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Influence of aging on cytoplasmic incompatibility, sperm modification and *Wolbachia* density in *Culex pipiens* mosquitoes

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Wolbachia are maternally inherited endocellular bacteria, widespread in invertebrates and capable of altering several aspects of host reproduction. Cytoplasmic incompatibility (CI) is commonly found in arthropods and induces hatching failure of eggs from crosses between *Wolbachia*-infected males and uninfected females (or females infected by incompatible strains). Several factors such as bacterial and host genotypes or bacterial density contribute to CI strength and it has been proposed, mostly from *Drosophila* data, that older males have a lower *Wolbachia* load in testes which, thus, induces a lighter CI. Here, we challenge this hypothesis using different incompatible *Culex pipiens* mosquito strains and show that CI persists at the same intensity throughout the mosquito life span. Embryos from incompatible crosses showed even distributions of abortive phenotypes over time, suggesting that host ageing does not reduce the sperm-modification induced by *Wolbachia*. CI remained constant when sperm was placed in the spermathecae of incompatible females, indicating that sperm modification is also stable over time. The capacity of infected females to rescue CI was independent of age. Last, the density of *Wolbachia* in whole testes was highly strain-dependent and increased dramatically with age. Taken together, these data stress the peculiarity of the *C.pipiens*/*Wolbachia* interaction and suggest that the bacterial dosage model should be rejected in the case of this association.
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Introduction

Cytoplasmic incompatibility (CI) caused by *Wolbachia*, a genus of maternally inherited α-proteobacteria, is a common phenomenon in arthropods (reviewed in Werren, 1997; Stouthamer *et al*., 1999). CI results from an inappropriate interaction between sperm and eggs, which leads to embryonic mortality in diploid species or to the production of male excess in haplodiploid species (reviewed in Tram *et al*. (2003)). CI occurs when infected males mate either with uninfected females or with females infected by incompatible *Wolbachia* strains. This has been usually interpreted as a result of two bacterial components, a *mod* function (for modification), which induces embryo death and a *resc* function (for rescue), which restores compatibility and is provided by the *Wolbachia* present in the egg (for critical approach, see Poinsot *et al*. (2003). As *Wolbachia* are found in testes but absent from mature sperm, it has been proposed that *Wolbachia* induce CI during sperm development (Bressac and Rousset, 1993; Clark *et al*., 2002), impairing the male pronucleus but not external sperm components (Presgraves, 2000). In incompatible crosses, the paternal chromosomes do not accurately segregate during the first zygotic mitosis, leading to aneuploid or haploid embryos blocked at distinct developmental stages (reviewed in Tram *et al*., 2003). In the mosquito *Culex pipiens*, incompatible crosses with uninfected females produced only embryos whose development fail at a very early stage whatever the *Wolbachia* variant infecting males (Duron and Weill, 2006a). By contrast, all incompatible crosses with infected females produce frequently embryos blocked at later developmental stages (Duron and Weill, 2006a).

The exact mechanisms by which *Wolbachia* induce CI are still unknown. Several factors have been found to modulate CI strength (i.e. egg hatchability), such as bacterial and host genotypes or bacterial density (reviewed in Weeks *et al*., 2002) and these factors may interact in complex ways. *Wolbachia* variants can act independently of the host genome (Montchamp-Moreau *et al*., 1991; Hoffmann *et al*., 1996; Duron *et al*., 2006b), but the host genome may also modulate CI expression (Boyle *et al*., 1993; Poinsot *et al*., 1998; Sinkins *et al*., 2005). CI intensity has been shown to decrease with male ageing in several hosts, such as the fruit fly (Turelli and Hoffmann, 1995; Reynolds and Hoffmann, 2002), the planthopper *Laodelphax striatellus* (Noda *et al*., 2001) and the mosquitoes *Aedes albopictus* (only if monoinfected, see Kittayapong *et al*., 2002) and *Armigeres sublbatus* (Jamnongluck *et al*., 2000). In contrast, no male ageing effect has been found in the planthopper *Sogatella furcifera* (Noda *et al*.,

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2001) nor in *Ae. albopictus* superinfected by two *Wolbachia* strains (Kittayapong *et al*., 2002). In *Drosophila*, in parasotoid wasps and in the Mediterranean flour moth, the lighter CI found in aged males is associated with a lower *Wolbachia* density, in particular in testes (Binnington and Hoffmann, 1989; Breeuwer and Werren, 1993; Bressac and Rousset, 1993; Clark *et al*., 2002, 2003; Ikeda *et al*., 2003; Veneti *et al*., 2003).

