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Insecticide resistance in the mosquito *Culex pipiens*: what have we learned about adaptation?

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Abstract

Resistance to organophosphate (OP) insecticide in the mosquito *Culex pipiens* has been studied for ca. 30 years. This example of micro-evolution has been thoroughly investigated as an opportunity to assess precisely both the new adapted phenotypes and the associated genetic changes. A notable feature is that OP resistance is achieved with few genes, and these genes have generally large effects. The molecular events generating such resistance genes are complex (e.g., gene amplification, gene regulation) potentially explaining their low frequency of *de novo* occurrence. In contrast, migration is a frequent event, including passive transportation between distant populations. This generates a complex interaction between mutations and migration, and promotes competition among resistance alleles. When the precise physiological action of each gene product is rather well known, it is possible to understand the dominance level or the type of epistasis observed. It is however difficult to predict *a priori* how resistance genes will interact, and it is too early to state whether or not this will be ever possible. These resistance genes are costly, and the cost is variable among them. It is usually believed that the initial fitness cost would gradually decrease due to subsequent mutations with a modifier effect. In the present example, a particular modifier occurred (a gene duplication) at one resistance locus, whereas at the other one reduction of cost is driven by allele replacement and apparently not by selection of modifiers.

Introduction

The mosquito *Culex pipiens*, common in temperate and tropical countries, is subjected to insecticide control in many places, particularly with organophosphate insecticides (OP). These insecticides inhibit the acetylcholinesterase (or AchE) in the central nervous system, inducing lethal conditions. Within a few years, this mosquito has developed various adaptations to this new and toxic environment. This example of micro-evolution has been thoroughly investigated as an opportunity to assess precisely both the new adapted phenotypes and the associated genetic changes. In addition, OP resistance in this mosquito was studied at its first appearance in some geographic areas, and was followed up in the context of a long term project. Changes of life-history traits, competition between resistance genes, evolution of the fitness cost, etc. have been documented during almost 30 years of insecticide selection. The most relevant features of our understanding of this example of adaptation are described below.

Mutation and migration

Genetics of resistance

Only three loci have developed major OP resistance alleles: *Est-2*, *Est-3* and *Ace.1*. The first two loci, *Est-2* and *Est-3*, code for detoxifying carboxylester hydrolases (or esterases), and are separated by an intergenic DNA fragment varying between 2–6 kb. Resistance alleles correspond to an esterase over-production (which

Table 1. Nomenclature for the various resistance genes and their products at *Ester* and *Ace.1* resistance loci

Allele	Amplified gene (s)	Enzyme (s)	Comments
$Ester^{0}$	None	'Null'	Class of normal activity esterases
$Ester^{1}$	None (?)	A1	Overproduced esterases
$Ester^{2}$	*Est-2* and *Est-3*	A2-B2	
$Ester^{4}$	*Est-2* and *Est-3*	A4-B4	
$Ester^{5}$	*Est-2* and *Est-3*	A5-B5	
$Ester^{8}$	*Est-2* and *Est-3*	A8-B8	
$Ester^{9}$	*Est-2* and *Est-3*	A9-B9	
$Ester^{B1}$	*Est-2*	B1	
$Ester^{B6}$	*Est-2*	B6	
$Ester^{B7}$	*Est-2*	B7	
$Ace.1^{S}$	None	AChE1S	OP-sensitive enzymes
$Ace.1^{R}$	None	AChE1R	OP-insensitive enzymes
$Ace.1^{RS}$	*Ace.1* (duplication)	AChE1S and AChE1R	

