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**Gene-dosage effects on fitness in recent adaptive
duplications: *ace-1* in the mosquito *Culex pipiens***

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Abstract

Gene duplications have long been advocated to contribute to the evolution of new functions. The role of selection in their early spread is more controversial.

Unless duplications are favored for a direct benefit of increased expression, they are likely detrimental. In this paper, we investigated the case of duplications favored because they combine already functionally divergent alleles. Their gene-dosage/fitness relations are poorly known, because selection may operate on both overall expression and duplicates relative dosage.

Using the well-documented case of *Culex pipiens* resistance to insecticides, we compared strains with various *ace-1* allele combinations, including two duplicated alleles carrying both susceptible and resistant copies. The overall protein activity was nearly additive, but, surprisingly, fitness correlated better with the relative proportion of susceptible and resistant copies rather than any absolute measure of activity. Gene dosage is thus crucial, duplications stabilizing an 'heterozygote' phenotype. It corroborates the view that these were favored because they fix a permanent heterosis, thereby solving the irreducible trade-off between resistance and synaptic transmission. Moreover, we showed that the contrasted successes of the two duplicated alleles in natural populations depend on genetic changes unrelated to *ace-1*, confirming the probable implication of recessive sublethal mutations linked to structural rearrangements in some duplications.

Key words: resistance gene, fitness cost, gene duplication, gene-dosage, overdominance.

Introduction

The role of gene duplications in the evolution of new functions, organismal complexity and adaptation has long been advocated (Ohno 1970; Lynch and Conery 2000; Conant and Wolfe 2008). Several authors suggested that selection plays a role in early duplication evolution, i.e. in their initial fixation (segregation avoidance models; Haldane 1954; Spofford 1969) rather than chance (Ohno 1970; Walsh 1995; Zhang 2003; Kondrashov and Kondrashov 2006). Today the evolution of duplication and new function remain controversial, owing to the large number of possible evolutionary scenarios that can operate (Bergthorsson et al. 2007; Labbé et al. 2007b; Hahn 2009; Innan and Kondrashov 2010; Kondrashov 2012).

Duplications are indeed likely to be non-neutral when they arise: unless they are tightly regulated, their immediate effect is to increase the duplicated gene expression. As it is the case for random mutation in general, such genetic change is likely to be deleterious, either because of the unnecessary overexpression cost or because of a disruption in gene-dosage (Papp et al. 2003; Wagner 2005; Kondrashov and Kondrashov 2006; Sopko et al. 2006; Conant and Wolfe 2008). For instance, in the human *PMP22* gene, an increased dosage by heterozygous duplication causes Charcot-Marie-Tooth type 1 disease, while a decreased dosage by heterozygous deletion causes an hereditary neuropathy (Lupski and Stankiewicz 2005).

However, in the subset of duplications that spread –and eventually fix– in populations, the change in gene-dosage may not be too large a handicap or may even be the reason of its selection. When expression is tightly regulated, duplications may have no strong phenotypic impact and spread neutrally despite the change in gene-dosage. However, the increased expression caused by duplications may be

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directly selected for this reason. This is a widespread mechanism (see Kondrashov et al. 2002). For instance, the number of copies of the amylase gene *AMY1* increases with starch amount in the diet (Perry et al. 2007). Understanding the gene-dosage impact on the fate of duplication is thus fairly straightforward when the two duplicates are identical to start with: in these cases, the evolution of new function requires subsequent divergence of the duplicates (Force et al. 1999; Lynch and Conery 2000; Otto and Yong 2002; Zhang 2003; Taylor and Raes 2004; Ward and Durrett 2004; Lynch and Katju 2004; Rastogi and Liberles 2005; Bergthorsson et al. 2007; Cusack and Wolfe 2007; Conant and Wolfe 2008; Storz 2009; Innan and Kondrashov 2010; Katju 2012).

Less straightforward is the case where duplications are initially favored because they combine already functionally divergent alleles (i.e. segregation avoidance models; Haldane 1954; Spofford 1969; Lenormand et al. 1998a; Labbé et al. 2007a; Remnant et al. 2013). In this case, the gene-dosage/fitness relations are very poorly known. When the two duplicates (say R and S) are divergent (with expression E_R and E_S), the gene-dosage fitness relationships become more complex, as selection may operate on overall expression level ($\sum E_R + \sum E_S$) as well as on relative dosage between R and S ($\sum E_R / \sum E_S$) in the various possible diploid genotypes. The latter possibility is particularly important in situations where selection favors expression of both R and S (which is the main condition favoring the fixation of duplication in segregation avoidance models; Haldane 1954; Spofford 1969). For instance, selection on overall expression level may have deleterious consequences (by causing a departure from the wild-type gene-dosage and expression), while co-expression of divergent duplicates may be favorable. In this (or similar situations), further evolution would be expected in order to tune overall expression as well as

relative duplicate expression. Such expression repatterning is expected to be fast when duplications are initially favored by the heterotic advantage of combining divergent duplicates.

Examples of duplication favored by this heterotic advantage are the *ace-1* duplications in the mosquito *Cx. pipiens* (Lenormand et al 1998, Labbé et al. 2007). In this system several recent and still polymorphic duplications have been selected worldwide, providing natural replicates of duplication early evolution. In this paper, we took advantage of this unique system to understand the gene-dosage/fitness relationships of young duplications under selection.

We briefly present this system as it is well described in Labbé et al. (2007b). *ace-1* duplications recently evolved (<40 years) in the context of resistance to organophosphate (OP) and carbamate (CX) insecticides in several mosquito species (Labbé et al. 2007a,b; Djogbénou et al. 2008, 2009; Alout et al. 2010; Osta et al. 2012). The target of these insecticides is a synaptic enzyme, the acetylcholinesterase (AChE1), encoded by the *ace-1* locus (Weill et al. 2002). A single nucleotide mutation (G119S) –which reduces AChE1 affinity for the insecticide molecules– has been repeatedly selected in treated natural populations of several mosquito species (Weill et al. 2003, 2004a; Alout et al. 2007). It is associated with more than 60 % activity reduction of the mutated AChE1, as compared to the susceptible one (Bourguet et al. 1997; Alout et al. 2008). This lower activity is probably the cause of this resistance allele (*ace-1^R*) high fitness cost in absence of pesticide revealed both by field surveys (Lenormand et al. 1998b) and laboratory experiments (Berticat et al. 2002; Bourguet et al. 2004; Duron et al. 2006). Duplications of the *ace-1* locus arose in the 90's, combining copies of both the resistant (*ace-1^R*) and the susceptible (*ace-1^S*) alleles on the same chromosome. As of today, 13 distinct duplicated alleles (globally named

ace-1^D) have been identified in both *Cx. p. quinquefasciatus* and *Cx. p. pipiens* subspecies (Bourguet et al. 1996b; Lenormand et al. 1998a; Labbé et al. 2007a; Alout et al. 2010; Osta et al. 2012); a similar duplication has been found in Western African *An. gambiae* (Djogbénou et al. 2008, 2009). These duplications do not segregate at detectable rates in laboratory crosses, they behave as ‘alleles’, at least at the scale of few generations (Labbé et al. 2007a,b). The present study focuses on two of these alleles. One arose in Martinique (D_1) and rapidly replaced *ace-1^R* in natural populations of *Cx. p. quinquefasciatus* (Yébakima et al. 1995, 2004). In natural populations of *Cx. p. pipiens* in the South of France, the other allele (D_3) was selected for when rare, but did not reach high frequency. The D_3D_3 homozygotes indeed have a particularly low fitness (Labbé et al. 2007b). This low fitness in homozygotes was hypothesized to be the consequence of D_3 being associated with an inversion carrying a recessive sub-lethal mutations (gene disruption at breakpoints or hitch-hiking deleterious allele; Lenormand et al. 1998a; Labbé et al. 2007b).

