npg

ORIGINAL ARTICLE

Rapid evolution of *Wolbachia* density in insecticide resistant *Culex pipiens*

P Echaubard[1], O Duron[2], P Agnew[1], C Sidobre[1], V Noël[1], M Weill[2] and Y Michalakis[1]

[1]Génétique et Evolution des Maladies Infectieuses (CNRS/IRD—UMR 2724), Montpellier, France and [2]Institut des Sciences de l'Evolution, CNRS - Université Montpellier 2, Montpellier, France

The maternally inherited symbiotic *Wolbachia* have been previously shown to have much greater densities in insecticide-resistant *Culex pipiens* mosquitoes than in insecticide-susceptible individuals. These high densities were shown to be at least partially responsible for the costs related to insecticide resistance in this species. We report here the rapid evolution, on the order of 50 generations, of bacterial densities both in laboratory and field populations. Along with other recently published studies, this report shows that *Wolbachia*–host interactions are very dynamic.

Heredity (2010) **104,** 15–19; doi:10.1038/hdy.2009.100; published online 5 August 2009

Introduction

Wolbachia are maternally inherited symbiotic bacteria, which manipulate their host's reproduction, either by modifying host sex ratio or by inducing cytoplasmic incompatibilities (Werren, 1997; Charlat *et al.*, 2003). Moreover, they have sometimes, but not always (for example, Poinsot and Mercot, 1997; Vavre *et al.*, 2002), been shown to have deleterious effects on their host's life history traits and fitness (Min and Benzer, 1997; McGraw *et al.*, 2002; Mouton *et al.*, 2004; Weeks *et al.*, 2007).

In the mosquito *Culex pipiens*, these bacteria cause cytoplasmic incompatibility (CI) where early embryo death results from crosses between infected males and uninfected females, or from crosses between males and females infected by incompatible bacterial strains (Yen and Barr, 1973; Duron *et al.*, 2006a). It was recently shown that mosquitoes resistant to organophosphate insecticides have higher *Wolbachia* loads than insecticide-susceptible mosquitoes (Berticat *et al.*, 2002) and that these higher loads are accompanied by deleterious effects on host life history traits, such as, preimaginal mortality, adult body size and fecundity (Duron *et al.*, 2006c). These observations were interpreted as resulting from a deregulation of control over bacterial replication in insecticide-resistant genomic backgrounds.

Recent studies on *Drosophila–Wolbachia* interactions showed that the virulence of these maternally transmissible symbionts can decrease very rapidly both in laboratory and field populations (McGraw *et al.*, 2002;

Weeks *et al.*, 2007). McGraw and colleagues showed that virulence attenuation was accompanied by a decrease in bacterial density.

The aim of this study was to investigate whether a similar phenomenon occurred in insecticide-resistant *C. pipiens* mosquitoes. To this end we analyzed *Wolbachia* density in the same strains that Berticat *et al.* (2002) analyzed 36 generations previously. We also measured bacterial densities in insecticide-susceptible and -resistant mosquitoes from the same field population as Berticat *et al.* (2002), approximately 55 generations after their study. Our results indicate that within these relatively short time intervals *Wolbachia* densities in insecticide-resistant mosquitoes have evolved to levels equivalent to those of susceptible mosquitoes, both in the lab and in the field.

Materials and methods

Mosquitoes

We used the same three strains of *C. pipiens* as used by Berticat *et al.* (2002): S-LAB, SA4 and SR. The S-LAB strain (Georghiou *et al.*, 1966) is an insecticide-susceptible strain. SA4 and SR are insecticide-resistant strains with an S-LAB nuclear and cytoplasmic (including *Wolbachia*) genetic background and homozygous for the resistance alleles *Ester⁴* at the *Ester* locus and *ace-1ᴿ* at the *ace-1* locus, respectively (for details on the strains, see Berticat *et al.*, 2002). We measured *Wolbachia* densities of both insecticide-resistant strains and compared them with those of the insecticide-susceptible strain, S-LAB. We analyzed 65 S-LAB females and 82 males, 70 SA4 females and 93 males, 76 SR females and 56 males 36 generations after Berticat *et al.* (2002), and asked whether *Wolbachia* densities had evolved in this time interval. The insecticide resistance of strain SR is periodically tested

through biochemical assays and its resistance properties have not evolved (M Weill, personal observation).

