Genet. Res., Camb. (2002), **79**, *pp.* 41–47. © 2002 Cambridge University Press
DOI: 10.1017/S001667230100547X Printed in the United Kingdom

Insecticide resistance genes induce a mating competition cost in *Culex pipiens* mosquitoes

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(*Received 23 May 2001 and in revised form 14 September 2001*)

Summary

Resistance to organophosphorus insecticides (OP) in *Culex pipiens* mosquitoes represents a convenient model for investigating the fitness cost of resistance genes and its origin, since both the environmental changes in nature and the adaptive genes are clearly identified. Two loci are involved in this resistance – the super-locus *Ester* and the locus *Ace.1* – each displaying several resistance alleles. Population surveys have shown differences in fitness cost between these resistance genes and even between resistance alleles of the same locus. In order to better understand this fitness cost and its variability, the effects of these resistance genes on several fitness-related traits are being studied. Here, through competition experiments between two males for the access to one female, we analysed the effect on paternity success associated with three resistance alleles – $Ester^4$, $Ester^1$ and $Ace.1^R$ – relative to susceptible males and relative to one another. The eventual effect of female genotype on male mating success was also studied by using susceptible and resistant females. The strains used in this experiment had the same genetic background. Susceptible males had a mating advantage when competing with any of the resistant males, suggesting a substantial cost of resistance genes to this trait. When competing against susceptible males, the paternity success did not vary among resistant males, whatever the genotype of the female. When competing against other resistant males, no difference in paternity success was apparent, except when the female was $Ester^1$.

1. Introduction

Genes responsible for an adaptation to a new environment are usually assumed to have a fitness cost, i.e. to be at a disadvantage in the previous environment (e.g. Fisher, 1958; Lande, 1983; Orr & Coyne, 1992; Carrière *et al*., 1994). This is based on the general view that resource reallocation occurs or that metabolic or developmental processes are affected, thus decreasing other fitness-enhancing characters (Davies *et al*., 1996). Cost can be a determinant in the evolution of adaptation since it can lead to allelic replacement (an allele is replaced by a less costly one) or to selection of modifier genes (Lenski, 1988*a*, *b*; Cohan *et al*., 1994). Few situations exist where both the environmental changes and the adaptive genes are clearly identified. Resistance to pesticides, and in particular resistance to organophosphorus insecticides (OP) in *Culex pipiens* L. mosquitoes, is one of them.

Two loci are involved in OP resistance in *Culex pipiens*: the super-locus *Ester* and the locus *Ace.1*. Several resistance alleles have been described at both loci (Raymond *et al*., 2001). *Ester* consists of two loci on chromosome II, *Est-3* and *Est-2*, separated by an intergenic DNA fragment of 2–6 kb (Heyse *et al*., 1996; Rooker *et al*., 1996; Guillemaud *et al*., 1997) and these loci encode detoxifying esterases A and B, respectively. In field studies, *Est-3* and *Est-2* loci have always been found in maximal linkage disequilibrium for alleles involved in resistance (see review in Raymond *et al*., 2001), which justifies the concept of the *Ester* super-locus. The resistance conferred by *Ester* is due to an esterase overproduction which is the

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result of two non-exclusive mechanisms: gene amplification of *Est-2* (e.g. *Ester*^{B1} allele) or of both *Est-2* and *Est-3* (for instance, *Ester*^{4}, *Ester*^{2} and *Ester*^{5} alleles), or a change in gene regulation (*Ester*^{1} allele) (for a review see Raymond *et al*., 1998). In at least one allele (*Ester*^{2}) a third unrelated functional gene is present in the *Ester* locus, and thus is amplified (Hemingway *et al*., 2000). The *Ace.1* locus codes for the OP target, acetylcholinesterase (AChE). Resistance alleles *Ace.1*^{R} code for an AChE with a reduced sensitivity towards OP, associated with modified catalytic properties (Bourguet *et al*., 1997).

Resistance genes have been studied in the Montpellier area for more than 28 years. Resistance first appeared in 1972 with the occurrence of *Ester*^{1}, followed by *Ace.1*^{R} in 1978, *Ester*^{4} in 1984, and *Ester*^{2} in 1990 (for a review see Chevillon *et al*., 1999). Population surveys have shown that *Ace.1* is associated with more highly deleterious effects than *Ester* regarding overall fitness cost (Lenormand *et al*., 1999; Lenormand & Raymond, 2000) and the survival of overwintering females (Chevillon *et al*., 1997; Gazave *et al*., 2001). The functional differences between these two genes could explain this phenomenon (Chevillon *et al*., 1997). The overproduction of esterases by the *Ester* locus should be at the expense of producing something else, with the resulting alteration in some fitness-related traits. The modified AChE could lead to changes in some behavioural fitness-related traits, since it alters the optimal functioning of cholinergic synapses of the central nervous system. It was observed during the 1990s that *Ester*^{4} had replaced *Ester*^{1} (Guillemaud *et al*., 1998). As *Ester*^{4} is known to confer a slightly lower OP resistance level, its advantage over *Ester*^{1} could possibly come from a lower cost (Guillemaud *et al*., 1998). The proximal causes of such a variability in the fitness cost between resistance alleles are unknown.

