late third- and early fourth-instar larvae of BK-PER and R-LAB *C quinquefasciatus*. In preliminary bioassays, the highest concentrations of permethrin and propoxur which produced no mortality (LC_0) were determined for each of the two strains of *C quinquefasciatus*. Each of the resistant strains of mosquitoes was exposed to the LC_0 permethrin and propoxur individually (positive control) and in combination with increasing levels of propoxur and permethrin, respectively. A log-probit analysis was performed for each insecticide individually and in combination, and their slopes were compared using a chi-squared parallelism test. Synergism ratios (SR) were calculated in order to determine the magnitude of increase or decrease of efficacy occurring with the permethrin and propoxur combinations. Synergism ratios were calculated as follows:

$$SR = \frac{LC_{insecticide1} \text{ without } LC_{0insecticide2}}{LC_{insecticide1} \text{ with } LC_{0insecticide2}}$$

Synergism ratios as well as their confidence intervals were given by the log-probit analysis software programmed by Raymond *et al*.[25] A SR significantly higher than 1 (ie confidence interval of SR did not include the value 1) indicated a synergistic effect, whereas a SR significantly lower than 1 indicated an antagonistic effect.

3 RESULTS

3.1 Pyrethroid resistant strain (BK-PER)

3.1.1 Toxicity of permethrin and propoxur alone

The relationships between log-concentration and probit-mortality with permethrin ($\chi^2 = 5.85, df = 5$)

and propoxur ($\chi^2 = 4.41, df = 5$) were statistically fitted by straight lines ($P > 0.05$) and mortality never exceeded 5% in the control. The slope of the regression line with permethrin [1.29 (±0.14)] confirmed the polyfactorial nature of resistance in the BK-PER strain. The LC_{50} and LC_{95} values of permethrin, 0.40 and 7.53 mg litre^{-1}, respectively (Table 1), also confirmed its high resistance level to pyrethroids. There was a resistance factor (RF) of >300 compared with the susceptible reference strain of *C quinquefasciatus*.[18] The slope of the regression line with propoxur [4.15 (±0.37)] was steeper than that for permethrin. The LC_{50} and LC_{95} values were 0.38 and 0.94 mg litre^{-1}, respectively (RF = 4).

3.1.2 *Toxicity of permethrin and propoxur in combination*

For propoxur, the LC_0 value was 0.1 mg litre^{-1} with the BK-PER strain. When this LC_0 was combined with increasing concentrations of permethrin, the slope for the mixture [1.82 (±0.15)] had increased significantly compared with that for permethrin alone [1.29 (±0.14)] (χ^2 parallelism test = 22.3, $df = 11$, $P = 0.02$). These results indicated that the heterogeneity of larval response to the mixture was slightly lower than to permethrin alone. The LC_{50} of the mixture (0.26 mg litre^{-1}) was approximately two fold less than that for permethrin (LC_{50} = 0.40 mg litre^{-1}). Significant synergism ratios started to appear at LC_{30} and increased with increasing permethrin concentrations (Table 1).

The LC_0 value for permethrin was 0.01 mg litre^{-1}. When this LC_0 was combined with increasing concentrations of propoxur, the slope for the mixture [3.51 (±0.19)] was not changed from that for propoxur alone [4.15 (±0.37)] (χ^2 parallelism test = 9.62, $df = 9$, $P = 0.38$). The LC_{50} of the mixture (0.35 mg litre^{-1}) was not significantly different from that of propoxur (0.38 mg litre^{-1}), indicating that there was no synergism with this combination.

Table 1. Efficacy of permethrin with and without propoxur at LC_0 against a pyrethroid-resistant strain of *Culex quinquefasciatus* (BK-PER)

	Permethrin (mg litre^{-1})		
Lethal concentration	Without propoxur (CI 95%)	With LC_0 propoxur (CI 95%)	Synergism ratio (CI 95%)
10	0.041 (0.029–0.054)	0.051 (0.042–0.060)	0.80 (0.068–0.94)
20	0.090 (0.070–0.112)	0.090 (0.078–0.102)	1.00[a] (0.88–1.13)
30	0.16 (0.13–0.19)	0.13 (0.12–0.15)	1.18 (1.07–1.30)
40	0.26 (0.22–0.29)	0.19 (0.17–0.21)	1.35 (1.25–1.47)
50	0.40 (0.37–0.45)	0.26 (0.24–0.28)	1.54 (1.44–1.65)
60	0.63 (0.57–0.70)	0.36 (0.33–0.39)	1.75 (1.65–1.87)
70	1.02 (0.92–1.15)	0.51 (0.47–0.55)	2.02 (1.89–2.17)
80	1.80 (1.57–2.11)	0.76 (0.68–0.85)	2.38 (2.18–2.60)
90	3.94 (3.25–4.97)	1.32 (1.15–1.54)	2.98 (2.65–3.37)
95	7.53 (5.88–10.16)	2.09 (1.78–2.54)	3.60 (3.09–4.19)

[a] Not significantly different from 1 (confidence interval of SR includes the value 1).

