

HIGH *WOLBACHIA* DENSITY CORRELATES WITH COST OF INFECTION FOR INSECTICIDE RESISTANT *CULEX PIPPIENS* MOSQUITOES

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Abstract.—In the mosquito *Culex pipiens*, insecticide resistance genes alter many life-history traits and incur a fitness cost. Resistance to organophosphate insecticides involves two loci, with each locus coding for a different mechanism of resistance (degradation vs. insensitivity to insecticides). The density of intracellular *Wolbachia* bacteria has been found to be higher in resistant mosquitoes, regardless of the mechanism involved. To discriminate between costs of resistance due to resistance genes from those associated with elevated *Wolbachia* densities, we compared strains of mosquito sharing the same genetic background but differing in their resistance alleles and *Wolbachia* infection status. Life-history traits measured included strength of insecticide resistance, larval mortality, adult female size, fecundity, predation avoidance, mating competition, and strength of cytoplasmic incompatibility (CI). We found that: (1) when *Wolbachia* are removed, insecticide resistance genes still affect some life-history traits; (2) *Wolbachia* are capable of modifying the cost of resistance; (3) the cost of *Wolbachia* infections increases with their density; (4) different interactions occurred depending on the resistance alleles involved; and (5) high densities of *Wolbachia* do not increase the strength of CI or maternal transmission efficiency relative to low *Wolbachia* densities. Insecticide resistance genes generated variation in the costs of *Wolbachia* infections and provided an interesting opportunity to study how these costs evolve, a process generally operating when *Wolbachia* colonizes a new host.

Key words.—Bacterial density, *Culex pipiens*, insecticide resistance gene, *Wolbachia*.

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Fisher (1958; pp. 41–44) predicted that mutations favoring adaptation to a new environment would be at a selective disadvantage in the previous environment. This is because they generally cause resource reallocation and metabolic or developmental processes to be affected, leading to reduced performance in some traits and a cost to overall fitness (Uyenoyama 1986; Roush and McKenzie 1987; Bergelson and Purington 1996; Davies et al. 1996; Coustau et al. 2000; Levin 2000). Few situations exist in which 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. Thirty years of organophosphorous (OP) insecticide use to control *Culex pipiens* has selected for various resistance alleles in this mosquito (Raymond et al. 2001; Weill et al. 2005). Monitoring of natural populations indicates that resistance genes spread and increase in frequency in treated areas and are strongly selected against in nontreated areas (Lenormand et al. 1999). This decrease in the nontreated areas reflects a substantial fitness cost, as predicted by Fisher. Numerous life-history traits are modified in insecticide resistant individuals, including increased larval development time, reduced predation avoidance, reduced male reproductive success, and lower female overwintering survival (Gazave et al. 2001; Berticat et al. 2002a, 2004; Bourguet et al. 2004), consistent with high fitness costs measured in natural populations (Lenormand et al. 1999).

Organophosphorous insecticides inhibit acetylcholinesterase (AChE) in the central nervous system, leading to death. The genetic basis of OP resistance involves two loci, both displaying major resistance alleles, the super-locus *Ester* and the locus *ace-1* (Raymond et al. 2001). The resistance con-

ferred by *Ester* results from increased detoxification through overproduction of esterases that degrade OPs. This overproduction is the result of two nonexclusive processes: gene amplification and changes in gene regulation (Raymond et al. 1998). Overproduced esterases account for up to 12% of the soluble proteins of insecticide resistant individuals, and are far less present in susceptible mosquitoes (Fournier et al. 1987). The metabolic cost of this overproduction varies depending on the *Ester* alleles involved (Berticat et al. 2002a). Resistance allele *ace-1^R* codes for a mutated AChE1 (Weill et al. 2003) that is less inhibited by OP, but which is associated with a 60% reduction in activity compared to the susceptible enzyme (Bourguet et al. 1997). Thus, modifications of AChE1 seem to alter the optimal functioning of cholinergic synapses of the central nervous system.

Culex pipiens is naturally infected by *Wolbachia*, a maternally inherited intracellular bacterium, that is widespread in arthropods and filarial parasitic nematodes (Werren 1997; Stouthamer et al. 1999). It is generally accepted that vertically transmitted microorganisms should tend to evolve towards a benign state, even bringing benefits to their hosts, because their fitness is linked to host fitness (Ebert and Herre 1996). These relations can develop towards a mutual interdependence when symbionts provide a novel function that enhances host fitness (Maynard-Smith 1989). Symbiosis will last only as long as the benefit of the interaction outweighs its cost for each partner (Bronstein 1994). Although *Wolbachia* seem to follow this type of evolutionary behavior in filarial nematodes (Hoerauf et al. 1999, 2001), they have evolved several different ways of manipulating the reproduction of arthropod hosts to their own advantage (Rousset and Raymond 1991; Werren 1997; Stouthamer et al. 1999). These manipulations allow *Wolbachia* to persist in host populations without being constrained to bring them a fitness advantage.

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TABLE 1. Resistance genes and infection status of strains used. Insecticide resistance alleles are in bold. *Wolbachia* density corresponds to the number of *Wolbachia* genomes relative to *Culex* genomes, adapted from Berticat et al. (2002b). Standard deviations in parentheses.

Strains	<i>Ester</i> allele	<i>ace-1</i> allele	<i>Wolbachia</i> densities		
			Larvae 4th instar	Males 5-days-old	Females 5-days-old
SLAB	<i>Ester</i> ⁰	<i>ace-1</i> ^S	0.31 (0.26)	0.25 (0.36)	1.62 (0.52)
SLABTC	<i>Ester</i> ⁰	<i>ace-1</i> ^S	—	—	—
SA1	<i>Ester</i>¹	<i>ace-1</i> ^S	1.83 (1.74)	3.29 (1.74)	4.47 (3.51)
SA1TC	<i>Ester</i>¹	<i>ace-1</i> ^S	—	—	—
SA2	<i>Ester</i>²	<i>ace-1</i> ^S	0.92 (0.89)	0.86 (1.45)	3.39 (1.76)
SA2TC	<i>Ester</i>²	<i>ace-1</i> ^S	—	—	—
SA4	<i>Ester</i>⁴	<i>ace-1</i> ^S	0.86 (0.85)	0.91 (1.75)	3.73 (2.48)
SA4TC	<i>Ester</i>⁴	<i>ace-1</i> ^S	—	—	—
SR	<i>Ester</i> ⁰	<i>ace-1</i>^R	1.47 (1.26)	2.46 (6.89)	3.43 (1.34)
SRTC	<i>Ester</i> ⁰	<i>ace-1</i>^R	—	—	—

The most frequent manipulation is cytoplasmic incompatibility (CI) leading to early embryo death in crosses between infected males and uninfected females, as well as in some crosses between individuals infected by incompatible *Wolbachia* strains. Thus, in a mixed population with infected and uninfected hosts, females carrying a *Wolbachia*-free cytoplasm are at a reproductive disadvantage when they copulate with infected males; this facilitates the spread and fixation of *Wolbachia* infections (Rousset and Raymond 1991).

