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Relating fitness to long-term environmental variations in natura

PASCAL MILESI,* THOMAS LENORMAND,† CHRISTOPHE LAGNEAU,‡ MYLÈNE WEILL* and PIERRICK LABBÉ*

*Institut des Sciences de l'Evolution de Montpellier (UMR 5554, CNRS-UM-IRD-EPHE), Université de Montpellier, Place Eugène Bataillon, 34095 Montpellier, Cedex 5, France, †CEFE UMR 5175, CNRS, Université de Montpellier, Université Paul-Valéry Montpellier, EPHE -1919 route de Mende, F-34293 Montpellier, Cedex 5, France, ‡Entente Interdépartementale pour la Démoustication du littoral méditerranéen, 34 rue du Nègue-Cat 34135, Mauguio, France

Abstract

Quantifying links between ecological processes and adaptation dynamics in natura remains a crucial challenge. Many studies have documented the strength, form and direction of selection, and its variations in space and time, but only a few managed to link these variations to their proximal causes. This step is, however, crucial, if we are to understand how the variation in selective pressure affects adaptive allele dynamics in natural settings. We used data from a long-term survey (about 30 years) monitoring the adaptation to insecticides of *Culex pipiens* mosquitoes in Montpellier area (France), focusing on three resistance alleles of the *Ester* locus. We used a population genetics model taking temporal and spatial variations in selective pressure into account, to assess the quantitative relationships between variations in the proximal agent of selection (amounts of insecticide sprayed) and the fitness of resistance alleles. The response to variations in selective pressure was fast, and the alleles displayed different fitness-to-environment relationships: the analyses revealed that even slight changes in insecticide doses could induce changes in the strength and direction of selection, thus changing the fitness ranking of the adaptive alleles. They also revealed that selective pressures other than the insecticides used for mosquito control affected the resistance allele dynamics. These fitness-to-environment relationships, fast responses and continuous evolution limit our ability to predict the outcome of adaptive allele dynamics in a changing environment, but they clearly contribute to the maintenance of polymorphism in natural populations. Our study also emphasizes the necessity of long-term surveys in evolutionary ecology.

Keywords: adaptation dynamics, insecticide resistance, population genetics model, selective pressure variations, time-series

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Introduction

Determining how selective pressures shape the adaptive response of organisms in natura has been a crucial challenge for 150 years and remains so today (Lewontin 1974; Endler 1986; Siepielski *et al.* 2009; Barrett & Hoekstra 2011). One of the principal difficulties involved is the measurement of fitness in the field. Natural

selection has been thoroughly investigated at phenotypic level, particularly since the development of multivariate methods (Lande & Arnold 1983). Many studies have documented the strength, form and direction of selection (Conner 2001; Kingsolver *et al.* 2001; Hereford *et al.* 2004) and its variation over space (reviewed in Kawecki & Ebert 2004; Siepielski *et al.* 2013) and time (reviewed in Siepielski *et al.* 2009, 2011; Bell 2010; but see also Morissey & Hadfield 2012). The interpretation of these variations is hindered by a series of well-known issues (Mitchell-Olds & Shaw 1987; Rausher

Correspondence: Pierrick Labbé, Fax: xx xxx; E-mail: pierrick.labbe@umontpellier.fr

1992; Bell 2008; Millstein 2008; Gallet *et al.* 2012; Lenormand *et al.* 2015), including the impact of drift, measurement error and trait plasticity. Another major problem is that natural selection can vary at different spatial and temporal scales. However, not all variations are relevant: for example, variations in selection over periods shorter than the generation time (e.g. between life stages, Schluter *et al.* 1991; seasons, Benkman & Miller 1996; or years for longer lived species, Schemske & Horvitz 1989; Grant & Grant 1995) and distances of less than the dispersal distance are not necessarily relevant for adaptation, as they can miss important trade-offs. Finally, natural selection may favour phenotypes that can cope with rare (relative to the scale of observation) and extreme events that are difficult to sample properly (Gutschick & BassiriRad 2003). Many of these events are central to the functioning of the ecosystem (fire, flood, storms, etc.) and must be understood if we are also to understand adaptation (Karlin & Lieberman 1974; Grant & Grant 1993). Repeated sampling, over sufficiently large geographical areas and time periods, is therefore required to ensure that these events are picked up.

Over and above these issues inherent to the measurement of fitness in the field, there is another major problem: it is generally more difficult to link selection to its causes than to quantify it (Wade & Kalisz 1990; Caruso *et al.* 2003; Siepielski *et al.* 2009). Like finding a needle in a haystack, it is indeed often extremely difficult to identify the *agent of selection* precisely, let alone determine its quantitative relationship to fitness, as phenotypic and environmental variations are both complex and multidimensional (Barrett & Hoekstra 2011). The agent of selection affecting the variation in a particular adaptive trait must be identified from a large number of correlated and interdependent variables acting on a similarly complex multivariate phenotype. Nevertheless, in many cases, the agent of selection can be reasonably inferred from field data (Bishop *et al.* 1975; Grant & Grant 1995; Losos *et al.* 1997; Carlson & Quinn 2007 see also references in Endler 1986) and further investigated by experimentation (Bishop 1972; Reznick & Bryga 1987; Losos *et al.* 1997; Rundle *et al.* 2003; Bradshaw *et al.* 2004).

However, the data required to assess the quantitative link between environmental and fitness variations, that is fine-scale measurements of well-identified agents of selection over time and space, are not generally available (but see, e.g., Bishop *et al.* 1975; Grant & Grant 1995; Carlson & Quinn 2007). Adaptations to environmental variation caused by humans (e.g. insecticide resistance, Whalon *et al.* 2008; Norris *et al.* 2015; antibiotic resistance, Nsanjabana *et al.* 2010; Gonzales-Canzels *et al.* 2011; heavy metal tolerance, Janssens *et al.*

2009) constitute a useful system for investigating the fitness response to changes in the environment. In such cases, the agent of selection is easier to identify and could, in principle, be quantified. Furthermore, the adaptive responses described so far to these anthropogenic selective pressures have a simple genetic determinism and can thus be traced in natural populations. However, even in these cases, the link between environmental variation and fitness remains mostly qualitative, semiquantitative at best: even in the best known examples, environmental variation is usually described in a binary fashion (e.g. mine vs pasture for *Holcus lanatus*, Macnair 1987; treated vs nontreated areas for insecticide resistance studies Lenormand *et al.* 1999), ignoring the continuous nature of the quantitative variation in selection pressure (e.g. concentration of particles in coal smoke, heavy metals or pesticides). In this study, we aimed to go beyond this simplified description, providing a quantitative explanation of the relationship between environmental variation (the agent of selection) and fitness in a natural setting.

We used insecticide resistance in *Culex pipiens* mosquitoes as a case study. Organophosphate (OP) insecticides were used in the Montpellier area (South of France) to control mosquito populations until 2007, when they were replaced by *Bacillus thuringiensis* var. *israelensis* (Bti) toxins in line with new EU regulations (European Commission 2007/393/EC 2007). The resistance of *Culex pipiens* mosquitoes to OPs in this area has been monitored for more than 40 years, providing one of the best-documented examples of adaptation to environmental modifications in natura.

Three different resistance alleles at the carboxyl esterase encoding *Ester* locus (*Ester*¹, *Ester*² and *Ester*⁴, Guillemaud *et al.* 1998) have been reported to segregate in natural populations since the beginning of OP treatments. Like most new adaptations, these alleles are associated with pleiotropic deleterious effects, a selective cost (Carrière *et al.* 1994; Chevillon *et al.* 1997; Berticat *et al.* 2002; Bourguet *et al.* 2004; Duron *et al.* 2006). Only the southern coastal strip of the Montpellier area was treated (Fig. 1). This resulted in antagonistic selective pressures: resistance alleles were selected for in the southern treated area (hereafter referred to as the TA), as they allowed survival, and selected against in the northern untreated area (hereafter referred to as the UTA), due to their selective costs. Along a south-north transect, this results in the clinal distribution of their frequencies, making it possible to quantify the key parameters driving the long-term dynamics of *Ester* resistance alleles (e.g. migration, fitness coefficients, dominance Guillemaud *et al.* 1998; Lenormand *et al.* 1998, 1999; Labbé *et al.* 2009), and their within-year variations in relation to seasons and OP usage Lenormand

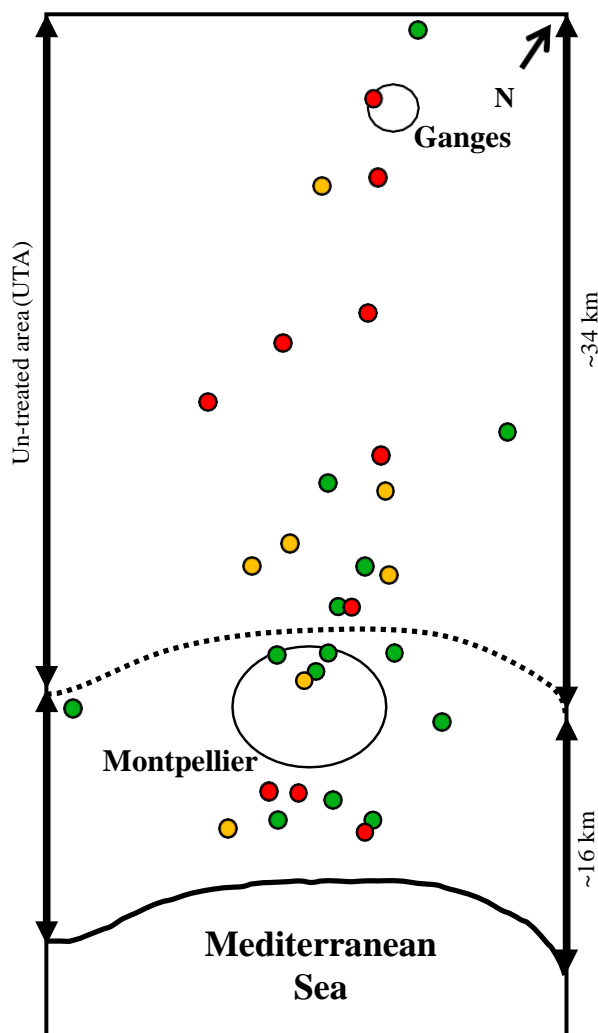


Fig. 1 Sampling transect map. Dots localize the various populations sampled over the 1986–2012 period. Colours indicate the number of years of sampling for each site (1 – green; 2–4 – yellow; >4 – red). The studied transect crosses two areas: a coastal strip treated with OPs (TA) and an inland untreated area (UTA); the approximate transition between these two areas is represented by the dotted line. The main towns in the TA and UTA are indicated by black circles.

et al. 1999; Lenormand & Raymond 2000; Gazave *et al.* 2001).

