

# Virulence, Multiple Infections and Regulation of Symbiotic Population in the *Wolbachia-Asobara tabida* Symbiosis

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## ABSTRACT

The density and regulation of microbial populations are important factors in the success of symbiotic associations. High bacterial density may improve transmission to the next generation, but excessive replication could turn out to be costly to the host and result in higher virulence. Moreover, differences in virulence may also depend on the diversity of symbionts. Using the maternally transmitted symbiont *Wolbachia*, we investigated how bacterial density and diversity are regulated and influence virulence in host insects subject to multiple infection. The model we used was the wasp *Asobara tabida* that naturally harbors three different *Wolbachia* strains, of which two are facultative and induce cytoplasmic incompatibility, whereas the third is necessary for the host to achieve oogenesis. Using insect lines infected with different subsets of *Wolbachia* strains, we show that: (i) some traits of *A. tabida* are negatively affected by *Wolbachia*; (ii) the physiological cost increases with the number of co-infecting strains, which also corresponds to an increase in the total bacterial density; and (iii) the densities of the two facultative *Wolbachia* strains are independent of one another, whereas the obligatory strain is less abundant when it is alone, suggesting that there is some positive interaction with the other strains.

THE genetic diversity of parasites co-infecting individual hosts is often thought to be an important factor in the evolution of their virulence (EWALD 1994; FRANK 1996a; GALVANI 2003). Theoretical studies have shown that lower relatedness among the parasites within the host could lead to increased virulence (FRANK 1994, 1996a; VAN BAALEN and SABELIS 1995). The classical idea, referred to as the “tragedy of the commons,” is that competition for a limiting resource puts faster exploiters at an advantage over more prudent ones (HARDIN 1968). However, recent articles have pointed out that in commonly used models, selection among parasites affects only the host exploitation rate, but that different outcomes could be reached when other types of competition are also considered (CHAO *et al.* 2000; READ and TAYLOR 2001; BROWN *et al.* 2002). For example, interference among parasites could lead to under-exploitation of hosts and hence to reduced virulence (CHAO *et al.* 2000). The relationship between virulence and multiple infection is thus still under debate and needs further empirical documentation.

So far these questions have received little study in vertically transmitted symbionts. There are two main

reasons for this. First, the vertical transmission of symbionts tends to limit multiple infection, because the oocytes of the host are colonized by only a few individuals, and the resulting bottleneck greatly reduces the genetic diversity of the symbiotic population (MIRA and MORAN 2002). Second, many vertically transmitted symbionts have evolved a mutualistic relationship with their hosts, and the benefit they confer considerably outweighs their cost; this makes the cost difficult to assess or even detect (THOMPSON 1988; BRONSTEIN 1994). However, these biological models could provide interesting data, because the closely linked evolutionary fates of host and symbionts should have led to the selection of mechanisms that reduce virulence to a minimum (LIPSITCH *et al.* 1995).

A good model for studying this question is the maternally transmitted symbiotic bacterium *Wolbachia*, which is able to induce cytoplasmic incompatibility (CI), leading to postzygotic reproductive isolation between any male infected by a *Wolbachia* strain and a female lacking this strain (for review see HOFFMANN and TURELLI 1997). This puts females with multiple infection at an advantage and promotes the spread and maintenance of multiple infection (FRANK 1998), which has proved rather common. Second, infected individuals benefit only indirectly from infection, as a result of the disadvantage suffered by uninfected females or females infected by only a subset of *Wolbachia* strains. Thus, the cost of

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infection (that we define here as the virulence of *Wolbachia*) and the advantage to the host are clearly distinguishable and can be measured separately in distinct individuals.

In this study, we used the association between *Wolbachia* and the parasitic wasp *Asobara tabida*, whose larvae develop as solitary endoparasites of *Drosophila* larvae. *A. tabida* individuals are co-infected by three different *Wolbachia* strains, *wAtab1*, *wAtab2*, and *wAtab3* (VAVRE *et al.* 1999). While both *wAtab1* and *wAtab2* are facultative parasites and induce CI (F. DEDEINE, personal communication), *wAtab3* is necessary for oogenesis to occur in this species: *A. tabida* females lacking *wAtab3* do not produce oocytes (DEDEINE *et al.* 2001). Using antibiotherapy, we created *A. tabida* lines that harbored different combinations of *Wolbachia* strains, but shared the same nuclear background.

This study addresses two main questions: (i) Is there any relationship between the diversity and virulence of *Wolbachia* and/or between *Wolbachia* density and virulence? and (ii) How are *Wolbachia* diversity and density regulated? The findings are discussed in the context of the evolution of virulence in multiple infections, by considering the peculiar selective pressures that act on this system.