In *C. pipiens*, Singh *et al*. (1976) showed that ageing reduced CI strength, whereas Rasgon and Scott (2003) found no effect. However, both studies were based on a single unidirectional cross, which does not allow one to draw general conclusions. In this species, nothing is known on the variation of the *Wolbachia* density with ageing, except that it is higher in adults than in larvae (Berticat *et al*., 2002). Here, we address the influence of ageing in *C. pipiens* males and females and the stability of sperm modification in both compatible and incompatible crosses between a large set of infected and uninfected laboratory strains. Field-collected mosquitoes were also used in experiments for testing the influence of environment on CI expression. In parallel, *Wolbachia* density in whole testes was estimated in two strains at different ages and was compared to other species. The data are discussed in the light of current hypotheses on the *modresc* system of *Wolbachia*.

Materials and methods

Mosquito strains

We used five infected *C. pipiens* laboratory strains of different forms, geographical origins and *Wolbachia* endosymbionts. Slab (Georghiou *et al*., 1966) and MaClo (Duron *et al*., 2006c) are *C. p. quinquefasciatus* collected in California in 1954 and 1984, respectively. LaVar (Duron *et al*., 2005) is a *C. p. pipiens* collected in France in 2003. Istanbul (Duron *et al*., 2005) and Tunis (Ben Cheikh *et al*., 1998) are *C. p. molestus* collected in Turkey in 2003 and in Tunisia in 1992, respectively. *Wolbachia* variants infecting these strains were found genetically different using the Tr1 transposable element (Duron *et al*., 2005) and WO prophage markers (Duron *et al*., 2006c). Field-caught *C. p. pipiens* pupae were collected in Viols-le-Fort (South of France) during summer 2005 and reared in the lab for emergence. Virgin males (VLF-05) were next used in crossing experiments. The uninfected SlabTC strain was generated by tetracycline treatment of the Slab strain as described in Duron *et al*. (2006d). To obviate artefactual effects of tetracycline on hatching rates or embryo phenotypes, the SlabTC strain was reared for at least four generations before crosses under standard laboratory tetracycline-free conditions. Mosquitoes were maintained in the laboratory at $23 \pm 2^{\circ}$C.

Molecular analysis

Mosquito DNA was extracted using the CTAB protocol (Rogers and Bendich, 1988). The infection status of VLF-05 and SlabTC samples was checked by *wsp* gene amplification using the specific primers wolpipdir and wolpiprev described by Berticat *et al*. (2002). DNA quality was controlled by amplifying the acetylcholinesterase *ace-2* gene, as described in Weill *et al*. (2000).

Measuring *Wolbachia* density

Testes of adult *C. pipiens* are pear-shaped bodies situated dorso-laterally in the fifth and sixth abdominal segments (Clements, 1992). Testes were collected by dissecting 2- and 30-day old males of Tunis and MaClo strains. DNA was extracted as described previously. Real-time quantitative PCR was carried out on a Roche Light Cycler to estimate the number of *Wolbachia* per mosquito testes. Two PCRs were performed on each mosquito, one specific of the host *ace-2* locus and the other specific of the *Wolbachia wsp* locus. Specific primers and procedures are described in Berticat *et al*. (2002). Standard curves were carried out using dilutions of a pBluescriptKS vector containing a single *ace-2* and *wsp* gene copy. Each DNA template was analysed in triplicate for *wsp* and *ace-2* quantification. As both genes are present as single copies per haploid genome, the ratio between the *wsp* and *ace-2* signals allows to estimate the relative number of *Wolbachia* genomes per *Culex* genome, thus correcting for mosquito size and DNA quality.