Using laboratory crosses of isogenic strains, we analyzed this system to specifically investigate three questions. First we investigated whether the quantity of protein activity is proportional to the gene copy number of each duplicate in various genotypes, in order to determine whether there was a specific regulation associated to duplicated alleles. Then, we investigated the fitness impact of the different diploid combinations of duplicates and single copies and their relations to gene-dosage and protein activity. This was done in presence (resistance measurements) or absence of pesticides (life history traits), as both environments are relevant to understand these duplications evolution. Third, we investigated how these results relate to the field evolution of these duplications. We finally examine how this case study informs us

more generally on the fitness impact of gene-dosage alterations on divergent duplicates.

Methods

Mosquito strains and crosses

Experiments were conducted with four homozygous strains, and five F1 offspring of crosses between these strains, i.e. on nine different *ace-1* genotypes (Supplementary Materials Fig.S1). The strains used were: SLAB, the reference susceptible strain (Georghiou et al. 1966), homozygous for *ace-1^S* (SS); SR, homozygous for the resistance allele *ace-1^R* (RR) (Berticat et al. 2002); BIFACE-DFix, homozygous for *ace-1^{D3}* (D₃D₃), a duplicated allele from Montpellier area (Labbé et al. 2007b); and DUCOS-DFix, isolated from the Martinique strain DUCOS and homozygous for the duplicated allele *ace-1^{D1}* (D₁D₁) (established following the protocol of Labbé et al. 2007b, using specific PCR tests described in Supplementary Materials S2 and Fig.S3). The five heterozygous genotypes (RS, D₁S, D₁R, D₃S and D₃R) were the F1 of mass crosses between these different strains (Slab/SR, DUCOS-DFix/Slab, DUCOS-DFix/SR, BIFACE-DFix/Slab and BIFACE-DFix/SR, respectively). SR and the two strains with duplicated alleles have been backcrossed for at least 14 generations with SLAB before fixation of their *ace-1* allele. Thus the nine genotypes studied shared the same genetic background and differed from one another almost only by their *ace-1* genotype.

Measure of AChE1 activity

The AChE1 activity was measured using the procedure described by Bourguet et al. (1996a) to test for an effect of gene-dosage. Briefly (details in Supplementary

Materials S4), ethanol and propoxur (CX, Baygon™, Chem-Service, 99 %) were added to two wells of a microtitration plate containing extracts from the same mosquito. After incubation, a substrate solution (DTNB + acetylthiocholine) was added to each well to measure AChE1 activity. The first well (ethanol) provides the total activity $A_{TOT} = A_S + A_R$ (A_S being the activity of AChE1S, susceptible, and A_R that of AChE1R, resistant), whereas the second one (propoxur) provides A_R only. Note that activity in the second well is never equal to 0: even susceptible individuals present a very low slope, due to the spontaneous degradation of DTNB.

We first analyzed AChE1R activity (A_R) using a linear model:

$$A_R = NR + ERS + ED_1S + ED_1R + ED_1D_1 + ED_3S + ED_3R + ED_3D_3 + \varepsilon$$

It includes the number of R copies (NR , reflecting the sum of activity of each R copy), the departures from additivity occurring in the different possible genotypes (E_{IJ} terms) and a normal error parameter (ε). The model without any E_{IJ} departures term (i.e. $A_R = NR$) was used to infer the activity of one R copy under a strictly additive model.

Similar models were then used to analyze AChE1S activity (A_S), as a function of the number of S copies (N_S) of each genotype, and the activity of one S copy under a strictly additive model.

For clarity, and because there are sex differences, we report separate analyses for males and females. This measure has been shown to be reproducible and independent of the mosquito size (Alout et al. 2008). Moreover, the densities and conditions of rearing were controlled to ensure they were similar for the various genotypes.

Insecticide resistance

To assess the impact of gene-dosage on resistance, bioassays were performed on batches of 20 young 4th instar larvae of the nine genotypes in 100 mL of water in plastic cups, as described in Raymond and Marquine (1994). Each bioassay included at least 4 replicates for at least 6 insecticide concentrations, inducing mortality between 0 and 100 %. The final concentration of solvent (ethanol) was systematically adjusted to 1 % for standardization. Mortality was recorded after 24 h exposure. Two of the most-used OP insecticides were tested, Temephos (Pestanal®, Riedel-de Haën, 96.4 %) and Chlorpyrifos-ethyl (Chem Service, 99.5 %).

Dose-mortality responses observed for the 9 genotypes were then compared using the GLM $MORT = GENO + \log(DOSE) + GENO.\log(DOSE) + \varepsilon$, where MORT is the proportion of dead larvae, GENO the genotype tested and DOSE the amount of insecticide. The “.” denotes the interaction of the two variables. ε is the error, following a quasi-binomial distribution to take overdispersion into account, if present. The lethal dose for 50 % of the individuals (LD_{50}) was computed for each genotype from the dose-mortality responses, using the dose.p function (MASS package, Venables and Ripley 2002) in the R free statistical software (v 2.15.1, <http://www.r-project.org>).

Pre-imaginal mortality

To assess the fitness cost associated with variation in gene-dosage, the various genotypes pre-imaginal mortality was measured, following the protocol developed by Agnew et al. (2004). Females' oviposition was synchronized for the nine genotypes, and single L1 larvae were isolated in standard *Drosophila* tubes

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containing 4 ml of mineral water. Food was provided once by adding 1 ml solution containing 2 mg TetraMin® powdered fish food/larva. Tubes were stored on racks (three racks per genotype) holding 40 tubes and arranged on a single shelf (25°C, 12:12h light:dark, >60 % humidity), with 3 racks (i.e. 120 larvae) per genotype ($N_{\text{total}} = 1080$ larvae). Racks were randomly distributed. Numbers of emerging adults were recorded. The percentage of mortality before emergence was estimated by the ratio of the number of emerging adults over the initial number of larvae in each rack.

Gene-dosage and fitness

To explore how gene-dosage impacts on fitness, we used regression models of resistance and cost proxies on various predictors connected to different biological explanations. All computations were performed using the R free software (v 2.15.1, <http://www.r-project.org>) and the models were simplified according to Crawley (2007) (i.e. using *F*-tests, non-significant terms were removed starting from the higher-order, and non-different factor levels of qualitative variables were grouped). When applicable, the residuals were tested for normality (Shapiro and Wilk 1965) and homoscedasticity (Breusch and Pagan 1979).

Results

AChE1 activities: close to additivity.