We also sampled in September 2005 mosquitoes from the same field site, situated at Ganges (43°55'02.08"N; 3°42'43.06"E), near Montpellier (France), that Berticat *et al.* (2002) had sampled in July 2001. This site is not treated by insecticides. As in Berticat *et al.* (2002), pupae and larvae isolated from the field were raised in the lab under standard conditions (25 °C and 70% humidity, given food *ad libitum*, with larval densities less than 1000 larvae per liter and with a water depth less than 2 cm) until 5-day-old adults were obtained. Using the same protocols as Berticat *et al.* (2002) we characterized insecticide resistance at the *Ester* and at the *ace-1* loci. The only difference in protocols was that instead of using an enzymatic bioassay to characterize resistance at the *ace-1* locus as Berticat *et al.* (2002), we used a PCR/RFLP test (Weill *et al.*, 2004). Using these protocols, we screened 120 individuals and isolated from the field sample two types of individuals: (1) insecticide-susceptible (S) and (2) insecticide-resistant mosquitoes carrying the *Ester⁴* allele but not the *ace-1ᴿ* allele (A4). We then estimated *Wolbachia* densities from 12 S male, 12 S female, 11 A4 male and 7 A4 female individuals and compared the *Wolbachia* load of the September 2005 samples to their July 2001 counterparts.

Estimating *Wolbachia* density

Bacterial density was estimated by real-time quantitative PCR using an Applied Biosystem 7300 machine (same machine as in Berticat *et al.*, 2002). All estimates were taken from 5-day-old male and female adults to standardize comparisons as *Wolbachia* density is known to vary with both mosquito gender and age (Berticat *et al.*, 2002). The experimental procedures were identical to those of Berticat *et al.* (2002). In short, two PCRs were performed on each mosquito's DNA: one specific for the *Culex ace-2* locus (Weill *et al.*, 2000), which is not involved in insecticide resistance and the other specific for the *Wolbachia wsp* locus (Berticat *et al.*, 2002). To obtain accurate estimates of the gene copy number, we plotted standard curves using dilutions of a pBluescriptKS vector containing one copy each of the *ace-2* and *wsp* fragments. The same dilutions as in the study of Berticat *et al.* (2002) were used to construct the standards.

Each DNA template was analyzed in duplicate or triplicate for *wsp* and a*ce-2* quantification. The ratio between *wsp* and *ace-2* arbitrary concentrations provided the number of *Wolbachia* genomes relative to *Culex* genomes, thus correcting for mosquito size and DNA extraction quality.

To assess the quality of our qPCRs we analyzed the efficiency and the repeatability of our measurements by considering amplification efficiency (AE) and the repeatability between replicates. Amplification efficiency represents the multiplication of PCR product increase during each cycle, which is between 1 and 2. The Percentile amplification efficiency (PAE) represents the percentage of full AE capacity, which is between 0 and 100%. AE and PAE are calculated through standard curves for C_t or fluorescent signal strength during the amplification process. A linear fit with a slope approximately between −3.1 and −3.6, equivalent to 90–110% reaction efficiency, is typically acceptable for most

applications requiring accurate quantification. In our study the average slope is −3.50 (s.d. 0.21) and the PAE is on average 93.44% (s.d. 7.09).

We assessed the repeatability of the measurements by comparing the C_t values of replicates of each sample. We calculated the mean C_t value, the standard deviation and the coefficient of variation for each sample. The median coefficient of variation was 0.00282 (2.5th percentile: 0.00015; 97.5th percentile: 0.02811) for lab-reared mosquitoes and 0.00064 (2.5th percentile: 0.000001; 97.5th percentile: 0.02552) for field mosquitoes.

Claire Berticat (ISEM, Montpellier, France) provided data from the Berticat *et al.* (2002) study.