Crossing experiments

Crossing relationships between the Slab, MaClo, LaVar, Tunis and Istanbul strains have been fully characterized and show a reproducible pattern of compatible or incompatible crosses when 2–5-day-old adults are mated (Duron *et al*., 2006b). Reciprocal mass crosses between 10 and 25 males and females reared in controlled conditions were used for each pair of strains. All individuals used were virgin. Age was assessed from the emergence for adults (day 0 = emergence) or from the copulation date for sperm (Table 1). Males can copulate only 24–48 h after emergence (Clements, 1992). Consequently, males used in this study were at least 2-day-old. Males and females were placed together only for 24 h to set the date of copulation. Females were blood-fed at least 4 days after copulation and allowed to oviposit on a water cup 5 days later. Egg-rafts (between 50 and 250 eggs per raft) were collected only during the 24 h following the introduction of the water cup. Each cross was characterized by (i) the

Table 1 Age of mosquito used in experiments

Effect measured	*Age at copulation*			*Age at fecundation*	
	Male	*Female*	*Sperm*	*Female*	*Sperm*
Male ageing					
Cross 1	2	2	0	11	9
Cross 2	10	2	0	11	9
Cross 3	30	2	0	11	9
Female ageing					
Cross 1[a]	2	2	0	11	9
Cross 2	2	9	0	18	9
Cross 3	2	16	0	25	9
Sperm ageing					
Cross 1[a]	2	2	0	11	9
Cross 2	2	2	0	18	16
Cross 3	2	2	0	25	23

Age of adults (in day) at the copulation is provided relatively to emergence from pupae (day $0_{\text{males, females}}$ = day of emergence). Age of sperm at the time of fecundation (~oviposition) is provided relatively to copulation date (Day 0_{sperm} = day of copulation).
[a]First crosses for each effect are equivalent.

mean proportion of hatched eggs which indicates the CI level; (ii) the total number of eggs; and (iii) the number of egg-rafts. The mean proportion of developed embryos and hatching rate were determined using a binocular magnifying loupe. Unhatched egg-rafts from infected mothers were checked for the presence of embryo 3 days after oviposition (compatible eggs need 36–48 h for hatching at 23°C) in order to assess correct insemination as described by Duron and Weill (2006a). Because no embryo development can be observed in crosses with uninfected females, their spermathecae were collected and dissected to check the presence of sperm. Egg-rafts from non-inseminated females were discarded.

Statistical analysis

We used generalized linear models (GLM) and Mann–Whitney tests to analyse hatching rates (HR) and the proportion of developed embryos (EMB) in egg-rafts. Each egg-raft was characterized by six to seven variables: HR, EMB, the paternal strain (MOD: seven levels), the maternal strain (RESC: six levels), the age of the male (AGM: three levels), the age of the sperm (AGS: three levels) and the age of the female (AGF: three levels) (for age levels, see Table 1). For the dependent variable HR and EMB, the linear models MOD × RESC × AGM × AGS × AGF and MOD × RESC × AGM were fitted, respectively. These models were simplified according to Crawley (1993). Normality of residuals of the minimal model was tested using a Shapiro–Wilk test. Calculations were performed using the R free software (R Development Core Team, 2004). *Wolbachia* density data in testes were analysed by a Mann–Whitney test.

Results

Compatibility status

Crossing data using 2-day-old adults from the six strains showed no significant differences with those described previously in Duron *et al*. (2006b). Incompatibility was never detected in intra-strain crosses, hatching rates all being over 90% (Table 2). Eight incompatible crosses were studied that produced none or few larvae (♀ Istanbul × ♂ Tunis and ♂ LaVar; ♀ Slab and ♀ SlabTC × ♂ MaClo ♂ Tunis and ♂ LaVar; Table 2). Males issued from field-caught pupae (♂ VLF-05) induced nearly 100% CI when crossed with ♀ Istanbul and ♀ SlabTC (Table 2). All ♂ VLF-05 ($n = 37$) were infected by *Wolbachia*, as monitored by *wsp* PCR analysis. Eggs from incompatible crosses were separated in three classes according to their phenotypes, as described in Duron and Weill (2006a). As expected, incompatible crosses between uninfected females and infected males all

Table 2 Incompatibility relationships between infected and uninfected (TC) strains in function of male ageing