The continuous sampling of mosquito populations along the transect provided us with a data set of *Ester* allele frequencies covering a period of about 30 consecutive years. We also compiled and made use of data for the amount of OPs used each year for mosquito control in the TA. This data set provided us with a quantitative spatial and temporal description of the environment, which allowed measuring the causal link between quantitative environmental variation and quantitative variation in fitness for several alleles at a locus. We first

described the strong correlations between variations in OP quantity and variations in allele frequencies. We then used a deterministic (*i.e.* no genetic drift) population genetics model to infer the specific fitness-to-environment response of each *Ester* resistance allele by explicitly and quantitatively linking environmental variation (*i.e.* the OP quantity, measuring the selective pressure variations) and fitness variation over the 1986–2012 period. It showed that even slight variations in insecticide doses induced changes in the strength and direction of selection acting on the various resistance alleles, thus changing the outcome of their dynamics.

Materials and methods

Ester resistance alleles in the Montpellier area

Carboxyl esterases (COEs) catalyse cleavage of the ester bond of many molecules, including OPs (Oakeshott *et al.* 2005; Labbé *et al.* 2011). In *Culex pipiens*, COE-mediated resistance is achieved by the overproduction of these enzymes due to upregulation or amplification of the genes encoding them (Rooker *et al.* 1996; Guillemaud *et al.* 1998). In the Montpellier area, three different *Ester* resistance alleles have been described: *Ester*¹ (upregulation), and *Ester*² and *Ester*⁴ (gene amplification).

As the various alleles are easy to identify by protein electrophoresis, their dynamics have been monitored since 1986 in the Montpellier area, by sampling a similar transect in late June or early July each year (Guillemaud *et al.* 1998; Lenormand *et al.* 1999; Labbé *et al.* 2005; this study). This transect covers about 50 km and runs in a south–north direction from the Mediterranean to inland areas (Fig. 1). Insecticide use varies over this transect, with a treated area (TA) running along the coast and about 16 km wide (Labbé *et al.* 2009) and an untreated area (UTA) further inland (Fig. 1). About ten larval *Cx. pipiens* populations have been collected almost each year along the sampling transect (some of the sites have changed, see Fig. 1) throughout the course of the survey. These larvae were reared to adulthood in the laboratory, and adults were stored in liquid nitrogen for further analyses.

Insecticide treatments

In the coastal TA (Fig. 1), the local mosquito control agency (*Entente Interdépartementale pour la Démoustication*, EID) regularly treated larval breeding sites with temephos (Abate®, Bayer), an OP insecticide, until 2007. EID provided us with the total quantities used per year from 1990 to 2007 (Table 1).

Table 1 Data collection from 1986 to 2012

Year	86*	87*	...	90	91*	92	93*	94	95*	96*	97	98	99†	00	01†	02†	03†	04†	05†	06†	07†	08†	09†	10†	12†	Total
N_{pop}	10	3	—	—	7	—	2	—	10	7	—	—	8	—	12	9	8	8	8	8	7	4	4	13	9	142
N_i	354	125	—	—	217	—	110	—	1203	512	—	—	411	—	736	521	464	464	466	464	406	236	722	582	722	8519
T	8.82	8.82	...	8.82	8.12	3.31	2.60	1.95	1.49	2.58	4.50	2.31	3.40	3.13	4.30	3.62	1.64	2.46	2.65	0.18	0.11	0.00	0.00	0.00	0.00	0.00

The number of populations collected (N_{pop}), the number of individuals analysed (N_i) and the total amount of OPs applied (T) each year in the treated area are presented. From 1986 to 1989, treatment applications (*i.e.* the size of the treated area and the amounts used) did not differ significantly from those in 1990 (EID 1992; Guillemaud *et al.* 1998). We therefore attributed the same amount of OPs to the years for which this information was missing (*italics*). The distribution of the insecticide within the treated area did not change significantly between 1990 and 2005 (Guillemaud *et al.* 1998, this study, data not shown). After 2007, temephos was no longer used, this product being replaced by Bti, a mixture of toxins extracted from *Bacillus thuringiensis* var. *israelensis*.

*Data from Guillemaud *et al.* 1998.

†Data from Labbé *et al.* 2009.

‡This study, see Table S1 (Supporting information).

Phenotyping

The 1986–2002 phenotype data set was already available (Guillemaud *et al.* 1998; Labbé *et al.* 2005, 2009). It was extended by phenotyping 58 mosquitoes from each of the 74 populations from 2003 to 2012. The *Ester* phenotype of each mosquito was obtained by starch gel electrophoresis (Tris-malate-EDTA 7.4 buffer, Pasteur *et al.* 1988). Overproduced esterases are dominant over non-overproduced esterases under our electrophoretic conditions. The nomenclature and the correspondence between genotypes and the observed phenotypes are given in Table S1 (Supporting information).

Allele frequencies and clines

Allele frequencies and their support limits (equivalent to 95% confidence intervals) were estimated from phenotypic data, independently for each population and each year, using the maximum-likelihood approach developed by Lenormand *et al.* (1999).

As the position and number of the sampled populations changed between years over the total period; synthetic parameters summarizing the cline were thus inferred to allow between-years cline comparisons. A geometric cline of allele frequencies (p_i) was fitted, by maximum-likelihood methods (see below), to all the samples for each sampling year. This cline, adapted from Lenormand *et al.* (Lenormand *et al.* 1998), was approximated by a negative exponential function:

$$p_i = h_i \cdot e^{-(a_i \cdot x^2)} \quad 1(a)$$

where h_i is the maximum frequency of each allele i . (MaxAF), a_i describes the shape of the cline for allele i and x is the distance to the sea. The parameters of this descriptive cline thus represent and summarize the observed data.

Population genetics model

To quantify the parameters influencing the allele dynamics at the *Ester* locus, we modified the model used in Labbé *et al.* (2009). This model is deterministic; that is, genetic drift was neglected: previous studies suggested, in particular due to the large size of mosquito populations, that drift plays, at best, a minor role in resistance alleles dynamics (Labbé *et al.* 2005). It is a stepping stone model (Lenormand & Raymond 1998, 2000; Lenormand *et al.* 1998, 1999) that considers 35 demes 2 km apart but connected by migration, and implements three successive steps at each cycle (*i.e.* each generation): reproduction, selection and migration. As described by Lenormand *et al.* (1999), we considered there to be 13 generations per year in *Cx. pipiens* from

southern France, corresponding to a total of 339 cycles for the period 1986–2012.

Reproduction

The allele frequencies in each generation were calculated from those in the previous generation, assuming panmixia independently in each deme, as inferred from the observed phenotypic data (Labbé *et al.* 2005). Each deme was considered to be an infinite population (no drift).

Selection

The fitness (w_{ij}) of a diploid genotype combining the *Ester* alleles i and j depends on the selective advantages (s_i and s_j) and costs (c_i and c_j) of each allele. We assumed codominance of advantages and of costs. w_{ij} was thus calculated as follows:

$$w_{ij} = 1 + \gamma[s_i + 0.5(s_j - s_i)] - [c_i + 0.5(c_j - c_i)], \quad (2)$$

where 1 is the fitness of a susceptible homozygote and c a variable indicating whether the considered deme is in the TA ($c = 1$) or the UTA ($c = 0$). Both the resistance

advantage s and the selective cost c affect fitness in the TA, whereas only c has an effect in the UTA. The frequency of each genotype after selection was calculated separately for each deme, as its frequency before selection multiplied by the ratio of its fitness to mean fitness (Lenormand & Raymond 2000; Labbé *et al.* 2009).

Migration

Migration between demes was calculated as an approximately Gaussian dispersal kernel with a parent-offspring distance standard deviation $\sigma = 6.6 \text{ km/generation}^{1/2}$ (Lenormand *et al.* 1998).

Initial conditions

As described by Labbé *et al.* (2009) and to allow more flexibility, the eqn 1a was slightly modified to infer the initial (*i.e.* in 1986) allele frequencies at the *Ester* locus as:

$$p_i = h_i \cdot e^{-(a_i \cdot x^2 + b_i \cdot x)} \quad (1b)$$

where b_i is an additional parameter defining the slope of the cline. As the *Ester*² allele was not yet present in 1986, we introduced it $t_{\text{app}2}$ generations after 1986, at a frequency of 0.01 in all demes in the TA ($t_{\text{app}2}$ is estimated in the model).

The various parameters were estimated by a maximum-likelihood approach.

Parameter estimations

Three types of inferences were thus computed independently from the phenotypic data: (i) the observed resistance allele frequencies, inferred in each sample independently, to plot the data, (ii) synthetic cline parameters, describing the observed resistance allele frequency at any position on the transect, inferred for each year independently, and (iii) fitness parameters driving the resistance allele dynamics (and their relation to treatment data), inferred using the population genetics model: deterministic recursions generated the predicted frequency of each allele at any point in time for the 1986–2012 period and at any position over the whole transect, for a given set of parameter values.

In each analysis, the inferred parameters allow computing the predicted phenotypic frequencies, which were then compared with the observed data of the relevant sample data set: (i) the phenotype frequencies in a given sample, (ii) the phenotype frequencies of all samples of a given year and (iii) the phenotype frequencies of all samples.

