Wolbachia infection and incompatibility dynamics in experimental selection lines

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Abstract

High and low levels of Wolbachia-induced cytoplasmic incompatibility (CI) were selected for in the parasitic wasp *Nasonia vitripennis,* in the single-infected strain Ti277. After nine generations of selection, males from lines selected for high incompatibility level (HI lines) were significantly more incompatible with uninfected females (AsymC) than the maternal strain. The reverse response, a full compatibility with AsymC, was observed in eight out of 12 lines selected for low incompatibility (LO lines), correlated with loss of *Wolbachia* infection. Bacterial density estimates in the eggs of some HI lines increased significantly. The procedure for line maintenance resulted in introgression of AsymC nuclear genome into the Ti277 background. Significant changes of CI level and bacterial density due to the introgression were also observed in the control lines, possibly reflecting an effect of host genotype on bacterial density and CI. After selection had been relaxed for six generations, bacterial density in the five high-infected HI lines declined back to a level comparable to the other lines. The data are consistent with the 'bacterial dosage' model, but with an upper threshold of bacterial infection above which there is no correlation between infection level and CI level. We further investigate the maternal transmission of bacterial density by a mother-daughter regression on bacterial density. The pattern observed is consistent with a density dependent regulation of bacterial numbers around an 'equilibrium' density, independent of any effects of CI. The equilibrium value is likely to be determined by both bacterial strain and host genotype.

Introduction

Intracellular bacteria of the genus *Wolbachia* cause cytoplasmic incompatibility (CI) in insects (Breeuwer & Werren, 1990; O'Neill & Karr, 1990; Rousset *et al.,* 1992; Solignac *et al.,* 1994). Although present in both the testes and the ovaries, they are vertically transmitted through the egg cytoplasm but not through the sperm. Wolbachia-induced CI results in the production of lethal embryos in diplodiploid organisms (Yen & Barr, 1973; O'Neill & Karr, 1990) and of all-male progenies in haplodiploid organisms (Breeuwer & Werren, 1990). Both phenomena result from an aborted karyogamy in early mitosis (Ryan *et al.,* 1985; O'Neill & Karr, 1990; Reed & Werren, 1995). *Wolbachia* distribution and biology are reviewed in Werren (1997).

Three main factors have been suggested to determine the direction and strength of cytoplasmic incompatibility: (1) bacterial strain, (2) bacterial density and (3) host genotype. Evidence supports a role for bacterial strain, such as occurrence of bidirectional incompatibility between hosts infected with different *Wolbachia* strains (Braig *et al.,* 1993; Mer~ot *et al.,* 1995; Rousset & Solignac, 1995; Perrot-Minnot *et al.,* 1996) and existence of nonfunctional *Wolbachia* strains (Holden *et al.,* 1993; Giordano *et al.,* 1995; Rousset & Solignac, 1995; Hoffman *et al.,* 1996). Bacterial density in both eggs (Hoffmann *et al.,* 1990; Boyle *et al.,* 1993; Breeuwer & Werren, 1993; Sinkins *et al.,* 1995; Bourtzis *et al.,* 1996) and sperm cysts

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(Bressac & Rousset, 1993; Solignac *et al.,* 1994) has been suggested to be important in determining CI level. Breeuwer £r Werren (1993) proposed a 'bacterial dosage model' in which incompatibility is determined by the relative bacterial dose in males and females (i.e. higher infected males are incompatible with lower infected females).

Host genotype may also influence CI, but there is so far little direct evidence for this. Boyle *et al.* (1993) suggest that the poor expression of CI *Wolbachia* from *Drosophila simulans* transfected into *D. melanogaster is* due to low CI expressivity of this host. Bordenstein & Werren (1997) have found that strength of expression of CI can be influenced by host species genotype in *Nasonia.*

Little is known about the inheritance pattern of *Wolbachia* infection level. There are certainly stochastic processes affecting the number of bacteria transmitted to eggs (Boyle *et al.,* 1993; Breeuwer £r Werren, 1993; Perrot-Minnot *et al.,* 1996; Guillemaud & Rousset, 1997), but it is unclear to what extent bacterial densities are inherited from one generation to the next.

The purpose of this study is to investigate (1) whether CI level can be changed by artificial selection, (2) whether changes in CI level correlate with changes in bacterial density and (3) how bacterial densities are inherited from one generation to the next.

Materials and methods

Laboratory strains

The following strains were used. Ti277: this strain carries an eye-colour mutation (ti277) and is singly infected with A-group *Wolbachia* (Perrot-Minnot *et al.,* 1996). Ti277, which shows intermediate bacterial densities and partial cytoplasmic incompatibility, was used in the selection experiment. AsymC: this strain was produced by antibiotic curing of the wild-type strain LablI in 1986, and has been maintained free of *Wolbachia* since.

Selection procedure

The experiment was designed to select for males with a high level (HI lines) or low level (LO lines) of incompatibility to uninfected females. The protocol for each selective regime involved the following steps: establishment of isofemale lines from the parental strain Ti277, maintenance of 50 lines per generation through female lineages, and selection of 10% of the lines at each generation based on male compatibility relationships with AsymC females.

Line establishment and maintenance

From the parental generation on, females used to establish new isofemale lines were individually mated to AsymC males and then set on two hosts for oviposition (fleshfly pupae). From the F2 to the F9 generation, five lines selected for high incompatibility with AsymC (HI lines) and five lines selected for low incompatibility (LO lines) were used to establish 50 lines of each type at the next generation. The selection criteria applied to chose five lines among 50 are detailed below. The females used per line were sisters of the males tested for compatibility. Lines were maintained under selection for nine generations. From the tenth to the sixteenth generation, selection was relaxed and lines were maintained by mass rearing.

