Cytoplasmic Incompatibility and Bacterial Density in *Nasonia vitripennis*

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ABSTRACT

Cytoplasmically (maternally) inherited bacteria that cause reproductive incompatibility between strains are widespread among insects. In the parasitoid wasp Nasonia, incompatibility results in improper condensation and fragmentation of the paternal chromosomes in fertilized eggs. Some form of genome imprinting may be involved. Because of haplodiploidy, incompatibility results in conversion of (diploid) female eggs into (haploid) males. Experiments show that bacterial density is correlated with compatibility differences between male and female Nasonia. Males from strains with high bacterial numbers are incompatible with females from strains with lower numbers. Temporal changes in compatibility of females after tetracycline treatment are generally correlated with decreases in bacterial levels in eggs. However, complete loss of bacteria in mature eggs precedes conversion of eggs to the "asymbiont" compatibility type by 3–4 days. This result is consistent with a critical "imprinting" period during egg maturation, when cytoplasmic bacteria determine compatibility. Consequent inheritance of reduced bacterial numbers in F_1 progeny has different effects on compatibility type of subsequent male *vs.* female progeny. In some cases, partial incompatibility occurs which results in reduced offspring numbers, apparently due to incomplete paternal chromosome elimination resulting in aneuploidy.

CYTOPLASMIC incompatibility in insects results in either embryo mortality or production of all male progeny between strains carrying different cytoplasmic factors (BREEUWER and WERREN 1990; CONNER and SAUL 1986; HOFFMANN 1988; KELLEN, HOFFMANN and KWOCK 1981; HOFFMANN, TURELLI and SIMMONS 1986; HSIAO and HSIAO 1985b; LAVEN 1957; O'NEILL and KARR 1990; SAUL 1961; TRPIS *et al.* 1981; WADE and STEVENS 1985). In a growing number of cases, the factors are identified as maternally inherited cytoplasmic bacteria or symbionts.

Evidence that cytoplasmic bacteria cause incompatibility comes from several sources, including cytological observations of bacteria in reproductive tissue that are associated with compatibility differences (BINNINGTON and HOFFMANN 1989; BREEUWER and WERREN 1990; HERTIG 1936; HERTIG and WOLBACH 1924; HSIAO and HSIAO 1985a; KELLEN, HOFFMANN and KWOCK 1981; O'NEILL 1989; O'NEILL and KARR 1990; TRPIS *et al.* 1981; WRIGHT and BARR 1980; YEN and BARR 1973), alteration of compatibility by antibiotic treatment or elevated rearing temperatures (BREEUWER and WERREN 1990; HOFFMANN 1988; HOFFMANN and TURELLI 1988; KELLEN, HOFFMANN and KWOCK 1981;MONTCHAMP-MOREAU, FERVEUR and JACQUES 1991; O'NEILL and KARR 1990; RICHARDSON, HOLMES and SAUL 1987; TRPIS *et al.* 1981; WADE and STEVENS 1985; YEN and BARR 1971) and amplification and identification of bacterial 16S rDNA from "infected" strains. Sequence comparison of 16S rDNA of cytoplasmic incompatibility bacteria from diverse insect taxa show over 95% sequence similarity, indicating that they are very closely related despite the fact that they are found in diverse insect taxa (BREEUWER *et al.* 1992; O'NEILL *et al.* 1992; ROUSSET, VAUGHIN and SOLIGNAC 1992; WOESE 1987). They belong to the alpha subdivision of the Proteobacteria (STACKEBRANDT, MURRAY and TRUPER 1988). Based upon sequence similarity to the mosquito incompatibility bacteria *Wolbachia pipiens*, it is probably reasonable to place all of these bacteria within the genus *Wolbachia* (O'NEILL *et al.* 1992).

Little is known about the mechanism of cytoplasmic incompatibility. In the parasitoid wasp Nasonia, it was demonstrated that the absence of female (diploid) offspring in incompatible crosses is the result of paternal chromosome elimination during early embryogenesis (BREEUWER and WERREN 1990; RYAN and SAUL 1968). This reconstitutes haploidy in the embryo, which develops into a male. Haploidization of fertilized eggs may also occur in diploid species complex *Culex pipiens*, following abnormal mitotic divisions (JOST 1970a,b). Aberrant early mitotic divisions have also been observed in fertilized eggs of incompatible crosses in *Drosophila simulans* (O'NEILL and KARR 1990).

Cytological examinations in several species reveal that cytoplasmic bacteria are absent in mature sperm (BINNINGTON and HOFFMANN 1989; O'NEILL 1989; WRIGHT and BARR 1980); they are shed from the

maturing spermatids with the excess of cytoplasm during the individualization process (BRESSAC and ROUSSET 1993; YEN and BARR 1974). However, sperm from carrier (symbiont) and non-carrier (asymbiont) males have different compatibilities. This suggests that cytoplasmic incompatibility involves some mechanism of chromosomal imprinting (BREEUWER and WERREN 1990; JABLONKA and LAMB 1989). More specifically, the chromosomal imprint of maturing sperm is altered by the presence of bacteria some time during development, such that in the case of unidirectional incompatibility, sperm of carrier males are incompatible with non-carrier eggs. Sperm chromosomes are "rescued" if eggs also carry the same bacterial strain.

Presence or absence of cytoplasmic bacteria in strains is not the only determinant of compatibility type. Unidirectional incompatibility is typically observed in crosses between symbiont and asymbiont (bacteria-free) strains: asymbiont females and symbiont males are incompatible, whereas the reciprocal cross (symbiont females and asymbiont males) is compatible and produces normal progeny. However, bidirectional incompatibility has been found in *Culex pipiens* (LAVEN 1957, 1967), *Drosophila simulans* (MONTCHAMP-MOREAU, FERVEUR and JACQUES 1991; NIGRO 1991; O'NEILL and KARR 1990), and between species of the parasitoid wasp Nasonia (BREEUWER and WERREN 1990). In these cases (in contrast to unidirectional incompatible crosses) both sexes carry cytoplasmic bacteria. Recently, it was demonstrated in *D. simulans* (NIGRO 1991) and Nasonia (BREEUWER and WERREN 1993) that bidirectionally incompatible strains harbor different bacterial strains.

