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Fitness cost in laboratory selected strain of *Culex pipiens* associated with resistance to the insecticide temephos

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Abstract: In Tunisia, decades of vectors control using organophosphates have led to dissemination of resistance. Although these insecticides have been employed for decades against *Culex pipiens* in the country, knowledge of the impact of temephos resistance on vector viability is limited. We evaluated several fitness parameters in a Tunisian *Culex pipiens* strain classified as temephos resistant. The insecticide-susceptible S-Lab strain was used as an experimental control. Two loci that possess alleles conferring organophosphate (OP) resistance were considered: *ace-1* coding for an acetylcholinesterase (AChE1, the OP target) and *Ester*, a ''super locus''. After 5 generations of pressure, the temephos resistance ratio increased to 60.51 at RR95, exhibited deficiency in the following two parameters: female fecundity ($\chi 2$=infini; dl=1; P<<0.05) and mortality rate (P<<0.05). Characterizations of resistance mechanisms indicate that Resistance *ace-1* alleles coding for a modified AChE1 were associated with a higher mortality rate and lower fecundity. These results are compared to previous research on field collected populations, and the impact of the fitness advantage of an insecticide resistance allele on insecticide resistance evolution and management is discussed.
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1. Introduction

One of the most important anthropogenic-based examples of natural selection is the development of resistance against insecticides. The origins, spread, and mechanisms of insecticide resistance have importance in both theoretical and practical issues (Hemingway, 2000; ffrench-Constant et al., 2004). From the beginning of the first insecticide treatment programs against insect pests, many insect species developed significant resistance levels against insecticides, and the number of resistant populations is still increasing (Georghiou, 1994; Denholm et al., 2002; Hemingway et al., 2002; Hardstone and Scott, 2010). Resistance against pesticides is seen as the product of 2 interacting forces. These are selection pressure acting on different genotypes in the presence or absence of the selecting agent (the insecticide) and gene flow, usually within a Mendelian population (May and Dobson, 1986).

Crow (1957) first pointed out that resistant and susceptible strains differ in fitness characteristics, such as development time, fecundity, and fertility. It is also generally assumed that resistant genotypes must have pleiotropic effects that result in reproductive disadvantage relative to susceptible genotypes, because in the absence of pesticides (i.e. selection agents), the resistant types are not common in pest populations before selection.

If the selective pressure is relaxed because of stabilizing selection, resistance alleles will decline in frequency (Crow, 1957; Roush and McKenzie, 1987; Carriere et al., 1994; McKenzie, 2000; Shi et al., 2004). By measuring reproductive, developmental, and behavioral fitness components of numerous resistant insect species, many studies have recorded the fitness costs of resistance alleles in the absence of insecticide selection pressure (Clarke and McKenzie, 1987; Rowland, 1991a, 1991b; Minkoff and Wilson, 1992; Boivin et al., 2001; Boivin et al., 2003; Foster et. al., 2003; Bourguet et al., 2004; Liu and Han, 2006).

The housefly, *Musca domestica* L. (Diptera: Muscidae), is an important mechanical vector of both human and animal diseases. The housefly's insecticide resistance has become a global problem, as it has developed resistance against almost every insecticide used against it (Georghiou and Mellon, 1983; Scott et al., 1989; Kristensen et al., 2000; Acevedo et al., 2009; Kaufman et al., 2010; Memmi, 2010). In addition, because of its high potential for insecticide resistance, *Musca domestica* is also a suitable model for studying the genetic and metabolic mechanisms of insecticide resistance. Two loci are involved in OP resistance in C.

pipiens, the super-locus Ester and the locus ace-1. Several resistance alleles have been described at both loci (for a review see Raymond et al., 2001). The resistance conferred by Ester is due to an esterase over-production which is the result of two non-exclusive mechanisms (Raymond et al., 1998): gene amplification (for instance, Ester4, Ester2 and Ester5 alleles), or change in gene regulation (Ester1 allele). The ace-1 locus codes for the OP target, acetylcholinesterase (AChE). Resistance alleles ace-1R code an AChE with a reduced sensitivity towards OP, associated with modified catalytic properties (Bourguet et al., 1997).

In the present study, we tested the hypothesis that biological parameters are impaired due to high levels of resistance to temephos in the *Culex pipiens* laboratory strain that is harboring the organophosphorus resistance mechanism.

