RELATIONSHIP BETWEEN *Wolbachia* DENSITY AND SEX-RATIO IN A *Trichogramma* STRAIN

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RESUMEN

RELACIÓN ENTRE LA DENSIDAD DE *Wolbachia* Y LA RAZÓN SEXUAL EN UNA LÍNEA DE *Trichogramma*

Un tratamiento térmico controlado se aplicó a una línea de *Trichogramma cordubensis* completamente infestada por endocitobiotos que pertenecen al género *Wolbachia*, y que inducen a un modo de reproducción de tipo telitoquia. La variación en la intensidad del tratamiento condujo a la producción de un número variable de machos en la progenie, lo que permitió analizar la correlación entre la razón sexual y la densidad bacteriana estimada por medio de la técnica de Dot-blot, utilizando el gen 18S de *Trichogramma* y el gen *wsp* de *Wolbachia*. Una baja correlación positiva se constató entre las dos variables. Un análisis estadístico suplementario permitió confirmar la relación entre la razón sexual del huésped y la densidad bacteriana, pero esta relación resultó parcialmente encubierta por problemas metodológicos.

PALABRAS CLAVE: bacteria, interacción, parasitoid de huevos, simbioto, Trichogrammatidae.

SUMMARY

A controlled thermal cure was applied to a *Trichogramma cordubensis* strain completely infected by endosymbionts belonging to the *Wolbachia* genus and which induce the thelytokous mode of reproduction. Varying the conditions of the cure led to the production of a variable number of males among the progeny, and it was possible to examine the correlation between sex-ratio and bacterial density estimated by means of the Dot-blot technique, using the *Trichogramma* gene 18S and the *Wolbachia* gene *wsp*. A slight positive correlation was observed between the two variables. An additional statistical analysis confirmed that a relationship exists between the host sex-ratio and bacterial density but that this relationship is partially concealed by methodological problems.

KEY WORDS: bacteria, egg parasitoid, interaction, symbiont, Trichogrammatidae.

INTRODUCTION

Bacteria Rickettsiaceae, of the genus *Wolbachia*, are endosymbionts living in many arthropods and nematodes. In arthropods, *Wolbachia* induce various reproductive alterations to improve their vertical transmission: cytoplasmic incompatibility, thelytokous parthenogenesis, male feminization, male killing (O'Neill *et al.*, 1997) and an increase in fecundity (Girin & Boulétreau, 1995).

Egg parasitoids of the genus *Trichogramma* (Hym.: Trichogrammatidae) are used in the biological control of some lepidopteran pests in various crops (Smith,1996).

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Their mode of reproduction is bisexual with diploid females and haploid males, or more rarely, parthenogenetic with production of females only (thelytoky). In most *Trichogramma* species, this phenomenon is induced by the presence of *Wolbachia* (Pintureau *et al.*, 2001), which impedes chromosome segregation during anaphase of the embryonic first mitotic division, causing the diploidisation of the embryo and its subsequent development as a female (Stouthamer & Kazmer, 1994).

Nevertheless, the intensity of the effect induced by *Wolbachia* in a host may be variable, especially depending on bacterial density, host genotype and degree of co-adaptation between host and symbiont (Boyle *et al.*, 1993; Bourtzis *et al.*, 1996). For example, thelytokous females of *Trichogramma* produce more males as they age (Jardak *et al.*, 1979), probably in relation to a decrease in *Wolbachia* density.

The aim of the present study was to improve description of the relationship between the intensity of the thelytokous mode of reproduction and *Wolbachia* density in *Trichogramma cordubensis* Vargas & Cabello. More precisely, our objective was to search for a correlation between bacterial density and host sex-ratio. It was hypothesized that a shift in rearing temperature from 28°C to 31°C has an impact on the number of *Wolbachia* endosymbionts and, consequently, on the mode of reproduction and sex-ratio in the host. This work was carried out using controlled thermal cures to induce variable sex-ratios, and the Dot-blot technique (three Dot-blots were achieved) to make a rough estimation of the bacterial density in the whole *Trichogramma* body. The thermal treatments were only performed to generate variable sex-ratios, and the treatment effect on the bacterial density was not tested (the number of lines studied in each treatment and Dot-blot was insufficient to analyse such a relationship).

MATERIAL AND METHODS

Biological material

The experiment was performed using the thelytokous strain 1032 of *T. cordubensis* (species completely infected by *Wolbachia* and completely thelytokous). This strain was established from a few individuals collected in June 1992 on São Jorge Island, Azores (Portugal), and its genetic variability is probably very low. The study focused on only one strain because different strains could present different genomes permitting variable *Wolbachia* densities. The strain was reared on *Ephestia kuehniella* Zeller (Lep.: Pyralidae) eggs, UV-irradiated and glued with arabic gum solution onto cardboard strips (4.8 x 0.8 cm). These strips were placed in glass tubes (8 cm in length x 1 cm in diameter) where *Trichogramma* adults were present, together with a drop of diluted honey as food.

