Evolutionan Ecology, 1996, 10, 593-607

Distribution and fitness effects of the son-killer bacterium in *Nasonia*

MICHAEL T. BALAS, MICHELLE H. LEE and JOHN H. WERREN*
Department of Biology. University of Rochester. Rochester, NY 14627. USA

Summary

Maternally inherited microorganisms that kill male (but not female) progeny are widespread in nature. Three hypotheses have been proposed for the evolution of male-killing microorganisms: inbreeding reduction, release of resources to remaining females and inoculum for horizontal transmission. The son-killer bacterium. *Arsenophonus nasoniae, is a* maternally inherited bacterium that causes lethality of male embryos of infected females in the parasitoid wasp, *Nasonia vitripennis.* In this paper we describe the geographical distribution and frequency of the son-killer bacterium in North American populations of N. *vitripennis* and *Nasonia longicornis.* We tested the resource release hypothesis using the body size measurements of infected and uninfected females from natural populations. No evidence was found for a fitness increase of females infected with the bacterium compared to uninfected females. We propose a modification of the existing models, termed the `incremental gain' hypothesis. According to this model, the bacteria are maintained in host populations due to horizontal transmission and male killing provides an incremental gain in the fitness of infected females relative to females infected with non-male-killing bacteria.

Keywords: parasitoid wasp; sex ratio distorter; male-killing microorganism; *Nasonia vitripennis; Nasonia longicornis; Nasonia giraulti*

Introduction

Microorganisms that are maternally inherited and kill the male progeny of infected females are widespread in insects (reviewed in Hurst *(1991)* and Ebbert *(1993)).* Examples include microsporida in mosquitoes (Andreadis, *1990),* spiroplasmas in *Drosophila* (Williamson, *1965, 1969;* Ebbert, *1991, 1995), Rickettsia* in ladybird beetles (Hurst *et al., 1992, 1993;* Werren *et al., 1994),* and an enterobacterium in wasps (Werren *et al., 1986;* Gherna *et al., 1991).* In plants, the conceptual analogue of male killing is cytoplasmically inherited male sterility (Charlesworth and Ganders, *1979).*

Previous models have predicted that maternally inherited male-lethal microorganisms with incomplete vertical transmission and no horizontal transmission require specific conditions for their maintenance in a population. They can be maintained stably only if male killing increases the fitness of infected females relative to uninfected females (Skinner, *1985;* Werren, *1987;* Hurst, *1991;* Ebbert, *1993).* Three major hypotheses have been proposed for the evolution of male-killing microorganisms. First, the resource release hypothesis (Skinner, *1985)* proposes that male death releases resources that would have gone to the males but are now available to infected sibling females.

The second explanation for the evolution of male killing is the `inbreeding avoidance' hypothesis (Shull, *1948;* Werren, *1987).* According to this model, male killing can be favoured

* To whom correspondence should be addressed.

0269-7653

because it reduces the frequency of sibling matings by infected females. Werren (1987) has demonstrated tLat even relatively low levels of inbreeding depression can selectively favour male killing by maternally inherited microorganisms.

The third major model for male killing is the `inoculum' hypothesis. According to this model, male killing by microorganisms is favoured because the microorganisms produced in the dead males can serve as an inoculum for horizontal transmission to new hosts while females serve as vehicles for vertical transmission. This phenomenon appears to be operating in the male-killing microsporida of mosquitoes (Andreadis, 1990).

Nasonia vitripennis is a gregarious parasitoid wasp that oviposits primarily upon the pupae of flies of the families Calliphoridae and Sarcophagidae (Whiting, 1967). Because they exhibit haplodiploid sex determination (unfertilized eggs become males and fertilized eggs become females), females have the ability to control their primary sex ratio by controlling the number of eggs they fertilize. Isolated females tend to produce female-biased sex ratios, but as the number of females in an ovipositing group increases the primary sex ratio approaches 50 : 50 (Werren, 1983). The progeny compete within the parasitized fly puparium for resources during development. In addition, O'Neill and Skinner (1990) showed that female fecundity is correlated with body size, so resource release would presumably improve the fitness of the female offspring.

