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Population genetics of insecticide resistance in the mosquito *Culex pipiens*

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Thirty years of control of the mosquito *Culex pipiens* using organophosphate insecticides (OP) has selected for OP-resistance alleles on a world-wide scale. As reviewed here, studies at the levels of gene and population allow identification of the main forces driving this process of adaptation. Three loci are involved in OP-resistance in *C. pipiens*. For two of these, adaptive mutations were found to be rare events, such that the ubiquitous distribution of certain resistance alleles could only be explained as deriving from a single origin by mutation followed by extensive migration. Population structure analyses confirmed that long-distance migration is frequent. Thus, different resistance alleles could accumulate and compete within populations soon after their origin by mutation. The different selection pressures acting on these alleles, i.e. their selective advantage in the presence of OP and their disadvantage (resistance cost) in absence of OP, were also analysed. Substantial differences in resistance cost among alleles present within the Mediterranean area were discovered. Long-term surveys of Mediterranean populations confirmed the pivotal importance of resistance cost in shaping the evolution of this adaptive polymorphism. Some hypotheses on the functional links between the nature of the initial mutation events and the subsequent evolution of polymorphism are discussed.

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INTRODUCTION

The process of adaptation to new environments is a major theme in evolutionary biology. However, the mechanisms involved remain poorly understood. The central questions are:

(a)   Does adaptation typically consist of the selection of a few mutations with major effects on fitness or of many mutations with minor effects?
(b)   Do alleles that are adaptive in certain environments typically have deleterious pleiotropic effects on fitness in different environments?
(c)   Are the adaptive and pleiotropic properties of alleles typically fixed or do they evolve, and if so, over what timescale and by which mechanisms?

These questions have been discussed extensively (Lande, 1983; Macnair, 1991; Orr & Coyne, 1992; Groeters, 1994; McKenzie & Batterham, 1994a, b; Tabashnik, 1994). Too few genetic studies of adaptation have been carried out to assess the relative importance of major-effect and minor-effect mutations adequately, although the occurrence of major adaptive mutations has been reported in several instances (Orr & Coyne, 1992). Consequently, comprehensive studies of adaptation at the gene, individual, and population levels are likely to greatly enhance our general understanding. Experimental studies of organisms with very short generation times, such as bacteria, have been fruitful. Such experiments have shown that pleiotropic fitness costs and their evolution may vary greatly among cases (e.g. Lenski, 1988a, b, Travisano, Vasi & Lenski, 1995; Vasi, Travisano & Lenski, 1995), although the adaptive changes have rarely been identified at the molecular level. Resistance to toxic chemicals, for which major mutations have frequently been identified (see for reviews Brown, 1996; Taylor & Feyereisen, 1996), can potentially provide excellent opportunities for integrated molecular and ecological studies of adaptation. However, such opportunities have rarely been exploited. Beside the case of *Culex pipiens* described below, a notable exception is the case of resistance to the insecticides dieldrin and diazinon in the sheep blow fly *Lucilia cuprina*. For each insecticide, field resistance has evolved through allelic substitution. At the *Rdl* locus, the allele involved in dieldrin resistance was associated with significant fitness cost (Clarke & McKenzie, 1987; McKenzie & Clarke, 1988; McKenzie, 1990). At the *Rop-1* locus, similar results were first found for the allele involved in diazinon resistance (R_{1A}), but this cost subsequently decreased due to the occurrence of a dominant modifier gene (e.g. McKenzie, Whitten & Adena, 1982; McKenzie & Purvis, 1984; Clarke & McKenzie, 1987; McKenzie & Clarke, 1988; McKenzie & O'Farrell, 1993; McKenzie & Batterham, 1994a; Davies *et al.*, 1996). The absence of evolution of dieldrin resistance cost is attributed to a shorter period of dieldrin use (2 years instead of 10 years for diazinon use), too short for selection of a modifier gene (Clarke & McKenzie, 1987).