Cross		*Hatching rate*		
		2-day-old	*10-day-old*	*30-day-old*
♀ Istanbul	♂ Istanbul	0.951 ± 0.061 (1402; 10)	—	0.940 ± 0.100 (902; 9)
♀ Istanbul	♂ Tunis	0.000 ± 0.000 (1230; 9)	0.000 ± 0.000 (848; 6)	0.004 ± 0.007 (807; 5)
♀ Istanbul	♂ LaVar	0.000 ± 0.000 (953; 7)	0.000 ± 0.000 (1291; 9)	0.000 ± 0.000 (601; 5)
♀ Istanbul	♂ VLF-05	0.002 ± 0.001 (1106; 7)	0.000 ± 0.000 (1402; 12)	0.000 ± 0.000 (301; 2)
♀ Slab	♂ Slab	0.969 ± 0.043 (2281; 15)	—	0.966 ± 0.031 (1558; 11)
♀ Slab	♂ Tunis	0.022 ± 0.054 (1637; 15)	0.038 ± 0.133 (1529; 13)	0.024 ± 0.057 (1155; 8)
♀ Slab	♂ MaClo	0.002 ± 0.005 (1761; 16)	0.000 ± 0.000 (1496; 12)	0.000 ± 0.000 (2402; 18)
♀ Slab	♂ LaVar	0.001 ± 0.003 (1266; 11)	0.000 ± 0.000 (1041; 10)	0.001 ± 0.003 (886; 7)
♀ SlabTC	♂ SlabTC	0.968 ± 0.014 (1661; 11)	—	0.969 ± 0.011 (2012; 15)
♀ SlabTC	♂ Tunis	0.000 ± 0.000 (2701; 10)	0.000 ± 0.000 (1582; 12)	0.000 ± 0.000 (1603; 12)
♀ SlabTC	♂ MaClo	0.000 ± 0.000 (1263; 10)	0.000 ± 0.000 (1468; 10)	0.000 ± 0.000 (1211; 9)
♀ SlabTC	♂ LaVar	0.000 ± 0.000 (1310; 11)	0.000 ± 0.000 (1112; 10)	0.000 ± 0.000 (347; 3)
♀ SlabTC	♂ VLF-05	0.000 ± 0.000 (720; 6)	0.000 ± 0.000 (420; 3)	0.000 ± 0.000 (934; 7)
♀ Tunis	♂ Tunis	0.935 ± 0.046 (991; 10)	0.901 ± 0.055 (1230; 15)	0.921 ± 0.078 (1102; 12)
♀ MaClo	♂ MaClo	0.950 ± 0.053 (1009; 8)	0.941 ± 0.080 (1620; 14)	0.943 ± 0.071 (1714; 14)
♀ LaVar	♂ LaVar	0.951 ± 0.071 (983; 6)	0.967 ± 0.069 (1448; 12)	0.960 ± 0.009 (1600; 13)

Two-day-old females have been used. Number of eggs and egg-rafts counted are in parenthesis.

Table 3 Proportion of developed embryos (second and third class) in incompatible crosses between infected and uninfected (TC) strains in function of male ageing

Cross		*Proportion of developed embryos*	
		2-day-old	*30-day-old*
♀ Slab	♂ Tunis	0.241 ± 0.110 (1637; 15)	0.294 ± 0.212 (1155; 8)
♀ Slab	♂ MaClo	0.474 ± 0.156 (1761; 16)	0.540 ± 0.184 (2402; 18)
♀ Slab	♂ LaVar	0.344 ± 0.187 (1266; 11)	0.300 ± 0.221 (886; 7)
♀ SlabTC	♂ Tunis	0.000 ± 0.000 (2701; 10)	0.000 ± 0.000 (1603; 12)
♀ SlabTC	♂ MaClo	0.000 ± 0.000 (1263; 10)	0.000 ± 0.000 (1211; 9)
♀ SlabTC	♂ LaVar	0.000 ± 0.000 (1310; 11)	0.000 ± 0.000 (347; 3)

Two-day-old females have been used. Number of eggs and egg-rafts counted are in parenthesis.