To this end, we calculated the log-likelihood L of observing all the data:

$$L = \sum_t \sum_i \sum_j n_{ijt} \ln(f_{ijt}), \quad (3)$$

with n_{ijt} and f_{ijt} the observed number (from phenotypic data) and the predicted frequency (from allelic frequencies/cline/model parameters), respectively, of individuals with phenotype i in population j at time t . It was simultaneously maximized (L_{max}) for all inferred parameters of a given equation/model over the whole relevant data set, with a simulated annealing algorithm (Lenormand & Raymond 1998, 2000; Lenormand *et al.* 1998, 1999; Labbé *et al.* 2009).

For each parameter, the support limits (SL) were calculated as the minimum and maximum values that the parameter could take without significantly decreasing the likelihood (Labbé *et al.* 2009); SL are roughly equivalent to 95% confidence intervals. Concretely, the upper (p_{max}) and lower (p_{min}) SL for each parameter p were inferred while all other parameters were allowed to change, ensuring that we find the actual p_{max} and p_{min} in the range of the multidimensional parameter landscape. We used the same equation/model and the same simulated annealing method, but instead of maximizing L , we maximized (p_{max}) or minimized (p_{min}) the values of p for which the likelihood was not significantly different from the maximum likelihood (*i.e.* L_{max} minus 1.96).

Overdispersion was calculated for each model as the residual deviance $D = -2L$ divided by the residual degrees of freedom (Dd.f.). The percentage of the total

deviance explained by a model was calculated as % TD = $(D_{\max} - D_{\text{model}})/(D_{\max} - D_{\min})$, with D_{\max} and D_{\min} the maximal and the minimal deviance, respectively. Recursions and likelihood maximization algorithms were written and compiled with LAZARUS v1.0.10 (<http://www.lazarus.freepascal.org/>).

Hypothesis testing using the population genetics model

The model developed by Labbé *et al.* (Labbé *et al.* 2009) considered 13 parameters (Table 2): two selection coefficients (s_i and c_i) for each resistance allele i , in addition to three initial allele frequency parameters (h_i, b_i, a_i) for

$Ester^1$ and $Ester^4$; $Ester^2$ was not yet present in 1986, so that the last parameter was its date of appearance ($t_{\text{app}2}$).

We added to this original model several parameters, which allowed testing for various hypotheses about how environmental variations could affect the *Ester* dynamics:

- 1 to test for reductions in the fitness costs of *Ester* alleles (c_{ri}), the model was allowed to fit different selective costs after generation $t_{\text{cost}r}$ a parameter estimated simultaneously with the others; from generation t_{cost} onwards, c_i was replaced by $c_i - c_{ri}$ in eqn 2 (see Methods section 'Migration-selection model').
- 2 to estimate the impact of selective pressures other than the insecticide used for mosquito control, the selective advantage (s_i) was broken down into two terms, s_{iT} and s_i^r : s_{iT} is the advantage due specifically to resistance to the OPs used for mosquito control; s_i^r is the selective advantage of the resistance alleles due

Table 2 Best population genetics model

Parameters			Estimate (SL)	F (Dd.f.)	
Initial conditions (1986)	MaxAF	h_1	0.55 (0.41–0.70)	19 314 (1)***	
	Slope	b_1	0.11 (0.07–0.15)	96 (1)***	
	Shape	a_1	0.00 (0.00–0.00)	0 (1) ^{n.s}	
	MaxAF	h_4	0.04 (0.02–0.07)	90 467 (1)***	
	Slope	b_4	0.02 (0.00–0.08)	3 (1) ^{n.s}	
	Shape	a_4	0.00 (0.00–0.00)	0 (1) ^{n.s}	
$Ester^2$ appearance generation		$t_{\text{app}2}$	55 (35–65)	8592 (1)***	
Costs change generation [#]		t_{cost}	261 (260–267)	15 (4)**	
Selective costs before t_{cost}		c_1	0.08 (0.07–0.10)	196 (1)***	
		c_2	0.11 (0.08–0.17)	67 (1)***	
		c_4	0.04 (0.03–0.05)	183 (1)***	
		c_{r1}	0.02 (0.003–0.05)	6 (1)*	
Selective cost reduction after t_{cost} [#]		c_{r2}	0.05 (0.01–0.10)	5 (1)*	
		c_{r4}	0.00 (0.00–0.01)	0 (1) ^{n.s}	
	Selective advantages	Treatment	s_{1T}	0.22 (0.19–0.26)	8 (3)*
			s_{2T}	0.32 (0.22–0.43)	72 (3)***
s_{4T}			0.21 (0.14–0.28)	77 (3)***	
	Background [#]	s_{1b}	0.13 (0.08–0.18)	325 (1)***	
		s_{2b}	0.08 (0.00–0.16)	2 (1) ^{n.s}	
		s_{4b}	0.12 (0.09–0.14)	217 (1)***	
		d_1/m_1	2.13 (1.96–2.39)/80.0 (2.25–80)	8 (2)*	
Dose-response parameters [#]		d_2/m_2	1.95 (1.79–2.27)/40.0 (2.30–40)	65 (2)***	
		d_4/m_4	3.66 (3.17–4.20)/1.26 (1.12–2.1)	77 (2)***	
	Relative fitnesses ($w_1:w_2:w_4$)		TA	1.14:1.19:1.16	
		UTA	0.92:0.89:0.96		
		%TD	92.5		
		Od	1.47		

The estimated value of the different parameters is given with their associated support limits (SL). The significance of each parameter (for their description, see Methods section 'Population genetics model' and 'Hypothesis testing using the population genetics model') was then tested by removing it and comparing the likelihood of the resulting model with that of the best model, using LRT_{od} (^{n.s}non-significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). The LRT_{od} (F) statistic and the difference in the number of degrees of freedom (Dd.f.) are also indicated for each parameter. The fitness of each resistance allele (w_i) before OP removal and relative to the susceptible allele ($w_0 = 1$) is also given, for both the TA and UTA. Finally, the log-likelihood (L), the total deviance explained (%TD) and the overdispersion (Od) of the best model are indicated. Parameters labelled with # were not included in the model described by Labbé *et al.* (48) (see Methods section 'Hypothesis testing using the population genetics model').

to the presence of other agents of selection. s_i could thus remain >0 even in the absence of insecticide treatment.

3 to take into account the quantitative variations in OP insecticide use over the 1986–2012 period, the selective advantage of *Ester* resistance alleles due to mosquito control (s_{iT}) was calculated as a function of the amount of OPs used each year t (T_t).

We took above points 2) and 3) into account by calculating the selective advantage s_i (eqn 2) as:

$$s_i = s_{iT} \cdot f(T_t) + s'_i, \quad (4)$$

We considered a flexible functional form for $f(T_t)$ (allowing for possible nonlinearity), assuming that it was monotonically increasing (selection intensity should increase with insecticide dose). We thus used a logistic function: $f(T_t) = 1 - (1/1 + e^{m_i \cdot (b_i + T_t)})$. This sigmoid curve is centred on dose b_i and has a maximum slope proportional to m_i ; these parameters are hereafter referred to as the dose–response parameters. We fitted m_i and b_i independently for each allele i but the model was constrained to accept only m_i/b_i combinations for which $f(T_t) < 0.0001$ when $T_t = 0$; so that $f(T_t) = 0$ in the absence of treatment. Thus, the advantage of the resistance alleles in the presence of mosquito control treatment varied from ~ 0 at low doses to s_{iT} at high doses.

Tests and control for overparameterization

The significance of each parameter was tested by comparing the likelihoods of the best model and of a model in which the tested parameter was withdrawn, using likelihood-ratio tests corrected for overdispersion [LRT_{od}; (Anderson *et al.* 1994)]. The best model includes 26 parameters. This number is fairly small given the size of the data set (994 phenotypic frequencies along a 50-km transect over 27 years, corresponding to over 8500 individuals sampled), but there is always a risk of overparameterization. One way to check for overparameterization is to check for structure in the model residuals: if they are randomly distributed, the inclusion of additional parameters would be superfluous.

We tested in particular whether the inclusion of the dose–response parameters resulted in overparameterization. A simplified model was implemented; this model included only a qualitative description of the environment, due to modification of the selective advantage (s_i), as $s_i = s_{iT} + s'_i$. The risk of overparameterization was then assessed by comparing the correlations of the residuals of the simplified and best models with the amounts of OPs applied.

For the period during which OPs were used, we calculated the simplified model residuals ϵ_j for all data points j as: $\epsilon_j = p_{j\text{obs}} - p_{j\text{mod}}$, where $p_{j\text{obs}}$ is the allelic frequency estimated from phenotypic data and $p_{j\text{mod}}$ the allelic frequency estimated with the model. Correlations between ϵ_j and T_t were tested by calculating Pearson's product-moments (R software v.3.1.1 <http://www.R-project.org/>).

Results

The 1986–2012 data set

Insecticide resistance. We estimated allele frequencies along the surveyed transect (Fig. 1), by phenotyping 58 individuals per sampled population per year by starch gel electrophoresis (Table S1, Supporting information). Over the entire 1986–2012 period, we analysed data from 8519 individuals from 142 sampled populations, with a mean of eight populations sampled per year (Table 1). The numbers of individuals displaying each phenotype have already been published for the 1986 to 2002 samples (Guillemaud *et al.* 1998; Labbé *et al.* 2005). For samples collected from 2003 to 2012, these data are presented in the Table S2 (Supporting information).

Insecticide treatments. The local mosquito control agency (EID) had used OPs for pest control since 1969. They are used essentially from March to October (seaside tourism), which has been shown to affect the allele dynamics within a year (Lenormand *et al.* 1999). The spatial distribution of treatments did not change significantly over time (Labbé *et al.* 2009; supplementary material; EID, personal communication). However, the amounts of OPs used annually in the treated area (TA) varied over the 1986–2012 period (Table 1). They varied according to changing general treatment policies: (i) first, from 1986 to 1991, temephos (an OP insecticide) was the only insecticide used, with relatively large quantities sprayed, around 8 L/km² (EID 1992; Guillemaud *et al.* 1998); unfortunately, precise information is unavailable for the years before 1990: the amounts used probably varied slightly around the mean, due to treatment adjustment to weather-linked variations in mosquito densities, but these variations were limited (EID 1992); (ii) in 1992, EID began to use new bacterial toxins [first, *Bacillus sphaericus* (Bs) then *Bacillus thuringiensis* var. *israelensis* (Bti)], and the amount of temephos used was decreased by a factor of more than two, to 1.5–4.5 L/km², with a mean value of about 3 L/km²; these variations again probably result from treatment adjustment (Table 1); (iii) finally, the amount of temephos applied was substantially decreased again in 2006 and 2007 (<0.2 L/km²), and this product was completely

withdrawn after 2007, in line with new European legislation (Table 1); OP insecticides have now been entirely replaced by Bti.