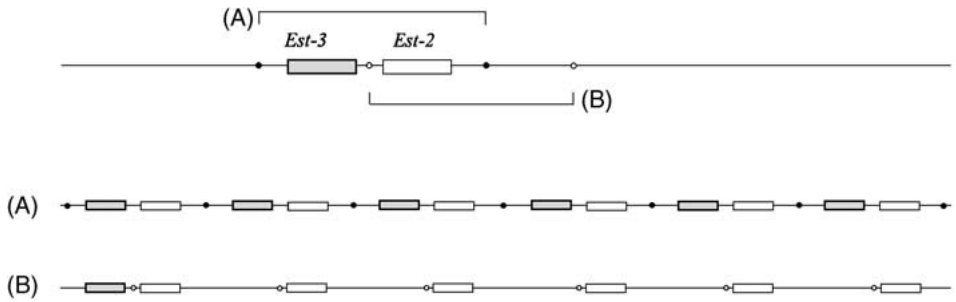

Figure 1. Amplification at *Ester* super locus. Two types of amplification are known, (A) one that co-amplifies *Est-2* and *Est-3* (materialized by black circles), (B) and one that amplifies only *Est-2* (materialized by empty circles). The resulting organisation of the amplicon is illustrated for a 6-fold amplification level.

binds or metabolizes the insecticide) relative to basal esterase production of susceptibility alleles. Several resistance alleles (each corresponding to a distinct over-produced allozyme) have been described at both loci (Table 1) (see for review Raymond et al., 1998; Chevillon et al., 1999). For most alleles, the overproduction of esterase is the result of gene amplification (i.e., several copies of the same gene are found in the same genome). This concerns either one locus or both (Figure 1). The latter situation, the co-amplification of two esterase loci, explains the tight statistical association of some electromorphs, like A2 and B2 (Guillemaud et al., 1996; Rooker et al., 1996). Although, strictly speaking, A4, A2 and A1 are coded by alleles of the *Est-3* locus, and B2 and B4 by alleles at the *Est-2* locus, A1, A4-B4 and A2-B2 behave as alleles of a single super locus (named *Ester*) due to the complete linkage disequilibrium between *Est-2* and *Est-3* co-amplification. Gene regulation is also present (i.e., esterase production is higher than expected by the amplification level), and is the major mechanism of overproduction of A1 (Rooker et al., 1996). There is an additional complexity: the level of gene amplification varies between the different amplified alleles: for $Ester^{B1}$, it could reach easily 100 copies in the field, whereas for $Ester^{4}$ it has never been found above few copies (Poirié, Raymond & Pasteur, 1992; Guillemaud et al., 1997; Weill et al., 2000). It varies also within and among populations for a given amplified allele, as shown for example for $Ester^{2}$ (Callaghan et al., 1998; Weill et al., 2000). Why the various amplified alleles seem to have distinct limits of amplification levels is unknown. A hint may lies in their variable tissue distribution, in particular in the brain where there is the insecticide target (e.g., $Ester^{B1}$ is expressed in the brain, contrarily to $Ester^{2}$, Pasteur, Nancé & Bons, 2001)). This variation may change the relationship between the advantage (related to the

esterase amount expressed where it reduces the insecticide concentration at the target location) and the cost (probably related to the overall esterase overproduction, independently of its location) for each amplified allele, potentially explaining the variable amplification levels observed.

The third locus, *Ace.1*, codes the insecticide target (acetylcholinesterase). The wild and susceptible form of this enzyme is inhibited by OP insecticides. Several resistance alleles $Ace.1^R$ have been described, with a reduced sensitivity towards OP, associated to modified catalytic properties (Bourguet et al., 1997a). They probably correspond to one or several point mutations in or around the active site, and their complete description awaits the cloning of *Ace.1*. A particular resistance mutant has been described ($Ace.1^{RS}$), corresponding to a duplication of *Ace.1* (i.e., there are two copies of the *Ace.1* locus), with one susceptible and one resistant allele (Bourguet, Capela & Raymond, 1996; Lenormand et al., 1998a). The advantage of this mutation is explained in the 'evolution of cost' section.