Table 4 Incompatibility relationships between infected strains in function of female ageing

Cross		*Hatching rate*		
		11-day old[a]	*18-day old*	*25-day old*
♀ Slab	♂ Slab	0.969±0.043 (2281; 15)	0.968±0.022 (814; 6)	0.980±0.012 (1340; 14)
♀ Slab	♂ Tunis	0.022±0.054 (1637; 15)	0.030±0.057 (836; 8)	0.035±0.066 (1145; 10)
♀ Slab	♂ MaClo	0.002±0.005 (1761; 16)	0.000±0.000 (1448; 12)	0.001±0.001 (1204; 11)

Two-day old males have been used.
[a]Results are the same to those in Table 1. Number of eggs and egg-rafts counted are in parenthesis.

produced only first class eggs, that is, identical to unfertilized eggs (Table 3). Second and third class eggs (i.e. containing embryos poorly developed or developed at any stage before hatching, respectively) were present in incompatible crosses involving infected females, although their frequency varied depending on strain combination (Table 3).

Ageing of males, females and sperm does not influence CI

Both compatible and incompatible crosses were repeated with older males, older sperm and older females in order to test the effect of ageing on CI strength. For male ageing, 2-, 10- and 30-day-old males were crossed with 2-day-old females (Table 1). For female ageing, 2-day-old males were crossed with 2-, 9- and 16-day-old females. At the time of fecundation, females were 11-, 18- and 25-day-old, respectively (Table 1). Lastly, to investigate sperm ageing, 2-day-old males and females were crossed and inseminated females were then divided in three groups: the first group was blood-fed at day 6, the second at day 11 and the third at day 18. Fecundation, that is, karyogamy with fusion of male and female pronuclei, occurs during the first hour following laying eggs (for a detailed synthesis, see Clements, 1992). At the time of fecundation (~time of oviposition), sperms were 11-, 16- and 23-day-old for the first, second and third groups, respectively (Table 1). Note that females here were 11-, 18- and 25-day-old, respectively (Table 1). Despite this large set of crosses, hatching rate (i.e. CI strength) was never found correlated with age, be it of males ($F=0.022$, $P=0.996$; Table 2), females ($F=0.014$, $P=0.994$; Table 4) or sperm ($F=0.220$, $P=0.832$, Table 5). In all incompatible crosses, hatching rate only correlated with the interaction between the nature of *Wolbachia* infecting males and females ($F=4.67$, $P=0.051$). In addition, the proportion of embryo class was measured in six incompatible crosses involving infected and uninfected females crossed with 2-day-old and 30-day-old infected males. Only first class eggs were observed when females were uninfected, and stable proportion of each class was observed within egg-rafts from infected females (Table 3). Again, proportion of embryos class was related to strain combination, that is, interaction between the nature of *Wolbachia* infecting males and females ($F=10.944$, $P=0.04$) but not to male ageing ($F=0.072$, $P=0.951$).

Wolbachia density in testes increased with ageing

In two strains inducing CI (Tunis and MaClo), we examined the density of *Wolbachia* in the testes of 8–10 individuals representing two different ages (Figure 1). In

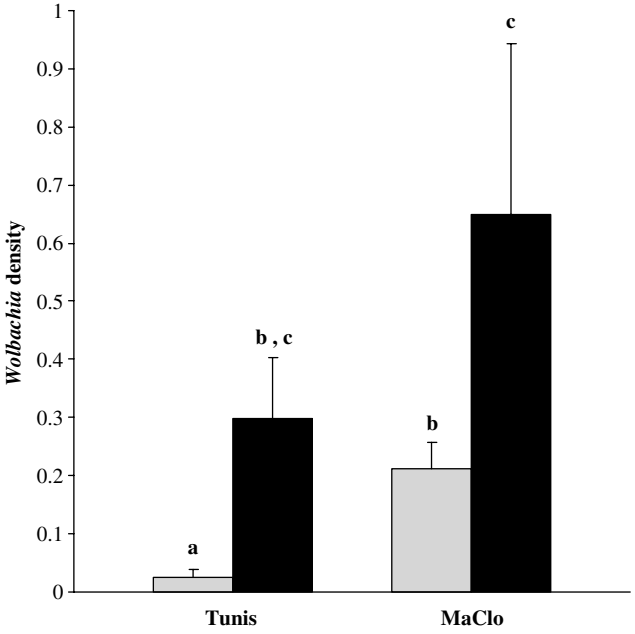

Figure 1 Variation of *Wolbachia* density in testes according to strain origin and male ageing. The *Wolbachia* density is provided by the ratio between the number of *Wolbachia* genomes relative to the *Culex* genomes (both estimated by real time quantitative PCR). Grey box, 2-day-old males; black box, 30-day-old males. a, b and c represent statistic groups.