The AChE1 activity was measured on single mosquitoes (Bourguet et al. 1996a) from nine different *ace-1* genotypes, combining two duplicated alleles (D_1 and D_3) as well as susceptible and resistant reference alleles (S and R). For each genotype and sex, the total AChE1 activity (A_{TOT} , see Supplementary Materials, Fig.S5; the number of individuals analyzed for each sex and genotype are indicated)

was decomposed into the activities due to the resistant (A_R) and susceptible (A_S) AChE1 (Fig.1), to assess whether A_{TOT} followed an additive model (i.e. the sum of each copy activity) or was regulated following gene-dosage modifications. This analysis confirmed that the AChE1 activity corresponding to one R copy is approximately a fifth of that corresponding to one S copy (Table 1, Bourguet et al. 1996a). However, it showed that, although close to it (Fig.1), A_S and A_R are not strictly the sum of the activity of each S or R copy present in the different genotypes. Despite large variance of activities within a genotype, there were indeed significant departures from the additivity hypothesis (Table 1; Fig.1) suggesting that the total AChE1 activity may somehow be slightly regulated. All genotypes including duplicated haplotypes indeed displayed lower activities than expected, with departures larger for males than females, and for AChE1S than AChE1R. Finally, individuals carrying $ace-1^{D1}$ displayed consistently slightly lower activities, either at homozygous or heterozygous states, than individuals carrying $ace-1^{D3}$ (Fig.1), suggesting some minor differences between the two duplicated haplotypes. Nevertheless, these departures again were generally mild and overall an increased number of copies proportionally increased the genotype total activity.

Resistance and fitness cost depend on the ratio of R and S copies

To understand how gene-dosage modifications impact fitness, we investigated the resistance (fitness advantage) and the larval mortality (fitness cost) of the nine genotypes. As it is very difficult to predict from existing information what should be the fitness effects of the genotypes carrying the duplications and single copy alleles various diploid combinations, we tested various hypotheses: fitness could be proportional to either the number of R copies (nR), or the percentage of R copies

[$\%R = nR/(nR+nS)$], or the resistant activity (A_R), or the percentage of resistant activity ($\%A_R = A_R/A_{TOT}$) or to the total AChE1 activity (A_{TOT}). We used regressions of resistance (LD_{50}) and cost (pre-imaginal mortality) proxies on these predictors to identify the most likely link between gene-dosage and fitness.

Resistance to two OPs, Chlorpyrifos and Temephos was measured through bioassays (see Supplementary Materials Fig.S6). The results of the regressions between the various genotypes LD_{50} (see Supplementary Material Table S7) and the gene-dosage predictors are presented in Table 2 (the LD_{50} have been log-transformed for linearity). It appeared that the best predictor was by large the $\%R$ (Spearman's correlation parameter $r = 0.96$, $p < 0.001$ and $r = 0.95$, $p < 0.001$, for Chlorpyrifos and Temephos, respectively). No strong difference appeared in terms of resistance between the genotypes carrying the duplicated alleles D_1 or D_3 , neither at the heterozygous nor at the homozygous states (Fig.2A). The duplicated homozygotes DD presented an intermediate resistance, similar to that of the single-copy heterozygotes RS, while DR and DS individuals were slightly more and slightly less resistant than RS, respectively (Fig.2A). In all cases, individuals carrying a duplicated allele were less resistant than RR individuals.

To assess the impact of gene-dosage on fitness cost, we used the percentage of pre-imaginal mortality as a proxy for the cost (Agnew et al. 2004). There was almost one third more emerging adults in SS (18 % mortality) than in RR (45 % mortality) (Supplementary Materials, Fig.S8). This is similar to previous studies (Duron et al. 2006; Berticat et al. 2008) and evidences the cost associated to the single-copy resistance allele. Similarly to resistance, the best predictor of the fitness cost was the $\%R$ (Spearman's correlation parameter $r = 0.8$, $p < 0.001$, without D_3D_3 , see below and Table 2). Heterozygotes carrying either D_1 or D_3 duplicated alleles

were less or more costly than standard heterozygotes RS when carrying, respectively, a susceptible (DS) or a resistant (DR) single-copy allele, while D_1D_1 displayed a cost similar to RS (Fig.2B). However, D_3D_3 individuals displayed more than 60 % mortality (>RR individuals), in agreement with the large cost already described for the duplicated alleles from Montpellier area (Labbé et al. 2007b). This was the only marked difference between the two duplicated alleles (Fig.2B). In general, except for D_3D_3 , individuals carrying duplicated alleles displayed a reduced cost compared to single-copy RR.

Discussion

This study aimed at understanding the impact of gene-dosage/fitness relations in the early evolution of divergent duplicates. We analyzed several duplicated alleles of the *ace-1* gene in *Cx. pipiens* that carry both susceptible and resistant copies, and thus result in both quantitative and qualitative changes.

AChE1 activity in duplicated alleles is close to additivity

An expected immediate effect of duplication is to increase gene-dosage and thus protein expression. Our results show that *ace-1* gene-dosage modifications have indeed a strong impact on overall AChE1 activity (Fig.1 and Supplementary Materials, Fig.S5). The expression of both the susceptible and the resistant AChE1 proteins is increased: a DD individual displays a higher activity than a RS individual. Finally, the total activity of a given genotype was close to the sum of the activities provided by each copy (Fig.1 and Table 1).

However, the genotypes including a duplicated allele generally displayed slightly (and significantly) lower activities than expected under a strict additive model

(Fig.1 and Table 1). These decreases were more pronounced for individuals carrying *ace-1*^{D1} than for those carrying *ace-1*^{D3}, but remained moderate (Fig.1).

These slight departures from the additive model may indicate that the overall AChE1 expression is partly regulated, suggesting the existence of some limited cost associated with this overexpression. Although unlikely (see Labbé et al. 2007b), it may also be due to other mutations in the *ace-1* gene. Interestingly, individuals carrying D₁ collected in 1994 (Bourguet et al. 1996b) showed a higher activity ratio – i.e. total activity of DD over total activity of SS– than those collected in 2003 for the present study (activity ratio = 1.15 ±0.11 vs. 0.88 ±0.31, resp., assuming a stable activity for the wild-type). This could indicate that this partial expression regulation is a secondary modification of D₁. As both the AChE1R and AChE1S activities are decreased in duplicated alleles, this could be due to a modification of either the promoters (regulation of the transcription), or the translation rate and/or in the proteins recruitment. More studies are required to pinpoint the actual mechanism, with the additional difficulty that *ace-1* is mostly transcribed in the early larval stages and not in adults (Huchard et al. 2006).

Gene-dosage effect on fitness depends on copy composition

In the case of a duplication combining functionally divergent alleles, although the overall expression change is expected to impact the fitness, the relative expression of the divergent alleles is also likely to be under selection. The combined impact of overall vs. relative expression on fitness is not trivial: changes in overall and relative expression can be both beneficial or have antagonistic effects. The latter situation is probably at work with *ace-1* duplications: while the increase in overall AChE1 expression appears to be slightly detrimental –so that minor down-regulation

is suspected—, the composition of the various genotypes in terms of R and S copies is the major determinant of their fitness in presence or in absence of insecticides.