The choice of AsymC males (uninfected) for maintenance of the lines was made to avoid any uncontrolled selection of compatibility level in the female lineages. Repeated crosses with AsymC males rapidly resulted in the complete replacement of the Ti277 nuclear genome by the genome of AsymC in experimental lines. Therefore, a set of control introgression lines, subsequently labelled 'CT[int] lines' was established. These CT[int] lines were started two generations later than the selected lines.

Selection criterion

The selection criterion was the compatibility level of males of each line with AsymC females. Normally, *N. vitripennis* females produce strongly female-baised sex ratios when ovipositing alone on hosts (Whiting, 1967). In contrast, incompatibility in *Nasonia* is expressed as production of all-male or nearly all-male families. This occurs because paternal chromosome loss results in (haploid) male production in this haplodiploid insect (Breeuwer & Werren, 1990). Thus, a sex ratio shift towards a higher proportion of males is the standard phenotypic assay for incompatibility. Test crosses were performed at each generation and for each of the 50 HI lines and 50 LO lines. Five to seven males were tested in single pair matings to uninfected (AsymC) females and the sex ratio of their progeny recorded (proportion of females). The selection criterion applied to LO lines was a smallest number of all-male progeny and an increase in sex ratio above the median level of 83% found in within-Ti277 crosses. For the HI lines, the criterion was the proportion of all-male progenies among the test crosses, since selection was directed towards complete incompatibility. No selection was applied to CT[int] lines.

Response to selection: compatibility relationships

After nine generations of selection, we determined the infection status of 15 HI lines, 12 LO lines and 18 CT[int] lines by test-crosses and bacterial infection estimates. Crosses of males from each line to AsymC were performed at the F10 generation to see if compatibility had changed. Reciprocal crosses between each line and Ti277 strain were performed to see whether selection on level

of compatibility to AsymC affected compatibility relationships with Ti277, the strain the lines were drawn from. Control crosses were performed simultaneously between Ti277 and AsymC individuals from stock cultures. The same test crosses were performed on the control lines (CT[int]) at the F10 generation, about 1 month after the ones above, together with another set of controls.

We also analysed the changes in CI level in the course of selection on HI and LO lines: sex ratio and CI expression were compared between generations from F3 to F8.

Correlated response in bacterial infection level

Density of bacteria was determined by counting bacteria in the egg cytoplasm under a light microscope, after fixation in Carnoy and lacmoid staining. Details of the procedure for the collection of eggs, fixation, staining and counting are given in Breeuwer & Werren (1993) and Perrot-Minnot et al. (1996). The total number of bacteria were scored in 15 10 x 10-mm columns in the posterior pole of the egg. Counts therefore are not an estimate of the total bacterial density in the egg.

For bacterial density analysis at the F10 and F16 generations, we sampled seven eggs per female and six females per line (for nine HI lines and seven LO lines) or three females per line (18 CT[int] lines). The changes in bacterial infection level during the selection were assessed by comparing bacteria counts at F3, F4, F5, F9 and F10 generations, in 6-15 lines, two females per line (except F3: one female per line) and eight eggs per female.

Statistical analysis on the selection experiment data

Three parameters have been analysed for the lines and the stock cultures: sex ratio, family size and bacterial density. Sex ratio data were transformed by taking the arcsin of square root (Sokal & Rolf, 1981). Bacteria counts in eggs show a frequency distribution close to a Poisson distribution; however, we chose to follow the Boxcox procedure to estimate the best transformation normalizing the count data (Sokal & Rolf, 1981; Crawley, 1993). Using the GLIM procedure for minimizing the residual deviance (Crawley, 1993), a cubic root was determined as the most appropriate transformation of bacterial count data. Therefore, we transformed all data of bacterial counts in cubic root.

Comparison of the parameters measured on the selected and the control lines to those of Ti277 involved several levels of variability. To compare data between the types of lines and the control Ti277, we needed to suppress the line effect from the analysis. This was done by performing a separate analysis of variance for each type of line, to test for differences between lines: (1) if the line effect was not significant, data from the different lines within each type were pooled, (2) if the differences between lines were significant, a multiple comparison test of Newman-Keuls was done to group the lines with no significant differences. In the latter case, the type of line (HI, LO or CT[int]) was split in as many groups as given after the Newman-Keuls test. These groups were subsequently labelled HI(I), HI(II), LO(I), LO(II), etc.

Analysis of variance were then performed to test for significant differences between groups, using a linear link function: one-factor ANOVA on sex ratio and family size data from the test crosses, and nested ANOVA on bacterial density, with the female effect nested in the group effect. If a significant group effect was evidenced in the ANOVAS, paired comparisons of mean sex ratio and family size between HI, LO, and the control Ti277 (A), and CT[int] and the control Ti277 (B), were performed with a Student t-test or Newman-Keuls test for unequal and equal variances, respectively. Mean bacterial density was compared between groups (at F10, between HI, LO, CT[int] and Ti277) by taking the mean per female of bacteria counts in seven eggs, after cubic root tranformation.

All statistical analysis were performed using GLIM software (version 3.77), except the Newman-Keuls multiple or paired comparison test (Logitheq software, after Sokal & Rolf, 1981). ANOVAs and Student t-tests were performed following GLIM procedure, with $P < 0.05$ (unless mentioned). After fitting a model to the transformed data with the linear link function, we checked the normality of errors and the goodness of fit by plotting the residuals against the standard normal deviates and against the fitted values, respectively (Crawley, 1993).

For simplicity of reading, data on sex ratio and bacterial density in tables are given as median and first quartiles (25-75%).