2. Materials and Methods

Two colonies of *Culex pipiens* were used in this study. A susceptible strain (S-Lab) which have been maintained in the insectarium of Unit of Genetics, University of Monastir for many years and have not been exposed to any insecticide and/or biological control agent. The resistant colony (Bou.tem5) was started from the Bou.nat (field population) colony and was subjected to continuous selection pressure with insecticide temephos. The selection procedure consisted of exposing a large number (10,000-20,000) of young 4th-stage larvae to temephos at concentrations ranging from the median lethal concentration to the 95 % lethal concentration for 5 generations.

To synchronise development, eggs were allowed to hatch and groups of 1,000 first instar larvae were then transferred to plastic basins containing 1 L dechlorinated water and 1 gram rabbit crop and were maintained in a laboratory. When rearing proceeded until the adult stage, food was replaced every three days. Adult mosquitoes were maintained in an insectary at 26 ± 1ºC under 80 ± 10% relative humidity.

Two technical-grade insecticides were used for selection and bioassay: the organophosphates temephos (9l%o; Ameican Cyanamid, Princeton, NJ) and the carbamate propoxur (997o; Mobay).

Temephos resistance levels were evaluated in larvae from both populations through dose response bioassays (WHO, 1963). In each assay five insecticide concentrations prepared with Temephos were tested. For each concentration, there were five replicates, each with 20 third instar larvae in 100 mL solution. Lethal concentrations (LCs) were calculated via probit analysis (Raymond et al, 1985). Resistance ratios (RR50 and RR95) were obtained by dividing the LC of the field population (Bou.tem5) by the equivalent LC from the S-Lab strain.

This test was similar to the bioassay tests except that 0.5 ml of the maximum sublethal concentration of an esterase inhibitor, S,S,S-tributylphosphorotrithioate, (0.5 µg/ml) was added to each cup with 0.5 ml of insecticide and piperonyl butoxide (pb), an inhibitor of mixed function oxidases. Esterase phenotypes were established by starch electrophoresis (TME 7.4 buffer system) as described by Pasteur et al. (1981, 1988) using homogenates of thorax and abdomen.

The parameters fecundity, fertility, development time and preadult survivorship were compared between the two colonies (S-Lab and Bou.tem5) to determine whether resistance to temephos was associated with any reproductive disadvantage.

Egg rafts were taken from female mosquitoes that had not been exposed to temephos during their larval stage. Fully bloodfed females were selected randomly from each S-Lab and Bou.tem5 colony and allowed to lay eggs. Fecundity was then measured by using egg rafts from each colony and determining the average number of eggs per raft at the first gonotrophic cycle.

Fertility was assessed as the mean number of first stage larvae (L1) and the percentage of eggs that hatched within 24 and 48 h after oviposition. Egg rafts were used from the S-Lab and Bou.tem5 colonies. Each egg raft was placed individually in a plastic cup containing 200 ml of distilled water. We have agreed to quantify a spawning of: Big if it gives a number of larvae greater than 150; Average if the number of larvae is between 150 and 100; Small if it gives less than 100 larvae.

Preadult development time and survivorship were assessed by accompanying larvae from egg rafts of each susceptible and resistant colony. Larvae from each egg raft were reared in a plastic pan filled with dechlorinated water and fed ground rabbit crop. To neutralize the effect of density, we conducted an environmental stress gradient through the establishment of three density ranges: Low density (50 larvae/500ml), Average density (100 larvae/500ml) and High density (200 larvae/500ml). The pupae were transferred daily to a 200 ml cup and placed in screen cages for adult emergence.

All the experiments described herein were repeated at least three times. Data obtained for each parameter evaluated were compared using *t* tests or $\chi 2$ analysis, as indicated in the results, except for longevity data that were subjected to Kruskal-Wallis followed by Dunn's Multiple Comparison Test. Graph-Pad Prism version 5.0 for Windows was adopted for all analyses (GraphPad Software, San Diego California USA) (graphpad.com).