Variation in bacterial density may also be a factor that determines compatibility relationships. SUBBARAO *et al.* (1977) suggest that variation in segregation of cytoplasmic factors among offspring from symbiont females may explain the observation that females produce offspring with different compatibility types. However, they dismiss this hypothesis based upon cytological examination of a few Culex strains that revealed very high bacterial levels (YEN and BARR 1974). In both *D. simulans* (HOFFMANN, TURELLI and SIMMONS 1986) and *C. pipiens* (SINGH, CURTIS and KRISHNAMURTHY 1976), the compatibility type of males changes with age and appears to be correlated with a reduction in the relative number of spermcysts in male testes that carry cytoplasmic bacteria (BINNINGTON and HOFFMANN 1989; BRESSAC and Rousset 1993). So far, no studies have explicitly examined the role of bacterial density in determining cytoplasmic incompatibility relationships.

This study investigates associations between bacterial density and compatibility in the parasitoid wasp Nasonia. Differences in bacterial density between strains and the effects of antibiotic treatment on bacterial density and compatibility relationships are investigated. Finally, the inheritance of altered compatibility in male and female F_1 offspring of antibiotic treated females is determined.

MATERIALS AND METHODS

A detailed description of the biology of Nasonia is given by WHITING (1967). In the laboratory, Nasonia are raised on fleshfly pupae, *Sarcophaga bullata*, at 25° under constant light. Generation time is 14 days under these conditions.

Strains: Three *Nasonia vitripennis* strains were used in the experiments: LabII, a bacteria-bearing laboratory strain from Leiden, The Netherlands (BREEUWER and WERREN 1990), *tinged-277* (*ti*), a second bacteria-bearing strain also carrying an R-locus eye color mutant strain (BREEUWER and WERREN 1990; RICHARDSON, HOLMES and SAUL 1985; SAUL 1961; SAUL *et al.* 1965; see Table 1C) and Asymc, a LabII strain cured of the cytoplasmic incompatibility bacteria by tetracycline treatment in 1986 (BREEUWER and WERREN 1990).

The compatibility of the LabII strain with *ti* and Asymc has been described earlier (BREEUWER and WERREN 1990; CONNER and SAUL 1986). LabII is unidirectional incompatible with both strains; LabII males are incompatible with females of the other strains, whereas LabII females are compatible. Cross compatibility between Asymc and *ti* was determined in the following way. Males and females were collected as pupae. Upon emergence, two replicates of 4 ♂ × 10 ♀ were allowed to mate for 24 hr. Next, males were removed and females were separated and provided with a single host. Subsequent offspring number and sex ratio were measured. Crosses were done for all possible combinations.

Terminology: Compatibility type (or cytotype) of an individual or strain is characterized by its compatibility with individuals of other strains or compatibility types. For example, a strain with the LabII compatibility type will be compatible with the LabII strain and show the same compatibility relationships as the LabII strain; *i.e.*, unidirectional incompatibility with the *ti* and Asymc strain. Note that compatibility type of an individual is actually determined by the compatibility of its gametes with the gametes of the opposite sex. Symbiont (=infected) and asymbiont (=uninfected) individuals refer to the presence or absence of cytoplasmic incompatibility bacteria in their respective cytoplasms. Offspring sex ratio is expressed as the percent females over the total offspring number. Absence of female offspring indicates incompatibility, because action of the bacteria results in loss of the paternal chromosomes, converting diploid females into haploid males.

Temporal effects of tetracycline: *Compatibility:* The effects of tetracycline on compatibility of eggs produced by symbiont females were determined in the following way. LabII virgin females were collected as pupae. Upon emergence, females were mated to LabII or Asymc males in groups of 4 ♂ × 10 ♀ for 24 hr. Next, males were removed and females were allowed to feed on a tetracycline solution (1 mg/ml in 10% sucrose) for an additional 24 hr. Then the females were separated and each was provided with one host. Every day for 9 consecutive days females were transferred onto new hosts. Subsequent offspring number and sex ratio were scored. In addition, a control cross between LabII ♂ × LabII ♀ was set up in a similar way, except that females were fed sucrose without tetracycline. This control cross was used to determine normal offspring numbers, sex ratio and bacterial densities in untreated eggs.

Bacterial density: In a subset of treated and control LabII females, hosts were replaced by fresh hosts two hours after oviposition. This was done every other day starting on day 1 post treatment. The parasitized hosts were cracked open, and the eggs were collected and fixed in Carnoy's (2.5:3:1 = chloroform:99% EtOH:acetic acid by volume) for 24 hr at 4°. Eggs were then placed on a microscope slide in a drop of 70% EtOH and a drop of 2.5% lacmoid [2.5% lacmoid (Pfaltz and Bauer Inc., Stamford, Connecticut) by weight in 1:1:1 = H_2O:acetic acid:lactic acid by volume]. The chorion was mechanically removed from the eggs by pressing down on the coverslip. Typically this would rupture the chorion. The slide was sealed with nail polish and stained overnight. Slides were examined with a light microscope (15 × 100, N.A. 1.25, phase, oil). The bacteria in Nasonia eggs are localized at the posterior end of the egg opposite the micropyle (BREEUWER and WERREN 1990) and are most prevalent in the cortex (BREEUWER 1993). Bacterial density was estimated by placing a 5 × 5 grid over the posterior end of the egg with one corner touching the posterior end. Each square in the grid is 10 × 10 μm (at the above magnification). All bacteria in every other square, 13 in total, were counted and added. During counting, the focal plain was adjusted so that all bacteria within the column of egg cytoplasm under the grid square were scored. Volume of the columns was not determined. However, there was no correlation between egg surface area and bacterial counts (Spearman rank correlation: $r = 0.1116$, $P = 0.34$). It should be noted that this method provides a measure of relative bacterial numbers. Absolute bacterial numbers in eggs were not determined.

