Variation of Dominance of Newly Arisen Adaptive Genes

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ABSTRACT

Newly arisen adaptive alleles such as insecticide resistance genes represent a good opportunity to investigate the theories put forth to explain the molecular basis of dominance and its possible evolution. Dominance levels of insecticide resistance conferred by insensitive alleles of the acetylcholinesterase gene were analyzed in five resistant strains of the mosquito *Culex pipiens*. Dominance levels were found to differ between strains, varying from partial recessivity to complete dominance. This variation was not explained by differences in catalytic properties of the enzyme, since four of the five resistant strains had identical inhibition properties for the insensitive acetylcholinesterase. Among these four laboratory strains and in individuals collected from natural populations, we found a correlation between increased acetylcholinesterase activities and higher dominance levels. We propose a molecular explanation for how variation in acetylcholinesterase activity may result in variation of dominance level. We also conjecture that the four resistant strains did not differ in their amino acid sequence in the catalytically active regions of acetylcholinesterase, but that the expression of the gene was regulated by either neighboring or distant sites, thereby modifying the dominance level. Under this interpretation, dominance levels may evolve in this system, since heritable variation in acetylcholinesterase activity was found.

IS evolution of dominance a general explanation for recessivity? This question has been a crucial point of one of the most famous debates between R. A. FISHER and S. WRIGHT to explain why 90% of deleterious mutations are recessive to their wild-type allele (FISHER 1958; SVED and MAYO 1970; WRIGHT 1977; CHARLESWORTH 1979; ORR 1991). In 1958, FISHER argued that the recessivity of recurrent deleterious mutations is the endproduct of natural selection (see also SVED and MAYO 1970). He claimed that most of these mutations are originally codominant and become progressively recessive through the accumulation of modifier alleles at other loci. WRIGHT alternatively proposed that dominance of the wild-type allele is a simple consequence of metabolism (WRIGHT 1977; SVED and MAYO 1970). He assumed that most deleterious mutations cause a reduction in enzymatic activity. If the wild-type allele has more activity than necessary, then the rate of reaction is likely to be substrate-limiting rather than enzyme-limiting. Thus, a deleterious allele that only slightly reduces enzyme activity should appear recessive or nearly recessive.

WRIGHT's physiological theory has gained favor among biologists. In the 80's, KACSER and BURNS (1981) and KEIGHTLEY and KACSER (1987) pushed WRIGHT's physiological theory one step further. They detailed why a change in enzyme concentration or activity at any one step in a multi-step metabolic pathway is unlikely to have a large effect on the output of the system. Furthermore, empirical studies (*e.g.*, ORR 1991) and theoretical predictions (*e.g.*, CHARLESWORTH 1979) provided evidence against FISHER's theory and thus strong support for the physiological theory of dominance. Thus, the widespread occurrence of recessive mutants may, to a large extent, be the inevitable consequence of the kinetic structure of enzymatic pathways. But the question still remains: to what extent has dominance evolved?

The physiological theory incorporates the idea that dominance relationships may evolve. For instance, KACSER and BURNS (1981 page 661) claimed "that every enzyme is, in principle, a 'modifier' of the dominance relationships of every other enzyme" and thus "it is perfectly possible that a *particular* dominance has been the subject of evolutionary modification." But they also added that "[evolution of dominance] is a historical question and hence not subject to experimental verification by reference to present observations." This led to the common supposition that because partial recessivity is the "default" state of deleterious mutations and the expected outcome, natural selection has had no influence. But some mutations involved in particular physiological processes have dominant deleterious effects (HODGKIN 1993; WILKIE 1994). Could these domi-

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nant mutations become recessives and if so what are the molecular mechanisms responsible of such modifications?

That dominance may evolve was in part rejected because selection for the increase in frequency of a single modifier was thought to be ineffective. This was the conclusion of a dozen theoretical analyses based on the mathematical model introduced by WRIGHT (1929) (see SVED and MAYO 1970). However, WAGNER and BÜRGER (1985) and MAYO and BÜRGER (1996) have shown that many of these analyses were incorrect, neglecting linkage disequilibrium or making unrealistic assumptions and thus unable to accurately predict the course of the change of the modifier frequency. Furthermore, BÜRGER (1983), using a complete nonlinear analysis of WRIGHT's mathematical model, proved that the modifier always goes to fixation. CURTSINGER *et al.* (1994) and MAYO and BÜRGER (1996) have recently reviewed cases of selection through visual predation on moth and butterflies that show evidence for the occurrence of modifiers of dominance (*e.g.*, MIKKOLA 1984). Both came to the conclusion that dominance relationships can be modified.

