Cross-Resistance to Pyrethroid and Organophosphorus Insecticides Induced by Selection with Temephos in the Potential Mosquito Vector of West Nile Virus (*Culex Pipiens)* from Tunisia

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Abstract: A sample of *Culex pipiens* from Northwestern Tunisia, North Africa, with a high level of temephos resistance, was subjected to temephos selection to evaluate the utility of this organophosphate insecticide for mosquito control. High resistance developed after three generations of selection (52x). High cross-resistance was observed for the pyrethroid permethrin (32x). Synergism tests showed the implication of cytochrome P450 monooxygenases but not of esterase in temephos and permethrin resistance. Pyrethroid resistance could be associated with a cytochrome P450 monooxygenases and/or glutathione-S-transferase mechanism but not the esterases. The cross-resistance to permethrin from temephos selection could limit the use of both insecticides for *Culex pipiens* control.

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1. Introduction:

Culex pipiens is one of the most important mosquito species in terms of geographical distribution and ability to transmit pathogens (Vinogradova et al., 2000). In North Africa, *Culex pipiens* is a competent vector of several pathogens infecting animals and humans including West Nile Virus (WNV) and Rift Valley Fever Virus (RVFV) (Meegan et al., 1980; Moutailler et al., 2008; Krida et al., 2011; Amraoui et al., 2012). In Tunisia, *Culex pipiens* is the most widespread mosquito species (Senevet et al., 1958; Senevet et al., 1960; Senevet et al., 1959; Amara Korba et al., 2016), and an efficient vector of WNV (Wasfi et al., 2016).

There are currently no effective drugs or vaccines for important diseases such as West Nile virus disease. The only way to control these diseases is to prevent transmission by insect vectors. Most vector control programs largely rely on the application of chemical insecticides. Although public health uses the account for only a very small fraction of overall insecticide quantities applied, many vector species of public health importance have already developed resistance to one or more insecticides. Development of resistance is a complex and dynamic process and depends upon many factors. Most commonly, when the frequency of resistant insects in a vector population increases, the efficacy of the treatment decreases up to the point where the insecticide has to be replaced by another one. Increasing the dosages in an attempt to maintain efficacy is not a recommended option because of environmental and safety concerns and increased cost of the insecticide.

Because of the relatively low mammalian toxicity and rapid knockdown effect on insects, pyrethroids are the most commonly used insecticides and constitute the only recommended class of insecticides for ITNs (Insecticide Treated Nets). However, insecticide exposure is a potent selective force, presenting a risk of generating resistance that would threaten the efficacy of control programs. Hence, preventing or delaying the emergence and development of resistance to pyrethroids is very important for vector control efforts. Improving vector management involves a better understanding of resistance mechanisms.

Global surveys have indicated that resistance of mosquitoes to pyrethroids mainly occurs through increased detoxification, as well as target site insensitivity (Nkya et al., 2012). Detoxification enzymes typically linked to insecticide resistance mainly include three major gene families, cytochrome P450 monooxygenases (P450s or CYPs), carboxyl/choline esterases (CCEs) and glutathione-S-transferases (GSTs). Numerous studies have associated these detoxification enzymes with pyrethroid resistance in mosquitoes (David et al., 2013; Lumjuan et al., 2011; Somwang et al., 2011). The primary target sites of pyrethroids, well known as knockdown resistance (kdr), encode voltage-

gated sodium channels, and single or multiple substitutions in the sodium channel gene can reduce neuronal sensitivity to pyrethroids (Hemingway & Ranson, 2000).

In this study, a field population of *Culex pipiens* was selected with temephos for six generations. We report the capacity of the population to develop temephos resistance and the cross-resistance that was conferred.

2. Materials and Methods:

2.1. Mosquito strains:

Two strains of mosquitoes were used in this study. The Bou.nat colony was derived from a collection of *Culex pipiens* eggs, larvae and pupae from Boussalem, Northwestern Tunisia, which was received in 2004. This colony was subjected to temephos selection and identified as Bou.tem6 after 6 generations in the laboratory. A long-established laboratory reference strain, S-Lab was used for comparisons.

2.2. Insecticides and synergists:

Three technical-grade insecticides were used for selection and bioassay: the organophosphates temephos (9l%o; American Cyanamid, Princeton, NJ), the pyrethroid permethrin (94.6Vo, ICI Americas, Inc., Richmond, CA), and the carbamate propoxur (997o; Mobay). Two synergists were used to help detect detoxification enzymes involved in resistance: S,S,S-ributyl phosphorothioate (DEF), an esterase inhibitor, and piperonyl butoxide (PB), an inhibitor of mixed function oxidases.