Statistical analysis

Data on *Wolbachia* densities were analyzed using fully factorial models, with insecticide resistance genotype (S-LAB, SA4 or SR for laboratory strains, S or A4 for field samples) and mosquito gender as fixed factors. As standard assumptions of analysis of variance were not met we used the nonparametric Scheirer-Ray-Hare extension of the Kruskal–Wallis test (H statistic; see Sokal and Rohlf, 2003, pp 446–447). Sums of squares based on rank transformed data were computed using JMP (SAS Institute, Cary, NC, USA).
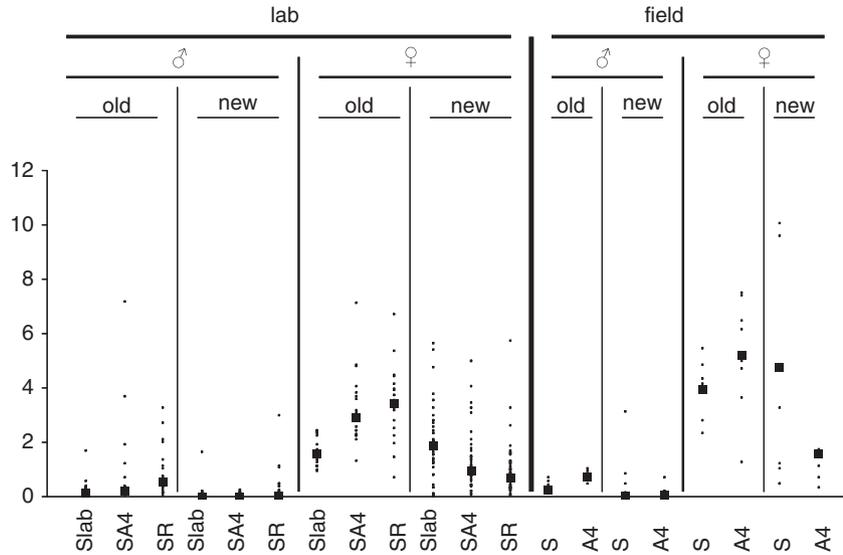
Results

Figure 1 summarizes our results. It is important to keep in mind that although Figure 1 exhibits *Wolbachia* densities, all the tests were performed on ranks.

Wolbachia density in lab strains

Across all strains, females have roughly 10 times more *Wolbachia* than males of the same age (1.388 vs 0.1068 *Wolbachia* mosquito⁻¹ genomes; $H = 186.66$, d.f. = 1, $P < 10^{-6}$). Contrary to Berticat *et al.* (2002), we found that S-LAB mosquitoes had the same *Wolbachia* load as resistant mosquitoes ($H = 0.61$, $P = 0.7371$). The interaction between sex and strain was significant ($H = 10.60$, $P = 0.005$), in that S-LAB females harbored relatively more *Wolbachia* than insecticide-resistant females, whereas S-LAB males harbored relatively less *Wolbachia* than insecticide-resistant males.

As we estimated, *Wolbachia* densities using exactly the same procedures as Berticat *et al.* (2002), it was possible to combine data from both studies to analyze bacterial density with a model including the factors strain, sex and time ('old' vs 'new'). As Figure 1 shows, overall *Wolbachia* densities decreased over time ('old' vs 'new' comparison; $H = 55.52$, $P < 10^{-6}$), the decrease being mostly due to change in the resistant strains. Mosquito sex was the only other factor with a significant effect on *Wolbachia* densities ($H = 151.6$, $P < 10^{-6}$).

The effects of factors strain and the interaction strain × time were close to the significance threshold ($P = 0.0626$ and $P = 0.0792$). As the differences between the two sexes are substantial, we performed separate analyses for each sex. We found that in females the strain effect was not significant ($H = 1.19$, d.f. = 2, $P = 0.5515$), whereas the time and time × strain interactions were highly significant (time: $H = 47.48$, d.f. = 1, $P < 10^{-6}$; time × strain: $H = 23.88$, d.f. = 2, $P < 10^{-6}$). In males, the strain effect ($H = 11.54$, d.f. = 2, $P = 0.003$) and the time effects were significant ($H = 52$, d.f. = 1, $P < 10^{-6}$) but the

Figure 1 *Wolbachia* density in laboratory strains and field samples. We present results for the samples analyzed by Berticat *et al.* (2002) ('old') and in this study ('new'). For each genotype/time of observation/gender/origin of samples, we present the individual ratios of *Wolbachia*/mosquito genome counts (black dots) and the median of each distribution (black squares). To increase the clarity of the figure we only present individuals whose ratio < 12. The following individuals are thus not represented: (1) one SR old lab male with a ratio ∼30; (2) one SA4 old lab female—ratio ∼15; (3) three S new field females—two with ratio ∼30 and one with ratio ∼20; (iv) two A4 new field females—one with ratio ∼40 and one with ratio ∼20. Please note that the figure shows the ratios whereas the tests mentioned in the text bear on their relative ranks.

