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# RESISTANCE GENE REPLACEMENT IN THE MOSQUITO *CULEX PIPIENS*: FITNESS ESTIMATION FROM LONG TERM CLINE SERIES

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*RHH.*— AMELIORATION AND GENE REPLACEMENT

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ABSTRACT

How adaptation appears and is later refined by natural selection has been the object of intense theoretical work. However the testing of these theories is limited by our ability to estimate the strength of natural selection in nature. Using a long-term cline series, we estimate the selection coefficients acting on different alleles at the same locus in order to analyse the allele replacement observed in the insecticide resistance gene *Ester* in the mosquito *Culex pipiens* in the Montpellier area, southern France. Our method allows us to accurately account for the resistance allele replacement observed in this area since 1986. A first resistance allele appeared early, which was replaced by a second resistance allele providing the same advantage but at a lower cost, itself being replaced by a third resistance allele with both higher advantage and cost. It shows that amelioration of the adaptation (here resistance to insecticide) through allele replacement was successively achieved by selection of first a generalist allele (i.e. with a low fitness variance across environments) and later of a specialist allele (i.e. with a large fitness variance across environments). More generally, we discuss how precise estimates of the strength of selection obtained from field data help understand the process of amelioration of adaptation.

"Without extensive knowledge of natural selection in the wild, we have no idea how relevant experiment or theory are to the evolution of natural populations" (ENDLER 1986). Twenty years after ENDLER's famous monograph, many advances have been made in the theory of adaptation. Some predictions made by this theory have been tested in the lab, but little insight has been gained into the process in nature. The main advance has been the shift from a strict micromutationnal view to one when mutations of larger effect also played a role. Several experimental studies support this view (see review in ORR 2005). When adaptation to a new habitat is like a sequential approach towards a phenotypic optimum, mutations of large effect will tend to occur early and be followed by mutations of smaller effects 'refining' the adaptation (HARTL and TAUBES 1996; BARTON 1998; ORR 1998; ORR 2000; BARTON and KEIGHTLEY 2002).

Adaptation to a new environment involves two types of traits; i) traits that change to match the new environmental challenge, and ii) traits that are not involved in this environmental change and have to remain unchanged. "Amelioration" of adaptation (sensu COHAN *et al.* 1994) occurs therefore in two directions: improving traits from set 1, and correcting the correlated (or 'pleiotropic') changes that may have occurred on traits from set 2 because of changes that occurred on traits in set 1. We will designate below these two modes of amelioration as being 'direct' or 'compensatory', respectively. Most new adaptive mutations affect these two sets of traits together, by producing traits that better match the environment but also modifying some traits that should not change, generating conflicting selection pressures and impeding the rate of adaptation (CASPARI 1952; WRIGHT 1969; CARRIÈRE *et al.* 1994). It is, however, difficult to disentangle these different selection pressures in nature. Moreover, this process of amelioration can take several routes at the genetic level: it can either involve several loci (e.g. a modifier gene with a compensatory effect) or only a single locus repeatedly (allele replacement) (COHAN *et al.* 1994).

Additionally, although less commonly appreciated, more complex molecular processes such as gene duplication may also be involved on a short time-scale (LABBÉ *et al.* 2007). While several examples of these processes have been described in laboratory studies (e.g. LENSKI 1988a; LENSKI 1988b; COHAN *et al.* 1994), it is difficult to study them in nature without precise methods to estimate selection coefficients and without the knowledge of the genetic bases of the adaptation.

In this paper, we developed an approach allowing us to precisely measure selection coefficients in order to study this process of amelioration in nature. We used a well-known case study, the evolution of organophosphate insecticide (OP) resistance in the mosquito *Culex pipiens* (vector of West Niles encephalitis, filariasis, etc.). In particular, we focused on the allele replacement that occurred at the *Ester* locus in the Montpellier area (Southern France) during the 1990's and 2000's (GUILLEMAUD *et al.* 1998; LABBÉ *et al.* 2005). The *Ester* superlocus codes for detoxifying carboxylester hydrolases (or esterases). The overproduction of these esterases is one of the major resistance mechanisms to OP in *C. pipiens* (see for review RAYMOND *et al.* 2001). Several resistance alleles, each corresponding to a distinct overproduced esterase, have been described. They are selected for in insecticide treated areas (i.e. a selective advantage as they survive better in this environment), but they are costly (i.e. confer a fitness disadvantage such as lower mating success, lower survival, etc. in absence of treatment) and thus selected against in non-treated areas (LENORMAND *et al.* 1998). No estimates of the relative fitness of these three resistance alleles are available so far.

We are still largely ignorant as to the precise mechanisms of amelioration operating in this example and would like to know if these allele replacements involved alleles increasing their advantage in treated area ($s$), decreasing their cost ($c$) or both. It is important to stress at this point that whether an allele replace another does not simply follow from $s$ and $c$ values, but also depends in a non-trivial way on gene flow and habitat spatial structure (NAGYLAKI

and LOU 2001). Although observing gene replacement allows qualitative inferences about the relative fitness of the competing alleles, only quantifying differences in fitness and their components (*s* and *c*) will enable us to discriminate between distinct scenarios of amelioration (direct or compensatory) and measure the net fitness change occurring during the process of amelioration.

The problem is that identifying such a subtle process requires precise methods to disentangle the relative values of *s* and *c* among adaptive alleles: while it is relatively easy to measure large fitness differences among two alleles (e.g. between susceptible and resistance alleles), it is more difficult when the differences are slight (e.g. between two resistance alleles) and a greater number of alleles are involved. In laboratory and model organism (especially microbes) studies it is possible to measure selection coefficient with a precision of less than 1% (e.g. DE GELDER *et al.* 2004; DE VISSER and ROZEN 2006). However, despite a long history of estimating the strength of natural selection in nature (see MANLY 1985; ENDLER 1986; HOEKSTRA *et al.* 2001, for review), this precision has never been reached with field data. The study of spatial and temporal frequency variation of adapted alleles has been one of the most accurate methods of estimating natural selection (e.g. HALDANE 1948; KETTLEWELL and BERRY 1961; MAY *et al.* 1975; MALLET and BARTON 1989; MALLET *et al.* 1990; MANI 1990; MANI and MAJERUS 1993; LENORMAND *et al.* 1998; COOK 2003; HOEKSTRA *et al.* 2004). However, at least two issues are crucial to obtaining accurate estimates with this method. The first issue is disentangling the effects of migration and selection on frequency variation (e.g. BRAKEFIELD and LIEBERT 1990). This problem can be overcome by estimating migration directly (e.g. using mark-recapture, BRAKEFIELD and LIEBERT 1990, or neutral genetic markers, HOEKSTRA et al. 2004), or by estimating migration from patterns of linkage disequilibria among locally adapted alleles (LENORMAND *et al.* 1998), an approach similar to that used to study hybrid zones (BARTON and HEWITT 1985;

MALLET and BARTON 1989). The second difficulty is that inference of selection depends on the validity of the underlying migration–selection model. In most cases it is necessary to assume that the observed spatial frequency pattern is at migration – selection equilibrium, precluding the study of transient allele replacement.