## MATERIALS AND METHODS

**Insect strains and rearing:** *A. tabida* (Hymenoptera: Braconidae) is a solitary endoparasitoid wasp of several *Drosophila* species. In the laboratory, parasitoids are reared on a *Wolbachia*-free strain of *Drosophila melanogaster* originating from Ste Foy-les-Lyon (France). Rearing and experiments were carried out without larval or adult competition at 20° under 12/12 light/dark (LD) cycle and 70% relative humidity.

*A. tabida* individuals are naturally infected with three *Wolbachia* strains, named *wAtab1*, *wAtab2*, and *wAtab3* (VAVRE *et al.* 1999). The triply infected line, named Pi(123), is an inbred line originating from Pierrefeu (Pi), France, which has been maintained by regular sib-mating for four generations. Derived *A. tabida* lines with different infection statuses were obtained using moderate antibiotic treatments (F. DEDEINE, personal communication). Since *wAtab3* is obligatory for reproduction in this species, only one singly infected and two doubly infected lines could be obtained and have proved stable in time: the singly infected line Pi(3) harboring *wAtab3* and the doubly infected lines Pi(13) and Pi(23) harboring *wAtab3* and *wAtab1* and *wAtab3* and *wAtab2*, respectively.

**Components of the fitness cost of infection:** Several fitness traits have been measured in individuals of each line of *A. tabida*.

**Offspring production and sex ratio:** After mating with 3- to 4-day-old males with the same infection status as themselves (checked by individual visual inspection), 1- to 2-day-old females were each provided with 150 *Drosophila* larvae (24 hr old) and allowed to parasitize hosts for 48 hr. The infested host larvae were then reared and allowed to develop. At emergence, the adult *Drosophila* and the wasps of both sexes were counted in each vial. We performed three series (*i.e.*, blocks) to test these traits (at least seven replicates per line for each block).

**Egg production:** *A. tabida* females are mainly proovogenic,

and so most of their oocytes are already mature at emergence. To estimate the oocyte load, newly emerged females were kept for 5 days with water and honey to allow oocyte maturation to be completed. The ovaries were dissected in physiological saline, transferred into neutral red solution for 5 min, and then gently squashed between the slide and cover glass to disperse the eggs. The stained eggs were then counted under the microscope with the help of a video system.

**Tibia length:** Left hind tibia was measured on the adult males and females of each line using a micrometer.

**Dry weight:** Emerging parasitoids of both sexes were sampled in each line and dried at 65° for 48 hr before weighing.

**Adult ability to survive starvation:** Two-day-old males and females were put in vials with moist cotton, but without food. Mortality was checked every day at the same time ( $\pm 1$  hr) until all the individuals had died. Six vials, each containing 10 parasitoids, were studied for each sex in each line.

**Locomotor activity pattern:** Individual locomotor activity was monitored using a video-tracking and image-analysis system to provide automatic continuous measurements of the insects over several days (ALLEMANT *et al.* 1994). Individual insects were isolated in experimental circular glass arenas with honey as food. The locomotor activity of each individual was measured every 3 min using binary data (1 if the wasp had moved during a 2-sec video recording and 0 if it had not), and the hourly activity was calculated as the percentage of active recordings obtained over 3 days with a 12/12 hr LD cycle. To evaluate the average daily pattern of activity, two independent parameters were estimated for each individual: the rate of locomotor activity, calculated as the average of active recordings over a 24-hr period, and the profile of the rhythm, which establishes the pattern of the total daily activity in terms of the hourly percentages. The rate of locomotor activity measures the locomotor performance of wasps, while the activity profile determines how this activity is organized throughout the day. Data are reported in terms of the Zeitgeber time (Zt, time within the environmental cycle); the light is turned off at Zt0 and turned on at Zt12.

**Real-time quantitative PCR: DNA extraction:** Insects or parts of insects were individually squashed in 150  $\mu$ l 5% (w/vol) Chelex solution (Bio-Rad, Richmond, CA) and proteinase K (Eurobio, Les Ulis, France; final concentration 0.5  $\mu$ g/ $\mu$ l) and kept at 56° for 6 hr. After 15 min at 95°, the samples were centrifuged at 16,000  $\times g$  for 4 min.

**Primers:** Quantification of *Wolbachia* bacteria was achieved by amplifying the *Wolbachia* surface protein gene *wsp*. To detect all the *Wolbachia* strains present we used the general forward primers 81F: 5'-TGG TCC AAT AAG TGA TGA AGA AAC-3' (BRAIG *et al.* 1998). Specific PCR detection of each *Wolbachia* strain was conducted using three other forward primers: 165'F (5'-TGG TAT TAC AAA TGT AGC-3') for *wAtab1*, 172F (5'-ACC TAT AAG AAA GAC AAG-3') for *wAtab2* (ZHOU *et al.* 1998), and Aso3 (5'-AAA GGG GAC TGA TGA TGT-3') for *wAtab3*. All these forward primers were used with the same reverse primer 691R: 5'-AAA AAT TAA ACG CTA CTC CA-3' (ZHOU *et al.* 1998).