Before the experiment, the rearing was performed at 23°C (70±5 % RH, 16:8 L:D), resulting in a developmental period of about 14 days. For the present experiment, a vast range of sex-ratios were necessary, and we thus submitted individuals of strain 1032 to higher temperatures. This thermal cure, which had to be controlled at a moderate level, was performed in 4 different conditions: 2 or 4 generations at 28° C (conditions A and C), and 2 or 4 generations at 31° C (conditions B and D). A minimum of 30 isofemale lines were treated in each of these conditions.

Prior to emergence of adults from the last generation submitted to a high temperature, 40 to 60 pupae were isolated in each line to obtain a minimum of 20 viable females. The fecundity of these females (in fact, the number of adult descendants from a virgin female during its entire life) and the sex-ratio of their progenies (expressed by the proportion of females) were estimated. To measure these traits, each female was provided with fresh *E. kuehniella* eggs only once. Other adults from non-isolated pupae were stored at -80° C to allow for DNA extraction and then to perform Dot-blots. Collection of these individuals was carried out as previously described (Pascal *et al.*, 2004) using several successive phototactic migrations.

DNA extraction

DNA extraction was performed using the Promega "Genomic DNA Purification kit" for plant DNA according to the manufacturer's specifications, with some modifications (Pascal *et al.*, 2004). The extraction was carried out on the homogenate of 2.4 mg of *Trichogramma* adults, i.e. 300 ± 5 individuals. The DNA extracted was maintained at 4° C for 24 hours prior to storage at –20° C.

DNA quality and quantity were estimated in each sample after electrophoretic migration on a 1% agarose gel followed by comparison with a control of known size and concentration. This method was used to prepare the three DNA quantities of each sample analysed in the Dot-blot 1 (0.50, 0.75 and 1µg). However, such an estimation of the DNA quantity appeared not to be very reliable. This is why, for Dot-blots 2 and 3, we decided to analyse four dilutions of the raw solution of total DNA extracted (5, 10, 15 and 20µl in Dot-blot 2; 1, 3, 6 and 9µl in Dot-blot 3) (Pascal *et al.*, 2004).

Estimation of the relative density of Wolbachia using Dot-blot

The Dot-blot technique allows a rough estimation of the relative frequency of one DNA sequence among a heterogeneous population of DNA sequences. Its application to *Trichogramma* has been described by Pascal *et al.* (2004), and only a summary is provided here. DNA extracted from each sample was diluted and, after heat denaturation, transferred onto nylon membranes. It was then fixed by heating the membranes for 2 hours at 80° C.

To estimate the ratio of *Wolbachia* DNA to host DNA, the same membrane was successively hybridized with two radioactive probes, *wsp* for *Wolbachia* and 18S for *Trichogramma* (Pascal *et al.*, 2004). Each radioactive probe was directly labelled by PCR from the total DNA of the strain Grey of *T. cordubensis*, originating from Alentejo (Portugal). Prehybridization and hybridization were carried out at 50° C, and the membranes were then washed, at 55° C, in 2X SSC to 0.1X SSC solutions.

Estimates of the quantity of radioactive DNA were carried out with a Storm apparatus (Molecular Dynamics, USA). Data collection and analysis were performed using ImageQuant software. Each replicate from each sample was characterized by its ratio of intensities *wsp*/18S.

A data filtration test was performed to eliminate data of low reliability. The correlation was then calculated for each sample between three (three quantities of DNA in Dot-blot 1) or four (four volumes of DNA solution in other Dot-blots) intensities of *wsp* spots and three or four intensities of corresponding 18S spots. Samples associated with non-correlated intensities ($p<0.05$) were not considered because an absence of correlation indicates a technical problem (pipette handling, signal saturation, ...). The different ratios of intensities obtained in each validated sample were considered as "replicates" used to calculate the correlation between the sex-ratio and the bacterial density, although any variability shown by these replicates does not reflect biological variability since only one DNA extraction was possible from one sample.

Dot-blot normalisation and statistical analysis

To analyse simultaneously all the data from the three Dot-blots performed, a normalisation was needed. We opted for intensity and ratio normalisations. For the intensity normalisation, radioactive intensities (*wsp* and 18S) were first scaled within the three or four replicates of each sample. Each scaled intensity was then divided by the mean intensity/10,000 of its corresponding Dot-blot. Ratios *wsp* normalised/18S normalised were then calculated. For the ratio normalisation, ratios were calculated without transformation but they were divided by the mean ratio of the corresponding Dot-blot.