Female-daughter correlation of bacteria density in eggs

To investigate inheritance patterns of bacterial density, seven mated 1-2-day-old females were taken from seven different HI lines at the F10 generation, chosen to obtain a large range of variation in bacterial density. Fifteen to 20 eggs were collected within a 2-4-h time frame for cytology, and the females were then set on hosts to produce daughters. At the next generation, five females were picked within each family after brother-sister mating, and eggs were collected following the same procedure as the previous generation. Bacterial density estimates in eight eggs per female were compared between mother and daughters after cubic root transformation.

Results

Ti277 strain characteristics: CI relationships and bacterial density

To select for high and low incompatibility, a strain with partial incompatibility is needed. Breeuwer & Werren (1993) reported partial compatibility and high variance in compatibility of Ti277 males crossed to uninfected females (AsymC), with a mean of 17% (± 21%) females produced. We further documented this variability within Ti277 in sex ratio (Table 1a) and in bacterial density (Table lb).

Moreover, Ti277 is singly infected with A type of *Wolbachia,* whereas most laboratory strains and natural populations of N. *vitripennis* are double-infected (Perrot-Minnot *et al.,* 1996). The latter characteristic avoids any confounding effect during selection due to a differential infection level with A and B types of *Wolbachia.*

Selection on compatibility level

The initial stage

Compatibility relationship of 33 lines established from Ti277 females crossed to AsymC males was assessed at the F1 generation, by crossing males individually to uninfected (AsymC) females. Sex ratio (proportion female) median for the 132 crosses was 0.11 (0.03-0.32 quartiles), confirming the partial compatibility of Ti277 with AsymC. A significant line effect was evidenced in the ANOVA ($F = 2.43$, 31 and 98 d.f., $P < 0.005$), with eight lines having a sex ratio median above 0.33, and six below 0.03. This heterogeneity among F 1 lines confirmed the within-strain variability of Ti277 on CI level with AsymC, and suggested sufficient heritable variation for selection to operate.

HI lines and LO lines CI changes

Incompatibility of HI lines with AsymC significantly increased from the F1 to F3 generation (Neuman-Keuls paired comparison test, $t = 5.74$, 182 d.f., $P < 0.01$) and from the F7 and F8 generations ($t = 3.72$, 270 d.f., $P < 0.01$) (Fig. la). No heterogeneity was detected with a multiple comparison test among the lines within each generation, except at F7.

A highly significant heterogeneity among LO lines at each generation masked divergent evolution towards high or low compatibility with AsymC (sex ratio median from 0.85 to 0.90 and from 0 to 0.09, respectively) (Fig. lb). This bimodal pattern of compatibility changes was evidenced in the drop of the proportion of partially compatible lines (sex ratio median from 0.1 to 0.8) from 57.5% at F1 generation to 19% at F8 generation (Fig. lb). Highly compatible lines appeared twice and were found to be uninfected (see below): at F3 and at F7.

HI and LO lines CI relationship at F10 generation compared to control introgression

HI and LO lines were characterized at the F10 generation by crosses with standard laboratory strains AsymC and Ti277. As expected, males from HI lines were significantly more incompatible with AsymC females than were males of the maternal line Ti277 (Table 2). One group of four LO lines (1) showed levels of incompatibility to AsymC females comparable to HI line males. The opposite pattern of CI changes was observed in the other eight LO lines (group II): males were fully compatible with AsymC females at a similar level as AsymC males, and females showed a high level of incompatibility with Ti277 males, similar to that observed between AsymC females and Ti277 males (Table 2).

The pattern of CI observed in CT[int] lines set as control for introgression was similar to that observed in HI lines and group I LO lines (Tables 2 and 3). Therefore, the increased incompatibility of HI males with AsymC females could simply be a result of introgression of AsymC genome into Ti277 cytoplasm. This interpretation is further supported by the occurrence of a similar response to introgression in group I LO lines, despite a selection for a decreased incompatibility of these lines with AsymC (i.e. in the opposite direction). Therefore, the only changes that can be unequivocably attributable to selection on CI level is the complete compatibility with AsymC of eight LO lines (group II), in response to a

Table la Characteristics of Ti277 strain of N. *vitripennis* (I a) Cl level and fecundity compared to laboratory strains AsymC and LabIl. Sex ratio of the progeny is given as the mean proportion of females. Family size corresponds to the adult offspring number.

Crosses male x female	Sample size	Family size mean (tSD)	Sex ratio mean (±SD)	Arcsin
Ti277 x Ti277	33	101.0 t 25.8	0.86 t 0.16	68.7 t 12.7
AsymC x Ti277	37	74.5 t 25.9	0.80 t 0.08	63.7 t 6.1
AsymC x AsymC	14	88.4 t 29.9	0.90 t 0.05	72.0+ 4.1
Labll x Labll	18	92.9 t 27.7	0.88 t 0.03	70.1 t 2.9

Table lb Comparison of bacterial densities. Bacterial density in a strain is given as the median and first quartiles (25% -75%) over all eggs. Sample size is the number of eggs (number of females in parentheses). A t-test was performed on the means of bacterial density, after a cubic root transformation. Infection type: A, A group of *Wolbachia;* B, B group of *Wolbachia;* AB, double infection with A and B group. Southern hybridization was used to determine infection type.