**Quantitative PCR:** Real-time quantitative PCR was performed using the LightCycler system (Roche). The 20- $\mu$ l reaction mixture consisted of 10% (vol/vol) LightCycler DNA master SYBR Green I (Roche Diagnostics), 3 mM MgCl<sub>2</sub>, 500 nM each primer, and 2  $\mu$ l of template DNA. The amplification consisted of 40 cycles of 15 sec at 95°, followed by 14 sec at 53° and 28 sec at 72° for 81F/691R and 11 sec at 52° and 22 sec at 72° for 165'F/691R, 172F/691R, and Aso3/691R.

Standard curves were drawn on clones of the three *Wolbachia* strains. Amplification with 81F/691R primers was performed on a triply infected female. PCR products were purified (GIBCO BRL, Gaithersburg, MD) and cloned into the

TABLE 1  
Fitness traits of the Pi(123), Pi(13), Pi(23), and Pi(3) lines

Sex/line	Productivity (total offspring)	Sex ratio (% males)	Fecundity (no. of eggs)	Tibia length (mm)	Dry weight (mg)	Survival (days)	Rate of locomotor activity (%)
Female/Pi(123)	181.6 ± 20.8 (8)	0.290 ± 0.017 (8)	254.2 ± 6.5 (n = 15)	0.704 ± 0.004 (n = 26)	0.174 ± 0.003 (a) (n = 30)	12.3 ± 0.2 (a) (n = 60)	19.2 ± 2.9 (n = 27)
	195.0 ± 11.8 (8)	0.330 ± 0.021 (8)					
	167.1 ± 15.6 (9)	0.370 ± 0.021 (9)					
Female/Pi(13)	179.8 ± 16.1 (8)	0.348 ± 0.022 (8)	259.5 ± 6.0 (n = 15)	0.711 ± 0.006 (n = 30)	0.180 ± 0.003 (b) (n = 29)	13.8 ± 0.2 (b) (n = 60)	21.7 ± 1.7 (n = 29)
	147.3 ± 21.1 (7)	0.372 ± 0.030 (7)					
	147.3 ± 6.4 (6)	0.414 ± 0.021 (6)					
Female/Pi(23)	163.3 ± 18.9 (10)	0.325 ± 0.025 (10)	247.9 ± 10.3 (n = 15)	0.715 ± 0.004 (n = 32)	0.181 ± 0.003 (b) (n = 28)	13.7 ± 0.2 (b) (n = 60)	17.4 ± 1.6 (n = 29)
	196.1 ± 14.2 (7)	0.371 ± 0.012 (7)					
	140.8 ± 17.7 (7)	0.350 ± 0.014 (7)					
Female/Pi(3)	197.2 ± 9.1 (10)	0.349 ± 0.012 (10)	255.0 ± 7.0 (n = 15)	0.716 ± 0.006 (n = 31)	0.195 ± 0.003 (c) (n = 32)	14.8 ± 0.2 (c) (n = 60)	22.5 ± 1.8 (n = 26)
	153.0 ± 21.4 (8)	0.375 ± 0.025 (8)					
	130.9 ± 9.5 (10)	0.351 ± 0.025 (10)					
Male/Pi(123)	—	—	—	0.717 ± 0.004 (n = 28)	0.126 ± 0.003 (d) (n = 30)	10.2 ± 0.2 (d) (n = 60)	10.0 ± 1.4 (a) (n = 14)
Male/Pi(13)	—	—	—	0.705 ± 0.004 (n = 28)	0.133 ± 0.004 (e) (n = 30)	11.9 ± 0.2 (e) (n = 58)	14.4 ± 1.3 (b) (n = 27)
Male/Pi(23)	—	—	—	0.715 ± 0.005 (n = 26)	0.133 ± 0.003 (e) (n = 30)	11.7 ± 0.2 (e) (n = 49)	10.4 ± 1.0 (a) (n = 20)
Male/Pi(3)	—	—	—	0.707 ± 0.005 (n = 25)	0.140 ± 0.003 (f) (n = 30)	12.1 ± 0.2 (e) (n = 64)	15.8 ± 1.4 (b) (n = 21)
ANOVA							
Sex	—	—	—	P = 0.523	P < 0.0001	P < 0.0001	P = 0.0003
Interaction	P = 0.292	P = 0.071	P = 0.760	P = 0.182	P < 0.0001	P < 0.0001	P < 0.0001
	—	—	—	P = 0.224	P = 0.489	P = 0.208	P = 0.815

Mean values ± SE (replicates) and statistical analysis of physiological traits (one- or two-way ANOVA) are shown. Analysis of the sex ratio and rate of locomotor activity were carried out after arcsine square root transformation. For productivity and sex ratio, three series of measures have been performed, and the *P*-value indicated corresponds to the factor line. The factor block was significant ( $F_{6,86} = 4.873$ ,  $P = 0.0099$  for total offspring;  $F_{2,86} = 4.605$ ,  $P = 0.0126$  for sex ratio), indicating that these traits are variable, but the interaction between block and line factors was not significant ( $F_{6,86} = 1.577$ ,  $P = 0.1636$  for total offspring;  $F_{6,86} = 1.018$ ,  $P = 0.4193$  for sex ratio). For dry weight, survival, and rate of locomotor activity, the means marked with the same letter are not significantly different (Student's *t*-tests,  $P = 0.05$ ).