Other loci are probably contributing to OP resistance, such as genes involved in mono-oxygenase detoxification or reduced penetration (Raymond et al., 1987; Raymond, Heckel & Scott, 1989). However, such resistance mechanisms are either absent in natural populations, or their contribution to OP resistance relatively minor compared to overproduced esterases or insensitive target (e.g., Pasteur & Sinègre, 1978; Pasteur, Sinègre & Gabinaud, 1981; Raymond et al., 1987), although this is not the case in Tunisia (Pasteur et al., 1999).

Frequency of 'beneficial mutations'

How many times has a mutation gene occurred (here mutation is a molecular event such as point mutation or gene amplification)? At the *Ester* locus, independent amplifications have occurred only a few times, as indicated by the known list of distinct amplified alleles (Table 2) and the huge polymorphism existing among non-amplified alleles (Raymond, Qiao & Callaghan, 1996). This relatively low number of independent *de novo* amplification events, inventoried on a world scale for a pest species with large population sizes, indicates that there is somehow a limitation on the rate of occurrence of new advantageous mutation. This does not originate from the limits to adaptation due to a too small treated area compared to gene flow (for theoretical developments see Slatkin, 1973, 1987), as the size of the treated areas are relatively large compared to estimates of migration variance (Lenormand & Raymond, 1998). Due to the advantage they provide in OP treated areas, these resistance genes have subsequently spread within populations, and then among populations (Table 2). The latter phenomenon is considerably facilitated by the fact that most OP treated areas are connected by plane or other transportation systems suitable to passive migration by mosquitoes (Highton & van Someren, 1970; Curtis & White, 1984; Pasteur et al., 1995; Chevillon et al., 1995a). Some resistance alleles such as $Ester^2$ or $Ester^{B1}$ are now found on several continents, after their first occurrence in one geographic location probably in the sixties (Raymond et al., 1991; Qiao & Raymond, 1995). Local invasions of $Ester^2$ have been documented in California and eastern France in the eighties (Raymond et al., 1987; Rivet, Marquine & Raymond, 1993), and a similar process has been recently observed for $Ester^5$ in northern Italy (Severini et al., 1997). Why some resistance alleles are distributed worldwide (e.g., $Ester^2$), and others have a more restricted range (e.g., $Ester^4$ and $Ester^5$ in west and east Mediterranean, respectively) is still unknown. Several factors are possibly at play, such as history (the first amplified allele has more time to spread) or selection (advantages and costs vary among alleles).

Interaction between migration and mutation

As an amplified *Ester* allele spreads within a local population subjected to OP treatments, the previous high polymorphism of non-amplified alleles (i.e., susceptible ones) observed at this locus decreases. Thus alternative alleles which could also undergo amplification are rapidly disappearing as the advantageous (generally amplified) alleles invade the treated population (e.g., only $Ester^2$ is found in some African samples, Curtis & Pasteur, 1981). The apparent rate of occurrence of a new amplification event thus slows down as resistance genes spread geographically: there is an interaction between the extent of migration and the apparent rate of new occurrence of beneficial mutations at the same locus.

This could be illustrated by the situation of the Corsica island (Raymond & Marquine, 1994). Insecticide treatments on this island have started few years later than in the nearby continent, and were performed with a distinct OP insecticides (temephos in Corsica and chlorpyrifos in southern France). Resistance genes in continental southern France have first developed

Table 2. Geographic distribution of *Ester* resistance alleles among major landmasses