	Sample size	Bacterial density median	quartiles	t-test (P < 0.05)	Infection type
T277	119(17)	79	52-157	b	A
Labll	91 (13)	189.5	146-275	a	AB
R511	98(14)	157.5	130-210	a	AB

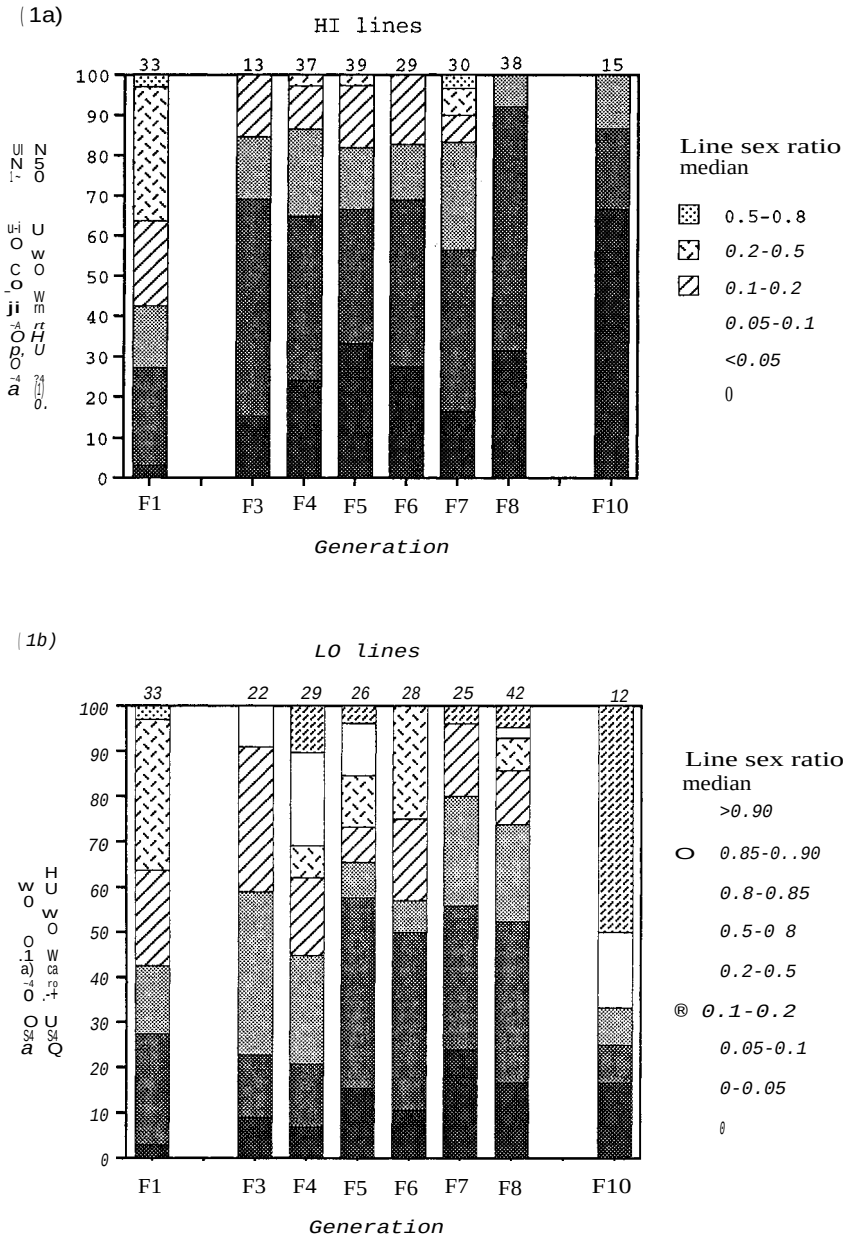

Fig. 1 Evolution of CI level in the selection lines. The diagram shows the distribution of CI levels of males from HI lines (a) and LO lines (b). CI level of a line was defined as the proportion of females among progeny of line males crossed to uninfected standard females. The proportion of lines in each class is shown, with the number of lines per generation given above each bar. Darker classes represent high to complete incompatibility (sex ratio lower than 0.1), lighter classes represent low incompatibility (sex ratio above 0.85).

decreased incompatibility from the original Ti277 strain. Such changes have occurred at least five times in the course of selection on LO lines.

Correlated response in bacterial density

The initial stage: bacterial density in FO (parental) females and in Ti277 females

Bacterial infection of 20 Ti277 females was estimated by egg counts. The infection level was found to be significantly lower than the one estimated from 17 Ti277 females several weeks later (75 ± 46, n = 140 vs. 122 ± 107, n = 119), and also lower than the bacterial density reported for the same strain 3 years ago (mean = 116 ± 34, n = 15) (Breeuwer & Werren, 1993) (Table 4). Heterogeneity within the Ti277 strain might be responsible for significant differences between samples.

LO and HI bacterial density at F10 generation: CT[int] lines at Fl O generation

HI and LO lines were significantly heterogeneous in bacterial density (multiple comparison test of Newman-Keuls, $P < 0.05$) and were therefore split in three and two groups, respectively. All females (15) examined from group II LO lines were uninfected (Table 4). In contrast,

Table 2 F10 generation compatibility relationships in crosses with AsymC and Ti277. Sample size is the number of crosses performed for each type of lines. Since line-effect was not significant among HI lines, crosses from all HI lines were pooled. LO lines were split into two groups (I,II) within which lines were not significantly different (see Methods section). Sex ratio corresponds to the proportion of females in the progeny, and family size to the number of emerging adults. Paired comparisons between crosses were performed on sex ratio and on family size means, within each group of crosses using either females or males from the same laboratory strain. Crosses with the same letter within a group are not significantly different ($P < 0.05$).