F_1 male and female incompatibility: In a second experiment temporal changes in compatibility of antibiotic treated parentals on their F_1 progeny were determined.

Parentals: Virgin LabII females ($n = 60$) were collected as pupae and upon emergence they were split into two groups. One group was mass mated to 12 LabII males and the other to 12 Asymc males for 24 hr. The following day, males were removed and females were provided with tetracycline solution (1 mg/ml in 10% sucrose) for 24 hr. Next, females from each group were distributed over 6 replicate vials (5 ♀ per vial) and provided with 5 hosts for egg laying. Every day for 10 successive days females were transferred to new hosts. Intrastrain control crosses for LabII, *ti* and Asymc were set up in the same way, except females were fed sucrose only. Offspring number and sex ratio of successive broods were scored when offspring were in the late pupal stage. This allowed collection of virgin F_1 females and males that were also tested for compatibility type by mating to appropriate strains.

F_1 offspring: Offspring of treated LabII females from successive days were further crossed to LabII, *ti*, and Asymc to determine their incompatibility. F_1 females offspring were collected from Asymc ♂ × treated LabII ♀ cross instead of the LabII ♂ × treated LabII ♀ cross to avoid the shortage of females in the latter cross due to changes in compatibility. F_1 males were collected from the LabII ♂ × treated LabII ♀ cross. Note that all F_1 crosses were only between offspring that were produced on the same day. For each cross 30 single pair matings were set up. After 24 hr the males were removed and each individual female was given a single host for egg laying. Subsequent offspring number and sex ratio were scored.

Statistics: The Mann-Whitney-U test (MWU) or Kruskal-Wallis one-way ANOVA (KW) is used for testing the null hypothesis for no differences, *i.e.*, $\mu_1 = \mu_2$ (SIEGEL 1956). The level of significance (α) is 0.01. In all the figures, 99% confidence interval of the means is defined by the mean ± $t_{\alpha,[n-1]} \cdot s \backslash n$, where t is Student-t statistic, s is sample standard deviation and n is sample size (SOKAL and ROLF 1969). Unless otherwise noted, the results of statistical analyses can be found in BREEUWER (1993).

TABLE 1

Compatibility relationships and bacterial density of LabII, *ti-277* and Asymc

A. Percent females[a]

	Female		
Male	LabII	*ti-277*	Asymc
LabII	86 ± 6	0 ± 0	0 ± 0
ti-277	86 ± 13	69 ± 13	17 ± 21
Asymc	84 ± 9	80 ± 8	85 ± 5

B. Offspring number[a]

	Female		
Male	LabII	*ti-277*	Asymc
LabII	44.2 ± 9.0 (17)	61.6 ± 11.7 (18)	40.8 ± 10.8 (13)
ti-277	38.7 ± 8.7 (19)	51.9 ± 9.1 (16)	34.9 ± 8.6 (15)
Asymc	42.2 ± 11.5 (16)	54.6 ± 8.3 (19)	39.7 ± 9.9 (20)

C. Bacterial density[a]

	292 ± 52 (13)	116 ± 34 (15)	0 ± 0 (11)

[a] Mean ± SD, and number of replicates in parentheses.

RESULTS

Strain compatibility type: The compatibility relationships between the three strains, LabII, *ti* and Asymc, are shown in Table 1. Compatibility (*i.e.*, percent female offspring produced) between strains is compared to intrastrain compatibility. LabII is unidirectionally incompatible with the two other strains: females are compatible, whereas males are incompatible with individuals from each of the other strains (Table 1A). This confirms previously reported compatibility relationships of LabII (BREEUWER and WERREN 1990; CONNER and SAUL 1986). In contrast, Asymc males are compatible with each of the other strains, whereas females are fully compatible only with their own strain. Crosses between *ti* males and Asymc females produced nearly all males, but a few female offspring (Asymc ♂ × Asymc ♀ *vs.* *ti* ♂ × Asymc ♀: MWU = 0, $P < 0.002$). Thus, *ti* males are partially compatible with Asymc females. The strains can be hierarchically ordered based upon compatibility, LabII > *ti-277* > Asymc, where males are incompatible and females are compatible with each lower ranked strain.

The offspring numbers in incompatible crosses were not significantly lower than compatible crosses (Table 1B). This is consistent with previous observa-

tions in Nasonia that cytoplasmic incompatibility does not result in offspring mortality, as is found in diploid species, but results in shifts in offspring sex ratio by converting diploid (fertilized) eggs to haploidy through paternal chromosome elimination (Breeuwer and Werren 1990; Ryan and Saul 1968). Interestingly, the incompatible cross LabII ♂ × *ti* ♀ produced more offspring than the (compatible) intrastrain cross *ti* ♂ × *ti* ♀, but the difference was not significant at $\alpha = 0.01$ (MWU = 79.5, $0.02 < P < 0.05$). This could be a side effect of the all male progeny produced in incompatible crosses. Offspring numbers of parasitized hosts that contain all male progeny tend to be slightly larger than those that have male and female progeny (Beukeboom 1993). Because males are smaller than females, single hosts can carry more offspring if these are all males. The partially incompatible cross *ti* ♂ × Asymc ♀ produced fewer offspring than the intrastrain Asymc cross, but the difference is not significant (MWU = 109, $P > 0.1$).

Intrastrain *ti* crosses produced a significantly lower percent female offspring than intrastrain Asymc (MWU = 39.5, $P < 0.002$) and LabII (MWU = 28.5, $P < 0.002$) crosses. The *ti* strain may have an inherently lower fertilization proportion than Asymc and LabII. However, *ti* females actually produced a greater proportion of females when mated to Asymc males (MWU = 75, $0.002 < P < 0.02$). This suggests an alternative explanation that *ti* is partially self-incompatible. Implications of this result will be discussed later.