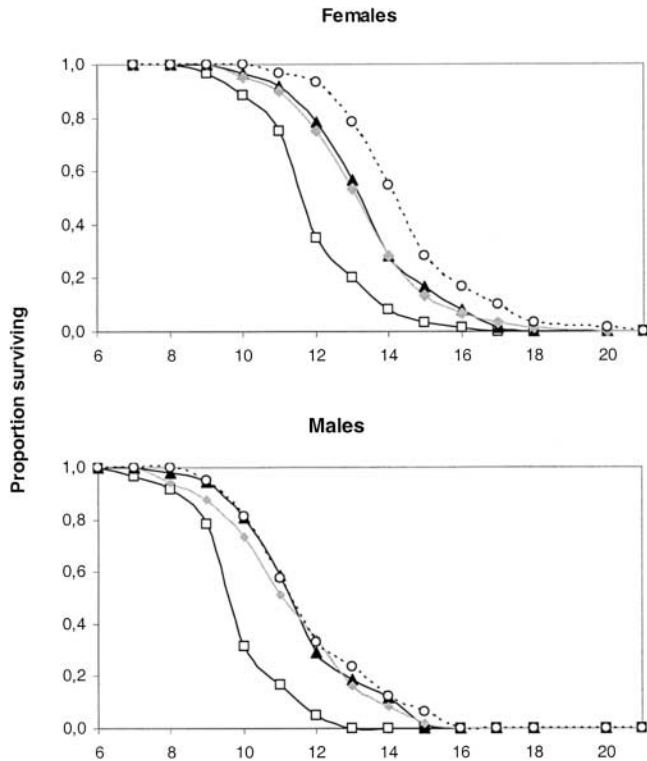


FIGURE 1.—Starvation survival curves of female and male wasps with differing *Wolbachia* infection statuses. (□) Pi(123), (▲) Pi(13), (◆) Pi(23), (○) Pi(3).

pDrive cloning vector (QIAGEN, Valencia, CA). Specific PCR assays were used to identify the *Wolbachia* strain present in the clone. The DNA concentration of each sample was measured by OD absorbance at 260 nm. Standard curves were plotted using five dilutions of this vector (from  $10^2$  to  $10^8$  copies) containing one copy of a specific *wsp* sequence, which is a single-copy gene (BRAIG *et al.* 1998). The number of *Wolbachia* cells was calculated as described in NODA *et al.* (2001). These values must be considered as semiquantitative estimates of *Wolbachia* cell numbers.

*Wolbachia* abundance was measured individually on 5-day-old males and females, either in whole bodies or separately in head plus thorax and abdomen of the same individual. Density was obtained by correcting the number of *Wolbachia* by the mean fresh weight of insects of the line. The ratio of *Wolbachia* cells in the abdomen to the sum of *Wolbachia* in head plus thorax and abdomen was calculated individually.

## RESULTS

**Infection cost:** Among the seven fitness components studied here, three do not vary according to the infection status: productivity, fecundity, and tibia length. For sex ratio, a marginally significant effect was detected, with a potentially higher proportion of females in the triply infected line. However, this trait is highly variable and no such effect was detected in the third block. Clear conclusions were obtained only for dry weight, adult survival, and rate of locomotor activity (Table 1).

Pi(3) individuals of both sexes were the heaviest, Pi(13) and Pi(23) had similar and intermediate weights,

