Interaction between host genotype and environmental conditions affects bacterial density in *Wolbachia* symbiosis

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Regulation of microbial population density is a necessity in stable symbiotic interactions. In *Wolbachia* symbiosis, both bacterial and host genotypes are involved in density regulation, but environmental factors may also affect bacterial population density. Here, we studied the interaction between three strains of *Wolbachia* in two divergent homozygous lines of the wasp *Leptopilina heterotoma* at two different temperatures. *Wolbachia* density varied between the two host genotypes at only one temperature. Moreover, at this temperature, reciprocal-cross F1 insects displayed identical *Wolbachia* densities, which were intermediate between the densities in the two parental lines. While these findings confirm that the host genotype plays an important role in *Wolbachia* density, they also highlight its interaction with environmental conditions, making possible the evolution of local adaptations for the regulation of *Wolbachia* density.

Keywords: host genotype; temperature; symbiosis; *Wolbachia*; density; quantitative PCR

1. INTRODUCTION

Symbiotic population density is a key factor of host-symbiont relationships, because it influences both the efficiency of transmission and the virulence of the symbiont. When symbionts are vertically transmitted, host and symbionts may act on the regulation of symbiont density to optimize the trade-off between these two parameters (Ebert & Bull 2003).

While theoretical works are numerous in this field, empirical studies are by far insufficient, especially in systems where symbionts are vertically transmitted. Recently, *Wolbachia* bacteria have become a recognized model system for the study of symbiotic regulation. *Wolbachia* are widespread bacterial endosymbionts of arthropods and filarial nematodes that display various types of interaction with their hosts, ranging from mutualism to reproductive parasitism, including cytoplasmic incompatibility (CI). Mechanisms that control *Wolbachia* density involve interactions between hosts

and bacteria (Kondo *et al.* 2005). Indeed, within a given multiply infected host, the density of each strain can differ, demonstrating that the *Wolbachia* genotype plays a role in the regulation process (Ijichi *et al.* 2002; Ikeda *et al.* 2003; Mouton *et al.* 2003, 2004). The involvement of the host genotype on the regulation of *Wolbachia* density has also been demonstrated, both at an interspecific level by transinfection experiments (Ikeda *et al.* 2003) and at an intraspecific level by cytological analysis in *Drosophila* species (Clark *et al.* 2003) and quantitative PCR in the adzuki bean beetle *Callosobruchus chinensis* (Kondo *et al.* 2005).

In addition to the genetic influence of both partners, environmental factors can also affect symbiont density. Among these factors, temperature is especially important in ectotherms, and its influence on host-symbiont interactions in general, and in *Wolbachia* symbiosis in particular, has been documented previously (reviewed in Thomas & Blanford 2003; Mouton *et al.* 2006). The combination of genetic and environmental factors can lead to complex genotype-by-genotype-by-environment interactions as already reported in other symbiotic systems (Greub *et al.* 2003), but data are lacking in *Wolbachia* symbiosis.

Here, we study the density of *Wolbachia* in two homozygous lines of the parasitic wasp *Leptopilina heterotoma* (Hymenoptera: Figitidae) at two different temperatures. These lines display a high level of genetic divergence (Fleury *et al.* 1995), but naturally harbour the same three CI-*Wolbachia* strains (Vavre *et al.* 1999). This study confirms the role of the host genotype on the bacterial population, but also demonstrates that environmental conditions may locally affect the evolution of the association, due to the existence of host genotype-by-environment interactions on the control of bacterial populations.

2. MATERIAL AND METHODS

(a) *Insect strains and rearing*

Leptopilina heterotoma, a solitary endoparasitoid wasp of *Drosophila* species, developed in a *Wolbachia*-free strain of *Drosophila melanogaster* originating from Lyon (France) on standard diet (David 1962) at 20 or 26°C with an LD cycle of 12 : 12 and 70% relative humidity.

Two inbred lines were used: A7, originated from Antibes (France, 43.5° N latitude), and SF4, originated from Lyon (France, 45.5° N latitude). These lines had been rendered homozygous by sib mating for 35 generations. They are infected with three CI-*Wolbachia* strains, *wLhet1*, *wLhet2* and *wLhet3*, which have, in both wasp lines, identical 16SrDNA, *ftsZ* and *wsp* gene sequences (Vavre *et al.* 1999; Mouton *et al.* 2005). Sequences of the ORF7 gene of the WO prophage, which has been shown to be highly efficient to distinguish *Wolbachia* strains (Duron *et al.* 2006), were identical in SF4 and A7 lines (Gavotte *et al.* 2004; H. Henri 2006, personal communication). Furthermore, A7 and SF4 individuals are fully mutually compatible.

