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High *Wolbachia* density in insecticide-resistant mosquitoes

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Wolbachia symbionts are responsible for various alterations in host reproduction. The effects of the host genome on endosymbiont levels have often been suggested, but rarely described. Here, we show that *Wolbachia* density is strongly modified by the presence of insecticide-resistant genes in the common house mosquito, *Culex pipiens*. The *Wolbachia* density was estimated using a real-time quantitative PCR assay. Strains harbouring different genes conferring resistance were more infected than a susceptible strain with the same genetic background. We show that this interaction also operates in natural populations. We propose that mosquitoes may control *Wolbachia* density less efficiently when they carry an insecticide-resistant gene, i.e. when they suffer from a physiological resistance cost.

Keywords: insecticidal resistance; fitness cost; endocellular bacteria; *Wolbachia*

1. INTRODUCTION

Wolbachia, a group of bacterial symbionts widespread among arthropods, are responsible for various effects including the feminization of chromosomal males, parthenogenesis and male killing (for a review, see Stouthamer *et al.* (1999)). In addition, in many insect species, *Wolbachia* induce cytoplasmic incompatibility (CI). This is an early embryo death that is observed in crosses between infected males and uninfected females, as well as in some crosses between individuals infected by different *Wolbachia* strains (Yen & Barr 1973). The effects of *Wolbachia* on their hosts are variable and variation in symbiont density within individual hosts may be one of the factors that modulate them.

Environmental factors, such as temperature, food quality or rearing density of host insects, affect symbiont densities within individual hosts and symbiont transmission from mother to offspring (Hoffmann *et al.* 1990; Sinkins *et al.* 1995). For a given *Wolbachia* strain, reduced CI expression may correlate with reduced bacterial densities in males (Clancy & Hoffmann 1998; Sinkins *et al.* 1995; Noda *et al.* 2001), although a causal link is difficult to demonstrate. Incompatibility decreases with male age as the *Wolbachia* load decreases (Binnington & Hoffmann 1989; Bressac & Rousset 1993; Noda *et al.* 2001). Host genomic effects on CI expression have also been indicated by transfection experiments between different host species (Boyle *et al.* 1993; Clancy & Hoffmann 1997; Bordenstein & Werren 1998; Poinsot *et al.* 1998; Dobson *et al.* 1999; McGraw *et al.* 2001). However, analyses of host genomic effects on single *Wolbachia* strains within host species have been inconclusive or negative (Rousset & Raymond 1991; Rousset & de Stordeur 1994; Hurst *et al.* 2001), with the exception of other non-Mendelian factors that are involved in the feminization process in an isopod (Rigaud *et al.* 1999). Thus, it is unclear how the between-species differences evolve in natural populations.

Wolbachia occur naturally in the common house mosquito, *Culex pipiens*, where they induce CI (Yen & Barr 1973). This host exhibits a high variability in CI expression, with complex patterns of unidirectional and bidirectional incompatibility between infected strains (Laven 1967; Magnin *et al.* 1987; Guillemaud *et al.* 1997). This variation is due at least in part to different *Wolbachia* strains, although some host genomic effects cannot be excluded (Rousset *et al.* 1991).

Here, we show that *Wolbachia* density is strongly modified by the presence of insecticide-resistant genes in *C. pipiens*.

2. MATERIAL AND METHODS

(a) *Mosquitoes*

The insecticide-susceptible strain, S-LAB (Georghiou *et al.* 1966), was used as a reference. We used several strains that are resistant to organophosphate (OP) insecticides. There are two main resistance mechanisms in *C. pipiens* (for a review, see Raymond *et al.* (2001)): overproduction of esterases (coded at the *Ester* locus) that bind to the insecticide, and target insensitivity (coded at the *ace-1* locus). The resistant strains are homozygous for one resistance allele at one of the two resistance loci. All of the resistant strains were introgressed with the S-LAB cytoplasmic (including *Wolbachia*) and nuclear genomes, through repetitive (more than 12) backcrosses (Berticat *et al.* 2002). Four resistance alleles at the first locus, $Ester^1$, $Ester^2$, $Ester^4$ and $Ester^5$, encode for overproduced esterase (Raymond *et al.* 1998) and are found in strains SA1, SA2, SA4 and SA5, respectively. One resistant allele at the second locus, $ace\text{-}1^R$, encodes for a modified (OP insensitive) insecticide-target site (Bourguet *et al.* 1996) and is found in the SR strain. All of the laboratory strains were reared in a controlled environment, with food given *ad libitum* and larval density less than 1000 larvae l^{-1} with a water depth lower than *ca.* 2 cm.

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A field sample of pupae was collected at a single breeding site in July 2001 in Ganges, near Montpellier (France), an area where both resistant and susceptible mosquitoes are found, and 5-day-old adults were obtained. Their resistant genes at the *Ester* locus were identified through starch gel electrophoresis (Pasteur *et al.* 1988) and at the *ace-1* locus through an enzymatic bioassay (Bourguet *et al.* 1996). Two classes of mosquitoes, for each sex, were identified for further analyses: susceptible mosquitoes at both loci (S) and resistant mosquitoes with just the $Ester^4$ allele (A4). Other classes were not represented in sufficiently large numbers for statistical comparisons.