In this paper, we developed a powerful approach that does not assume migration – selection equilibrium, and which comes close to the precision obtained under laboratory conditions. We used this approach to estimate the selection coefficients of the *Ester* alleles in *C. pipiens* from the pattern of resistant allele replacement at this locus in Montpellier area. We used the 15-year *Ester* allele frequency series to estimate the magnitude of the two fitness components, cost ($c$) and selective advantage ($s$), conferred by each resistance *Ester* allele, by combining the information of both spatial and temporal frequency variation. We also investigated the effect of dominance among the different alleles on the quality of our estimates. In addition we looked for evidence that the selection regime had changed or that compensatory modifiers had spread in these populations. Finally, we discuss what this case study tells us about the processes of amelioration in natural populations.

## MATERIAL AND METHODS

In the Montpellier area, the mosquito *Culex pipiens* is treated with OP insecticides on a coastal belt delimiting two areas (Figure 1): a treated area close to the Mediterranean Sea, where resistant alleles tend to be frequent due to their selective advantage ($s$), and a non-treated area more inland where resistant alleles are less frequent due to their cost ($c$). The frequency of resistant alleles thus display a clinal shape along a transect from the sea (treated) to the inland (untreated). At equilibrium between migration and selection, the rate of decline

in the frequency of resistance is proportional to the intensity of migration ($\sigma$), and depends on the magnitude of selection in the two areas.

In this study, we thus present a model using spatial information from clines to estimate selective advantages and costs, but also temporal information from long term survey (~15 years) to estimate the selection coefficients of each allele in each environment. With this approach, we focused on the long term trend of allele replacement. For a proper comparison among years, we used data from only one season (summer), ignoring within years variations that are not directly relevant to the long term trend. Importantly, this approach does not make any equilibrium assumption: the initial situation is fitted by a description of the clines of the first year in the series (1986), and all the clines in the following years depend on these initial frequencies, migration rate and selection coefficients of the different alleles (See Supplementary materials for a review of previous models).

**Data collection**: The dataset is a compilation of published *Ester* starch-gel electrophoresis phenotype frequencies from samples collected in the Montpellier area from 1986 to 2002 (GUILLEMAUD *et al.* 1998; LABBÉ *et al.* 2005). We used samples from years 1986 (9 populations, $N = 354$), 1987 (3 populations, $N = 193$), 1991 (9 populations, $N = 217$), 1993 (2 populations, $N = 110$), 1995 (8 populations, $N = 1203$), 1996 (9 populations, $N = 512$), 1999 (9 populations, $N = 582$), 2001 (9 populations, $N = 736$) and 2002 (9 populations, $N = 521$) to perform our analysis, considering only samples collected during the summer. A total of 4428 individuals were analyzed.

**Insecticide treatment:** The size of the treated area is pivotal to estimating the selection parameters from cline analysis (LENORMAND *et al.* 1998). In order to obtain good estimation of this size, we used data of total insecticide quantities per district provided by the local

insecticide treatment agency (EID, Entente Interdépartementale de Démoustication) from 1990 to 2002. In previous years (1986 to 1989), treatment applications (i.e. treated area size and quantities used) did not change significantly (EID 1992; GUILLEMAUD *et al.* 1998). We used GIS data to estimate the quantities of insecticide applied in the Montpellier area. The total area analyzed was 40 km wide (20 km each side of the sampling transect) and 35 km long, perpendicularly to the coast. We divided this area in 2 km wide stripes, parallel to the coast. The OP quantity used in each stripe, $q_j$, expressed in liter.km$^{-2}$ (L/km$^2$), was computed as

$$q_j = \sum_i \frac{A_{ij}}{A_i} q_i \Big/ \sum_i A_{ij} \quad (1),$$

where $A_{ij}$ is the area of each district $i$ within the stripe $j$, $A_i$ and $q_i$ the total area and the quantity of insecticide used in this district $i$, respectively. We determined the width of the treated area as the distance from the sea where the quantities of insecticides treatments used drops for most years to 0 L/km².

**Migration-Selection Model:** In order to estimate the relative costs and selective advantages of each the three alleles at the *Ester* locus we used a deterministic stepping stone-like model to follow frequency changes. The order of life cycle was assumed to be reproduction-selection-migration. We assumed 13 generations per year according to LENORMAND *et al*. (1999) and a constant population density across treated and non-treated areas (we will discuss this last hypothesis below). Algorithms of migration and selection were checked using analytical results for one locus provided by equations 32-33 in NAGYLAKI (1975).

*Reproduction.* Each generation was computed from the previous one assuming Hardy-Weinberg proportions in each deme independently (each deme being considered as an infinite population), as it has been shown that data are congruent with this assumption (LABBÉ et al. 2005).

*Selection*. The fitnesses of the *Ester* alleles were computed as follow: let $s_i$ and $c_i$ be the fitness advantage conferred by resistance alleles in treated area and the fitness cost of the allele *i*, respectively; let $h^s_{ij}$ be the dominance of the benefit for resistance and $h^c_{ij}$ the dominance of the cost of the allele *i* over the allele *j*. The fitness $w_{ij}$ of the diploid genotype (*ij*) was computed as

$$w_{ij} = 1 + \gamma\,[s_i + h^s_{ij}\,(s_j - s_i)] - [c_i + h^c_{ij}\,(c_j - c_i)] \qquad (2),$$

with $\gamma = 1$ in the treated area and $\gamma = 0$ in the non-treated area and where the fitness of a susceptible homozygote $w_{00} = 1$ (i.e. $s_0 = c_0 = 0$). For $\gamma = 1$, $[s_i + h^s_{ij}\,(s_j - s_i)]$ represents the overall advantage of the resistance genotype in the treated area. $[c_i + h^c_{ij}\,(c_j - c_i)]$ represents its cost in both the treated and the untreated area. $h^s_{ij}$ ranges from 0 (total dominance of *i* over *j*) to 1 (total dominance of *j* over *i*), with 0.5 representing codominance. $h^c_{ij}$ ranges from 0 to a value >1 (overdominance), providing that $c_j > c_i$. As there are three resistance alleles at *Ester* locus, 18 parameters were used to describe selection at this locus: the costs, $c_1$, $c_2$, $c_4$, the advantage of resistance $s_1$, $s_2$, $s_4$ (for $Ester^1$, $Ester^2$ and $Ester^4$, respectively) and the 6 dominance parameters for the cost and the advantage of resistance of the four alleles ($Ester^0$, $Ester^1$, $Ester^2$ and $Ester^4$).

To consider indirect selection due to genetic association of *Ester* alleles with the other *C. pipiens* main OP resistance locus, *ace-1*, we used fitness parameters estimated for *ace-1* in previous study ($s_R = 0.33$ and $c_R = 0.11$, LENORMAND *et al.* 1999, ignoring possible fitness differences between *ace-1* resistance alleles) and assumed codominance at this locus. We also assumed multiplicative fitness between the *ace-1* and *Ester* loci and used the recombination rate between the two loci ($r = 14.5\%$) estimated from laboratory crosses (LENORMAND *et al.* 1998).

