# Energetic Cost of Insecticide Resistance in *Culex pipiens* Mosquitoes

A. RIVERO,[1] A. MAGAUD, A. NICOT, AND J. VÉZILIER

Génétique et Evolution des Maladies Infectieuses, CNRS, UMR 2724, Centre de Recherche IRD, 911 Avenue Agropolis, 34394 Montpellier, France

J. Med. Entomol. 48(3): 694–700 (2011); DOI: 10.1603/ME10121

**ABSTRACT** The extensive use of insecticides to control vector populations has lead to the widespread development of different mechanisms of insecticide resistance. Mutations that confer insecticide resistance are often associated to fitness costs that prevent them from spreading to fixation. In vectors, such fitness costs include reductions in preimaginal survival, adult size, longevity, and fecundity. The most commonly invoked explanation for the nature of such pleiotropic effects of insecticide resistance is the existence of resource-based trade-offs. According to this hypothesis, insecticide resistance would deplete the energetic stores of vectors, reducing the energy available for other biological functions and generating trade-offs between insecticide resistance and key life history traits. Here we test this hypothesis by quantifying the energetic resources (lipids, glycogen, and glucose) of larvae and adult females of the mosquito *Culex pipiens* L. resistant to insecticides through two different mechanisms: esterase overproduction and acetylcholinesterase modification. We find that, as expected from trade-off theory, insecticide resistant mosquitoes through the overproduction of esterases contain on average 30% less energetic reserves than their susceptible counterparts. Acetylcholinesterase-modified mosquitoes, however, also showed a significant reduction in energetic resources (20% less). We suggest that, in acetylcholinesterase-modified mosquitoes, resource depletion may not be the result of resource-based trade-offs but a consequence of the hyperactivation of the nervous system. We argue that these results not only provide a mechanistic explanation for the negative pleiotropic effects of insecticide resistance on mosquito life history traits but also can have a direct effect on the development of parasites that depend on the vector's energetic reserves to fulfil their own metabolic needs.

**KEY WORDS** life history trade-offs, carboxylesterase, acetylcholinesterase, glycogen, lipids

Insecticide resistance is one of the largest hurdles in the fight against vector-transmitted diseases. Broadly, two main mechanisms of insecticide resistance have been described: metabolic resistance (the overproduction of specific enzymes, through gene amplification or gene expression, that sequester, metabolize, or detoxify the insecticide; Hemingway and Karunaratne 1998) and target site resistance (through point mutations that render the neural targets of the insecticide less sensitive to the active ingredient; Weill et al. 2003). The insecticide resistance alleles underlying these two insecticide resistance mechanisms confer a large fitness benefit to the bearer and have been shown to rapidly increase in frequency after the onset of insecticide treatment programs (Guillemaud et al. 1998, Hartley et al. 2006). However, in the absence of insecticide, reduced fitness of resistant insects is frequently reported, with the consequent reduction (and eventually disappearance) of resistant allele frequencies (Lenormand et al. 1999).

In insects, the fitness costs of resistance are thought to be the result of pleiotropic effects of the insecticide resistant genes (or of genes closely linked with them as a result of hitchhiking) and include decreases in preimaginal survival (Berticat et al. 2008, Djogbenou et al. 2010), adult longevity (Boivin et al. 2001, Agnew et al. 2004), fecundity (Duron et al. 2006, Foster et al. 2007), and increased predation risk (Berticat et al. 2004, Foster et al. 2007). Resource-based trade-offs are often invoked as the proximate pleiotropic mechanism underlying these costs (Roush and McKenzie 1987, Chevillon et al. 1997, Foster et al. 2003, Agnew et al. 2004). In particular, the production of large amounts of detoxifying enzymes (up to 50 times more in some cases; Raymond et al. 2001) is widely assumed to deplete the energetic stores of insects, reducing the energy available for other biological functions and generating energetic trade-offs between insecticide resistance and key life history traits (Roush and McKenzie 1987). In some insecticide resistant insects, these overproduced detoxifying enzymes can represent up to 3% of the total body proteins (Devonshire and Moores 1982). Lipids are likely victims of this large overinvestment in proteins, because they are an important source of the acetyl groups needed to synthe-

[1] Corresponding author, e-mail: rivero@mpl.ird.fr.

size the enzymes' constitutive amino acids (Rivero et al. 2010).

