

# Insecticide resistance genes confer a predation cost on mosquitoes, *Culex pipiens*

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## Summary

Newly occurring adaptive genes, such as those providing insecticide resistance, display a fitness cost which is poorly understood. In order to detect subtle behavioural changes induced by the presence of resistance genes, we used natural predators and compared their differential predation on susceptible and resistant *Culex pipiens* mosquitoes, using strains with a similar genetic background. Resistance genes were either coding an overproduced detoxifying esterase (locus *Ester*), or an insensitive target (locus *ace-1*). Differential predation was measured between susceptible and resistant individuals, as well as among resistant mosquitoes. A backswimmer, a water measurer, a water boatman and a predaceous diving beetle were used as larval predators, and a pholcid spider as adult predator. Overall, the presence of a resistance gene increased the probability of predation: all resistance genes displayed predation costs relative to susceptible ones, at either the larval or adult stage, or both. Interestingly, predation preferences among the susceptible and the resistance genes were not ranked uniformly. Possible explanations for these results are given, and we suggest that predators, which are designed by natural selection to detect specific behavioural phenotypes, are useful tools to explore non-obvious differences between two classes of individuals, for example when they differ by the presence or absence of one recent gene, such as insecticide resistance genes.

## 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 assumption 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 important 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 *C. pipiens*, the super-locus *Ester* and the locus *ace-1*. Several resistance alleles have been described at both loci (for a review see Raymond *et al.*, 2001). The resistance conferred by *Ester* is due to an esterase over-production which is the result of two non-exclusive mechanisms (Raymond *et al.*, 1998): gene amplification (for instance, *Ester*<sup>4</sup>, *Ester*<sup>2</sup> and *Ester*<sup>5</sup> alleles), or change in gene regulation (*Ester*<sup>1</sup> allele). The *ace-1* locus codes for the OP target, acetylcholinesterase (AChE). Resistance alleles *ace-1*<sup>R</sup> code 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 30 years. Resistance first appeared in 1972 with the occurrence of *Ester*<sup>1</sup>, followed by *ace-1*<sup>R</sup> in 1978, *Ester*<sup>4</sup> in 1984 and *Ester*<sup>2</sup> in 1990 (Guillemaud *et al.*, 1998). Estimations of overall fitness costs from population surveys have shown that *ace-1* is associated with higher deleterious effects than *Ester* (Lenormand *et al.*, 1999; Lenormand & Raymond, 2000). This difference is also observed for a

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specific life history trait, survival during the overwintering period (Chevillon *et al.*, 1997; Gazave *et al.*, 2001). The functional differences between the two loci could explain this phenomenon (Chevillon *et al.*, 1997). The over-production of esterases by the *Ester* locus could be at the expense of producing something else, with the resulting alteration of 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 has been observed that, during the 1990s, *Ester<sup>A</sup>* has replaced *Ester<sup>I</sup>* (Guillemaud *et al.*, 1998). As *Ester<sup>A</sup>* is known to confer a slightly lower OP resistance level, its advantage over *Ester<sup>I</sup>* could possibly come from a lower cost (Guillemaud *et al.*, 1998). The proximal causes of such variability in the fitness cost between resistance alleles are still unknown.

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, using strains sharing the same genetic background. In a recent study, a mating competition cost associated with *Ester<sup>I</sup>*, *Ester<sup>A</sup>* and *ace-1<sup>R</sup>* resistance alleles was demonstrated, but no cost difference between them was detected (Berticat *et al.*, 2002a). Here, we investigate how these three resistance alleles affect the probability of predation at larval and adult stages, relative to susceptible alleles. We also attempt to compare the resistance alleles with one another. Avoiding predation is an important fitness component of *C. pipiens* (Sih, 1986), and confrontation with a predator could constitute a risky situation, liable to amplify the physiological differences between the resistance genotypes, thus potentially allowing us to detect cost difference between the resistance alleles.