(b) Quantification

DNA was extracted from the whole body of 5-day-old individuals using Chelex solution (Biorad) as in [Mouton *et al.* \(2003\)](#).

Wolbachia cell number was measured using the quantitative PCR LightCycler System (Roche Diagnostics). The *Wolbachia* surface protein gene *wsp* was amplified using either generalist primers or the specific primers of each *Wolbachia* strain as described in Mouton *et al.* (2003). *Wolbachia* load was calculated by correcting the numbers of *Wolbachia* cells by the fresh weight of each insect. This allows size variations of the wasps associated with their genetic background and their rearing temperature to be taken into account.

(c) *Experiment 1: influence of temperature on host genotype–Wolbachia strain interaction*

Host genotype–*Wolbachia* strain interaction
We studied the influence of host genotype on *Wolbachia* regulation within two environments by measuring the total and specific

Table 1. *Wolbachia* load in A7 and SF4 females. (Total number of *Wolbachia* cells per mg of wasp measured in *L. heterotoma* females of A7 and SF4 lines reared at 20 and 26°C (mean ($\times 10^3$) $\pm$ s.e.; $n=5$), and statistical analyses by two-way ANOVA (significant effects in bold). Each mean was compared with the other three by Student's *t*-test, and those marked with the same letter are not significantly different ($p=0.05$).)

	20°C	26°C
genotype		
A7	44.27 $\pm$ 7.25 (a)	80.35 $\pm$ 5.11 (b)
SF4	82.62 $\pm$ 5.90 (b)	77.39 $\pm$ 12.37 (b)
ANOVA		
genotype	$F_{1,16}=4.70$	$p=0.046$
temperature	$F_{1,16}=3.57$	$p=0.077$
interaction		
genotype $\times$ temperature	$F_{1,16}=6.41$	$p=0.022$

Wolbachia densities in female wasps of both strains reared either at 20 or 26°C (five individuals measured for each condition). Both lines had been kept at these temperatures for at least two generations before the experiments were performed.

(d) **Experiment 2: *Wolbachia* density in reciprocal F1 hybrids**

For each cross (parental lines and reciprocal F1), 1–2 day old virgin females were mated with 4–5 day old males (15 repetitions per condition). After mating (visually checked), three females were each provided with 100 *Drosophila* larvae and allowed to parasitize them for 24 h. After developing at 20°C, the wasps that emerged were individually tested to determine their *Wolbachia* load as above.

3. RESULTS

(a) **Experiment 1: influence of temperature on host genotype–*Wolbachia* strain interaction**

The *Wolbachia* load was determined in A7 and SF4 females at 20 and 26°C (table 1). At 20°C, SF4 females harbour more *Wolbachia* than A7 females, but in contrast there is no difference between the two genotypes at 26°C. This demonstrates a clear interaction between the host genotype and the environment (ANOVA2: $F_{1,16}=6.41$, $p=0.022$).

We also measured the specific load of the three *Wolbachia* strains (figure 1). Globally, the effect on each strain is comparable to the effect observed on total load with a reduced load only in A7 females reared at 20°C. Consequently, the proportion of each strain remains constant whatever the conditions (table 2). *wLhet3* accounted for the highest fraction of the total *Wolbachia* load, and *wLhet2* the lowest, which is consistent with data obtained previously at 20°C on A7 individuals (Mouton et al. 2003).

(b) **Experiment 2: *Wolbachia* density in reciprocal F1 hybrids at 20°C**

As in the first experiment, SF4 females harbour more *Wolbachia* than A7 females (Student's *t*-test: $p=0.0015$). The *Wolbachia* load of the two reciprocal F1 hybrid females are equivalent ($p=0.616$) and intermediate between the values found for the A7 and SF4 parent females (figure 2).

Figure 1. Load of each *Wolbachia* strain in A7 and SF4 females. Numbers of cells per mg host of each *Wolbachia* strain in five triply infected individuals of the A7 and SF4 genotypes reared at 20 or 26°C. In one modality, each symbol represents the *Wolbachia* density in one individual.