The frequency of genotype *k* in deme *l* after selection $g'_{kl}$ was computed from its frequency before selection $g_{kl}$ as:

$$g'_{kl} = g_{kl}\, w_{kl} / W_l \qquad (3),$$

where $w_{kl}$ is the fitness of genotype $k$ in deme $l$ and $W_l$ the mean fitness in deme $l$. We first computed selection coefficients by assuming that all alleles were codominant (i.e $h^s_{ij} = h^c_{ij} =$ 0.5 for all alleles): this is the COD model. We then relaxed this hypothesis by allowing dominance parameters to differ from 0.5: this is the NOCOD model.

*Migration.* One-dimensional clines were simulated by a series of demes connected by migration (one deme every 2 kms, 35 demes in total). The migration distribution was reflected at one edge of the stepping-stone, to simulate a semi-infinite environment and take into account the presence of the sea (LENORMAND *et al.* 1998). We used an approximately gaussian dispersal kernel with a parent-offspring distance standard deviation $\sigma$ = 6.6 km.generation$^{-1/2}$; this value has been estimated by LENORMAND *et al.* (1998) using the spatial pattern of linkage disequilibrium between resistance locus. This method does not require assuming migration–selection equilibrium at the selected loci as far as the linkage disequilibrium equilibrates faster than frequency changes. This latter situation will be easily met if the resistance alleles are not too far from an equilibrium or if their frequency change slowly, provided that the loci are not too tightly linked (this in the well-studied quasi linkage equilibrium situation, BARTON and GALE 1993; NAGYLAKI 1993). This situation is very likely to be met in our case as the recombination rate between resistance loci is quite large ($r \sim 15\%$) and the important frequency changes at these loci occurs at the scale of hundreds of generations.

*Initial conditions***.** Only phenotype data are available due to the dominance of overproduced esterases in the identification method (starch-gel electrophoresis, see *Parameters estimations*). Thus, to obtain initial conditions, we estimated (simultaneously with the selection coefficients) the distribution of the frequency of each allele in 1986 (first year of sampling available) using a descriptive model as in previous studies (LABBÉ *et al.* 2005;

LENORMAND and RAYMOND 2000). In 1986, only *Ester*$^{1}$ and *Ester*$^{4}$ alleles were present, as *Ester*$^{2}$ was detected for the first time in 1990 in the Montpellier area (one heterozygote individual found among all populations sampled, RIVET *et al.* 1993). Frequency clines for the two first resistance alleles $i$ ($i$ = 1 or 4 for *Ester*$^{1}$ and *Ester*$^{4}$, respectively) were simultaneously fitted to a scaled negative exponential

$$p_i = k_i \exp[-(a_i x^2 + b_i x)] \qquad (4),$$

where $x$ is the distance from the coast, and $k_i$, $b_i$ and $a_i$ are the estimated parameters. $k_i$ is the frequency of resistance allele $i$ for $x$ = 0 (i.e. at the coast). As the *Ester*$^{2}$ allele was not yet present in 1986, we introduced it $t^*$ generations after 1986, at a frequency of 0.001 in all demes. $t^*$ was estimated together with the other parameters. As all the parameters (initials conditions, selection coefficients and $t^*$) are fitted simultaneously, the model is not particularly susceptible to the initial conditions and choosing another year to start the estimation would not change them significantly (of course, the longest the period considered, the better the estimation).

**Parameters estimations:** The method of estimation is a standard maximum likelihood approach. Deterministic recursions described above generate the predicted clines of each allele at any point in time for a given set of parameter values. It is then straightforward to compute the probability of observing a sample at any location in any year given this prediction. Assuming that the different samples are independent, the likelihood of a full 15 year scenario can then readily be obtained. The only difficulty, however, is the computer time needed to maximize this likelihood given that a single 15 years prediction requires simulating ~200 generations in a relatively large stepping stone. Recursions and likelihood maximisation algorithms were written and compiled using Delphi™ v. 7 (Borland Software Corporation).

The *Ester* phenotype of each mosquito was obtained using starch-gel electrophoresis. This technique does not allow complete identification of genotype (GUILLEMAUD *et al.* 1998; LENORMAND *et al.* 1998; LABBÉ *et al.* 2005) because the presence of a susceptible allele cannot reliably be detected in an individual with an overproduced resistance allele. The phenotype was thus considered to be a seven-state random variable ([0], [1], [2], [4], [12], [14], [24], where phenotype [i] corresponds to genotypes $Ester^{i}/Ester^{0}$ or $Ester^{i}/Ester^{i}$, and phenotype [ij] correspond to genotype $Ester^{i}/Ester^{j}$, see LENORMAND *et al.* 1998). The log-likelihood of a sample was computed from the phenotypic multinomial distribution. Let $n_{ij}$ and $f_{ij}$ be the observed number and expected frequency of individuals having phenotype $i$ in population $j$, respectively. The log-likelihood $L$ of observing all the data is proportional to

$$L = \sum_{i} \sum_{j} n_{ij} \ln\left(f_{ij}\right) \qquad (5).$$

It was maximized for parameters joint estimation, using a simulated annealing method (using a Metropolis algorithm, see LENORMAND AND RAYMOND 2000).

**Model comparison and tests:** For the complete COD model (model COD-A), a total of 13 parameters needed to be estimated: (i) $s_1$, $s_2$, $s_4$, the selective advantages of $Ester^{1}$, $Ester^{2}$ and $Ester^{4}$ respectively, (ii) $c_1$, $c_2$, $c_4$, the selective costs of $Ester^{1}$, $Ester^{2}$ and $Ester^{4}$ respectively, (iii) $k_1$, $k_4$, $a_1$, $a_4$, $b_1$ and $b_4$, the parameters of the initial frequency clines in 1986 of $Ester^{1}$ and $Ester^{4}$ respectively, and (iv) $t^*$, the date of apparition of $Ester^{2}$. When the codominance hypothesis is relaxed (NOCOD model), 12 additional parameters are needed: (vi) $h^s_{10}$, $h^s_{20}$, $h^s_{40}$, $h^s_{21}$, $h^s_{41}$ and $h^s_{24}$, for the dominance of the resistance fitness benefit and (vii) $h^s_{10}$, $h^s_{20}$, $h^s_{40}$, $h^s_{21}$, $h^s_{41}$ and $h^s_{24}$, for the dominance of the cost (where *0*, *1*, *2* and *4* represent $Ester^{0}$, $Ester^{1}$, $Ester^{2}$ and $Ester^{4}$ respectively).