## 2. Materials and methods

### (i) Mosquito strains

Four strains sharing the same genetic background and only differing by their genotype at *Ester* and/or *ace-1* locus were used: the insecticide-susceptible strain S-LAB, homozygous for *ace-1<sup>S</sup>* and *Ester<sup>O</sup>* (Georghiou *et al.*, 1966); the resistant strains SA1 and SA4, homozygous for *ace-1<sup>S</sup>* and for the resistance alleles *Ester<sup>I</sup>* and *Ester<sup>A</sup>*, respectively; and finally, the resistant strain SR, homozygous for *Ester<sup>O</sup>* and for the resistance allele *ace-1<sup>R</sup>* (Berticat *et al.*, 2002a). Before all experiments, all strains were reared under the same standardized conditions for a minimum of 5 generations, preventing possible maternal effects.

### (ii) Predation on adult mosquitoes

The adult predator used in this experiment was a spider, *Holocnemus pluchei* (Scopoli) (Araneae, Pholci-

dae), a common inhabitant of homes, which is known to feed on flying insects, including *C. pipiens* (Déom, 1990). *H. pluchei*, through vibrations of its web, locates its prey, which is eventually immobilized and rapidly packed with silk threads. Then *H. pluchei* injects its digestive saliva into a captured insect, and ingests the content. The external skeleton of an empty individual remains, tightly packed like a mummy, allowing easy detection of eaten adults. *H. pluchei* used here were locally collected in one University building.

Differential predation between two strains was assessed by introducing, into the same cage (20 × 20 × 20 cm<sup>3</sup>), 20 one-day-old male mosquitoes from each of the two strains considered, together with one *H. pluchei*. Predators were starved for 10 days before each experiment. Every day, predated adults ('mummies') were collected, and the spider was replaced by a new starved one. This procedure ensured that the predation rate did not decrease due to satiation. The experiment was ended when approximately 50% of all adults were eaten. In order to recognize the strain of origin of each mummy, adults of each strain were marked just before the start of an experiment, using fluorescent powders of different colour (yellow or orange). For each experiment, at least two replicates were performed by switching the colour of each strain. Additionally, experiments with adults marked with orange or yellow from the same strain were conducted for all strains. The different experiments performed and their number of replicates are indicated in Table 1.

### (iii) Predation on mosquito larvae

The larval predator used in this experiment was the pigmy backswimmer, *Plea minutissima* Leach (Hemiptera, Pleidae), which is about 2 mm in size. This insect is a common inhabitant of ponds of the Palearctic, and feeds on small aquatic prey such as other small insects or crustaceans. *P. minutissima* is a potential predator of *C. pipiens*, as both often co-occur in the same breeding sites (Laird, 1988), and *P. minutissima* readily feeds on young (L1 or L2) *C. pipiens* larvae in the laboratory. *P. minutissima* injects its digestive saliva into a captured larvae, and ingests the contents. The external skeleton of an empty larva remains, allowing easy detection of captured larvae. *P. minutissima* used here were collected locally (around the Montpellier area) and reared in the laboratory.

Differential predation between two strains was assessed by introducing, into the same container, an equal number of L2 larvae from the two strains considered, together with two or three *P. minutissima*. The experiment was ended when approximately 50% of all larvae had been preyed upon, and eaten larvae of each strain were recorded. Predators were starved for 10 days before each experiment. In order to

Table 1. Adult predation. (A) Effect of powder coloration on each strain, (B) effect of resistance genes compared with a susceptible one, and (C) effect of different resistance genes between them

