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European populations of *Diabrotica virgifera virgifera* are resistant to aldrin, but not to methyl-parathion

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cyclodiene, insecticide resistance monitoring, multiple introductions of adaptive characters, organophosphate, vial bioassay, western corn rootworm

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Abstract

The western corn rootworm, *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae), is a major pest of cultivated corn in North America and has recently begun to invade Europe. In addition to crop rotation, chemical control is an important option for *D. v. virgifera* management. However, resistance to chemical insecticides has evolved repeatedly in the USA. In Europe, chemical control strategies have yet to be harmonized and no surveys of insecticide resistance have been carried out. We investigated the resistance to methyl-parathion and aldrin of samples from nine *D. v. virgifera* field populations originating from two European outbreaks thought to have originated from two independent introductions from North America. Diagnostic concentration bioassays revealed that all nine *D. v. virgifera* field populations were resistant to aldrin but susceptible to methyl-parathion. Aldrin resistance was probably introduced independently, at least twice, from North America into Europe, as there is no evident selection pressure to account for an increase of frequency of aldrin resistance in each of the invasive outbreaks in Europe. Our results suggest that organophosphates, such as methyl-parathion, may still provide effective control of both larval and adult *D. v. virgifera* in the European invasive outbreaks studied.

Introduction

The western corn rootworm, *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae), is one of the most destructive pests of cultivated corn, *Zea mays* L. Corn crops are damaged principally by larval feeding on the root system, disrupting water and nutrient uptake, leaving the plants prone to lodging and difficult to harvest (Kahler et al. 1985). This pest has rapidly expanded its range from the southwestern region of the US Corn Belt in the 1950s to the east coast of North America during the 1980s (Metcalf 1983; Spencer et al. 2005).

Diabrotica virgifera virgifera was recently introduced into Europe where it was first observed near

Belgrade, Serbia, in 1992. An international network has since monitored its spread throughout Europe (Kiss et al. 2005). Two types of infested area have been recorded: a continuously expanding area in Central and South-Eastern (CSE) Europe and several disconnected outbreaks in Western Europe, including a smaller spreading area in North-Western (NW) Italy. The continuously expanding CSE European area now includes parts of 11 countries, extending from Austria to the Ukraine and from Southern Poland to Northern Bulgaria. The first documented infestation away from this area was discovered near Venice, Italy, in 1998. A number of outbreaks have been detected almost every year since in various countries including Italy, France, Switzerland, Belgium,

the United Kingdom, the Netherlands (Edwards and Kiss 2007) and Germany (Anonymous 2007). However, economic losses have been reported only in Italy and in certain parts of CSE Europe such as Serbia, Romania, Hungary and Croatia. Recent population genetic studies suggested that intracontinental redistribution is not the only method of spread of this pest in Europe (Miller et al. 2005; Ciosi et al. 2008). These studies suggested that transatlantic introductions of this insect probably occurred repeatedly (Miller et al. 2005; Ciosi et al. 2008), accounting for the initiation of several European outbreaks, including the CSE European and the NW Italian outbreaks.

In addition to crop rotation, chemical control is an essential management strategy against *D. v. virgifera*. Cyclodiene insecticides belonging to the chlorinated hydrocarbon class of insecticides were recommended for *D. v. virgifera* control in the USA in the early 1940s (Hill et al. 1948). They were widely used until the early 1960s when it became apparent that corn rootworm control was no longer effective and resistance had become widespread. This development of resistance coincided with a rapid eastward expansion, such that, by the 1980s, cyclodiene resistance was common throughout the US Corn Belt, including areas in which cyclodiene insecticides had never been used (Metcalf 1986). Aldrin and dieldrin were banned by the US Environmental Protection Agency in 1974 due to widespread environmental contamination and persistence problems (Metcalf 1983), and insecticides from this family have not been used for rootworm control in the USA for almost 40 years. Variation in resistance levels in natural populations has been detected, based on assessments of survival at a diagnostic aldrin concentration, and high levels of resistance are still observed in Corn Belt populations (Parimi et al. 2006). The results of these assays suggest that the frequency of resistance is higher in eastern US populations, despite the more limited use of cyclodiene insecticides against *D. v. virgifera* in these areas.