Both proxies of resistance (LD_{50}) and cost (pre-imaginal mortality) are indeed better correlated with the percentage of R copies (%R) among the various genotypes than with the number of R copies or any AChE1 activity predictor (Table 2). Thus, in spite of increasing the produced protein quantity, the relative proportions of the heterogeneous duplicate products are more important in terms of fitness than their absolute quantity. Indeed, DD and RS genotypes, which differ in the produced protein quantity ($DD > RS$) but have similar ratios for R and S copies (1:1), display similar resistances and costs (Fig.2). Moreover, in heterozygotes carrying a D allele, the nature of the single-copy allele associated is decisive: an R confers more resistance but a higher cost (reduced activity), while an S reduces both resistance and cost (Fig.2).

Synaptic AChE1 is probably limited

One possible interpretation of this unexpected pattern affecting both investigated fitness traits (resistance, cost) is that there is only room for a limited number of AChE1 molecules in the synapse, so that increasing the quantity of protein would reduce the relative part of each of them, i.e. like a dilution effect. For example, so many AChE1 molecules only could be released in the synapse or exposed on the membrane of the post-synaptic neuron (Bourguet et al. 1997), randomly picked from the pool produced in the cell (the one we measure). With such a mechanism, the phenotype will depend only on %R but not on total protein production (the number of protein 'slots' available in the synapse being fixed and independent of the protein quantity produced in the cell). As a duplicated allele produces both AChE1R and

AChE1S, the relative quantity of AChE1R would be reduced, thereby explaining the observed fitnesses.

This mechanism would thus explain how the proportion of R copies is the main determinant of fitness. However, while of much more limited fitness impact, the overall AChE1 expression may also be under selection. A decrease in overall activity, although limited, was indeed observed for individuals carrying both D₁ or D₃ (Fig.1). In a situation where overexpression of AChE1 proteins is wasteful (as it would be the case if protein production exceeds the quantity that can be packed in the synapse), we would expect such overall decrease in protein expression for the duplicated alleles, and thus in activity, to decrease the production cost. Confirming this expression regulation requires further studies, distinguishing the amount of proteins in synapses from the overall quantity produced in the cell, which may prove challenging.

In a larger perspective, it appears that the effect of gene-dosage on fitness is less straightforward when the duplicates are functionally different than when they are identical. When they are identical, only quantity matters: the gene-dosage change can be detrimental, e.g. the human *PMP22* gene (Lupski and Stankiewicz 2005), or selected for, e.g. increased quantity of detoxifying proteins through duplication is a common resistance mechanism in arthropod pests (reviews in Oakeshott et al. 2005; Labbé et al. 2011). Conversely, the *ace-1* duplications associate different copies: their net effects on fitness depend not on the overall activity, but on their ratio, a larger proportion of R copies increasing resistance and a larger proportion of S copies reducing the cost. A probable explanation is that the number of AChE1 proteins that can be present in the synapse is limited and drawn from a cytoplasmic pool where R and S are represented in proportion the number of gene copies.

Further (and limited) evolution of the expression of each duplicate independently may fine-tune the resistance/cost balance of duplicates in different ecological situations, explaining their widespread worldwide success.

D₃D₃ deleterious phenotype is unrelated to *ace-1*

One notable departure from the pattern described so far concerns D₃ homozygotes. When heterozygous, D₁ and D₃ are indeed very similar for resistance and display comparable costs. But while D₁D₁ individuals display only a moderate cost, D₃D₃ genotype is sublethal (Fig.2). However, the various genotypes, including D₃D₃, display AChE1 activities corresponding to their number of S and R copies. These observations thus confirm our previous hypothesis that D₃D₃ extreme deleterious effect is most probably independent of the *ace-1* locus itself, and probably due to a recessive sublethal mutation associated to *ace-1* during the chromosomal rearrangement that produced the duplication. As proposed by Labbé et al. (2007b), this chromosomal rearrangement could be an inversion, a phenomenon often associated with duplications (Katju and Lynch 2003; Ranz et al. 2007). By reducing recombination and preventing the break-up of the R-S heterotic combination, such inversion may even be favored. However, one major evolutionary drawback of inversions is that they can disrupt close genes or regulatory regions, or hitch-hike unbanishable deleterious mutations (Kirkpatrick and Barton 2006). D₁ appears to have escaped this unfortunate fate, explaining its success in Martinique (Yébakima et al. 2004), while D₃ stagnates below 20 % in Montpellier area (Labbé et al. 2007b). Thus, similar molecular processes can have dramatically different outcomes, and it remains to be known which of the successful or the unfit duplications are the most frequent.

Early rise of *ace-1* duplication is favored by overdominance

In general, individuals carrying duplicated alleles (D_1 or D_3) displayed resistances and costs very similar to those of standard heterozygotes, i.e. intermediate between RR and SS individuals (except for D_3D_3). Consequently, these duplications were not selected because they confer similar resistance level than RR homozygotes at a lower cost, as previously hypothesized (Bourguet et al. 1997; Weill et al. 2004b; Alout et al. 2008). They were advantaged because they confer a more favorable resistance/cost balance across treated and non-treated zones (overdominance). This result strongly corroborates the view that these duplications were favored because they allow the fixation of a permanent heterosis in this polymorphic gene (Haldane 1932; Spofford 1969), thereby solving the irreducible trade-off between resistance (R copy, treated areas) and optimal synaptic transmission (S copy, non-treated areas).

However, duplication remains a risky genomic rearrangement. First, it will disrupt the initial protein balance, which is likely to be deleterious. In our case, this effect is probably mild, as the number of AChE1 protein that can be packed in the synapse seems to be limited and independent of the quantity produced. Second, duplications are likely to arise in combination with chromosomal inversions that could associate them with deleterious mutations and prevent their fixation in natural populations, as shown by D_3 . This two effects could contribute to explain the discrepancies between the low rate of duplication measured in inter-specific comparisons and the comparatively large intraspecific diversity of copy-number variation uncovered by genomics (Freeman et al. 2006; Korbelt et al. 2008 and refs

39 to 55 in Katju 2012): only duplications passing the sieve of short-term selection would fix and become new material for longer-term evolution.

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TABLE 1. Additivity of AChE1 activities. The models tested were $AX = NX + ERS + ED_1D_1 + ED_3D_3 + ED_1S + ED_1R + ED_3S + ED_3R + \varepsilon$, where AX is the response variable (AR or AS), Nx is the number *ace-1* copies (NR or NS, respectively), and EIJ the specific effect of the genotype IJ, and ε the normal error (see Methods). Sexes were analyzed separately: males and females are indicated respectively by M and F in the Sex column. The magnitudes \pm the standard errors of the activity of one R or one S copy activities are indicated respectively in the R and S columns. Magnitudes \pm the standard errors and significances (^{NS} non significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) of the various genotype effects are also indicated for each model; they correspond to the increase or decrease in activity (if positive or negative, respectively) observed in the given genotype as compared to a strict additivity. Significant departures from a strict additivity are bolded. *b* is the intercept of the model, to account for the activity due to the spontaneous degradation of the substrate (see Methods). A dash (-) indicates that the effect was irrelevant, and thus not included, in the corresponding model. The percentage of the total deviance explained by the model (%TD) is also presented.