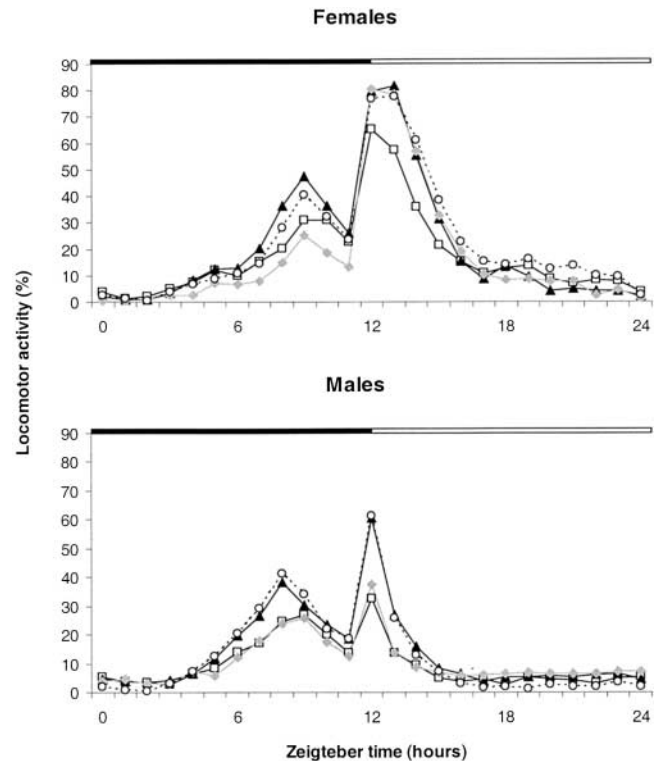


FIGURE 2.—Mean curves of the locomotor activity rhythms of *A. tabida* lines Pi(123), Pi(13), Pi(23), and Pi(3). Males and females were measured for 3 days under LD 12/12 (Zeitgeber time) with light off at Zt0 and light on at Zt12. The black-and-white rectangles at the top of the figure represent night and day, respectively. (□) Pi(123), (▲) Pi(13), (◆) Pi(23), (○) Pi(3).

and Pi(123) were the lightest. Thus, the greater the diversity of *Wolbachia* lineages harbored by the insects, the less they weighed. More diversity in *Wolbachia* also led to a shorter life span of the host (Table 1; Figure 1). Life span of doubly infected wasps was intermediate for both sexes, but the difference from simply infected wasps was not significant in the males. The rate of locomotor activity also varied with the infection status of individuals (Table 1). As for dry weight and life span, singly infected wasps had greater locomotor activity than triply infected ones, but in this case the difference was not significant for the females. In contrast, the profile of the rhythm was the same regardless of infection status (ANOVA, d.f. = 3189,  $F = 1.763$ ,  $P = 0.16$ , Figure 2).

**Total *Wolbachia* density:** Previous findings have shown that infection cost increases with bacterial diversity. To find out whether this is linked to density variations, we measured the number of *Wolbachia* cells in male and female Pi(3), Pi(13), Pi(23), and Pi(123) (Table 2). In all these lines, the numbers of *Wolbachia* and their relative densities (number of cells per milligram of fresh weight) were higher in females than in males (Mann-Whitney test,  $P < 0.001$ ).

Despite the fact that the cell numbers obtained by real-time quantitative PCR were rather low and may have

TABLE 2  
Wolbachia density in *A. tabida* species

Sex	Line	No. of Wolbachia cells per wasp ( $10^3$ )	No. of Wolbachia cells per milligram of wasp ( $10^3$ )
Female	Pi(123)	45.2 $\pm$ 2.8 (a)	259.7 $\pm$ 16.3 (a)
	Pi(13)	22.6 $\pm$ 1.8 (b)	125.8 $\pm$ 9.8 (b)
	Pi(23)	39.6 $\pm$ 3.1 (a)	218.5 $\pm$ 17.4 (a)
	Pi(3)	10.4 $\pm$ 1.2 (c)	53.2 $\pm$ 6.2 (c)
		Kruskal-Wallis test: $P < 0.0001$	$P < 0.0001$
Male	Pi(123)	15.6 $\pm$ 2.3 (d,e)	123.8 $\pm$ 18.1 (d,e)
	Pi(13)	10.9 $\pm$ 1.3 (d)	82.2 $\pm$ 10.1 (d)
	Pi(23)	16.7 $\pm$ 1.2 (e)	125.9 $\pm$ 9.0 (e)
	Pi(3)	3.9 $\pm$ 0.5 (f)	27.7 $\pm$ 3.6 (f)
		Kruskal-Wallis test: $P = 0.0002$	$P = 0.0001$

Mean values  $\pm$ SE and statistical analysis of the total number of Wolbachia cells per wasp (eight replicates per sex and line) are shown. The analyses were carried out using the Kruskal-Wallis nonparametric test. Means marked with the same letter are not significantly different (Mann-Whitney tests,  $P = 0.05$ ).

been underestimated, in both sexes the total Wolbachia density depended on the combination of the Wolbachia strains co-infecting the same individual host (Kruskal-Wallis test,  $P < 0.005$ ). Singly infected individuals of the Pi(3) strain had the lowest density, which was less than one-quarter of that in Pi(123). In females, the density in the two doubly infected lines was intermediate between those of the singly and triply infected lines, but it was lower in Pi(13) than in Pi(23). In contrast,

there was no significant difference between males of the two doubly and the one triply infected lines.

Overall, these findings demonstrate that increasing the mixture of Wolbachia strains results in a higher physiological cost to the host and also leads to higher Wolbachia density. This means that the cost of infection is also positively correlated with bacterial density, as shown in Figure 3.