To analyse sex-ratio and fecundity variation in the different lines, nested and mixed multiway ANOVA were performed using JMP 3.2.2 software (SAS Institute). The lines were defined from a combination of the two factors, temperature (28 or 31° C) and number of generations at high temperature (two or four), and a complete factorial analysis could not be performed. Hence, the line factor was nested under the two other factors.

RESULTS

Sex-ratio and fecundity in the lines after a thermal cure

From the 120 lines established at the beginning of the experiment, only 99 survived to all the rearing steps and were analysed: 29 lines for each of treatments A and B, 27 for treatment C, and 14 for treatment D. In each line submitted to one thermal condition, the fecundity of several isolated virgin females and the sex-ratio of their offspring were recorded. The number of females studied (replicates) generally varied between 20 and 25 but, exceptionally, it was fewer than 10, with a minimum of 6, in some lines due to a high preimaginal mortality (in 1, 3 and 3 lines maintained in conditions B, C and D, respectively). Three-way mixed and nested ANOVAs were carried out on the sex-ratio (after arcsine$\sqrt{}$ transformation) and on the fecundity of these 99 lines. The first two fixed factors of the ANOVAs are the length (2 and 4 generations) and heat intensity (28 and 31° C) of the thermal cure, leading to four modalities (A, B, C and D). The third factor, the lines, was nested within the previous factors and selected at random to be submitted to the different conditions.

The ANOVA showed that sex-ratio (proportion of females) differences exist between the rearing conditions (treatment duration and temperature) and between the lines within the rearing conditions. Nevertheless, the differences caused by the rearing conditions are clearly greater than the differences between the lines (Table 1). Figure 1 shows that the four rearing conditions caused four different sex-ratios, the most drastic condition (4 generations at 31°C) leading to the lowest sex-ratio and the least drastic condition (2 generations at 28° C) leading to the highest sex-ratio. However, the sex-ratio seems to be more influenced by the heat intensity (28 or 31° C) than by the exposure time at high temperature (2 or 4 generations). A

TABLE 1. Three-way mixed and nested ANOVAs to compare two biological traits in different lines of *T. cordubensis* reared in four conditions (two or four generations at 28° C or 31° C). The factors «Generation» and «Temperature» are fixed, whilst the factor «Line» is nested within the previous factors.

Trait	Source of variation	Degree of freedom	Mean square	F	p
Sex-ratio	Generation	1	28.68	191.1	<0.0001
	Temperature	1	256.14	1706.7	<0.0001
	Generation X Temperature	1	1.49	9.9	0.0021
	Line	95	0.16	1.7	0.0001
Fecundity	Generation	1	9767.15	45.4	<0.0001
	Temperature	1	95.43	0.4	0.51
	Generation X Temperature	1	31.26	0.1	0.70
	Line	95	238.42	2.8	<0.0001

decline in sex-ratio when temperature increases is probably caused by the de-activation of more *Wolbachia*, although this phenomenon could be slightly counterbalanced by a reduction in female longevity, leading to higher sex-ratio (on average, male eggs are laid later than female eggs). Progeny of several females being considered in each line, the effect of lifetime may be lower when lines are compared.

To carry out our experiment, a sufficient number of lines was needed and, thus, fecundity had to be high enough in all the thermal conditions applied. The ANOVA showed only moderate differences in fecundity according to the rearing condition and the line (F values are low, although they sometimes reach the significant threshold; Table 1). Among treatment duration and temperature, only the first factor reveals significant differences in fecundity. As for sex-ratio, the differences caused by the treatment duration are clearly greater than the differences between lines. Figure 1 shows that females kept for two generations at a high temperature (either 28° C or 31° C) are more fertile than females staying for four generations at these temperatures. Fecundity is thus influenced more by the duration of time spent at a high temperature than by the heat level.

Estimation of bacterial density

The quantity of DNA obtained from about 300 *Trichogramma* adults was estimated on an agarose gel to be about 2μg. Such a quantity appears to be relatively low but it allowed us to obtain the three or four spots of increasing intensity which are necessary to correctly calculate the ratio of *Wolbachia* to host DNA and to assess the quality of the hybridization.

In Dot-blot 1, 16 lines were analysed. These lines showed sex-ratios (proportions of females) from 0.01 to 1.00, but only two had sex-ratios above 0.5 (Table 2). Only 10 lines gave significant coefficients of correlation between the intensities of the three spots *wsp* and the three spots 18S ($p<0.05$). Each of these 10 lines allowed for the calculation of three ratios of intensities *wsp*/18S.