Crosses male x female	Sample size	Family size mean (SD)	t-test	Sex ratio median (quartiles)	t-test
Ti277 x AsymC (A)	28	88.21 (28.8)	n.s.	0.06 (0.01-0.36)	b
HI x AsymC	90	85.3 (25)		0(0-0.02)	a
LO(I) x AsymC	24	91.8 (28.9)		0 (0--0.04)	a
LO(II) x AsymC	48	97.4 (27)		0.91 (0.88-0.92)	c
Ti277 x Ti277 (A)	26	118.8 (19.4)	n.s.	0.87 (0.85-0.89)	n.s.
HI x Ti277	90	121 (20.3)		0.89 (0.84-0.91)	
LO(I) x Ti277	24	122 (17.3)		0.89 (0.79-0.91)	
LO(II) x Ti277	48	121.1 (24.4)		0.88 (0.82-0.91)	
Ti277 x Ti277 (A)	26	118.8 (19.4)	a	0.87 (0.85-0.89)	b
Ti277 x HI	60	107.8 (14.3)	b	0.91 (0.89-0.92)	a
Ti277 x LO(I)	16	97.1 (21)	c	0.91 (0.89-0.92)	a
T277 x LO(II)	32	92.8 (19.5)	c	0.06 (0.01-0.17)	c
AsymC x Ti277 (A)	27	115.7 (31.9)	a	0.90 (0.89-0.93)	n.s.
AsymC x HI	60	85.7 (27.8)	b	0.90 (0.88-0.91)	
AsymC x LO(I)	16	82.1 (23.2)	b	0.88 (0.87-0.92)	
AsymC x LOII)	32	87.3 (21.6)	b	0.89 (0.88-0.90)	
AsymC x AsymC (A)	29	91.2 (28)		0.91 (0.89-0.92)	

Table 3 FIO generation compatibility relationships of CT[int] lines compared to the Ti277 strain. The crosses from the 18 CT[int] lines were pooled, since the line-effect was not significant. Significant differences between crosses with CT[intl and the corresponding control were all at $P < 0.001$.

Crosses male x female	Sample size	Family size mean (SD)	t-test	Sex ratio median (quartiles)	t-test
Ti277 x AsymC (13)	33	62 (21.4)	n.s.	0.04 (0-0.16)	b
CT[int] x AsymC	108	70.6 (28.4)		0(0-0.03)	a
Ti277 x Ti277 (13)	33	108.2 (27.8)	n.s.	0.85 (0.80-0.87)	n.s.
CT[int] x Ti277	108	107 (27.8)		0.86 (0.76-0.90)	
Ti277 x T277 (13)	33	108.2 (27.8)	a	0.85 (0.80-0.87)	b
Ti277 x CT[Int]	72	91.4 (20.5)	b	0.90 (0.88-0.93)	a
AsymC x Ti277 (B)	32	107.9 (28.9)	a	0.83 (0.68-0.88)	b
AsymC x CT[int]	72	86.4 (25.1)	b	0.90 (0.88-0.92)	a
AsymC x AsymC (B)	28	90.3 (25.2)		0.90 (0.89-0.92)	

the bacterial densities had increased in females in the other LO lines (group I), the HI lines and the CT[int] lines. This increase was significant compared to Ti277 FO females (for all groups except the lowest infected HI lines (group III)), and to Ti277 females from the mass rearing (for group I LO lines and group I HI lines) (Table 4). Consequently, one interpretation is that the increase in bacterial infection in most F 10 infected lines is an effect of the introgression of AsymC nuclear genome in a Ti277 cytoplasm.

The changes in bacterial load during the course of selection were followed from F3 to F8 for HI and LO lines. For both HI and LO infected lines, some fluctuations could be observed from one generation to the next, despite a steady tendency to increase during the first five generations, and to stabilize further on (Fig. 2).

Comparative changes in CI and infection level

The pattern of increased bacterial density observed in all the F10 infected lines (HI, LO (group I) and CT[int]) was consistent with the increased incompatibility of these lines with AsymC females. Therefore, our data are consistent with the view that the level of incompatibility

Table 4 Bacterial infection levels in different lines. Sample size is the number of eggs, with number of females in parentheses. Bacterial infection level is given as the median and first quartiles over all eggs. Paired comparisons by the Newman-Keuls test were performed, after cubic root transformation ($P < 0.05$). Types of lines with the same letter are not significantly different in paired comparison. Groups with significant line-effect were split into groups with no significant differences (1, 11 and 111) before paired comparisons. Percentage compatibility corresponds to the proportion of female offspring in crosses of males to AsymC females.

Type of line generation (no. of lines)	Sample size	Bacterial density median (quartiles)	t-test	Compatibility with AsymC females (median-quartiles)	% ali-male progenies
Ti277	119(17)	79(52-157)	c	4(0-29)	27.9 (61 crosses)
FO generation	140(20)	63 (37.5-108)	d		
F1 generation		–		12(3-32)	0(0-25)
F10 generation					
CT[int] (9)	189(27)	121 (88-182)	be	0(0-3)	75 (50-87.5)
HI (I) (5)	210(30)	248(175-330)	a	0(0-3)	83.3 (41.7-83.3)
HI (II) (3)	112 (16)	132 (107-197)	be	0(0-3)	75 (50-83.3)
HI (III) (1)	42(6)	103 (79-131)	bcd		
LO (I) (3)	126(18)	150 (107-197)	b	0(0-4)	58.3 (25-79.2)
LO (II) (5)	105(15)	0	-	91 (88-92)	OO⁻0)
F16 generation					
HI (9)	371 (53)	128 (88-195)	be		
LO (I) (3)	90(15)	150.5 (106-205)	be		
LO (II) (5)	105(15)	0	-		

was a function of bacterial density in the males. This correlation was particularly clear when comparing the changes in CI and infection level during the course of the selection /introgression from FO to F9 (Figs la and 2).