Effect tested	Confronted strains		No. of replicates	P values	$\hat{\beta}$ of the strain mentioned
	Orange	Yellow			
(A) Effect of coloration	S-LAB	S-LAB	4	0.1345	–
	SA1	SA1	3	1	–
	SA4	SA4	3	0.74	–
	SR	SR	2	0.88	–
	All		–	0.69	–
(B) Effect of resistance vs susceptible genes	SA1	S-LAB	2	0.018	–
	S-LAB	SA1	2	0.03	–
	All		–	0.001	SA1 <b>0.67</b> (0.048)
	SA4	S-LAB	2	0.48	–
	S-LAB	SA4	2	0.001	–
	All		–	0.02	SA4 <b>0.64</b> (0.076)
	SR	S-LAB	2	0.25	–
	S-LAB	SR	2	0.89	–
(C) Effect of different resistance genes	All		–	0.59	SR 0.50 (0.075)
	SA1	SA4	2	0.56	–
	SA4	SA1	2	0.22	–
	All		–	0.36	SA4 0.41 (0.044)
	SR	SA1	2	0.93	–
	SA1	SR	2	0.13	–
	All		–	0.35	SR 0.44 (0.060)
	SR	SA4	2	0.32	–
	SA4	SR	2	0.76	–
All		–	0.57	SR 0.57 (0.033)	

The *P* value refers to a two-sided (A and C) or a one-sided test (B), when the alternative hypothesis is a higher predation rate for resistant mosquitoes. For all cases, the *P* value refers to a global exact test across replicates. Estimates of average predation coefficients ( $\hat{\beta}$ ) refer to the strain mentioned and bold characters indicate  $\hat{\beta}$  values significantly ( $P < 0.05$ ) higher than 0.5. SE is given in parentheses. See text for explanations.

recognize the strain of origin of each larva, two protocols were used. For the first protocol, each experiment was conducted in 100 ml of tap water (water depth 1.5 cm), with a total number of 40 larvae. No refugium was available for the mosquito larvae. Larvae of one of the strains considered were stained just before the start of an experiment, using diluted methylene blue. For each experiment, two replicates were performed by switching the stained strain. Additionally, experiments with stained and unstained larvae from the same strain were conducted for all the strains. The number of replicates of the different experiments are indicated in Table 2. For the second protocol, when larvae from the SR strain were involved, a propoxur (a carbamate insecticide) concentration of 5 mg/l was applied during 24 h to the non-eaten larvae. In this case, each experiment was conducted in 500 ml of tap water (water depth 1 cm), with a total number of 200 larvae and no refugium was available for the mosquito larvae. This dose kills in a few hours only those larvae without the *ace-1<sup>R</sup>* resistance gene (i.e. all individuals except those from the SR strain), as the propoxur concentration required to kill SR larvae after 24 h exposure is more than 100-fold higher (Bourguet *et al.*, 1997). This

procedure allowed the identification of SR individuals among non-eaten larvae. As a control, the same propoxur dose was simultaneously applied only to susceptible *ace-1<sup>S</sup>* (S-LAB, or SA1 or SA4) and only to *ace-1<sup>R</sup>* resistant (SR) larvae. The number of replicates of the different experiments is indicated in Table 3. The same procedure could not be used for the other resistant strains, as their relatively low OP resistance level does not allow the use of a discriminative dose.

The larval predation cost of SR relative to S-LAB was further evaluated using three additional predators: a water boatman *Sigara lateralis* (Leach) (Hemiptera, Corixidae), a predaceous diving beetle *Guignotus pusillus* Fabricius, 1781 (Coleoptera, Dytiscidae) and the water measurer *Hydrometra stagnorum* (Linnaeus, 1758) (Hemiptera, Hydrometridae). Their size is approximately 5–6, 2 and 10 mm, respectively. All these predators are commonly found in mosquito breeding sites around the Montpellier area, and also at a larger scale (Laird, 1988). They can feed only on young (L1 or L2) *C. pipiens* larvae in laboratory conditions, and inject their digestive saliva into a captured larva in order to ingest its content. Water boatmen appear to be very effective predators, and seem to hunt like *P. minutissima*. In comparison with

Table 2. Larval predation by *Plea minutissima*. (A) Effect of dye on each strain, and (B) effect of resistance genes compared with a susceptible one