In Dot-blot 2, 22 lines were analysed. These lines showed sex-ratios from 0.01 to 0.95, and 12 had sex-ratios above 0.5 (Table 3). The sex-ratios were thus better spread out in this Dot-blot than in the previous one. Only 15 lines gave significant coefficients of correlation between the intensities of the four spots *wsp* and the four spots 18S ($p<0.05$). Each of these 15 lines allowed for the calculation of four ratios of intensities *wsp*/18S.

In Dot-blot 3, 24 lines were analysed. These lines showed sex-ratios from 0.03 to 0.98, correctly spread out since 10 lines had sex-ratios above 0.5 (Table 4). Only 17 lines gave significant coefficients of correlation between the intensities of the three (lines 13 and 15) or four (other lines) spots *wsp* and the three or four spots 18S ($p<0.05$). Each of these 17 lines allowed for the calculation of three or four ratios of intensities *wsp*/18S. These ratios are obviously higher than in the preceding Dot-blots, the cause being a

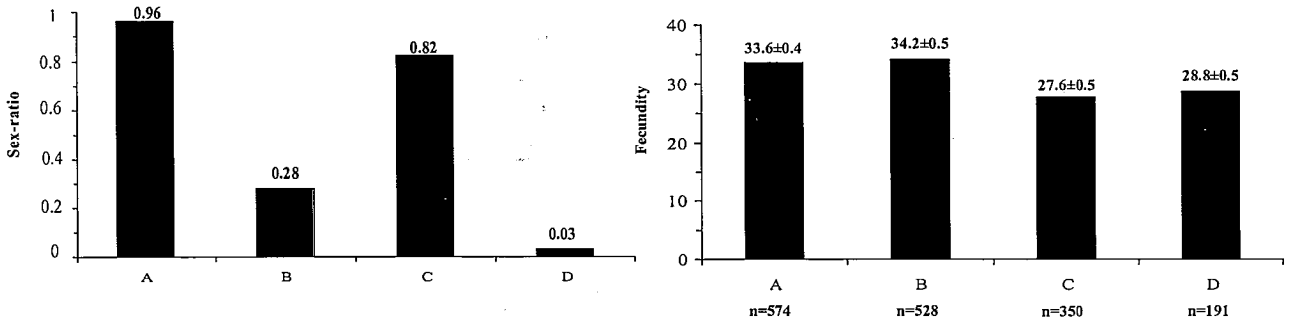

FIG. 1. Sex-ratio (number of females/number of individuals) and fecundity ± s.e. (number of adult descendants from a virgin female) of the strain 1032 of *T. cordubensis* in each rearing condition.
Condition A: two generations at 28°C, B: two generations at 31°C, C: four generations at 28°C, D: four generations at 31°C. n: number of progeny studied.

TABLE 2. Analysis of Dot-Blot 1 performed with 16 lines of *T. cordubensis*, strain 1032, including 15 strains submitted to a controlled thermal cure (28 or 31°C for two or four generations).

Line	Sex-ratio (1)	Coefficient of correlation *wsp* -18S (2)	Ratio of intensities *wsp* /18S		
			0.50 (3)	0.75	1
1	0.01	0.95			
2	0.03	0.72			
3	0.03	0.38			
4	0.04	0.94			
5	0.04	0.98*	0.54	0.40	0.37
6	0.05	0.71			
7	0.05	0.98*	0.43	0.41	0.32
8	0.31	0.99*	0.54	0.46	0.42
9	0.31	1.00*	0.69	0.69	0.70
10	0.33	0.98*	0.49	0.64	0.57
11	0.34	0.99*	0.50	0.58	0.50
12	0.46	0.99*	0.62	0.56	0.64
13	0.48	0.99*	0.59	0.62	0.47
14	0.50	0.97*	0.61	0.42	0.40
15	0.57	0.21			
16	1.00	0.99*	0.58	0.84	1.50

(1) Number of females/number of individuals, calculated from 20 progenies of virgin females in each line (a progeny included 5 to 46 individuals).
(2) Correlation between the intensities of the *wsp* spots and the intensities of the 18S spots in the three replicates performed using three quantities of DNA.
(3) Quantity (μg) of DNA.
* $p<0.05$.

TABLE 3. Analysis of Dot-blot 2 performed with 22 lines of *T. cordubensis,* strain 1032, submitted to a controlled thermal cure (28 or 31° C for two or four generations).