Organophosphates and carbamates were introduced for *D. v. virgifera* control to get around the problems caused by the emergence of cyclodiene resistance and because they were less persistent in the environment than cyclodienes. These insecticides successfully replaced chlorinated hydrocarbons for rootworm control and were extensively used in the US Corn Belt from the 1970s to 1990s. Both organophosphates and carbamates are still used as soil insecticides for larval control and as foliar sprays for adult management programs. Failure of adult control to prevent egg laying with methyl-parathion was first reported in the

mid-1990s, and resistance to organophosphates (methyl-parathion) and carbamates (carbaryl) has been documented in rootworm adults from a number of Nebraskan populations (Meinke et al. 1998). This region has seen a significant increase in both the distribution and intensity of resistance over a 4-year period (Zhou et al. 2002; Miller et al. 2008).

In Europe, organophosphates, carbamates and pyrethroids are currently used as soil insecticides (Van Rozen and Ester 2006). A second management tactic involves the spraying of foliar insecticides to control *D. v. virgifera* adults. Foliar treatment was first used in the 1950s, in the USA, when resistance to chlorinated hydrocarbons became apparent and farmers were obliged to seek new control strategies. In Europe, organophosphates and pyrethroids are used for foliar treatment (Van Rozen and Ester 2006). A third insecticide application method is seed treatment for which insecticides are directly applied to seeds. Most of the insecticides used for seed treatments are neonicotinoids (Van Rozen and Ester 2006). Cyclodiene insecticides (e.g. endosulfan) have rarely been used as foliar treatments in Europe (Van Rozen and Ester 2006).

In Europe, chemical control strategies have not yet been harmonized and no surveys of insecticide resistance have been carried out, but control failures have been reported occasionally (Furlan et al. 2006). Moreover, most chemical treatments have been combined with crop rotation in Western European outbreaks, so that the management success does not necessarily imply chemical control success. Insecticide resistance in *D. v. virgifera* in Europe therefore remains largely unknown. The recent invasion history of *D. v. virgifera* may impact the probability of insecticide resistance occurring in Europe. Due to the multiple introduction events resulting in several European outbreaks (Miller et al. 2005; Ciosi et al. 2008), it is possible that resistances to both cyclodienes and organophosphates have been introduced from the USA on several occasions.

The aim of this study was to determine the resistance status of European field populations of adult *D. v. virgifera* to insecticides for which resistance had been recorded in the USA: (i) cyclodienes, which were extensively used for rootworm control in the USA over a period of more than 20 years (Metcalf 1983) but have seldom been used in Europe (Van Rozen and Ester 2006) and (ii) organophosphates, which are used in both the USA (Meinke et al. 1998; Wright et al. 2000) and Europe (Van Rozen and Ester 2006). Aldrin, a cyclodiene insecticide, and methyl-parathion, an organophosphate insecticide were used in

diagnostic concentration bioassays to evaluate the susceptibility of *D. v. virgifera* adults collected from several European field populations, in comparison with a known susceptible laboratory strain.

Materials and Methods

Diabrotica virgifera virgifera populations

Diabrotica virgifera virgifera adults were collected from nine populations in cornfields in the CSE European and NW Italian outbreaks. At least 300 adults were collected from two sites in each of Serbia, Romania and Hungary (CSE Europe), and from three sites in NW Italy, during June and July 2007 (table 1; fig. 1). Adults were shaken from corn plants into a funnel attached to a homemade gauze bag. They were then transported in a cool box to the laboratory. Samples were not collected from other isolated European populations due to their low densities after relatively recent introductions or effective control measures (Kiss et al. 2005a). A non-diapausing *D. v. virgifera* strain from the US Department of Agriculture (USDA Agricultural Research Service, Brookings, SD, USA) was reared as described by Branson et al. (1975). This laboratory strain was generated from a diapausing colony established in