Model COD-A was then simplified using likelihood ratio tests corrected for over-dispersion (*F*-test, LEBRETON *et al.* 1992; ANDERSON *et al.* 1994) to find the best adequate

model. We first determined whether the selective advantages $s_i$ and $s_j$ of alleles $i$ and $j$ were significantly different by setting $s_1 = s_2$, $s_1 = s_4$, $s_2 = s_4$ in models COD-$B_1$, COD-$B_2$ and COD-$B_3$, respectively, all other parameters being freely estimated. Similarly, we then determined whether costs $c_i$ and $c_j$ of alleles $i$ and $j$ were significantly different. We computed the models COD-$C_1$, COD-$C_2$ and COD-$C_3$ by setting $c_1 = c_2$, $c_1 = c_4$, $c_2 = c_4$, respectively, with all other parameters being freely estimated. Models combining more complex hypotheses (i.e. constraining both s and c values to be identical among some alleles) were then computed as models COD-D, with all other parameters again being freely estimated.

Over-dispersion was computed from model COD-A as the ratio of residual deviance (the deviance equals -2 x ln[likelihood]) over residual degrees of freedom. We computed the percentage of total deviance explained by a model (%TD) as

$$\%TD = (D_{max} - D_{model}) / (D_{max} - D_{min}) \qquad (6),$$

where the maximal deviance ($D_{max}$) is obtained by fitting a minimal model in which the frequency of each allele in each population is set to its average along the transect and over years, and the minimal deviance ($D_{min}$) obtained by fitting a maximal model in which the frequency of each allele in each population is set to its observed frequency. Models were compared using *F*-tests in order to correct for overdispersion. All deviances were also corrected for overdispersion to estimate the support limits of each parameter $p$. These were computed by maximising or minimizing the value of $p$, for upper limit ($p_{max}$) and lower limit ($p_{min}$), respectively. All other parameters were allowed to change, ensuring us to find the actual $p_{max}$ and $p_{min}$ in the range of the multidimensional parameter landscape where likelihood is not significantly different from the maximum likelihood of the model, using the same simulated annealing method.

We used the same process when relaxing the overdominance hypothesis (NOCOD models) and simply tested whether each dominance parameter was significantly different from 0.5 (codominance) or not, all other parameters being again freely estimated.

## RESULTS

**Insecticide treatment:** The treatment practices for the period 1990-2002 are presented in Supplemental Figure 1 (Supplementary Materials). Over the 12 years analyzed, the treated area size is roughly constant and runs 16 km inland from the sea, although treatment extension and intensity was higher in the first years analysed (Supplemental Figure 1). The total amount of insecticides used is variable over the years, from 124.3 to 733.1 $L.km^{-2}$ (Supplemental Figure 2, Supplementary Materials). The treated area is not evenly treated: immediately close to the sea, treatments are less intense, due to less suitable breeding sites for *C. pipiens*. This mosquito needs freshwater to reproduce and brackish lagoons near the sea largely reduce the area with potential breeding sites. However, for simplicity we considered the treated area as an approximately uniformly treated surface in the model, as we have no clue about how the dose of insecticide relates to fitness in the field.

**Model comparison and tests:** Model selection was performed using likelihood ratio tests corrected for overdispersion (*F*-test, LEBRETON *et al.* 1992; ANDERSON *et al.* 1994) to determine whether the selection coefficients were different among alleles. Results of each comparison are detailed in Table 1. Under the codominance hypothesis, in the simplest adequate model (model COD-D) the selective advantages of $Ester^1$ and $Ester^4$ and the costs of $Ester^1$ and $Ester^2$ are not significantly different (i.e. $s_1 = s_4$ and $c_1 = c_2$, resp.), but the cost is

lower for $Ester^4$ (i.e. $c_4 < c_2 = c_1$). Relaxing the codominance hypothesis modifies the results, such that neither the selective advantages nor the costs of $Ester^1$ and $Ester^4$ are significantly different (i.e. $s_1 = s_4$ and $c_1 = c_4$, model NOCOD-D).

**Parameters estimations**: The first six parameters of our models are those describing the initial clines (1986): the initial maximum frequencies ($k_1$ and $k_4$) and the rates of decline ($a_1$, $a_4$, $b_1$ and $b_4$) of each allele present ($Ester^1$ and $Ester^4$). This initial cline is best described by an $\exp(-x^2)$ shape, where $x$ is the distance from the coast ($k_1 = 0.461$, $k_4 = 0.233$, $a_1 = 0.095$, $a_4 = 0.071$, $b_1 = b_4 = 0$).

Under the codominance hypothesis, the simplest model (COD-D) is built with four other parameters describing selective advantage and cost: $s_1 = s_4$, $s_2$, $c_1 = c_2$ and $c_4$. The best value of each of these and corresponding support limits are indicated in Table 2A. This model explains 70.3% of the total deviance with low overdispersion (~1.52).

Relaxing the codominance hypothesis introduces 12 additional parameters of dominance associated to $s$ and $c$, $h^s_{ij}$ and $h^c_{ij}$. Among these only one, $h^s_{20}$, the advantage dominance of $Ester^2$ over $Ester^0$, is significantly different from 0.5 (codominance) ($h^s_{20} = 0.076$, Table 2B). Under this hypothesis, the simplest model (NOCOD-D) is built with four parameters describing selective advantage and cost: $s_1 = s_4$, $s_2$, $c_1 = c_4$ and $c_2$, the differences in cost and advantage dominances, $h^c$ and $h^s$, alone explaining the allele replacement. The best value of each of these and corresponding supporting limits are indicated in Table 2B. This model explains 72.9% of the total deviance with low overdispersion (~1.43).

The last parameter, the time to $Ester^2$ appearance $t^*$, is estimated to 45 and 48 generations (with and without the codominance hypothesis respectively), which corresponds to ~1989 with support limits being 30-60 generations, i.e. 1988-1990.

The predicted cline of each allele (spatial frequencies variation) is presented in Figure 2, for each year for which samples are available, under the NOCOD-A model. The computed variation over the period 1986-2002 (temporal frequencies variation) of the maximal frequency of each allele is presented in Figure 3 (NOCOD-A model). It shows the replacement of $Ester^{1}$ by $Ester^{4}$ that occurred during the 1990s. It also predicts the replacement of $Ester^{4}$ by $Ester^{2}$ over the period 2002-2024 (Figure 3), providing that treatment practices will not change during this period.

To assess whether there was a consistent variation of selection coefficients over time, we used two estimators of the goodness of fit, overdispersion and percentage of deviance explained by the model, for each sampling year. The variability over the 1986-2002 period of these two measures is presented in Supplemental Figure 3. Each sampled year independently is well fitted with more than 50% and up to 82% of the total deviance explained and an overdispersion inferior to 2 for each of them.

## DISCUSSION

Natural selection is notoriously difficult to measure in the wild, and this imposes an important limit on our ability to study evolution and to test *in natura* theories of adaptation. However, the study of cline series is a particularly informative situation since temporal and spatial frequency variation can be combined to estimate selection. We illustrate this approach with the evolution of insecticide resistance in the mosquito *Culex pipiens*: the resistance allele replacement observed at the *Ester* locus in the Montpellier area since 1986 allows us to analyse the process of amelioration with a degree of precision more typical of laboratory than field studies (down to a precision of ~2% for some selection coefficients). In addition, this is

the first time, to our knowledge, that estimation of the relative fitness of more than two alleles at a single locus has been performed using field data (our model is nevertheless in accordance with previous average measures of fitness on this system, see Comparison with previous estimations, Supplementary Materials). This approach enabled us to study in more detail the process of allele replacement in the field and to determine whether it was driven by direct or compensatory amelioration. It also enables us to quantify the amount of fitness variation occurring at the different stages of the process.