In order to better understand this fitness cost and the origin of its variability, we have undertaken to study how these various resistance alleles affect some important life history traits. Here, we focused on the effect of resistance genes on male mating competition, using strains sharing the same genetic background. In *Culex pipiens*, male mating competitive ability is probably a particularly important fitness component, as the preferential monogamy of females (Vinogradova, 2000) and the potential polygyny of males give a low fitness to non-competitive males. Through competition experiments between two males for the access to one female, we report how resistance alleles *Ester*^{4}, *Ester*^{1} and *Ace.1*^{R} can affect male paternity success, relatively to susceptible individuals (*Ester*^{0} and *Ace.1*^{S} alleles). We also investigated how these resistant individuals are ranked when competing with one another. In order to detect an eventual effect of female genotype on male mating success, these competitions were performed with several female genotypes (*Ester*^{0}, *Ester*^{1} and *Ester*^{4}).

2. Material and methods

(i) *Mosquito strains*

(*a*) *Parental strains*. The strains used in the experiment were derived from: S-LAB, the susceptible reference strain, homozygous for *Ester*^{0} and *Ace.1*^{S} alleles (Georghiou *et al*., 1966); VIM, homozygous for *Ester*^{4} and *Ace.1*^{S} alleles (Poirié *et al*., 1992); BARRIOL, homozygous for *Ester*^{1} and *Ace.1*^{R} alleles (Chevillon *et al*., 1995); and CYPRUS, homozygous for *Ester*^{5} and *Ace.1*^{S} alleles (Poirié *et al*., 1992). In order to obtain a strain carrying *Ester*^{1} and *Ace.1*^{S} alleles, CYBAR, the genome of BARRIOL was introgressed by that of CYPRUS until recombination between *Ester*^{1} and *Ace.1*^{R} alleles of the BARRIOL strain occurred, i.e. until the *Ace.1*^{R} allele of BARRIOL was replaced by the *Ace.1*^{S} allele of CYPRUS. At each generation, heterozygotes *Ester*^{1}/*Ester*^{5} were selected using starch gel electrophoresis (Pasteur *et al*., 1988). Recombinants were observed at the fifth generation. Homozygosity for *Ester*^{1} and *Ace.1*^{S} alleles was then achieved by family selection. The strain CYBAR derived from five families.

(*b*) *Derived strains*. To obtain resistant strains sharing the same genetic background, the genome of the strains VIM, CYBAR and BARRIOL was introgressed by that of S-LAB through repeated backcrossing (15, 14 and 14, respectively). At each generation of backcrossing, a discriminating insecticide dose was applied to select for resistant heterozygotes, and the surviving females were crossed to S-LAB males. During the last generation of backcross, the surviving males were crossed to S-LAB females in order to introduce the S-LAB cytoplasm into the introgressed strains. Finally, homozygosity of the strains for the resistance alleles considered was achieved by analysing parents or their offspring, using the molecular test of Berticat *et al*. (2000) for the strains derived from VIM and CYBAR, and the microplate test of Bourguet *et al*. (1996) for the strain derived from BARRIOL. It must be noted that during the introgression of the BARRIOL strain, its *Ester* allele was replaced by that of S-LAB leading to an introgressed strain carrying *Ester*^{0} and not *Ester*^{1}. For a given allele of an introgressed strain, we can define the probability P that, at the end of i backcrosses, this allele is still associated with the selected resistance allele, i.e. no recombination event has occurred between the two genes. If r is the recombination rate between both genes, then $P = (1-r)^{i}$. This allows the computation of the genetic distance around the selected gene which has not been replaced by the S-LAB genome, e.g. around $1-(e^{(\ln(\alpha)/i)})$, α being the risk level. This leads

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Table 1. *Genotypes at resistance genes of the strains used. All share the S-LAB genetic background*

Strains	*Ester* genotype	*Ace.1* genotype
S-LAB	$Ester^0/Ester^0$	$Ace.1^S/Ace.1^S$
SA1	$Ester^1/Ester^1$	$Ace.1^S/Ace.1^S$
SA4	$Ester^4/Ester^4$	$Ace.1^S/Ace.1^S$
SR	$Ester^0/Ester^0$	$Ace.1^R/Ace.1^R$