From F10 to F16 generation, the 15 HI lines and 12 LO lines were maintained without selection in mass rearing. The five uninfected LO lines at F10 were still free of *Wolbachia* at F16, therefore showing that the curing of bacteria was complete (Table 4). Changes in bacterial infection from F10 to F16 were significant only in the highly infected HI lines (group I) with a decrease of the median over all eggs from 248.5 (145-305.5; $n = 210$) to 108 (79-163; $n = 210$) (Table 4). Bacterial density in the other HI lines and in infected LO lines slightly increased or remained constant, respectively (Table 4). The infection levels of these lines at F16 was homogeneous (multiple comparison test on all HI and infected LO lines, n.s.) and was very close to that found in the CT[int] lines at F10, suggesting that an equilibrium had been reached, possibly associated with AsymC genotype.

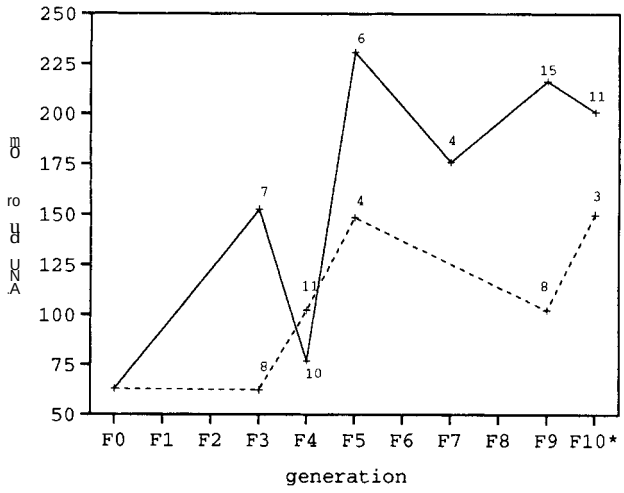

Fig. 2 Evolution of bacterial density in HI lines (plain lines) and LO lines (broken lines). Bacterial load is given as the median of bacteria density in eggs over all lines (seven eggs per female, two females per line). Only the infected LO lines (group 1) are represented, the group 11 LO lines were consistently uninfected.

Inheritance pattern of *Wolbachia* infection in HI females

Comparisons between bacterial densities in eggs of females and their daughters were made to investigate maternal inheritance pattern of bacterial infection (Table 5 and Fig. 3).

Both a maternal effect on the infection level of the daughters and within-family differences between females were detected by the nested ANOVA ($F = 6.36$, 6 d.f., $P < 0.01$ and $F = 11.47$, 26 d.f., $P < 0.01$, respectively). However, this maternal effect and the within-family heterogeneity of bacterial infection were different from one mother/family to the other. When comparing the median of infection level of the daughters to the median of the mother in Table 5, it is clear that bacterial density has increased in some families and decreased in others, and that the range of bacterial infection in daughters was also variable from one family to another. We therefore calculated an index of transmission bias to check whether a consistent trend characterizes maternal transmission of

Table 5 Maternal inheritance pattern of *Wolbachia* infection. Bacterial infection in mothers is given as the median and minimum-maximum of bacterial density over all eggs (eight per female). Bacterial infection of daughters is the median over the medians of the 4-5 females analysed per family (eight eggs per female). Min-max are the median infection level of the weakest and the highest infected daughters. Mother/daughter comparison shows the number of daughters having a significantly higher or lower infection level than the mother (paired comparison test on bacterial density means after cubic root transformation, $P < 0.05$). Transmission bias in maternal-daughter bacterial density is also shown (see text for explanation). The mean transmission bias is shown, based on calculation of transmission bias per each mother-daughter pair.

Mother (M) median (min-max)	Daughters (D) median (min-max)	Mother/daughter comparison	Transmission bias mean (SD)
99.5 (80-176)	142 (57.5-250)	3/4	0.49 (0.9)
133.5 (79-161)	267(103.5-315.5)	3/5	0.7 (0.66)
164(111-257)	255 (202.5-412.5)	3/5	0.72 (0.5)
167.5 (130-254)	111.5 (89.5-118.5)	4/4	-0.36 (0.08)
176 (114-347)	146.5 (125-169)	1/5	-0.16 (0.09)
204(122-342)	96 (54.5-115.5)	5/5	-0.54 (0.12)
266(162-424)	141 (89.5-193.5)	4/4	-0.45 (0.15)

infection level. The transmission bias was defined as the deviation of the daughter bacterial density median (D) to the bacterial density median of the mother (M), divided by the bacterial density median of the mother. Mean transmission bias was positive in the three weakest infected mothers, ranging from 0.49 to 0.72, and was negative in the four highest infected mothers, ranging from -0.16 to -0.54 (Fig. 3, Table 5). The dispersion of the daughters within a family around the mean value of transmission bias was also higher when the bias was positive (Fig. 3, Table 5).

Results suggest a density-dependent regulation of bacterial density. Based on the data, an equilibrium value of bacterial infection can be roughly estimated to fall around 165-170. This intermediate level, around which bacterial density of a female lineage would fluctuate, is fairly close to the average infection level of **150** found in F16 LO lines, and slightly higher than infection level in the control lines and HI F16 lines after several generations of mass rearing.

Discussion

We have found the following: (1) selection for low male incompatibility resulted in complete compatibility and loss of bacteria in some lineages; (2) selection for high male incompatibility resulted in an increase of incompatibility and an increase in bacterial density; (3) increases in incompatibility and bacterial density also occurred in the control introgression lines; and (4) there is a transmission bias between the bacterial density in mothers' eggs and density in daughters' eggs, the sign of which depends upon infection level in the mother.