time × strain interaction was not ($H = 0.45$, d.f. $= 2$, $P = 0.7985$). In brief, the above statistical tests lead to the following observations: (1) for both males and females, the *Wolbachia* density of insecticide-susceptible individuals was similar in the 'old' and 'new' samples; (2) for both males and females, 'old' insecticide-resistant mosquitoes had higher *Wolbachia* densities than 'new' mosquitoes; furthermore, as reported by Berticat *et al.* (2002), in the 'old' sample for both males and females insecticide-resistant individuals had higher *Wolbachia* densities than insecticide-susceptible individuals, whereas in the 'new' sample *Wolbachia* densities did not differ with respect to the insecticide resistance genotypes.

Wolbachia density in field samples

The analysis of the field samples revealed similar results to that of the lab strains: females harbored 30 times more *Wolbachia* than males ($H = 23.78$, $P < 0.0001$), but there were no differences in *Wolbachia* load according to insecticide resistance genotype ($H = 0.16$, $P = 0.69$). The interaction between gender and insecticide resistance genotype is not significant ($H = 0.016$, $P = 0.9$).

The comparison of 'old' and 'new' samples also revealed similar results: the full factorial analysis, including factors genotype (S vs A4), gender and time ('old' vs 'new'), revealed a very strong gender effect (females having much higher *Wolbachia* densities than males; $H = 42.92$, d.f. $= 1$, $P < 10^{-6}$) and a time effect (overall *Wolbachia* density has decreased in the field; $H = 4.846$, d.f. $= 1$, $P = 0.028$), all other factors having very little effect (associated P values > 0.35) except for the time × genotype interaction ($H = 2.068$, d.f. $= 1$, $P = 0.15$). Because of the very strong gender effect, we repeated the analysis separately for males and females. We found that in males the 'new' samples had lower *Wolbachia* densities ($H = 14.41$, d.f. $= 1$, $P = 0.0001$) but *Wolbachia* densities

were not affected by the insecticide resistance genotype ($H = 0.892$, d.f. $= 1$, $P = 0.3449$) nor the interaction time-genotype ($H = 0.1469$). In females, only the time × genotype interaction had a marginally significant effect (genotype: $H = 0.06$, d.f. $= 1$, $P = 0.8064$; time: $H = 0.97$, d.f. $= 1$, $P = 0.3246$; time × genotype: $H = 3.48$, d.f. $= 1$, $P = 0.0621$), indicating that although *Wolbachia* densities remained the same in insecticide-susceptible field females they decreased in A4 females.

Discussion

As in previous studies involving several species (for example, *C. pipiens* (Berticat *et al.*, 2002); *Aedes albopictus* (Dobson *et al.*, 1999); *Drosophila simulans* (Bourtzis *et al.*, 1998); two planthopper species (Noda *et al.*, 2001)) we found a higher *Wolbachia* load in females than in males. This gender difference could be due to several factors. As proposed by Berticat *et al.* (2002) it could simply result from an organ size effect. Another explanation derives from their mode of transmission: *Wolbachia* are vertically transmitted through the eggs. *Wolbachia* in male mosquitoes participate in the invasion of the host population by these bacteria through the phenomenon of CI. However, there is no evidence of a link between CI intensity and *Wolbachia* density in *C. pipiens* (Duron *et al.*, 2006c).

Like Berticat *et al.*, we found higher *Wolbachia* densities in females sampled in the field than in the laboratory strains. This comparison, however, is not very meaningful as *Wolbachia* density is known to depend not only on host and symbiont genotypes but also on the environment in which individuals grow (for example, Mouton *et al.*, 2006, 2007). The fact that larval environment is not controlled for the field samples must be at least partially responsible for the higher variance we observed. Another likely factor contributing to this higher variation in the field is the potential greater

variation in *Wolbachia* and mosquito genotypes. For example, this population contained in 2001 at least 10 different *Wolbachia* genotypes (Duron *et al.*, 2006b).