We set up to test this assumption, namely, that insecticide resistant vectors contain less energetic resources than their susceptible counterparts. A reduction of energetic resources may have drastic consequences for parasite transmission by altering several of the parameters that determine vector competence (Rivero et al. 2010). For this purpose, we carried out an energetic analysis in the mosquito *Culex pipiens* L., a species where the fitness costs of insecticide resistance have been particularly well investigated, both in the field (Chevillon et al. 1997, Gazave et al. 2001) and in the laboratory (Berticat et al. 2002; Agnew et al. 2004; Berticat et al. 2004, 2008). *Culex* mosquitoes are important vectors of diseases such as filariasis, West Nile, and Japanese encephalitis. The mechanisms of insecticide resistance found in *Cx. pipiens*, esterase overproduction and acetylcholinesterase modification, also have been found in other important mosquito vectors such as *Anopheles* and *Aedes* (Weill et al. 2003, Hemingway et al. 2004). We compared four different isogenic strains: a fully susceptible strain (SLAB), two strains resistant to insecticides through the overproduction of esterases (SB1 and SA4B4), and one strain with an insensitive acetylcholinesterase but no overproduced esterases (SR). We predict that 1) insecticide resistant strains should contain lower energetic reserves than their susceptible counterparts and 2) this effect should be more marked in SA4B4 and SB1 strains as a result of the reallocation of resources toward esterase overproduction.

## Materials and Methods

**Mosquitoes.** The four different isogenic strains used in the experiment (SLAB, SB1, SA4B4, and SR) shared the same genetic background and only differed by their genotype at the *Ester* (esterase over-production) or the *ace-1* (insensitive acetylcholinesterase) loci (Table 1). These strains were created several years ago by a series 14–15 generations of backcrossing and selection at the Institute des Sciences de l'Evolution de Montpellier (ISEM), Montpellier, France (see Berticat et al. 2002 for details) and have been since kept under identical conditions. Synchronized *Cx. pipiens* mosquito egg cohorts from the four different strains were obtained from the ISEM and placed under our standard insectary conditions (25 ± 1°C, 75 ± 5% RH, and a photoperiod of 12:12 [L:D] h). On hatching day (day 0), larvae ($n$ = 95 for SLAB, SA4B4, and SR; $n$ = 110 for SB1) were individually transferred into tubes (90 by 20 mm) containing 5 ml of mineral water (Eau de Source, Carrefour, Montpellier, France) in which 2.2 mg of fish food (Tetramin, Melle, Germany) had been dissolved. Tubes were individually labeled and randomly allocated a place in the tube racks. On day 5, 28 larvae from each genotype were randomly sampled, dried on a sheet of absorbent laboratory paper, weighed (Mettler Toledo MX 5 microbalance, Mettler-Toledo Inc., Columbus, OH), and frozen at −80°C for the energetic analysis. At this stage mosquito larvae are at their fourth and final larval stage before pupation. The rest of the larvae were left on the tubes until the emergence of the adult.

**Table 1. Isogenic insecticide resistant and susceptible strains used in the experiment**

| Strain | IR mechanism | Alleles | Genetic background |
|---|---|---|---|
| SLAB | None | $Ester^0$, $ace\text{-}1^S$ | SLAB |
| SB1 | Overproduction esterase B1 | $Ester^{B1}$, $ace\text{-}1^S$ | SLAB |
| SA4B4 | Overproduction esterases A4 and B4 | $Ester^4$, $ace\text{-}1^S$ | SLAB |
| SR | Insensitive acetylcholinesterase | $Ester^0$, $ace\text{-}1^R$ | SLAB |

The overproduction of esterases is controlled by a superlocus consisting of two loci (*esterase A* and *esterase B*) in complete linkage disequilibrium (Rooker et al. 1996). Alleles for this locus are the wild-type susceptible $Ester^0$ or the insecticide resistant $Ester^{1B}$ (overproduces the esterase B1 isozyme) and $Ester^4$ (overproduces the esterase 4 isozyme; Raymond et al. 1998). The modification of the acetylcholinesterase is controlled by the locus *ace*-1. Alleles for this locus are the wild-type susceptible $ace\text{-}1^S$ and the insecticide resistant $ace\text{-}1^R$ (which contains a single GGC→AGC point mutation that renders the acetylcholinesterase insensitive to the insecticide (Weill et al. 2003, 2004).