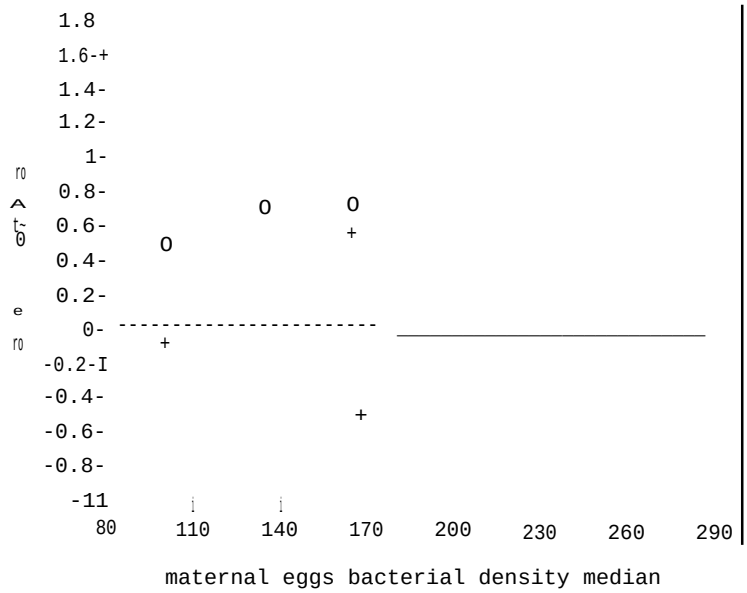

Fig. 3 Relationship between bacterial density in a female's eggs to the bias in infection transmission to the eggs of their female offspring. For each mother, the circle is the mean transmission bias, and the crosses are the bias for each daughter (see text for the definition of the transmission bias). Maternal bacterial density is the median over eight eggs per female.

Results of the selection experiment revealed two phenomena. First, there was a rapid loss of infection and resulting high compatibility occurring in some of the LO lines. Second, there was a general rapid increase in CI level and bacterial density in infected lines. The latter phenomenon was independent of the selection regime, since it occurred in infected LO lines, HI lines and control CT[int] lines. We have therefore not been able to demonstrate a response to selection for high incompatibility to uninfected females in Ti277 lines (HI lines). Instead, a possible response to selection on CI phenotype in isofemale lines of *N. vitripennis* might have been masked by the strong effect of the introgression of AsymC nuclear genome into Ti277 cytoplasm.

Three other studies have addressed the effectiveness of selection for increased cytoplasmic incompatibility, two of which gave a positive response, in the mosquito Culex *pipiens* (French, 1978) and in *D. melanogaster* (Boyle *et al.*, 1993). At a time where the aetiological agent was only suspected to be a rickettsia-like symbiont, French (1978) designed a selection experiment for increased cytoplasmic incompatibility on the basis of existing heterogeneity between and within populations of *C. pipiens.* Complete incompatibility was reached in three generations of selection, thereby showing a strict maternal inheritance pattern, and the segregation of hereditary CI factors responsible for variable CI level. Boyle *et al.* (1993) also gained a positive response to selection for increased CI level in *D. melanogaster* lines, starting from a partially incompatible line established from the interspecific transfer (by microinjection) of *D. simulans* cytoplasm into *D. melanogaster* eggs. In both studies, females used to establish the lines were crossed to their brothers. In contrast, Hoffmann *et al.* (1994) failed to increase incompatibility levels by selection on isofemale lines of *D. melanogaster,* despite initial polymorphism of CI level among the isofemale lines.

The selective response in the LO lines may be interpreted as the consequence of stochastic loss of bacteria. In support of this is the absence of lines with intermediate infection and CI levels. Moreover, an uninfected line was also found at F4 generation in HI lines, despite an opposite direction of selection. Following several authors, we therefore assume that curing can occur by the stochastic loss of bacteria in few oocytes within an infected individual (Hoffmann *et al.*, 1990; Turelli *et al.*, 1992; Boyle *et al.*, 1993; Mergot *et al.*, 1995).

The effect may be influenced by the baseline bacterial density. In males, the production of a low proportion of sperm cycts devoid of bacteria has already been demonstrated cytologically in *Drosophila* (Bressac & Rousset, 1993; Mergot *et al.*, 1995), and is probably responsible for partial compatibility with uninfected females (Hoffmann *et al.*, 1990; Breeuwer &r Werren, 1993; Bressac & Rousset, 1993; Solignac *et al.*, 1994; Mer~ot *et al.*, 1995). Our data also confirm that loss of *Wolbachia* may be more likely in lines, strains or populations with weak to intermediate infection level (Hoffmann *et al.*, 1990; Turelli fr Hoffmann, 1995). Uninfected lines appeared more often among LO lines than among HI lines.

Bacterial dosage model

Despite the confounding effect of introgression on the interpretation of the selection experiment, some interesting conclusions can be drawn from the data presented in this paper. The consistent pattern of bacterial density changes with changes in CI level lends support for the bacterial dosage model, i.e. elevated incompatibility with increased bacterial infection, and increased (partial) compatibility with low bacterial densities or low proportion of infected cysts.

Several authors have interpreted cytoplasmic incompatibility induced by bacteria as a quantitative phenomenon, depending on bacterial infection level, although bacterial density was not estimated (French, 1978; Hoffmann *et al.*, 1990; Montchamp-Moreau *et al.*, 1991). Since then, experimental evidence for this has been accumulating in *Drosophila,* relying on interspecific or interstrain comparisons of CI level and bacterial infection (Solignac *et al.*, 1994; Sinkins *et al.*, 1995; Bourtzis *et al.*, 1996), on the effect of male ageing (Bressac & Rousset, 1993), and on laboratory manipulation of females' infection level through backcrosses to uninfected males (Rousset fr De Stordeur, 1994; Mer~ot *et al.*, 1995), and transfection of cytotypes (Boyle *et al.*, 1993). Incompatibility level was correlated with bacterial density in *N. vitripennis* females variably infected, after partial antibiotic curing (Breeuwer & Werren, 1993) or prolonged diapause (Perrot-Minnot *et al.*, 1996). In all these studies, bacterial infection differences were associated with differences in unidirectional incompatibility level in standard CI crosses.