Arsenophonus nasoniae is a maternally transmitted bacterium that causes the son-killer trait in *N.* vitripennis (Werren et al., 1986; Gherna et al., 1991). Infected females produce highly female-biased sex ratios due to the mortality of approximately 80% of the male eggs laid (Skinner, 1985). The bacteria are found in the tissues of infected individuals (Huger et al., 1985). Transmission occurs through ingestion by developing larvae of bacteria inoculated into a host puparium by the wasp during oviposition (Huger et al., 1985; Werren et al., 1986). Thus, A. nasoniae has the potential to be transmitted both vertically from the mother to the offspring and horizontally through superparasitization (Skinner, 1985). The transmission rate of A. nasoniae from a carrier female to the larvae is approximately 95% (Skinner, 1985). Skinner (1983) reported that 4% of females in a Utah population carry the son-killer trait.

In this study, we document the distribution of A. nasoniae in *N.* vitripennis populations in North America, as well as the first report of A. nasoniae infection in the sibling species *N.* longicornis. We also report the frequencies of A. nasoniae in western North American populations of N. vitripennis during the years 1990 and 1991, which confirm that the frequency of A. nasoniae-infected females is at least as high as Skinner (1983) reported and possibly higher. We compare the relative size of infected females to uninfected females as a test of the resource-release hypothesis and show that infected females are not significantly larger than uninfected females, contrary to the expectations of the resource-release hypothesis.

Methods

Field collections of Nasonia

Nasonia wasps typically parasitize fly pupae found in bird nests (Darling and Werren, 1990) or underneath animal carcasses. Wasps of each of three species of Nasonia, N. vitripennis, N. longicornis and Nasonia giraulti, were obtained either by collection directly from the field or by collection of parasitized fly pupae. Adult wasps were collected from either animal carcasses or from baits. These baits consisted of pupae of the flesh fly Sarcophaga bullata and cow liver consumed by S. bullata larvae as an attractant. These wasps were individually placed on two S. bullata pupae and maintained at 25°C.

Parasitized fly pupae were obtained from either (1) bird nests collected from the field soon after the nestlings had fledged, (2) fly pupae collected from underneath carcasses or (3) S. *bullata* pupae from the baits described above, retrieved after 1 week in the field. A collection of pupae from a single discrete location is hereafter called a `patch'. The pupae collected from each patch were placed into closed buckets with collection vials on the top. If wasps emerged from the material, they were removed from the vials and one out of every five females was placed on one or two S. *bullata* pupae and maintained at 25°C. Lineages established from single females by either of these methods will hereafter be called iso-female lines.

Emerging F, progeny were scored for the sex ratio and wasp species. In some cases, a sample of F, progeny was frozen at - 70°C for future DNA studies. A second sample of F, females was provided with hosts under a short photoperiod (6 h light : 18 h dark) and cool temperature (20°C). Under these conditions developing larvae typically enter diapause, which is the natural overwintering condition of the wasp. Diapause permits storage of a field-collected strain under refrigeration for up to 2 years (Whiting, 1967). Lines whose larvae did not enter diapause were maintained for one or two more generations until diapause occurred.

Assays for son killer

Three different assays for the presence of son-killer bacteria, bacterial, molecular and phenotypic, were performed and the results of each subsequently compared. The protocols for each assay are as follows.

Bacterial assay. *To* test for the presence of bacteria, wasp pupae were surface sterilized by immersing them in a 50% chlorine bleach solution for 60 s, rinsing in sterile water for 30 s, immersing in 70% ethanol for 5 min, and rinsing again with sterile water for 1 min. Pupae were then cut with a sterile lancet and haemolymph and tissue was spread on agar containing GC media plus Kellog's supplement (Werren *et al.*, 1986; Gherna *et al.*, 1991). The agar plates were incubated at room temperature (20-25°C) for 3-5 days. Colonies of *A. nasoniae* have a distinctive morphology (Gherna *et al.*, 1991). The presence of son-killer bacteria was scored based on the presence of these distinctive colonies.