The most interesting aspect of our results concerns the evolution of *Wolbachia* density over time. Berticat *et al.* (2002) showed that insecticide-resistant strains, sharing the same cytoplasmic and nuclear background with an insecticide-susceptible strain, harbored much higher *Wolbachia* loads. An analysis of field samples confirmed this result. This difference was interpreted as being due to a less efficient control of *Wolbachia* load by insecticide-resistant mosquitoes, which could result from physiological costs associated with insecticide resistance genes. The higher *Wolbachia* density, in the extent that it is correlated to insecticide resistance, could feedback and increase the physiological costs of insecticide resistance. This was indeed reported by Duron *et al.* (2006c) on the same lab strains as in Berticat *et al.* (2002) analyzed 12 generations after the original density measurements by Berticat *et al.* (2002).

As *Wolbachia* are vertically inherited, it is expected that their deleterious effects should decrease over time. This was indeed observed in *Drosophila* both in the laboratory (McGraw *et al.*, 2002; Weeks *et al.*, 2007) and in the field (Weeks *et al.*, 2007). If these negative effects are correlated with *Wolbachia* density, we would expect the bacterial load to evolve to lower levels, and such a decrease of bacterial density was indeed observed in laboratory *Drosophila* populations (McGraw *et al.*, 2002). Our results show that *Wolbachia* density evolved in the insecticide-resistant laboratory strains within a maximum of 36 generations, whereas it remained constant in the insecticide-susceptible strain (it is worth noting that the fact that *Wolbachia* densities do not differ between 'old' and 'new' insecticide susceptible samples *a posteriori* corroborates our use of the qPCR technique to estimate and compare microbial densities). The analysis of the field samples corroborated this finding. Within the 50 months separating the two sampling points, roughly corresponding to 55 generations (13 generations per year (Lenormand *et al.*, 1999)), the *Wolbachia* load of insecticide-resistant mosquitoes had evolved to levels comparable to those of insecticide-susceptible mosquitoes. The relative rate of evolution of *Wolbachia* loads in the laboratory vs field populations would depend on (1) whether the rate of the process is mostly limited by the rate of *de novo* mutations or by the strength of selection; (2) the control of *Wolbachia* density (host factors, bacterial factors, interaction between the two). It is highly likely that evolution is mostly limited by *de novo* mutations in the laboratory, because all strains share the same cytoplasmic host and bacterial backgrounds as well as most of the nuclear genomic background. Moreover, in laboratory populations a given cytoplasmic background is confronted with the same nuclear background in each generation. This is not the case in field populations, which are polymorphic for insecticide resistance, where a given cytoplasm may alternate between insecticide-resistant and -susceptible nuclear genomic backgrounds over generations. The strength of selection in favor of *Wolbachia* density control mechanisms is thus probably weaker in the field, although, because of the huge population densities and high migration rates that these mosquitoes exhibit, mutation is probably less limiting.

We cannot tell whether the rapid evolution we observe is due to changes in the host or the bacterial genome, or to coevolution between the two. Weeks *et al.* (2007) were able to demonstrate that in their case evolution of the bacteria was responsible for the observed changes. Future studies are needed to elucidate the mechanisms underlying the rapid evolution of *Wolbachia* density in insecticide-resistant mosquitoes, as well as to verify that the decrease in density has resulted in a decrease of associated physiological costs. Nevertheless, this study, in agreement with the studies on *Wolbachia* in *Drosophila* (McGraw *et al.*, 2002; Weeks *et al.*, 2007) and in a nymphalid butterfly (Hornett *et al.*, 2006; Charlat *et al.*, 2007), demonstrates that host–*Wolbachia* interactions can evolve extremely rapidly.