Environmental variations affect the dynamics of Ester alleles

Using this unique data set, we were able to carry out a precise survey of the dynamics of *Ester* resistance alleles.

First, the typing method gives only access to the phenotypes in each sample: we cannot differentiate *Ester* resistance allele homozygotes from heterozygotes carrying one resistant and one susceptible alleles (see Methods and Fig. S1, Supporting information). We thus used a maximum-likelihood approach to infer the allelic frequencies from these observed phenotypic frequencies, independently for each sample (Fig. S3, Supporting information).

Secondly, the position and number of the sampled populations changed between years over the total period; synthetic parameters were thus required for between-years cline comparison. A geometric cline was thus fitted for each resistance allele to the observed phenotypic data of all samples of each year (eqn 1a, Methods section 'Allele frequencies and clines', Table S4, Supporting information). These clines provide us for each resistance allele i with its maximum frequencies, or MaxAF $_i$ (*i.e.* its frequency at the coast), and a parameter a_i describing the shape of the cline, with their associated support limits (see Methods section 'Parameter estimations'). Both approaches provide purely descriptive representations of the spatial distribution and dynamics of the resistance alleles.

Resistance allele frequencies are correlated with the amount of OPs applied. As previously reported (Guillemaud *et al.* 1998; Labbé *et al.* 2009), during the period of OPs use, *Ester*¹ was initially replaced by *Ester*⁴, until the invasion of *Ester*² in the 1990s. As *Ester*² fitness appeared superior to that of the other resistance alleles in the presence of insecticide, Labbé *et al.* (2009) fore-told its increase in frequency (providing continuation of the OP treatments), eventually reaching fixation. Unexpectedly, the frequency of *Ester*² actually peaked in 2002, even since OPs were still used until 2006; thereafter, the *Ester* allele frequencies remained globally stable until 2005, with some marked differences between years (Fig. 2).

We investigated whether variations in the amounts of OPs applied could account for these allele frequency variations and for *Ester*² not increasing further in frequency. We first focused on the 1995–2008 period, when

all three resistant alleles were present in the area studied and the amount of OPs applied ranged from 0.11 to 4.5 L/km² (Table 1). There is on average 13 mosquito generations per year, and several studies have shown that the selection-migration equilibrium is rapidly restored each year, after insecticide treatments resume in spring (Lenormand *et al.* 1999; Labbé *et al.* 2009); we thus chose to directly analyse the relations between the summer *Ester* allele frequencies and the annual amount of OPs. Globally, *Ester* allele frequencies (resistance and susceptible alleles) in a given year were significantly correlated with the amounts of OPs used in the previous year (Fig. 3), but not with those used in the same year (Table S5, Supporting information). A highly significant negative correlation was found between susceptible allele (*Ester*⁰) frequencies at the sea and the amounts of OPs applied the previous year (Pearson's coefficient correlation $r = -0.81$, $t = -4.16$, d.f. = 9, $P < 0.01$, Fig. 3A). However, different correlations were observed when each resistance allele was considered separately. The MaxAFs of the *Ester*² and *Ester*⁴ alleles were correlated with the amounts of OPs used ($r = 0.74$, $t = 3.33$, d.f. = 9, $P < 0.01$ and $r = 0.70$, and $t = 2.95$, d.f. = 9, $P < 0.05$, respectively, Fig. 3B), but the correlation was not significant for *Ester*¹, despite a similar trend being identified ($r = 0.37$, $t = 1.2$, d.f. = 9, $P = 0.26$, Fig. 3B). If we considered the 1986–2008 period (*i.e.* when OPs were used), the frequency of the *Ester*¹ allele was greatly affected by the decrease in the amounts of OPs used after 1991 (from 8.12 to 3.31 L/km², Table 1 and Fig. 2). These relations were further investigated, by considering the allele frequencies directly inferred from the phenotypic data, over the whole transect, for the 1986–2008 period. Similar correlations were identified, demonstrating the relation between the resistance allele frequencies and the year-to-year variation in selective pressure on the whole transect (not only in the TA close to the coast; Fig. S6, Supporting information).

Resistance allele frequencies decreased after the withdrawal of OPs, but are now moving towards a new equilibrium. A major shift occurred around 2005. Anticipating the Europe-wide ban on OPs due to come into force in 2007, EID substantially decreased the amounts of OPs used between 2005 and 2007, when these products were withdrawn completely. As expected, the withdrawal of OP insecticides had a considerable effect on the dynamics of the *Ester* resistance alleles. After 2005, MaxAFs decreased very rapidly, with the frequencies of all the resistance alleles together falling from 0.85 to 0.37 between 2005 and 2010, before stabilizing again. The withdrawal of OPs thus led to a 56% decrease in

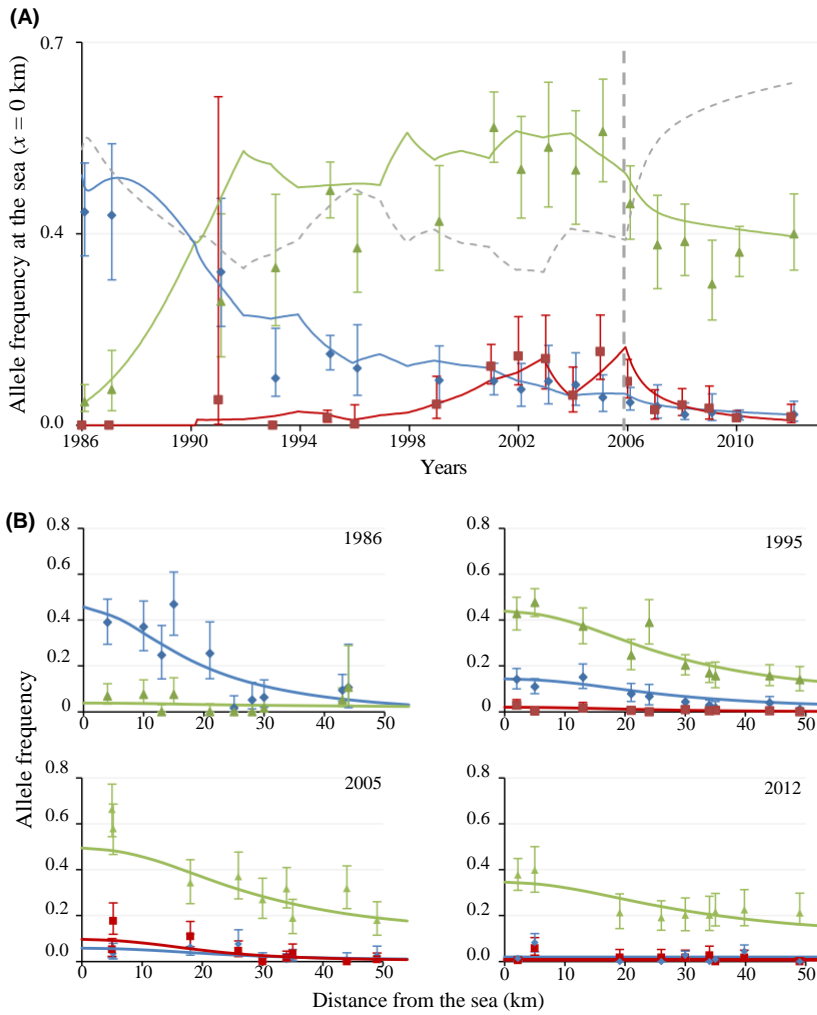


Fig. 2 *Ester* resistance allele dynamics over the period 1986–2012 in (A) time and (B) space. (A) Evolution of the resistance allele frequencies from 1986 to 2012. For each sampled year, the dots represent the maximum allele frequencies (MaxAFs, *i.e.* at the sea) for *Ester*¹ (blue diamonds), *Ester*² (red squares) and *Ester*⁴ (green triangles), synthesizing the observed phenotypic data. The evolution of the allele frequencies predicted by the best population genetics model (complete set of parameters with the maximum likelihood) is represented by the solid lines for the resistance alleles and by the grey dashed line for the susceptible. The grey vertical dashed line indicates the date at which the model re-estimated the cost of each resistance allele (t_{cost}). (B) Distribution of *Ester* resistance allele frequencies along the sampling transect. Dots represent the allele frequencies (with their support limits) inferred from the observed phenotypic frequencies for each sampled population. Lines represent the frequency cline predicted by the best population genetics model. The colour code is conserved. Only four representative years are figured (1986, 1995, 2005 and 2012, see Fig. S3, Supporting information for the others).

resistance allele frequency, highlighting the high selective costs of these alleles.

Over the entire sampling transect, all *Ester* allele frequencies followed a similar clinal distribution until 2005 (Labbé *et al.* 2009; and Fig. S3 and Table S4, Supporting information). After 2005, the overall decrease in the frequency of all resistance alleles softened these clines (*i.e.* the frequencies became more homogeneous between TA and NTA), as expected, due to spatial homogenization of the environment, before a new stabilization between 2010 and 2012 (Figs 2 and S3 and Table S4, Supporting information). Consequently, in 2012, *Ester*¹ and *Ester*² frequencies appeared to be uniform and low (around 0.01) over the entire transect: a_i parameters (see Methods section ‘Allele frequencies and clines’ eqn 1a), which describe the shape of the clines, were not significantly different from zero ($\text{LRT}_{\text{od}}, F = 0.64, \text{Dd.f.} = 1, P = 0.43$ and $F = 0.88, \text{Dd.f.} = 1, P = 0.48$, respectively, for *Ester*¹ and *Ester*²). By contrast, the clinal shape remained significant for *Ester*⁴ ($a_4 > 0$,

$\text{LRT}_{\text{od}}, F = 10.11, \text{Dd.f.} = 1, P < 0.001$) (Fig. S3, Supporting information). The frequency of this allele was relatively high over the entire transect, stabilizing at about 0.35 close to the sea and 0.16 inland (Fig. S3, Supporting information).