Insecticide resistance has been reported to influence the outcomes of interactions with organisms inhabiting mosquitoes (McCarroll et al. 2000; Agnew et al. 2004). *Wolbachia* density is strongly increased by the presence of insecticide resistance genes in both laboratory strains and natural populations (Berticat et al. 2002b). Increased bacterial density is observed for the two distinct resistance mechanisms to OP insecticides (i.e., increased detoxification and target insensitivity), suggesting that perturbations of host physiology at a global level induce a higher susceptibility to *Wolbachia*. Several factors affected by bacterial density may influence the spread of *Wolbachia* infection. *Wolbachia* density must be sufficiently high to induce an efficient level of CI and to ensure efficient transovarial transmission, while being low enough to avoid host disease (Werren 1997). Artificial selection for increased *Wolbachia* densities and transinfection experiments suggest that density is controlled by a complex interplay of host and bacterial factors (Boyle et al. 1993; Poinot et al. 1998; McGraw et al. 2002). It can therefore be expected that *Wolbachia* would adjust their densities to balance the cost of infection and the efficiency of maternal transmission. According to this view, recent *Wolbachia*-host associations are likely to be poorly adapted relative to older ones (McGraw and O'Neill 1999).

The main aim of this study was to determine the relationship between *Wolbachia* density and fitness cost of infection, considering host genetic variants (i.e., susceptible and resistant alleles). Because insecticide resistance appeared recently, resistant mosquitoes offer a new physiological environment to *Wolbachia*, in which bacterial responses could be less well adapted relative to an ancestral host (i.e., insecticide susceptible mosquitoes). We tested this hypothesis within the association *C. pipiens*-*Wolbachia*. We measured several life-history traits, including insecticide resistance level, larval mortality, female size, fecundity, predation avoidance, and mating competition, considering both host genotype and *Wol-*

bachia infection. Crossing experiments were performed to determine the effect of bacterial density on CI intensity.

MATERIAL AND METHODS

Mosquito Strains

Five strains were used: the insecticide susceptible strain SLAB (Georghiou et al. 1966), and four insecticide resistant strains, SA1, SA2, SA4, and SR. These four strains share the same genetic background as SLAB (each was backcrossed for at least 14 generations with SLAB, for details see Berticat et al. 2002a), and possess the same SLAB cytoplasmic genome (including *Wolbachia*). These strains differ by the presence or absence of resistance alleles at two loci, *Ester* and *ace-1*, as detailed in Table 1. All strains are homozygous at both *Ester* and *ace-1* loci.

Wolbachia densities for each developmental stage and for the various strains were previously published by Berticat et al. (2002b). The original data, provided by C. Berticat, are reported in Table 1. During the experiments described below (that began approximately 12 generations after the measures of Berticat et al. 2002b), *Wolbachia* densities in insecticide resistant and susceptible strains were measured again and found to be similar (data not shown). For each strain, a sub-strain was derived, and cured of *Wolbachia* infection according to Portaro and Barr (1975). Larvae were reared in an antibiotic solution (tetracycline hydrochloride) for three generations (10^{-4} M for first generation, 2.10^{-4} M for second generation, and 5.10^{-4} M for third generation). The absence of *Wolbachia* was tested by PCR assay using *wsp* primers as described in Berticat et al. (2002b). *Wolbachia*-free substrains were named with the suffix TC. Thus, SLABTC, SA1TC, SA2TC, SA4TC, and SRTC are uninfected substrains derived from SLAB, SA1, SA2, SA4, and SR, respectively (Table 1).

To insure that life-history trait differences between infected and uninfected strains were not due to antibiotic toxicity, uninfected substrains were reared for at least two generations in standard laboratory conditions, without tetracycline, before beginning the experiments. All strains were reared in standard laboratory conditions within the same room. Individuals used in experiments were randomly sampled from several containers of a given strain, to minimize any eventual rearing bias.

TABLE 2. Effects of *Wolbachia* infection on preimaginal mortality according to host strain and experimental groups (A to G). Results are given for both sexes. Standard deviations in parentheses. All significant *P*-values are in bold and indicate a cost associated with *Wolbachia* infection.

Group	Strains	Number of replicates	Initial number (per replicate)	Mean number of adults per replicate (males)	<i>P</i> -value	Mean number of adults per replicate (females)	<i>P</i> -value	Pre-imaginal mortality rate	<i>P</i> -value
A	SLAB	7	500	196.6 (14.9)	0.74	191.7 (29.1)	0.67	0.22 (0.07)	0.54
	SLABTC	10	500	192.7 (11.9)		192.5 (14.6)		0.22 (0.03)	
B	SLAB	9	500	219.4 (39.5)	0.44	196.3 (44.4)	0.40	0.12 (0.02)	0.16
	SLABTC	10	500	232.5 (13.2)		207.1 (15.5)		0.12 (0.02)	
C	SA1	9	500	115.9 (28.8)	0.40	102.3 (19.6)	0.01	0.56 (0.09)	0.04
	SA1TC	10	500	123.9 (10.5)		124.8 (25.4)		0.50 (0.06)	
D	SA1	8	500	68.7 (24.2)	<10⁻²	76.0 (25.1)	0.02	0.71 (0.09)	<10⁻²
	SA1TC	4	500	113.5 (13.3)		110.5 (12.4)		0.55 (0.03)	
E	SR	10	500	159.8 (23.3)	0.80	164.3 (21.6)	0.03	0.35 (0.08)	0.28
	SRTC	10	500	160.4 (15.9)		183.5 (14.7)		0.31 (0.05)	
F	SR	10	500	143.5 (19.9)	0.07	154.3 (22.9)	0.09	0.40 (0.07)	0.04
	SRTC	10	500	165.6 (25.9)		175.7 (25.8)		0.32 (0.09)	
G	SLAB	8	100	42.1 (6.7)	0.38	39.2 (5.9)	0.23	0.19 (0.06)	0.72
	SLABTC	8	100	39.0 (4.8)		42.2 (3.1)		0.19 (0.05)	
G	SA1	7	100	11.3 (3.1)	<10⁻²	12.3 (3.3)	0.04	0.76 (0.05)	0.01
	SA1TC	8	100	17.4 (3.9)		14.1 (3.4)		0.68 (0.05)	
G	SA2	8	100	43.5 (6.4)	0.08	38.4 (7.8)	0.28	0.18 (0.09)	0.16
	SA2TC	8	100	47.9 (5.6)		37.7 (5.1)		0.14 (0.07)	
G	SA4	8	100	36.4 (5.4)	0.01	32.6 (4.5)	<10⁻²	0.31 (0.07)	<10⁻²
	SA4TC	8	100	43.6 (4.2)		43.6 (7.3)		0.13 (0.09)	
G	SR	8	100	20.7 (3.4)	<10⁻³	19.4 (5.0)	<10⁻²	0.60 (0.07)	<10⁻³
	SRTC	8	100	30.6 (4.3)		29.2 (3.9)		0.40 (0.05)	

Insecticide Resistance Analysis

Insecticide bioassays were performed on fourth instar larvae of the strains SA1, SA1TC, SR, and SRTC as described in Raymond and Marquine (1994). Four to five replicates of six insecticide concentrations (20 larvae per concentration), causing mortality between 0 and 100%, were done with chlorpyrifos (an organophosphate insecticide) for SA1, and propoxur (a carbamate insecticide) for SR. *Ester* resistance alleles allow resistance to chlorpyrifos (but not to propoxur) whereas *ace-1^R* displays a higher resistance to propoxur than chlorpyrifos.