the mid-1960s from adults collected in a field near Brookings, SD, USA. After six generations of rearing in the laboratory, the non-diapausing strain was selected, as described by Branson (1976), and reared continuously in the absence of exposure to insecticides and constitutes the susceptible laboratory strain of reference for insecticide resistance studies in *D. v. virgifera* (Parimi et al. 2006; Miller et al. 2008). Adults from the non-diapausing strain and adults collected in the field were held in rearing cages in a quarantine laboratory (18°C at night, 24°C during the day, 50% RH; L : D 14 : 10 h). They were supplied with unlimited access to food, in the form of soft corn kernels and zucchini squash (Organic Production, Migros Comp., Delémont, Switzerland). Agar cubes (1.5% agar) were supplied as a source of water. Samples of the nine field populations and the non-diapausing laboratory strain were maintained in identical rearing conditions for at least 10 days before insecticide bioassays. Bioassays were performed, as the rearing, at the quarantine laboratory of CABI-Europe in Delémont, Switzerland.

Tested insecticides

Technical grade methyl-parathion (98.4% purity) and aldrin (98.1% purity) were used (Sigma-Aldrich,

Table 1 *Diabrotica virgifera virgifera* samples and strains tested for their susceptibility to insecticides

Geographic area	Field sample or strain name	Nearby city	First observation year	GPS coordinates	Collection date
Central and South Eastern Europe	Serbia 1	Crvenka, Serbia	1995	45°39.351'N 19°28.480'E	26 June 2007
	Serbia 2	Backa Topola, Serbia	1995	45°49.412'N 19°40.915'E	26 June 2007
	Romania 1	Lovrin, Romania	1996	45°56.516'N 20°51.549'E	5 July 2007
	Romania 2	Lovrin, Romania	1996	45°56.400'N 20°51.821'E	5 July 2007
	Hungary 1	Szekszard, Hungary	1997	46°30.992'N 18°37.962'E	25 July 2007
	Hungary 2	Kondoros, Hungary	1998	46°44.886'N 20°49.201'E	July 2007
North Western Italy	Piedmont	Momo, Italy	2000	45°36.492'N 8°32.492'E	20 July 2007
	Lombardy 1	Rovato, Italy	2001	45°34.456'N 10°02.176'E	27 June and 21 July 2007
	Lombardy 2	Rovato, Italy	2001	45°34.486'N 9°57.263'E	27 and 28 June 2007
South Dakota, USA	Non diapausing strain	Brookings, SD, USA	Mid 1960s		Laboratory rearing