Quantifying the effects of environmental variations on fitness

Changes in fitness costs following OP withdrawal. One possible explanation for the persistence of resistance alleles after the withdrawal of OPs is changes in their fitness costs. We tested this hypothesis by fitting a selective cost reduction (c_{ri}) after t_{cost} generations, a number of generations also estimated by the model: the selective cost of the allele i after t_{cost} is thus $c_i - c_{ri}$ (see Methods section ‘Hypothesis testing using the population genetics model’, Table 2). Despite the low frequencies of the *Ester*¹ and *Ester*² alleles, which reduces the statistical power, the best fit suggested a significant decrease in

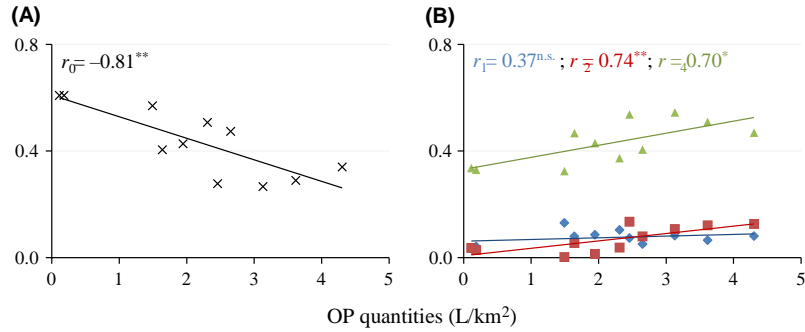


Fig. 3 *Ester* allele frequencies vs amounts of insecticide used for (A) the susceptible allele and (B) the resistance alleles. The data points indicate the allele frequencies close to the sea, inferred from phenotypic data. They are presented as a function of the amounts of OPs used in the previous year. (A): for *Ester*⁰ (black crosses) and (B): for *Ester*¹ (blue diamonds), *Ester*² (red squares) and *Ester*⁴ (green triangles). Straight lines represent the linear regressions between the two factors. The correlation coefficient r_i (Pearson's product-moment correlation) between f_i and the amount of OPs is provided, together with a P value indicating the significance of this correlation (n.s., $P > 0.05$; *, $P < 0.05$; **, $P < 0.01$; *** $P < 0.001$).

their associated costs: respectively, $c_{r1} = 0.02$, that is a 25% reduction (LRT_{od}, F -test = 6, Dd.f. = 1, $P = 0.02$) and $c_{r2} = 0.06$, that is over 50% reduction (LRT_{od}, F -test = 5, Dd.f. = 1, $P = 0.02$) after $t_{\text{cost}} = 261$ generations (Table 2). This number of generation corresponds to year 2006, when the amounts of OPs used were strongly decreased, anticipating the ban on these products (Table 1 and Fig. 2). However, no significant change in the cost of *Ester*⁴ was detected ($c_{r4} = 0.00$; Table 2).

Previous studies have suggested that the level of amplification of some *Ester* resistance alleles may vary (*i.e.* copy number variation) in response to selection pressures, with larger numbers of copies resulting in higher resistance, but also in higher costs (Weill *et al.* 2000; Berticat *et al.* 2002). Both *Ester*² and *Ester*⁴ result from amplifications (whereas *Ester*¹ results from the constitutive overexpression of a single copy). We therefore also quantified the level of amplification of these alleles, before and after the withdrawal of OPs, by quantitative real-time PCR on genomic DNA (for details, see Appendix S7, Supporting information). However, no change in amplification level was detected for either *Ester*² or *Ester*⁴ (Appendix S7, Supporting information).

Mosquito control treatments are not the only selective agent. Another explanation for the persistence of the *Ester* resistance alleles after OP withdrawal could be the presence in the environment of others compounds that could select these alleles. To test this hypothesis, the selective advantages of the resistance allele in the model, s_i , were partitioned into a selective advantage s_{IT} due to the OPs used for mosquito control (for which we know the doses used in the years studied) and another component, denoted s'_i corresponding to the potential

effects of other selective pressures (see Methods section 'Genetic model', eqn 4). The s'_i values estimated were significantly different from zero for *Ester*¹ and *Ester*⁴ (respectively, $s'_1 = 0.13$, LRT_{od}, F -Test = 325, Dd.f. = 1, $P < 0.001$ and $s'_4 = 0.12$, LRT_{od}, F -Test = 219, Dd.f. = 1, $P < 0.001$), but not for *Ester*² (Table 2). It thus appeared that *Ester*¹ and *Ester*⁴ still conferred a selective advantage, even after the withdrawal of OPs. The mosquito control treatments were therefore not the only selective agents acting on these alleles.

Fitness-to-environment relationships shape resistance allele dynamics. For quantification of the fitness-to-environment responses of the various alleles, the selective advantage due to mosquito control (s_{IT}) was calculated as a logistic function of the amounts of OPs used in each year t (T_i) over the period 1986–2012, with the addition of two dose-response parameters for each allele (see Methods section 'Genetic Model', 3). This best model fitted the data significantly better than the simplified model with constant s_{IT} (see Methods section 'Tests and control for overparametrization'; LRT_{od}, F -test = 33, Dd.f. = 6, $P < 0.001$).

Despite the highly significant result obtained, we checked whether the estimation of the dose-response parameters could have been driven by a few outlier samples. We ruled out such overparameterization by analysing the residuals of both the simplified and best models, with respect to the amounts of OPs applied (see Methods section 'Tests and control for overparametrization'). The residuals of the simplified model appeared to be structured and correlated with OP levels ($r = 0.15$, $t = 2.7$, d.f. = 324, $P = 0.007$), whereas those of the best model were not ($r = 0.09$, $t = 1.7$, d.f. = 324, $P = 0.095$; Fig. S8, Supporting information). Together with the correlations illustrated in Fig. 3, this confirms

that adding the dose-response parameters captured actual fitness responses to dose variations and that this improvement in fit was driven by the bulk of the data.

The parameter estimates indicated that the fitness-to-environment responses differed between the *Ester* resistance alleles (Fig. 4A): the selective advantages of these alleles were differently affected by the variations in OP quantities, confirming the correlations observed (Figs 3 and S6, Supporting information). The advantage of all alleles was significantly and positively dependent on OP levels (*Ester*¹: LRT_{od} , F -test = 8, Dd.f. = 2, P = 0.021; *Ester*²: F -test = 66, Dd.f. = 2, P < 0.001 and *Ester*⁴: F -test = 78, Dd.f. = 2, P < 0.001). However, the fitness-to-environment response was steep for *Ester*¹ and *Ester*² (Fig. 4A): they appeared to be disproportionately affected by slight variations in treatment in the 0 to ~2 L/km² range, but expressed their full advantage for higher OP levels (Fig. 4A). By contrast, *Ester*⁴ was affected over the whole 0 to 9 L/km² range, but the curve was smoother than for the other two alleles (Fig. 4A).

Overall, this quantitative analysis suggests that the fitness of the various resistance alleles is finely tuned to quantitative changes in the amounts of OPs applied, but also depends on changes in costs and on other selective forces. The overall net effect was that the allele with the highest fitness in the TA differed between treatment intensities (Fig. 4B): *Ester*² was more

advantageous at moderate to high doses of OPs, whereas *Ester*⁴ and *Ester*¹ were more advantageous at low doses. However, *Ester*⁴ was the fittest resistance allele in the UTA, due to its lower fitness cost (Table 2). Following the withdrawal of OPs, and thanks to continuing secondary sources of selection, *Ester*¹ and *Ester*⁴ appeared to confer similar and higher levels of fitness in the former TA (Fig. 4B). In the UTA, *Ester*⁴ remained the fittest resistance allele, despite the decrease in the fitness cost of *Ester*¹ and *Ester*² (Table 2).

Discussion

To investigate how the variations in the amounts of OPs applied (*i.e.* selection intensity) affected the dynamics of the *Ester* resistance alleles (*i.e.* the adaptive alleles), we first needed a quantitative description of the environment at a scale compatible with the scale at which the allele frequency variations were measured. Fortunately, the treated area was only slightly greater than the area over which mosquitoes can disperse, so the heterogeneous distribution of selection intensities within the treated area (due to spatial variations in insecticide use) could be ignored. We were also able to ignore the within-year variations, because such variations were not directly relevant to the long-term trend: no insecticide treatment occurred from October to March–April (leading to a decrease in