Allele	First detection	Current distribution				Reference
		Africa	Eastern Eurasia	Western Eurasia	Americas	
Ester^{1}	France (1972)	–	Israel	Spain, France, Italy	–	(Pasteur, Iseki & Georghiou, 1981; Severini et al., 1993; Chevillon et al., 1995b; Eritja & Chevillon, 1999)
Ester^{2}	Japan? Burma? (1969)	Most countries	Most countries	France, (Spain), (Italy)	Most countries	(Raymond et al., 1991, 1998; Pasteur et al., 2001)
Ester^{4}	France (1984)	North Africa	–	Spain, France, Italy		(Chevillon et al., 1995b; Silvestrini et al., 1998; Eritja & Chevillon, 1999)
Ester^{5}	Cyprus (1987)	Tunisia	Mediterranean countries	Italy	–	(Severini et al., 1997; Silvestrini et al., 1998; Berticat et al., 2000)
Ester^{8}	China (1994)	–	China	–	–	(Qiao et al., 1998)
Ester^{9}	China (1994)	–	China	–	–	(Weill et al., 2001)
Ester^{B1}	USA (1975)	–	China, Japan	–	Most countries	(Georghiou & Pasteur, 1978; Qiao & Raymond, 1995; Pasteur et al., 2001)
Ester^{B6}	China (1992)	–	China	–	–	(Xu, Qu & Liu, 1994)
Ester^{B7}	China (1992)	–	China	–	–	(Xu, Qu & Liu, 1994)

The place and year of first detection are indicated as well as the currently known distribution. No information is available from Australia. Countries from which resistance genes have disappeared are in parentheses. Spread of resistance genes (rather than independent mutations) is documented by molecular studies (see e.g., Raymond et al., 1991; Qiao & Raymond, 1995; Guillemaud et al., 1996; Severini et al., 1997).

(e.g., *Ester*^{1} and *Ace.1*^{R}) providing a fair resistance level to chlorpyrifos, and have subsequently migrated to Corsica. These resistance genes provided, by chance, only a low resistance level toward temephos (other resistance genes, not found in western Mediterranean, such as *Ester*^{B1} or *Ester*^{5}, induce a very high protection level toward temephos). They have nevertheless invaded the Corsican treated area, due to the non-null temephos protection they confer, thus removing most genetic variability at the *Ester* locus, and reducing the chances of occurrence of a more suitable amplified allele (at least by mutation). As a result, temephos resistance in Corsica has remained low after 17 years of treatments.

Deciphering gene interaction

The precise knowledge of both phenotypes (resistance) and the corresponding genes (resistance genes) is a useful situation to study how genes interact and if general rules can emerge. Specifically, this question could be addressed to intra-locus and inter-locus interactions (respectively dominance and epistatis).

Dominance

If a resistance gene is dominant (v.s. recessive), resistant homozygotes (v.s. susceptibles) and heterozygotes display the same resistance level. Can we predict the dominance level of a resistance gene? The general answer is no, unless we know the precise physiological role of this gene, and its mode of interaction with the insecticide. When a resistance gene is involved in an enzymatic pathway, the metabolic theory proposed by Wright applies (which takes into account kinetics properties of metabolic systems)(see Keightley, 1996 and Kacser & Burns, 1981). In this case, resistance is predicted to be dominant (Bourguet & Raymond, 1998): this probably explains the observed dominance of resistance alleles at the *Ester* locus. However, no clear prediction exists for other situations (e.g., receptors, ion channels, non-linear enzymatic pathways, etc.), and a relevant understanding is to be sought on a case by case basis (Bourguet & Raymond, 1998).

The situation at the *Ace.1* locus could be used to illustrate this latter point, as resistance conferred by insensitive acetylcholinesterase (or AChE) varies from semi-recessivity to dominance (Bourguet et al., 1997a). This is partly explained by the positive correlation between survival and AChE activity (Hoffmann, Fournier & Spierer, 1992). AChE activity of resistant *Ace.1* alleles is often altered (see for review Fournier & Mutero, 1994) so that, in R/S heterozygotes (which possess only half the quantity of insensitive AChE present in R/R homozygotes), the insensitive AChE (remaining uninhibited) accounts for less than 50% of the total AChE activity. The consequence is a low survival of heterozygotes compared to resistant homozygotes, hence a low level of dominance. This may explain the recessive resistance found in a strain of *Culex pipiens* where the activity of the insensitive AChE is only one-fourth that of the wild-type enzyme (Bourguet et al., 1997a). The same resistance mechanisms (insensitive AChE target) can display a complete dominance in some conditions, for example if the insecticide used inhibits a second target at high concentrations. If the modified AChE is completely insensitive to this insecticide, mortality will be induced by the inhibition of this second target. When this second target is equally sensitive in the susceptible and resistant strains, R/S and R/R strains present the same mortality curves, that is resistance will be fully dominant (for a detailed example, see Bourguet et al., 1997b).