The mosquito *C. pipiens*, common in temperate and tropical countries, has been the subject of organophosphate (OP) insecticide control in many countries since the 1960s. World wide surveys have shown that major resistance alleles occur at only three loci (*Ace.1*, *Est-2* and *Est-3*). Contrary to the case of *L. cuprina*, OP resistance in *C. pipiens* involves more than one allele at each locus. Long-term studies of this adaptive polymorphism have been carried out over the whole species range and at the local geographic scale. We discuss the insights these studies provide on the evolutionary mechanisms that shape these adaptive polymorphisms.

GENETIC DIVERSITY INVOLVED IN RESISTANCE

The *Ace.1* locus of *C. pipiens* encodes the OP-targeted enzyme acetylcholinesterase, the adaptive alleles of which produce enzymes insensitive to OP inhibition (Raymond *et al.*, 1986; Bisset *et al.*, 1991; Khayrandish & Wood, 1993; Bourguet *et al.*, 1996b, 1997a). No molecular assay has been developed to further investigate the allelic diversity at this locus. The number of independent mutation events leading to this type of OP-resistance is therefore unknown, although it is suspected to be quite low (Bourguet *et al.*, 1997a). Wild-type alleles coding for OP-sensitive forms of the enzyme are designated $Ace.1^S$ and resistance alleles are designated $Ace.1^R$. A biochemical test performed on individual mosquitoes allows discrimination of phenotypes carrying $Ace.1^S$ and/or $Ace.1^R$ (Bourguet *et al.*, 1996a). In the Caribbean and in southern France, difficulty in assigning a precise genotype to the *Ace.1* locus arises from the occurrence of a gene duplication (designated $Ace.1^{RS}$) containing one copy of $Ace.1^S$ and one copy of $Ace.1^R$ (Bourguet *et al.*, 1996b; Lenormand *et al.*, 1998a). The biochemical test cannot identify all possible genotypes when $Ace.1^R$, $Ace.1^S$ and $Ace.1^{RS}$ co-occur, so allelic frequencies cannot be directly deduced from phenotypic frequencies. The cloning and sequencing of the *Ace.1* gene of *C. pipiens* is required for a molecular assay to overcome this problem.

Besides *Ace.1*, two tightly-linked loci (*Est-2* and *Est-3*) code for detoxifying esterases. These genes confer OP-resistance through protein overproduction, which is achieved either by gene up-regulation or by gene amplification (Rooker *et al.*, 1996). When overproduced, esterases coded by *Est-2* and *Est-3* are designated Bi and Ai, respectively; i being a reference number. In the case of A1, overproduction results from gene up-regulation. For all other overproduced esterases, gene amplification is involved. The amplification encompasses either the two esterase loci (e.g. haplotypes A2-B2; A4-B4; A5-B5, A8-B8; Wirth *et al.*, 1990; Poirié, Raymond & Pasteur, 1992; Qiao *et al.*, 1999) or a single locus (such as B1; Mouchès *et al.*, 1986). As a result, the combination of *Est-2* and *Est-3* behaves as a single 'super locus' (*Ester*). The *Ester* polymorphism can be studied using protein electrophoresis or molecular assays such as Restriction Fragment Length Polymorphism (RFLP) or DNA sequencing.

MUTATION: A FEW ADAPTIVE MUTATION EVENTS

Resistance to OP has developed throughout the entire distribution of *C. pipiens* during the last few decades of intensive OP control. The success of this adaptation could be due to the appearance of many resistance alleles, i.e. to high mutation rates producing alleles conferring OP resistance. This hypothesis can be tested by comparison of the genetic diversity of OP-resistant and OP-susceptible alleles. No such data are available for the acetylcholinesterase locus. At the *Ester* locus, eight alleles conferring OP-resistance have been found: B1, A1, B6, B7, A2-B2, A4-B4, A5-B5 and A8-B8 (Wirth *et al.*, 1990; Poirié *et al.*, 1992; Xu, Qu & Liu, 1994; Guillemaud *et al.*, 1997; Qiao *et al.*, 1999). All but two of these can be identified by protein electrophoresis. To discriminate between A4-B4 and A5-B5 requires RFLP assays or DNA sequencing (Poirié *et al.*, 1992). Overproduced B esterase alleles derived from different continents but with similar electrophoretic patterns displayed identical restriction maps for 13 restriction enzymes clustered around the structural

gene *Est-2* (see for example B1 and B2; Raymond *et al.*, 1991; Qiao & Raymond, 1995). Likewise, samples of the overproduced esterase allele A2 from Asia, Africa or North America showed an identical 57 bp sequence in intron IV of the *Est-3* locus (Guillemaud *et al.*, 1996).

