**Wolbachia Mediate Variation of Host Immunocompetence**

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**Abstract**

**Background:** After decades during which endosymbionts were considered as silent in their hosts, in particular concerning the immune system, recent studies have revealed the contrary. In the present paper, we addressed the effect of *Wolbachia*, the most prevalent endosymbiont in arthropods, on host immunocompetence. To this end, we chose the *A. vulgare-Wolbachia* symbiosis as a model system because it leads to compare consequences of two *Wolbachia* strains (*wVulC* and *wVulM*) on hosts from the same population. Moreover, *A. vulgare* is the only host-species in which *Wolbachia* have been directly observed within haemocytes which are responsible for both humoral and cellular immune responses.

**Methodology/Principal Findings:** We sampled gravid females from the same population that were either asymbiotic, infected with *wVulC*, or infected with *wVulM*. The offspring from these females were tested and it was revealed that individuals harbouring *wVulC* exhibited: (i) lower haemocyte densities, (ii) more intense septicaemia in their haemolymph and (iii) a reduced lifespan as compared to individuals harbouring *wVulM* or asymbiotic ones. Therefore, individuals in this population of *A. vulgare* appeared to suffer more from *wVulC* than from *wVulM*. Symbiotic titer and location in the haemocytes did not differ for the two *Wolbachia* strains showing that these two parameters were not responsible for differences observed in their extended phenotypes in *A. vulgare*.

**Conclusion/Significance:** The two *Wolbachia* strains infecting *A. vulgare* in the same population induced variation in immunocompetence and survival of their hosts. Such variation should highly influence the dynamics of this host-symbiont system. We propose in accordance with previous population genetic works, that *wVulM* is a local strain that has attenuated its virulence through a long term adaptation process towards local *A. vulgare* genotypes whereas *wVulC*, which is a widespread and invasive strain, is not locally adapted.

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**Introduction**

Investigations on the consequences of endosymbionts on their host's fitness have revealed that some of them exhibit variable effects, blurring the distinction between mutualism and parasitism. It is now admitted that symbionts are deeply involved in the evolutionary process of their hosts and that variations in symbiont genotypes may trigger more important differences in host life history traits than variations of the host genotypes themselves [1]. Thus, the entity that undergoes selection is clearly the extended phenotype of symbionts in their host. Within such a conceptual framework, the studies that focus on an understanding of host population dynamics need to consider symbiosis as a central parameter. Of the endosymbionts known to exhibit various effects on their host fitness, *Wolbachia* are the most prevalent in arthropods. The diversity of the interactions between *Wolbachia* and their hosts is mainly illustrated by the various strategies these endosymbionts exhibit in order to secure their vertical transmission. Hence, in some host species *Wolbachia* decrease the fitness of uninfected individuals (i.e. cytoplasmic incompatibility) while in others they increase female ratio in populations (i.e. parthenogenesis, male-killing and feminization) [2,3]. As the transmission of *Wolbachia* is vertical, their fitness is directly linked to the fitness of their hosts. Therefore, such situations could be seen as favourable to evolve towards obligate symbiosis and therefore mutualism. However, such obligate *Wolbachia* symbioses have only been described in filarial nematodes and in the parasitic wasp *Asobara tabula* [4,5]. In both models, aposymbiotic females failed to produce mature oocytes showing that *Wolbachia* are obligate for reproduction [4,5]. Other studies have shown that *Wolbachia* may be mutualists by improving development, survival and reproduction of their hosts [6–10]. However, these effects can vary over time or with respect to host genotypes and cause continuous evolutionary changes [7,10].