Insecticide resistance conferred by modification of the pesticide target site is a suitable model for investigating dominance relationships (BOURGUET *et al.* 1996a; BOURGUET and RAYMOND 1997). One of these pesticide targets is acetylcholinesterase (AChE; acetylcholine acetylhydrolase, EC 3.1.1.7), an enzyme of the central nervous system of insects that degrades the synapse neurotransmitter acetylcholine (see TOUTANT 1989 for review). This enzyme is inhibited by organophosphorous and carbamate insecticides, and insensitive AChE is due to one or more point mutations (*e.g.*, MUTÉRO *et al.* 1994). Insensitive AChE have been reported in >25 insect species (FOURNIER and MUTÉRO 1994). The mosquito *Culex pipiens* possesses two acetylcholinesterases, AChE1 and AChE2, which are thought to be produced by distinct genes, *Ace.1* and *Ace.2* (BOURGUET *et al.* 1996b). Only *Ace.1* is involved in insecticide resistance and two types of alleles can be distinguished: $Ace.1^S$ and $Ace.1^R$ coding for sensitive and insensitive AChE1s, respectively (BOURGUET *et al.* 1996c). We have already investigated the molecular basis for the dominance relationships of pesticide resistance conferred by modified target-sites including modifications of acetylcholinesterase (BOURGUET and RAYMOND 1997). We showed why resistance conferred by $Ace.1^R$ alleles is usually codominant to dominant as predicted by the physiological theory. However, some $Ace.1^R$ alleles also conferred a recessive resistance making difficult a general prediction of dominance of insecticide resistance. In this article, we further investigate dominance relationships of pesticide resistance by sampling several resistant strains bearing an $Ace.1^R$ allele from various geographical areas. Our goal was to detect variation in dominance levels and to study their molecular basis. We addressed the following points: (1) is there variation in the dominance level of resistance due to AChE insensitive targets? (2) what is the molecular basis of this variation? and (3) can dominance evolve in this system?

MATERIALS AND METHODS

Mosquito strains: Six strains of mosquitoes were used: S-LAB, an insecticide-susceptible strain from California (GEORGHIOU *et al.* 1966); MSE, collected from southern France in 1979 (RAYMOND *et al.* 1986; BOURGUET *et al.* 1996d); ESPRO, collected in Tunisia in 1993 (BEN CHEIKH and PASTEUR 1993); SUPERCAR, collected from Ivory Coast in 1994 (F. CHANDRE, unpublished data); ACE-R, collected in Cyprus in 1993 (WIRTH and GEORGHIOU 1996) and PADOVA, derived from a natural population collected in Padova (Italy) in 1994 by C. SEVERINI. This last population was mass-selected during >30 generations by exposing fourth instars to propoxur doses that induced 60–90% mortality. Finally, offspring from four eggrafts, which were all $Ace.1^{RR}$ (homozygous for resistance), were raised separately to generate the PADOVA strain. For simplicity, these six strains are subsequently designated as S, R1, R2, R3, R4 and R5 for, respectively, S-LAB, MSE, ESPRO, SUPERCAR, PADOVA and ACE-R.

The susceptible strain S possesses only the sensitive AChE1 allele (and is thus homozygous $Ace.1^{SS}$) and the resistant strains (R1–R5) possess only insensitive AChE1 (and are homozygous $Ace.1^{RR}$). To obtain heterozygous individuals $Ace.1^{RS}$, males of each resistant strain were mass-crossed with S females. Offspring were designated as R1-F1, R2-F1, R3-F1, R4-F1 and R5-F1 depending on the resistant strain used as male parent. Males R1-F1 and R3-F1 were backcrossed with S females. Offspring were designated as R1-BC and R3-BC.

In addition, two natural populations were sampled: "Viols le Fort" (VLF, 43.46N, 3.27E) and "Notre Dame de Londres" (NDL, 43.49N, 3.47E) both from Hérault (Southern France). Larvae were collected on 15 May 1996 at VLF and 17 May 1996 at NDL, and fourth instars were submitted to insecticide bioassays the day of collection.

Insecticide bioassays: Insecticide bioassays were performed in plastic cups on fourth instars using propoxur (Bayer, Leverkusen, Germany). In all bioassays, 20 larvae, in 100 ml of tap water solution, were exposed to the insecticide for 24 hr, and the final concentration of solvent (alcohol) was systematically adjusted to 1%. Mortality curves of each strain and their F_1 progeny were established by bioassays performed with propoxur concentrations giving between 0 and 100% mortality. Five replicates were done for each insecticide concentration tested. A control, where larvae experienced the same environmental conditions except for the presence of the insecticide, was run in each experiment. Mortality data were analyzed using the Log-Probit program of RAYMOND *et al.* (1993) based on FINNEY (1971). Mortality lines were considered identical when their parallelism was not rejected at the 0.05 probability level, and the 95% confidence limits of the resistance ratio included the value 1.

For each of the two field samples (NDL and VLF) and each of the two backcrosses (R1-BC and R3-BC), 10 bioassays (200 larvae tested) were performed at 30 mg/L propoxur. At this concentration, frequency of $Ace.1^{SS}$ genotypes can be estimated after 15 min by counting dead larvae since they do not survive more than 5 min (D. BOURGUET, personal observation). Once dead, $Ace.1^{SS}$ individuals were removed, leaving only $Ace.1^{RS}$ larvae (for R1-BC and R3-BC) and $Ace.1^{RS}$ and $Ace.1^{RR}$ larvae (for NDL and VLF). Mortality was then recorded after 24 hr and survivors reared to the adult stage to determine their *Ace.1* genotypes and their AChE1 activities.

Dominance of insecticide resistance: Dominance levels were measured as previously described by BOURGUET *et al.* (1996a) and BOURGUET and RAYMOND (1997): $D = (LC_{F1} - LC_S)/(LC_R - LC_S)$, where LC_S, LC_R and LC_{F1} are the insecticide concentrations needed to obtain a given mortality level for susceptible, resistant and F_1 mosquitoes, respectively. *LC* values are expressed in terms of their logarithms. When mortality curves were not linear, *LC*s were estimated directly from the curves at different mortality levels. *D* varies linearly between 0 (complete recessivity) to 1 (complete dominance).