**Strain-specific Wolbachia density:** We have shown that

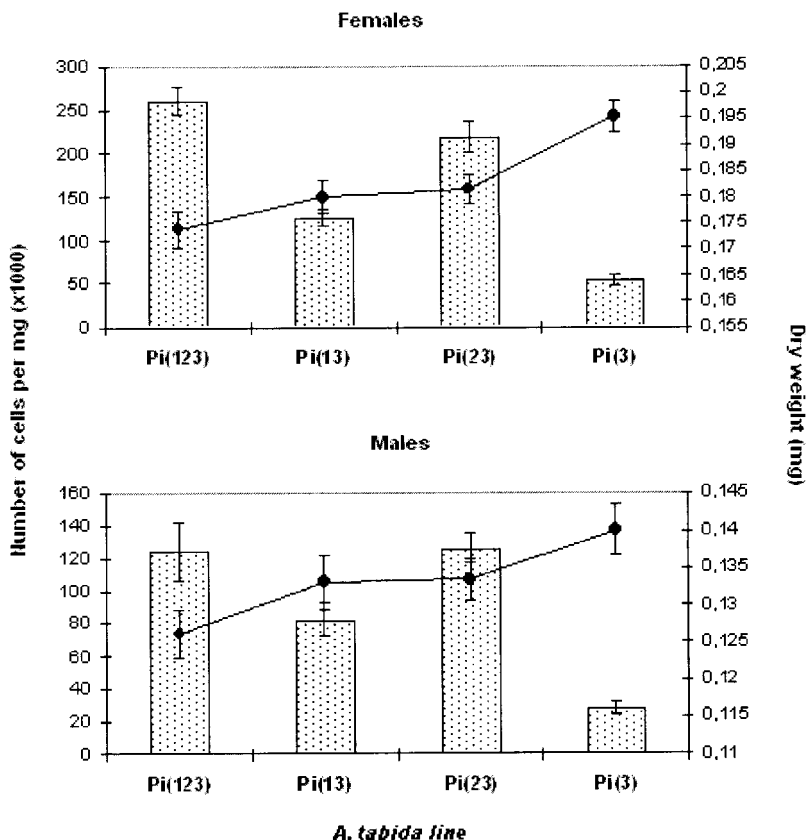


FIGURE 3.—Wolbachia density and dry weight. The Wolbachia density (histogram) and dry weight (curve) of Pi(123), Pi(13), Pi(23), and Pi(3) individuals are presented. Values correspond to the average of eight individuals for density and of 30 individuals for dry weight. Bars show the standard error.