4. DISCUSSION

(a) **Influence of the host genotype on *Wolbachia* density**

At 20°C, SF4 females harbour more *Wolbachia* than A7 females, and reciprocal F1 hybrid females show the same level of infection, intermediate between the two parental lines. Thus, variation between the two insect lines must be attributable to intrinsic differences in the host genotype, as previously demonstrated by Kondo et al. (2005). The host may control the population of bacteria either directly, by limiting the nutrients provided to them or by actively eliminating bacterial cells, or indirectly through the response of the bacterial population to its own physiological state. Whatever the mechanism involved, it is clear that host genetic characteristics do influence *Wolbachia* density.

(b) **Influence of temperature on host genotype–*Wolbachia* strain interaction**

The higher *Wolbachia* density in SF4 than in A7 females is observed at 20°C but not at 26°C. This difference is due to the lack of any response of the SF4 females to temperature, since they harbour the same *Wolbachia* load at 20 and 26°C. On the other hand, the effect of the A7 genotype on *Wolbachia* differs between these two temperatures, suggesting a different ability to influence bacterial density according to temperature. Moreover, despite the fact that the relative proportions of the three strains do not vary with temperature conditions, they do not contribute equally to the total *Wolbachia* density since each strain has a specific density, indicating the role of their genotype on this basal level. These observations indicate that *Wolbachia* load depends on the *Wolbachia* genotype, the host genotype and the environment.

Table 2. Proportion of each *Wolbachia* strain in A7 and SF4 females. (Percentage of each *Wolbachia* strain measured in A7 and SF4 females of *L. heterotoma* reared at 20 or 26°C (mean $\pm$ s.e.; $n=5$). We fit a generalized linear model using two categorical explanatory variables, the host genotype and the temperature, considering a multinomial error structure and taking the log-linear function as the link function. Neither the factor genotype nor temperature added first to the null model reduced the deviance significantly. Therefore, there are no differences in the relative proportion of *Wolbachia* strains between the two genotypes and the two temperatures. Adding both factors together with or without their interaction gives the same results).

		percentage of each strain		
		<i>wLhet1</i>	<i>wLhet2</i>	<i>wLhet3</i>
temperature	genotype			
20°C	A7	38.42 $\pm$ 1.19	11.67 $\pm$ 1.60	49.91 $\pm$ 1.97
	SF4	36.99 $\pm$ 1.34	9.17 $\pm$ 0.98	53.83 $\pm$ 0.88
26°C	A7	32.59 $\pm$ 2.86	13.26 $\pm$ 0.58	54.15 $\pm$ 2.63
	SF4	37.02 $\pm$ 0.99	12.01 $\pm$ 0.80	50.96 $\pm$ 1.67
model		resid. dev.	resid. d.f.	p (chi)
null model		3153.27	38	
genotype (or temperature)		3149.98 (3150.03)	36	0.192 (0.198)
genotype + temperature		3147.54	34	0.295
interaction genotype $\times$ temperature		3144.60	32	0.231

Figure 2. *Wolbachia* load in A7, SF4 and F1 hybrid females at 20°C. A and S correspond to the A7 and SF4 genotypes, respectively. AS females derived from crosses between A7 females and SF4 males, and SA females from the reciprocal crosses. Bars show standard errors ($n=7$). Means indicated with the same letter are not significantly different (Student's t -test, $p=0.05$).

(c) How can we account for within-species differences in *Wolbachia*?

Two recent studies have demonstrated a positive correlation between *Wolbachia* density and the cost of infection (McGraw *et al.* 2002; Mouton *et al.* 2004). Moreover, temperature has been shown to play a major role in *Wolbachia* density and, as a consequence, in its transmission (Hurst *et al.* 2001). Influence of bacterial density on the phenotypic expression of the association, together with the influence of host genetic background on bacterial load in a given environment, provides the necessary conditions for the evolution of local adaptations. In the present case, the average annual temperatures of the original locations of insect lines are 20°C (Antibes, A7) and 17°C (Lyon, SF4), respectively (data: Météo France). Global physiological adaptation of the insects to local climatic differences could account for their differing capacities to regulate bacterial density in response to temperature change. It is of course premature to speculate about field

situations, and further experiments involving more than two populations of *L. heterotoma* and a wider range of temperatures are needed.

Bacterial density is of great importance in the evolution of symbiotic associations, and this study highlights that interactions between hosts and *Wolbachia* strongly depend on the host genotype, but also on the temperature conditions, as had been claimed previously (Weeks *et al.* 2002). Therefore, host genotype-by-environment interactions could play a major role in the expression of the phenotype, and thus on the evolution of host-*Wolbachia* associations.

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