3. Results

Culex pipiens was placed under selection pressure and the resistant strain (bou.tem5) was tested for susceptibility to temephos (Table 1). Under selective pressure the resistance ratio was approximately a 4-fold increase in the LD95 (60.51 at LD95). Our results showed that neither esterases (or GST) inhibited by DEF nor P450 cytochrome mediated monooxygenases inhibited by PB played a role in the observed resistance of Bou.tem5. This conclusion was confirmed by Starch gel electrophoresis that did not disclose any overproduced known esterase in the resistant strain (Bou.tem5). C*ulex pipiens* of selection temephos showed resistance to Propoxur wich indicates an acetyl cholinesterase insensitive (*Ace-1R*).

The observations for egg fertility, female fecundity, mortality rate and egg-to-adult development time are shown in Figure 1 and Table 2, 3, 4. There was a significant difference within the resistant/susceptible groups between the different populations for the two parameters examined: female fecundity ($\chi 2$=infini ; dl=1 ; P<<0.05; Table 2) and mortality rate (P<<0.05; Tables 3, 4). Despite egg fertility ($\chi 2$=0.03; dl=1 ; P>>0.05; Table 2) and development time (P>0.05; Tables 3, 4) did not differ between resistant and susceptible populations. Moreover, we note that the number of eggs given by the S-Lab females (61) is significantly higher than that given by Bou.tem5 (29). Also we note that the two strains tend to give more small eggs (number of larvae <100) than big larvae (number of larvae> 150) and medium (150 <number of larvae <100). Development time seems to be affected by density. The emergence of mosquitoes from low density larvae is faster than those of high densities showing longer development. All high densities have a high mortality rate compared to average and low densities. It should be noted that, on average, resistant individuals have a shorter development time compared to susceptible individuals. The high mortality rates of Bou.tem5 explain the difference in development time between the two strains. Whatever the resistance status (resistant or sensitive strain), males developed faster than females: compared to males, the development of females is slowed from one to three days, but this difference is not significant (P> 0.05, Tables 3 and 4). On the other hand, within each strain, the sex ratio is unbalanced, ie the number of females emerged is greater than that of males. This deficiency in males is significant only for Bou.tem5 (Bou.tem5: P <0.05, S-Lab: P> 0.05).

4. Discussion

Studies on fitness components of resistant individuals in the absence of chemical treatment were rather neglected until the 1970s (Roush and McKenzie, 1987) and remain insufficient (Taylor and Feyereisen, 1996; Coustau et al., 2000). In addition, most published data suffer from three weaknesses. First, comparisons generally involve unrelated resistant and susceptible strains (e.g., Baker et al., 1998; Alyokhin and Ferro, 1999). As pointed out 50 years ago by Varzandeh et al. (1954), resistant and susceptible strains may differ in many other genes than those involved in resistance. This is particularly relevant because populations from different geographical origins often differ in trait life history (Whitehead et al., 1985). In addition, reference susceptible strains have usually been maintained in the laboratory for decades and therefore may be adapted to laboratory conditions. Unless compared in a similar genetic background, there is no guarantee that the effects measured are truly due to the resistance alleles. Unfortunately, only a few studies have properly controlled for the effect of the genetic background. One way is to repeatedly backcross the resistant individuals with the susceptible one so that the genetic background of the resistant strain is replaced by that of the susceptible one (e.g., Amin and White, 1984; Argentine et al., 1989). A second procedure is to analyze the correlation between insecticide susceptibility and life-history traits in different natural populations, strains, or crosses (e.g., Campanhola et al., 1991; Raymond et al., 1993; Hollingworth et al., 1997; Foster et al., 1999). The second weakness of studies on fitness costs is that they have generally been performed in optimal conditions (but see McKenzie et al., 1982; Heather, 1982; Zhu et al., 1996). However, the metabolic and/or physiological modifications induced by resistance alleles may be more deleterious in adverse conditions. Third, most studies have been conducted in artificial laboratory conditions and consequently suffer from the problem of uncertain relevance to field conditions (Roush and McKenzie, 1987; Roush and Daly, 1990). Therefore, there is still a crucial need for direct estimations of the fitness costs of insecticide resistance alleles in natural populations.