Bacterial density: Consistent with the compatibility tests, bacterial densities in the egg cytoplasm were significantly different between the three strains (KW = 32, df = 2, $P < 0.001$; Table 1C). Based upon the total bacterial number in 13 10 × 10 μm columns, cytoplasmic bacteria were absent in Asymc and were most prevalent in LabII eggs (292 ± 52). Bacterial densities in *ti* eggs were intermediate (116 ± 34). The *ti* compatibility type (complete unidirectional with LabII and partially unidirectional with Asymc) may be correlated with its intermediate bacterial density.

Temporal changes following tetracycline treatment: In this experiment, mated LabII females were fed either sucrose or a tetracycline sucrose solution and then allowed to lay eggs over successive days. Shifts in sex ratio and offspring number among F_1 progeny were recorded. Additional control crosses involved like-treated females who had been mated to Asymc males.

The temporal shift in sex ratio of sucrose fed *vs.* tetracycline fed LabII females mated to LabII males is shown in Figure 1A. Sucrose fed females produced around 90 % females throughout the experiment. In contrast, percent females in successive offspring of tetracycline fed females showed a very different pattern over the 9 day post treatment period. Initially (days 1–3), treated and control females produced similar sex ratios (Figure 1A). However, during days 6 and 7 post treatment, the percent female progeny of antibiotic fed females (mated to LabII males) dropped dramatically from 80–90% to almost 0% on days 8 and 9. In contrast, no shift in compatibility was observed in tretacycline treated females mated to Asymc males (Figure 1A). Percent females in this cross were not significantly different from the sucrose fed controls, except on days 4 and 5. This rules out the possibility that sex ratio shifts were caused by adverse effects of antibiotic treatment upon sperm vitality, and indicates that the drop in percent females is due to specific interactions between LabII (symbiont) sperm and tetracycline treated LabII eggs.

Temporal shifts in offspring number were also ob-

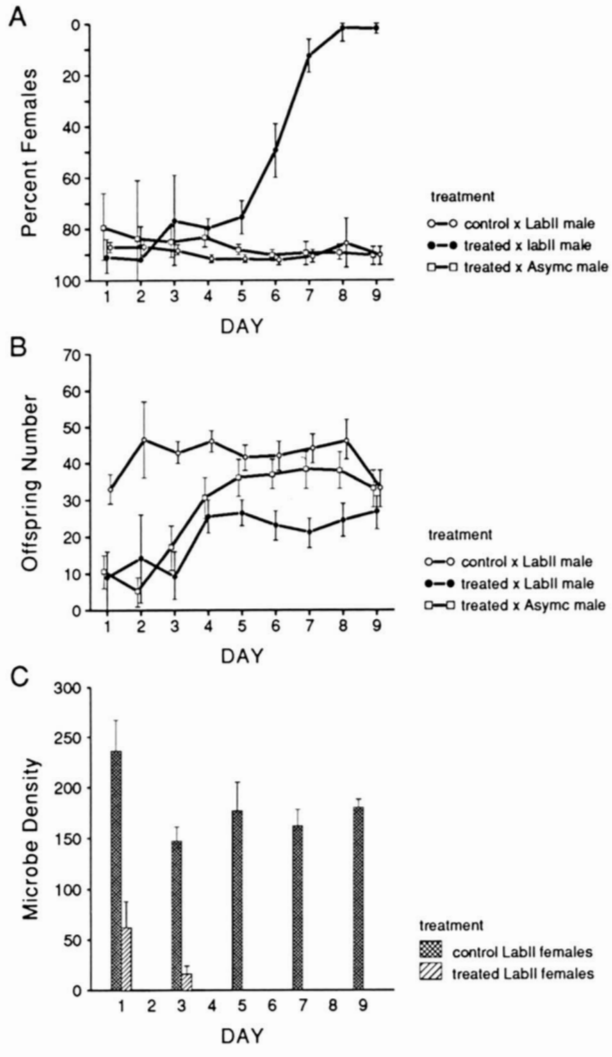

Figure 1.—Temporal changes in compatibility following antibiotic treatment of symbiont (LabII) females. Daily mean percent females (A), offspring number (B) and bacterial density in the egg cytoplasm (C) produced by control (sucrose only) LabII females × LabII males and treated (tetracycline + sucrose) LabII females × LabII or Asymc males. Error bars show 99% confidence interval around mean. Statistics are shown in Breeuwer (1993).

served (Figure 1B). First, antibiotic treated LabII females (mated to LabII or Asymc males) produced significantly fewer offspring on days 1–4 post treatment than did untreated LabII females, but the difference decreased over time. This was probably due to negative side effects of tetracycline on the physical condition of treated females, in particular egg production. Tetracycline inhibits bacterial protein synthesis, but has also small inhibitory effects on mitochondrial function (GOODMAN and GILMAN 1970; PORTER 1970).

Treated LabII females produced similar offspring numbers when mated to LabII *vs.* Asymc males early in the experiment, but significantly fewer offspring on days 4–9 when mated to LabII males (Figure 1B). The reduction in offspring numbers in the cross between treated females and symbiont males coincided with the drop in compatibility between days 4 and 9. The drop in offspring number was not sufficient to account for the dramatic sex ratio shift and almost complete absence of females on days 8 and 9. Therefore, the change in sex ratio when mated to LabII males was clearly due to a shift to incompatibility, converting diploid female eggs to haploid males. In conclusion, the temporal change in sex ratio after tetracycline treatment resulted in changes in compatibility between eggs of treated symbiont females and sperm of symbiont males.

Bacterial density: The results of bacterial counts in egg cytoplasm are presented in Figure 1C. Sample sizes were between 13 and 31 eggs. Microbe densities in eggs from untreated symbiont (LabII) females were high. Day 1 eggs had the highest bacterial levels, and day 3 and 7 eggs the lowest (KW = 50.5, d.f. = 4, $P < 0.001$). These variations may reflect differences in physiological conditions of the mother, such as age or egg maturation rate, which may in turn affect replication of cytoplasmic bacteria. Nevertheless, compared to antibiotic treated females, bacterial densities in eggs of these females were relatively constant throughout the 9-day experiment.