AChE1 purification: To analyze the kinetic properties of AChE1, AChE was purified from heads of adult mosquitoes that almost lack AChE2 (BOURGUET *et al.* 1996b). For each strain, ≈1000 heads were homogenized in 20 ml extraction buffer (20 mM Tris pH 7.0, containing 0.1% Triton X-100) with a glass pestle. Homogenates were centrifuged at 10,000 × *g* for 5 min. Supernatants were loaded on affinity chromatography columns containing procainamide (Sigma), a specific ligand of the AChE active site (PASTEUR *et al.* 1996). Columns were washed with 20 mM Tris-HCl pH 7.0, 0.1% Triton X-100, and the bound enzyme was eluted with 50 mM Tris-HCl pH 9.5, 0.4% CHAPS (Sigma), 2.5 mM decamethonium (Sigma). The eluted fractions were dialyzed three times during 2 hr against the extraction buffer resulting in a 10^6 fold dilution of small molecules. The resulting purified AChE was used for kinetic analyses of AChE1.

Evaluation of catalytic parameters: AChE1 activities were determined by the photometric method of ELLMAN *et al.* (1961). AChE1 enzymatic activity of each strain (five replicates) was measured using acetylthiocholine (AcSCh), butyrylthiocholine (BuSCh), propionylthiocholine (PrSCh) and ortho-nitrophenyl acetate (Onpa) at a concentration of 1 mM (all substrates from Sigma). AChE1 substrate preferences were calculated using AcSCh as reference, *i.e.*, by computing the following activity ratios (in $\mu M \cdot min^{-1}$): (BuSCh/AcSCh), (PrSCh/AcSCh) and (Onpa/AcSCh). For each strain, bimolecular rate constants (*ki*'s) for three carbamates (eserine, aldicarb and propoxur) and two oxon forms of organophosphates (malaoxon and paraoxon) were estimated for AChE1 following the dilution method of ALDRIDGE (1950). Briefly, purified AChE1 were incubated with the inhibitor for various times before adding 100 μl of these inhibition mixtures into 1 ml of a substrate-reagent solution [final concentration: 1.7 mM 5,5′-dithiobis-2-nitrobenzoic acid (DTNB); 2.5 mM AcSCh]. The plot of the natural logarithm of residual activity (Ai/Ao) against time, for a given inhibitor concentration, is linear when only one AChE1 is present (sensitive or insensitive). The slope of the line divided by the inhibitor concentration gives the *ki*.

AChE1 activity and identification of *Ace.1* genotypes in single mosquitoes: In single mosquitoes, the relative activity of sensitive and insensitive AChE1 can be estimated by using two discriminating concentrations of propoxur (BOURGUET *et al.* 1996c). (1) At 0.1 mM propoxur, sensitive AChE1 is fully inhibited whereas insensitive AChE1 and AChE2 are not affected, and (2) at 10 mM propoxur, AChE2 is inhibited whereas insensitive AChE1 is still not affected. Single adult mosquitoes were homogenized in 250 mM phosphate buffer containing 1% Triton X-100 and centrifuged for 5 min at 10,000 × *g*. Supernatants were used to estimate AChE activities both without insecticide (A0) and in the presence of 0.1 mM (A1) and 10 mM (A2) propoxur. Activities were measured on a Spectramax 250 (Molecular Devices) at 412 nm over a period of 15 min as indicated by BOURGUET *et al.* (1996c).

Genotypes were determined using AcSCh as a substrate (3 mM). Absence of A2 activity (*i.e.*, absence of insensitive AChE1) corresponds to $Ace.1^{SS}$ genotypes. If A2 is not null, A0 and A1 are compared: identical activities (*i.e.*, indicating an absence of sensitive AChE1) corresponds to $Ace.1^{RR}$ genotypes, whereas a lower A1 activity (*i.e.*, indicating the presence of sensitive AChE1) is characteristic of $Ace.1^{RS}$ genotypes. Sensitive and insensitive AChE1 activities correspond to (A0 - A1) and A2, respectively. Activities were measured either using AcSCh (1 mM) or Onpa (1 mM) as a substrate, and were weighted by the protein concentration using the bicinchoninic acid protein assay (Pierce, Interchim).

RESULTS

Dominance of propoxur resistance: Propoxur mortality lines for parental strains and their F_1s are presented in Figure 1. All mortality curves from the parental strains were linear following Log-Probit transformations ($P > 0.1$) and their parallelism was not rejected ($P > 0.9$). For F_1s, mortality curves were linear functions of the insecticide dose for R1-F1, R3-F1, R4-F1 and R5-F1 ($P > 0.1$) but not for R2-F1 ($P < 0.001$), which rose and fell with dosage. This latter phenomenon is neither artefactual nor due to the heterogeneity of parental strains (BOURGUET *et al.* 1997)

R3-F1 and R4-F1 heterozygotes displayed mortality lines not different from their resistant parental strains (parallelism not rejected: for R3-F1, $\chi^2 = 0.11$, d.f. = 3, $P > 0.9$ and for R4-F1 $\chi^2 = 0.26$, d.f. = 3, $P > 0.9$; the ratio of the LC_{50}s being not different from 1, $P > 0.1$). Thus, dominance of propoxur resistance was ≈1 and constant for R3 and R4 strains (Figure 1, C and D). Mortality curves displayed by larvae from R1-F1 and R5-F1 were distinct from their resistant parental strains (parallelism rejected: for R1-F1, $\chi^2 = 27.3$, d.f. = 4, $P < 0.0001$ and for R5-F1, $\chi^2 = 9.62$, d.f. = 4 , $P < 0.05$; the ratio of the LC_{50}s being different from 1, $P < 0.05$) so that dominance levels were monotonic functions of mortality (Figure 1, A and E). For the R2 strain, dominance was a non-monotonic function of the dose (Figure 1B).