In this study, we did not observe any significant difference in egg fertility and development time between the resistant and the susceptible populations. However, Resistance seems to increase the mortality rate of individuals and decrease the fecundity of females. Similar trends have been observed by many previous studies (Guillemaud et al., 1998; Lenormand et al., 1998). Our results suggested that temephos resistance was not associated with monooxygenase and esterases or (GST). However, evidence was found of insensitive acetylcholinesterase in the resistant strain (Bou.tem5). We noted that the increase of mortality rate and the decrease of fecundity females were probably due to the modified acetylcholinesterase that appears associated with a higher cost than that associated with overproduced esterase (Lenormand et al., 1998; Lenormand et al., 1999; Lenormand &

Raymond, 2000). A fitness cost as a consequence of the resistance to OP was further studied in the mosquito *Culex pipiens*. By monitoring laboratory and field populations in the southern part of France, it was found that three loci are involved in the resistance to OP. Two tightly linked loci, *Est-2* and *Est-3*, encode detoxifying esterases A and B and confer resistance to OP through overexpression. This is achieved by the modification of gene regulation or by gene amplification (Rooker et al., 1996). The third locus, *Ace*, encodes the target site of OP, acetylcholinesterase. Variants of this locus are insensitive to the inhibitory action of OP. Studies on populations with these different loci conferring resistance to OP found that they suffer different fitness cost effects and selection pressures. For example, some variants in the *Ace* locus showed differential survival rates during winter time, indicating their impaired ability to avoid predation and other selection pressures to which the overwintering females are exposed (Rooker et al., 1996). Bourget et al. 2004 showed that in natural populations all *ace-1* and *Ester* resistance alleles induced a longer larval developmental time (up to about 15%). However, this cost is variable: the effects of *Ester*1 and *Ester*4 varied with larval density and were not additive with those of *ace-1R* and *ace-1RS*. Conversely, the effect of these two *ace-1* resistance alleles was constant across density levels. Previous field studies also suggested that environmental conditions influence the pleiotropic effects of *Ester* resistance alleles, although this is not apparent for *ace-1* (Chevillon et al., 1997; Gazave et al., 2001).

Several studies have shown that in the absence of insecticide selection pressure, resistance genes have a cost in the biology of organisms such as low fertility and fecundity rates (Mandla et al., 2001, Arnaud et al., 2002), reduced survival, prolonged development (Voordouw et al., 2009), reduced body size, altered wing morphology, oviposition behavior, fluctuating asymmetry (Mandla et al., 2001), as well as mating competitiveness (Rowland, 1991a). But there have been exceptions where resistance genes have been reported to confer a fitness advantage. In *Anopheles funestus*, the resistant mosquitoes were reported to have both higher fecundity, as well as more viable eggs, when compared with the susceptible strain. In terms of development, no significant differences in pupation and emergence rates, or adult longevity between the resistant and susceptible strains were ob-served (Okoye et al., 2007). Developmental time and body size are key life history traits which affect fitness of organisms. Similarly, the degree of expression of many genes is influenced by environmental conditions. Higher temperatures can affect aquatic larvae and terrestrial adults.

Because the application of chemical insecticides still plays an important role in the control of several insect vectors, a detailed analysis of the effect of resistance on the biology of these species can directly contribute to the development of novel control strategies as well as to the management of resistance in natural vector populations.

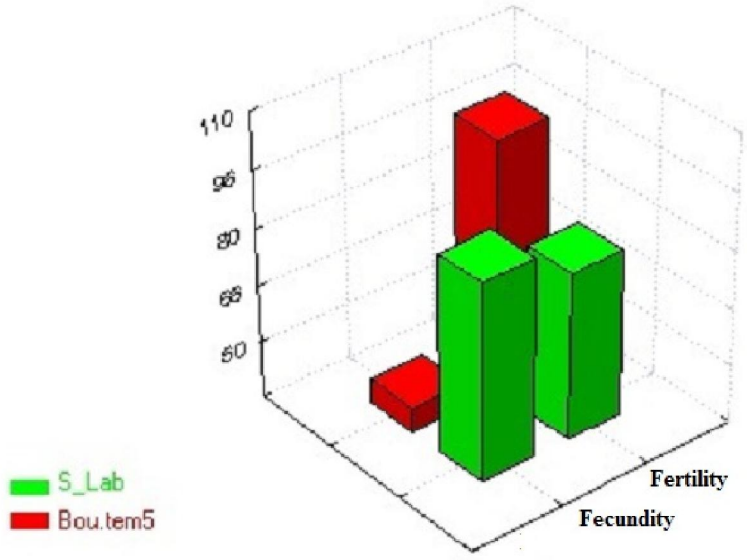

Figure1. Female fecundity and fertility of sensitive (S-Lab) and resistant (Bou.tem5) strains