Tested effect	Confronted strains		No. of replicates	P value	$\hat{\beta}$
	Not stained	Stained			
(A) Effect of coloration	S-LAB	S-LAB	16	<10 <sup>-5</sup>	–
	SA1	SA1	4	0.14	–
	SA4	SA4	5	0.24	–
	SR	SR	3	1	–
	All		–	<10 <sup>-4</sup>	–
(B) Effect of resistance vs susceptible genes	SA1	S-LAB	8	0.68	0.49 (0.038)
	SA4	S-LAB	12	<10 <sup>-5</sup>	<b>0.63</b> (0.050)
	SR	S-LAB	12	<10 <sup>-8</sup>	<b>0.71</b> (0.050)

The P value refers to a two-sided (A) or a one-sided test (B), when the alternative hypothesis is a higher predation rate for resistant mosquitoes. For all cases, the P value refers to a global exact test across replicates. Estimates of average predation coefficients ( $\hat{\beta}$ ) refer to the resistant strain and bold characters indicate  $\hat{\beta}$  values significantly ( $P < 0.05$ ) higher than 0.5. SE is given in parentheses. See text for explanations.

Table 3. Larval predation by *Plea minutissima*, using an insecticide for genotype identification

Confronted strains	No. of replicates	P value	$\hat{\beta}$
SR S-LAB	5	<10 <sup>-8</sup>	<b>0.65</b> (0.014)
SR SA1	5	0.22	0.52 (0.024)
SR SA4	5	<10 <sup>-8</sup>	<b>0.86</b> (0.036)

The P value refers to a two-sided (lines 2 and 3) or a one-sided test (line 1), when the alternative hypothesis is a higher predation rate for SR mosquitoes. For all cases, the P value refers to a global exact test across replicates. Estimates of average predation coefficients ( $\hat{\beta}$ ) refer to the SR strain and bold characters indicate  $\hat{\beta}$  values significantly ( $P < 0.05$ ) higher than 0.5. SE is given in parentheses. See text for explanations.

other Dytiscidae, adults of *Guignotus pusillus* are very small, and feed only on tiny prey. The water measurer walks slowly onto the water surface, usually among vegetation, and spears small prey under the water surface with its long rostrum. Differential predation between S-LAB and SR was assessed with the same protocol described above with *P. minutissima*, although only L1 larvae were used, and only one predator per replicate. Experiments were conducted in 250, 50 and 50 ml of tap water, with a total number of larvae of 200, 100 and 40 for the water boatman, water beetle and water measurer, respectively. Non-eaten larvae were assigned to each strain by treating them with a discriminating dose of propoxur (5 mg/l), as described above. The numbers of replicates of the different experiments are indicated in Table 4.

(iv) Statistics

A predation experiment corresponds to sampling without replacement. The null hypothesis ( $H_0$ ) is that

Table 4. Estimates of average predation coefficients ( $\hat{\beta}$ ) for resistant larvae (SR strain) compared with susceptible ones (S-LAB strain), in the presence of various predators (SE in parentheses)

Predator	No. of replicates	P value	$\hat{\beta}$
<i>Sigara lateralis</i>	9	0.22	0.56 (0.020)
<i>Guignotus pusillus</i>	9	<10 <sup>-2</sup>	<b>0.69</b> (0.043)
<i>Hydrometra stagnorum</i>	11	<10 <sup>-8</sup>	<b>0.68</b> (0.033)

The P value refers to a one-sided test, when the alternative hypothesis is a higher predation rate for SR larvae. For all cases, the P value refers to a global exact test across replicates. Estimates of average predation coefficients ( $\hat{\beta}$ ) refer to the SR strain and bold characters indicate  $\hat{\beta}$  values significantly ( $P < 0.05$ ) higher than 0.5. SE is given in parentheses. See text for explanations.