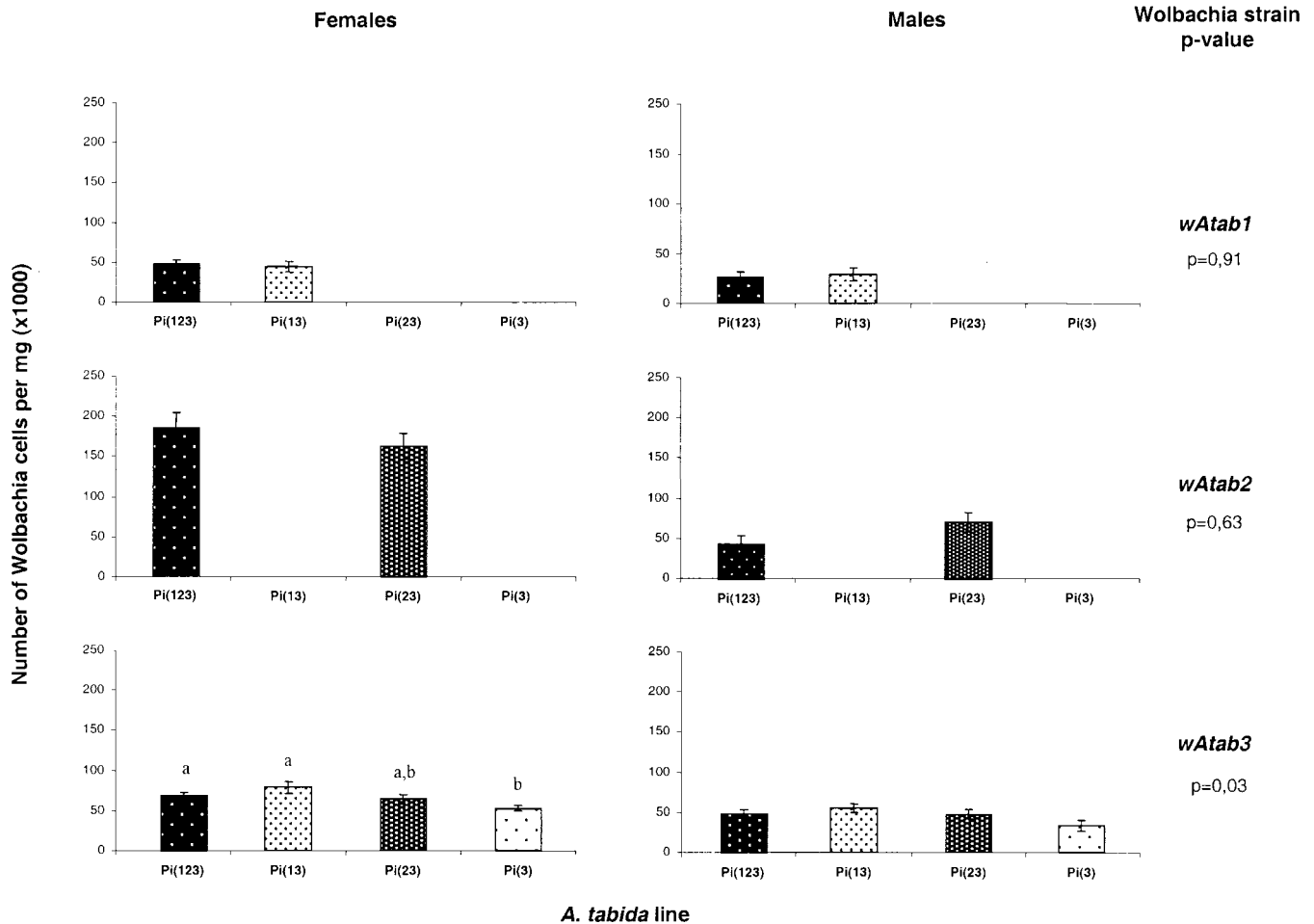


FIGURE 4.—Specific densities of *wAtab1*, *wAtab2*, and *wAtab3*. Specific densities of *wAtab1*, *wAtab2*, and *wAtab3* in females and males of various infection statuses are presented. Values correspond to the average of eight individuals per sex and line. Bars show the standard error. *P*-values of Kruskal-Wallis tests are indicated. For *wAtab3*, means marked with the same letters are not significantly different (Mann-Whitney tests,  $P = 0.05$ ).

total bacterial density increases with bacterial diversity, but we still do not know how each Wolbachia strain responds to the presence of other Wolbachia strains. We therefore measured the specific density of each Wolbachia strain co-infecting the same individual in all *A. tabida* lines (Figure 4).

First, the sums of the specific densities are equal to the estimations of total densities in all lines (Wilcoxon rank test,  $P = 0.11$ ), thus indicating the reliability of the method and ruling out any concern about significant bias.

In both sexes, we found that the specific densities of *wAtab1* and *wAtab2* were the same in the Pi(123) and Pi(13) lines and the Pi(123) and Pi(23) lines (Mann-Whitney test,  $P > 0.05$ ), respectively, and are thus independent of the infection status of the individuals. The density of *wAtab3* was lower in Pi(3) than in multiply infected lines and, whereas the differences were not significant in the males (Kruskal-Wallis test,  $P = 0.11$ ), they were significant in the females ( $P = 0.02$ ), with the Pi(13) and Pi(123) lines harboring more *wAtab3* than Pi(3) lines (Mann-Whitney test,  $P \leq 0.02$ ).

Considering the relative abundance of Wolbachia strains in triply infected individuals, *wAtab1* was found to be the least represented in both sexes (16% in females and 22% in males), whereas the most abundant strains were *wAtab2* in females (61%) and *wAtab3* in males (near 41%). Despite the different abundances of *wAtab1* and *wAtab2* in the host, they induced similar infection costs.

**Wolbachia distribution in the host body:** The localization of Wolbachia may have an influence on the infection cost (McGraw *et al.* 2002). We then studied the preferential localization of these bacteria in *A. tabida* species of each line by measuring the total number of Wolbachia cells in head plus thorax and in abdomen of the same host body. Comparison of the percentage of Wolbachia in abdomen compared to the entire body in the four lines demonstrates that Wolbachia are preferentially localized in abdomen in males as well as in females whatever the infection status (Table 3). However, the percentage of Wolbachia in abdomen is higher in females than in males (Student's *t*-test,  $P < 0.0001$ ). This repartition does not differ between lines (ANOVA,

TABLE 3

Percentage of *Wolbachia* cells in abdomen compared to the entire body in *A. tabida* females and males

Sex	<i>A. tabida</i> line	% of <i>Wolbachia</i> cells in A compared to H + T + A
Female	Pi(123)	86.65 ± 2.02
	Pi(13)	86.75 ± 1.91
	Pi(23)	84.62 ± 0.83
	Pi(3)	83.86 ± 2.73
		ANOVA: $P = 0.5821$
Male	Pi(123)	68.58 ± 2.37
	Pi(13)	73.71 ± 4.52
	Pi(23)	72.32 ± 5.24
	Pi(3)	57.44 ± 5.73
		ANOVA: $P = 0.0769$

Mean percentage ±SE and statistical analysis of total *Wolbachia* cells in abdomen compared to the sum of densities in head (H), thorax (T), and abdomen (A) in five males and five females of each *A. tabida* line are shown. One-way ANOVA analyses were carried out after arcsine square root transformation ( $P = 0.05$ ).