Epistasis

Can we predict the type of epistasis occurring between resistance genes, that is how the resistance provided by each resistance gene will combine when they are in the same individual? The general answer is no, unless we know which resistance mechanisms are involved. A compartment model has been developed (Raymond, Heckel & Scott, 1989), which takes into account the kinetics of the insecticide concentration within the larvae, with or without the various resistance mechanisms (reduced penetration, increased detoxification, reduced transportation, target insensitivity). It allows to derive predictions on how the resistance mechanisms will interact to reduce the inhibition of the target. This model, supported by empirical data, predicts that a reduced penetration mechanism will combine multiplicatively with any other resistance mechanism, whereas an increased detoxification and target insensitivity will combine additively (such as esterase overproduction at *Ester* and insensitive AChE at *Ace.1*). This variety of interactions indicates that a fair understanding of physiological and molecular processes is required to understand gene interaction. Apparently, this diversity of results applies also to other types of mutations (e.g., Clark & Wang, 1997; de Visser, Hoekstra & Van den Ende, 1997; Elena & Lenski, 1997).

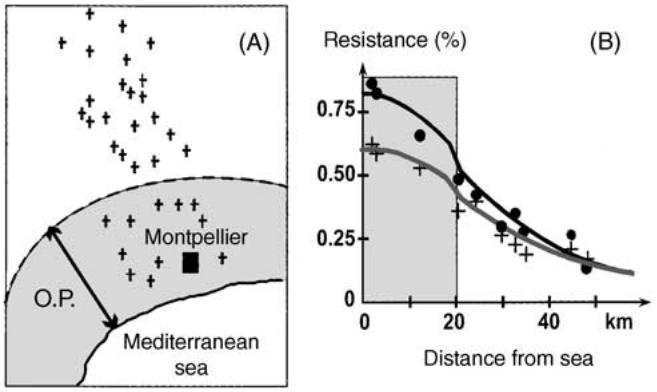

Figure 2. Analyzing migration/advantage/cost balance. (A) Location of the transect crossing the OP treated (shaded) and non treated areas (crosses = sampling locations) (B) Cline of resistance gene frequency during the summer at *Ester* (dots) and *Ace.1* (crosses) loci along the transect. Curves are fitted values (see Lenormand et al., 1998b).

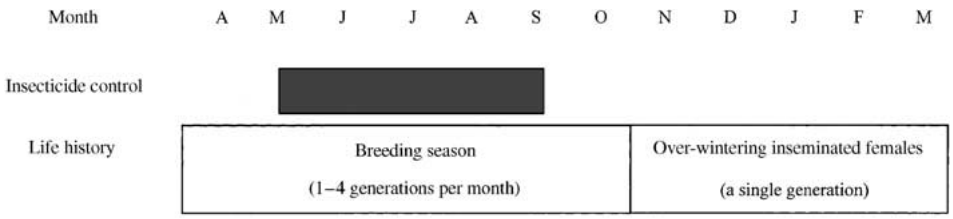

Figure 3. Life history traits of *Culex pipiens* in southern France and timing of insecticide treatments. Mosquito control (directed against larvae) takes place from mid-May to mid-September. In October, mated females enter caves or cellars for over-wintering.

How costly are insecticide resistance genes?