**Selection coefficients:** The selection coefficient estimations provided for the various resistance alleles (Table 1) accurately explain the allele replacements observed in Montpellier area (70% of the total deviance is explained). With the susceptible allele fitness being $w_0$=1, the overall resistance allele fitness orders are $Ester^2$ ($w_2$ = 1.250) > $Ester^4$ ($w_4$ = 1.154) > $Ester^1$ ($w_1$ = 1.112) in the treated area and $Ester^4$ ($w_2$ = 0.964) > $Ester^1$ ($w_4$ = 0.922) > $Ester^2$ ($w_1$ = 0.880) in the non-treated area (COD-A model, Table 2). The fitnesses differences detected between resistant alleles can thus be relatively small compared to the differences between susceptible and resistant (e.g. in treated area $Ester^4$ fitness is $w_4$ = 1.038 relative to $Ester^1$, i.e. if $w_1$ = 1, although $Ester^2$ differences with $Ester^4$ and $Ester^1$ are still quite large, e.g. in treated area $w_2$ = 1.124 relative to $Ester^1$).

During the 1990s, $Ester^4$ replaced $Ester^1$ (Figure 3, GUILLEMAUD *et al.* 1998; LABBÉ *et al.* 2005). We show here that $Ester^1$'s selective advantage is not significantly different from that of $Ester^4$ (relative to $s_1$, $s_4$ = 0, SL = -0.026 – 0.019), but that its cost is higher under the codominance hypothesis (relative to $c_1$, $c_4$ = -0.045, SL = -0.055 – -0.035). Thus, the lower cost of $Ester^4$ could be pivotal in explaining the replacement of $Ester^1$ by $Ester^4$. However, if dominances are different from 0.5, the estimated costs of $Ester^1$ and $Ester^4$ are not distinguishable (relative to $c_1$, $c_4$ = -0.002, SL = -0.032 – 0.059). In this case, the allele

replacement is due to the cost being more dominant for $Ester^1$ than $Ester^4$ (the cost of ($Ester^1$/$Ester^0$) heterozygotes is closer to that of ($Ester^1$/$Ester^1$) than ($Ester^0$/$Ester^0$) homozygotes, whereas the cost of ($Ester^4$/$Ester^0$) heterozygotes is closer to that of ($Ester^0$/$Ester^0$) than ($Ester^4$/ $Ester^4$) homozygotes). However dominances remain difficult to precisely estimate using these data (Table 2B). Fitting dominances only marginally increases the overall goodness of fit, explaining no more than 3% more of the deviance than the codominance model. Finally, laboratory data provide support for $Ester^1$ having a higher cost than $Ester^4$ (see Comparison with laboratory experiments, Supplementary Materials), suggesting a compensatory amelioration in this first allele replacement.

$Ester^2$ was first detected in Southern France near Marseille (~150 km from Montpellier) in 1986, but it was first detected at a very low frequency in the Montpellier area in 1990 (RIVET *et al.* 1993). Our study estimates its first occurrence near Montpellier during the year 1989, which indicates a quite fast spread of this allele in the south of France (c.a. 50 km per year in approximately 3 years). This alone suggests a strong fitness advantage for this resistance allele. It remained at low frequency (coastal frequency < 0.1) until 1999 (GAZAVE *et al.* 2001; GUILLEMAUD *et al.* 1998; LABBÉ *et al.* 2005) and then progressively increased in frequency (coastal frequency ~ 0.2 in 2002), leading to a decrease in the frequency of $Ester^1$ and the stabilization of the frequency of $Ester^4$ (LABBÉ *et al.* 2005). As shown in this study, this seems to be explained by a strong selective advantage leading to a higher fitness of $Ester^2$ in the treated area compared to the previous alleles (relative to $s_1$, $s_2$ = 0.158, SL = 0.072 – 0.282; relative to $s_4$, $s_2$ = 0.158, SL = 0.077 – 0.288), despite a higher cost (relative to $c_1$, $c_2$ = 0.188, SL = 0.118 – 0.329; relative to $c_4$, $c_2$ = 0.155, SL = 0.082 – 0.302; see Table 2). Thus, the increased advantage of $Ester^2$ in the treated area compensates for its increased cost in the non-treated area, indicating that this second allele replacement is most probably due to a direct amelioration (see also Comparison with laboratory experiments, Supplementary

Materials). Finally, our estimations (Figure 3) indicate that without any modification in the insecticide treatment practices, *Ester*$^{2}$ would eventually replace both alleles in about 20 years, the approximate time from the appearance of *Ester*$^{4}$ until now.

**Limit of the approach:** In this study, we used two main assumptions by neglecting the intra annual variation and the density variation between treated and non-treated areas (other parameters, including the initial conditions, are fitted in the model or were obtained independently; see Migration-Selection Model, Material and Methods). For practical reasons of computational time, intra annual variations of selection coefficients were not taken into account in our model. Because we ignored them, we cannot determine if the different resistance alleles are selected differently in different season (e.g. if differences in the fitness cost are due to difference in mortality during female overwintering or to differences of larval development time during summer; GAZAVE *et al.* 2001; LENORMAND *et al.* 1999; LENORMAND and RAYMOND 2000). Thus, the estimates we give have to be understood as annual averages. However, the cline observed in summer is relatively independent of what happened during the rest of the year, so that the hypothesis of a temporary migration-selection equilibrium reached each year at the end of summer is reasonably accurate (see Comparison with previous estimations in Supplementary Material), although the allele replacement modifies this equilibrium from one year to another. Density differences between treated and non-treated areas may bias our estimates of selection by causing an asymmetrical gene flow between the two habitats (NAGYLAKI 1978). This is true in particular for the relative selection coefficients between susceptible and resistance alleles. Like for selection, the density pattern may also vary seasonally or among years. Thus, the estimates we give have to be understood as if density was constant. For instance, if density is lower in the treated area (which is not necessarily the case, LENORMAND *et al.* 1998), larger *s* would be required to maintain the

same clines. However, it is important to underline that because the different resistance alleles experience the same density variation across treated and non treated area and because they have very similar spatial distribution (see Figure 2), this bias is minimal as far as resistance alleles are compared to one another, as it is the case in our study (i.e. the values of $s$ and $c$ relative to the susceptible allele might be biased, but the differences between $s_1$, $s_2$ and $s_4$ on one hand, or between $c_1$, $c_2$ and $c_4$ on the other hand, cannot be strongly affected by density effects). The same arguments hold for demographic, topological or treatment intensity variations that could occur perpendicular to our transect (although we are unaware of such variation over few tens of km East or West of our transect). Globally, our model explains more than 70% of the total deviance (TD) observed in the evolution of *Ester* resistance genes in Montpellier area (with a low global overdispersion, ~1.43). There is no indication of a trend towards increase or decrease of %TD or overdispersion during allele replacement (Supplemental Figure 3), as would be the case if selection changes with time. This would be the case, for example, if a modifier gene appeared at another locus during the course of the replacement, as was seen in *Lucilia cuprina* (for a review see MCKENZIE 1996). Such a modifier could increase the fitness of resistant *Ester* alleles by reducing their deleterious side-effects. Although our power to detect a compensatory modifier was limited, especially if it had a weak effect, a modifier gene is not necessary to explain the trends observed in natural populations through this long-term study.