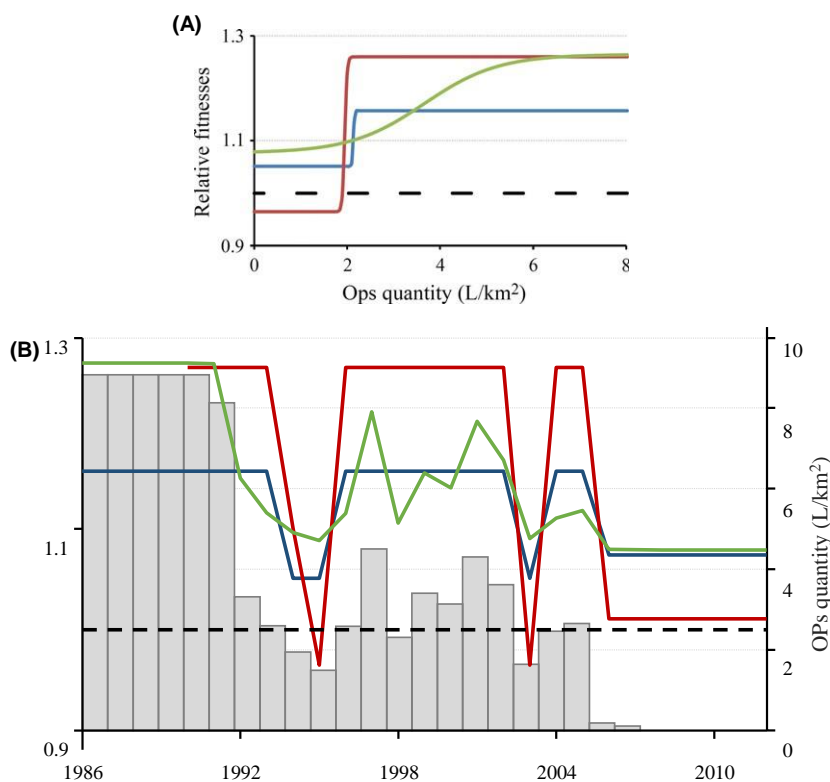


Fig. 4 Fitness-to-environment relationships and relative fitness of *Ester* alleles in the treated area over the 1986–2012 period. (A) The relative fitnesses of the different *Ester* alleles are represented as a function of the OP quantities (L/km²), *Ester*⁰ (susceptible allele) - dashed black line, *Ester*¹ - blue line, *Ester*² - red line and *Ester*⁴ - green line. (B) The solid lines represent the relative fitness (left axis) in the insecticide-treated area (TA) for each resistance allele (*Ester*¹ in blue, *Ester*² in red and *Ester*⁴ in green), estimated from the best model (with treatment-dependent fitnesses). The dotted line represents the fitness of the susceptible allele (*Ester*⁰ in black, $w_0 = 1$). For each year, fitness is calculated as: $1 + s_{iT} + s_i^d - c_i$ with s_{iT} , s_i^d and c_i the advantages (depending on the amount of OPs applied or other selective pressures) and the cost of the allele i (equal to $c_i - c_{i0}$ after t_{cost} , see text), respectively. The barplot shows the total amount of OPs (L/km², right axis) used each year in the treated area over the 1986–2012 period.

resistance allele frequency during this period due to the fitness costs of these alleles, Lenormand *et al.* 1999; Raymond *et al.* 2001), but it was previously shown that a selection-migration equilibrium was rapidly reached each year between susceptible and resistance alleles, after a few rounds of treatment (Lenormand *et al.* 1999; Labbé *et al.* 2009). We also took into account the total amounts of OPs used in the TA in each sampling year (therefore not taking into account the precise timing of treatment during the year). Thus, spatial and temporal variations in selective pressure were described quantitatively, at scales relevant to the long-term trends. Consistent with this description of the environment, we used *Ester* frequency data from only one season (early summer). Fitness estimates based on these data must therefore be considered as averages over the treatment period.

Adaptive responses are tuned to selective pressure variations

Annual OP use was found to be strongly correlated with summer resistance allele frequencies: the resistance allele frequencies in any given year were more strongly related to the amount of OPs applied in the previous year than to the amount of OPs applied in the same year, reflecting the longer timescale of resistance allele replacement. As stated above, in a year t , most of the treatments were applied from June to August, on five to eight mosquito generations. Then, after one or two more generations, a single generation of mosquito females entered caves to overwinter until March–April, producing one or two more generations before the recommencement of treatment in year $t + 1$ (Lenormand *et al.* 1999; Raymond *et al.* 2001). Our samples were collected in late June of year t (*i.e.* after only one or two rounds of treatment), but only a few generations (about three) after the end of the previous treatment campaign ($t-1$). Considering the long-term trend, and as we analysed the total amount of OPs applied in each campaign (*i.e.* a dozen rounds of treatment), the *Ester* frequencies in our June samples probably reflected the previous ($t-1$) year of selection more strongly than the current (t) year of selection, accounting for the time lag observed in the correlation.

This first analysis revealed that yearly variations in insecticide treatment intensity clearly affected the frequencies of the *Ester* alleles, confirming the high speed of the response to selection, that is detectable within a few generations (Lenormand *et al.* 1999). It thus suggested a quantitative and sharp direct relationship between the dose of insecticides and the fitness of the *Ester* resistance alleles, relationship that we further investigated.

The long-term survey reveals that Ester evolution is more complex than anticipated

Using a migration-selection population genetics model with few parameters (and checking for overparameterization, see Fig. S8, Supporting information), we were able to explain most of the total deviance (%TD = 92.5), with a low level of overdispersion (od = 1.47), for a long-term survey data set corresponding to 994 phenotypic frequencies measured along a 50-km transect over a period of 27 years.

This long-term survey covers a period during which a major environmental change occurred: anticipating new European Union regulations banning the use of OPs, EID substantially decreased the amounts of OP applied after 2005, before their complete withdrawal after 2007. From this date onwards, the dynamics of *Ester* resistance alleles should have been determined solely by their selective costs: the resistance alleles were expected to disappear, being replaced by the susceptible allele (the fittest in the absence of treatment), as already observed in other situations of selection pressure removal (Parrella & Trumble 1989; Casimiro *et al.* 2006a,b; Sharp *et al.* 2007). In line with these predictions, the withdrawal of OPs did indeed result in a rapid decrease in *Ester* resistance allele frequencies over the whole transect. Our yearly sampling made it possible to quantify the rate of decrease: resistance allele frequency fell by 51% in the first three years. This rapid change suggested that resistance management strategies based on the temporal alternation of insecticides (see Labbé *et al.* 2011) could be effective in natural populations, as long as resistance alleles remain costly. However, the following years showed that the Montpellier situation was actually more complex and that both evolution and human activities could impede resistance management, as shown thereafter.

Selective costs allow resistance management, but they might change over time. Changes in the costs of resistance alleles pose a major threat to resistance management: cost reductions have been documented after the withdrawal of selective pressure in cultures of prokaryotes or protozoa (Lenski 1988; Andersson & Hughes 2010; Duncan *et al.* 2011), although examples in natural populations of metazoans remain scarce (McKenzie 1996). This study is original in that the best model suggests that a decrease in the fitness costs of *Ester*¹ and *Ester*² could have occurred. These compensatory changes would coincide with the withdrawal of OPs and might thus involve a direct alteration of the expression level of *Ester*¹ and *Ester*² (rather than the occurrence of a modifier elsewhere in the genome). The cost of *Ester*⁴, however, appeared stable: this difference did not appear to be

related to overexpression (*Ester*¹) vs gene amplification (*Ester*² and *Ester*⁴), and the levels of *Ester*² and *Ester*⁴ amplification did not appear different before and after OP withdrawal (note, however, that sample size is limited for *Ester*², Appendix S7, Supporting information). Such decreases, if confirmed, would in any case slow resistance allele disappearance, thereby complicating resistance management.

Other human activities can prevent the elimination of resistance. Our study also had important implications for resistance management: from 2009 to 2012, *Ester* resistance allele frequencies remained relatively stable, rather than decreasing further. The frequencies of *Ester*¹ and *Ester*² were very low, but *Ester*⁴ remained frequent over the entire survey transect (Fig. S3, Supporting information). We inferred from the model that a significant proportion of the selective advantage of *Ester*¹ and *Ester*⁴ resistance alleles was unrelated to

mosquito control (s_1^{\dagger} and s_4^{\dagger} parameters were significantly different from zero, Table 2). These results suggest that selective pressures other than the OPs used for mosquito control affected the dynamics of *Ester*¹ and *Ester*⁴ resistance alleles, allowing their maintenance after the withdrawal of OPs, and thus impeding resistance management.

In 2012, *Ester*⁴ presented a clinal distribution similar to that observed before the withdrawal of OPs (Fig. S3, Supporting information). This clinal distribution of the resistance indicates that the selective pressures implicated are local and contemporary. The distribution of the persisting selective pressures moreover seems to be similar to that of EID insecticide treatments: concentrated in the area closest to the sea. Esterases do not confer cross-resistance to Bti, the insecticide now used by EID. Nevertheless, while we cannot rule out the persisting (and illegal) use of OPs either for personal protection or for crop treatments, many other compounds could select esterase resistance. Two anthropic factors have indeed such a distribution in this area: urbanization and agriculture. The habitat of *Cx. pipiens* consists of water bodies rich in organic matter and effluent from human activities. The larvae are thus probably exposed to many residuals from agricultural or urban activities, so high levels of detoxifying enzymes, such as esterases, may be advantageous. Such pollutants have already been shown to select for resistance mechanisms (e.g. resistance to insecticides in *An. gambiae*; see review in Nkya *et al.* 2013; Reid & McKenzie 2016). The particular environmental preferences of *Cx. pipiens* may make it an interesting sentinel organism for the detection of chronic pollution through the monitoring of insecticide resistance allele levels. However, the precise agents of *Ester* allele selection remain elusive and may prove

difficult to identify: we could analyse the water in the breeding sites, but many xenobiotics can be effective selective agents without being detectable (notably OPs, Eritja & Chevillon 1999); moreover, selection could actually result from a mix of compounds rather than one in particular.

Different fitness-to-environment responses explain Ester resistance allele dynamics. The dynamics of the *Ester* resistance alleles after OP withdrawal were thus consistent with both changes in fitness costs and the persistence of a selective advantage in the environment concerned, but how were these dynamics influenced before the withdrawal of the insecticide?

A previous study showed that the dynamics of the different resistance alleles reflected differences in their selective advantages and costs (Labbé *et al.* 2009). The *Ester*¹ resistance allele was first replaced by *Ester*⁴, a more generalist allele (with the same advantage, but at a lower cost), and *Ester*² later invaded the Montpellier area due to the greater advantages associated with this allele, despite a relatively high cost. In the absence of any major change in trends, *Ester*² was expected to replace *Ester*⁴ by about 2007 (Guillemaud *et al.* 1998; Labbé *et al.* 2009) (Fig. S9, Supporting information).

However, our findings indicate that selective advantages actually vary with the amount of insecticide used. Moreover, the three alleles showed different patterns of reaction to variations in selective pressure: the relationship between dose and fitness advantage was smooth for *Ester*⁴, whereas that for the other alleles was a more binary response, with a full advantage over a threshold dose and none below (Fig. 4A).