Despite these few examples of dependency or mutualism between *Wolbachia* and their hosts, *Wolbachia* are mostly described as facultative endosymbionts that negatively influence their hosts' life history traits, including body size [11], fecundity [11–13],
survival [13–15], larval competitiveness [16], male fertility and sperm cyst production [17] and mating choice [18]. Recently, Fytrou et al. (2006) [19] hypothesized that Wolbachia may also immunodepress their hosts. Indeed, they observed that Drosophila infected by Wolbachia showed less encapsulation of parasitic wasp eggs than cured ones. The capacity of Wolbachia to interact with the arthropod immune system has also been recently suggested by (i) the discovery of an intracellular sensor of Gram (c) bacteria in Drosophila and (ii) the observed modifications of the immune response in Drosophila melanogaster and Aedes aegypti cell lines due to the presence of Wolbachia [20–22]. Moreover, molecular evolution studies within Wolbachia-infesting insects have revealed that the Wolbachia outer membrane protein wsp, which has been shown to play a role in filarial nematode infection success [23], is under strong positive selection thus suggesting that invertebrate immune response may be an important selection factor for Wolbachia [24]. In invertebrates, immune cells (i.e. haemocytes) are essential effectors of immunity [20]. They are responsible for both cellular (encapsulation, phagocytosis etc.) and humoral (antimicrobial peptides, phenoloxidase cascade etc.) responses. Wolbachia have been observed within host haemocytes in only one species: the terrestrial isopod Armadillidium vulgare [25]. In light of this observation and also due to the even more central role of haemocytes in crustacean immune systems [26], the A. vulgare-Wolbachia association appears to be a very pertinent biological model to study the influence of symbiosis on host immunocompetence. In addition, A. vulgare is of particular interest because individuals from the same population are mono-infected (to date, no naturally co-infected individuals have been observed) by one of three different strains of Wolbachia (wVulM, wVulC and wVulP) [27,28]. wVulP, which has only been identified recently and which is less prevalent than the others, seems to be the result of a recombination event between wVulM and wVulC suggesting that co-infections do occur but are unstable. This would explain why co-infected individuals are never observed in sampled populations [28]. Both wVulC or wVulM are feminizing strains since offspring from sampled A. vulgare females are highly female-biased [27]. A. vulgare lineages infected with wVulC or wVulM are maintained in laboratory for decades showing that the symbiotic transmission and feminizing phenotypes of these two Wolbachia strains persist through generations (Bouchon et al., unpublished data). However, despite these similarities, population genetic studies suggest that wVul strains exhibit different strategies [27,29]. wVulM would be the most invading strain able to replace previous Wolbachia strains including wVulM [27,29], whereas wVulM would be the resident strain, more locally adapted to host genotypes. As immunocompetence is obviously a primordial parameter in host dynamics, it is thus of great interest to compare the effect of these two Wolbachia strains on host immune capacities.

In the present study, a comparison was made of the extended phenotype of wVulM and wVulC in A. vulgare. To this end, we evaluated in A. vulgare individuals infected by wVulC or wVulM: (i) the titer of Wolbachia in ovaries by qPCR; (ii) the presence of Wolbachia in haemocytes by electronic microscopy; (iii) the density of haemocytes; (iv) the intensity of natural septicemia (i.e. number of CFU obtained from haemolymph); (v) their survival over a 7 month period. These results were used to detect differences in immunocompetence and survival of A. vulgare as a function of Wolbachia genotype.

Materials and Methods

A. vulgare lineages

Gravid females (F0) of A. vulgare were sampled in the natural park of Chizé (Western France 46°06′05″N-0°24′21″W) and brought back to the laboratory. The infection status of each gravid female (infected with Wolbachia wVulC or wVulM or asymbiotic i.e. noninfected by Wolbachia) was determined as described below. To avoid any maternal effect in further experiments at least three gravid females (F0) for each infection status were used to start lineages. Their offspring (F1) were born and reared in the laboratory. For each infection status, one hundred virgin females from the F1 generation were kept and placed individually with one male (F1) in individual boxes. Over a 7 month period, the survival of these F1 females was monitored every three days and their progenies (F2) were collected. The virgin F2 females grew during two years before immunocompetence experiments. In order to simplify the reading of the paper, virgin asymbiotic females were called A females, virgin females infected with wVulC were called C females, virgin females infected with wVulM were called M females. All of these lineages were grown at 20°C on moistened potting mix derived from peat from sphagnum moss (pH = 6.4 and conductivity = 50.0 mS/m) with dead leaves and carrot slices as a food source.

Additionally, a lineage of females experimentally infected by wVulC (herein called injC females) was created. For this, the ovaries of 10 C females (F0) were collected and crushed into 1ml of Ringer solution. The resulting suspension was filtered through a 1.2 μm pore membrane, and 1 μl of filtrate injected into non gravid A females (F0) using a thin glass needle (Bouchon et al., 1998). F0 injC females were then crossed with asymbiotic males for two generations in order to produce two year old F2 virgin injC females.