The final concentration of solvent (ethanol) was systematically adjusted to 1% for standardization. Mortality data were analyzed using the log-Probit program of Raymond et al. (1993) based on Finney (1971). Mortality lines were considered identical when their parallelism was not rejected at the 0.05 probability level, and the 95% confidence limits of the resistance ratio (RR) between the LC₅₀s included the value 1. LC₅₀ corresponds to the lethal concentration that killed 50% of individuals.

Preimaginal Mortality

Mortality was measured for low and high larval density treatments. For high larval density, 500 first-instar larvae of a strain were transferred just after hatching to containers with 200 ml of water with 0.1 g of yeast food. The water and food of each container were changed daily from three days post-hatching and onwards. Preimaginal mortality was estimated by the difference between the initial number of larvae and the total number of emerging adults. For each adult, its sex was recorded. Four to 10 replicates were performed for each strain or substrain. For low larval density, 100 first-instar larvae were transferred just after hatching to containers with

500 ml of water with 0.1 g of yeast, and they were otherwise treated as in the high density treatment. Seven to eight replicates were performed for each strain or substrain.

Experiments were pooled into groups designed by a letter (A to G, see Table 2), each group corresponding to experiments conducted simultaneously. Results were analyzed by means of generalized linear models (GLM) and Mann Whitney tests. In the GLM, we analyzed survival frequency (*p*) of the 61,400 larvae involved in the experiment by means of models with a logit link and binomial error structure (e.g., McCullagh and Nelder 1983) in a model with two-nested random effects: groups, and replicates within strain within each group. The full model concerning the fixed part of the model was:

$$\log \frac{p}{1-p} = \mu + \text{GEN} \times \text{INF}$$

where μ is a mean survival frequency among all groups, GEN is the effect of mosquito genotype, INF is the infection status of the strain, and “ \times ” indicates additive effects and interactions between variables. This model was then compared with models with less or no interactions. Changes in likelihood were compared by means of χ^2 tests. The likelihood was computed using the GLLAMM function (available from www.gllamm.org) in Stata version 8 (Stata Corporation 2005).

Associations between preimaginal mortality and *Wolbachia* density was measured using Spearman's rank-correlation coefficient (Siegel and Castellan 1988).

Female Size and Fecundity

Fecundity was assessed by introducing, into the same cage (25 × 30 × 40 cm), one-day-old adult females from both an

infected strain and an uninfected substrain, and an excess of uninfected males from the same substrain. To easily recognize the infection status of individuals, females of each strain and substrain were marked just before the start of an experiment, using fluorescent powders of different colors (yellow or orange). To control for an eventual color effect, a replicate cage was run simultaneously, with the color swapped between the strain and the substrain. The adults were provided access ad libitum to a honey solution. Seven days later, females were blood fed, then individually isolated, and egg-rafts collected. For each female, a posterior leg was taken, and tibia length was measured using a measuroscope (Measuroscope 10 NIKON, digital counter CM 6S NIKON). Tibias were measured twice independently by the same experimenter. The correlation between both measures indicates good agreement ($R^2 = 0.98$), and the mean of the two measures was used for further analyzes.

The effect of *Wolbachia* infection on mean tibia length and fecundity was estimated as follows; for each egg-raft, the number of larvae was recorded just after hatching. Each female was characterized by four variables: genotype (qualitative variable GEN, two levels), *Wolbachia* infection (qualitative variable INF, two levels), color of the fluorescent powder (qualitative variable COL, two levels), mean tibia length (quantitative variable SIZ), and number of offspring (quantitative variable LARV). For the dependent variable SIZ, the linear model GEN \times INF \times COL (where “ \times ” indicates additive and interactive effects between variables) was fitted to the data. For the dependent variable LARV, the linear model GEN \times INF \times SIZ \times COL was fitted to the data. This model was then simplified according to Crawley (1993). Normality of residuals from the minimal model was tested using Lilliefors test (Dallal and Wilkinson 1986). Calculations were performed using the R free software (R Development Core Team 2004).

Predation

Three insect species and the mosquitofish *Gambusia affinis* were used as larval predators. Insect species were the pigmy backswimmer *Plea minutissima* (Hemiptera, Pleidae), the water boatman *Sigara lateralis* (Hemiptera, Corixidae), and the water measurer *Hydrometra stagnorum* (Hemiptera, Hydrometridae). Their sizes were approximately 2 mm, 6 mm, and 10 mm, respectively. They feed on small aquatic prey, such as small arthropods, and are commonly found in mosquito breeding sites (Laird 1988). They capture *C. pipiens* larvae, inject digestive saliva into the larva and subsequently ingest its contents. The external skeletons of empty larvae remain, readily allowing their detection. Mosquitofish are voracious predators widely used for the biological control of mosquito populations.

Differential predation was assessed by introducing, into the same container, an equal number of fourth instar larvae (for mosquitofish) or first instar larvae (for insect predators) from each pair of strains considered, plus predator(s). Experiments were conducted in 750, 500, 250, and 50 ml of tap water, with a total number of larvae of 200, 200, 200, and 40 for the mosquitofish, the pigmy backswimmer, the water boatman, and the water measurer, respectively. No refuge

was available for mosquito larvae. Mosquitofish and predatory insects were starved for two or 10 days before each experiment, respectively. Experiments were terminated when about 50% of all larvae were preyed upon, and the number of eaten larvae of each strain was recorded. Equal numbers of individuals from two strains, one harboring a resistance allele *ace-1^R* (i.e., strains SR or SRTC) and the other without (i.e., strains SLAB, SLABTC, SA1, and SA1TC) were placed in the presence of predators. To recognize individuals among nonpredated larvae, a propoxur (a carbamate insecticide) concentration of 5 mg/L was applied for 24 h to all noneaten larvae. This dose kills all larvae lacking the *ace-1^R* resistance allele within a few hours. The effect of *Wolbachia* infection was inferred by comparing predation of SLAB and SLABTC in the presence of SR.