Line	Sex-ratio (1)	Coefficient of correlation *wsp* -18S (2)	Ratio of intensities *wsp* /18S			
			5 (3)	10	15	20
1	0.01	0.41				
2	0.02	0.99*	0.78	0.61	0.56	0.55
3	0.02	0.58				
4	0.03	0.19				
5	0.05	0.98*	0.84	0.67	0.60	0.52
6	0.36	1.00*	0.48	0.50	0.53	0.52
7	0.37	0.95*	0.66	0.57	0.48	0.59
8	0.38	1.00*	0.47	0.56	0.64	0.66
9	0.39	0.93*	0.54	0.43	0.52	0.54
10	0.48	0.99*	0.73	0.72	0.65	0.59
11	0.59	1.00*	0.79	0.59	0.62	0.57
12	0.63	1.00*	0.61	0.54	0.49	0.49
13	0.64	0.85				
14	0.69	0.90*	0.64	0.73	0.53	0.60
15	0.82	0.99*	0.72	0.82	0.92	0.93
16	0.88	0.76				
17	0.89	0.12				
18	0.90	0.99*	0.66	0.68	0.68	0.73
19	0.90	0.90*	0.79	0.70	0.63	0.52
20	0.93	0.76				
21	0.95	0.97*	0.57	0.71	0.86	0.89
22	0.95	0.90*	0.77	0.61	0.40	0.37

(1) Number of females/number of individuals, calculated from 7 to 23 progenies of virgin females in each line (a progeny included 16 to 101 individuals).
(2) Correlation between the intensities of the *wsp* spots and the intensities of the 18S spots in four replicates performed using four volumes of DNA solution.
(3) Volume (μl) of DNA solution.
* $p<0.05$.

lengthening of the hybridization period with the *wsp* probe but not with the 18S probe.

Correlation between the sex-ratio and bacterial density

Dot-blot 1 allowed for the analysis of 10 lines and 30 replicates. Nevertheless, one replicate with a high sex-ratio appears to be aberrant (Fig. 2) and this could modify the relationship between the *Trichogramma* sex-ratio and the *Wolbachia* density. So, we decided to study this relationship with and without this aberrant data point. Dot-blot 2 allowed for the analysis of 15 lines and 60 replicates, while Dot-blot 3 allowed for the analysis of 17 lines and 66 replicates (Fig.2). Therefore, after the Dot-blot normalisation, either by intensity normalisation or by ratio normalisation, the correlation analysis involved 42 lines and 156 replicates, or 155 replicates when the aberrant data point was excluded.

TABLE 4. Analysis of Dot-blot 3 performed with 24 lines of *T. cordubensis,* strain 1032, submitted to a controlled thermal cure (28 or 31° C for two or four generations).

Line	Sex-ratio (1)	Coefficient of correlation *wsp* -18S (2)	Ratio of intensities *wsp* /18S			
			1 (3)	3	6	9
1	0.03	0.84				
2	0.04	0.90*	2.58	2.07	2.72	3.20
3	0.25	0.96*	2.64	2.74	2.51	2.10
4	0.30	0.98*	2.51	2.61	2.71	2.36
5	0.30	0.66				
6	0.31	0.90*	2.58	2.83	2.78	3.55
7	0.32	0.99*	1.51	2.30	2.40	2.88
8	0.32	1.00*	1.99	2.45	2.30	2.33
9	0.35	0.82				
10	0.35	0.57				
11	0.35	0.91*	2.47	3.17	2.98	2.53
12	0.36	0.83				
13	0.39	0.99*		2.32	2.62	3.01
14	0.44	0.94*	2.16	2.70	2.17	2.16
15	0.65	0.97*		2.85	2.86	3.42
16	0.69	0.92*	2.80	2.99	2.87	2.46
17	0.71	0.98*	2.36	2.99	2.17	2.26
18	0.73	0.98*	3.26	3.05	2.88	3.07
19	0.76	0.54				
20	0.78	0.86				
21	0.81	0.92*	3.09	2.72	2.56	3.17
22	0.83	0.99*	2.15	2.61	2.70	2.99
23	0.86	0.99*	2.20	2.51	2.68	2.54
24	0.98	0.98*	2.45	2.54	2.49	2.40

(1) Number of females/number of individuals, calculated from 11 to 22 progenies of virgin females in each line (a progeny included 14 to 84 individuals).
(2) Correlation between the intensities of the *wsp* spots and the intensities of the 18S spots in three or four replicates performed using three or four volumes of DNA solution.
(3) Volume (μl) of DNA solution.
* $p<0.05$.