On the day of emergence (days 9 and 10), a random sample of the adult females ($n$ = 20 for SLAB, SA4B4, and SR; $n$ = 13 for SB1) was collected, weighed (as above), and frozen at −80°C for energetic analysis. The percentage of larvae that successfully completed their development to the adult stage ranged between 75 and 80%, except for the SB1 strain where it was 54%. The rest of the larvae either died before pupation or, in the case of SB1, failed to pupate (they were still larvae on day 12). The sex ratio (% males) ranged between 45 and 50%, except for SB1 where it was 70%. These two facts explain why we could only obtain 13 SB1 females for the adult energetic analysis, despite a higher initial replication number (see above).

In a separate experiment, an additional set of SLAB, SB1, and SA4B4 of eggs were set up to emerge under identical experimental conditions. The individuals were sampled either on day 5 (L4 larvae, $n$ = 30 of each strain) or on day 9 (newly emerged females, $n$ = 25 of each strain). These individuals were used to quantify the activity of the esterases (see below).

**Energetic Resources.** The three main energetic resources of mosquitoes (lipids, glycogen, and sugars) were quantified in larvae and newly emerged females using colorimetric techniques modified from van Handel (1988) as described in previous studies (Rivero and Ferguson 2003, Rivero et al. 2007). The energetic value of glycogen and sugars was calculated as 16.74 $J.mg^{-1}$, and that of lipids as 37.65 $J.mg^{-1}$ (Clements 1992). Analyses were done both on total energetic reserves (sum of lipids, glycogen, and sugars) and separately for each of the resources by correcting the energetic value of the individual by their body weight (also called "size-specific caloric content"; Timmermann and Briegel 1999).

**Esterase Activity.** Nonspecific esterase activity was quantified in single mosquitoes, by using colorimetric

**Table 2. Results of the generalized linear models for the effects of strain (SLAB, SB1, SA4B4, and SR) and stage (larva and adult) on the amount of lipids, glycogen, sugars, and total energy content per mg body wt in *Cx. pipiens***

| | Lipids | Glycogen | Sugars | Energy |
|---|---|---|---|---|
| Strain | $F_{3,177} = 3.23$; $P = 0.023$ | $F_{3,177} = 1.23$; $P = 0.29$ | $F_{3,177} = 3.77$; $P = 0.011$ | $F_{3,177} = 3.66$; $P = 0.014$ |
| Stage | $F_{1,177} = 37.19$; $P < 0.0001$ | $F_{1,177} = 23.19$; $P < 0.0001$ | $F_{1,177} = 8.01$; $P = 0.005$ | $F_{1,177} = 48.65$; $P < 0.0001$ |
| Strain × stage | $F_{3,177} = 1.48$; $P = 0.22$ | $F_{3,177} = 0.63$; $P = 0.59$ | $F_{3,177} = 0.07$; $P = 0.97$ | $F_{3,177} = 1.27$; $P = 0.28$ |

techniques as described in Guillemaud et al. (1999) and Martin et al. (2002). In brief, 10 $\mu$l of a centrifuged supernatant of each insect was incubated with 90 $\mu$l of phosphate buffer, pH 6.5, in a microplate well for 10 min at room temperature. Then, 100 $\mu$l of a solution containing 3 ml of phosphate buffer, 8.4 ml of distilled water, and 600 $\mu$l of $\alpha$-naphthyl acetate (0.3 M), was added to the wells, and the mixture was incubated for 30 min at room temperature. The reaction was stopped by addition of 100 $\mu$l of a 0.8 mg/liter Garnet salt solution. After a further 10-min incubation time, the microplates were read against a blank at 550 nm, and the amount of $\alpha$-naphthol produced was estimated from a calibration curve. The total amount of proteins was obtained by mixing 10 $\mu$l of the centrifuged supernatant with 145 $\mu$l of Coomassie Plus protein assay reagent and 145 $\mu$l of distilled water. The mixture was incubated at room temperature for 5 min, and the microplates were read against a blank at 590 nm. The esterase activities are reported as micromoles of $\alpha$-naphthol produced per minute per milligram of protein.

**Statistical Analysis.** Statistical analysis was carried out using generalized linear modeling techniques available in the JMP 7.0.1 statistical package (SAS Institute, Cary, NC), by using mosquito stage (larva and adult) and strain (SLAB, SB1, SA4B4, and SR) as fixed explanatory variables. For the analysis of esterase activity, the microplate effect was added as a random factor. The analyses were carried out by building up a full model containing all potential explanatory variables and their interactions. The significance of each element was assessed by removing it from the model and analyzing the resulting change in deviance (Crawley 2007). The significant values given in the text are for the minimal model (the model containing only significant terms and interactions), whereas nonsignificant values are those obtained before the deletion of the variable from the model. Due to the nonnormality of errors, the duration of metamorphosis was analyzed using the nonparametric Kruskal–Wallis test, also available in JMP.