TABLE 1

Ax	Sex	Model											%TD
		b	R	S	ERS	ED ₁ D ₁	ED ₃ D ₃	ED ₁ S	ED ₁ R	ED ₃ S	ED ₃ R		
AR	M	1.4 ±0.2^{***}	4.4 ±0.2		-0.6 ±0.3 ^{NS}	-0.4 ±0.4 ^{NS}	-1.0 ±0.4^{**}	-0.2 ±0.3 ^{NS}	-1.2 ±0.4^{**}	-0.5 ±0.3 ^{NS}	-1.0 ±0.4[*]	84.26	
	F	1.2 ±0.2^{***}	3.5 ±0.2		-0.8 ±0.3^{**}	-0.3 ±0.3 ^{NS}	-0.1 ±0.3 ^{NS}	0.0 ±0.3 ^{NS}	-0.4 ±0.3 ^{NS}	-0.2 ±0.3 ^{NS}	-0.4 ±0.3 ^{NS}	80.88	
AS	M	0.4 ±1.2 ^{NS}		23.8 ±0.8	-1.0 ±1.2 ^{NS}	-15.2 ±1.3^{***}	-5.1 ±1.3^{***}	-9.5 ±1.3^{***}	-8.4 ±1.2^{***}	-8.4 ±1.3^{***}	-2.8 ±1.2[*]	88.38	
	F	0.4 ±1.0 ^{NS}		19.4 ±0.7	-2.8 ±1.1[*]	-10.8 ±1.2^{***}	-1.0 ±1.2 ^{NS}	-7.2 ±1.2^{***}	-6.3 ±1.1^{***}	-3.6 ±1.2^{**}	-2.4 ±1.1[*]	87.62	

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TABLE 2. Variance explained by the correlations between fitness components and gene-dosage or AChE1 activity. Fitness components tested are i) the resistance level (LD_{50} ; data were log-transformed for linearity) for Chlorpyrifos and Temephos insecticides, and ii) the cost, estimated by the pre-imaginal mortality (Mortality). The correlations between these fitness components and various predictors were independently measured by linear regressions. For each genotype, the predictors were the number of R copies (nR), the percentage of R copies (%R), the resistant activity (A_R), the total activity (A_{tot}) and the percentage of resistant activity (% A_R) (see text for details). For each predictor the percentage of the total variance explained (R^2) and the regression significance are indicated (p-value: *** < 0.001; ** < 0.01; * < 0.05; ^{NS} > 0.05). The best predictor, i.e. the regression with the maximum of variance explained, is bolded and illustrated in Fig.2 (note that these regressions remain significant when only DR, DS and DD genotypes are considered).

TABLE 2

Predictors	R ²		
	Ln(LD ₅₀) Chlorpyrifos	Ln(LD ₅₀) Temephos	Mortality
nR	0.583*	0.666**	0.587*** (0.50***) ^b
%R	0.917***	0.897***	0.636*** (0.29**) ^b
A _R	0.600*	0.699**	0.607*** (0.51***) ^b
%A _R	0.629* (0.77**) ^a	0.797** (0.81**) ^a	0.505*** (0.00 ^{NS}) ^{a,b}
A _{tot}			0.400*** (0.00 ^{NS}) ^b

^a %A_R of the RR genotype is much larger than %A_R of the others genotypes. Its weight in the regression was tested by removing it. The percentage of total variance explained by the model with RR is given between brackets.

^b The D₃D₃ genotype induces a much larger cost than the others genotypes. Its weight in the regression was tested by removing it. The percentage of total variance explained by the model with D₃D₃ is given between brackets.

Fig.1: Mean AChE1R and AChE1S activities of the various genotypes. The mean activities (given as the variation in optical density (OD) per minute) and associated standard errors for each genotype are presented for both AChE1R and AChE1S, for males (top) and females (bottom). The predicted activity of one S copy and one R copy were inferred from the strictly additive model (see text). These activities and those of two copies are indicated by horizontal (S) and vertical (R) dotted lines.

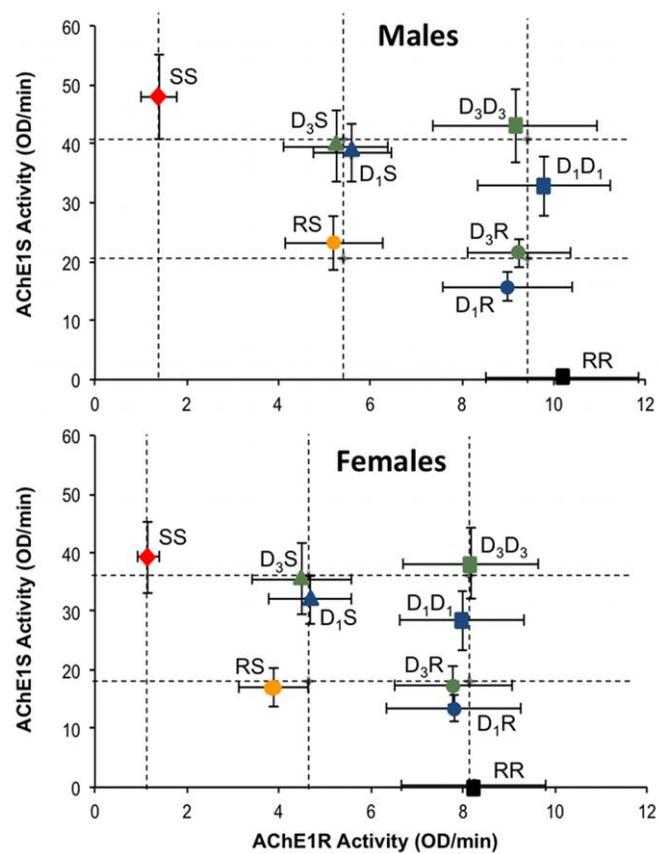
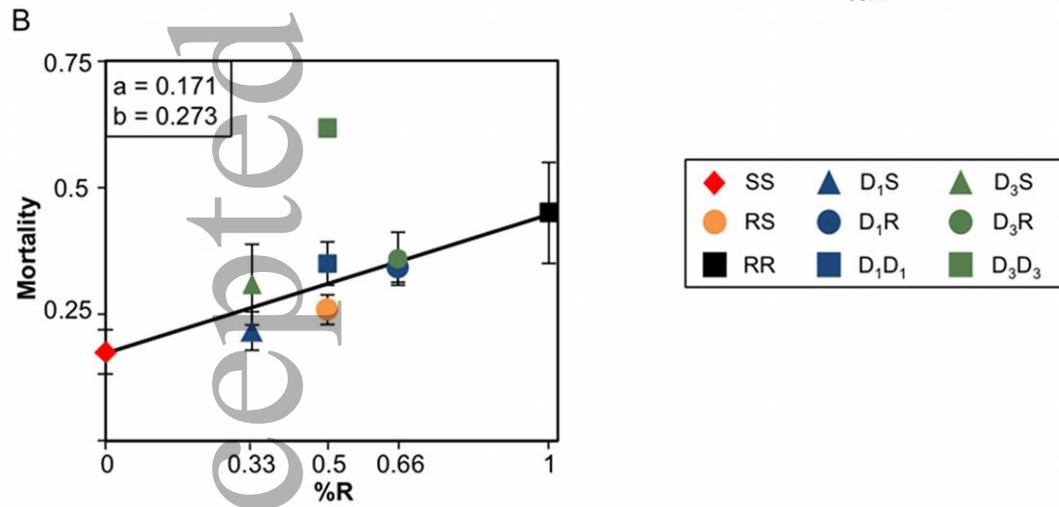
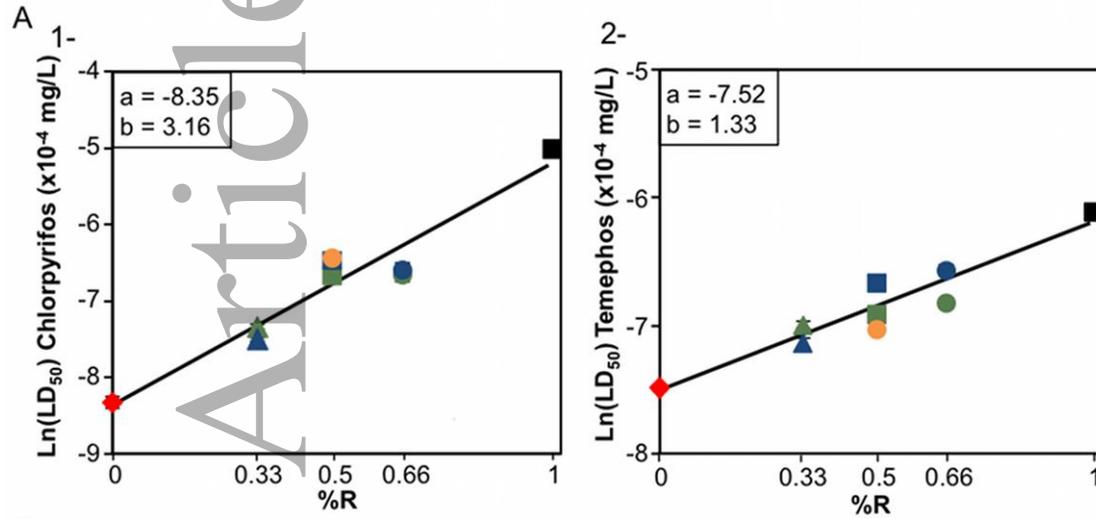