To understand the physiological basis underlying these different dominance levels, AChE1 properties of susceptible and resistant strains were compared.

Comparison of AChE1 properties in susceptible and resistant strains: AChE1s of resistant and susceptible strains were purified and their catalytic properties analyzed. For each strain and insecticide (propoxur, eserine, malaoxon, paraoxon and aldicarb), the plot of the natural logarithm of residual AChE1 activity against time was linear (details not shown). This indicates that all strains are homozygous at the *Ace.1* locus. For all insecticides, the inhibition constant, *ki*, of AChE1 purified from resistant strains was lower ($P < 0.0001$) than that from the susceptible strain S (Table 1). This confirms that all resistant strains carried a modified AChE1 with less efficient binding to insecticides. Abilities to degrade the substrate were also significantly ($P < 0.0001$) affected as these modified AChE1s degrade propionylthiocholine and butyrylthiocholine less efficiently and ortho-nitrophenyl acetate more efficiently

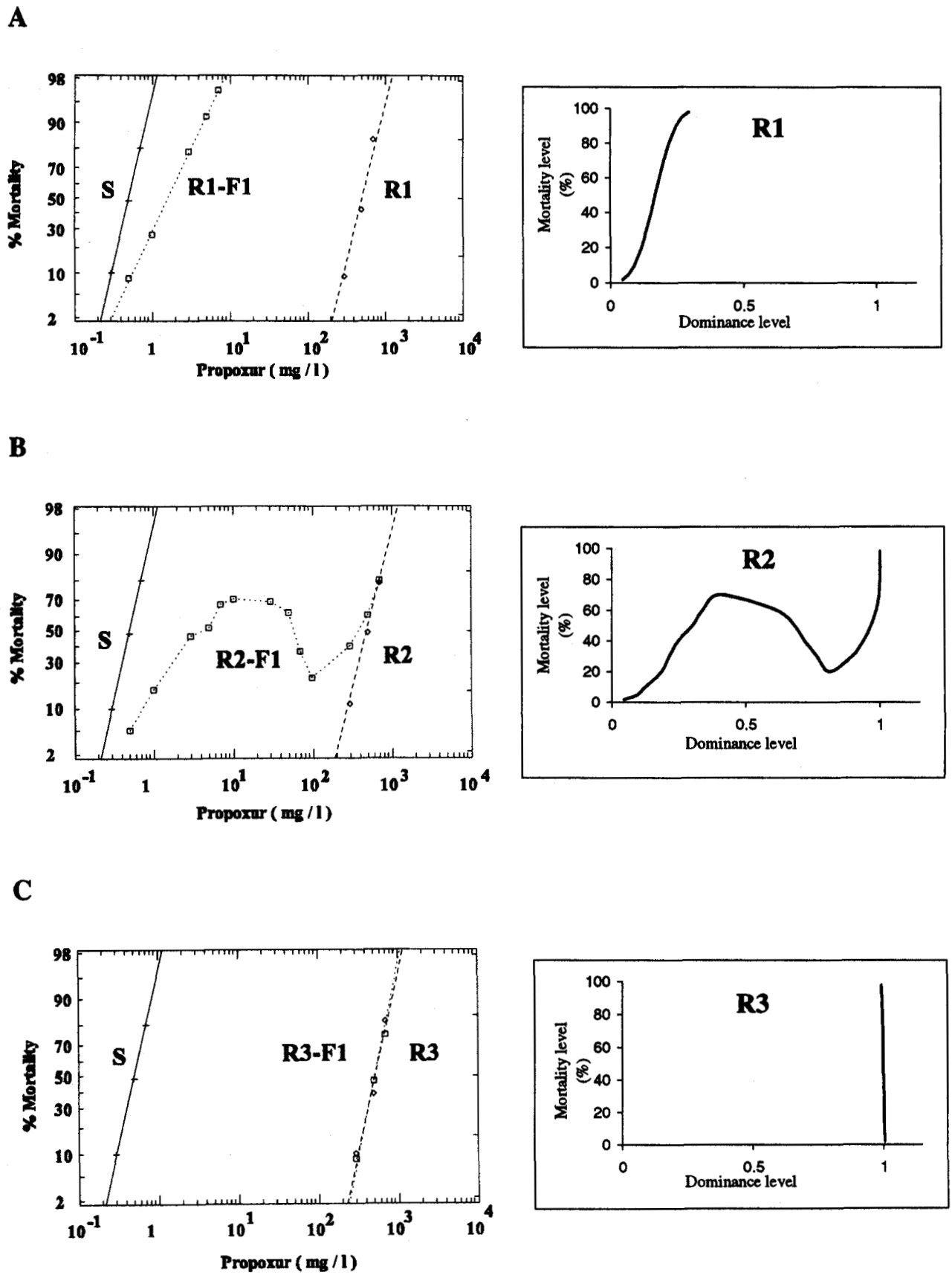

FIGURE 1.—Insecticide (propoxur) mortality curve (left panels, Log-Probit scale) and dominance level (right panels, linear scale) for the different resistant strains using the susceptible strain S (S-LAB) as reference. (A) R1, MSE; (B) R2, ESPRO; (C) R3, SUPERCAR; (D) R4, PADOVA; (E) R5, ACE-R.