## Results and Discussion

The amount of energetic resources depended strongly on both the stage and the strain of the individuals (Table 2). On average, recently emerged adult females had significantly less lipids (−27%), glycogen (−39%), glucose (−63%), and consequently, also less total energetic reserves (−30%) per milligram of body weight than larvae. Lipids, glycogen, and sugars have been reported previously as being fuels for metamorphosis in several holometabolous insects (Wheeler and Buck 1992, Odell 1998, Reim et al. 2009), including mosquitoes (Lang 1963, Nishiura et al. 2007). The strain effect came through insecticide resistant mosquitoes, and in particular SA4B4 mosquitoes, having significantly less lipids, sugars, and consequently also less total energetic reserves than insecticide susceptible mosquitoes. This strain effect spanned across the larval and adult stages (i.e., there was no significant strain × stage interaction; Table 2), although visual inspection of the data (Fig. 1) suggested that it was stronger in the adult stage. To confirm this observation, we carried out separate analyses for the larval and adult stages. As expected, the trend toward lower energetic resources in insecticide resistant mosquitoes was not significant in larvae (lipids: $F_{3,108} = 0.25$, $P = 0.85$; glycogen: $F_{3,108} = 1.22$, $P = 0.30$; sugars: $F_{3,108} = 1.63$, $P = 0.85$; and overall energetic resources: $F_{3,108} = 0.25$, $P = 0.85$) but was highly significant in adults. In particular, insecticide resistant SA4B4 and SR females had significantly less energetic resources ($F_{3,69} = 5.69$; $P = 0.001$) than susceptible (SLAB) females or females of the insecticide resistant SB1 strain (−30 and −20% for the comparison SA4B4-SLAB and SR-SLAB, respectively). This reduction is due to the combined effect of a lipid ($F_{3,69} = 4.41$; $P = 0.007$) and a sugar depletion ($F_{3,69} = 4.78$; $P = 0.004$), although the effect is only statistically significant for SA4B4 females (Tukey's honestly significant difference [HSD] test; see Fig. 1). The amount of adult glycogen did not contribute to explain the differences between the strains ($F_{3,69} = 1.23$; $P = 0.30$).

The energetic costs of insecticide resistance thus arise only in adults, a result that may seem surprising given that larvae had a higher esterase activity than adults (0.59 ± 0.04 and 0.44 ± 0.05 $\mu$mol/min/mg protein, respectively; $F_{1,164} = 154$, $P < 0.0001$) (Fig. 2). A post hoc power analysis (Jennions and Moller 2003) revealed that the statistical power of the experiment was sufficiently large to have detected a significant result in the larvae equivalent to that found in adults, had there been one (power = 0.89, $\alpha = 0.05$). The nonsignificance of the results is therefore biologically relevant and not simply the consequence of insufficient replication. The most likely explanation for these results is, in our opinion, that the amount of food provided to the larvae was sufficiently abundant to compensate for the costs associated to the deployment of insecticide resistance and that, as a result, the costs of insecticide resistance were only paid once the larvae stopped eating and entered metamorphosis. *Cx. pipiens* spend on average 48 h on the pupal stage, irrespective of the strain (Kruskal–Wallis test $\chi^2_3 =$