2-day-old males, density was lower in Tunis males ($n=10$) than in MaClo males ($n=8$; $P>10^{-3}$). The difference was no longer significant in 30-day-old males ($n=9$ for Tunis and MaClo; $P=0.14$). Interestingly, in both strains, 30-day-old males displayed a *Wolbachia* density in testes significantly higher than two-day-old males ($P=0.01$ and 0.05, for Tunis and MaClo, respectively). *Wolbachia* density in testes thus varied widely depending on male ageing and mosquito strain.

Discussion

The aim of our study was to evaluate the contribution of host ageing as one of the life traits that maintain the high CI complexity observed in *C. pipiens* mosquitoes. Host ageing is a feature shown to affect CI in most species (Turelli and Hoffmann, 1995; Jamnongluck *et al*., 2000; Noda *et al*., 2001; Kittayapong *et al*., 2002; Reynolds and Hoffmann, 2002). We have sampled mosquitoes widely, from six distinct origins, including field-caught individuals, infected by at least five different *Wolbachia* variants. Nearly 100% hatching failure was observed in all incompatible crosses, in agreement with previous

C. pipiens CI data (Laven, 1967; Guillemaud *et al*., 1997; Rasgon and Scott, 2003; Duron *et al*., 2006b).

Ageing has no effect on CI strength

Unexpectedly, host ageing had no effect on CI rate. In contrast to most host species, *C. pipiens* males displayed a constant capacity over time to induce full CI, and females, to rescue CI. In addition, matings between infected males issued from field-caught pupae and lab strain females were unproductive, indicating that rearing conditions – field or laboratory – have little influence if any on CI strength, as reported for the superinfected mosquito *Ae. albopictus* (Kittayapong *et al*., 2002). Male ageing not only did not reduce hatching failure, but it also did not modify the distribution of aborted embryo classes. Indeed, CI may induce lethality at various developmental stages, from the immediate early case (class I), in which embryos are phenotypically similar to unfertilized eggs and are aneuploid, to later stages (class II and III), in which embryos present evidence of cell differentiation and are haploid (Callaini *et al*., 1996; Duron and Weill, 2006a). The absence of a male ageing effect on the distribution strongly suggests that the paternal contribution (*mod* factor) is stable over time. This is consistent with the long-term efficacy of sperm to induce CI, stable for at least 3 weeks before fecundation. The absence of a female ageing effect on hatching rate indicates that the *resc* factor is equally stable over time. The only significant variation in the distribution of aborted embryos correlated with the strain types, which indicates that CI rate in *C. pipiens* is mainly driven by *mod-resc* factors. The stability over time of *mod* and *resc* functions in *C. pipiens* has probably greatly favoured the spread of *Wolbachia* and contributed to its fixation (Duron *et al*., 2005). This contrasts with the situation in *Drosophila* wherein CI expression decreases with ageing, thus lowering the infection spread (Hoffmann *et al*., 1998).