2.3. Selection and bioassay procedures:

The selection procedure for the Bou.nat strain involved exposing groups of late third or early 4th-stage larvae to temephos for 24 h. Survivors were transferred to clean water and used to continue the colony.

Bioassay tests utilized standard methods (Raymond et al., 1986) which are briefly described here. Groups of 20 late third or early 4th in stars were placed in 177-mI waxed cups in 99 ml of tap water and 1 ml of insecticide solution in acetone. Control cups received acetone without insecticide. Five or more concentrations of insecticide, providing mortality between 50 and 75% after 24 h, were replicated on 5 different days. Data were subjected to probit analysis (Finney, 1971) using a BASIC program (Raymond, 1993). Resistance ratios were calculated at the median lethal concentration (LC50) and LC95 by comparing with those of the susceptible strain.

Synergism tests were similar to the bioassay tests except that 0.5 ml of the desired concentration of synergist was added to each cup, followed by the concentration of insecticide.

2.4. Esterase assay protocol:

Total esterase activity in individual frozen adult mosquitoes (3 days postemergence) from Bou.nat, Bou.tem4 and Bou.tem6 strains was determined according to the method of Pasteur et al. (1981, 1988).

2.5. Acetylcholinesterase activity assay protocol:

The AChE activity in individual mosquitoes was observed in the presence of propoxur and in the control (ethanol).

3. Results

In the present study, *Culex pipiens* larvae were selected with temephos for 6 generations and each generation was tested for susceptibility to permethrin using the dosage- mortality relationship (Table 1). Under continuous selection pressure, the LC50 and LC95 values fluctuated widely during the six generations. The only significant spike in LC95 was observed in the F3 generation (510.56). An approximately 36-fold increase in LC95 was observed based on the RR95 (Table 1). The slopes of the regression curves for each generation were calculated and varied from 1.33-5.64 (Table 1). The highest value was observed in the F3 generation and the lowest in the F0 generation. The linearity of concentration-mortality curves was accepted ($P<0.05$) only for Bou.tem1.P, Bou.tem2.P, and Bou.tem3.P (Tables 1).

To test the hypothesis that synergists can improve the toxic effect of insecticides on mosquitoes, two synergists DEF, and PB were applied in conjunction with the insecticides. The mortality rates of the strains of *Culex pipiens* larvae, in response to various combinations of insecticide and synergist, are shown in Table 2. No significant changes in mortality were found when DEF treatment was applied in conjunction with permethrin. Bio chemical analysis confirmed these results and esterases were not detected. PB treatment led to an increase in the mortality rate of *Culex pipiens* affected by permethrin. In summary, cytochrome P450 monooxygenases are involved in resistance to permethrin. Except for Bou.nat, *culex pipiens* of selection temephos showed resistance to Propoxur which indicates an acetyl cholinesterase insensitive.

4. Discussion

Tunisian *Culex pipiens* is able to develop high levels of resistance to temephos after intensive selection pressure. However, despite the use of this insecticide for larval control, there have been a few reports of resistance to temephos in this species (Daaboub et al., 2008).
Temephos resistance in our selected strain was associated with a cross-resistance to pyrethroid insecticide. Similar results were reported by Wirth et al. (1999) in *Aedes aegypti* strain selected with temephos for 13 generations.

Table 1: *Permethrin resistance evolution of Bou.tem larvae under selection pressures with temephos. Bou: Boussalem; nat: natural population; tem: temephos*

Name of population	LD50 (a)	LD95 (a)	Slope (b)	H (df)	RR50 (c)	RR95 (c)
S-Lab.P	0.00041 (0.0003-0.0044)	0.0009 (0.0007-0.0011)	4.7± (0.55)	1 (1)	-	-
Bou.nat.P	0.0069 (0.0051-0.0092)	0.0135 (0.0076-0.0243)	5.64± (1.68)	7.94 (3)	16.84 (8.64-32.81)	14.74 (3.92-55.34)
Bou.tem1.P	0.0063 (0.0054-0.0072)	0.0428 (0.0327-0.0619)	1.98± (0.16)	1 (4)	15.42 (12.58-18.91)	46.66 (29.7-73.32)
Bou.tem2.P	0.0071 (0.0056-0.0088)	0.1058 (0.0715-0.1796)	1.40± (0.12)	1 (3)	17.40 (14.09-21.48)	115.12 (72.96-181.65)
Bou.tem3.P	0.0276 (0.0217-0.0347)	0.4693 (0.3118-0.8069)	1.33± (0.10)	1 (3)	67.35 (54.47-83.28)	510.56 (324.34-803.69)
Bou.tem4.P	0.0056 (0.0029-0.0108)	0.0193 (0.0051-0.0740)	3.09± (0.94)	14.72 (3)	13.82 (6.28-30.40)	21.07 (4.08-108.75)
Bou.tem5.P	0.0084 (0.0044-0.0161)	0.0397 (0.0074-0.2174)	2.45± (0.80)	10.76 (3)	20.66 (10.91-39.12)	43.22 (8.40-222.36)
Bou.tem6.P	0.0086 (0.0045-0.0165)	0.0528 (0.0095-0.3629)	2.09± (0.42)	3.68 (2)	21.05 (12.76-34.71)	57.49 (15.63-211.45)