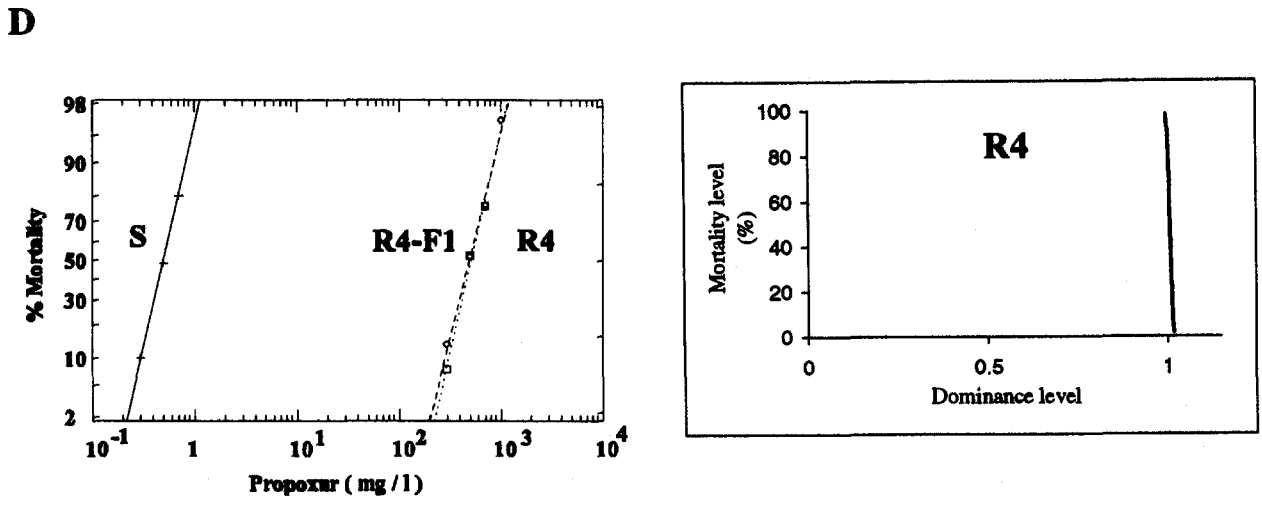

FIGURE 1. *Continued*

than the sensitive AChE1 of the susceptible strain S (Table 2).

AChE1s purified from four of the five resistant strains displayed identical catalytic properties: their inhibition constants (Table 1) for the five inhibitors were not different ($P > 0.96$) as well as their substrate preferences (Table 2) ($P > 0.87$). The only exception was the strain R5, with distinct inhibition constants and distinct substrate preferences.

Differences between resistant strains possessing an AChE1 enzyme with identical modified catalytic properties (*i.e.*, R1, R2, R3 and R4) were further investigated

TABLE 1

Bimolecular rate constants for different insecticide inhibitors of AChE1 for the reference susceptible strain S and for the different resistant strains from various geographical origins

		ki (M^{-1} min^{-1})				
Strain	Origin	Propoxur	Eserine ($\times 10^{-3}$)	Malaoxon	Paraoxon	Aldicarb
S	California	145,000 ± 5,000	15,000 ± 760	1,200 ± 100	160,000 ± 10,000	6,400 ± 150
R1	Southern France	0.58 ± 0.10	53 ± 6	170 ± 10	230 ± 10	540 ± 30
R2	Tunisia	0.55 ± 0.06	57 ± 4	170 ± 10	240 ± 15	540 ± 10
R3	Ivory Coast	0.55 ± 0.03	57 ± 5	180 ± 20	230 ± 5	540 ± 20
R4	Italy	0.56 ± 0.05	53 ± 4	180 ± 20	240 ± 15	530 ± 20
R5	Cyprus	650 ± 20.00	730 ± 40	170 ± 15	12,000 ± 1,000	57 ± 5

Values are ± SE. *ki*, rate constants.

TABLE 2

Activity ratio of AChE1 for different substrates for the reference susceptible strain S and for the different resistant strains, R1–R5, relative to the acetylthiocholine activity

Strain	P/A	B/A	O/A
S	0.747 ± 0.008	0.149 ± 0.007	1.75 ± 0.06
R1	0.246 ± 0.004	0.096 ± 0.003	6.92 ± 0.51
R2	0.250 ± 0.011	0.097 ± 0.003	7.75 ± 0.85
R3	0.247 ± 0.005	0.096 ± 0.004	7.30 ± 0.28
R4	0.247 ± 0.006	0.095 ± 0.008	7.81 ± 0.85
R5	0.264 ± 0.005	0.143 ± 0.005	—

P, AChE1 activity using propionylthiocholine; A, AChE1 activity using acetylthiocholine; B, AChE1 activity using butyrylthiocoline; O, AChE1 activity using ortho-nitrophenyl acetate.

by studying activity in single individuals for acetylthiocholine (AcSCh) and ortho-nitrophenyl acetate (Onpa) (Table 3). To control for variation in size of each individual, enzymatic activities were weighted by the amount of total protein. When AcSCh was used as a substrate, AChE1 activity was lower in the resistant strains than in susceptible strain. The activity displayed by R1 or R2 mosquitoes represented ≈20% of that of susceptible (Table 3). This percentage reached 40% in R4 mosquitoes and 80% in R3. When Onpa was used as a substrate, activity was not different in S, R1 and R2 ($P > 0.5$) but it was higher in the two other resistant strains ($P < 0.001$).