Molecular assay. Molecular screening for the presence of *A. nasoniae* bacteria in individual females was performed using DNA hybridization by a method similar to that of Beukeboom and Werren (1992) for the PSR chromosome. The total genomic bacterial DNA to be used as a molecular probe was isolated from the strain of *A. nasoniae*, type SKI4 (Gherna *et al.*, 1991). Wasps to be tested for the presence of son killer were ground either individually or in groups of five in 100 Wl homogenization buffer (0.2 m NaCl, 0.2 M Tris, 0.02 M EDTA, 2% SDS, pH 7). This solution was mixed with 10 Wl 2.5 mg ml –' proteinase K and incubated at 50°C for 1 h. The DNA was denatured with 20 Rl NaOH, incubated for 5 min, then neutralized with 20 p,l Tris and 20 Rl HCI. The solution (1.5 ELl) was dotted onto a nitrocellulose filter that was then dried and baked for 1.5 h at 80°C in a vacuum oven. Filters were then pre-hybridized for 3 h and hybridized overnight with the SKI4 probe at 65°C. Pre-hybridization and hybridization solutions were 2x SSC, 5x Denhardt's, 1 % sodium pyrophosphate, 25 mM sodium phosphate, 250 mg ml –' denatured calf thymus DNA and 1 % SDS in distilled water. The SK14 clone was labelled by either nick translation or by random priming (Amersham kit) with ^{32}P-labelled ATP. Following hybridization, filters were rinsed sequentially in four decreasing salt concentrations (4x, 2x, lx and O.lx SSC plus 0.1% SDS in each solution). After drying, filters were exposed to autoradiographic film for 4-5 days at - 70°C. A wasp was scored positive based on visual hybridization to the SK14 DNA. Under these conditions, the SKI4 DNA does not cross-hybridize

to the DNA of either *Escherichia coli* or the closest known relative of A. *nasoniae, Proteus vulgaris.*

Phenotypic assay. Because *Nasonia* exhibits haplodiploid sex determination, virgin females produce only male progeny. Therefore, the presence of the son-killer microorganism in an ovipositing virgin female is expected to cause a dramatic reduction in family size relative to mated females. In addition. infected virgin females are expected to produce smaller family sizes than uninfected virgin females and mated females infected with son killer should produce more female-biased sex ratios than uninfected mated females.

To test for phenotypic expression of son killer within an iso-female line, four sets of two virgin *N. vitripennis* females each were provided with one host pupa for oviposition. Four sets of two mated N. *vitripennis* females were also provided with hosts. Females were mated to control wild type males from laboratory strains (Lb II or R5-11). Emerging progeny were scored for family size (0-10, 11-20 or >20 wasps) and sex ratio (percent males: 0, >0-5 or >5%; Table 1).Some of the settings did not produce progeny, so each iso-female line is typically represented by three rather than four replicates.

For statistical analysis, the family size and sex ratio categories were converted into numerical scores (family size: <_ 10 = 0, 11-20 = 1, > 20 = 2; sex ratio: 0% = 0, > 0-5% = 1 and > 5% = 2) and the mean scores for each iso-female line were determined. Tests for differences in family size between virgin and mated female settings of the same iso-female lines were performed using the Wilcoxon signed-ranks test. Tests for differences in the family size and sex ratio between different iso-female lines were performed using the Mann-Whitney U-test.

Comparisons of the bacterial, molecular and phenotypic assays

The following tests were performed to determine whether positive results from the molecular assay are correlated with the ability to isolate the bacteria as well as phenotypic expression of A. *nasoniae.* Sixty-five iso-female lines of N. *vitripennis* that produced 100% females among their F, progeny were identified from field collections made in New York state during the summer of 1987. These lines were mated to standard wild type laboratory strains (either Lb II or R5-11) to perpetuate the line. One sample of F, female progeny from each iso-female line was placed under diapause conditions while a second sample was frozen at - 70°C.