Fig.2: Linear regressions between gene-dosage of each genotype and the resistance level (A) or the mortality (B). For each genotype (see legend), the percentage of R copies (%R) is represented as a function A) of the resistance level (LD_{50} ; data were log-transformed for linearity) for Chlorpyrifos (A1) and Temephos (A2) insecticides, and B) of the pre-imaginal mortality (Mortality). Standard errors are indicated. For each linear regression (solid line), the slope (b) and the intercept (a) are indicated (they are all significant, p -value <0.01). The R^2 of these regressions are given in Table 2 (note that only the regression without D_3D_3 is represented in Fig.2B, see Table 2).

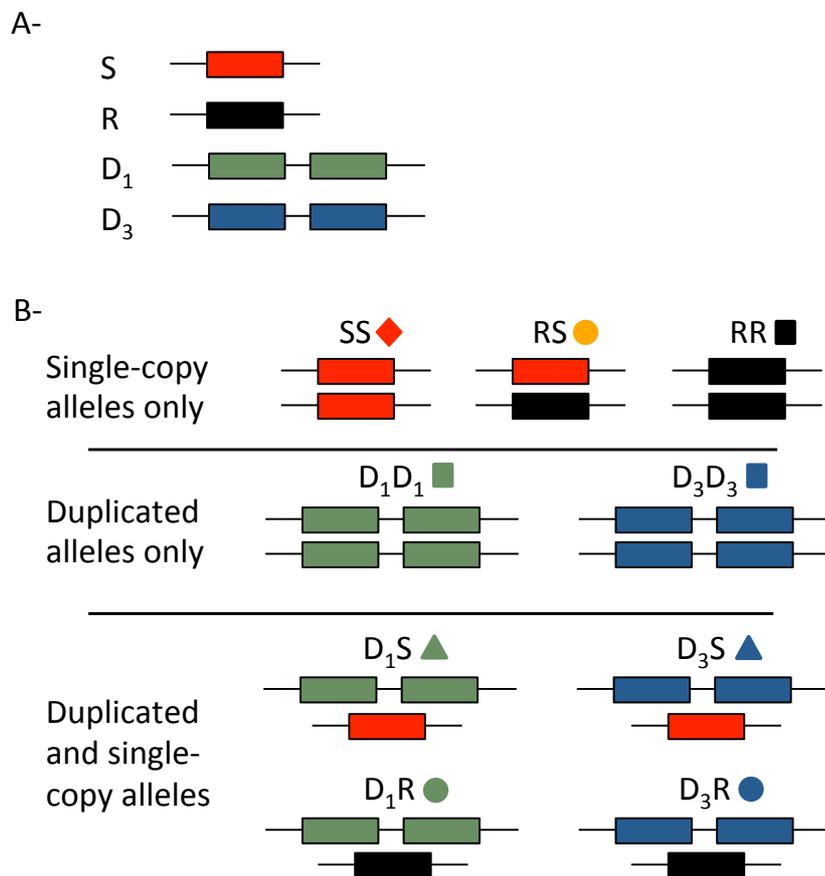


Gene-dosage effects on fitness in recent adaptive duplications: *ace-1* in the mosquito *Culex pipiens*

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Supplementary Materials

Fig.S1: The various *ace-1* alleles and resulting genotypes. A- The various alleles studied, with the corresponding nomenclature: S = *ace-1*^S, R = *ace-1*^R, D₁ = *ace-1*^{D₁} (Genbank: JX007772.1 and JX007773.1, for the susceptible and the resistant copy, resp.) and D₃ = *ace-1*^{D₃} (Genbank: JX007766.1 and JX007767.1, *idem*). B- The different combinations of the four alleles in diploid genotypes, with the corresponding nomenclature and the corresponding symbols used in the main article figures.



S2: Specific PCR protocols

Partial sequences of *ace-1* exon-3 identified only 5 nucleotides differences between the susceptible copy of the duplicated allele and S_{SLAB}. Individuals from DUCOS and SLAB strains indeed belong to the same subspecies, *C. p. quinquefasciatus*, which displays low genetic variability at the *ace-1* locus (Labbé et al. 2007a). Two pairs of primers amplifying specifically a fragment within this exon 3 were designed: Ex3dirDUCOS 5'-ACA-CTG-GAA-GCG-CCT-AGC-3' and Ex3revDUCOS 5'-CGA-GGC-CAG-CGT-CCG-G-3' (leading to a fragment of 359pb) and, Ex3dirSLAB 5'-TTC-CGT-ACG-CGC-AGC-CC-3', Ex3revSLAB 5'-TGT-GCC-CAG-GAA-GAG-AAA-C-3' (leading to a fragment of 382pb). Using specific PCR conditions (30 cycles, 93 °C for 30s, 60 °C for 30s and 72 °C for 1 min), the first pair amplifies only the three different copies originating from DUCOS (D₁ resistant and susceptible copy and R), whereas the second couple is specific for S_{SLAB} (Fig.S3).