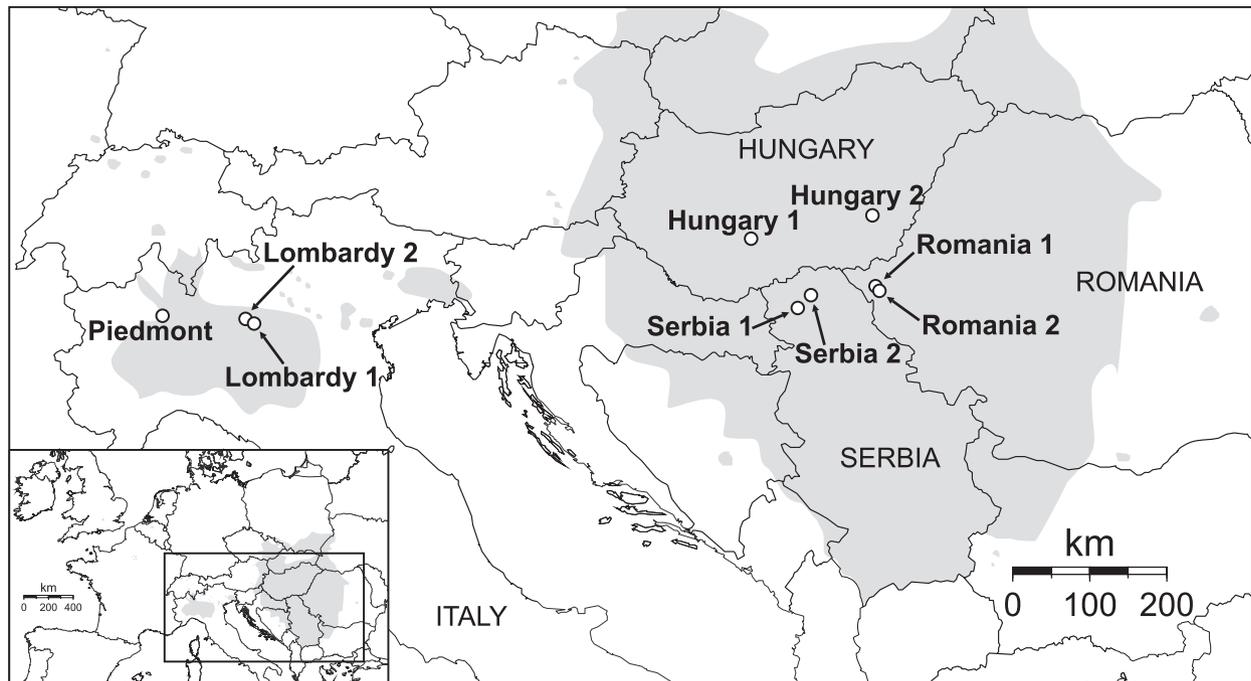


Fig. 1 Location of sampling sites and geographic distribution of *Diabrotica virgifera virgifera* in Europe in 2006. Distribution area, shown in grey, is defined as areas in which *D. v. virgifera* has been observed for at least 1 year (Edwards and Kiss 2007). Names of the countries in which insects were collected are shown in capital letters.

St Louis, MO). Insecticides were dissolved in reagent grade acetone (>99% purity; Acros Organics, Geel, Belgium) to establish stock solutions for subsequent dilution. Stock solutions were prepared at concentrations of 20 mg/ml for aldrin and 10 mg/ml for methyl-parathion, respectively, and stored at -20°C .

Resistance bioassays

We evaluated the susceptibility of *D. v. virgifera* adults to insecticides under laboratory conditions (see above), in a vial residue bioassay (Brindley 1975). The nine samples from the field populations and the non-diapausing laboratory strain were exposed to the LC_{99} of the susceptible laboratory colony for both insecticides. For aldrin, the diagnostic concentration of $16.5 \mu\text{g}/\text{vial}$ was determined based on a modified version of the protocol of Parimi et al. (2006), and for methyl-parathion, $0.5 \mu\text{g}/\text{vial}$ was used (Zhou et al. 2002). In these tests, 0.5 ml of the desired concentration of insecticides was added to 20-ml glass scintillation vials (e.g. Scharf et al. 1999) (VWR, West Chester, PA). Control vials were coated with acetone only. Vials were placed in a fume hood on a commercial hot dog roller, to evaporate off the solvent and ensure that the inner surface of the vial was evenly coated with insecticide. Coated vials

were used within 24 h after acetone evaporation. Before use, they were allowed to equilibrate at room temperature for at least 4 h. They were then left open for 10 min before adding adults. Ten replicate vials for each population or strain, plus one vial for the control, with 10 unsexed adults per vial, were used per insecticide. This resulted in the use of 110 adults per population sample or strain and per insecticide, with the exception of the Piedmont sample (60 adults for methyl-parathion and 70 adults for aldrin) and the non-diapausing strain (60 adults for methyl-parathion and 80 adults for aldrin) (table 2). Vials were maintained at 24°C and adult mortality, defined as a lack in coordinated movement (Parimi et al. 2006), was assessed after 4 h for methyl-parathion and after 12 h for aldrin.

Data analysis

Data were corrected for control mortality (Abbott 1925). We tested the effect of the following factors on mortality: invasive outbreak (NW Italy or CSE Europe), population within an outbreak, and replicate vial within a population. These random effects were tested by fitting a nested generalized linear model to the data with STATISTICA software (Statsoft, 2007). A binomial probability distribution was used

Table 2 Mortality of *Diabrotica virgifera virgifera* adults collected from European corn fields in 2007 or belonging to a susceptible laboratory strain, and exposed to a diagnostic dose of methyl parathion or aldrin