F2 females (A, C, M) were used to assess: Wolbachia titer, haemocytes density and intensity of natural septicemia. Due to a small number of individuals, injC females (F2) were only used to highlight the assessment of the effect of wVulC on haemocyte density.

Infection status and Wolbachia titer

The infection status of each A. vulgare female used in experiments was determined by a PCR-RFLP assay. Individuals were dissected and total DNA extracted from the ovary as previously described [30]. PCR amplifications were then performed to test for presence/absence of Wolbachia using specific primer sets for the wsp gene [31] and conditions as previously described [27]. In order to discriminate each Wolbachia strain, a PCR-RFLP test was performed based on the analysis of wVulC and wVulM wsp sequences (wVulM: AJ419984 and wVulC: AJ419987). Two restriction enzymes were used: BsuI, which cuts wsp amplicons in wVulC but not in wVulM, and MfeI, which cuts wsp amplicons in wVulM but not in wVulC.

Comparative analysis of the titer of Wolbachia in ovaries between C females and M females was performed using qPCR of the wsp gene. Total DNA from the ovaries of 20 females of each infection status were individually extracted [30]. To prepare the standard, 7 μl of purified wsp gene PCR product were directly ligated into a pGEM-T-easy vector (Promega) and one site was cut with NcoI, which cuts wsp amplicons in wVulC but not in wVulM, and MfeI, which cuts wsp amplicons in wVulM but not in wVulC.

Haemolymph sampling

Haemolymph was sampled in the same way for all following experiments: cuticles were disinfected by immersing individuals for
placed in wash buffer for 2h at 4

for 1 min, and then stained with lead citrate. Sections were contrasted by incubation in 1% uranyl acetate in 50% ethanol stained with 1% toluidin blue. Thin sections (90nm) were

resin (Spurr, Polyscience Inc.). Thick sections (0.5

graded series of acetone solutions, infiltrated, and embedded in 45 min. Haemocytes were subsequently dehydrated through a

inclusion in a 2% agar gel (37°C) and 1mm³ plugs were cut and placed in wash buffer for 2h at 4°C following which they were post fixed into 4% OsO₄, 0.3M sodium cacodylate, 5.5% NaCl for 45 min. Haemocytes were subsequently dehydrated through a graded series of acetone solutions, infiltrated, and embedded in resin (Spurr, Polyscience Inc.). Thick sections (0.5 μm) were stained with 1% toluidin blue. Thin sections (90nm) were contrasted by incubation in 1% uranyl acetate in 50% ethanol for 1 min, and then stained with lead citrate. Sections were observed using a transmission electronic microscope (JEOL 100C).

Haemocyte density in haemolymph

The haemolymph (10 μl) of 35 females of each infection status (A females, C females and M females) was individually sampled and added to 10 μl of MAS and 60 μl of 0.4% Trypan blue to discriminate dead haemocytes from living ones. The actual number of living haemocytes in each sample was evaluated using a Thoma counting chamber.

Natural septicaemia assessment

The haemolymph (10 μl) of 60 females of each infection status (A females, C females and M females) was individually sampled and added to 290 μl of LB medium. An aliquot of 100 μl of this suspension was streaked onto one plate of each of the three different solid agar media used: (i) a non selective chocolate medium (Biomérieux) on which most bacteria, even fastidious ones, can grow, (ii) the Columbia Nalidixic Acid Agar (CNA) (Biomérieux) in order to preferentially select Gram (+) bacteria and (iii) the Mueller-Hinton Agar (MHA) (Biomérieux) (53g/l) with 10% sheep’s blood and 10 μl/l vancomycin in order to preferentially select Gram (−) bacteria. After 3 days at 28°C, the number of colony forming units (CFUs) on each plate was determined.

Statistical analyses

All statistical analyses were performed using JUMP software (JMP, 2001, ver.4.03; SAS Institute, Cary, NC, USA). Survival estimates were assessed by a Kaplan-Meier analysis followed by a univariate Survival Analysis using a Wilcoxon test. As haemocyte density and natural septicaemia data showed homoscedasticity of variance (Levene test p>0.05), difference in mean responses was tested by an ANOVA followed by PLSD Fisher post-hoc test.

Results

Infection status and Wolbachia titer

All C, injC and M females used in this experiment were controlled positive for Wolbachia. The two strains of Wolbachia

exhibited a similar titer (~7,640×10⁶ bacteria per μg total DNA) in the host (ANOVA, F₁,₃₅ = 1.92, p = 0.17). However, aVulM tended to show higher titer than aVulC (Fig. 1).