**The process of amelioration:** This work enables us to understand the causes of the resistance allele replacement observed at the *Ester* locus in the Montpellier area. Our model and previous laboratory experiments (see Supplementary Materials) suggest that $Ester^1$ has most probably been replaced by $Ester^4$ because $Ester^4$ is less costly (compensatory amelioration). Currently, a second replacement is occurring: $Ester^2$ is replacing $Ester^4$ despite a higher

fitness cost, due to a higher selective advantage in the treated area (direct amelioration). Thus, contrary to the first replacement during which a more 'generalist' allele was selected, the second replacement involves an allele which is more 'specialist' to treated areas, leading to the reinforcement of local adaptation. There are two ways by which evolution may proceed ultimately if the insecticide treatments are maintained. The first option would involve further evolution towards specialist alleles that confer high resistance but with strong pleiotropic effects, such as *Ester*$^{2}$. This type of situation occurs when there is a strong trade-off between conflicting selection pressures in the different habitats. In such a situation a stable polymorphism is likely to be maintained and may lead to the evolution of distinct niches. The second option would involve further evolution towards generalist alleles that confer resistance with little pleiotropic effects, such as *Ester*$^{4}$. This type of situation occurs when there is a weaker trade-off between the conflicting selection pressures in the different habitats. In this scenario the polymorphism is likely to be lost rapidly, with the fixation of a 'cost-free' resistance allele, which corresponds to an extension of the niche. It is interesting to note that in our case both options have occurred successively in combination with a changing environment. The evolution at the *Ester* locus is certainly influenced by treatment practices, and it is possible that if insecticide treatment intensity decreases *Ester*$^{4}$ may be favoured again due to its lower cost, with the stronger resistance of *Ester*$^{2}$ then being less advantageous. This emphasizes the role of local treatment practices, which have been shown to be crucial in the competition of *Ester* alleles on a worldwide scale (LABBÉ *et al.* 2005).

**The theory of adaptation**: From a more general point of view, the evolution of insecticide resistance at the *Ester* gene represents a first step to study theories of adaptation (ORR 2005) in nature. Although amelioration has been reported from field studies (e.g. MCKENZIE 1993), our study allows quantitative estimation of the selection coefficient involved with a clear

knowledge of the history and genetic bases of the adaptive changes. Viewed at first as a slow process of accumulation of small mutations toward a fitness optimum (FISHER 1928; HARTL and TAUBES 1996), the process of adaptation is now thought to imply larger fitness effect mutations, occurring early in the process, and smaller mutations occurring subsequently to refine the adaptation (COHAN *et al.* 1994; BARTON 1998; ORR 1998; ORR 2000; BARTON and KEIGHTLEY 2002; ORR 2005). This theory has been supported by laboratory studies (e.g. COHAN *et al.* 1994; OXMAN *et al.* 2008), but ours gives new support from field data: the first mutation, *Ester*$^{1}$, was indeed a mutation of large fitness effect (relatively to ancestral susceptible alleles), the second, *Ester*$^{4}$, had a smaller fitness effect and refined the adaptation by lowering the cost, and the third, *Ester*$^{2}$, had again a large effect on fitness (Table 2). Albeit representing few steps, this field study is consistent with the theory formalized by ORR (1998). Interestingly, this study also provides some insights about the fact that adaptation is local, and thus that several strategies are possible in the course of adaptation to a heterogeneous environment. It is difficult to predict whether local adaptation will reinforce itself and lead to the evolution of specialist strategies or whether a generalist strategy that can exploit all habitats will emerge. The course of events obviously depends on the selective properties of the new resistance alleles that occur (and the underlying trade-off). Our study illustrates this point precisely: *Ester*$^{4}$ was more generalist than *Ester*$^{1}$ (low *c*), but it is now being replaced by *Ester*$^{2}$ a more specialist allele (high *s*, high *c*). The theory of adaptation tends to focus primarily on evolution in a single population. It will benefit from taking into account the spatial and temporal variability of the environment.

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## TABLE 1

**Model selection.**

| Model | Simplification | Dev[1] | Df[2] | %TD[3] | *F-test* | *P-value* | |
|---|---|---|---|---|---|---|---|
| COD-A | complete model | 6215.98 | 7 | 0.705 | - | - | |
| COD-B1 | $s_1 = s_2$ | 6230.86 | 6 | 0.694 | 14.88 | 0.000 | *** |
| COD-B2 | $s_1 = s_4$ | 6216.38 | 6 | 0.704 | 0.40 | 0.526 | n.s. |
| COD-B3 | $s_2 = s_4$ | 6238.40 | 6 | 0.689 | 22.43 | 0.000 | *** |
| COD-C1 | $c_1 = c_2$ | 6217.86 | 6 | 0.703 | 1.88 | 0.171 | n.s. |
| COD-C2 | $c_1 = c_4$ | 6233.88 | 6 | 0.692 | 17.90 | 0.000 | *** |
| COD-C3 | $c_2 = c_4$ | 6222.20 | 6 | 0.700 | 6.22 | 0.013 | * |
| COD-D | $s_1 = s_4$ *and* $c_1 = c_2$ | 6218.48 | 5 | 0.703 | 1.25 | 0.288 | n.s. |
| NOCOD-A | complete model | 6179.95 | 19 | 0.730 | - | - | |
| NOCOD-B1 | $s_1 = s_2$ | 6193.16 | 18 | 0.720 | 13.20 | 0.000 | *** |
| NOCOD-B2 | $s_1 = s_4$ | 6179.95 | 18 | 0.730 | 0.00 | 1.000 | n.s. |
| NOCOD-B3 | $s_2 = s_4$ | 6187.23 | 18 | 0.725 | 7.28 | 0.007 | ** |
| NOCOD-C1 | $c_1 = c_2$ | 6183.88 | 18 | 0.727 | 3.93 | 0.048 | * |
| NOCOD-C2 | $c_1 = c_4$ | 6180.00 | 18 | 0.730 | 0.05 | 0.830 | n.s. |
| NOCOD-C3 | $c_2 = c_4$ | 6184.57 | 18 | 0.726 | 4.62 | 0.032 | * |
| NOCOD-D | $s_1 = s_4$ *and* $c_1 = c_4$ | 6179.96 | 17 | 0.730 | 0.00 | 0.997 | n.s. |

The models are described in the text. They correspond to different simplifications of the complete model (COD-A and NOCOD-A, respectively with and without codominance hypothesis), indicated in the "Simplification" column. *F*-test statistic values and *P*-values indicate whether the deviance of the model considered is significantly different from that of the complete model A ([n.s.] *P*-value > 0.05, * *P*-value < 0.05, ** *P*-value < 0.01, *** *P*-value < 0.001). Each dominance parameter was set to 0.5 (COD) or freely estimated (NOCOD).