3.2 Carbamate resistant strain (R-LAB)

3.2.1 *Toxicity of permethrin and propoxur alone*

The relationships between log-concentration and probit-mortality with permethrin ($\chi^2 = 8.82, df = 6$) and propoxur ($\chi^2 = 7.07, df = 6$) were well fitted by straight lines ($P > 0.05$) and mortality never exceeded 5% in the control batches. The slopes of the regression lines of propoxur [9.8 (±0.96)] and permethrin [8.4 (±0.90)] were steep, indicating a strong homogeneity of the mosquitoes with respect to the toxic effect of the two insecticides.

The high LC_{50} and LC_{95} values of propoxur (180 and 266 mg litre^{-1}, respectively) confirmed the high level of resistance of this strain to carbamates, which is 1900 times that of the susceptible reference strain of *C quinquefasciatus* (S-LAB).[18]

Conversely, the R-LAB strain displayed a great susceptibility to permethrin. The LC_{50} and LC_{95} values (1.2×10^{-3} and 1.9×10^{-3} mg litre^{-1}, respectively) were comparable with those for the susceptible reference strain S-LAB; LC_{50} and LC_{95} values for the latter, were 1.5×10^{-3} and 2.5×10^{-3} mg litre^{-1}, respectively.[18]

3.2.2 *Toxicity of permethrin and propoxur in combination*

The LC_0 value of permethrin was 4×10^{-4} mg litre^{-1}. A significant antagonistic effect appeared when this LC_0 was combined with increasing concentrations of propoxur. The LC_{50} of propoxur increased from 180 mg litre^{-1}, when used alone, to 269 mg litre^{-1}, when used in combination with permethrin at LC_{50} (Table 2). Because both regression lines were parallel (χ^2 parallelism test = 13.2, $df = 8$, $P = 0.10$), the synergism ratios were statistically the same for all the lethal concentrations (SR = 0.67).

The LC_0 value of propoxur was 80 mg litre^{-1}. When this LC_0 was combined with increasing concentrations of permethrin, the slope for the mixture [8.4 (±0.90)] did not significantly change compared with that for permethrin alone [9.2 (±0.99)] (χ^2 parallelism test = 19.0, $df = 11$, $P = 0.06$). In addition, there was no significant difference between the LC_{50} values of permethrin alone and permethrin mixed with propoxur (1.2×10^{-3} mg litre^{-1} for each).

Table 2. Efficacy of propoxur with and without permethrin at LC_0 against a carbamate resistant strain of *Culex quinquefasciatus* (R-LAB)

	Propoxur (mg litre^{-1})		
Lethal concentration	Without permethrin (CI 95%)	With LC_0 permethrin (CI 95%)	Synergism ratio (CI 95%)
10	133.4 (128.7–137.5)	202.2 (191.4–211.1)	[a] —
20	148.0 (144.2–151.3)	223.1 (214.1–230.6)	[a] —
30	159.5 (156.3–162.4)	239.5 (231.9–245.9)	[a] —
40	170.0 (167.2–172.7)	254.4 (248.1–260.0)	[a] —
50	180.5 (177.6–183.3)	269.2 (263.7–274.4)	0.67 (0.61–0.74)
60	191.5 (188.3–194.9)	284.9 (279.6–290.3)	[a] —
70	204.1 (200.2–208.6)	302.6 (296.7–309.4)	[a] —
80	219.9 (214.8–226.2)	324.8 (317.2–334.4)	[a] —
90	243.9 (236.3–253.4)	358.4 (346.8–373.6)	[a] —
95	265.7 (255.6–278.4)	388.7 (372.9–409.8)	[a] —

[a] Synergism ratios were identical for all lethal concentrations since the propoxur regression lines with and without LC_0 permethrin were parallels ($P > 0.05$).

4 DISCUSSION

In this study, bioassays were carried out to evaluate the insecticidal activities of permethrin and propoxur, alone and in combination, against pyrethroid- and carbamate-resistant larvae of *C quinquefasciatus*. The results indicated three types of relationship between the insecticides, depending on the resistance mechanisms displayed by the mosquito strains:

- With the pyrethroid–resistant strain (BK-PER), the pyrethroid toxicity was significantly increased when adding a sub-lethal concentration of the carbamate insecticide. This increase, which was more acute at LC_{95}, resulted from a synergistic interaction between permethrin and propoxur.
- With the carbamate-resistant strain (R-LAB), the opposite situation was observed. The carbamate efficacy decreased when combined with a sub-lethal concentration of pyrethroid (antagonism).
- Finally, neither synergistic nor antagonistic interactions occurred with either strain when mosquitoes were resistant to the insecticide used at non-toxic concentrations.