both morphs (here strains) are equally preyed upon. At the end of the experiment, the number of eaten individuals of each morph follows a hypergeometric distribution, and the probability of the observed data, under  $H_0$ , is:  $P_{obs} = (C_{A_1}^{r_1} C_{A_2}^{r_2}) / C_{A_1+A_2}^{r_1+r_2}$ , where  $A_j$  denotes the total number of morph  $j$  at the beginning of the experiment,  $r_j$  is the number of morph  $j$  remaining after predation, and  $C_i^j = i! / (j!(i-j)!)$ . To test  $H_0$ , a hypergeometric exact test was constructed. The P value is defined as:  $P = \sum_{P_i \leq P_{obs}} P_i$ , where  $P_i$  is the probability (under  $H_0$ ) of all  $i$  cases describing all possible ways of distributing the observed number of eaten individuals among both morphs, with the total number of individuals of both morphs kept constant. When an alternative hypothesis was present (e.g. resistant individuals were more preyed upon than susceptible ones), a one-sided test was performed. When no alternative hypothesis was obvious (e.g. when differently coloured adults of the same strain were together), a two-sided

test was done. A quick-basic program was written to perform these tests, and was checked by comparison with hand calculations. A global test across replicates was performed by generating the joint distribution, and computing the  $P$  value as  $P = \sum_{P_j \leq P_{g_{obs}}} P_j$ , where  $P_j$  is the probability of element  $j$  of the joint distribution, and  $P_{g_{obs}}$  is the joint probability of the observed data. When a specified alternative hypothesis was present (e.g. type 1 individuals were more preyed upon than type 2), the  $P$  value was  $P = \sum_{N_j \geq N_{obs}} P_j$ , where  $N_j$  is the total number of type 1 preyed upon individuals in element  $j$  of the joint distribution, and  $N_{obs}$  is the total number of observed type 1 preyed upon individuals across replicates. A quick-basic program was written to perform the global exact test for up to five replicates, using the complete enumeration method. A PowerBasic program was written to perform the global exact test for an unspecified number of replicates, using the resampling method to estimate the  $P$  value. Program checking was done by comparing the  $P$  values generated by the two programs (which use very different algorithms) when used on the same data, for 2–5 replicates. When the number of resamplings was 500 000, the estimated  $P$  values diverged by less than 0.4% from the computed exact values. The exact  $P$  value was computed for cases with 2–4 replicates, and also for 5 replicates when the number of assayed individuals was lower than 40. In all other cases, the exact  $P$  value was estimated using 500 000 resamplings.

Preference was measured using the index proposed by Manly (1974, 1985):

$$\beta_i = \frac{\log_e(r_i/A_i)}{\sum_{j=1}^K \log_e(r_j/A_j)},$$

where  $K$  is the number of morphs (here  $K=2$ ). This measure is appropriate for experiments in which the prey are not replaced during the experiment. This index varies between 0 and 1, and  $\sum_i \beta_i = 1$ . The absence of preference between two morphs corresponds here to  $\beta = 1/2$ .

### 3. Results

#### (i) Adult predation

Each predation experiment lasted about 3 days (range 1–4 days). In order to recognize susceptible and resistant mosquitoes in the experimental cage, adults were marked with a fluorescent powder, either yellow or orange. The colour of the powder had no significant effect ( $P > 0.69$ ) on the predation frequency, for all the strains used (Table 1). When susceptible and resistant adults were in the same cage, the latter were significantly more preyed upon than the former (SA1:  $P < 0.001$ ,  $\beta = 0.67 \pm 0.048$ ; SA4:  $P = 0.02$ ,

$\beta = 0.64 \pm 0.076$ ). However, no difference in predation rate relative to susceptible individuals was apparent for the SR strain (Table 1). When the resistant strains were confronted pairwise within the same cage, predation was not different ( $P > 0.3$ ) according to the resistance genes present.

#### (ii) Larval predation

Each predation experiment lasted about 2 days (range 1–3 days). In order to recognize susceptible and resistant mosquitoes in the experimental container, larvae were stained with a blue dye. This dye slightly increased the risk of predation by *P. minutissima* for the susceptible strain (Table 2). As the hypothesis considered is a higher predation for resistant larvae compared with susceptible ones, only assays where the susceptible strain is stained are presented, in order to be conservative (assays where the resistant strain is stained are all supportive of the hypothesis tested, but they are not conclusive due to the dye bias). Despite this disadvantage, stained susceptible larvae were significantly less preyed than resistant ones ( $P < 10^{-5}$ ), with the exception of SA1 larvae (Table 2).