A predation experiment corresponds to sampling without replacement. Predator preference was measured using the predation coefficients ($\hat{\beta}$) proposed by Manly (1974; 1985):

$$\hat{\beta}_i = \frac{\log_e(r_i/A_i)}{\log_e(r_i/A_i) + \log_e(r_j/A_j)}$$

where $\hat{\beta}_i$ is the predation coefficient of the morph i , i and j being two different morphs; A_i denotes the total number of morphs i at the beginning of the experiment, and r_i is the number of morphs i remaining after predation. This measure is appropriate for experiments in which prey are not replaced during the experiment. This index varies between 0 and 1, and $\hat{\beta}_i + \hat{\beta}_j = 1$. The absence of preference between two morphs corresponds to $\hat{\beta}_i = 1/2$. A Mann-Whitney two-sided test was used to compare $\hat{\beta}_i$ between an infected strain and its corresponding uninfected substrain when they were confronted against the same reference strain. The reference strain was SR when SLAB and SLABTC (or SA1 and SA1TC) were compared; and was SLAB when SR and SRTC were compared.

Mating Competition

Two virgin males (one from an infected strain and the other from its derived uninfected substrain) were placed in competition to fertilize a virgin female from the same substrain. Competition took place in a 125 cm³ glass vial for six days. All adults were one day old at the beginning of the competition. There was ad libitum access to a honey solution. The female was then removed and blood fed. Egg-rafts were individually collected, with the spermathecae of the corresponding female being dissected to check for fertilization. Paternity assignment was determined by measuring egg hatching: crosses between infected males and uninfected females are incompatible. Fifty-five to 116 replicates were performed for each pair of strains.

Paternity success of each male type was defined as the percentage of females that it fertilized among replicates. The null hypothesis was that paternity success equaled 0.5 for both competing males. For each pair of strains, deviation from the null hypothesis was tested using an exact binomial test.

Crossing Experiments

Crosses were performed to determine whether bacterial density affected the intensity of cytoplasmic incompatibility

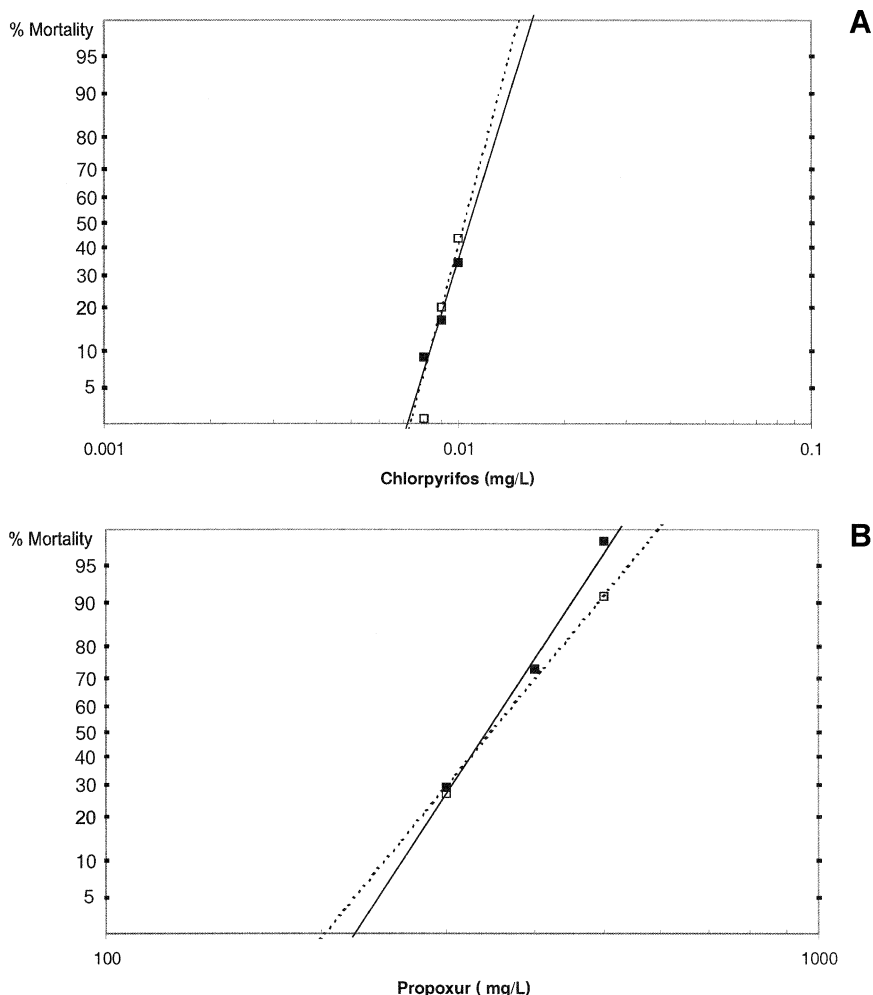


FIG. 1. Effect of *Wolbachia* on insecticide resistance. Mortality according to insecticide concentration was obtained in bioassays with (A) chlorpyrifos for SA1/SAITC, and (B) propoxur for SR/SRTC. Black squares and solid lines: infected strains; empty squares and dashed lines: uninfected strains (TC strains).

(CI). SLAB and SR were used as references for low and high *Wolbachia* density, respectively. Males of these two strains were crossed (1) with uninfected females SLABTC and SRTC, and (2) with infected females belonging to mosquito strains from various geographic origins (SELAX-B, California; BISMUTH, Tunisia; KUNU, Crete; KEO, Cyprus; BIFA and LA VAR, France; DUCOS, Martinique; KARAOKE and BBJT, China). These strains are infected with different *Wolbachia* strains (for more details see Duron et al. 2005). Mass crosses between 50 males and 50 females reared in controlled conditions were performed for each cross, using two to five-day-old males. Six days later, females were blood fed. Egg-rafts were collected daily and isolated individually. Hatching rate (i.e., corresponding to a compatible or incompatible cross) was approximately quantified by visual estimation using a binocular magnifying glass. When an egg-raft did not produce larvae, the spermathecae of the corresponding female were checked for insemination. Egg-rafts from noninseminated females were discarded.

RESULTS

Insecticide Resistance

Resistance alleles at the *Ester* locus provide resistance to OP insecticides. Mortality due to chlorpyrifos (an OP) was compared for the SA1 and SAITC strains (Fig. 1A). Linearity of the response curve was not rejected ($P > 0.5$), for both curves. Both lines did not differ, as parallelism was not rejected ($P = 0.97$), and the resistance ratio (RR) was not different from 1 (RR = 0.90, 95% confidence interval 0.61–1.32). Resistance alleles at the *ace-1* locus provide a high resistance to carbamates. Mortality due to propoxur (a carbamate) was compared for the SR and SRTC strains (Fig. 1B). Linearity of the response curves was not rejected ($P > 0.3$), for both curves. Both lines were not different, as parallelism was not rejected ($P = 0.12$), and the RR was not different from one (RR = 1.02, 95% confidence interval 0.84–1.22). Thus, the presence or absence of *Wolbachia* does not seem to affect the strength of insecticide resistance.