$P > 0.05$  for both sexes), even though the percentage of *Wolbachia* in abdomen of males for the Pi(3) line seems to be lower.

## DISCUSSION

Three main conclusions can be drawn from our findings:

- i. Despite the vertical transmission of *Wolbachia* and the high selective pressures that tend to reduce infection costs, some traits of *A. tabida* are still negatively affected by *Wolbachia*. However, the resulting costs are low, may have little influence on the fitness of adults in the wild, and probably do not affect the maintenance of multiple infection. This finding is consistent with numerous studies on the cost of *Wolbachia* where a high variability among species and traits exists (HOFFMANN *et al.* 1990, 1994, 1998; GIORDANO *et al.* 1995; GIRIN and BOULÉTREAU 1995; TURELLI and HOFFMANN 1995; WADE and CHANG 1995; BOURTZIS *et al.* 1996; CLANCY and HOFFMANN 1997; MIN and BENZER 1997; POINSOT and MERÇOT 1997; HOERAUF *et al.* 1999; VAVRE *et al.* 1999; FLEURY *et al.* 2000; DOBSON *et al.* 2002; FRY and RAND 2002). On the other hand, the nearly significant effect on sex ratio might promote multiple infections through increase in the proportion of females.
- ii. The cost of infection depends directly on the diversity of *Wolbachia* and increases with the number of *Wolbachia* strains within the insect host. The same localization has been observed between individuals of all infection statuses; therefore the differences of infection cost observed in the various lines of *A. tabida* cannot be explained by a difference of localization in the host body. These results mean that even

though variations in density are due to the differing diversity of the *Wolbachia* strains infecting individuals, higher bacterial densities also correlate with an increase in the cost of infection. A relationship between density and virulence has already been documented by MCGRAW *et al.* (2002), who demonstrated that the fitness cost associated with the *popcorn* strain was reduced in a new transfected *Drosophila* host harboring a lower *Wolbachia* density. Therefore, with results of MCGRAW *et al.* (2002), the data presented here strongly support the existence of a relationship between bacterial density and infection cost, which has rarely been demonstrated hitherto, but is generally accepted since more symbionts can be expected to require more energy (THOMPSON 1988). However other factors, such as the particular traits of each strain, may affect the impact of bacteria on their host. For example, the same cost is induced by *wAtab1* and *wAtab2*, in spite of the lower density of *wAtab1*. Thus, all differences in the cost of infection cannot be attributed to bacterial density alone.

- iii. The densities of the two facultative strains *wAtab1* and *wAtab2* are specifically regulated and do not depend on the presence of other strains. The situation is less clear for the obligatory bacterium *wAtab3*, suggesting that there may be some positive interaction with other strains. Such specific bacterial regulation seems to be rather common in multiple infections by *Wolbachia*, as suggested by studies in *D. simulans*, *Ephestia kuehniella*, and *Leptopilina heterotoma* (ROUSSET *et al.* 1999; IKEDA *et al.* 2003; MOUTON *et al.* 2003).

The puzzling question that now arises is how the infection cost for *A. tabida* has persisted despite selective pressures toward reduced virulence that act on both the bacteria and the insects. Is multiple infection responsible for this persistence? The strain-specific regulation of bacterial density suggests that strains do not compete with one another for limited resources within the host, and this should prevent the increased virulence that is usually expected from multiple infection (FRANK 1996a). The maintenance of infection cost in *A. tabida* probably expresses specific constraints, such as the classical trade-off between virulence and bacterial transmission (for review, see FRANK 1996b). On the one hand, bacterial density should be kept as low as possible to reduce fitness costs to the host, but on the other hand, the intracellular density of microorganisms must be high enough to ensure transmission to the next generation. Moreover, convergent selective pressures also act on the host (TURELLI 1994; VAVRE *et al.* 2003), since strain loss can have dramatic indirect effects on individual fitness. In *A. tabida*, this is obvious for the obligatory *wAtab3* strain, since loss of this strain results in female sterility. It is also true to some extent for the two facultative strains, both of which induce high CI levels (>70%; F.

DEDEINE, personal communication). Losing one or both of them can be expected to expose females to CI and counterselection, whereas it should have no effect on males. Consequently, selective pressures do promote the maintenance of diversity of infection, and this could explain how the specificity of density regulation has evolved in spite of other types of competition, such as interference between Wolbachia strains. In contrast, the density data for *wAtab3* suggest that there may be some positive interaction between this obligatory strain and the two facultative ones, since the density of *wAtab3* is lower when it is the only strain present.

Finally, the bacterial density regulating system exhibited by *A. tabida* and other multiply infected species may limit both competition for resources and interference between different Wolbachia strains, and this may reflect the peculiar selective pressures acting on the system. Multiple infection by Wolbachia can be viewed as a criminal conspiracy among bacteria, with each partner relying on the others for its own fitness. Clearly, the success of the plot relies on the reciprocal agreement among accomplices, and reciprocal damage would be unacceptable.

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