Tetracycline strongly altered the abundance of cytoplasmic bacteria in successive eggs of treated symbiont (LabII) females (Figure 1C). Already the very first eggs ($n = 13$) showed significantly reduced bacterial densities compared to eggs from untreated symbiont females ($n = 12$) (MWU = 1, $P < 0.002$). No bacteria were observed in the cytoplasm of eggs from day 5 on.

A most interesting result is that the reduction in cytoplasmic bacteria preceded the shift in offspring sex ratio (*i.e.*, compatibility) by 3–4 days (Figure 1). Complete incompatibility (days 8 and 9) was not reached until 3–4 days after bacteria were undetectable in the egg cytoplasm. The same basic result was obtained from a second experiment (BREEUWER 1993); sex ratio shifts were not observed until 3 days after bacteria were almost completely absent in the egg cytoplasm. In this second experiment, shifts in bacterial density and sex ratio occurred earlier. Such differences could reflect differences in tetracycline uptake by females, developmental stage of oocytes at the time of treatment or other variables not controlled in the experiments.

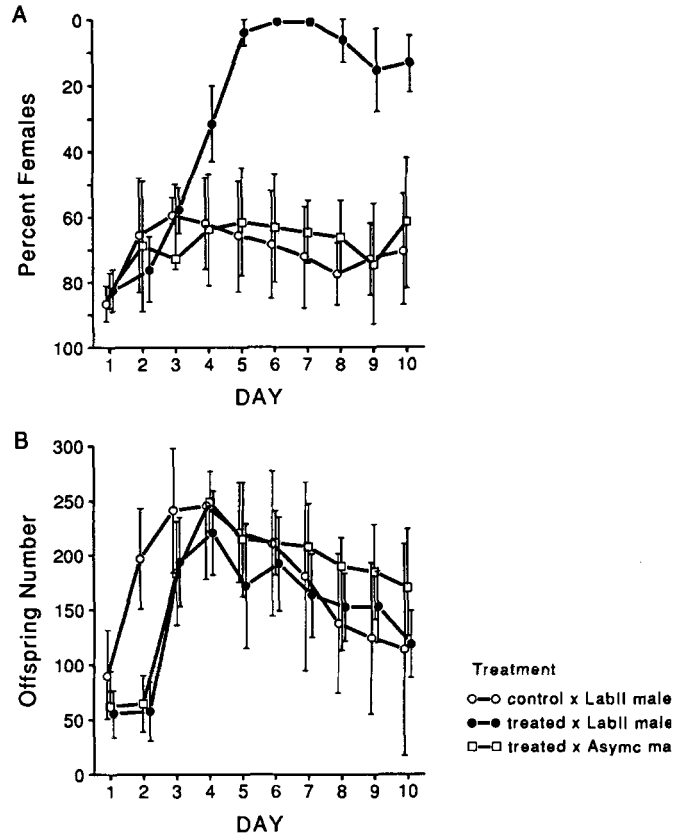

FIGURE 2.—Compatibility changes of symbiont (LabII) females following antibiotic treatment. Daily mean percent females (A) and offspring number (B) produced by control (sucrose only) LabII females × LabII males and treated (tetracycline + sucrose) LabII females × LabII or Asymc males. Error bars show 99% confidence interval around mean. Statistics are shown in BREEUWER (1993).

Clearly, the results show that the shift to incompatibility between symbiont sperm and tetracycline exposed eggs is roughly correlated with bacterial density in the egg, although there appears to be a delay before complete loss of the bacteria results in complete incompatibility.

F_1 male and female compatibility: In the next experiment, changes in bacterial density were not recorded, but the effects of antibiotic treatment of mothers on the compatibility relationships of their F_1 progeny were determined. Each cross consisted of six replicates of five females on five hosts.

Parentals: The untreated cross LabII females × males produced daily broods with 60–80% females (Figure 2A). Sex ratios in this experiment were generally lower than in the previous experiment, because

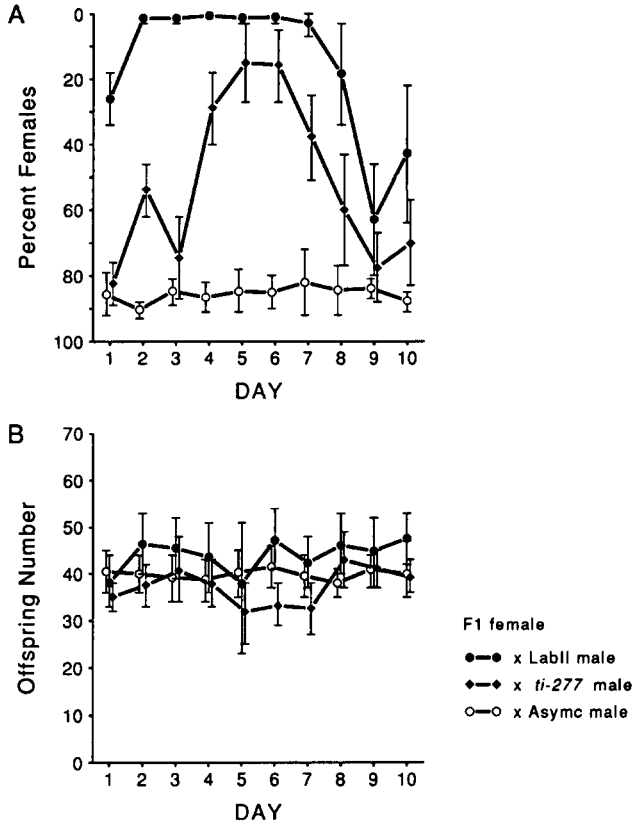

FIGURE 3.—Compatibility of F_1 females. Mean percent female offspring (A) and offspring numbers (B) produced by successive F_1 female progeny of treated LabII females. F_1 females were mated to LabII, *ti-277* or Asymc males. Error bars show 99% confidence interval around mean. Statistics are shown in BREEUWER (1993).

females were hosted in groups instead of individually. Fertilization rate of individual females is influenced by the presence of other females (WERREN 1983).