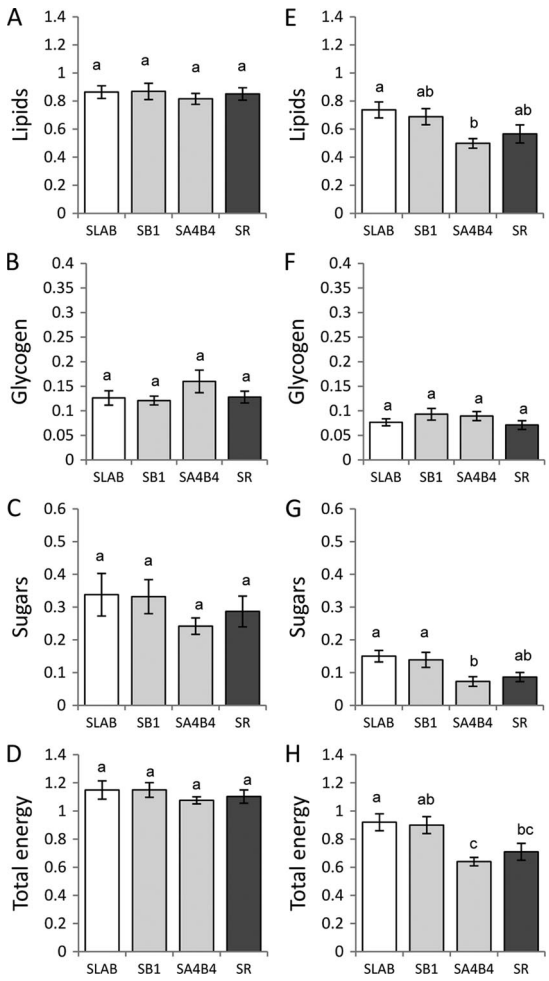


**Fig. 1.** Amount (mean ± SE) of lipids, glycogen, sugars, and total energetic resources ($J.mg^{-1}$ body weight) in larvae (A–D) and adults (E–H) of insecticide susceptible (SLAB) and resistant (SB1, SA4B4, and SR) *Cx. pipiens* strains. Strains not connected by the same letter are significantly different ($\alpha = 0.05$; Tukey's HSD test).

3.72, $P = 0.29$) during which time they do not feed. Our results therefore suggest that the energetic costs of metamorphosis are higher for insecticide resistant strains and that this effect is stronger in SA4B4 females that are resistant to insecticides through the overproduction of esterases.

As expected, the activity of nonspecific esterases was significantly higher in SA4B4 (mean ± SE, 0.77 ± 0.05 μmol/min/mg protein) and SB1 individuals (0.68 ± 0.05 μmol/min/mg protein) than in SLAB individuals (0.10 ± 0.05 μmol/min/mg protein; $F_{2,164} = 154$, $P < 0.0001$) (Fig. 2). There were, however, no significant differences in activity between SA4B4 and SB1 individuals ($\alpha = 0.05$, Tukey's HSD test). If resource based trade-offs are acting on SA4B4 females, why is this effect not found in SB1 females given that esterase levels were similar in both strains? One potential explanation is that, for reasons unknown, the conditions under which the larvae were reared imposed a strong selection on SB1 female larvae as only a small proportion of them made it through to the adult stage (see Materials and Methods). The energetic analysis was therefore carried out in a small subsample of the females that managed to pass this developmental filter and thus does not reflect the population as a whole.

In *Cx. pipiens*, the modification of the acetylcholinesterase reduces the activity of the enzyme resulting in an excess of acetylcholine in the synapses and in a hyperactivity of the nervous system (Bourguet et al. 1997). This hyperactivity is likely to underlie the behavioral and developmental alterations observed in this species (Berticat et al. 2004, 2008) but was hitherto not thought to have any resource-based costs (Chevillon et al. 1997, Raymond et al. 2001, Djogbenou et al. 2010). Our results contradict this assumption: acetylcholinesterase-modified (SR) females contained significantly less metabolic resources than insecticide-susceptible (SLAB) females (Fig. 1). This reduction is likely to be a direct consequence of the hyperactivity of the nervous system. Indeed, recent work carried out in *Drosophila* has shown that a hyperactivation of the nervous system results in a depletion of fat stores by increasing metabolic rate and decreasing fatty acid synthesis (Al-Anzi et al. 2009). In our results, the decrease in overall energetic reserves arises through the combined effects of a decrease in lipids and sugars (Fig. 1), but the underlying mechanism remains to be established (work is in progress to quantify the activity patterns and metabolic rates of SR mosquitoes).

Hardstone et al. (2010) also found significant reductions of energetic resources in *Cx. pipiens* resistant to insecticides through a different (P450) detoxification mechanism, although the interpretation of their results was complicated by the different geographical origins of the insecticide-resistant and susceptible strains compared (see Rivero et al. 2010 for why this is a problem). Such a decrease in energetic resources associated to insecticide resistance can have drastic consequences for the vectorial capacity of female mosquitoes (Rivero et al. 2010). Lipids are the main fuel for insect survival (Clements 1999), and insecticide resistant *Cx. pipiens* have been shown to have significantly shorter life spans than their susceptible counterparts (Agnew et al. 2004). Longevity is, arguably, the most important parameter in disease transmission as it increases the potential for infective bites to hosts and is particularly important for parasites that have a long incubation period in the vector (Rivero et al. 2010). Carbohydrates, such as sugars and glycogen, are the fuels for mosquito flight and distance flown by mosquitoes has been shown to be dependent on sugar availability (Clements 1999). A decrease in the energetic reserves also may alter mosquito behavior by switching the feeding preference of vectors away from hosts. In *Aedes aegypti* (L.) and *Cx. nigripalpus* Theobald, for example, resource deprivation renders mosquitoes more responsive to sugar-rich odors such as honey and less responsive to host odors (Clements