To investigate the inheritance of variation in AChE1 activity, only R1 and R3 were chosen due to their difference in AChE1 activity. For such purpose, heterozygotes from S × R1 and S × R3 crosses as well as from S × (S × R1) and S × (S × R3) backcrosses were analyzed for their insensitive AChE1 fraction and their insecticide resistance. The percentage of insensitive AChE1 was not different between R1-F1 and R1-BC heterozygotes ($P > 0.3$) and was ≈21% of total AChE1 activity (Table 4). Similarly, the insensitive AChE1 fraction was not

TABLE 3

AChE1 activity using acetylthiocholine or Onpa as a substrate in the susceptible strain S and in different resistant strains, R1–R4

Strain	N^a	Activity ($\mu M \cdot min^{-1}$ per individual) AcSCh	Onpa	Ratio Onpa[b]
S	100	32.2 ± 0.8	57.1 ± 2.5	1
R1	100	6.8 ± 0.6	47.9 ± 6.2	0.8 ± 0.1
R2	100	7.2 ± 0.2	55.4 ± 4.2	1.0 ± 0.1
R3	100	25.7 ± 1.0	188.3 ± 7.9	3.3 ± 0.2
R4	100	13.4 ± 1.0	104.2 ± 4.2	1.8 ± 0.1

AcSCh, acetylthiocholine; Onpa, ortho-nitrophenyl acetate.
[a] Number of individuals analyzed.
[b] Onpa activity of the strain considered/Onpa activity of S.

TABLE 4

Percentage of insensitive AChE1 activities and mortality at 30 mg/L propoxur in heterozygotes derived from crosses between the susceptible strain S and the resistant strains, R1 or R3

Strain	Crosses[a]	Percentage insensitive AChE1[b]	Percentage mortality[b]
R1-F1	S × R1	21.4 ± 3.1 (122)	100 (60)
R1-BC	S × (S × R1)	20.8 ± 1.3 (35)	100 (152)
R3-F1	S × R3	43.8 ± 3.7 (85)	0 (60)
R3-BC	S × (S × R3)	42.5 ± 2.3 (31)	0 (142)

[a] Indicating the female parent first.
[b] Number of individuals analyzed in parentheses.

different ($P > 0.1$) in R3-F1 and R3-BC, and was higher ($P < 0.0001$) than in R1-F1 or R1-BC, representing >40% of total AChE1 activity (Table 4).

Insensitive AChE1 and survival to insecticide exposure: To investigate the relationship between the insensitive AChE1 fraction in heterozygotes and survival to insecticide exposure, bioassays (at 30 mg/L of propoxur) were performed on heterozygous larvae from laboratory crosses. None of the heterozygotes from R1-F1 and R1-BC (with ≈21% of insensitive AChE1) crosses survived the insecticide exposure (Table 4). Conversely, all heterozygotes from R3-F1 and R3-BC (with ≈40% of insensitive AChE1) survived at this insecticide concentration (Table 4).

To further investigate the relation between insecticide resistance and the relative activity of insensitive AChE1, an analysis of natural populations was undertaken. Before insecticide exposure, percentage of insensitive AChE1 was determined on adults emerged from VLF and NDL field larvae samples. Part of these field larvae were also subjected to insecticide bioassays at 30 mg/L propoxur. As expected, all susceptible *Ace.1*^{SS} mosquitoes were killed, whereas *Ace.1*^{RR} were unaffected. Mortality of *Ace.1*^{RS} (individuals displaying sensitive and insensitive AChE1) were 42% and 46% for NDL and VLF, respectively. Distributions of percentage of insensitive AChE1 in *Ace.1*^{RS} individuals before and after insecticide exposure were different in both sites (Mann-Whitney test: $P < 0.005$ for NDL and $P < 0.0001$ for VLF), and survivors were those displaying the highest percentage of insensitive AChE1 (Figure 2, A and B).

DISCUSSION

Our results show that the dominance level of insecticide resistance conferred by an insensitive AChE varies between recessivity to dominance. This situation is an opportunity to understand the molecular basis of variation of dominance level and the possible modality of its evolution.