The correlation between the arcsine$\sqrt{}$ transformed sex-ratio of each line and the intensities *wsp*/18S of the different replicates of each line is significant ($p<0.01$), whether the aberrant data point was included or not (Table 5). However, this correlation appears to be slight (Fig. 3), and an additional analysis (ANOVA) was performed to test the difference in *Wolbachia* density among four classes of sex-ratios, i.e. 0-0.25, 0.26-0.50, 0.51-0.75 and 0.76-1. In this ANOVA, the intensity normalisation was applied to the ratios of intensities *wsp*/18S and the aberrant data point was excluded. A significant difference in bacterial density was recorded ($p=0.002$), the mean class ratios being 0.92, 0.95, 1.02 and 1.07 from the low class to the high class, respectively.

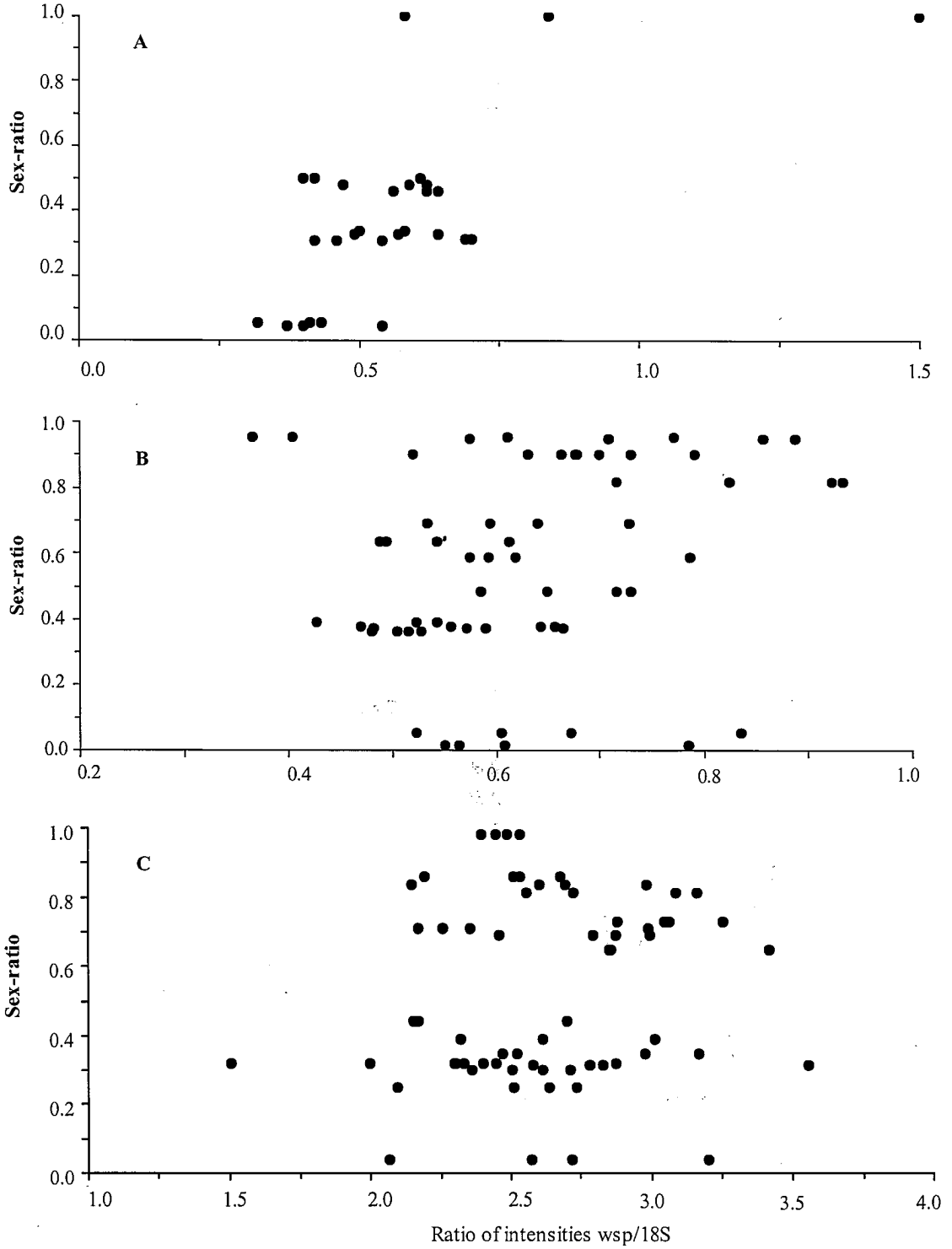

FIG. 2. Sex-ratios plotted against ratios of intensities *wsp*/18S calculated in the three Dot-blots.
A: Dot-blot 1, with three quantities of DNA analysed for each sex-ratio (i.e. each line of *T. cordubensis* studied). B: Dot-blot 2, with four volumes of the DNA solution analysed for each sex-ratio. C: Dot-blot 3, with three or four volumes of the DNA solution analysed for each sex-ratio.