Fig.S3: Sequence alignment of susceptible ($D_1(S)$) and resistant ($D_1(R)$) copies of the duplicated allele $ace-1^{D1}$ (Martinique), the single martiniquan R copy (all present in DUCOS) and the susceptible copy of SLAB, S_{SLAB} . The primers used for DUCOS- and SLAB-specific PCR amplification are highlighted in grey and black, respectively. The position of the G119S mutation (box) distinguishing resistant and susceptible copies is indicated. The first position corresponds to the first nucleotide of exon 3.

							Ex3dirDUCOS	80
D1 (S)	GCCACCGACT	CGGACCCACT	GGTCATAACG	ACGGACAAAG	GCAAAATCCG	TGGAACGACA	CTGGAAGCGC	CTAGCGGAAA
D1 (R)	-----	-----	-----	-----	-----	-----	-----	-----
R	-----	-----	-----	-----	-----	-----	-----	-----
S _{SLAB}	-----	-----	-----	-----G-	-----	-----	-----	-----T
								160
D1 (S)	GAAGGTGGAC	GCATGGATGG	GCATTCCGTA	CGCGCAGCCT	CCGCTGGGTC	CGCTCCGGTT	TCGACATCCG	CGACCCGCCG
D1 (R)	-----	-----	-----	-----	-----	-----	-----	-----
R	-----	-----	-----	-----	-----	-----	-----	-----
S _{SLAB}	-----	-----	-----	-----C	-----	-----	-----	-----
				Ex3dirSLAB				
								240
D1 (S)	AAAGATGGAC	CGGTGTGCTG	AACGCGACCA	AACCGCCCAA	CTCCTGCGTC	CAGATCGTGG	ACACCGTGTT	CGGTGACTTC
D1 (R)	-----	-----	-----	-----	-----	-----	-----	-----
R	-----	-----	-----	-----	-----	-----	-----	-----
S _{SLAB}	-----	-----	-----	-----	-----	-----	-----	-----
								320
D1 (S)	CCGGGGGCCA	CCATGTGGAA	CCCGAACACA	CCGCTCTCGG	AGGACTGTCT	GTACATCAAC	GTGGTCGTGC	CACGGCCCAG
D1 (R)	-----	-----	-----	-----	-----	-----	-----	-----
R	-----	-----	-----	-----	-----	-----	-----	-----
S _{SLAB}	-----	-----	-----	-----	-----	-----	-----	-----
								400
D1 (S)	GCCCAAGAAT	GCCGCCGTCA	TGCTGTGGAT	CTTCGGGGGT	GCTTCTACT	CCGGGACTGC	CACGCTGGAC	GTGTACGACC
D1 (R)	-----	-----	-----	-----	A	-----	-----	-----
R	-----	-----	-----	-----	A	-----	-----	-----
S _{SLAB}	-----	-----	-----	-----	-----	-----	-----	-----
					G119S			
								480
D1 (S)	ACCGGACGCT	GGCCTCGGAG	GAGAACGTGA	TCGTAGTTTC	GCTGCAGTAC	CGTGTCGCAA	GTCTTGGTTT	TCTCTTCTCG
D1 (R)	-----	-----	-----	-----	-----	-----	-----	-----
R	-----	-----	-----	-----	-----	-----	-----	-----
S _{SLAB}	-T-----	-----	-----	-----	-----	-----	-----	-----G
								Ex3revSLAB
D1 (S)	GGCACA							
D1 (R)	-----							
R	-----							
S _{SLAB}	-----							

S4: Detailed AChE1 activity measure protocol

Each adult mosquito was decapitated and the head was placed in an eppendorf tube with 400 μ l of extraction buffer (20 mM Tris-HCl, pH 7.0, 1 % Triton X-100). It was then homogenized using a pestle and the mix was centrifuge for 1 min at 10,000 rpm. For each mosquito, 100 μ l of supernatant were then distributed into two wells of a microtitration plate. 10 μ l of EtOH were added to the first well and 10 μ l of propoxur (a carbamate insecticide, at 10^{-1} M in EtOH) to the second. The plate was then incubated for 15 min at room temperature. Then, 100 μ l of substrate solution (25 mM sodium phosphate, pH 7.0, 0.2 mM DTNB, 0.35 mM sodium bicarbonate, 2.5 mM acetylthiocholine) were added to each well. The active AChE1 present in the supernatant cleaves the acetylthiocholine into a yellow colored product. Optical density at 412 nm kinetics was recorded every minute for 15 min using a Microplate Reader EL 800 (Bio-Tek Instruments, Inc.). The mean slope of each reaction was computed using the analysis software KCjunior v1.41.4 (Bio-Tek Instruments, Inc.), and was used as a measure of AChE1 activity.

Fig.S5: Total AChE1 activity for males and females of the different genotypes. For each genotype, the distribution of total AChE1 activity (given as the variation in optical density per minute) is represented by a box with a horizontal line for the median value and bottom and top of the box for the 25th and 75th percentiles, respectively. The vertical dashed lines represent either the maximum value or 1.5 times the interquartile range, whichever is the smaller. Rounds indicate outliers. For genotype D₃D₃, D₃R, D₃S, D₁D₁, D₁R, D₁S, RR, RS and SS a total of 28, 28, 25, 27, 27, 25, 16, 26 and 29 males (shaded boxes), and 29, 30, 29, 28, 29, 29, 20, 29 and 27 females (zebra boxes) were analyzed, respectively. The average activity (\pm SE) of each genotype was 49.2 (\pm 8.0), 27.8 (\pm 4.8), 42.3 (\pm 7.1), 39.4 (\pm 6.9), 23.0 (\pm 3.9), 40.1 (\pm 6.4), 9.5 (\pm 1.9), 24.4 (\pm 6.0) and 45.0 (\pm 8.2), respectively.

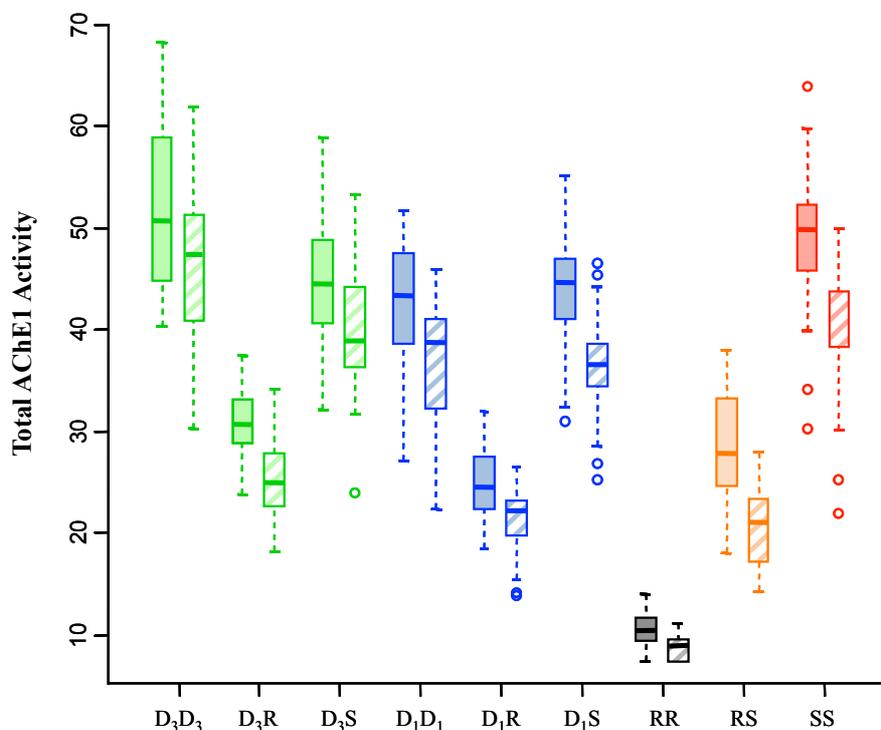


Fig.S6: Insecticide bioassays. The mortality (%) in relation to the insecticide dose is presented for 9 genotypes (see legend) and for the two insecticides used (Temephos, top, and Chlorpyrifos, bottom). The left and right columns respectively present the *ace-1^{D1}* and *ace-1^{D3}* genotypes, SS, RS and RR genotypes being used as references.