Table 2. *Number of replicates for each mating competition. The competitions are organised in three data sets for the statistical analyses*

		Females			
Data set	Males competing	S-LAB	SA1	SA4	Totals
SET-1	S-LAB vs SA1	60	39	29	128
	S-LAB vs SA4	58	40	29	127
	S-LAB vs SR	39	31	28	98
SET-2	SA1 vs SR	30	26	18	74
	SA4 vs SR	32	33	34	99
SET-3	SA1 vs SA4	43	42	55	140

to 19 and 18 cM for 14 and 15 backcrossing generations, respectively, at the 0·05 risk level.

With this protocol, we obtained three new resistant strains sharing the same genetic background as S-LAB (Table 1): SA1, derived from CYBAR, was homozygous for $Ace.1^S$ and $Ester^1$ alleles; SA4, derived from VIM, was homozygous for $Ace.1^S$ and $Ester^4$ alleles; SR, derived from BARRIOL, was homozygous for $Ace.1^R$ and $Ester^0$ alleles. These strains were subsequently raised without known insecticide exposure.

For all the following experiments, differential maternal effects were prevented by maintaining S-LAB and the resistance strains under the same standardized conditions for a minimum of 5 generations.

(ii) *Mating competition*

Same-aged (1-day-old) adults were used in mating competitions. They were distributed in triad as follows. Two virgin males and one virgin female were placed for 6 days in a 125 cm^3 glass vial with access *ad libitum* to a honey solution. Females were then removed and blood-fed. All the females were fertilized and laid eggs. Egg-rafts were individually collected, and offspring were bred until adulthood, when they were frozen for further analyses. All possible male–male competitions were performed in the presence of either S-LAB, SA1 or SA4 females, and each was replicated 37 times on average (Table 2).

(iii) *Paternity assignation of female offspring*

Multipaternity has rarely been observed in *Culex pipiens* mosquitoes (Kitzmiller & Laven, 1958; Bullini *et al*., 1976; Thank *et al*., 1977; Vinogradova, 2000), thus only one individual per female offspring was analysed for paternity assignation. Three techniques were used depending on mother genotypes and putative fathers genotypes. For competitions involving males carrying $Ace.1^R$ (SR strain), the microplate test of Bourguet *et al*. (1996) was used to determine the transmission of either $Ace.1^R$ or $Ace.1^S$ alleles. To determine the transmission of *Ester* alleles, starch gel electrophoresis was used (Pasteur *et al*., 1988). With this technique, $Ester^1$ and $Ester^4$ are dominant over $Ester^0$, thus preventing paternity assignation when, in the triad, one male is homozygous for $Ester^0$ (S-LAB strain) and the two other partners are either both homozygous for $Ester^1$ (SA1 strain) or both homozygous for $Ester^4$ (SA4 strain). In these cases, we used the molecular test of Berticat *et al*. (2000) to identify *Ester* alleles.

(iv) *Statistical analyses*

Paternity success of each male genotype was defined, in each competition, as the percentage of females that it fertilized among replicates.

Three independent sets of data were considered (Table 2). The first data set (SET-1) allowed investigation of the effects of the three resistance alleles on paternity success, relative to susceptible individuals. The second and third data sets allowed investigation of how the three types of resistant males were ranked when competing with one another ($Ace.1^R$ relatively to $Ester^1$ and $Ester^4$ for SET-2, and $Ester^1$ relatively to $Ester^4$ for SET-3).

(*a*) *Variation in paternity success*. Variation in paternity success of S-LAB males competing against the resistant males (SET-1, Table 2) was tested using a logistic regression. Two qualitative variables were defined corresponding to the genotype of the competitor (COMP) and to the genotype of the female (FEM). The model was COMP + FEM + COMP. FEM ('.' represents the interaction between two qualitative variables). This model was simplified according to Crawley (1993): interaction effect was first tested and removed if not significant ($P > 0{\cdot}05$) allowing the separate testing of COMP and FEM effects. This process gave the minimal adequate model. Variation in paternity success of SR males competing against SA1 or SA4 males (SET-2, Table 2) was tested following the same principle. Variation in paternity success of SA1 when competing against SA4 males (SET-3, Table 2) was tested slightly differently since the genotype of the competitors did not vary. In this

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case, the model was FEM only. These analyses were performed using GLIM version 4 (Baker, 1987).