TABLE 5. Coefficients of correlation between sex-ratios and ratios of intensities *wsp*/18S in the strain 1032. Sex-ratios were arcsine$\sqrt{}$ transformed. The three Dot-blots performed were normalised according to two methods (see text).

	Intensity normalisation		Ratio normalisation	
	n	Correlation	n	Correlation
All data used	156	0.333***	156	0.325***
After removal of an aberrant datum	155	0.287**	155	0.277**

**: p<0.001.
***: p<0.0001.

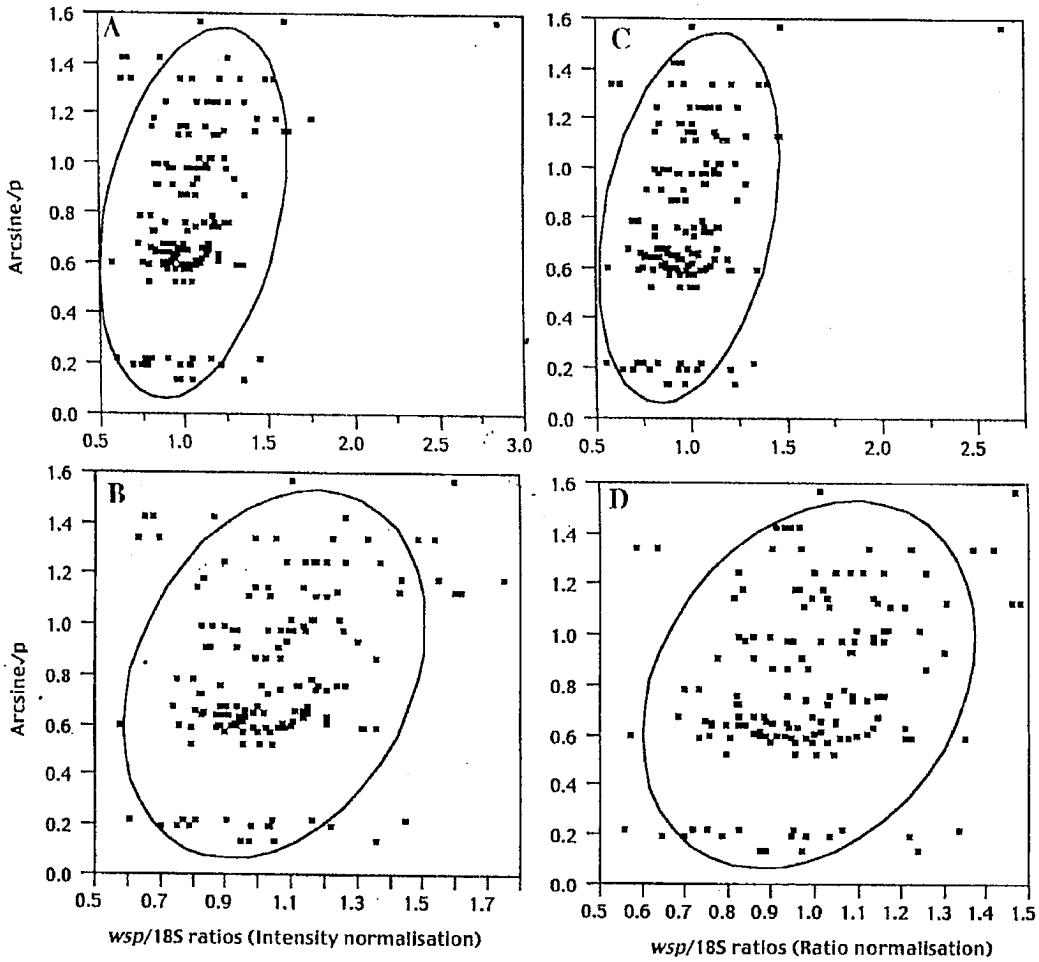

FIG. 3. Arcsine$\sqrt{}$ transformed sex-ratios plotted against ratios of intensities *wsp*/18S with 90% ellipse density.
A: after intensity normalisation of the three Dot-blots (all data used). B: after intensity normalisation of the three Dot-blots and removal of an aberrant replicate performed in Dot-blot 1. C: after ratio normalisation of the three Dot-blots (all data used). D: after ratio normalisation of the three Dot-blots and removal of an aberrant replicate performed in Dot-blot 1.