Costs of resistance genes have been thoroughly investigated in the Montpellier area (southern France). OP treatments started in 1968, and were restricted to a 20–25 km wide belt along the coast (Figure 2A). Resistance first appeared in 1972 with the occurrence of $Ester^{1}$, followed by $Ace.1^{R}$ in 1978, $Ester^{4}$ in 1984, $Ester^{2}$ in 1990 and the $Ace.1^{RS}$ duplication in 1993 (see for review Chevillon et al., 1999). Resistance genes spread and increased in frequency in the treated area, and migrated also into the non treated area, where they were selected against due to their fitness cost. A cline of frequency observed across the treated and non treated areas (Figure 2B) is the result of a balance between selection (i.e., fitness advantages and costs) and migration (Nagylaki, 1975). Insecticide selection varies through the year in the treated area: first and last breeding generations (as well as the overwintering generation) escape OP treatments (Figure 3). The clines at *Ester* and *Ace.1* evolve according to this variation, which allows a precise estimate of both migration and selection (advantage and cost) (Table 3). Fitness costs were rather large, with mean advantage/cost ratios in summer of 1.9 and 2.7 for *Ace.1* and *Ester*, respectively. How costs of resistance genes at these loci combine (e.g., additively or multiplicatively) is currently unknown.

How are resistance genes generating a fitness cost? The overproduction of esterase by the *Ester* locus should be at the expense of producing something else, with the resulting alteration of some fitness related traits. The modified AChE alters the optimal functioning of cholinergic synapses of the central nervous system, with probable changes in some behavioral fitness related traits. Some of these altered fitness traits have been identified: one or several resistance genes are associated with for example a decrease of overwintering survival (Chevillon et al., 1997; Lenormand et al., 1999; Gazave et al., 2001), a lower adult size (Bourguet et al., in prep.), an increased predation and developmental time, and a decreased male reproductive success (Berticat et al., 2001). Interestingly, these traits are differently modified for *Ester* and *Ace.1*.

Evolution of cost in southern France

On a long term basis, the following feature emerged: the most prevalent *Ester* allele during the seven-

Table 3. Fitness advantage and fitness cost (in % of the fitness of a susceptible gene in the non-treated area) of resistance genes at the *Ester* and *Ace.1* loci in the Montpellier (France) area, estimated per generation during the breeding season (OP treated) and the over-wintering period (no insecticide treatments)

Locus	Breeding season		Over-wintering generation	
	Advantage	Cost	Advantage	Cost
Ace.1	30	11	0	50–60
Ester	16	6	0	Variable (overall: 26)

Data from Chevillon et al., 1997; Lenormand et al., 1998b, 1999; Gazave et al., 2001; Lenormand & Raymond, 2000.

ties/eighties, $Ester^1$, has been replaced by $Ester^4$ during the nineties (Guillemaud et al., 1998), indicating that the advantage/cost/migration balance varies among the resistance genes at *Ester*, and that it overall favors $Ester^4$. As $Ester^4$ is known to confer a slightly lowest OP resistance level, its advantage over $Ester^1$ could possibly be its substantial lower cost, as recent laboratory experiments appear to confirm (Berticat et al., unpublished data). A similar phenomenon was observed at the *Ace.1* locus: the $Ace.1^{RS}$ allele has quickly been replacing $Ace.1^R$ since 1993 (Lenormand et al., 1998a). For the $Ace.1^{RS}$ allele, the additional S copy does not modify the resistance provided by the R copy, thus its advantage is probably a lower cost, as the additional AChE activity provided by the S copy probably compensates for deficiency of AChE activity of the R copy. Therefore, the S copy can be considered as a modifier for the cost generated by the R copy. The $Ace.1^{RS}$ can thus represent an incipient epistatic supergene (Kelly, 2000), with the emergence of new function through gene duplication (Hughes, 1994).