(b) *Measuring the* Wolbachia *density*

Real-time quantitative PCR was performed with a Roche light cycler and was used to estimate the number of *Wolbachia* in each mosquito. Two PCRs were performed on each mosquito's DNA: one was specific for the *Culex Ace-2* locus (Weill *et al.* 2000), which is not involved in insecticidal resistance (Malcolm *et al.* 1998), and the other was specific for the *Wolbachia wsp* locus (Braig *et al.* 1998). The *wsp* specific primers (wolpipdir 5′-AGAATTGACGGCATTGAATA-3′ and wolpiprev 5′-CGTCGTTTTTGTTTAGTTGTG-3′) amplified a 151 bp fragment. The quantitative PCR performed with these primers respected all of the criteria required for an accurate estimate of the gene copy number (Weill *et al.* 2000). Standard curves were plotted using dilutions of a pBluescriptKS vector containing one copy of each of the *Ace-2* and *wsp* gene fragments. Each DNA template was analysed in triplicate for *wsp* and *Ace-2* quantification. Assuming that both genes are present in a single copy per haploid genome of the host and the symbiont, the ratio between the *wsp* and *Ace-2* arbitrary concentrations provided the number of *Wolbachia* genomes relative to the *Culex* genomes, thus correcting for mosquito size.

3. RESULTS

The *Wolbachia* density varied according to the developmental stage (higher in adults than in larvae or pupae) and sex (higher in females than in males) (Mann–Whitney, all $p < 10^{-3}$; figure 1). Fourth-instar larvae and 5-day-old adults (males and females) were used to compare the *Wolbachia* density between strains. For each strain, ten mosquitoes were analysed at each stage, and for two separate rearings. No heterogeneity was found between these two replicates, and thus they were pooled in later analyses. All resistant strains displayed a significantly higher *Wolbachia* density than the susceptible reference strain, for the larvae and adults of both sexes (Mann–Whitney, all $p < 10^{-4}$; figure 2). *Wolbachia* density did not differ among the resistant strains, except for the SA1 males who displayed a significantly higher density (Mann–Whitney, $p < 0.002$). These results clearly show an interaction between resistant genotypes and *Wolbachia* density in insects with the same nuclear and mitochondrial genetic background. To determine whether such an interaction also operates in natural populations, we examined the *Wolbachia* density of 5-day-old males and females derived from pupae collected in a breeding site where susceptible and resistant mosquitoes are present. Male and female genotypes at the *Ester* and *ace-1* loci were determined and the *Wolbachia* density was estimated in ten mosquitoes of each sex with susceptible alleles at both loci and in mosquitoes carrying the $Ester^4$ allele. As with laboratory

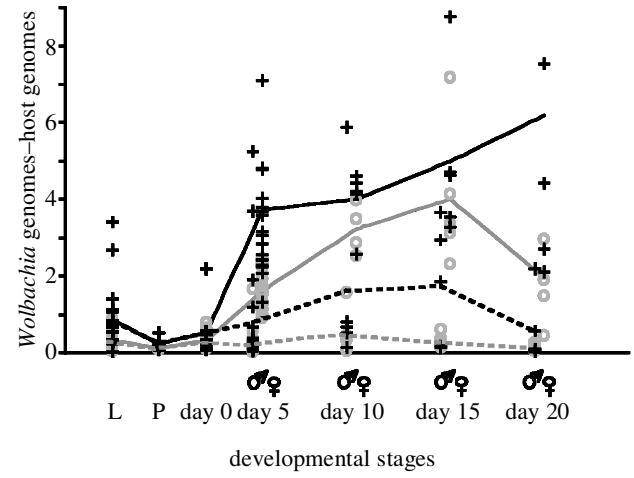

Figure 1. Variations in *Wolbachia* density according to the developmental stages of its host. Each point refers to the mean of a triplicate measure of one individual. The means of each distribution are connected by lines. Two extreme points are not represented for SA4 females (day 5, at $y = 12.4$, and day 20, at $y = 14.2$). Dashed lines, males; solid lines, females. Pluses, SA4; empty circles, S-LAB. Abbreviations: L, fourth instar larvae; P, pupae; day 0–20, adults of both sexes and of known age (day 0 = emergence), for one insecticide-susceptible (S-LAB; grey lines) and one insectide-resistant strain (SA4; black lines).