Our results suggest that an upper threshold exists for bacterial dosage model effect on CI level. Bacterial densities in some HI lines were as high as twice the level of the control lines and the other HI lines, but with a similar CI level (close to maximum). The range of bacterial infection variability in these lines was therefore still above the threshold of infection below which CI level would decrease with decreasing bacterial infection.

Host genetic and epigenetic factors

The other conclusion drawn from the present study is that introgression of AsymC into Ti277 increased bacterial density and CI expression. AsymC is a LabII strain of *N. vitripennis* cured from *Wolbachia* by tetracycline treatment. LabII females harbour significantly higher bacterial density than Ti277, and the bacterial load of these two strains is stable over time: mean bacterial densities reported here for LabII (215 ± 94.5, $n = 88$) and Ti277 (122.2 ± 106.5, $n = 119$) are comparable to the

estimates made 3 years before on the same strains and with the same protocol for egg counting (292 t 52, $n = 13$ and 116 t 34, $n = 15$, respectively) (Breeuwer & Werren, 1993).

The influence of host nuclear genes on symbiont density and CI expression has been proposed to explain changes following interspecific transfer of *Wolbachia* through microinjection from a *D. simulans* donor to a *D. melanogaster* recipient egg (Boyle *et al.,* 1993). In addition, host genomic effects on strength of CI expression of *N. vitripennis* A group *Wolbachia* have been found following introgression of *N. girauld* nuclear genome into *N. vitripennis* cytoplasmic background (Bordenstein & Werren, 1997).

There are at least two alternative explanations for the increase in bacterial density during introgression of AsymC into the infected Ti277 cytoplasm. Rather than being due to a permissive nuclear genotype, an increase in bacterial density may have been stimulated by introduction of chromosomes from uninfected males. A second possibility is that larval crowding was less under the experimental regime than in standard cultures, and the reduced crowding permitted increases in bacterial density. Sinkins *et al.* (1995) have suggested larval crowding effects on CI in *D. simulans.*

Maternal transmission and regulation of bacterial density

Based on our results, we suggest that a density-dependent regulation of bacterial density is operating. When and how such regulation could occur is unclear. *Wolbachia* are obligate endosymbiotic bacteria, self-replicating in the host cells. From an initial population of bacteria in the egg cytoplasm to populations of bacteria in the gametocysts of the mature adult, two main processes occur that will affect the final infection level of the cysts: segregation among dividing host cells and replication within host cells. Both may determine the average bacterial density in the cysts and the variance among cysts. One part of the process, segregation of bacteria among dividing embryonic nuclei, has been described by Callaini *et al.* (1994). According to these authors, bacteria occur in close association with the mitotic apparatus during mitosis, thus ensuring segregation of bacteria among dividing nuclei. We further suggest that at low bacterial density, stochastic association of the bacteria with the opposite poles of mitotic spindles may be less balanced than at high bacterial density, eventually increasing bacterial density variance among the eggs. Conversely, elevated number of bacteria in highly infected females may tend to homogenize infection levels in daughters, resulting in a more balanced segregation of bacteria among stem cells and/or limitation in the number of bacteria accumulating at a pole.

We do not know whether bacterial replication is a continuous process during host development to adulthood, or if peaks of replication occur at some stage of the host life, for instance during larval development and gametogenesis. Indirect evidence for an effect of the individual physiological state on bacterial replication stem from the dramatic decrease of bacterial density in larvae of *N. vitripennis* artifically maintained two years in diapause (Perrot-Minnot *et al.,* 1996), from the effect of ageing on male infection level in *Drosophila* (Hoffmann *et al.,* 1990; Montchamp-Moreau *et al.,* 1991; Bressac & Rousset, 1993; Solignac *et al.,* 1994; Turelli & Hoffmann, 1995), and from decrease infection level with nutritional stress during larval crowding (Sinkins *et al.,* 1995).

Our results clearly show stochastic variation in the number of bacteria received by individual eggs of a female. In addition, individual females differ in the 'central tendency' of bacterial density among their eggs. This stochastic variation introduces the possibility that lineages with different bacterial densities can evolve strictly due to intergenerational changes in bacterial numbers, coupled with incompatibility effects. Guillemaud & Rousset (1997) have proposed a model that assumes stochastic variation in bacterial density (with a bias toward bacterial reduction), and selection against female lineages with low bacterial densities due to incompatibility with males containing higher bacterial density (the bacterial dosage effect). Their model does not assume any mechanism of density regulation of bacterial numbers and does assume epigenetic inheritance of bacterial density (i.e. bacterial densities of progeny are similar to that of the mother).

Although epigenetic inheritance of bacterial density through the cytoplasm seems reasonable, our data suggest that such 'heritability' of bacterial density may be low. In contrast, our preliminary data support some form of intrinsic density-dependent regulation of bacterial density. Females that produce eggs below an 'equilibrium' density tend to produce daughters with eggs of higher bacterial density and those above the 'equilibrium' tend to produce daughters with lower egg bacterial densities.

Some form of bacterial autoregulation of density is to be expected since these bacteria are vertically transmitted and will be selected to reduce negative fitness effects on females in which they occur (Fine, 1978; Turelli, 1994; Werren & O'Neill, 1997). Thus, they may be expected to regulate replication rate to minimize negative effects on the host, while still maintaining sufficient bacterial density to protect against Cl. Min & Benzer (1997) recently reported a strain of *Wolbachia* in *D. simulans* that has apparently lost this autoregulation, showing overreplication that causes neural degeneration and early death of adults. If autoregulation of bacterial density is correct, then the 'set point' for equilibrium bacterial density could also be influenced by host genotype, which may explain why bacterial densities change during introgression of AsymC into Ti277.

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