Presence of Wolbachia in haemocytes

Wolbachia cells were observed by transmission electronic microscopy in haemocytes of all C and M females tested. In haemocytes, Wolbachia were included in a vacuole and did not seem to be undergoing any type of degradation process suggesting that they may survive and perhaps even multiply within such cells (Fig. 2).

Effect of Wolbachia on host survival

Comparison of survival plots between A females, C females and M females revealed significant differences (Wilcoxon test,
C Females survived significantly less (19% mortality, mean time before death = 177.4 ± 4.4 days) than A females (6% mortality, mean time before death = 189.1 ± 2.7 days) (Wilcoxon test, $\chi^2 = 9.39$ $df = 1$ $p = 0.002$) whereas survival of M females (11% mortality, mean time before death = 177.8 ± 2.1 days) was not significantly different from that of A females (Wilcoxon test, $\chi^2 = 4.05$ $df = 1$ $p = 0.044$). Finally, C females survived significantly less than M females (Wilcoxon test, $\chi^2 = 1.56$ $df = 1$ $p = 0.212$) (Fig. 3).

**Effect of Wolbachia on haemocyte density**

Global comparison of haemocyte densities in A females, C females, M females and injC females exhibited significant heterogeneity (ANOVA, $F_{3,141} = 18.91$, $p < 0.0001$) (Fig. 3). Statistical analysis revealed that A females exhibited significantly higher haemocyte densities (mean: 29,731 haemocytes per μl) than (i) C females (mean: 11,760 haemocytes per μl) (Fisher’s PLSD test: $p < 0.0001$), (ii) M females (mean: 22,805 haemocytes per μl) (Fisher’s PLSD test: $p = 0.0232$) and injC females (mean: 15,722 haemocytes per μl) (Fisher’s PLSD test: $p < 0.0001$). However, M females exhibited higher haemocyte densities than either C females (Fisher’s PLSD test: $p < 0.0001$) or injC females (Fisher’s PLSD test: $p < 0.009$) (Fig. 4). C females and injC females showed similar haemocyte densities (Fisher’s PLSD test: $p = 0.104$).

**Effect of Wolbachia on natural septicaemia**

On CNA [selective medium for Gram (+) bacteria], the mean number of CFUs obtained for haemolymph samples from (i) A females, (ii) C females and (iii) M females showed heterogeneity (ANOVA, $F_{2,174} = 3.961$, $p = 0.0208$). The mean CFUs was significantly higher in the haemolymph from C females (mean: 81 bacteria/μl) than in A females (mean: 18 bacteria/μl) (Fisher’s PLSD test: $p = 0.0133$) or M females (mean: 21 bacteria/μl) (Fisher’s PLSD test: $p = 0.0179$). Differences between mean CFUs on MHA [selective medium for Gram (-) bacteria] and chocolate medium (‘‘non-selective’’ medium) were not significant (ANOVA, $F_{2,174} = 0.769$, $p = 0.4650$ and $F_{2,174} = 2.850$, $p = 0.0606$, respec-

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**Figure 3. Effect of Wolbachia on host survival.** Comparison of survival plots between A females, C females and M females during 7 months revealed that C females survived significantly less than A females and M females.

**Figure 4. Effect of Wolbachia on haemocyte density.** Global comparison of haemocyte densities in haemolymph of A. vulgare females infected or not by Wolbachia revealed that A females exhibited significantly higher haemocyte densities than C females, M females and injC females.

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tively) (Fig. 5). However, in all media, C females tend to harbour more bacteria in their haemolymph than other females (Fig. 4).