In January 1988 the 65 iso-female lines were tested by the molecular assay. Thirty-eight of these lines were successfully reared from diapause and tested by both the bacterial and phenotypic assays. The results of all three assays were then compared.

Estimates of relative fitnesses of infected and uninfected females

Two studies using field-collected wasps were conducted to test the resource-release hypothesis. The first study compared the sizes of infected and uninfected females collected directly from the field. The second study measured the relative sizes of infected and uninfected females emerging from pupae in field-collected bird nests.

Field-collected adult females. Adult females collected directly from the field in both 1990 and 1991 had their head widths measured to the nearest 0.024 mm after they had parasitized pupae in the lab. Five female offspring from each successfully parasitized puparium were ground up together and tested for bacterial presence using the molecular assay. A field-collected female was classified as being infected if her daughters tested positive in this assay and uninfected if her daughters tested negative. The head widths of infected and uninfected females were then compared.

The data were analysed using two different methods. First, the difference in the median head width between infected and uninfected females was determined for each patch from which five or

more females were collected and for which at least one female tested positive by the molecular assay. The relative sizes of infected and uninfected females were compared by the Wilcoxon signed-ranks test. If the resource-release hypothesis is correct, infected females are expected to be consistently larger.

The data were also analysed by comparing the head widths of all of the infected and uninfected wasps collected during each year using the Mann-Whitney U-test. *Nasonia vitripennis* and N. *longicornis* were treated separately for this anaiysis.

Females emerging from field-collected nests. Five nests collected during the summer of *1989* that contained both infected and uninfected N. *vitripennis* females were selected. Fifty to *80* females that had emerged from fly pupae in those nests and were subsequently frozen had their head widths measured and were tested for the presence of bacteria using the molecular assay. Head widths of infected and uninfected females were then compared on a nest by nest basis.

Male killing in a New York strain of A. nasoniae

The percentage of males killed by an A. *nasoniae* strain collected near Albany, New York was determined by infecting a scarlet-eyed laboratory strain (ScDr; Saul *et al., 1967)* through superparasitization (Skinner, *1985).* Both infected and uninfected females were mated, then placed individually on a single pupa for life. The sex ratio of the offspring was then determined and the percent reduction in male family size %vas determined according to the method of Skinner *(1985).*

Results

Comparison of molecular, phenotypic and bacterial assays

Of the *65* iso-female lines of N. *vitripennis* tested for the presence of son killer by the molecular assay, 22 were scored positive. Of the *38* lines successfully reared from diapause and completely tested by all three assays, ten lines were positive by both the molecular and bacterial assays, four lines were positive by the molecular assay but negative by the bacterial assay and 24 were negative by both assays. None of the lines scored negative by the molecular assay and positive by the bacterial assay.

Table 1 summarizes the results of the comparisons between the three assays. Son-killer females are expected to produce significantly smaller family sizes when virgins, due to high mortality among their haploid sons. The comparison of family sizes produced by virgin and mated females shows that lines that tested positive by both assays produced family sizes consistent with the son-killer phenotype. Virgin females from such lines consistently produced smaller family sizes than mated females from the same lines. However, virgin and mated females from iso-female lines that were positive only by the molecular assay produced similar family sizes, as did virgin and mated females from lines that tested negative by both assays (Table 1).

When only the family sizes produced by virgin females are considered, iso-female lines testing positive by both assays produced smaller family sizes than iso-female lines testing positive by only the molecular assay or lines negative by both assays. The same comparison for mated females shows no significant differences between family sizes (Table 1). These findings are consistent with the interpretation that male lethality reduces the family size only in lines that test positive by both assays. The failure to detect a reduction in family size produced by infected, fertilized females compared to uninfected, fertilized females is probably because under the conditions of the experiment, fertilized *Nasonia* typically produce highly female-biased sex ratios. Therefore, a reduction in male production would not greatly reduce the total family size and, thus, may not have been detectable by the statistical methods used.