When SR individuals were used, they could be recognized within the non-eaten larvae as they survive a high concentration of propoxur. Thus no dye was required in these experiments. SR larvae were significantly more preyed upon than susceptible individuals ( $P < 10^{-8}$ ,  $\beta = 0.65 \pm 0.014$ ). SR larvae were also significantly more eaten than SA4 ( $P < 10^{-8}$ ,  $\beta = 0.86 \pm 0.036$ ), although no difference ( $P = 0.22$ ) in predation rate was apparent when SR and SA1 were together (Table 3).

To evaluate whether the differences detected by *Plea minutissima* were also detected by other larval predators, SR were confronted with S-LAB larvae in the presence of the three other aquatic predators. For these predators, SR larvae were significantly more preyed upon than susceptible ones (diving beetle:  $P < 10^{-2}$ ,  $\beta = 0.69 \pm 0.043$ ; water measurer:  $P < 10^{-8}$ ,  $\beta = 0.68 \pm 0.033$ ; Table 4), with the exception of the water boatman ( $P = 0.22$ ,  $\beta = 0.56 \pm 0.020$ ; Table 4).

### 4. Discussion

Overall, the presence of a resistance gene increased the probability of predation, at both the larval and the adult stage: there is thus a 'predation cost' associated with these genes.

#### (i) Origin of the predation cost

Hunting techniques of backswimmers and water boatmen (families Notonectidae, Corixidae and Pleidae) rely essentially upon prey motion (Murphey & Mendenhall, 1973; Sih, 1979). Behaviour underlying

backswimmers' preferences seems to be stereotyped and inflexible (Scott & Murdoch, 1983). Many mosquito larvae, including those of *C. pipiens*, are natural prey items for several backswimmer species, and thus share an evolutionary history with them (e.g. Sunish & Reuben, 2002; Chesson, 1984; Blaustein, 1998; Mogi *et al.*, 1999). It is thus not surprising that upon a backswimmer attack, mosquitoes try most of the time to escape by becoming motionless, although other strategies are also occasionally observed (such as wriggling away) (Scott & Murdoch, 1983; Sih, 1979). *C. pipiens* larvae are apparently able to detect chemicals released by conspecifics which have been preyed upon by backswimmers, and adjust their behaviour to reduce the predation risk by choosing a less risky microhabitat (a vegetation refugium, the edge of the breeding site, etc.) and moving less (Sih, 1986). Similarly, prey motion is reduced following the introduction of a dytiscid (Kruuk & Gilchrist, 1997). This behavioural change is probably an adaptation to escape predators using motion and/or vibration to detect and locate their prey.

The higher predation cost inflicted by three larval predators could be explained if resistant larvae are more active, and thus are detected more frequently by the predator. Another possibility is that resistant larvae are not changing their microhabitat and/or their moving frequency after conspecifics have started to be preyed upon, unlike susceptible individuals. SR larvae display a distinct feeding behaviour, as they replace their gut contents at a faster rate than the other strains (Agnew *et al.*, 2004). This is consistent with the former hypothesis (resistant larvae are more active), although a direct measurement is required to confirm this. The absence of predation cost in the presence of the water boatman is surprising, and suggests that its hunting technique is different. The identification of this difference could potentially shed some light on the modified behaviour of resistant larvae.

The pholcid spider's principal means of capturing prey is to throw silk with the aid of its hind legs. This method is used to immobilize mosquitoes which are entangled in the standing web, or to catch flying mosquitoes directly (Strickman *et al.*, 1997; Déom, 1990). Once a mosquito has been in contact with the web, it could escape a spider attack. Apparently, mosquitoes possessing *Ester*<sup>1</sup> or *Ester*<sup>4</sup> have a higher predation probability (Table 1), suggesting that they are either more active (thus with a higher probability of flying near the web or the spider), or have fewer chances to escape an attack by *H. pluchei*. However, possessing *ace-1*<sup>R</sup> does not seem to affect predation probability. There are several physiological differences between susceptible and resistant mosquitoes. For example, susceptible adults live longer (Agnew *et al.*, 2004), and have a lower density of endocellular *Wolbachia* (Berticat *et al.*, 2002b). *Wolbachia* affect

locomotive performance, at least in a parasitic wasp (Fleury *et al.*, 2000), and thus may represent a causal link between the effect of a resistant gene and the predation cost. Further experiments, using aposymbiotic strains, could settle this issue.