[1]The residual deviance of each model is scaled to the overdispersion of model A.

[2]df is the number of degrees of freedom.

[3]%TD is the part of the total deviance explained by each model.

## TABLE 2

**Best fitted parameters**

A-

| Parameter | Best value | Support limits |
|---|---|---|
| $s_1$ | 0.19 | 0.15 - 0.24 |
| $s_2$ | 0.37 | 0.28 - 0.53 |
| $s_4$ | 0.19 | 0.16 - 0.21 |
| $c_1$ | 0.078 | 0.058 - 0.10 |
| $c_2$ | 0.12 | 0.050 - 0.28 |
| $c_4$ | 0.036 | 0.027 - 0.045 |
| $t^*$ | 45 | 30 - 60 |

B-

| Parameter | Best value | Support limits |
|---|---|---|
| $s_1$ | 0.17 | 0.13 - 0.21 |
| $s_2$ | 0.51 | 0.35 - 0.72 |
| $s_4$ | 0.18 | 0.15 - 0.24 |
| $c_1$ | 0.057 | 0.040 - 0.079 |
| $c_2$ | 0.20 | 0.084 - 0.52 |
| $c_4$ | 0.059 | 0.027 - 0.11 |
| $t^*$ | 48 | 30 - 60 |
| $h^s_{10}$ | 0.19 | 0.00 - 0.57 |
| $h^s_{40}$ | 0.29 | 0.00 - 0.59 |
| **$h^s_{20}$** | **0.076** | **0.00 - 0.29** |
| $h^s_{14}$ | 1.00 | 0.00 - 1.00 |
| $h^s_{12}$ | 1.00 | 0.44 - 1.00 |
| $h^s_{24}$ | 0.54 | 0.095 - 1.00 |
| $h^c_{10}$ | 0.65 | 0.44 - 1.02 |
| $h^c_{40}$ | 0.21 | 0.00 - 0.52 |
| $h^c_{20}$ | 0.36 | 0.00 - 0.86 |
| $h^c_{14}$ | 2.86 | 0.00 - 3.00 |
| $h^c_{12}$ | 0.00 | 0.00 - 0.88 |
| $h^c_{24}$ | 0.64 | 0.00 - 1.92 |

For each parameter, the best value fitted is indicated, associated to the corresponding support limits (see text), A) for codominance hypothesis (COD-A model) and B) dominance

parameters being freely estimated (NOCOD-A model). In the last case, the estimated value of the dominance parameters, for selection, $h^s_{ij}$, or for cost, $h^c_{ij}$, are also indicated (values significantly different from 0.5, i.e codominance, are bolded).

FIGURE LEGENDS:

FIGURE 1. Sample site locations in the northwest southeast transect in the Montpellier area. Samples are indicated with black circles. The dashed line represents approximately the border between treated and untreated areas (Labbé et al. 2005). *C. pipiens* is present in the whole area.

FIGURE 2. Cline fitting of the best model. The expected patterns of frequency variation along the sampling transect are presented for each year of sampling. Samples frequencies of each allele are represented: $Ester^1$ with diamonds, $Ester^4$ with black triangles and $Ester^2$ with crosses. Lines represent the expected clines under the NOCOD-A model (see Material and Methods): $Ester^1$ with solid line, $Ester^4$ with interrupted line and $Ester^2$ with dotted line. The susceptible allele is not represented as its frequency is equal to 1- Σ(resistant allele frequencies).

FIGURE 3. Interannual variation of *Ester* alleles frequencies. The frequencies presented here are the maximum frequencies, i.e. at the coast. The first allele to appear was $Ester^1$ (with white diamonds), then replaced by $Ester^4$ (black triangles) (GUILLEMAUD *et al.* 1998). Recently, a third allele, $Ester^2$, invaded the Montpellier area (crosses) (LABBÉ *et al.* 2005). We used the estimation of coastal frequencies of the different alleles provided by LABBÉ *et al* (2005). Lines represent the fitted values according to the NOCOD-A model (see Material and Methods). The susceptible allele is not represented as its frequency is equal to 1- Σ(resistant allele frequencies).

FIGURE 1.

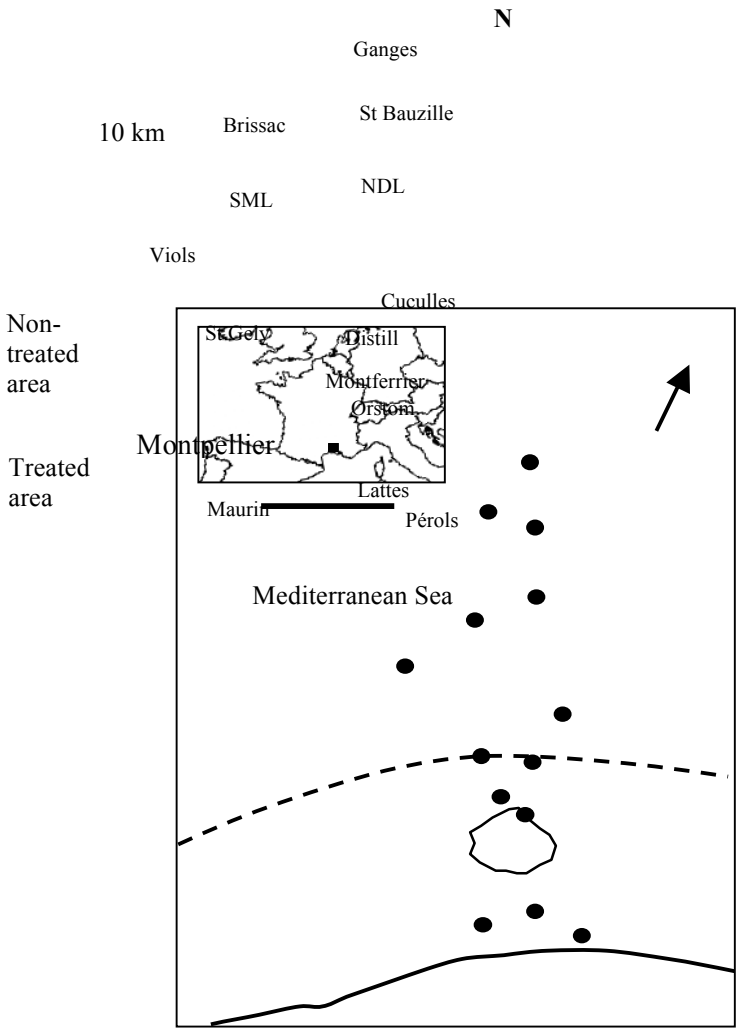

N
Ganges
10 km
Brissac
St Bauzille
NDL
SML
Viols
Cuculles
Non-treated area
Treated area
St Gely
Distill
Montferrier
Orstom
Montpellier
Lattes
Maurin
Pérols
Mediterranean Sea

FIGURE 2.