In contrast, both esterase loci display high levels of genetic diversity in OP-susceptible populations of *C. pipiens*. More than 14 protein electrophoretic patterns and 24 RFLP profiles were detected, using acrylamide gels and a single restriction enzyme, respectively, in a sample of 72 susceptible mosquitoes (Raymond, Qiao & Callaghan, 1996). Furthermore, two restriction maps from susceptible mosquitoes from the same population shared only 40% of their restriction sites (Raymond *et al.*, 1996). Finally, the *Est-3* locus displays one of the highest nucleotide diversities described so far with an estimate of $\pi = 0.17$ in intron IV (Guillemaud *et al.*, 1996).

These data indicate that each esterase resistance allele derives from one of about eight independent mutation events. This estimate is based on the analysis of mosquitoes sampled over a wide geographic range of OP-resistant populations (including North and South America, Caribbean, China, Thailand, Vietnam, Polynesia, North, East and South Africa, and Mediterranean countries). Although additional adaptive mutation events will probably be detected, the total number of esterase alleles known to cause OP resistance is not expected to increase significantly in the near future.

MIGRATION: AN IMPORTANT DRIVING FORCE ON A WORLD-WIDE SCALE

The geographical spread of these few OP-resistance alleles has been the major event during the 40 years of less of their existence. For instance, A1 and A4-B4 have spread within the western Mediterranean (Poirié *et al.*, 1992; Chevillon *et al.*, 1995b; Severini *et al.*, 1997; Ben Cheikh *et al.*, 1998), A5-B5 has spread within the eastern Mediterranean (Poirié *et al.*, 1992; Severini *et al.*, 1997), and A8-B8 has spread within China (Qiao *et al.*, 1999). Two other OP-resistance alleles, B1 and A2-B2, have been even more successful in having spread between continents (Raymond *et al.*, 1991; Qiao & Raymond, 1995). There are several non-exclusive mechanisms to explain these differences in the degree of geographical spread. One possibility is that the overall balance occurring between their advantage in OP-controlled populations and their disadvantage in OP-free populations greatly differs among alleles. In this case, B1 and A2-B2 would be the alleles conferring the highest average fitness among these contrasted environmental situations. Another possibility is that different alleles have been subject to different levels of passive dispersal. Lastly, it is possible that the degree of geographical spread is determined mainly by the age of the alleles. In the latter two cases, the present geographical pattern is a transient state unrelated to long-term equilibrium.

Opportunities for long-distance gene flow are indeed common in *C. pipiens*. There is direct evidence for long-distance migration through passive transport by man (Highton & von Someren, 1970), and the presence of one A2-B2 female in an aircraft has been established (Curtis & White, 1984). Indirect evidence for long-distance gene flow emerged from long-term studies of OP-resistance composition in the western Mediterranean. A2-B2 has occurred *de novo* three times in the vicinity of international airports: in California before 1984 (Raymond *et al.*, 1987), near

Marseille in southern France in 1986 (Rivet, Marquine & Raymond, 1993) and near Barcelona in Spain in 1993 (Eritja & Chevillon, 1999). In the two former cases A2-B2 subsequently invaded the local population of *C. pipiens* (Raymond *et al.*, 1987; Rivet *et al.*, 1993; Chevillon *et al.*, 1995c). In the latter case, the introduction appeared to have been unsuccessful, as A2-B2 was not detected the following year (Eritja & Chevillon, 1999). Analyses of population genetic structure based on non-OP-selected polymorphism have confirmed that passive transport by man generates high levels of gene flow (Chevillon *et al.*, 1995a; Pasteur *et al.*, 1995).