Preimaginal Mortality

With the assumption of a primary sex ratio of 0.5 in each strain (Clements 1992), mortality was compared in independent GLMs for each sex. Removing the interaction (between GEN and INF) from the full model had a significant effect ($P < 10^{-3}$ for both males and females). However, the genotype/infection interaction was not significant, in particular in the SLAB genomic background ($P = 0.76$ and 0.57 for males and females, respectively). Thus, the infection effect was significant only for certain genotypes, justifying separate analyses for each genotype. Mann-Whitney two-sided tests were used to compare preimaginal mortality between pairs of strains. Multiple testing was taken into account using the sequential Bonferroni procedure, according to Hochberg (1988). For the same strain, variation in preimaginal mortality across independent experiments (performed at different times) was significant (Mann-Whitney two-sided test, $P < 0.01$ for all comparisons). Thus, further comparisons were made only within similar experimental groups, and global tests across groups were performed by combining P -values using Fisher's method (Manly 1985).

For the insecticide susceptible strain SLAB, the absence or presence of *Wolbachia* did not affect preimaginal mortality in three independent experiments (groups A, B, and G; Table 2), or globally (global test, $P = 0.48$). For the resistant strains, results differed according to the resistance allele. For the strain SA1 (allele *Ester^l*), the presence of *Wolbachia* resulted in a higher preimaginal mortality, which was found in three independent experiments (C, D, and G groups; Table 2), and globally (global test, $P < 10^{-3}$). Both males and females were affected by the presence of *Wolbachia* (global test, for each sex, $P < 10^{-3}$). For the strain SA4 (allele *Ester^A*), a similar result was found although only one experiment was performed (G group; Table 2). For the strain SA2 (allele *Ester²*), preimaginal mortality was not affected by presence of *Wolbachia* ($P = 0.16$). For the strain SR (allele *ace-1^R*), two experiments (F and G groups) indicated a higher preimaginal mortality associated in the presence of *Wolbachia* and no difference was found in a third one (E group). Overall, *Wolbachia* had an effect on mortality (global test, $P < 10^{-3}$), for both males (global test, $P < 10^{-2}$), and females (global test, $P < 10^{-3}$).

Several interstrain comparisons were possible in the experimental group G, and comparisons between infected strains were performed. SLAB and SA2 strains had the lowest preimaginal mortality rates, and differences between them were not significant ($P = 0.65$). However, pairwise comparisons between SLAB and the insecticide resistant strains SA4, SA1, and SR were significantly different (for all comparisons, $P < 10^{-2}$; Table 3), showing costs of the insecticide resistance alleles *Ester^A*, *Ester^l*, and *ace-1^R*. Preimaginal mortality also differed among SA4, SA1, and SR: higher mortality rates were recorded for SA1 and SR relative to SA4 (both $P < 10^{-3}$; Table 3), and for SA1 relative to SR ($P = 0.001$; Table 3).

The same comparisons were performed among uninfected substrains. Preimaginal mortality of SLABTC, SA2TC, and SA4TC was not significantly different (for all comparisons, $P > 0.05$; Table 3). Comparisons between SLABTC and

TABLE 3. Two-sided P -values of preimaginal mortality comparisons between strains for infected (above-left) or uninfected mosquitoes (below-right) in group G. The bold characters indicate significant P -values (< 0.05), taking into account multiple testing.

	SLAB	SA1	SA2	SA4	
SR	<10⁻³	0.001	<10⁻³	<10⁻³	
SA4	0.005	<10⁻³	0.010		SA4TC
SA2	0.64	<10⁻³		0.80	SA2TC
SA1	<10⁻³		<10⁻³	<10⁻³	SA1TC
		<10⁻³	0.05	0.16	SLABTC
					SA1TC SA2TC SA4TC SRTC

SA1TC or SRTC remained significant (both $P < 10^{-3}$; Table 3). Thus, when *Wolbachia* were removed, cost of insecticide resistance alleles was detectable only for *Ester^l* and *ace-1^R*, whereas differences between SLAB and SA4 were suppressed (Table 3). Thus, the cost of increased preimaginal mortality for SR and SA1 (Tables 2 and 3) is partially explained by the presence of *Wolbachia*.

Wolbachia density was found to be negatively correlated with the number of emerging adults in group G (Spearman's rank order correlation, adults of both sexes: $r_s = -0.72$; two-sided test: $P < 0.05$) (Fig. 2).

Female Size and Fecundity

To recognize mosquito strains in experimental cages, females were marked with a fluorescent powder, either yellow or orange. Tibia length and offspring number were recorded for SLAB ($n = 32$), SLABTC (53), SR (44), and SRTC (44) females.

The minimal model describing the tibia length was GEN \times INF. Thus, tibia length is related to interaction between genotype and infection ($F_{1,170} = 13.40$, $P < 10^{-3}$). No effect of *Wolbachia* infection was observed for SLAB and SLABTC ($F_{1,85} = 0.07$, $P = 0.79$), in contrast to results between SR and SRTC ($F_{1,85} = 24.24$, $P < 10^{-5}$). Thus, *Wolbachia* effect on host size was detected only for the highly infected strain SR that displayed tibia smaller than SRTC females (Fig. 3A). An effect of genotype was detected between SLAB and SR ($F_{1,93} = 53.14$, $P < 10^{-9}$), but not for SLABTC and SRTC ($F_{1,77} = 1.50$, $P = 0.22$). The susceptible strain SLAB had a significantly greater mean tibia length than the resistant strain SR (Fig. 3A), but this effect disappeared when uninfected: SRTC individuals had a mean tibia length similar to

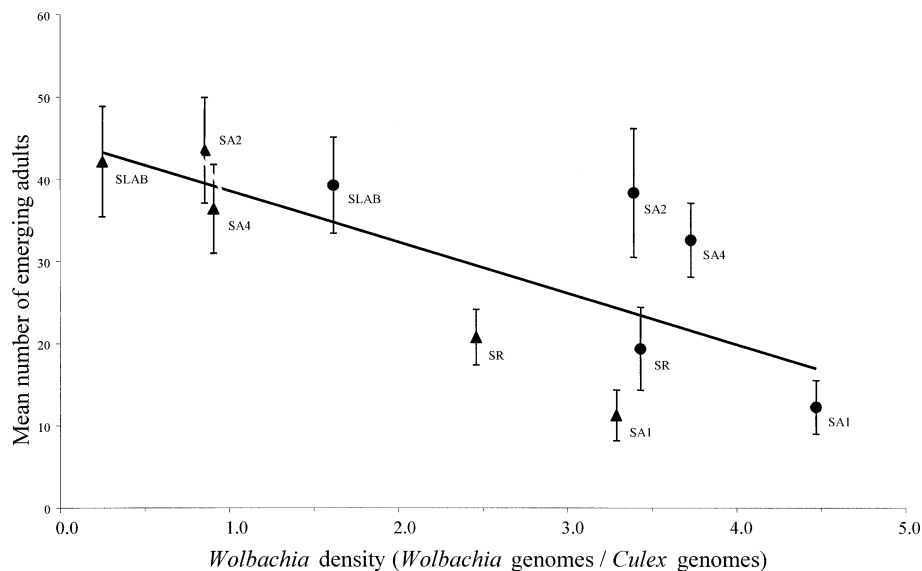


FIG. 2. Relationship between preimaginal mortality and *Wolbachia* density. Triangles and circles represent males and females, respectively. Strain names are indicated.