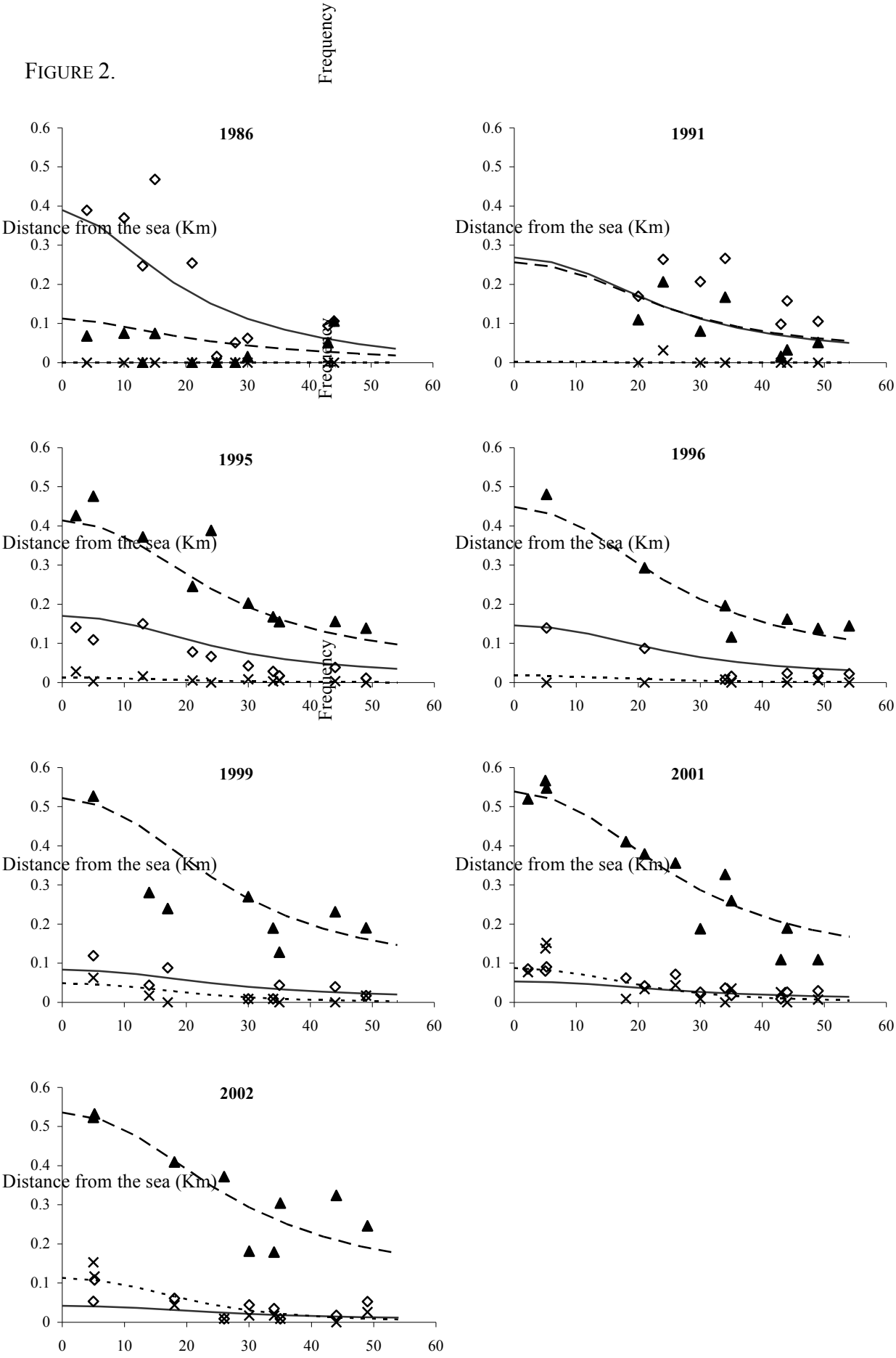
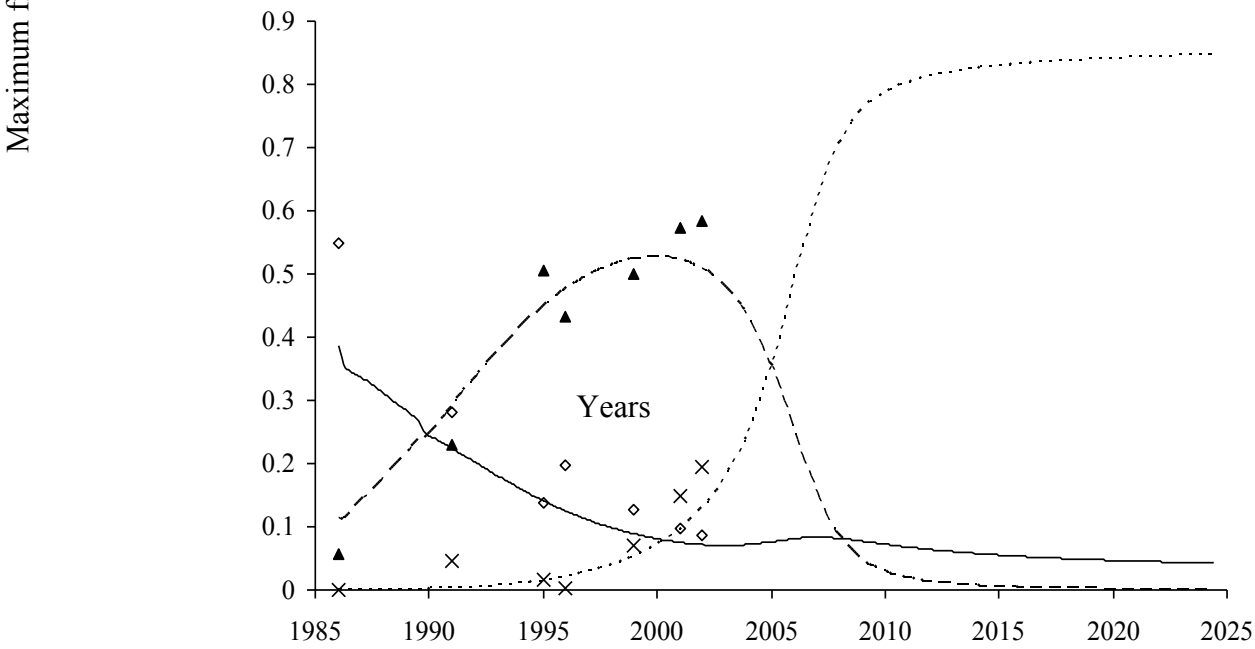

Frequency
1986
1991
1995
1996
1999
2001
2002
Distance from the sea (Km)

FIGURE 3.


Maximum frequency
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Years
1985
1990
1995
2000
2005
2010
2015
2020
2025