Geographic area	Field sample or strain name	Methyl parathion experiment			Aldrin experiment		
		Mortality rate (N _{ind})	SD	Control mortality rate (N _{ind})	Mortality rate (N _{ind})	SD	Control mortality rate (N _{ind})
Central and South Eastern Europe	Serbia 1	93% (100)	1.57	0% (10)	1% (100)	0.32	0% (10)
	Serbia 2	98% (100)	0.42	0% (10)	0% (100)	0	0% (10)
	Romania 1	99% (100)	0.32	0% (10)	1% (100)	0.32	0% (10)
	Romania 2	100% (100)	0	0% (10)	0% (100)	0	0% (10)
	Hungary 1	100% (100)	0	0% (10)	3% (100)	0.48	0% (10)
	Hungary 2	96% (100)	0.84	0% (10)	1% (100)	0.32	0% (10)
North Western Italy	Piedmont	100% (50)	0	0% (10)	3.33% (60)	0.52	0% (10)
	Lombardy 1	99% (100)	0.32	0% (10)	4% (100)	0.52	0% (10)
	Lombardy 2	98% (100)	0.42	0% (10)	6% (100)	0.7	10% (10)
South Dakota, USA	Non diapausing strain	96% (50)	0.89	0% (10)	98.57% (70)	0.38	10% (10)

SD, standard deviation of the mortality rate among vials; N_{ind}, total number of individuals analysed per population or sample in each treatment.

and a logit link function was applied to the dependent variable, i.e. the proportion of adults that were dead. When a significant effect was detected, Fisher's exact tests were used for multiple comparisons of mortalities among *D. v. virgifera* field populations. As these tests involve non-orthogonal and multiple comparisons, sequential Bonferroni correction was applied (Sokal and Rolf 1995: p. 236).

Results

All European field populations were susceptible to methyl-parathion. Mortality rates of at least 93% were observed at the diagnostic concentration, for

each sample (fig. 2). Only 17 of the 850 (2%) adults exposed to this insecticide survived (table 2). Mortality rates seemed to be similar in the various field population samples, ranging from 93% in the Serbia 1 sample to 100% in the Romania 2, Hungary 1 and Piedmont samples (mean mortality across field populations = 98.1%, SD = 2.3%). No effect of 'replicate vial within a population sample' was detected ($\chi^2 = 60.04$, d.f. = 76, $P = 0.910$). However, a significant 'population within outbreak' effect was observed ($\chi^2 = 19.46$, d.f. = 7, $P = 0.007$). Only two multiple comparisons were significant, adults from the Serbia 1 sample being slightly less susceptible (93% mortality) than adults from the Romania 2

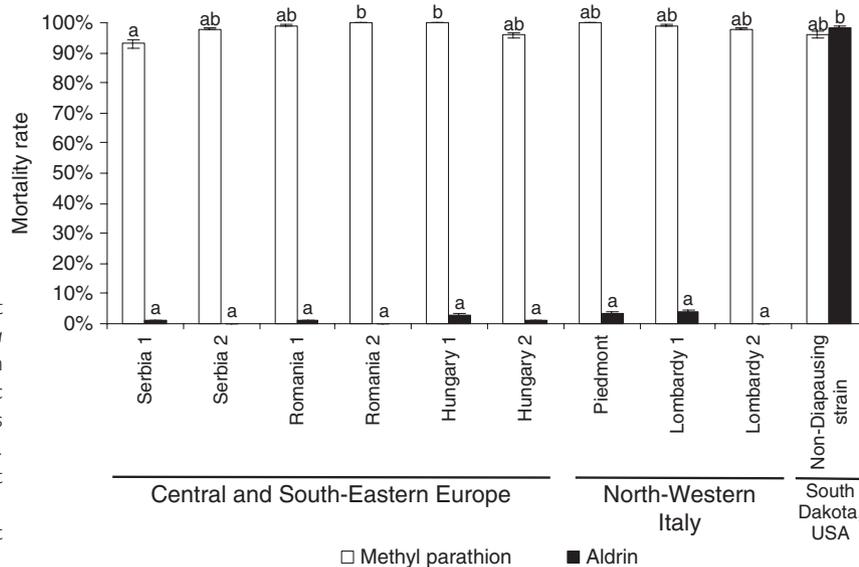


Fig. 2 Mortality [corrected using the Abbot formula (Abbott 1925)] of *Diabrotica virgifera virgifera* adults, collected from European corn fields in 2007 and exposed to a diagnostic dose of methyl parathion or aldrin. Error bars correspond to standard errors of the mean. Letters above the columns indicate significant differences between samples of field populations or strains for each insecticide treatment according to Fisher's exact tests, at $P < 0.05$.