Catalytic properties, AChE1 activity and dominance

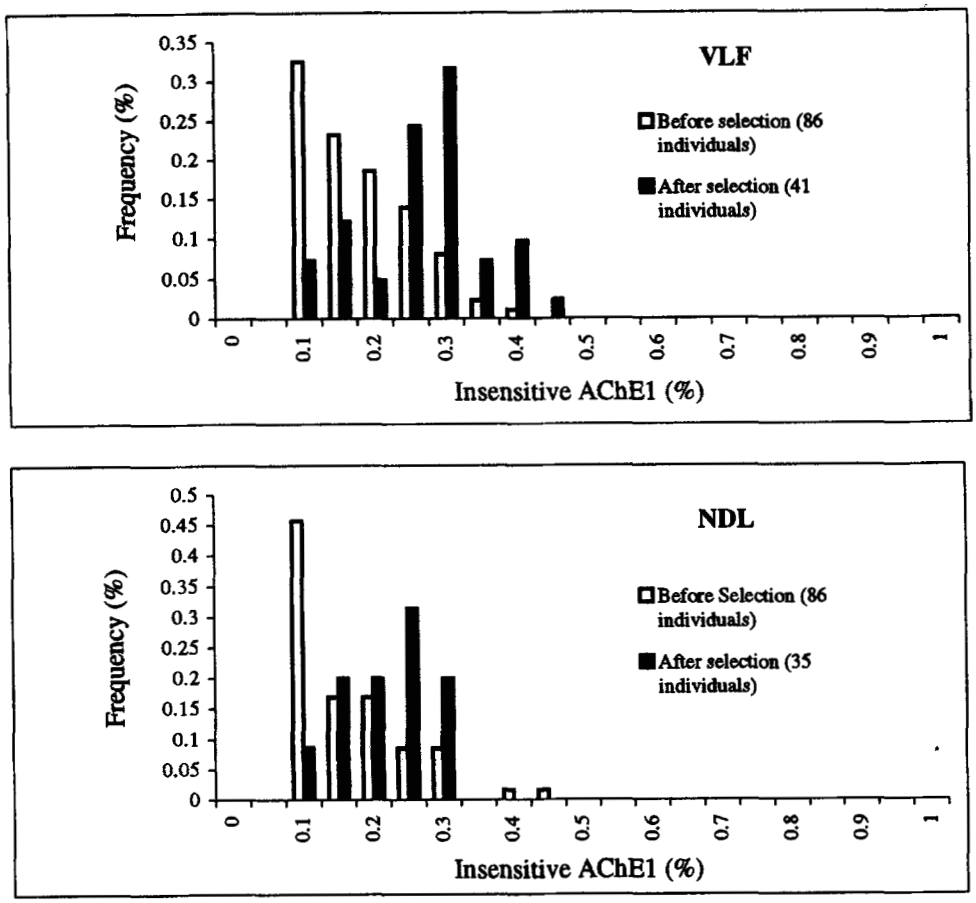

FIGURE 2.—Percentage of insensitive AChE1 in *Ace.1*^{RS} individuals from two field samples from Southern France: NDL ("Notre Dame de Londres") and VLF ("Viols le Fort").

level: Four of the five resistant strains (*i.e.*, R1, R2, R3 and R4) possess an insensitive AChE1 with identical catalytic properties, while the R5 strain possessed a differently modified AChE1 enzyme. Thus, if difference in catalytic properties may explain the variation in dominance level among *Ace.1*^{R} alleles (*e.g.*, the *Ace.1*^{R} allele in the R5 strain *vs.* those of the other strains), other explanations are required to explain differences in dominance level among R1, R2, R3 and R4. For these strains, our results suggest a link between AChE1 activity and dominance level. R3 and R4 display completely dominant-resistance alleles (Figure 1, C and D) and possess the highest AChE1 activity (Table 3). The correlation between AChE1 activity and dominance level is further suggested by the selection experiment of wild larvae since lower mortalities of *Ace.1*^{RS} (thus a higher dominance levels) are associated with higher AChE1 percentages (Figure 2).

A physiological explanation for variation of dominance level: Differences in dominance level were the consequence of variability in heterozygous mortality only, as the four resistant strains studied (*i.e.*, R1, R2, R3 and R4) displayed similar mortality curves (Figure 1). At first glance, it is puzzling that variation in AChE1 activity can change the insecticide survival of heterozygotes *Ace.1*^{RS} without affecting the insecticide survival of homozygotes *Ace.1*^{RR}. A logical explanation can however be proposed.

The modified AChE1 in strains R1-R4 is ≈300,000-fold less sensitive to propoxur than the sensitive AChE1 (Table 1). We have shown that in homozygotes *Ace.1*^{RR}, the lowest propoxur concentrations giving 100% mortality do not inhibit the insensitive AChE1, and that mortality is due to the inhibition of another target: the choline acetyltransferase (ChAT, E.C.2.3.1.6) (BOURGUET *et al.* 1997). As a consequence, mortality of homozygotes *Ace.1*^{RR} is independent of AChE1 activity, and the identical mortality curves of the four resistant strains indicates that the susceptibility of their secondary ChAT target is similar.

Heterozygotes *Ace.1*^{RS} possess both sensitive and insensitive AChE1s. When propoxur concentration increases, there is a progressive inhibition of the sensitive fraction until only the insensitive AChE1 remains active. For R1-F1 and R2-F1, the activity provided by this insensitive fraction (≈21% of the AChE1 activity) is probably not sufficient to maintain larvae alive. This conclusion is consistent with the minimal AChE activity necessary for life in other insect species: 30% in *Spodoptera littoralis* (ZAAZOU *et al.* 1973) and 25% in *Drosophila melanogaster* (HOFFMANN *et al.* 1992). For R3-F1 and R4-F1, the higher insensitive AChE1 activity (≈40%) is sufficient to maintain larvae alive once the sensitive AChE1 is fully inhibited. At higher propoxur concentrations, mortality occurs via the inhibition of the ChAT enzyme. This is consistent with the similar mortality curves of R3 and

R3-F1 as well as R4 and R4-F1 (Figure 1) and the complete dominance ($D \approx 1$) of $Ace.1^R$ in these strains.

In conclusion, a difference in AChE1 activity does not modify $Ace.1^{RR}$ survival but affects $Ace.1^{RS}$ mortality: depending on the insensitive AChE1 activity, heterozygotes may or may not survive the complete inhibition of the sensitive AChE1. This explains why differences in AChE1 activity produce variation in dominance level.

Hypothesis for variation of AChE1 activity: Variation in AChE1 activity has two possible origins. It can result from differences in the amino acid substitutions that confer resistance or from additional mutation(s) that do not alter AChE1 insecticide sensitivity. The former hypothesis implies that distinct amino acid replacements modifying AChE1 insecticide sensitivity have been selected in R1-R4 resistant strains. This hypothesis cannot be formally rejected, but seems difficult to reconcile with the identical constant of affinity found for five insecticides belonging to two different classes of insecticides (carbamates and organophosphates) and the similar enzymatic activity for four different substrates. Mutations of pesticide target sites conferring insecticide resistance have been reviewed for acetylcholinesterase (FOURNIER and MUTÉRO 1994), acetolactate synthase (LEE *et al.* 1988; GUTTIERI *et al.* 1996), sodium channel (WILLIAMSON *et al.* 1996), chloride channel (FFRENCH-CONSTANT *et al.* 1996) and the D1 protein of photosystem II (ROCHAIX and ERICKSON 1988; KLESS *et al.* 1994; TREBST 1996). All these reviews show that each amino acid substitution gives a specific pattern of insecticide sensitivity. Conversely, a specific amino acid replacement is always associated with the same resistance pattern, even across species.