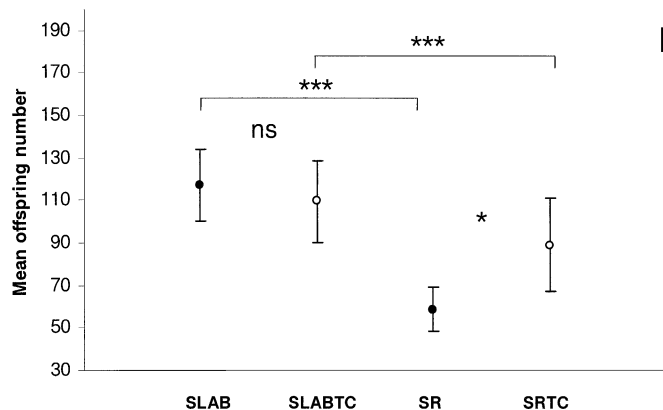
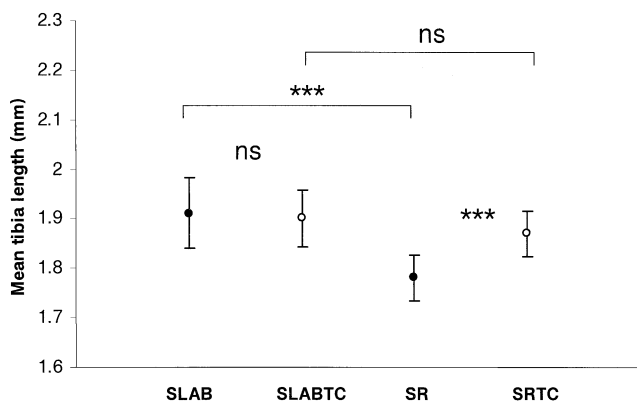


FIG. 3. Costs of insecticide resistance and *Wolbachia* infection. (A) Mean tibia length and (B) mean offspring number for infected (filled circles) and uninfected (empty circles) females. ns, $P > 0.05$; * $P < 0.05$; *** $P < 0.001$.

A

SLABTC individuals (Fig. 3A). Normality of the residuals for all models was not rejected (Lilliefors normality test, all $P > 0.20$).

The minimum adequate model describing the number of larvae was GEN + INF + SIZ + GEN:INF + INF:SIZ (where “:” indicates interaction between variables). Thus, female fecundity is related to an interaction between genotype and infection ($F_{1,168} = 6.83, P < 10^{-2}$), and an interaction between infection and tibia length ($F_{1,84} = 5.41, P = 0.02$). For the insecticide susceptible strain SLAB and its uninfected substrain SLABTC, fecundity is only related to female tibia length ($F_{1,85} = 28.72, P < 10^{-6}$). For the insecticide resistant strain SR and its uninfected sub-strain SRTC, fecundity is related to an interaction between tibia length and infection ($F_{1,84} = 5.77, P = 0.02$). An effect of *Wolbachia* on fecundity was thus detected for the highly infected strain SR, and it was deleterious (reduced fecundity) (Fig. 3B). No *Wolbachia* effect on fecundity was detected for the SLAB strain (Fig. 3B). Significant effects of both genotype and size were observed for infected ($F_{1,92} = 35.92, P < 10^{-7}$; and $F_{1,92} = 19.69, P < 10^{-4}$, respectively) as for uninfected mosquitoes ($F_{1,76} = 3.87, P = 0.05$; and $F_{1,76} = 44.50, P < 10^{-8}$, respectively). Thus, differences did not disappear when mosquitoes were uninfected: SRTC individuals had a lower fecundity than SLABTC individuals (Fig. 3B). Normality of the residuals was not rejected for all models based on fecundity analysis (Lilliefors normality test, all $P > 0.30$).

B

Predation

When individuals were infected, insecticide resistant larvae were significantly more likely to be preyed upon than susceptible individuals (data not shown), in agreement with previously published results (Berticat et al. 2004). To evaluate whether the differences in predation were influenced by the presence of *Wolbachia*, predation coefficients ($\hat{\beta}$) between infected and uninfected strains were compared. We analyzed

TABLE 4. *Wolbachia* effects on larval predation avoidance. Pairs of strains (infected or uninfected) were compared for predator avoidance, measured by the predator coefficient $\hat{\beta}$, standard error (SE) indicated in parenthesis. See text for details. *P*-values refer to comparisons between $\hat{\beta}$ for infected and the corresponding uninfected substrains.

Predator	Pair of strains						<i>P</i> -value
	Infected	Replicates	$\hat{\beta}$ (SE)	Uninfected	Replicates	$\hat{\beta}$ (SE)	
<i>Gambusia affinis</i>	SLAB (vs. SR)	10	0.56 (0.11)	SLABTC (vs. SR)	6	0.52 (0.05)	0.37
	SR (vs. SLAB)	10	0.44 (0.11)	SRTC (vs. SLAB)	9	0.52 (0.12)	0.24
<i>Plea minutissima</i>	SLAB (vs. SR)	7	0.43 (0.06)	SLABTC (vs. SR)	7	0.46 (0.05)	0.99
	SR (vs. SLAB)	7	0.57 (0.06)	SRTC (vs. SLAB)	7	0.63 (0.05)	0.97
<i>Sigara lateralis</i>	SR (vs. SLAB)	9	0.56 (0.06)	SRTC (vs. SLAB)	10	0.56 (0.08)	0.99
<i>Hydrometra stagnorum</i>	SLAB (vs. SR)	11	0.32 (0.10)	SLABTC (vs. SR)	7	0.26 (0.05)	0.13
	SA1 (vs. SR)	10	0.33 (0.08)	SA1TC (vs. SR)	10	0.39 (0.09)	0.12
	SR (vs. SLAB)	11	0.68 (0.10)	SRTC (vs. SLAB)	11	0.57 (0.17)	0.70

predatory avoidance of 14,960 larvae. For all predators and all infected/uninfected pairs of strains, no difference ($P > 0.12$) in predation rate was found (Table 4). Thus, presence or absence of *Wolbachia* does not seem to affect predation risk of its host.