Discussion

Infection dynamics of vertically transmitted endosymbiotic bacteria is highly dependant on their host’s reproductive success. However, symbionts such as *Wolbachia* often lead to physiological alterations which can negatively impact host fitness. Among potentially important effects, the impact on immunocompetence seems of particular interest in light of its fundamental role on host fitness. Two arthropod bacterial endosymbionts (*Serratia symbiotica* and *Hamiltonella defensa*) have been demonstrated to increase pea aphid resistance towards parasitoids showing that some vertically transmitted symbionts are able to improve immunocompetence [1,33]. For *Wolbachia*, which are the most frequent endosymbiotic bacteria in arthropods, two recent studies suggest that they may interact with the host immune system and thus modify the host’s ability to overcome infection by other parasites [19,22]. In the present study, we showed that reduction of haemocyte density in *A. vulgare* was due to *Wolbachia* and not to difference in host genotypes. Such reduction is an indication of immunodepression as haemocyte load is a determinant factor in the ability of crustaceans to mount an efficient immune response against parasites [26]. However, differences in the effects of the two *Wolbachia* strains on *A. vulgare* were observed: C females had less haemocytes but also more intense septicaemia than M females. These results highly suggest that haemocyte density and intensity of septicaemia are linked and that, in *A. vulgare*, *wVulC* is a more important immunodepressing biotic factor than *wVulM*.

Three non exclusive hypotheses can be proposed to explain how *wVulC* triggers a decrease in haemocyte density. A first hypothesis involves a direct negative effect of *Wolbachia* on haemocytes survival via toxins. Such *Wolbachia*-toxins could for example interfere with apoptosis in haemocytes as previously described for other cell types [34,35]. A second hypothesis is that the decrease in haemocyte density is due to the impact of *Wolbachia* load in haemocytes whereby high symbiotic densities in a cell would lead to its destruction as previously described in other tissues for *wPop* [36]. This hypothesis is further supported by previous studies in which *wVulC* has been shown to generate effects comparable to those of *wPop* when injected into foreign recipient hosts [3,37]. However, haemocytes observed here by transmission electronic microscopy mainly exhibited low bacterial loads. A third hypothesis would be that the global physiological cost of *Wolbachia* on their hosts leads to a decrease in their immunocompetence due to a drop in haemocyte production and a reduced capacity to cure bacteria from the haemolymph.

Our data revealed that the differences in immunocompetence and survival, in the population of *A. vulgare* we studied, are due to *Wolbachia* strains they harbour. The strain *wVulC* was the most immunodepressing and also reduced host lifespan the most, suggesting that these two life history traits may be linked and showing that *wVulC* is clearly more virulent than *wVulM*. This difference in virulence between *wVulC* and *wVulM* seems not due to different tier or location in haemocytes between these two *Wolbachia* strains but can be interpreted in the light of population genetic works conducted on the same populations [27,29]. Such studies showed that the *wVulC* strain was widely distributed and associated with all *A. vulgare* mitochondrial lineages while *wVulM* was restricted to particular host mitochondrial lineages. In the area where gravid females (i.e. F0) were sampled, the mitochondrial lineages associated with *wVulM* are very frequent [27,29]. Taking into account these data, Cordaux et al. (2004) proposed a scenario in which *wVulM* is a locally adapted strain (i.e. resident) while *wVulC* is invasive and widely distributed all over the world. Such a scenario, associated with expected evolutionary trends, would suggest that local adaptation occurred between *wVulM* and local host genotypes leading to the observed attenuation of its virulence compared to *wVulC*.

Even if virulence seems to decrease during local adaptation processes, we have demonstrated here that *Wolbachia* symbiosis is costly and can lead to a reduced lifespan for *A. vulgare*. It is hard to understand selective forces which would promote and maintain such genomic conflicts between symbionts and their hosts in the context of vertically transmitted symbioses. This discrepancy can be seen as the consequence of various strategies adopted by symbionts in order to invade host populations. While symbionts such as those in the pea aphid may spread by increasing their host’s immunocompetence [1,33], *Wolbachia* rely on manipulating host reproduction which can generate indirect costs. Such costs

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**Figure 5. Effect of *Wolbachia* on CFUs obtained from haemolymph samples.** Haemolymph samples from *A. vulgare* females infected or not by *Wolbachia* were streaked onto several agar media (CNA, MHA and Chocolate). On CNA [selective medium for Gram (+) bacterial], the mean number of CFUs obtained for haemolymph samples from C females was significantly higher than in A females or M females.

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would tend to keep the prevalence of Wolbachia at lower levels than those predicted by feminizing effects alone and could help explain the low frequency of symbiotic females observed in natural populations of A. vulgare [29].

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Author Contributions
Conceived and designed the experiments: CBV ML YC DB SS. Performed the experiments: CBV ML JH MJ SS. Analyzed the data: CBV ML MJ SS. Contributed reagents/materials/analysis tools: CBV JH. Wrote the paper: CBV ML MJ DB SS.