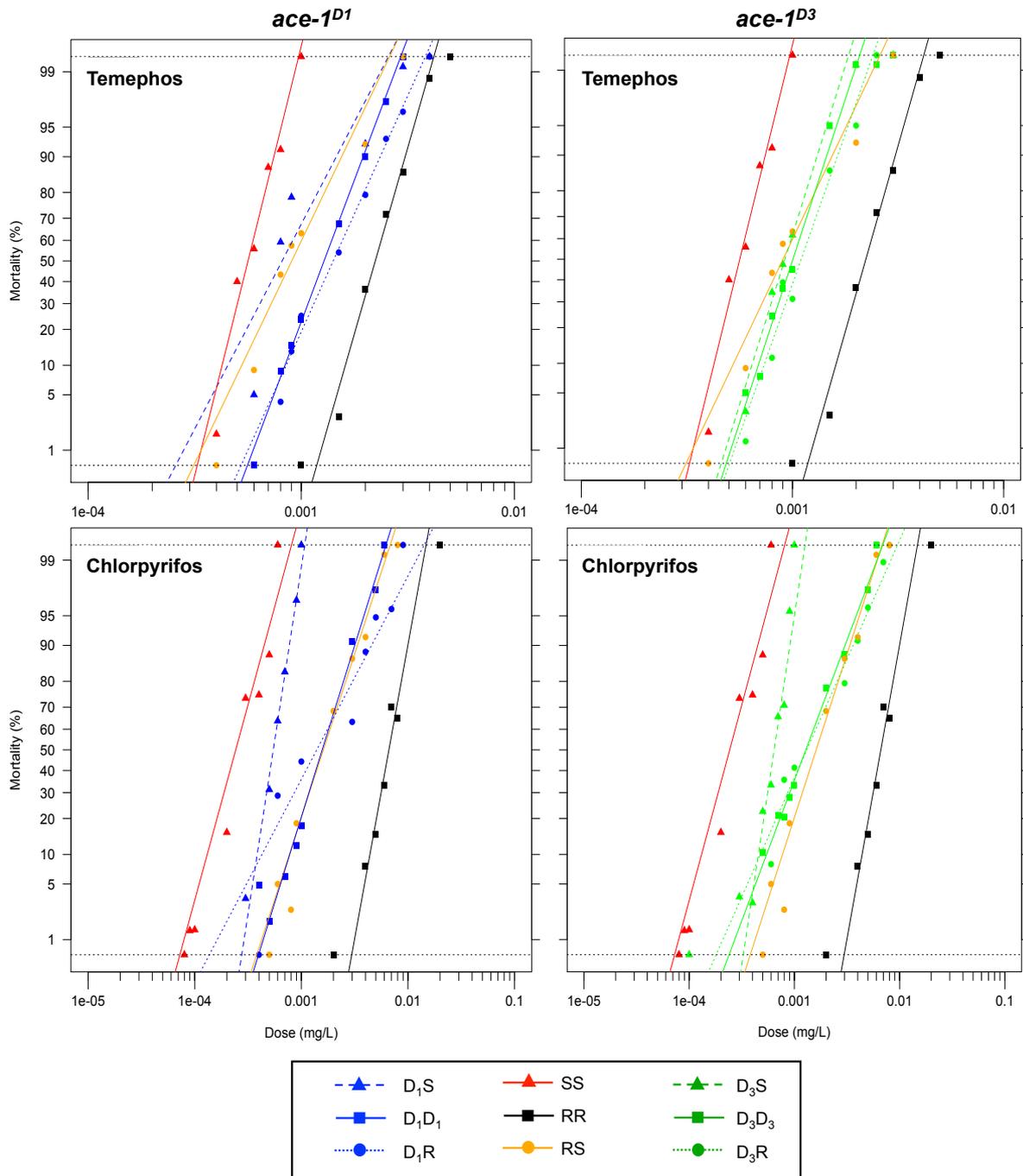


TABLE S7: LD₅₀ for the various genotypes with Temephos and Chlorpyrifos. The 50 % lethal doses (LD₅₀), i.e. the doses expressed in mg/L at which half of the individuals that died are presented for the 9 genotypes. The 95 % confidence intervals are within brackets. The resistance ratios *rr* are also indicated: they correspond to the ratio of the LD₅₀ of the corresponding genotype over the LD₅₀ of the SS genotype.

Genotypes	Temephos		Chlorpyrifos	
	LD ₅₀ (x10 ⁻⁴ mg/l)	<i>rr</i>	LD ₅₀ (x10 ⁻⁴ mg/l)	<i>rr</i>
SS	5.61 (5.47-5.75)	-	2.42 (2.24-2.60)	-
RS	8.89 (8.55-9.24)	1.6	16.0 (14.9-17.0)	6.6
RR	22.2 (21.3-23.1)	4.0	66.3 (63.6-69.1)	27.4
D ₃ D ₃	9.95 (9.67-10.2)	1.8	12.7 (11.8-13.7)	5.3
D ₃ S	9.15 (8.89-9.42)	1.6	6.48 (6.23-6.69)	2.7
D ₃ R	10.9 (10.5-11.3)	1.9	12.7 (11.7-13.8)	5.3
D ₁ D ₁	12.7 (12.2-13.3)	2.3	15.5 (14.5-16.5)	6.4
D ₁ S	7.96 (7.63-8.30)	1.4	5.51 (5.36-5.66)	2.3
D ₁ R	14.1 (13.5-14.6)	2.5	13.7 (12.4-15.1)	5.7

Fig.S8: Pre-imaginal mortality. The mean percentage of individuals dead before emergence for each genotype is indicated, with the standard errors (3 racks of 40 individuals per cross). The data were analyzed using the GLM: $MORTALITY = GENO + BLOC + GENO.BLOC + \epsilon$, where GENO is the genotypes, BLOC the racks of 40 individuals and ϵ the error parameter following a binomial distribution. An identical letter labeling the bar indicates that the number of emerging adults is not different between two genotypes. No BLOC effect was observed. Four groups of genotypes emerged from the highest to the lowest recorded mortality: a) D_1D_1 , D_1R , D_3S and D_3R (30-35% mortality), b) D_1S , SS and RS (18-25% mortality), c) RR (45% mortality), d) D_3D_3 (61.7% mortality).