Migration appears to be a major driving force in shaping the evolution of OP resistance in *C. pipiens*. Indeed, mutation and migration are the only evolutionary forces involved in the occurrence of a novel resistance allele in a given population. We have described how fewer than ten independent mutation events can account for the entire range of esterase OP-resistance alleles observed throughout the world. Both active and passive dispersal have spread these alleles widely. It is worth noting that the frequency of resistance alleles would increase rapidly following immigration into OP-controlled populations because of their strong selective advantage. As a result, migration is expected to induce regular selective sweeps within OP-controlled areas, at least during the initial phase of the evolution of resistance. Both immigration and rapid increases in frequency of distinct OP-resistance alleles within OP-controlled populations have been documented in the Mediterranean Basin (Severini *et al.*, 1993; Chevillon *et al.*, 1995c; Lenormand *et al.*, 1998a).

VARIATION IN SELECTION PRESSURES AND EVOLUTION ON A LOCAL SCALE

By definition, OP resistance alleles are selected for in OP-controlled populations. Such alleles are expected to impose a disadvantage (resistance cost) in the absence of OP compounds, according to the theoretical framework of adaptive changes (e.g. Fisher, 1958; Lande, 1983; Macnair, 1991; Orr & Coyne, 1992). Much of the debate on mechanisms of genetic adaptation is concerned with how fitness cost evolves. Two alternative mechanisms have been proposed. The first involves a two-step process whereby an adaptive allele has a high fitness cost when it first appears. This cost is subsequently reduced by selection for cost-reduction alleles at a different modifier gene. The alternative mechanism is allele replacement, in which an existing costly allele is replaced by a novel allele conferring a lower fitness cost. Both hypotheses have received some support from the few adequate empirical models that exist, such as OP resistance in *L. cuprina* (McKenzie & Game, 1987; McKenzie & Clarke, 1988; Davies *et al.*, 1996), and bacterial adaptations (Lenski, 1988a, b; Cohan, King & Zawadzki, 1994; Schrag & Perrot, 1996).

The dynamics of OP resistance in *C. pipiens* in southern France have provided an excellent opportunity to study the complex interplay between environmental and genetic factors in the evolution of adaptation. The overproduced esterase A1 has been present in this area since 1972, four years after OP control began (Pasteur & Sinègre, 1975). The subsequent order of appearance of OP-resistance alleles was: *Ace.1^R* in 1977, A4-B4 in 1984, A2-B2 in 1991 and the duplication *Ace.1^{RS}* in 1993 (see for details Raymond *et al.*, 1986; Poirié *et al.*, 1992; Chevillon *et al.*, 1995c; Lenormand *et al.*, 1998a). The spread of each new OP-resistance allele followed an invasive dynamic, with the notable exception of A2-B2, which remained at low

frequency (Guillemaud *et al.*, 1998). These successive appearances allowed comparison of the selective pressures acting on OP-resistance alleles at different loci as well as on competing alleles of the same locus. Since 1969, the intensive use of OP insecticides has been restricted to a strip approximately 20 km wide along the Mediterranean coast. The notable change in the level of OP-mediated selection is its gradual decrease due to the progressive replacement of OP-based insecticides by *Bacillus sp.* toxins since 1991. A cline in the frequency of each OP-resistance allele from the OP-controlled to the OP-free areas was expected if their selective advantage and resistance costs are sufficiently high relative to migration (Nagylaki, 1975).

The overall selection intensities acting on OP-resistance alleles were compared between loci, assuming no selective differences among alleles within loci. This assumption was supported by experimental data on the level of resistance (selective advantage) associated with A1 and A4-B4, and with *Ace.1^R* and *Ace.1^{RS}*. Lenormand *et al.* (1998b) developed a migration/selection model that allowed the impacts of migration and selection to be differentiated, and that simultaneously estimated migration, selective advantage and resistance cost acting on each locus, using data on levels of linkage disequilibrium between *Ace.1* and *Ester* resistant alleles. These authors showed that the clines observed in the summer of 1995 were consistent with an overall fitness cost per generation of 11% for *Ace.1^R* and *Ace.1^{RS}* (modified OP target enzymes), and of 6% for A1 and A4-B4 (overproduced esterases). The overall selective advantage per generation was estimated as 30% for the modified OP target enzymes, and 16% for the overproduced esterases.