Compatibility changes in the cross LabII ♂ × treated LabII ♀ followed a pattern similar to the experiment above (Figure 1). Again, no changes in sex ratios were observed when treated LabII females were crossed to asymbiont (Asymc) males. Also, there were no significant sex ratio differences between this cross and the control cross LabII ♂ × untreated LabII ♀. Complete incompatibility (absence of female offspring) between LabII males and treated LabII females was reached on day 6, 2 days earlier than in the previous experiment. This may be due to differences in tetracycline intake by the females between experiments. After 8 days, some female offspring reappeared, indicating that eggs were again compatible with sperm from symbiont (LabII) males. This suggests that the effect of tetracycline decreased over time.

Tetracycline treatment had a negative effect on offspring production; treated females produced significantly fewer offspring on day 2 (Figure 2B).

F_1 females: Compatibility type of successive F_1 females were determined in crosses to Asymc, LabII and *ti* males (Figure 3). Recall that *ti* males show an intermediate compatibility type and are derived from eggs with intermediate bacterial densities; *i.e.*, these males are compatible with LabII and *ti* females, and partially incompatible with Asymc females.

F_1 females showed dramatic changes in compatibility, dependent upon which egg batch they were derived from following tetracycline treatment of their symbiont (LabII) mother. F_1 females from each day crossed to asymbiont (Asymc) males, produced 80–90% female offspring (Figure 3A). This cross was expected to remain unaffected by tetracycline treatment because asymbiont (Asymc) males are compatible with females from all three strains (Table 1A; BREEUWER and WERREN 1990).

In contrast, F_1 females that were mated to symbiont (LabII and *ti*) males showed a very different pattern. Day 1-derived F_1 females mated to LabII males produced very few female progeny and F_1 females derived from later days (day 2–7) produced almost exclusively male offspring (Figure 3A); percent female offspring from F_1 females mated to LabII *vs.* Asymc males were significantly different on every day, except for day 9. Thus, these F_1 females from treated symbiont (LabII) mothers were incompatible with symbiont (LabII) males. These data suggest that F_1 females inherited few or no cytoplasmic incompatibility bacteria from their treated symbiont mother and therefore lost the LabII compatibility type. This is consistent with the fact that after treatment of females, cytoplasmic bacteria were almost absent in the eggs that gave rise to F_1 females in the previous experiment.

Compatibility changes between F_1 females and *ti* males were more gradual with incompatibility increasing in day 3–6-derived F_1 females (Figure 3A). Compatibility of day 6-derived females to *ti* males dropped to a level comparable to that between Asymc females and *ti* males (Table 1A). Thus, F_1 females did not have the Asymc compatibility type initially, as suggested by the absence of females in crosses of day 2-derived F_1 females mated with LabII males. The result indicates, however, that compatibility type of F_1 females from successive egg batches gradually changed; first to *ti* and then to Asymc compatibility type by day 6. F_1 females from each day were significantly more compatible, *i.e.*, produced more female offspring, when mated to *ti* males than when mated to LabII males, except day for 9. This corresponds to the intermediate bacterial density of the *ti* strain.

Females from later time periods (days 8–10) were partially compatible with both symbiont *ti* and LabII males. This occurred at the same time period when female offspring reappeared in the parental LabII ♂ × treated LabII ♀ cross (Figure 2A). This suggests that bacterial density among eggs of treated (LabII) females rebounded at the end of the experiment.

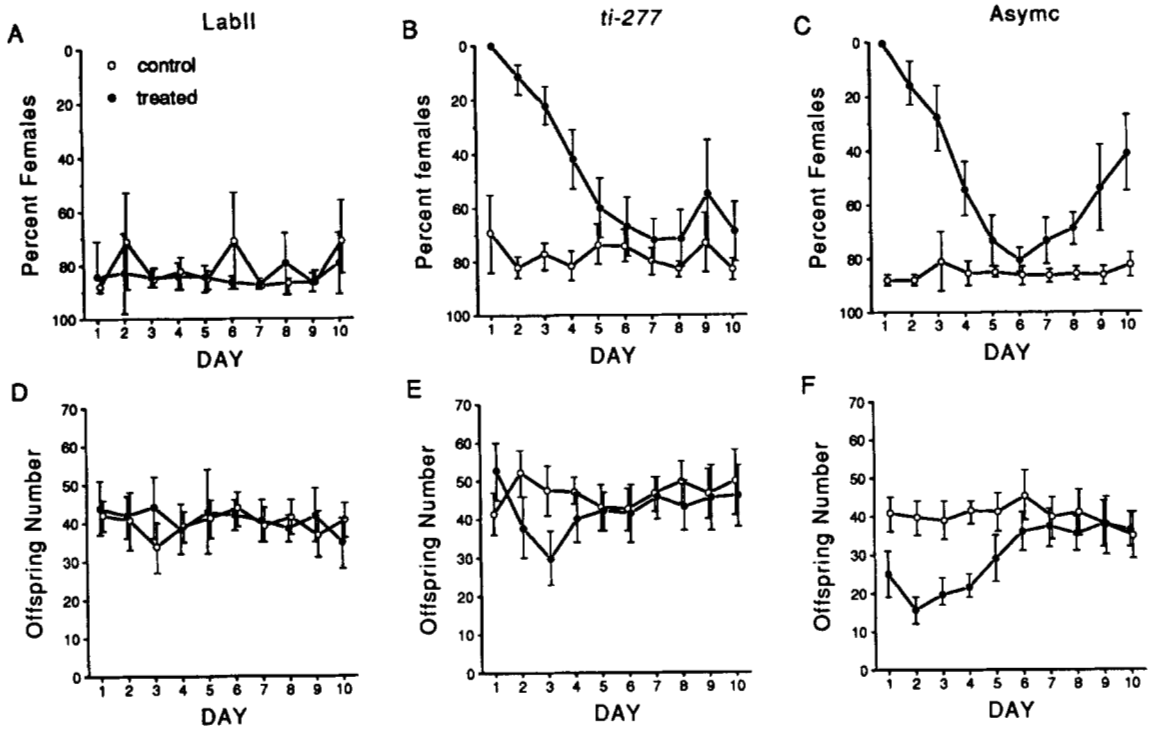

FIGURE 4.—Compatibility of F_1 males. Mean percent females (A–C) and offspring numbers (D–F) produced by successive F_1 male progeny of treated LabII females (treated). F_1 male progeny were mated to LabII (A, D), *ti*-277 (B, E), or Asymc females (C, F). Controls represent intrastrain crosses of F_1 offspring from untreated LabII, *ti*-277, and Asymc females. Error bars show 99% confidence interval around mean. Statistics are shown in BREEUWER (1993).