Acknowledgements

References

Berticat C, Rousset F, Raymond M, Berthomieu A, Weill M (2002). High *Wolbachia* density in insecticide-resistant mosquitoes. *Proc Biol Sci* **269**: 1413–1416.
Bourtzis K, Dobson SL, Braig HR, O'Neill SL (1998). Rescuing *Wolbachia* have been overlooked. *Nature* **391**: 852–853.
Charlat S, Hornett EA, Fullard JH, Davies N, Roderick GK, Wedell N *et al.* (2007). Extraordinary flux in sex ratio. *Science* **317**: 214.
Charlat S, Hurst GD, Mercot H (2003). Evolutionary consequences of *Wolbachia* infections. *Trends Genet* **19**: 217–223.
Dobson SL, Bourtzis K, Braig HR, Jones BF, Zhou W, Rousset F *et al.* (1999). *Wolbachia* infections are distributed throughout insect somatic and germ line tissues. *Insect Biochem Mol Biol* **29**: 153–160.
Duron O, Bernard C, Unal S, Berthomieu A, Berticat C, Weill M (2006a). Tracking factors modulating cytoplasmic incompatibilities in the mosquito *Culex pipiens*. *Mol Ecol* **15**: 3061–3071.
Duron O, Fort P, Weill M (2006b). Hypervariable prophage WO sequences describe an unexpected high number of *Wolbachia* variants in the mosquito *Culex pipiens*. *Proc Biol Sci* **273**: 495–502.
Duron O, Labbe P, Berticat C, Rousset F, Guillot S, Raymond M *et al.* (2006c). High *Wolbachia* density correlates with cost of infection for insecticide resistant *Culex pipiens* mosquitoes. *Evolution* **60**: 303–314.
Georghiou GP, Metcalf RL, Gidden FE (1966). Carbamate-resistance in mosquitos. Selection of *Culex pipiens fatigans* Wiedemann (=*C. quinquefasciatus* Say) for resistance to Baygon. *Bull World Health Organ* **35**: 691–708.
Hornett EA, Charlat S, Duplouy AM, Davies N, Roderick GK, Wedell N *et al.* (2006). Evolution of male-killer suppression in a natural population. *PLoS Biol* **4**: e283.
Lenormand T, Bourguet D, Guillemaud T, Raymond M (1999). Tracking the evolution of insecticide resistance in the mosquito *Culex pipiens*. *Nature* **400**: 861–864.
McGraw EA, Merritt DJ, Droller JN, O'Neill SL (2002). *Wolbachia* density and virulence attenuation after transfer into a novel host. *Proc Natl Acad Sci USA* **99**: 2918–2923.
Min KT, Benzer S (1997). *Wolbachia*, normally a symbiont of *Drosophila*, can be virulent, causing degeneration and early death. *Proc Natl Acad Sci USA* **94**: 10792–10796.
Mouton L, Dedeine F, Henri H, Bouletreau M, Profizi N, Vavre F (2004). Virulence multiple infections and regulation of symbiotic population in the *Wolbachia-Asobara tabida* symbiosis. *Genetics* **168**: 181–189.

Mouton L, Henri H, Bouletreau M, Vavre F (2006). Effect of temperature on *Wolbachia* density and impact on cytoplasmic incompatibility. *Parasitology* **132**: 49–56.

Mouton L, Henri H, Charif D, Bouletreau M, Vavre F (2007). Interaction between host genotype and environmental conditions affects bacterial density in *Wolbachia* symbiosis. *Biol Lett* **3**: 210–213.

Noda H, Koizumi Y, Zhang Q, Deng K (2001). Infection density of *Wolbachia* and incompatibility level in two planthopper species, *Laodelphax striatellus* and *Sogatella furcifera*. *Insect Biochem Mol Biol* **31**: 727–737.

Poinsot D, Mercot H (1997). *Wolbachia* infection in *Drosophila simulans*: does the female host bear a physiological cost? *Evolution* **51**: 180–186.

Sokal RR, Rohlf FS (2003). *Biometry: 3rd Edition*. WH Freeman and Company: New York, NY.

Vavre F, Fleury F, Varaldi J, Fouillet P, Bouletreau M (2002). Infection polymorphism and cytoplasmic incompat-ibility in Hymenoptera-*Wolbachia* associations. *Heredity* **88**: 361–365.

Weeks AR, Turelli M, Harcombe WR, Reynolds KT, Hoffmann AA (2007). From parasite to mutualist: rapid evolution of *Wolbachia* in natural populations of *Drosophila*. *PLoS Biol* **5**: 997–1005.

Weill M, Berticat C, Raymond M, Chevillon C (2000). Quantitative polymerase chain reaction to estimate the number of amplified esterase genes in insecticide-resistant mosquitoes. *Anal Biochem* **285**: 267–270.

Weill M, Malcolm C, Chandre F, Mogensen K, Berthomieu A, Marquine M *et al*. (2004). The unique mutation in ace-1 giving high insecticide resistance is easily detectable in mosquito vectors. *Insect Mol Biol* **13**: 1–7.

Werren JH (1997). Biology of *Wolbachia*. *Annu Rev Entomol* **42**: 587–609.

Yen JH, Barr AR (1973). The etiological agent of cytoplasmic incompatibility in *Culex pipiens*. *J Invertebr Pathol* **22**: 242–250.