These differences do not result from the genetic architecture underlying the resistance mechanisms, as *Ester*¹ and *Ester*² result from different mechanisms (overexpression vs gene amplification), but show similar and limited fitness-to-environment responses. Instead, the observed differences probably reflect the nature of the overproduced esterases (*i.e.* the differences in amino acid sequence between the proteins encoded by the various alleles), although further studies are required to explore the molecular basis of these differences.

Nevertheless, these differences in the shape of the fitness-to-environment relationship have major consequences, as variations in selection pressures, altering the intensity and direction of the selection for the different alleles, thereby changing the ranking of the alleles in terms of fitness. Even within the small range of inter-annual differences in the amounts of OPs applied, changes between years were observed (e.g. *Ester*⁴ was the fittest allele in 2003, with 1.64 L/km² OPs, whereas

*Ester*² is the fittest in 2004, with 2.46 L/km², Fig. 4B). Such changes in relative fitness may temporarily modify the direction of frequency changes for the various alleles. Labbé *et al.* (2009), considering a purely qualitative description of the environment over the 1986–2002 period, anticipated *Ester*² invasion (Fig. S9, Supporting information). The relative fitnesses of the different resistance alleles were estimated at 1.12/1.25/1.16 in the TA and 0.92/0.88/0.96 in the UTA, for *Ester*¹, *Ester*² and *Ester*⁴, respectively. The mean relative fitnesses (calculated from the fitnesses estimated each year over the 1986–2007 period) inferred from the best model in this study are consistent with these values (TA: 1.14/1.19/1.16 and UTA: 0.92/0.89/0.96, respectively, Table 2), highlighting the robustness of the general approach (the robustness of these estimations and of the subsequent conclusions were also confirmed by analysing only a subset of years, the 1993–2012 period, data not shown). However, *Ester*⁴ (the least costly and the most generalist allele) seems to have won the battle for supremacy in the Montpellier area, rather than *Ester*². This finding can be entirely explained by the variations in OP selective pressure intensity, with low OP doses changing the ranking of the alleles in terms of relative fitness, thus altering *Ester* allele dynamics.

Finally, fitness-to-environment responses associated with relatively large selection coefficients allowed fast responses to selective pressure variations. The resistance allele frequencies were thus adjusted over only a few generations (as seen in yearly variations in insecticide intensities Lenormand *et al.* 1999), and these adjustments were detectable even for only slight variations in insecticide pressure (e.g. an increase of 0.2 L/km² in the amount of OPs applied resulted in an 8% increase in the frequency of the *Ester*² allele between 2004 and 2005, Fig. 2).

Conclusion: measuring selection in natural populations requires long-term surveys

We have seen in the introduction that measuring fitness in natural settings can prove difficult, because (i) identifying the selective agent is not trivial, and (ii) the data allowing the assessment of the quantitative link between environmental and fitness variations are not generally available. While our model allows circumventing these fundamental issues, it also reveals several other difficulties.

Our study first illustrates that the responses to selection can be fast. However, even with large selection coefficients, there is a lag for its effects to be detectable in the adaptive allele dynamics (as shown here by the better correlations with previous-year treatments), which requires repeated samplings to be demonstrated.

Similarly, even in our case study (or similar situations) where the selective agent is thought to be known and quantified, other causes of selection can be present and impact the adaptive allele dynamics. Moreover, these secondary sources, far from being anecdotal, can become the major players in the adaptive process once the primary one is removed (as shown by the maintenance of resistance alleles following OP removal). Only a careful analysis and long-term data can reveal their presence. They can be easily missed if the data available remain limited to a few generations. Finally, our study challenges the naïve vision of selection coefficients as more or less fixed parameters; they should rather be considered as dynamic variables, quantitatively related to selective pressure intensities. We showed that the various adaptive alleles can present different fitness-to-environment relationships. Consequently, when the selective environment varies on relatively long time-scales, it can cause frequent reorderings of the fitness ranking of the various alleles; that is, the fittest allele is not always the same, which prevents the fixation of a single allele. This study thus shows how environmental variations can largely contribute to the maintenance of polymorphism in natural populations. However, these fitness-to-environment relationships (and fast responses) also point out that obtaining good evolutionary predictions on the long run requires to precisely document and forecast environmental variation (Lenormand *et al.* 2009).

This study thus emphasizes the paramount importance of long-term surveys in evolutionary ecology. Only data covering many generations in changing environments allow identifying the different sources of selection and understanding the fitness-to-environment relationships of adaptive alleles in natural settings. This is crucial in a context in which environmental pollution due to human activities is increasing and organisms are chronically exposed to varying, but generally low doses of xenobiotics.

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Competing financial interests

The authors have no competing financial interests to declare.

References

- Anderson DR, Burnham KP, White GC (1994) AIC model selection in overdispersed capture-recapture data. *Ecology*, 75, 1780–1793.
- Andersson DI, Hughes D (2010) Antibiotic resistance and its cost: is it possible to reverse resistance?. *Nature Reviews Microbiology*, 8, 260–271.
- Barrett RDH, Hoekstra HE (2011) Molecular spandrels: tests of adaptation at the genetic level. *Nature Reviews Genetics*, 12, 767–780.
- Bell G (2008) *Selection*. Oxford Univ. Press, Oxford.
- Bell G (2010) Fluctuating selection: the perpetual renewal of adaptation in variable environments. *Philosophical Transactions of the Royal Society B*, 365, 87–97.
- Benkman CW, Miller RE (1996) Morphological evolution in response to fluctuating selection. *Evolution*, 50, 2499–2504.
- Berticat C, Boquien G, Raymond M, Chevillon C (2002) Insecticide resistance genes induce a mating competition cost in *Culex pipiens* mosquitoes. *Genetical Research*, 79, 41–47.
- Bishop JA (1972) An experimental study of the cline of industrial melanism in *Biston betularia* (L.) (Lepidoptera) between urban Liverpool and rural North Wales. *Journal of Animal Ecology*, 41, 209–243.
- Bishop JA, Cook LM, Muggleton J, Seaward MRD (1975) Moths, Lichens and air pollution along a transect from Manchester to North Wales. *Journal of Applied Ecology*, 12, 83–98.
- Bourguet D, Guillemaud T, Chevillon C, Raymond M (2004) Fitness costs of insecticide resistance in natural breeding sites of the mosquito *Culex pipiens*. *Evolution*, 58, 128–135.
- Bradshaw WE, Zani PA, Holzapfel CM (2004) Adaptation to temperate climates. *Evolution*, 58, 1748–1762.
- Carlson SM, Quinn TP (2007) Ten years of varying lake level and selection on size-at-maturity in sockeye salmon. *Ecology*, 88, 2620–2629.
- Carrière Y, Deland J-P, Roff DA, Vincent C (1994) Life-history cost associated with the evolution of insecticide resistance. *Proceedings of the Royal Society of London B*, 258, 35–40.
- Caruso CM, Brook Peterson S, Ridley CE (2003) Natural selection on floral traits of *Lobelia* (Lobeliaceae): spatial and temporal variation. *American Journal of Botany*, 90, 1333–1340.
- Casimiro S, Coleman M, Hemingway J, Sharp B (2006a) Insecticide resistance in *Anopheles arabiensis* and *Anopheles gambiae* from Mozambique. *Journal of Medical Entomology*, 43, 276–282.
- Casimiro S, Coleman M, Mohloai P, Hemingway J, Sharp B (2006b) Insecticide resistance in *Anopheles funestus* (Diptera: Culicidae) from Mozambique. *Journal of Medical Entomology*, 43, 267–275.
- Chevillon C, Bourguet D, Rousset F, Pasteur N, Raymond M (1997) Pleiotropy of adaptive changes in populations: comparisons among insecticide resistance genes in *Culex pipiens*. *Genetical Research*, 68, 195–203.
- Conner JK (2001) How strong is natural selection? *Trends in Ecology and Evolution*, 16, 215–217.
- Duncan AB, Fellous S, Kaltz O (2011) Reverse evolution: selection against costly resistance in disease-free microcosm populations of *Paramecium caudatum*. *Evolution*, 65, 3462–3474.
- Duron O, Labbé P, Berticat C *et al.* (2006) High *Wolbachia* density correlates with cost of infection for insecticide resistant *Culex pipiens* mosquitoes. *Evolution*, 60, 303–314.
- Endler JA (1986) *Natural Selection in the Wild*. Princeton University Press, Princeton, NJ.
- Eritja R, Chevillon C (1999) Interruption of chemical mosquito control and evolution of insecticide resistance genes in *Culex pipiens* (Diptera: Culicidae). *Journal of Medical Entomology*, 36, 41–49.
- European Commission 2007/393/EC (2007) *Commission decision of 6 June 2007 concerning the non-inclusion of diazinon in Annex I to Council Directive 91/414/EEC and the withdrawal of authorisations for plant protection products containing that substance*. 2007/393/EC.
- Gallet R, Cooper TF, Elena SF, Lenormand T (2012) Measuring selection coefficients below 10^{-3} : method, questions, and prospects. *Genetics*, 190, 175–186.
- Gazave E, Chevillon C, Lenormand T, Marquine M, Raymond M (2001) Dissecting the cost of insecticide resistance genes during the overwintering period of the mosquito *Culex pipiens*. *Heredity*, 87, 441–448.
- Gonzales-Candels F, Comas I, Martinez JL, Galan JC, Baquero F (2011) The evolution of antibiotic resistance. In: *Genetics and Evolution of Infectious Diseases* (ed. Tibayrenc M), pp. ???–???. Elsevier, London, UK.
- Grant BR, Grant PR (1993) Evolution of Darwin's finches caused by a rare climatic event. *Proceedings of the Royal Society B: Biological Sciences*, 251, 111–117.
- Grant PR, Grant BR (1995) Predicting microevolutionary responses to directional selection on heritable variation. *Evolution*, 49, 241–251.
- Guillemaud T, Lenormand T, Bourguet D *et al.* (1998) Evolution of resistance in *Culex pipiens*: allele replacement and changing environment. *Evolution*, 52, 443–453.
- Gutschick VP, BassiriRad H (2003) Extreme events as shaping physiology, ecology, and evolution of plants: toward a unified definition and evaluation of their consequences. *New Phytologist*, 160, 21–42.
- Hereford J, Hansen TF, Houle D (2004) Comparing strengths of directional selection: how strong is strong? *Evolution*, 58, 2133–2143.
- Janssens TKS, Roelofs D, van Straalen NM (2009) Molecular mechanisms of heavy metal tolerance and evolution in invertebrates. *Insect Science*, 16, 3–18.
- Karlin S, Lieberman U (1974) Random temporal variation in selection intensities: case of large population size. *Theoretical Population Biology*, 6, 355–382.
- Kawecki TJ, Ebert D (2004) Conceptual issues in local adaptation. *Ecology Letters*, 7, 1225–1241.
- Kingsolver JG, Hoekstra HE, Hoekstra JM *et al.* (2001) The strength of phenotypic selection in natural populations. *The American Naturalist*, 157, 245–261.
- Labbé P, Lenormand T, Raymond M (2005) On the worldwide spread of an insecticide resistance gene: a role for local selection. *Journal of Evolutionary Biology*, 18, 1471–1484.
- Labbé P, Sidos N, Raymond M *et al.* (2009) Resistance gene replacement in the mosquito *Culex pipiens*: fitness estimation from long-term cline series. *Genetics*, 182, 303–312.
- Labbé P, Alout H, Djogbénou D *et al.* (2011) Evolution of resistance to insecticide in disease vectors. In: *Genetics and*