Consequently, F_1 progeny collected on these days did not express the Asymc compatibility type, *i.e.*, incompatibility with symbiont males (Figure 3A). No consistent differences in offspring numbers were observed in any of these crosses (Figure 3B).

F_1 males: Compatibility type of successive F_1 males was assayed in crosses to LabII, *ti* and Asymc females (Figure 4). Recall that LabII males are incompatible with both *ti* (intermediate bacterial density) and Asymc (zero bacteria) females. Control intrastrain crosses (*ti* ♂ × *ti* ♀, Asymc ♂ × Asymc ♀, LabII ♂ × LabII ♀) were also conducted. As expected, crosses between F_1 males and symbiont (LabII) females were not affected by antibiotic treatment of the paternal mother (Figure 4, A and B). This is consistent with the observation that LabII females are compatible with both symbiont (LabII and *ti*) and asymbiont (Asymc) *N. vitripennis* males. Percent females and offspring numbers were not different from those produced in the control cross between symbiont LabII ♂ × LabII ♀. This shows that antibiotic treatment of the mother did not inherently alter fertility of F_1 males, independent of their compatibility relationships.

Compatibility of F_1 males derived from successive days after antibiotic treatment of their mother gradually increased over time in matings to *ti* and Asymc females; day 1 derived F_1 males sired no or very few female offspring, whereas day 6-derived F_1 males sired an almost similar percent females as day 6 control crosses (Figure 4, B and C).

In contrast to the pattern of F_1 females, F_1 males showing partial incompatibility to *ti* (days 2–4) and Asymc (days 1–6) also had reductions in offspring numbers compared to controls (Figure 4, E and F). This is most evident in crosses to Asymc females and to a lesser extent in crosses to *ti* females; offspring numbers were significantly reduced between days 1 and 6 in crosses to Asymc females and between days 2 and 4 in crosses to *ti* females compared to intrastrain (Asymc ♂ × Asymc ♀ and *ti* ♂ × *ti* ♀, respectively) crosses. The fact that these males did not show fertility reductions when mated to LabII females (Figure 4A) indicated that it was due to compatibility effects rather than fertility problems. Thus, incompatibility of F_1 males with Asymc and *ti* females may be partially due to female mortality. This is in sharp contrast to compatibility changes observed in F_1 females, which resulted only in sex ratio shifts (Figure 3). It may reflect the different effects variable bacterial densities have on the determination of compatibility type in males *vs.* females.

DISCUSSION

The "bacterial dosage" model: Based upon the results of our experiments, we propose the following "bacterial dosage" model. Cytoplasmic incompatibility

apparently involves an action of the bacteria in the male (*e.g.*, "imprinting" of sperm chromosomes) and a counteraction in the egg (*e.g.*, production of a "rescue" substance). It is an interaction between these two effects that determines whether the sperm chromosomes are fragmented and lost. We propose that unidirectional compatibility is strongly influenced by the "dose" of bacteria in the male (*e.g.*, in spermatocytes) relative to the dose in the egg. Sperm will be incompatible with an egg when the number of bacteria in the male strain is greater than in the female strain. Conversely, a cross is compatible when the paternal strain harbors equal (*e.g.*, intrastrain crosses) or lower numbers of cytoplasmic bacteria.

The pattern of incompatibility in the three strains examined (LabII, *ti-277* and Asymc) is consistent with this hypothesis. If the paternal strain has a higher bacterial density than the maternal strain the cross is incompatible. For example, *ti-277* has intermediate bacterial numbers. Males are compatible with the strain with greater numbers (LabII) and incompatible with the strain with fewer (Asymc). Similarly, Asymc males are compatible with females of all other strains. Furthermore, the pattern of shifts in compatibility and bacterial numbers following antibiotic treatment is also consistent with this model.

The "imprinting" of sperm is likely to occur in developing spermatocytes, although this has not yet been shown. Exactly how the number of bacteria in unfertilized (male) eggs translates into bacterial numbers (and imprinting level) in a developing spermatocyte is still unclear. In contrast, our data do show that female (egg) compatibility appears to be determined during oogenesis.

As suggested by the "bacterial dosage" model above, compatibility relationships between *N. vitripennis* strains appear to be correlated with differences in relative bacterial infection levels in the paternal and maternal strain. Transient partial incompatibility in field collected strains of *D. simulans* have been suggested to reflect low levels of bacterial infection in females (HOFFMANN and TURELLI 1988; HOFFMANN, TURELLI and HARSHMAN 1990). Also in *C. pipiens*, it was suggested that bacterial densities could underlay compatibility types, but bacterial densities were not determined (BARR 1980; SUBBARAO *et al.* 1977). Factors (*e.g.*, chromosomal or bacterial) that control strain specific bacterial density of LabII and *ti* in *N. vitripennis* remain to be determined.