(*b*) *Paternity success compared with chance*. The null hypothesis was that paternity success equalled 0·5 for both competing males. For each competition, deviation from the null hypothesis was tested separately by a binomial exact test. A global test was then performed across the competitions leading to the same paternity success (for the male considered in each data set) by combining the *P*-values using Fisher's method (Manly, 1985).

3. Results

Paternity success of S-LAB males (SET-1) was influenced neither by the genotype of the competitor (COMP, $\chi^2 = 4{\cdot}46$, df = 2, $P = 0{\cdot}11$; Table 3), by that of the female (FEM, $\chi^2 = 1{\cdot}57$, df = 2, $P = 0{\cdot}45$; Table 3), nor by the interaction of the two (COMP. FEM, $\chi^2 = 13{\cdot}74$, df = 8, $P = 0{\cdot}08$; Table 3), i.e. the minimal model was the null one. A slight overdispersion was observed (scaled deviance/residual df = 1·41). All the binomial exact test *P* values corresponding to this data set were combined using Fisher's method to compare their average paternity success (= 0·76; Table 3) with chance. Paternity success of S-LAB males is significantly higher than that expected by chance ($P < 10^{-5}$, Table 3).

When SR males competed against SA1 or SA4 males (SET-2), their paternity success depended on the genotype of the female ($\chi^2 = 7{\cdot}03$, df = 2, $P = 0{\cdot}029$; Table 3). This minimal model explained 75·8 % of the total deviance and displayed no overdispersion (scaled deviance/residual df = 0·74). Consequently, the binomial exact test *P* values corresponding to this data set were combined separately, according to the genotype of the female, i.e. their average paternity success with each type of female was compared with chance. When the female was S-LAB or SA4, the paternity success of SR males did not differ from that expected by chance ($P > 0{\cdot}05$ for both females; Table 3). By contrast, when the female was SA1, paternity success of SR males (= 0·25; Table 3) was significantly lower than that expected by chance ($P = 0{\cdot}001$; Table 3).

Finally, when SA1 males competed against SA4 males (SET-3), their paternity success was not influenced by the genotype of the female ($\chi^2 = 0{\cdot}04$, df = 2, $P = 0{\cdot}98$; Table 3). The best model was the null one. It displayed a slight overdispersion (scaled deviance/residual df = 2·01). All the binomial exact test *P* values corresponding to this data set were combined to compare their average paternity success with chance. Paternity success of SA1 males (and consequently of SA4 males) did not deviate from chance ($P > 0{\cdot}05$; Table 3).

4. Discussion

Susceptible males had a mating advantage when competing with any of the resistant males. This suggests that the three resistance alleles studied are associated with a strong fitness cost on paternity success, relative to susceptible individuals. In other insect species this trait has not always been proved discriminative, resistant males displaying either a similar (e.g. *Metaseiulus occidentalis*: Roush and Hoy, 1981), a lower (e.g. *Anopheles gambiae*: Rowland, 1991*a*) or a higher (e.g. *Anopheles albimanus*: Gilotra, 1965; *Tribolium castaneum*: Arnaud *et al*., 1999) mating success than the susceptible ones.

No variability in fitness cost associated with the three resistance alleles was observed, neither when competitions occurred with susceptible males, nor when competitions involved two resistant males, except for one situation: when the female was $Ester^1$, $Ace.1^R$ appeared more costly than $Ester^1$ and $Ester^4$. The similar effect on paternity success of the *Ester* and

Table 3. *Statistical results. For each data set, P values of model terms explaining the paternity success are shown, as well as results concerning comparison to chance of paternity success that followed from them. Bold characters correspond to significant values* ($P < 0{\cdot}05$)

Data sets	*P* values from the model tested			Best model	Paternity success compared with chance and *P* values		
	COMP[a]	FEM[b]	COMP.FEM[c]				
S-LAB against all (SET-1)	$P = 0{\cdot}11$	$P = 0{\cdot}45$	$P = 0{\cdot}08$	Null	0·76	$\boldsymbol{P < 10^{-5}}$	
SR against SA1 or SA4 (SET-2)	$P = 0{\cdot}51$	$\boldsymbol{P = 0{\cdot}029}$	$P = 0{\cdot}37$	FEM	With female S-LAB	0·48	$P = 0{\cdot}55$
					With female SA1	0·25	$\boldsymbol{P = 0{\cdot}001}$
					With female SA4	0·35	$P = 0{\cdot}11$
SA1 against SA4 (SET-3)	–	$P = 0{\cdot}98$	–	Null	0·46	$P = 0{\cdot}27$	

[a] Effect of the male competitor genotype.
[b] Effect of the female genotype.
[c] Interaction between the male competitor and the female genotypes.

https://doi.org/10.1017/S001667230100547X Published online by Cambridge University Press

Ace.1 resistant males, despite the functional difference between the two genes involved, could have several origins. First, if a very small difference exists between their associated cost on this trait, the number of replicates could be too low to detect it. Second, these results could suggest that the pleiotropic effects associated with the two resistance genes are similar on this trait. This hypothesis cannot be rejected since *Ester* function is unknown (Leinweber, 1987), and we do not know the proximal causes of the selective disadvantage observed.