and Hungary 1 samples (100% mortality for both) (Fisher's exact tests, $P = 0.014$ for both tests, non-significant after sequential Bonferroni correction). These differences were not due to variations in adult mortality in untreated vials (0% mortality in each case; see table 2). Overall, no difference was found between samples of field populations and the non-diapausing laboratory strain (Fisher's exact tests, $P > 0.11$ for each test), and mortality rates were similar in the two outbreaks (between outbreak effect: $\chi^2 = 1.27$, d.f. = 1, $P = 0.259$).

All samples from European field populations were resistant to the cyclodiene aldrin: mortality rates at the diagnostic concentration never exceeded 4% (fig. 2), whereas the mortality rate of the susceptible non-diapausing laboratory strain at this concentration reached almost 99%. Mortality rates were similar between samples of field populations, ranging from 0% in the Serbia 2, Romania 2 and Lombardy 2 samples to 4% in the Lombardy 1 sample (mean mortality = 1.5%, SD = 1.5%). The mean mortality rate obtained for untreated vials of field population samples was, at 1.1% (SD = 3.14%), similar to the mean mortality rate observed in treated vials (Fisher's exact tests, $P > 0.05$ for each field population). Mortality rates were similar between vials, populations and outbreaks (outbreak effect: $\chi^2 = 2.07$, d.f. = 1, $P = 0.150$; population within outbreak: $\chi^2 = 12.61$, d.f. = 7, $P = 0.082$; replicate vial within population: $\chi^2 = 33.66$, d.f. = 77, $P = 1$).

Discussion

Almost all individuals from the nine samples of *D. v. virgifera* populations originating from the NW Italian and CSE European invasive outbreaks were resistant to aldrin and susceptible to methyl-parathion. No heterogeneity in aldrin resistance was detected in either the CSE European or the NW Italian outbreaks. Thus, given the very low rates of mortality observed in this study, this resistance may be considered fixed in the field samples examined.

Susceptibility to methyl-parathion differed significantly between the field populations sampled within the CSE European outbreak (7% of the individuals from the Serbia 1 sample survived, whereas all individuals from the Romania 2 and Hungary 1 samples died). No difference in mortality between these samples was observed in the absence of insecticide. However, only one vial per population was used as an untreated control, and this may have been insufficient for the detection of heterogeneity in natural mortality rates. Moreover, no significant differences

were observed between the European field collections and the susceptible control strain. We therefore conclude that the observed heterogeneity in susceptibility to methyl-parathion is not associated with genetic variability, but is instead more likely to reflect slight differences in the physiological condition of adults originating from different field populations.

Pyrethroids are the most widely used insecticides for *D. v. virgifera* control or eradication in Europe, although organophosphates, such as methyl-parathion, are also frequently used (Van Rozen and Ester 2006). In contrast to the reporting of adult resistance to methyl-parathion in Nebraska (Meinke et al. 1998), our results suggest an absence of methyl-parathion resistance in samples from CSE Europe and NW Italy. The use of methyl-parathion against adult *D. v. virgifera* may therefore still be an effective management option in Europe. However, cyclodienes, such as aldrin, should not be used in CSE Europe and NW Italy. It should be noted that cyclodiene insecticides are not currently used and were rarely used in the past in Europe (Van Rozen and Ester 2006).

We detected no clear spatial heterogeneity in insecticide susceptibility within Europe, but we cannot conclude that the entire CSE Europe and NW Italian populations are resistant to aldrin and susceptible to methyl-parathion. Undetected geographic variability may exist in the unsampled regions of each outbreak. However, given the low level of genetic variability within each European outbreak (Miller et al. 2005; Ciosi et al. 2008) and the geographically broad sampling scheme used in this study (six populations in CSE Europe and three populations in NW Italy), the hypothesis of undetected geographic variability of resistance is unlikely.