How can differences in bacterial densities between mates explain the partial incompatibility observed between *ti* males and Asymc females? Compatibility type of an individual is likely to be determined by the compatibility of individual eggs and sperm. In this view, the presence of female offspring in the cross *ti* ♂ × Asymc ♀ indicates that a certain proportion of *ti* sperm are compatible with Asymc eggs. This may be the result of unequal assortment of the cytoplasmic bacteria among spermatocytes or developing spermatids (SUBBARAO *et al.* 1977). In particular, if bacterial densities are low, some gametes may not have been exposed to or inherit the cytoplasmic bacteria at all, and result in partial incompatibility. Cytological observations in *D. simulans* (BINNINGTON and HOFFMANN 1989; BRESSAC and ROUSSET 1993) support the proposition of unequal assortment of bacteria among gametes. In these species, a low proportion of spermatocytes of infected males is devoid of cytoplasmic bacteria. Moreover, the proportion of uninfected spermatocytes increases with age of infected males and correlates with changes in compatibility of these males (BINNINGTON and HOFFMANN 1989; BRESSAC and ROUSSET 1993; HOFFMANN, TURELLI and SIMMONS 1986). Testes of *ti* males were not examined for variation among spermatocytes in presence or absence of cytoplasmic bacteria.

Tetracycline treatment of symbiont females had a dramatic effect on bacterial densities in their eggs; in two experiments microorganism densities were already greatly reduced or absent in eggs on the first day after treatment. This clearly shows that tetracycline effectively eliminates cytoplasmic bacteria. The most interesting result is that compatibility of treated symbiont females with symbiont males did not change until several days after bacteria were undetectable in the egg cytoplasm. In other words, the drop in microbe density did not immediately result in incompatibility between "cured" eggs and "symbiont" sperm. Eggs can show the LabII compatibility type, *i.e.*, compatibility with sperm from symbiont (LabII) males, even if bacteria are absent in the egg cytoplasm at the time of fertilization. This strongly suggests that cytoplasmic incompatibility bacteria influence compatibility of an egg some time before oviposition, and that their presence is not required at the time of fertilization to express the symbiont compatibility type. In other words, there is a critical "imprinting" period prior to oviposition during which compatibility is determined.

It is interesting that a dramatic reduction in bacterial numbers in eggs can be seen after only 1 day of tetracycline treatment. It is unknown how long it takes a primary oogania to become a mature egg. However, since females can mature 20–40 eggs per day at peak egg production (WERREN 1983), egg maturation is apparently rapid. It is likely that bacteria are rapidly replicating at this time. Indirect evidence for this is the observation of many bacteria that appear to be dividing in recently laid eggs. Tetracycline therefore could reduce bacterial numbers simply by interfering with their replication.

We do not know whether the lacmoid stain is de-

tecting only living bacteria, or whether significant numbers of dead bacteria are also detected in the assay. However, since the compatibility shifts occur after complete loss of lacmoid staining bacteria in the eggs, the possibility of detecting dead bacteria reinforces the view that an imprinting period occurs during oogenesis. A second, and more critical, concern is whether the lacmoid stain detects all bacteria in the egg. It is possible that certain stages of the bacteria are resistant to this stain and therefore go undetected. Lacmoid is not a direct DNA stain, but rather stains protein complexes associated with DNA, although its specific affinities have not been determined. We consider it unlikely that large numbers of bacteria were undetected.

An interesting observation is that the temporal change in compatibility of treated symbiont females with symbiont males is gradual and shows little offspring mortality. In other words, the reduction in bacterial density gradually lowers the number of compatible fertilizations and consequently percent female offspring. Apparently, treated symbiont females produce two types of eggs in changing proportions; ones that are compatible with "symbiont" sperm and ones that are not. This suggests a bacterial threshold density in eggs that determines compatibility type. If bacterial density in the egg is above the threshold density, the egg will be completely compatible with "symbiont" sperm; *i.e.*, the effect in eggs is all or none. If it is below this threshold, the egg will be completely incompatible with "symbiont" sperm.

The change in compatibility type of symbiont (LabII) females after antibiotic treatment is inherited by their offspring. In both male and female offspring compatibility types appeared to change from LabII to *ti* and then to the Asymc type among F_1 progeny derived from eggs laid on successive days after treatment of their symbiont (LabII) mother. The gradual change is most obvious in females, who are initially compatible with *ti* and Asymc. Subsequent F_1 females become incompatible with *ti*. Percent females of day 5–6 females are typical for crosses between *ti* males and Asymc females (Table 1A), indicating that these females have acquired the Asymc compatibility type.

Compatibility type of successive F_1 males also changed gradually and day 6-derived males exhibit the Asymc compatibility type. However, in contrast to F_1 females, the change in compatibility type (percent females) of successive F_1 males is accompanied by significant reductions in offspring numbers in crosses with both *ti* and Asymc females. The reductions in offspring number were most dramatic in crosses to Asymc females and encompass the entire compatibility transition period from day 1 to 6 (Figure 4F). The drop in offspring numbers in crosses to *ti* males is less pronounced (Figure 4E). One explanation is that reduced offspring numbers are the result of incomplete chromosome destruction in fertilized eggs, causing lethal aneuploid embryos. Partial elimination of cytoplasmic incompatibility bacteria in males may result in incomplete imprinting of the paternal chromosomes. This, in turn, would result in incomplete chromosome destruction in fertilized eggs leading to aneuploid and lethal embryos. This explanation is supported by the observation that incompatible crosses in Nasonia occasionally produce male progeny that have extra chromosomal fragments (BEUKEBOOM and WERREN 1993; RYAN, SAUL and CONNER 1985). Thus, in contrast to eggs, compatibility of spermatozoa does not appear to be an all or nothing phenomenon, but may vary between individual sperm.

The restoration of the original symbiont compatibility type in later time periods is probably due to diminishing effects of tetracycline over time. It suggests that bacterial densities in developing oocytes rebounds and consequently eggs become compatible again with symbiont sperm. This is also consistent with the increase of symbiont-like compatibility type among F_1 offspring produced during the last 3 days of the experiment; females became more compatible with symbiont males and males become more incompatible with asymbiont females. Interestingly, reversion of F_1 males to the LabII compatibility type is not associated with reduced offspring numbers. The reason for this is obscure, but may be related to effects of actively replicating cytoplasmic bacteria during male development.

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