The modified AChE encoded by $Ace.1^R$ could lead to changes in some behavioural fitness traits, since it alters the optimal functioning of cholinergic synapses of the central nervous system. Thus, in this experiment, behavioural components involved at mating time could be affected by $Ace.1^R$. If the absence of variability in fitness cost observed here is due to similar pleiotropic effects associated with the two resistance genes, consequently, the same behavioural components would be affected by *Ester* resistance alleles. The fact that the *Ester* gene could be involved in behavioural components of mating is not surprising. Indeed, an esterase involved in the reproductive system of *Drosophila melanogaster*, esterase 6, present in the seminal fluid of males, has been suspected of being involved in a pheromone system which influences female reproductive behaviour (Richmond & Senior, 1981; Richmond *et al*., 1990). The importance of mating behaviour in the relative reproductive success of resistant and susceptible males has been analysed in some insects. In *Tribolium castaneum*, a resistant male (and not a susceptible one) preferentially copulates with females mated by other males rather than with females it has already fertilized. As the last male to copulate fathers the majority of the progeny in this species, this contributes to its higher reproductive success (Arnaud & Haubruge, 1999). In *Anopheles stephensi*, resistant males and females tend to mate assortatively in mating competition experiments because of an activity out of synchrony with that of susceptible individuals (Rowland, 1991*a*). A possible physiological mechanism underlying this phenomenon is that a change in the cyclodiene receptor on the chloride channels (leading to the resistance to dieldrin) could increase their permeability to chloride ions, causing hyper-inhibition of the nervous system (Rowland, 1991*b*).

In the present experiment, issue of the competitions, i.e. paternity success, can be determined either by the males (for instance, their competitive ability), the females (choice of sexual partner), or both. It is not possible to discriminate between these possibilities with the current experimental setting. However, some results are consistent with the existence of a female choice, i.e. the interaction between $Ace.1^R$ males and $Ester^1$ females. $Ester^1$ females could always discriminate against $Ace.1^R$ males, whether the competitor genotype was susceptible or *Ester* resistant. Susceptible and $Ester^4$ females could then behave differently: they would discriminate against SR males only when facing a S-LAB male, but would not differentiate the three types of resistant males. Further experiments are required to settle this point.

The effects on paternity success of two resistance alleles of *Ester* gene were investigated not only in males but also in females. $Ester^1$ and $Ester^4$ females had a different effect on paternity success. A recent study (Pasteur *et al*., 2001) suggests that the distribution of the esterases encoded by these alleles could be different in the tissues where they are synthesized or stored. It would then be possible that this putative difference in expression of these alleles is associated with a difference in the reproductive phenotype of females.

Finally, population surveys suggest a difference in fitness cost associated with *Ester* and *Ace.1* genes (Lenormand *et al*., 1999; Lenormand & Raymond, 2000) and in fitness cost associated with $Ester^1$ and $Ester^4$ alleles (Guillemaud *et al*., 1998). In the present experiment, a substantial mating cost associated with the *Ester* and *Ace.1* resistance alleles was shown, although cost differences between them was not apparent. Maybe there is no cost difference between $Ace.1^R$, $Ester^1$ and $Ester^4$ alleles, at least for this life history trait. Pleiotropic effects associated with resistance are a consequence of the biochemical and physiological changes associated with the resistant phenotype. Consequently, it is possible that some life history components are affected by resistance in different ways. The study of other fitness traits (e.g. overwintering survival, larval developmental time, predation avoidance) seems to confirm this point (Gazave *et al*., 2001; Bourguet *et al*., in preparation; Berticat *et al*., in preparation). The comparison between these various affected traits will allow us to better understand the physiological and biochemical causes of the pleiotropy of adaptive genes.

We are very grateful to N. Pasteur for helpful comments on the manuscript, to M. Marquine, C. Bernard and G. Pistre for technical assistance, and to Bayer for providing propoxur. This work was financed in part by the PNETOX program and the Ministère de l'Amenagement du Territorie et de l'Environnement. Contribution 2001.121 of the Institut des Sciences de l'Evolution de Montpellier (UMR CNRS 5554).

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https://doi.org/10.1017/S001667230100547X Published online by Cambridge University Press