Mating Competition

We tested 55, 66, and 116 mating competitions of SLAB/SLABTC, SA1/SA1TC, and SR/SRTC males, respectively. For all pairs of strains, paternity success was not affected by the presence or absence of *Wolbachia* ($P = 0.59, 0.27$ and 0.11 , respectively; Fig. 4). Thus, uninfected males do not

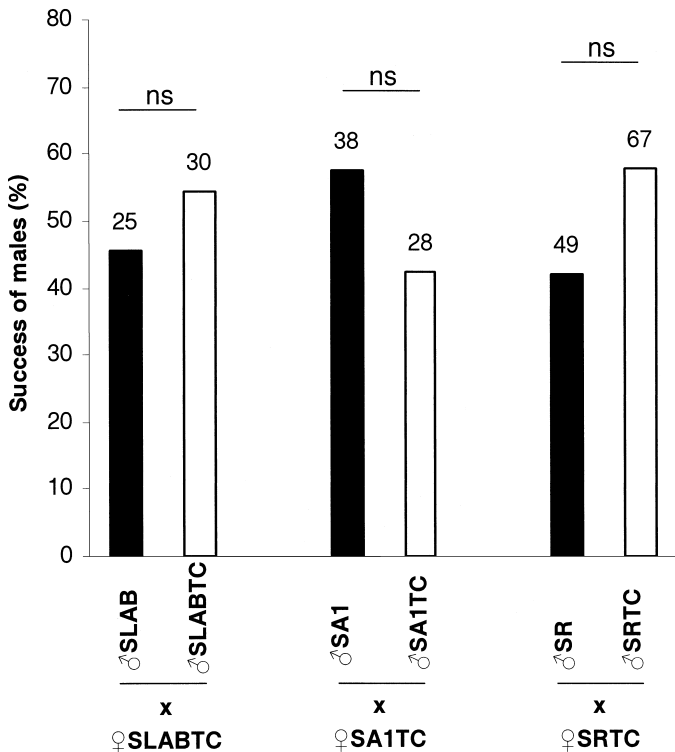


FIG. 4. Mating competition. Success of males was deduced from numbers of incompatible and compatible egg-rafts recorded when uninfected females were mated with infected males (filled bars) or uninfected males (empty bars), respectively. The number of observations is given on the top of the bars; ns, $P > 0.05$.

show a mating advantage when competing against infected males.

Crossing Experiments

All possible crosses between infected males (strains SLAB or SR) and uninfected females (strains SLABTC or SRTC) were completely incompatible (no larvae obtained; Table 5). All crosses with SLAB males and infected females from various strains were compatible (all hatching rates were estimated above 0.90; Table 5). Similar results were found when SLAB males were replaced by SR males. However, the protocol did not allow us to distinguish small effects on hatching rates (<5%). Within this limit, the difference in *Wolbachia* density (low in SLAB, high in SR) does not affect the expression of CI.

DISCUSSION

Insecticide resistant mosquitoes display an obvious fitness cost (Lenormand et al. 1999). The aim of this study was to measure the role of *Wolbachia* in the fitness cost associated with *C. pipiens* insecticide resistance, and to assess *Wolba-*

TABLE 5. Cytoplasmic incompatibility intensity in relation to *Wolbachia* density. SLAB and SR were the references for low and high levels of *Wolbachia*, respectively, and both have been crossed with different strains. Crossing experiments used SLAB and SR males. Number of egg-rafts analyzed is reported, and outcomes of the crosses (compatible crosses correspond to hatching rates >0.90, and incompatible crosses correspond to hatching rate equal to 0). Except for SLABTC and SRTC, all strains were infected.

Females	Males			
	SLAB		SR	
	Cross	Egg-rafts	Cross	Egg-rafts
SLABTC	incompatible	30	incompatible	40
SRTC	incompatible	14	incompatible	23
SELAX-B	compatible	43	compatible	26
BISMUTH	compatible	29	compatible	19
KUNU	compatible	30	compatible	16
KEO	compatible	26	compatible	24
BIFA	compatible	29	compatible	9
LA VAR	compatible	8	compatible	20
DUCOS	compatible	19	compatible	9
KARAOKE	compatible	7	compatible	7
BJBJT	compatible	11	compatible	11

TABLE 6. Overview of insecticide resistance costs and *Wolbachia* effects on life-history traits. Insecticide resistance costs are reported relative to the reference strain SLAB (insecticide susceptible strain). All *Wolbachia* effects refer to deleterious effects (costs). ^aBerticat et al. (2004); ^bBerticat et al. (2002a).

Strains	Insecticide resistance costs					Insecticide resistance	<i>Wolbachia</i> effects				
	Pre-imaginal mortality	Female size	Female fecundity	Predation avoidance	Male mating competition		Pre-imaginal mortality	Female size	Female fecundity	Predation avoidance	Male mating competition
SLAB	—	—	—	—	—		no	no	no	no	no
SA1	yes			yes ^a	yes ^b	no	yes			no	no
SA2	no						no				
SA4	yes			yes ^a	yes ^b		yes				
SR	yes	yes	yes	yes ^a	yes ^b	no	yes	yes	yes	no	no

chia adaptation in regard to its cost of infection, CI intensity and maternal transmission (see below).

Insecticide Resistance Cost

Resistant alleles were associated with a greater preimaginal mortality and smaller female size, relative to susceptible alleles (overview on Table 6). These results indicate that insecticide resistant alleles are at a selective disadvantage in the absence of insecticides. This disadvantage is detectable for both loci *Ester* and *ace-1*, each with different mechanisms of resistance, suggesting that a fitness cost could be expected to be found for various types of adaptations. Fisher's prediction (recent adaptive genes have a fitness cost) has been verified for herbicide, pathogen, and herbivore resistance in plants (reviews in Simms and Rausher 1987; Simms and Triplett 1994; Bergelson et al. 1996), for antibiotic resistance in bacteria (review in Levin 2000), and pesticide resistance in arthropods (reviews in Roush and McKenzie 1987; Coustau et al. 2000).

Cost is a crucial parameter in the evolution of adaptation since variation in fitness cost among adaptive alleles can lead to allelic replacement, with less costly alleles replacing those with greater cost. Our results indicate variable cost among resistance alleles. For example, preimaginal mortality was lower for *Ester*² individuals than *Ester*⁴ or *Ester*¹. Similarly, pre-imaginal mortality was lower for *Ester*⁴ than *Ester*¹. These results are in agreement with field surveys, which show that *Ester*⁴ replaced *Ester*¹ during the 1980s, and that *Ester*² is now increasing in frequency (Guillemaud et al. 1998; Lenormand et al. 1999; Labbé et al. 2005).

Wolbachia Involvement in Insecticide Resistance Cost

Berticat et al. (2002b) showed that resistant mosquitoes are more heavily infected by *Wolbachia* whatever the mechanism implied. Due to the various deleterious effects of *Wolbachia* on their hosts, this increased density could contribute to the fitness cost of resistance. Experiments comparing life-history traits of infected and uninfected strains were performed to evaluate the possible involvement of *Wolbachia* in the cost associated with insecticide resistance. *Wolbachia* were eliminated by treating mosquitoes with tetracycline, but this treatment may have eliminated other vertically transmitted bacteria (although none have been described so far). Experiments were conducted at least two generations after this treatment, so that the overall bacterial flora (except for vertically transmitted bacteria) was probably not greatly af-

fected. Thus, we compared mosquito strains sharing a similar genetic background, except for the presence or absence of resistance alleles and/or *Wolbachia* infection.