DISCUSSION AND CONCLUSION

Interactions between host and symbiont genomes can modify *Wolbachia* density and effect on the host (Boyle *et al.*, 1993; Bordenstein & Werren, 1998; Pintureau *et al.*, 2000; Bertícat *et al.*, 2002). Therefore, the description of a relationship between a certain density and a certain effect from different populations or species is difficult. For example, in such genetically heterogeneous material, if the infection level is often consistent with the expression of cytoplasmic incompatibility (Breeuwer & Werren, 1993; Noda *et al.*, 2001; Clark & Karr, 2002), we can assume that the rule presents numerous exceptions (McGraw *et al.*, 2001; Clark & Karr, 2002).

Inside a population, the relationship between bacterial density and effect should be stronger, as it appeared to be for the sex-ratio obtained under different intensities of male-killing in a *Drosophila* species (Hurst *et al.*, 2000). In *Trichogramma cordubensis*, it seems that a slight correlation exists between the sex-ratio produced after thelytoky of variable intensity and *Wolbachia* density. A stronger correlation would, however, be expected and some hypotheses can be proposed to explain such a weak relationship.

A certain variability in sex-ratio was recorded in the progeny of different females from the same line, and the sex-ratio and bacterial density were measured on different progeny from the same line. Therefore, although no trait measurement was restricted to one progeny (mean sex-ratio, DNA extraction from several progeny), two slightly different samples might sometimes have been used to estimate sex-ratio and bacterial density.

Moreover, the DNA extractions were performed from all male and female individuals of a line. Although in *Trichogramma*, it is known that *Wolbachia* are present in males without transmission by spermatozoids (Boléat *et al.*, 2000), the bacterial density could be different in the two sexes. For instance, Noda *et al.* (2001) recorded differences between males (9.1 million *Wolbachia*/adult) and females (28.9) in the homopteran species *Sogatella furcifera* using quantitative PCR, but Ijichi *et al.* (2002) observed no obvious differences at the whole insect level in the coleopteran species *Callosobruchus chinensis*. *Wolbachia* density could also be different in the whole individual and in ovaries or oocytes where thelytoky is induced. Although the numbers of *Wolbachia* present in ovaries and in other tissues could be correlated, possible divergences may exist and this may explain the weak relationship recorded between sex-ratios and bacterial density in the present study. For instance, Dobson *et al.* (1999) observed obvious differences in bacterial density in reproductive tissues and other female tissues of some dipteran species using the Western-blot technique. Likewise, Ijichi *et al.* (2002) recorded density differences between tissues and organs of a beetle using quantitative PCR. Moreover, McGraw *et al.* (2002) suggested that rate of *Wolbachia* replication depends on the type of tissue infected.

It is also possible that we selected a *Trichogramma* species showing low bacterial densities. In species infected by a higher number of *Wolbachia*, thermal treatments could lead to a larger range of densities, which would allow easier assessment of correlation with sex-ratio. Other species have thus to be tested, especially partly infected species (including infected and non-infected individuals) suspected to harbour higher bacterial densities.

The weak relationship between bacterial density and sex-ratio could also originate from the fact that the Dot-blot technique was not sensitive enough. Several authors used a more sensitive technique, namely quantitative PCR and/or confocal microscopy, to quantify bacterial density in various insects (Sinkins *et al.*, 1995; Noda *et al.*, 2001; Bertícat *et al.*, 2002; Clark & Karr, 2002; Kondo *et al.*, 2002). In subsequent experiments, although expensive and difficult to use with a large number of lines, such techniques would probably allow a better estimation of the quantity of *Wolbachia* from a lower number of *Trichogramma*.

Finally, a difference could exist between the quantity of *Wolbachia* present in the hosts, estimated by Dot-blot, and the quantity of active *Wolbachia*, i.e. able to alter the sex-ratio. Indeed, the thermal cure can correspond to a *Wolbachia* de-activation or elimination of *Wolbachia* (Pintureau *et al.*, 2002). We can then suppose that the removal of the de-activated *Wolbachia*, assuming that it is possible, would increase the strength of the relationship between bacterial density (active bacteria) and *Trichogramma* sex-ratio. Although physiologically active *Wolbachia* can be detected by the F.I.S.H. technique (Pintureau *et al.*, 2000), they cannot be quantified by this technique because they are too minute and dispersed in the cell.

In spite of some problems, we showed that the effect of *Wolbachia* on host reproduction depends on the bacterial density, which is probably regulated by the host itself. In that way, the host can decrease or increase the intensity of some symbiont effects and, as a result, reduce or promote the symbiont prevalence. Such a process could notably lead to *Wolbachia* suppression.

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