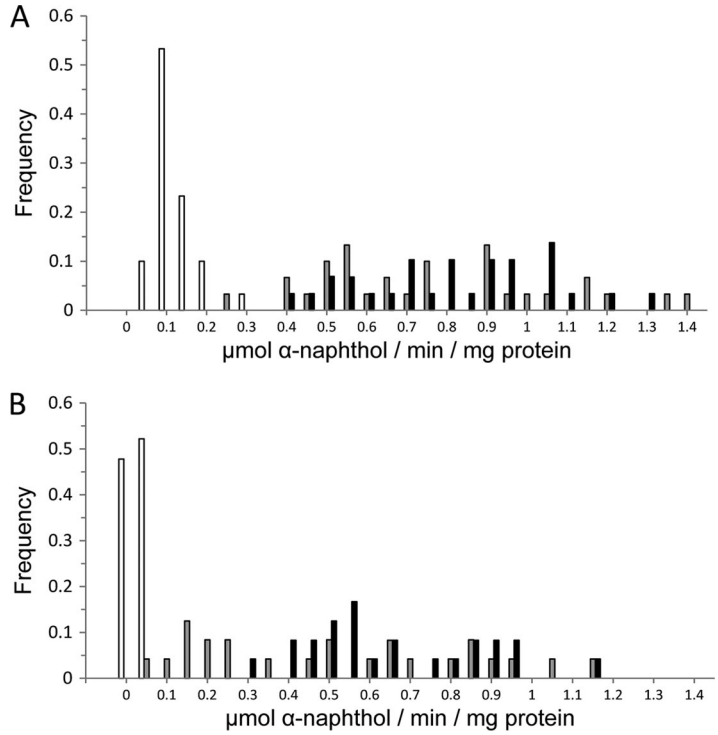


**Fig. 2.** Nonspecific esterase activities in larvae (A) and adults (B) of the SLAB (white bars), SB1 (gray bars), and SA4B4 (black bars) *Cx. pipiens* strains. Mean and SE values are given in the text.

1999). Indeed, insecticide resistant mosquitoes may thus be able to compensate for the low teneral reserves by feeding on sugar sources if these are available. Aside from the effects on mosquito life history traits of key importance for transmission, a reduction of metabolic resources may also have a direct effect on parasite development in at least two different ways. First, by interfering with the ability of mosquitoes to mount an immune response. There is indeed plenty of evidence that there are significant resource costs involved in the deployment and maintenance of the immune system (reviewed in Povey et al. 2009). Energetically deficient female mosquitoes may be less immunocompetent, therefore favoring the development of parasites. Quite surprisingly, however, to our knowledge no study has explicitly investigated the effects of insecticide resistance on mosquito immunity. Second, by limiting the development of parasites that depend on the hosts energetic resources to fulfill their own metabolic needs. Several studies have shown that parasites within mosquitoes have high carbohydrate and lipid demands (Atella et al. 2006, Rivero et al. 2007, Samsa et al. 2009). In addition, there is ample evidence that parasite production is positively correlated with resource availability in several invertebrate species (Bedhomme et al. 2004, Pulkkinen and Ebert 2004). In these systems, the reduction of energetic resources is likely to impair the ability of parasites to develop inside the vectors.

To conclude, our results could extend to other vector species (such as *Anopheles gambiae* Giles, where the acetylcholinesterase-based resistance is identical to that of *Cx. pipiens;* Weill et al. 2003) and insecticide resistance mechanisms, particularly those based on the overproduction of detoxifying enzymes (such as glutathione S-transferases; Hemingway et al. 1998) with potentially important consequences for the vectorial capacity of these insects.

## Acknowledgments

We thank Mylène Weill's laboratory for providing the mosquito isogenic lines; Fabrice Chandre for the esterase activity protocol; and Stephane Cornet, Sylvain Gandon, and Nicole Pasteur for useful discussions on the subject. A.R. is funded through a project grant from the Agence Nationale de la Recherche (ANR, France) and J.V. through a Graduate Program in Areas of Basic and Applied Biology (GABBA, Portugal).

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*Received 10 May 2010; accepted 31 October 2010.*