For the insecticide susceptible strain (SLAB), no differences in life-history traits were detectable when *Wolbachia* were removed (Table 6). This absence of infection cost has been described in other *Wolbachia*/host interactions (e.g., in *Drosophila melanogaster*—see Turelli and Hoffmann 1995; Hoffmann et al. 1996; Poinso and Merçot 1997). In general, there is considerable variability (parasitism to mutualism) among *Wolbachia*/host interactions (Dedeine et al. 2001; Dobson et al. 2002; Girin and Boulétreau 1995; Min and Benzer 1997; Vavre et al. 1999), thus it is not surprising to find that sometimes *Wolbachia* reduces fitness in some strains (Girin and Boulétreau 1995; Min and Benzer 1997; Vavre et al. 1999). However, in the mosquito *C. pipiens*, the cost of infection (reduction in fecundity and preimaginal survival) was detected only in strains carrying insecticide resistance alleles (Table 6). The insecticide resistant strain SA2 represents an exception because no fitness decrease was detected when infected. The absence of cost for the SA2 strain suggests a peculiar interaction between *Wolbachia* and the *Ester*² allele, as the *Ester*² allele differs from other insecticide resistance alleles. Interestingly, *Ester*² has a broad geographic distribution that seems to be explained by a competitive advantage of *Ester*² relative to other *Ester* resistance alleles (Labbé et al. 2005). This suggests that *Ester*² is a less costly allele in natural populations, the absence of *Wolbachia* infection cost may enhance this situation.

Although these results suggest that *Wolbachia* are somehow responsible for the fitness cost of insecticide resistance, there is still a fitness cost associated with resistance genes when *Wolbachia* are removed. For some uninfected insecticide resistant strains, preimaginal mortality remained high relative to the susceptible strain (Table 2). Removing *Wolbachia* did not increase the performance of insecticide resistant individuals in predation avoidance or mating competition, although substantial costs of insecticide resistance have been reported for these traits (Berticat et al. 2002a, 2004). Thus, *Wolbachia* infection induces an additional cost of resistance for particular traits.

Infection cost appears related to *Wolbachia* density, suggesting that its virulence is linked to bacterial population size. A relationship between density and virulence has also been documented by McGraw et al. (2002) in *Drosophila simulans* and Mouton et al. (2004) in the wasp *Asobara tabida*, both studies demonstrating that the cost associated with

Wolbachia infection was reduced when density decreased. The increase of *Wolbachia* density reported in resistant mosquitoes suggests that perturbations of host physiology in a general sense play a major role in *Wolbachia* susceptibility. *Wolbachia* density in SA2 is not particularly low compared to other insecticide resistant strains (Berticat et al. 2002b), thus a lack of infection cost for SA2 appears surprising. *Wolbachia* density is measured for the whole organism, and does not take into account variability in tissue distribution. *Wolbachia* are often concentrated in gonads, but for *C. pipiens* they may be found in most host tissues (Dobson et al. 1999). The resistance conferred by *Ester* results from an overproduction of esterases, but the location of these enzymes varies according to the *Ester* allele (Pasteur et al. 2001). Thus, the ratio of *Wolbachia* to esterases may vary from tissue to tissue and influence the expression of any costs associated with their interaction.

Fitness studies of insecticide resistant arthropods have not generally controlled for infection by parasites like *Wolbachia*. These bacteria have been detected in most insect orders, infecting at least 15% of all insect species worldwide, and they are believed to be a pervasive endosymbiont (Jeyaprakash and Hoy 2000; Werren and Windsor 2000), particularly in mosquito species (Kittayapong et al. 2000). Studies on life-history traits in relation to insecticide resistance must take into account the presence of *Wolbachia*. This possibility also extends to other parasites in which density is modified by the presence of insecticide resistance genes (see Agnew et al. 2004). In addition, the outcome of the association may change significantly under particular environmental conditions, as reported in *D. simulans*, in which different effects occurred in wild versus laboratory reared flies carrying *Wolbachia* (Hoffmann et al. 1990; Turelli and Hoffmann 1995).

Wolbachia Interactions with Insecticide Resistant Mosquitoes

No host fitness advantage due to *Wolbachia* was revealed by our experiments. Only a few studies have found *Wolbachia* to be beneficial for their arthropod hosts: an obligatory mutualism has been reported for *Asobara tabida* wasps (Dedeine et al. 2001) and for a particular mutant strain of *D. melanogaster* (Starr and Cline 2002). A slight enhancement of reproductive success has been reported for *Aedes albopictus* mosquitoes (Dobson et al. 2002) and *D. melanogaster* (Fry et al. 2004).

For some strains (SLAB and SA2), no differences in life-history traits were detectable when *Wolbachia* were removed, whereas infection cost was present for other strains (SA1, SA4, and SR; Table 6). This variability in infection cost depends on the host genotype at insecticide resistance loci. Insecticide resistance genes for OP are new for the mosquito *C. pipiens*, as they appeared during the 1970's (Raymond et al. 1998, 2001). Their occurrence has apparently disturbed the *Wolbachia*/host interaction, resulting in increased *Wolbachia* density and thus an increase in the cost of infection. In this species, the cost of resistance genes evolves mainly through allele replacement at the *Ester* locus (Guillemaud et al. 1998; Labbé et al. 2005). It is worth noting that the less costly resistance gene, *Ester*², is not associated with an in-

creased cost of infection, suggesting an interaction between both types of costs (resistance gene and *Wolbachia* infection).

A model developed by Turelli (1994) suggests that significant reductions in host fitness may be stable in a population if they are linked to gains in *Wolbachia* transmission. Theory also predicts that *Wolbachia* will minimize their cost by reducing their density in order to maximize maternal transmission (McGraw and O'Neill 1999). However, investigations based on *wsp* PCR using primers described in Berticat et al. (2002b) have never found uninfected individuals in our laboratory susceptible colony of mosquitoes ($n = 200$), demonstrating strong transmission efficiency. *Wolbachia* density thus appears in excess in insecticide resistant mosquitoes, at least in laboratory conditions. Moreover, *Wolbachia* loads show no major effect on CI expression: no variation in hatching rates was detected for our *C. pipiens* strains, whatever the *Wolbachia* density in males. CI was complete in incompatible crosses with SLAB males and uninfected females, with high *Wolbachia* density in SR males being useless in this case. All crosses with infected females were compatible using SLAB males, but no hatching rate decrease was observed with SR males. This result contrasts with studies finding that reduced CI expression correlates with reduced bacterial densities in males of some insect species (Sinkins et al. 1995; Clancy and Hoffmann 1998; Noda et al. 2001). Variation of CI intensity in *C. pipiens* might relate to the *Wolbachia* variants involved and eventually to their interactions with host gene(s) rather than to density, at least in laboratory conditions (Guillemaud et al. 1997; Rousset et al. 1991). Thus, factors implicated in CI expression correspond to qualitative variables (i.e., *Wolbachia* variant or host restorer genes), and apparently not to quantitative variables (i.e., *Wolbachia* density).

Insecticide resistance genes are thus host genes influencing the outcome of the host-*Wolbachia* association. As insecticide resistant mosquitoes display an infection cost without an increase of CI, the invasion dynamics of *Wolbachia* could be modified. This could limit the efficiency of *Wolbachia* infections as potential biological control agents capable of driving changes in the gene pool of disease vectoring mosquito populations (Sinkins 2004).

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