Wolbachia density in whole testes increased with ageing

The observation that male ageing is associated with a lower *Wolbachia* load in testes is thought to result from a reduced number of infected spermatocytes, a factor critical for CI rate (Binnington and Hoffmann, 1989; Bressac and Rousset, 1993; Clark *et al*., 2002, 2003; Veneti *et al*., 2003). This is probably true for *Drosophila*, the biological model which provides almost all previous data, but this cannot be extended straightforwardly to *C. pipiens*, in which *Wolbachia* showed a three fold to 10-fold increase in density within whole testes as males aged from 2- to 30-day-old (Figure 1). The level of *Wolbachia* infection in testes is strain-dependent in *C. pipiens*, as illustrated by the MaClo and Tunis strains. Interestingly, whereas MaClo males are threefold more infected than Tunis males, this does not correlate with higher CI rates: ♂ MaClo and ♂ Tunis are compatible and incompatible with ♀ Istanbul, respectively (Duron *et al*., 2006b), whereas CI induced on ♀ Slab is less severe with ♂ Tunis than ♂ MaClo (see Table 2). In addition, we previously reported that CI rates remained constant whatever the *Wolbachia* density in males harbouring the same variant (Duron *et al*., 2006d). This could strengthen the notion that *Wolbachia* density is not a factor critical for CI rate in *C. pipiens* and suggest that the bacterial dosage model proposed by Breeuwer and Werren (1993) – according to which CI strength correlates with relative infection levels in males and females – cannot be applied to this species. However, in *Drosophila*, high CI rates correlate with high levels of *Wolbachia* only when spermatocytes and/or spermatids are infected whereas infection of somatic cyst cells, even at high levels, has no effect (Clark *et al*., 2003). The increase in *Wolbachia* observed in *C. pipiens* may affect either spermatocytes/spermatids or somatic cyst cells and the distrubtion of *Wolbachia* within tissues in testes is necessary to evaluate the evolution with age of the fraction that participates to CI.

In fact, different groups of associations can be detected, which need different threshold infection levels to express similar levels of CI (Bourtzis *et al*., 1996). Even if density increases with ageing in spermatocytes/spermatids, one cannot definitely rule out a threshold effect, above which CI rate does no longer correlate with density, as proposed for the wasp *Leptopilina heterotoma* (Mouton *et al*., 2006). For example, Kittayapong *et al*. (2002) could not detect a male ageing effect in superinfected *Ae. albopictus* but did find an effect of male ageing when using single-infected strain. This strain has been shown to be infected at much lower densities than superinfected strains (Sinkins *et al*., 1995) and might be more susceptible to density-related ageing effects. This might suggest that the *Wolbachia* density in *C. pipiens* males is always sufficient to induce almost complete CI, contrasting with the *Drosophila* situation. Indeed, It has been also shown in *Drosophila* that higher *Wolbachia* loads are associated with higher costs, as exemplified by a lower sperm production in infected flies vs uninfected ones (Snook *et al*., 2000). This does not seem to be the case in *C. pipiens*, as compatible crosses involving infected or uninfected as young or aged males produce same proportion of fertilized eggs (Table 2). This result suggests that sperm production and quality seem not to be sufficiently affected by the density of *Wolbachia* to induce a decrease in egg hatchability. Another possibility is that the variations of *Wolbachia* density could be

Table 5 Incompatibility relationships between infected strains in function of sperm ageing

Cross		*Hatching rate*		
		9-day-old[a]	*16-day-old*	*23-day-old*
♀ Slab	♂ Slab	0.969±0.043 (2281; 15)	0.980±0.019 (2507; 19)	0.957±0.034 (1045; 11)
♀ Slab	♂ Tunis	0.022±0.054 (1637; 15)	0.017±0.088 (1171; 10)	0.024±0.049 (703; 6)
♀ Slab	♂ MaClo	0.002±0.005 (1761; 16)	0.001±0.002 (1059; 9)	0.001±0.001 (1183; 11)

[a]Results are the same to those in Table 1. Number of eggs and egg-rafts counted are in parenthesis.

associated with phage WO density as observed in *Nasonia* wasp (Bordenstein *et al*., 2006). Thus, phages might replicate independently from *Wolbachia* and play a significant role in the expression of CI.

In conclusion, the identification of factors that modulate CI rate remains a pivotal step toward the understanding of the basic mechanisms responsible for incompatibility. Our data in *C. pipiens* differ from those obtained in other insect species, suggesting that hypotheses drawn from the *Drosophila* model cannot be generalized directly. Host ageing and probably bacterial density appear to contribute relatively little to CI strength, compared to the major influence of the endosymbiont genotype. Additional experiments such investigating the distribution within tissues are nonetheless needed to definitely exclude bacterial density as driving forces of CI in *C. pipiens*. It would appear wise to conduct experimental studies on a wide range of hosts/*Wolbachia*/phage associations before constructing a general model of the host-reproductive parasite interactions.

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