Differences in fitness cost between competing OP-resistance alleles have been clearly demonstrated by spatio-temporal analysis of these clines (Guillemaud *et al.*, 1998; Lenormand *et al.*, 1998a). Guillemaud *et al.* (1998) analysed a long-term dataset on the distribution of overproduced esterases since the appearance of A1 in 1972. It appears that A1 exhibited a steep cline in frequency when it was the sole OP-resistance allele present. In 1978, one year after the invasion of *Ace.1^R* began, this cline was still present, with a maximum frequency of about 0.60. The A1 cline became progressively shallower after the appearance of A4-B4 in 1986. There has been a general decrease in the frequency of A1 across the whole area to below 0.15 in 1996. The evolution of A4-B4 within the same populations was very different. This allele did not display a cline when it first appeared in 1986, but was homogeneously distributed with a frequency of about 0.05. Its frequency has progressively increased since 1986, and averaged over 0.50 in both 1995 and 1996. Thus, A4-B4 is progressively replacing A1, i.e. an allele replacement is occurring over a timescale of approximately 10 years. Both alleles confer approximately the same degree of OP resistance, either alone or in association with *Ace.1^R*. The process of replacement is therefore being driven by differential resistance cost, i.e. an older costly allele is being replaced by a novel less costly one.

An even more rapid allele replacement has been documented at the acetylcholinesterase locus within the same populations (see for details Lenormand *et al.*, 1998a). The duplication *Ace.1^{RS}* was apparently absent from the area in April 1993. Two years later, *Ace.1^{RS}* reached frequencies as high as 0.30 near the coast. Regular analyses of the *Ace.1* phenotypic distributions from 1995 to 1997 allowed estimation of the selective pressures acting on both alleles. Lenormand *et al.* (1998a) showed that both *Ace.1^R* and *Ace.1^{RS}* impose a resistance cost, and that the cost of *Ace.1^{RS}* is approximately 3% to 6% lower than that of *Ace.1^R*.

DIFFERENCES IN RESISTANCE COSTS: TOWARDS FUNCTIONAL HYPOTHESES

One possible mechanism by which resistance costs are generated is the disruption of metabolic equilibria (Uyenoyama, 1986). The evolution of OP resistance at the *Ace.1* locus in *C. pipiens* supports this hypothesis. This OP target enzyme plays a role in the function of the central nervous system by regulating the turnover of the neurotransmitter acetylcholine (ACh). The amount of ACh present in the synapses is regulated by the opposing actions of acetylcholinesterase, which degrades ACh, and cholinacetyltransferase, which synthesizes ACh. The maintenance of a steady-state level of ACh in synapses appears crucial in determining fitness since both a deficit and an excess of ACh can cause mortality (Bourguet *et al.*, 1997b). A physiological model of the fitness costs imposed by *Ace.1^R* proposes that excess levels of ACh build up in synapses. The enzyme encoded by *Ace.1^R* is indeed about four-fold less active than that encoded by *Ace.1^S* (Bourguet *et al.*, 1997a). The replacement of *Ace.1^R* by the duplication *Ace.1^{RS}* further supports this model. The enzymes encoded by *Ace.1^{RS}* are expected to possess increased acetylcholinesterase activity relative to those encoded by *Ace.1^R*, and hence to reduce the levels of ACh in synapses.

Esterase alleles A1 and A4-B4 confer OP-resistance through protein over-production, suggesting that their resistance cost may be mediated by resource limitation. Although the rate of protein overproduction could be the key factor linking both resistance cost and OP resistance, A1 and A4-B4 have different mechanisms of overproduction. A1 overproduction results from gene up-regulation, while that of A4-B4 results from gene amplification. The tendency of A4-B4 to replace A1 in Mediterranean countries (France, Spain and Italy; Guillemaud *et al.*, 1998; Severini *et al.*, 1993; Eritja & Chevillon, 1999) could reflect the different molecular basis of the mutations involved. In addition, the level of amplification varies among individuals carrying the same amplified allele in natural populations (see Callaghan *et al.* (1998) for A2-B2, and Guillemaud *et al.* (1999) for B1). Mother-offspring comparisons indicated that significant variations in amplification level occur within a single generation, probably due to unequal cross-over (Guillemaud *et al.*, 1999). This suggests that under gene amplification, protein dosage could be quickly adjusted to a new equilibrium value, i.e. determining a new balance between the selective advantage and cost associated with a particular haplotype.