Thus, the identical catalytic properties of AChE1 found among the four resistant strains of *C. pipiens* strongly suggest that these enzymes share an identical amino acid replacement(s) conferring insecticide resistance. Similarly, the unique catalytic properties of the insensitive AChE1 enzyme of the R5 strain suggest different amino acid replacement(s). We infer that higher AChE1 activity in R3 and R4 strains (compared with R1 and R2 strains) results from additional mutations that increase AChE1 amount. Overproduction could result from alteration of regulation either by *cis*-acting factors, or from gene duplication as it was already found in Caribbean's populations of *C. pipiens* (BOURGUET *et al.* 1996e).

Several examples of overproduction of AChE enzymes have already been described. An increased AChE activity in resistant strains has been found in *D. melanogaster* strains selected for resistance to parathion and fenthion (EL-ABIDIN SALAM and PINSKER 1981). The increase in resistance was accompanied by a correlated increase in AChE activity that, after several generations of selection, was almost 10 times higher than before selection. Identically, TSAKAS (1977) reported an increase of the acetylcholinesterase gene dosage in the fruit fly *Dacus oleae* selected with organophosphates. Furthermore several authors have reported an increase in Vm of AChE from resistant strains (*e.g.*, VOSS 1980; OI *et al.* 1990) that may in some cases reflect an overproduction of the enzyme (FOURNIER and MUTÉRO 1994). Finally, FOURNIER *et al.* (1992) have shown that insecticide susceptibility is correlated with acetylcholinesterase amounts.

Toward an evolutionary explanation: WRIGHT (1929) defined the margin of error for an enzyme as the maximum decrease of the enzyme activity that can be tolerated without affecting the phenotype. Within this margin, deleterious mutations are recessive. HALDANE (1930) proposed that this "safety margin" results from selection, since a reduction of activity in the presence of mutations, environmental fluctuations, or both, would be at a disadvantage. Thus any mutant producing an increased amount of enzyme would be selected. KACSER and BURNS (1981), in their detailed biochemical theory of dominance, showed that the safety margin does not require natural selection. Their key conclusion was that wild-type enzyme activity is far in excess of that necessary as an *inevitable* consequence of the kinetic properties of metabolic pathways.

This safety margin is also found for enzyme targets of pesticides. Less than 30% of AChE wild-type activity is sufficient to ensure viability in several species (see FOURNIER and MUTÉRO for review). Similarly *Saccaromyces cerevisiae* mutants with only 10% of the wild-type acetolactate synthase (an herbicide target-site) activity are viable (FALCO and DUMAS 1985). This excess of enzyme activity explains why most of enzyme target-modifications conferring insecticide resistance are mostly semi-dominant (BOURGUET and RAYMOND 1997). However, in this paper, we show that this safety margin is not always sufficient to keep heterozygotes alive so that resistance may appear as semi-recessive. This insufficiency in heterozygotes is the result of mutations associated with a strongly reduced enzymatic activity.

For such mutations, our data suggest that the safety margin may be increased by enzymatic overproduction. By conjecturing that R1-R4 resistant strains possess the same mutation(s) conferring insecticide resistance, additional mutation(s) or mechanisms that increase AChE1 production would formally be considered modifiers of dominance. Interestingly, such modifiers will probably also be selected in the absence of insecticide as they may decrease the fitness cost associated with AChE1 insensitivity (unpublished data).

Dominance modifiers have been previously described in pesticide-resistance systems. They correspond to additional resistance mechanisms affecting homozygotes (SS and RR) and heterozygotes (RS) differentially. GRIGOLO and OPPENOORTH (1966) and RUPES and PINTEROVA (1975) have shown that, in houseflies, a detoxification mechanism increased the dominance level of an insensitive target gene. They found that recessive resis-

tance to DDT conferred by sodium channel modification (*kdr*) was rendered more dominant in the presence of a DDT-ase, an enzyme of detoxification giving by itself a low resistance level only.

Therefore insecticide resistance conferred by modification of target sites gives new insights on variation of dominance and its possible evolution. From this perspective, dominance of insecticide resistance resembles the dominance of industrial melanisms where evolution of dominance is either caused by closely linked modifiers or by competition between dominant and nondominant alleles (reviewed by MAYO and BÜRGER 1996). This strengthens the hypothesis of HALDANE (1956) who suggested that when a gene sweeps through a population as a result of natural selection, heterozygotes are very frequent, providing opportunities for more intense selection for modification of the heterozygote and hence rapid evolution of dominance. For example, the frequency of heterozygote $Ace.1^{RS}$ in the southern France treated area was between 15% and 50% during the decade 1986–1996 (MAGNIN 1986; CHEVILLON *et al.* 1995, T. LENORMAND, T. GUILLEMAUD, D. BOURGUET and M. RAYMOND, unpublished data), probably due to the constant influx of susceptible mosquitoes from the adjacent nontreated area. This indicates that $Ace.1^{RS}$ frequency is not a limiting condition for the possible evolution of dominance modifiers in the mosquito *C. pipiens*.

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