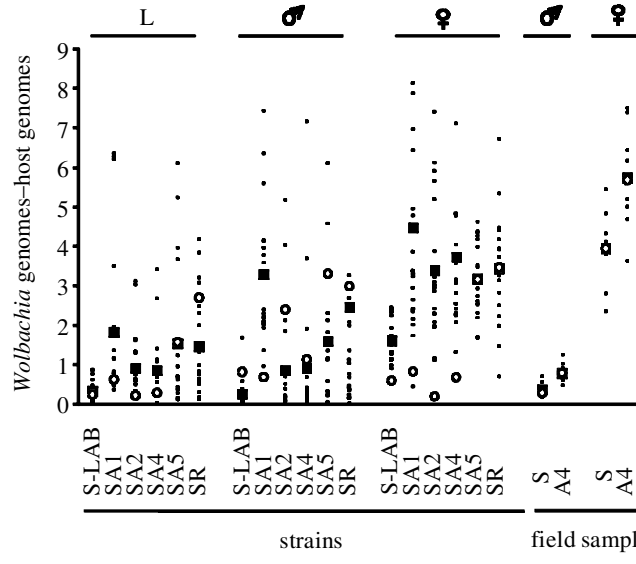

Figure 2. Variations in *Wolbachia* density among susceptible and resistant mosquitoes. The strain comparison involves the susceptible strain (S-LAB), the strains homozygous for a resistant gene at *Ester* (SA1, SA2, SA4 or SA5) and a strain homozygous for a resistant gene at *ace-1* (SR). Mosquitoes from the field are either without the resistant genes (S) or bearing only the $Ester^4$ resistant gene (A4). All measures are performed on fourth-instar larvae (L) or 5-day-old males and females. Each point refers to the mean of a triplicate measure of one individual. The squares and circles refer to the means and medians, respectively, of the distribution of individual measures.

strains, the resistant mosquitoes showed a significantly higher infection rate than the susceptible mosquitoes (Mann–Whitney for males and females separately, both $p < 0.006$; figure 2).

4. DISCUSSION

The *Wolbachia* load was higher in females than in males, as observed in the fruitfly *Drosophila simulans* (Bourtzis *et al.* 1998; Rousset *et al.* 1999), the mosquito *Aedes albopictus* (Dobson *et al.* 1999) and two planthoppers, *Laodelphase striatellus* and *Sogatella furcifera* (Noda *et al.* 2001). Although *Wolbachia* may be found in most host tissues (Dobson *et al.* 1999), they are often concentrated in the gonads. The higher infection load of the females may therefore be explained by the much larger size of the ovaries relative to the testes. The variation in the *Wolbachia* load with the developmental stage may, in part, be due to the variation in the size of gonads relative to other host tissues.

Our results indicate a clear interaction between the presence of resistant genes and *Wolbachia* density. Resistant mosquitoes are more infected than susceptible mosquitoes, despite the fact that they share the same genetic background and the same *Wolbachia* strain. Our results point to a direct involvement of the *Ester* and *ace-1* alleles. The alternative hypothesis, that they are due to closely linked loci, would imply that each of the five recently and independently derived resistance alleles at two loci are linked to alleles that increase *Wolbachia* density. In the case of filarial infection (McCarroll *et al.* 2000), *Ester*-resistant mosquitoes were less infected than susceptible mosquitoes, and McCarroll *et al.* (2000) proposed that esterase could provide some direct protection against infection. In our study, we find that resistant mosquitoes are more infected by *Wolbachia*, regardless of whether the resistance is due to *Ester* or *ace-1* alleles. Previous studies have shown that these two genes are associated with various physiological and fitness costs (Lenormand *et al.* 1999). We propose that mosquitoes may control *Wolbachia* density less efficiently when they carry an OP-resistant gene, i.e. when they suffer from a physiological resistance cost. Higher *Wolbachia* infection levels may in turn have deleterious effects on the host (Min & Benzer 1997), thus increasing the cost of OP resistance. Furthermore, the variation amongst the SA strains may be explained by variation in the physiological cost of different *Ester*-resistant alleles, possibly mediated by different tissue expression (Pasteur *et al.* 2001). It is interesting to note that the most infected strain (SA1) is the most costly one when reared in competition with other alleles (Berticat 2001).

Field mosquitoes were more infected than laboratory strains. However, this comparison is not informative, as these differences may be due to several factors, including different environments, different *Wolbachia* strains (polymorphism is known to occur at a low spatial scale in the Montpellier area (Magnin *et al.* 1987)), as well as host genomic effects over the OP resistance alleles.

These results give experimental proof that *Wolbachia* infection is influenced by the host genome in natural populations of one host, and give insight into the fitness cost of insecticide-resistant genes. Due to the fact that resistant mosquitoes appear unable to control *Wolbachia* loads, the fitness costs of resistance may be amplified by interactions with *Wolbachia*. Further experiments comparing infected and uninfected strains will test this hypothesis. Our results may also have implications for the evolution of the *Wolbachia*–host symbiosis. However, males from the least infected strain (S-LAB) are highly incompatible with uninfected females and no strong incompatibility effects were detected in crosses between the different strains used in this work (preliminary observations). Thus, the *Wolbachia* load has no major effect on CI expression in these strains. The relationship between *Wolbachia* density and CI remains to be determined in *C. pipiens* field populations.

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