CONCLUSION

Only a small number of resistance alleles have occurred in *C. pipiens* in response to OP selection throughout the world, at least in the case of the esterase genes. Owing to the advantage they provide in OP-controlled areas, they have rapidly spread within populations, and have subsequently invaded wider geographic areas. As a result, these resistance alleles have accumulated rapidly within certain OP-controlled populations, leading to allelic competition. Depending on the levels of OP resistance, the associated cost and the regime of OP-insecticide use, one particular allele may eventually replace the others. This process is well illustrated in southern France by the replacement of A1 by A4-B4, and of *Ace.1^R* by *Ace.1^{RS}* during the progressive withdrawal of OP use. The replacement of A1 by A4-B4 has also been reported in Spain (Eritja & Chevillon, 1999) and in Italy (Villani & Hemingway, 1987;

Severini *et al.*, 1993). Thus, the process of adaptation to OP insecticides in *C. pipiens* does not appear to involve genes that modify the original resistance cost. This is contrary to what has been observed in the Australian sheep blowfly *L. cuprina* (for the identification of a modifier see Davies *et al.*, 1996; McKenzie & Game, 1987). However, change in gene dosage may be a key factor driving allelic replacement in *C. pipiens*.

Besides *C. pipiens* and *L. cuprina*, the only model in which adaptation to toxic chemicals has been intensively studied at both the gene and population levels is the peach aphid, *Myzus persicae*. The overproduced detoxifying esterases E4 and FE4 appeared to be the main genetic factors conferring resistance to a wide range of insecticides (Devonshire & Moores, 1982; Devonshire, 1989). Overproduction was achieved by gene amplification, and E4 and FE4 probably derive from only two original amplification events of a single gene located on chromosome 3 (Blackman *et al.*, 1995, 1996). In addition, variation in resistance levels is usually associated with variation in gene copy numbers (Devonshire, 1989), although it is not the unique mechanism involved as described below. As in the case of *C. pipiens*, it appears that adaptation was first driven by rare mutation events and extensive migration, and subsequently modified by changes in gene dosage. In addition, resistance allele E4 appears to impose strong deleterious effects such as reduced overwinter survival (Foster *et al.*, 1996) and disruption of the life cycle (Blackman *et al.*, 1996). *M. persicae* is holocyclic, i.e. it reproduces both by apomictic parthenogenesis and by sexual reproduction, although the rate of sexual reproduction is geographically variable (Blackman, 1974). The comparison of resistance costs under clonal re-production is difficult because both E4 and FE4 will be completely associated with many other genes determining life history traits. Moreover, copies of the amplified resistance alleles may be dispersed throughout the genome (Blackman *et al.*, 1995), so that both E4 and FE4 could coexist in the same apomictic clone (Blackman *et al.*, 1996). Finally, changes in DNA methylation are known to be involved in both partial and total reversion to susceptibility in E4-carrying clones, and in the potential re-appearance of resistance in the progeny of such revertant clones (Ffrench-Constant *et al.*, 1988; Field *et al.*, 1989). If esterase dosage in *M. persicae* modulates both the selective advantage and the resistance cost, as suspected in *C. pipiens*, then genes regulating DNA methylation are good candidates for possible modifiers. However, one would not expect such modifiers to be as efficient as those described in *L. cuprina* because E4 revertants continue to suffer from a low rate of overwinter survival (Foster *et al.*, 1989). These two last phenomena (dispersion of multiple gene copies and DNA methylation) were not detected in the *C. pipiens* situations.

In conclusion, as demonstrated in bacteria (e.g. Cohan *et al.*, 1994), the process of adaptive evolution shows many parallels among these three case studies of insecticide resistance concerning the initial invasive step, but shows striking differences concerning subsequent evolution. Integrated studies at the gene and population levels are expected to shed further light on these fascinating processes.

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