Thus, within a decade (i.e., of the order of 100 generations), allele replacement has taken place and the most costly resistance genes are being replaced by more recent and less costly ones. Could the less costly genes have occurred first? This is possible for the *Ester* locus, but this is not the case for the *Ace.1* locus, as the occurrence of the less costly allele, which requires probably an unequal crossing over, cannot occur before the most costly. The other way to reduce the cost, a selection of a modifier gene such as that described for OP resistant esterase in *Lucilia cuprina* (see for review Davies et al., 1996), is not documented in *Culex pipiens*. It is too early to know why modifier genes do not appear important at the *Ester* locus in *Culex*. It is however worthy of note that the esterase gene involved in resistance in *Lucilia* has apparently several functions, as it operates also during embryogenesis and metamorphosis (Davies et al., 1996; Clarke, Yen & McKenzie, 2000). If the cost results from the perturbation (due to the qualitative change responsible for resistance) of this function, it is perhaps easier to reduce it by another compensatory mutation which does not affect resistance. If products of the *Ester* locus in *Culex* have no other function than detoxification (which is currently not known), there is a necessary link between resistance and cost (both resulting from the overproduction level). This link might be different for the different *Ester* alleles (e.g., they are expressed differently in the various tissues, in particular in the brain where is located the insecticide target, Pasteur, Nancé & Bons, 2001), explaining that allele replacement, instead of occurrence of modifiers, takes place to reduce the cost.

Conclusion

Insecticides and insecticide resistance generate an evolutionary arms race that provides a system to study the genetics of adaptation in the wild. What have we learned with the example of resistance to OP-insecticides in the mosquito *Culex pipiens*?

A noticeable feature is that OP resistance is achieved with few genes (and at three loci), and these genes generally have a large effect. There is an endless debate on the number of mutation events required to achieve a successful adaptation (e.g., Lande, 1983; MacNair, 1991; Orr & Coyne, 1992), and the present example of a few mutations with large effects seems to represent a situation far from being uncommon (for direct and indirect supports see e.g., some adaptations to environmental changes: Hilbish, Bayne & Day, 1994; Parsch et al., 2000, and a distribution of QTL effects: Orr, 1998; Bost, Dillman & de Vienne, 1999).

The molecular events generating such resistance genes are complex (e.g., gene amplification, gene regulation) potentially explaining the low frequency of *de novo* occurrence. In contrast, migration is a frequent

event, including passive transportation between distant populations. This generates a complex interaction between mutations and migration, and promotes worldwide competition between resistance alleles.

The spread of resistance genes has occurred independently in all treated areas, and in the regions where detailed monitoring was conducted (e.g., southern France, Catalonia, north-eastern Italy and California), it was observed that repetitive spreads at the same locus were taking place (Raymond et al., 1987; Severini et al., 1997; Guillemaud et al., 1998; Lenormand et al., 1998a; Eritja & Chevillon, 1999). Thus it seems that the spread of advantageous mutations is rather common for this example of environmental change in a large population, which is to be considered within the current controversy concerning the phase three of Wright's shifting balance theory (Coyne, Barton & Turelli, 1997). Estimates of fitness advantage and fitness cost of the *Culex pipiens* resistance genes are similar to estimates for other genes providing resistance to xenobiotics (McKenzie, 1996), or for selected genes in classical models of natural selection, such as industrial melanism (Haldane, 1924; Lees, 1981).

When the precise physiological action of each gene product is well known, it is possible to explain the dominance level or the type of epistasis observed. It is however difficult to predict *a priori* how resistance genes will interact, and it is too early to state whether or not this will be ever possible. A minimum knowledge about gene function is required to predict some gene interactions (e.g., epistasis) and a fair understanding of what occurs in the 'metabolic world' around the gene product is required for other types of interaction (e.g., dominance).

These resistance genes are costly, and the cost is variable among them. It is usually believed that the initial fitness cost would gradually decrease due to subsequent mutations with a modifier effect. In the present example, the cost at one locus (*Ace.1*) has evolved with a particular modifier (a gene duplication a the same locus), whereas at the other one (*Ester*) a reduction of cost took 10 years to became widespread, driven by allele replacement and apparently not by selection of modifiers.

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