#### (ii) Variability of the predation cost

All the resistance genes studied present a predation cost relative to susceptible ones, at either the larval or adult stage, or both.

For the *ace-1* locus, the predation cost of the resistance alleles seems to be restricted to the larval stage: spiders seem to capture susceptible and resistant adult mosquitoes equally. This indicates that the high survival cost associated with the *ace-1*<sup>R</sup> gene during the overwintering period (Chevillon *et al.*, 1997; Gazave *et al.*, 2001), could not be attributed to pholcid predation. However, it is still possible that other spider species use distinct cues or use different catching techniques which are more discriminatory towards the behavioural changes between mosquitoes resistant and susceptible at the *ace-1* locus. It is also possible that the predation cost is only apparent in female mosquitoes (which were not used in the experiments), as only females overwinter in caves. Only empirical data using the most common spider predators in local caves (*Meta bourneti* (Simon, 1922), *Tegenaria parietina* (Fourcroy, 1785), *Pholcus phalangioides* (Fuesslin, 1775)) could settle this point. The first two species have already been observed catching hibernating *C. pipiens* (M. Michaud, personal communication), although no quantitative data are yet available.

As regards the *Ester* locus, the allele *Ester*<sup>4</sup> displays a predation cost in both larvae and adults, although *Ester*<sup>1</sup> induces a cost only in adults. This absence of predation cost in larvae must be considered with caution, as the procedure used was very conservative: it could be safely concluded only that the predation cost of *Ester*<sup>1</sup> in larvae is not significantly higher than that induced by the staining procedure in susceptible individuals.

There is one example of transitivity for predation preferences (e.g. if the preference is ranked as  $A < B$  and  $B < C$ , then  $A < C$ ): adults with *Ester*<sup>1</sup> or *Ester*<sup>4</sup> are equally more preyed upon than susceptible mosquitoes ( $\beta = 0.67$  and  $0.64$ , respectively), and thus adults with *Ester*<sup>1</sup> or *Ester*<sup>4</sup> are equally preferred when they are presented together to the predator ( $\beta$  values not different from  $0.5$ ). However, this transitivity is not always observed: for example, larvae with *Ester*<sup>4</sup> or *ace-1*<sup>R</sup> are approximately equally preferred to susceptible mosquitoes ( $\beta = 0.63$  and  $0.65$ – $0.71$ , respectively), although larvae with *ace-1*<sup>R</sup> are strongly preferred when the alternative is larvae with *Ester*<sup>4</sup> ( $\beta = 0.86$ ). The other possible example of non-transitivity

in larval predation, involving individuals with *Ester*<sup>1</sup>, *ace-1<sup>R</sup>* and susceptible, is not conclusive because  $\beta$  for the pair SA1/S-LAB is probably underestimated (see Section 3). The non-transitivity observed for both larval and adult predation suggests that several phenotypic traits of the prey are affected by the resistance genes, and that the predator uses these cues differently according to environmental conditions.

In conclusion, predators seem to be useful tools to detect behavioural changes that are caused by these genes of recent origin. There is a large variety of potential predators for any given insect species, each with its own detection method, stimulus type and capture strategy (Lima & Dill, 1990). It is likely that any phenotypic variation will result in differential predation for at least one type of predator. We suggest that predators, which are designed by natural selection to detect specific behavioural phenotypes, are useful tools to explore non-obvious differences between two classes of individuals, for example when they differ by the presence or absence of a gene such as insecticide resistance.

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