Genetic differentiation between the various European outbreaks has been documented (Miller et al. 2005; Ciosi et al. 2008). The observed frequency of aldrin and methyl-parathion susceptibility therefore cannot be extrapolated to all other European outbreaks. Nevertheless, the North-Eastern (NE) Italian outbreak is probably a secondary outbreak originating from the CSE Europe outbreak (Miller et al. 2005; Ciosi et al. 2008). It is therefore highly likely that the NE Italian population also displays a high frequency of aldrin resistance and methyl-parathion susceptibility. However, the outbreaks observed near Paris, near London and in Eastern France probably resulted from introductions from North America independent of the CSE and NW Italian outbreaks (Miller et al. 2005; Ciosi et al. 2008), and their resistance status is unknown.

There are several possible explanations for the absence of methyl-parathion resistance in both the NW Italy and CSE Europe outbreaks. Firstly, this resistance may have been absent from the source populations of both outbreaks. Indeed, a genetic polymorphism for resistance to methyl-parathion has been observed in North America. This polymorphism is characterized by geographic variability in resistance levels, although resistant populations have not been reported outside of Nebraska (Meinke et al. 1998; Zhou et al. 2002). In other words, both methyl-parathion susceptible and resistant populations exist in North America, and susceptible populations may have been the sources of the two outbreaks analysed here. Alternatively, resistance may have been present in the source populations but lost through genetic drift. The small size of the population during the early phases of the invasion process may increase the impact of stochastic variation in allele frequencies between generations (Hartl and Clark 1997). This process may lead to the loss of an allele at a polymorphic locus, even if it is not rare. A third possibility is that resistance was present in the source population but lost through natural selection. This would imply a fitness cost of the resistance, with individuals displaying resistance less fit than susceptible individuals in the absence of the insecticide (Coustau et al. 2000). No such fitness cost associated with methyl-parathion has yet been documented in *D. v. virgifera* in North America and methyl-parathion resistance has been stable for more than six generations in the absence of selection (Parimi et al. 2006). This hypothesis therefore seems less likely than the others. Although the susceptible source population hypothesis is the most parsimonious, a wide-scale survey of the distribution of methyl-parathion resistance in North America would allow us to determine which of the first two hypotheses are correct.

An eastward increase in aldrin resistance frequency among natural populations from Nebraska (77.1% mortality at aldrin LC₉₉) to Pennsylvania (5.6% mortality at aldrin LC₉₉) (H. Wang, unpublished data) has been observed. The frequency of resistance to aldrin observed in CSE Europe and NW Italy is thus consistent with those reported in North-Eastern USA.

A between-population polymorphism exists in the US Corn Belt for aldrin and methyl-parathion resistance (Meinke et al. 1998; Parimi et al. 2006; Miller et al. 2008). Resistance in invasive European outbreaks might constitute a genetic marker for the identification of the source population in North

America. Given the high frequency of aldrin resistance in the CSE European and Italian outbreaks, our results are consistent with a probable source area in North-Eastern USA. Extensive monitoring of aldrin and methyl-parathion resistance throughout the US Corn Belt might narrow down identification of the most probable source population of the European outbreaks. However, as changes in allele frequency and even allele fixation or loss may occur during the introduction and settlement of a population, a quantitative analysis taking genetic drift into account, such as the ABC method used by Miller et al. (2005), should be used for this purpose.

This study shows that resistance to aldrin is present in both the CSE European and NW Italian outbreaks, which are believed to have resulted from two independent transatlantic introductions (Miller et al. 2005; Ciosi et al. 2008). Cyclodiene insecticides are seldom used in Europe, thus high selection pressures cannot explain an increase in aldrin resistance frequency from a spontaneous mutation or from a few resistant migrants originating from another outbreak. It is therefore extremely likely that aldrin resistance was introduced independently at least twice into Europe from North America. Our work suggests that the knowledge of the introduction routes of *D. v. virgifera* could facilitate identification of the origin of adaptive characters in the populations invading Europe. The probability of insecticide resistance, rotation tolerance or adaptation to control strategies existing in Europe depends on the genetic characteristics of the source populations and on the number of introductions from North America into Europe.

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