(a) In mg/liter, 95% CI in parentheses.
(b) Standard errors in parentheses.
H: Heterogeneity, (df): testing linearity of the probit mortality/log dose response.
(c) RR, resistance ratio (LC50 of the population considered / LC50 of S-Lab); 95% CI in parentheses.

Table 2: *Responses of Bou.tem strains of Culex pipiens to permethrin with and without synergists*

Name of population	LD50 (a)	LD95 (a)	Slope (b)	H (df)	RR_{50} (c)	RR_{95} (c)	SR_{50} (d)	SR_{95} (d)
S-Lab.P	0.0004 (0.0003-0.00044)	0.0009 (0.0007-0.0011)	4.7± (0.55)	1 (1)	-	-	-	-
Bou.tem6.P	0.0086 (0.0045-0.0165)	0.0528 (0.0095-0.3629)	2.09± (0.42)	3.68 (2)	21.05 (12.76-34.7)	57.49 (15.63-211.45)	-	-
S-Lab.P.DEF	0.0004 (0.0002-0.0007)	0.0090 (0.0033-0.0860)	1.22± (0.25)	1 (1)	-	-	0.99 (0.73-1.33)	0.10 (0.04-0.23)
Bou.tem6.P.DEF	0.0086 (0.0045-0.0167)	0.0411 (0.0082-0.2309)	2.43± (0.53)	4.92 (2)	20.98 (11.06-39.82)	4.53 (0.80-25.64)	0.99 (0.56-1.76)	1.28 (0.28-5.71)
S-Lab.P.PB	0.0001 (0.00009-0.00019)	0.0010 (0.0005-0.0030)	1.80± (0.26)	1 (2)	-	-	3.08 (2.28-4.16)	0.85 (0.42-1.69)
Bou.tem6.P.PB	0.0020 (0.0000-2.8515)	0.0198 (0.0000-273483)	1.68± (0.91)	22.71 (1)	15.73 (4.17-59.27)	18.33 (0.70-474.31)	4.12 (1.09-15.61)	2.66 (0.09-73.63)

(a) In mg/liter, 95% CI in parentheses.
(b) Standard errors in parentheses.
H: Heterogeneity, (df): testing linearity of the probit mortality/log dose response.
(c) RR, resistance ratio (LC50 of the population considered / LC50 of S-Lab); 95% CI in parentheses.
(d) SR, synergism ratio (LC50 observed without synergist / LC50 observed with synergist); 95 CI in parentheses.

Biochemical tests indicated that elevated esterase activity is not related to temephos resistance in our selected strain. However this resistance mechanism is associated with chlorpyrifos and temephos resistance in *Culex pipiens* from Tunisia (Ben Cheikh et al., 1993; 2008). Wirth et al. (1999) reported temephos resistance in *Aedes aegypti* associated with elevated esterase using both synergist and biochemical tests but failed to identify the specific esterase and inhibitor studies.

Synergist studies did not implicate the esterase mechanism in the temephos/permethrin cross-resistance apparent after selection with temephos. The cross-resistance could be associated with the elevated P450 and GST activity, which resulted from the selection, but this needs further confirmation. Wirth et al, (1999) found resistance and/or cross-resistance to the pyrethroid permethrin after temephos selection. Pyrethroid resistance occurs in *Aedes aegypti* from Puerto Rico (Hemingway et al. 1989); the Dominican Republic (Mekuria et al., 1991); Venezuela (Mazarri and Georghiou, 1995).

Resistance to pyrethroid has been associated with cross resistance to DDT (Chadwicky et al., 1977; Prasittisuk and Busvine, 1977; McDonald and Wood, 1979). Hemingway et al. (1989) found that the pyrethroid resistance in Puerto Rico was caused by an altered target site (Kdr nerve insensitivity) rather than being metabolically based.

5. Conclusion

The cross-resistance to permethrin has serious implications for the use of pyrethroid in *Culex pipiens* control programs, as temephos is the most widely used insecticide for *Culex pipiens* control.

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Conflicts of Interest:

Authors declared they have no conflicts of interest

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