A general model has been developed to explain synergism between insecticides.[26] The model indicated that 'one toxicant interferes with the metabolic detoxification of the second toxicant, thereby potentiating the toxicity of the latter compound'. Indeed, Kulkrani and Hodgson[27] demonstrated that pyrethroid and organophosphate insecticides may be competitive substrates for the same oxidase, thus increasing the toxicity of the mixture. In addition, Gunning *et al*[28] demonstrated that synergism between fenvalerate and organophosphate insecticides in the cotton pest *Helicoverpa armigera* (Hübn) was due to an inhibition by organophosphates of esterases involved in pyrethroid resistance.

In our study, it is likely that a similar phenomenon occurred with the pyrethroid-resistant strain BK-PER which exhibited an increased metabolic detoxification by the cytochrome P450-dependant monooxygenases.[3] The monooxygenase action on propoxur would prevent (or delay) the degradation of permethrin, hence providing a level of synergism by competitive substrate inhibition. The non-specific esterases (NSE) were probably not involved in synergism since Chandre *et al*[3] demonstrated that efficacy of permethrin was unchanged in BK-PER after addition of DEF (*S*,*S*,*S*-tributyl phosphorotrithioate), an esterase inhibitor.

Conversely, the mechanism by which permethrin antagonized the propoxur in the carbamate-resistant strain R-LAB appeared more complex. It is obvious that an inhibition of metabolic detoxification (esterase or oxidase activities) by one of the two compounds of the mixture cannot explain such interaction. In a previous study, we have shown that similar combinations of propoxur with permethrin displayed synergistic interactions against susceptible larvae of *C quinquefasciatus* (S-LAB).[18] Moreover, this synergism was maintained even when oxidase activity was inhibited by piperonyl butoxide, an oxidase inhibitor (V Corbel, unpublished data). Thus, it is surprising to note that synergism occurred in the susceptible reference strain S-LAB whereas antagonism appeared in the carbamate resistant strain R-LAB, both having an identical enzymatic background, only differing by an insensitive acetylcholinesterase (AChE).

Bourguet *et al*[29] reported that in the resistant strain R-LAB, AChE (EC 3.3.3.7) (the primary target of carbamates) was unaffected by propoxur, even at concentrations giving 100% mortality, because of its complete insensitivity to carbamates. These authors showed that, when AChE was highly insensitive to carbamates, another enzyme responsible for the synthesis of acetylcholine (choline acetyl transferase or ChAT EC 2.3.1.6) became the second target of propoxur, and insect death occurred through a lack of acetylcholine in the synapses. We thought that inhibition of either AChE (for S-LAB) or ChAT (for R-LAB) by propoxur would explain the opposite interactions observed with the insecticide mixtures in *C quinquefasciatus*.

Salgado *et al*[30] demonstrated that the repetitive firing of nerves induced by pyrethroids stimulated an acetylcholine release within the synaptic gap, even when low concentrations of permethrin were used. Consequently, with the R-LAB strain, the acetylcholine released by permethrin at LC_0 may

counterbalance the deficit of acetylcholine due to ChAT inhibition by propoxur, leading to an antagonistic interaction. Conversely, with the susceptible strain (S-LAB), the acetylcholine released by permethrin at LC_0 may strengthen the acetylcholine accumulation due to AChE inhibition by propoxur, leading to synergistic interaction. This phenomenon would confirm the concept that the pivotal step for insecticide toxicity is not the acetylcholinesterase activity but the amount of acetylcholine present in the synaptic junctions. In order to verify and quantify the physiological mechanisms involved in these binary insecticide interactions, electrophysiological techniques will be performed on the dorsal ganglion of the mosquito larvae. A better understanding of these phenomena could contribute to a more effective control of mosquito populations, particularly in areas of strong resistance to insecticides.

In conclusion, the occurrence of pyrethroid resistance in the vectors of human diseases[31] has recently raised great interest in the search for strategies to prevent or overcome resistance in the field. Insecticide mixtures may offer interesting perspectives for controlling vectors of diseases, especially if synergistic interactions occurred between the insecticides.[32] However, we showed that some resistance mechanisms in mosquitoes, such as the highly insensitive AChE present in our *C quinquefasciatus* strain, may negate the advantages of insecticide combinations. In similar cases, alternatives strategies such as mosaics or rotations should be considered. Then, there is a need to strengthen basic and operational researches on interaction between insecticides (and between pesticide target sites) to set up adequate resistance management strategies in the field.

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