Table 1. Insecticide resistance of resistant (Bou.tem5), reference (S-Lab) and original (Bou.nat) strain

Name of population	LD_{50} (a)	LD_{95} (a)	Slope (b)	H (df)	RR_{50} (c)	RR_{95} (c)
S-Lab.T	0.0012 (0.0011-0.0014)	0.0062 (0.0047-0.0094)	2.34± (0.22)	1 (3)	-	-
Bou.nat.T	0.0266 (0.0237-0.0301)	0.0934 (0.0741-0.1283)	3.02± (0.27)	1 (2)	21.45 (17.63-26.10)	14.90 (9.15-24.28)
Bou.tem5.T	0.0202 (0.0058-0.0738)	0.3792 (0.0188-10.3333)	1.29± (0.24)	3.96 (2)	16.29 (10.44-25.43)	60.51 (19.93-183.76)

Bou: Boussalem; nat: natural population; tem: temephos
(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. Female fecundity and egg fertility of sensitive (S-Lab) and resistant (Bou.tem5) strains

	Number of eggs	Egg size (a)	Bridges by female (%)	Big eggs (%)	Average eggs (%)	Small eggs (%)
S-Lab	61	79.03 ± (40.36)	87.14	3.27	32.78	63.93
Bou.tem5	29	101.24 ± (70.45)	41.42	24.13	20.68	55.17

(a) Standard errors in parentheses.

Table3: Average number of larvae, percentage of emerged adults, mortality rate and development time of resistant strain (Bou.tem5)

Density	Larvaes	Adults	Males	Females	Mortality rate	Development time (h) Male	Development time (h) Female
ALDR	50± (0.00)	50± (6.92)	16.66± (1.15)	33.33± (7.57)	50.00± (6.92)	282.66± (15.14)	
						268.00± (18.33)	285.00± (15.02)
AADR	100± (0.00)	41.33± (12.89)	15± (4.58)	26.33± (11.06)	58.66± (12.89)	375.55±(7.34)	
						394.47± (53.88)	381.66± (12.50)
AHDR	200± (0.00)	29.66± (14.02)	13.16± (7.21)	16.5± (7.54)	71.33± (14.02)	419.66±(14.79)	
						372.00± (16.80)	430.86± (15.41)
AA	116.66± (76.37)	40.33± (10.20)	14.94± (1.75)	25.38± (8.45)	59.99± (10.72)	359.29±(69.93)	
						344.82± (67.47)	365.88± (74.13)

h :hours
Standard errors in parentheses.
ALDR : Average of the three Low Density Repetitions
AADR : Average of the three Average Density Repetitions
AHDR : Average of the three High Density Repetitions
AA : Average of Averages

Table 4. Average number of larvae, percentage of emerged adults, mortality rate and development time of sensitive **strain (S-Lab)**

Density	Larvaes	Adults	Males	Females	Mortality rate	development time (h) Male	development time (h) Female
ALDR	50± (0.00)	90.66± (6.42)	46± (3.46)	44.66± (8.32)	9.33 ± (6.42)	292.00± (6.92)	302.85± (12.98) 316.22± (14.20)
AADR	100± (0.00)	87.33± (4.50)	38.66± (3.21)	48.66± (2.08)	12.66± (4.50)	370.18± (17.61)	375.46± (15.10) 409.04± (38.39)
AHDR	200± (0.00)	38± (9.64)	10± (2.50)	28± (7.85)	62± (9.64)	463.88± (20.41)	485.07± (13.41) 526.66± (24.11)
AA	116.66± (76.37)	71.99± (29.49)	31.55± (19.02)	40.44± (10.96)	27.99± (29.49)	408.68± (49.03)	387.80± (91.73) 417.31± (99.46)

h :hours
Standard errors in parentheses.
ALDR : Average of the three Low Density Repetitions
AADR : Average of the three Average Density Repetitions
AHDR : Average of the three High Density Repetitions
AA : Average of Averages

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