- Evolution of Infectious Diseases*(ed Tibayrenc M), pp. 363-409. Elsevier, London, UK.
- Lande R, Arnold SJ (1983) The measurement of selection on correlated characters. *Evolution*, 37, 1210-1226.
- Lenormand T, Raymond M (1998) Resistance management: the stable zone strategy. *Proceeding of the Royal Society, London, B*, 265, 1985-1990.
- Lenormand T, Raymond M (2000) Analysis of clines with variable selection and variable migration. *American Naturalist*, 155, 70-82.
- Lenormand T, Guillemaud T, Bourguet D, Raymond M (1998) Evaluating gene flow using selected markers: a case study. *Genetics*, 149, 1383-1392.
- Lenormand T, Bourguet D, Guillemaud T, Raymond M (1999) Tracking the evolution of insecticide resistance in the mosquito *Culex pipiens*. *Nature*, 400, 861-864.
- Lenormand T, Roze D, Rousset F (2009) Stochasticity in evolution. *Trends in Ecology & Evolution*, 24, 157-165.
- Lenormand T, Rode N, Chevin LM, Rousset F (2015) Valeur sélective : définitions enjeux et mesures *in press*. In: *Biologie Evolutive* (eds Lefèvre T, Thomas F, Raymond M), pp. ???-???. de boeck, ???.
- Lenski RE (1988) Experimental studies of pleiotropy and epistasis in *Escherichia coli*. I. variation in competitive fitness among mutants resistant to virus T4. *Evolution*, 42, 425-432.
- Lewontin RC (1974) *The Genetic Basis of Evolutionary Change*. Columbia University Press, New York, NY.
- Losos JB, Warheit KI, Schoener TW (1997) Adaptive differentiation following experimental island colonization in *Anolis* lizards. *Nature*, 387, 70-73.
- Macnair MR (1987) Heavy metal tolerance in plants: a model evolutionary system. *Trends in Ecology & Evolution*, 2, 354-359.
- McKenzie JA (1996) *Ecological and Evolutionary Aspects of Insecticide Resistance*. Academic Press, Austin, TX.
- Millstein RL (2008) Distinguishing drift and selection empirically: "The great snail debate" of the 1950s. *Journal of the History of Biology*, 41, 339-367.
- Mitchell-Olds AT, Shaw RG (1987) Regression analysis of natural selection: statistical inference and biological interpretation. *Evolution*, 41, 1149-1161.
- Morrissey MB, Hadfield JD (2012) Directional selection in temporally replicated studies is remarkably consistent. *Evolution*, 66, 435-442.
- Nkya TE, Akhouayri I, Kisinza W, David JP (2013) Impact of environment on mosquito response to pyrethroid insecticides: facts, evidences and prospects. *Insect Biochemistry and Molecular Biology*, 43, 407-416.
- Norris LC, Main BJ, Lee Y *et al.* (2015) Adaptive introgression in an African malaria mosquito coincident with the increased usage of insecticide-treated bed nets. *Proceedings of the National Academy of Sciences of the United States of America*, 112, 201418892.
- Nsanabana C, Hastings IM, Marfurt J *et al.* (2010) Quantifying the evolution and impact of antimalarial drug resistance: drug use, spread of resistance, and drug failure over a 12-year period in Papua New Guinea. *The Journal of Infectious Diseases*, 201, 435-443.
- Oakeshott JG, Devonshire AL, Claudianos C *et al.* (2005) Comparing the organophosphorus and carbamate insecticide resistance mutations in cholin- and carboxyl-esterases. *Chemico-Biological Interactions*, 157-158, 269-275.
- Parrella MP, Trumble JT (1989) Decline of RESISTANCE in *Liriomyza trifolii* (Diptera: Agromyzidae) in the absence of insecticide selection pressure. *Journal of Economic Entomology*, 82, 2-5.
- Pasteur N, Pasteur G, Bonhomme F, Britton-Davidian J (1988) ????. In: *Practical Isozyme Genetics* (ed. EH Ltd.), pp. ???-???. ????, Chichester, UK.
- Rausher MD (1992) The measurement of selection on quantitative traits: biases due to environmental covariances between traits and fitness. *Evolution*, 46, 616-626.
- Raymond M, Berticat C, Weill M, Pasteur N, Chevillon C (2001) Insecticide resistance in the mosquito *Culex pipiens*: what have we learned about adaptation? *Genetica*, ???, 287-296.
- Reid MC, McKenzie FE (2016) The contribution of agricultural insecticide use to increasing insecticide resistance in African malaria vectors. *Malaria Journal*, 15, 107.
- Reznick DN, Bryga H (1987) Life-history evolution in guppies (*Poecilia reticulata*): 1. Phenotypic and genetic changes in an introduction experiment. *Evolution*, 41, 1370-1385.
- Rooker S, Guillemaud T, Bergé J, Pasteur N, Raymond M (1996) Coamplification of *esterase A* and *B* genes as a single unit in *Culex pipiens* mosquitoes. *Heredity*, 77, 555-561.
- Rundle HD, Vamosi SM, Schluter D (2003) Experimental test of predation's effect on divergent selection during character displacement in sticklebacks. *Proceedings of the National Academy of Sciences of the United States of America*, 100, 14943-14948.
- Schemske DW, Horvitz CC (1989) Temporal variation in selection on a floral character. *Evolution*, 43, 461-465.
- Schluter D, Price TD, Rowe L (1991) Conflicting selection pressures and life history trade-offs. *Proceedings of the Royal Society B: Biological Sciences*, 246, 11-17.
- Sharp BL, Kleinschmidt I, Streat E *et al.* (2007) Seven years of regional malaria control collaboration-Mozambique, South Africa, and Swaziland. *The American Journal of Tropical Medicine and Hygiene*, 76, 42-47.
- Siepielski AM, Dibattista JD, Carlson SM (2009) It's about time: the temporal dynamics of phenotypic selection in the wild. *Ecology Letters*, 12, 1261-1276.
- Siepielski AM, DiBattista JD, Evans JA, Carlson SM (2011) Differences in the temporal dynamics of phenotypic selection among fitness components in the wild. *Proceedings. Biological sciences/The Royal Society*, 278, 1572-1580.
- Siepielski AM, Gotanda KM, Morrissey MB *et al.* (2013) The spatial patterns of directional phenotypic selection. *Ecology Letters*, 16, 1382-1392.
- Wade JW, Kalisz S (1990) The causes of natural selection. *Evolution*, 44, 1947-1955.
- Weill M, Berticat C, Raymond M, Chevillon C (2000) Quantitative polymerase chain reaction to estimate the number of amplified *esterase* genes in insecticide-resistant mosquitoes. *Analytical Biochemistry*, 285, 267-270.
- Whalon MEE, Mota-Sanchez D, Hollingworth RMM (2008) ????. In: *Global Pesticide Resistance in Arthropods* (eds Whalon ME, Mota-Sanchez D, Hollingworth RM), pp. ???-???. CABI publishing, Cambridge, MA.

Data accessibility

All the data used for this study are available in the main document or as supplementary materials. Insecticide quantities used each year over the 1986–2012 period are presented in the Table 1 and phenotyping data of individuals of each sampled population from 2003 to 2012 are available as supplementary materials in the Table S2 (Supporting information). Those from 1986 to 1996 are already published in Guillemaud *et al.* 1998 as those from 1997 to 2002 in Labbé *et al.* 2009. They are also available on DRYAD: doi:10.5061/dryad.1cv0n.

P.M., T.L. and P.L. designed research; P.M. and P.L. designed and computed the model; C.L. provided treatment data and analyses; P.M., M.W., T.L. and P.L. analysed data; P.M., T.L. and P.L. wrote the first draft; C.L. and M.W. reviewed the manuscript.

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1. Resistance mechanisms and nomenclature at the *Ester* locus.

Table S2. *Ester* phenotypes of individuals collected between 2003 and 2012.

Figure S3. Observed and predicted *Ester* resistance allele frequencies as a function of the distance from the sea from 1986 to 2012.

Table S4. Synthetic cline parameters inferred for each sampled year independently, from 1986 to 2012.

Table S5. *Ester* allele frequencies vs OP quantities of year t or $t-1$.

Figure S6. *Ester* allele frequencies vs insecticide quantities over the whole sampling transect.

Appendix S7. Evolution of copy numbers in *Ester* amplified allele.

Figure S8. The best model is not over-parameterized.

Figure S9. Predictions from Labbé *et al.*'s model.