Table 1. Family sizes and sex ratios produced by iso-female lines tested by the molecular, bacterial and phenotypic assays

Iso-female line category	Family size (number of offspring)								Sex ratio (percent males)			
	Unmated			Significance group	Mated			Significance group	Wilcoxon signed-rank z-score	0	> 0-5	> 5	Significance group
	<_ 10	11-20	> 20		<_ 10	11-20	> 20						
Positive by both molecular and bacterial assays (ten lines)	20	8	2	a	1	8	22	a	2.81 ($p < 0.01$)	7	19	4	a
Positive by molecular assay only (four lines)	0	1	12	b	0	2	11	a	0.45 (ns)	0	6	7	b
Negative by both assays (24 lines)	0	8	74	b	0	4	76	a	0.00 (ns)	6	38	36	b

Family sizes and sex ratios of unmated and mated sets of females were compared for three categories of iso-female lines: those that tested positive by both the molecular and bacterial assay, those that were positive by the molecular assay but negative by the bacterial assay and those that were negative by both assays. Different lower case letters within significance groups indicate that the categories of iso-female lines differ significantly with $p < 0.01$. ns, not significant.

Mated females from iso-female lines testing positive by both assays produced more female-biased sex ratios than did lines testing positive only by the molecular assay and lines negative by both assays (Table 1). Lines testing positive by the molecular assay alone and negative lines produced similar sex ratios. These findings are consistent with the interpretation that female-biased sex ratios resulting from male death occur only in lines that test positive by both assays. These results indicate that lines that probe positive for son-killer genomic DNA but from which the bacterium was not isolated do not express the son-killer phenotype. This may be due to reduced bacterial densities in these lines or the presence of non-male-killing bacteria that could not be isolated by the methods used.

Distribution and frequency of A. nasoniae in the United States

Figure 1 shows the collection locations for wasps that were tested for the presence of the bacterium, with the individual locations listed in Table 2. Arsenophonus nasoniae-infected wasps were found in several populations of *N.* vitripennis in the Northeast and Rocky Mountain regions of North America, with one strain found in Minnesota. Arsenophonus nasoniae was also found in samples of *N.* longicornis from Idaho, Utah and Nevada. No A. nasoniae-infected wasps were found in Virginia, Ohio, Wyoming, Montana, Colorado and California. However, sample sizes from these states were small, so this does not mean that the bacterium is absent from these locales.

The frequencies of A. nasoniae in field-collected adult females in western North American populations of *N.* vitripennis and *N.* longicornis were 11.0% (21 out of 191) in 1990 and 7.6%

Figure 1. Distribution in North America of female wasps of the genus **Nasonia infected** with the son-killer bacterium, **A. nasoniae.** Closed symbols indicate collection locations where at least one female tested positive for the presence of bacteria (Table 2); open symbols indicate locations from which wasps were collected but no evidence of infection was found. Wasp species are indicated as follows: circles, *N.* **vitripennis;** triangles, **N. giraulti;** squares, *N.* **longicornis.**

Table 2. Numbers of iso-female lines tested for the presence of A. *nasoniae* for the locations mapped in Fig. 1

Year	State	Location	Type of test	Species	Number of iso-female lines tested		Number of lines positive for A. *nasoniae*	
					Field	Laboratory	Field	Laboratory
1987	CO	Bellvue	M + B	NV	–	1	–	0
	MT	Ronan	M + B	NL	–	1	–	0
		Plains	M + B	NL	–	1	–	0
		Red Rock Lake	M + B	NL	–	1	–	0
	NY	Geneseo	M + B	NV	–	43	–	13
		Springville	M + B	NV	–	5	–	4
		Mumford	M + B	NV	–	1	–	1
	UT	Salt Lake City	M + B	NV	–	5	–	0
1989	CA	Walnut Creek	M	NL	–	7	–	0
	MN	Park Rapids	M	NV	–	55	–	25
	NV	Reno	M	NL	–	34	–	0
	NY	Albany	M	NV	–	12	–	6
	OH	Cambridge	M	NV, NG	-	15 NV	–	0
					–	3 NG	–	0
	PA	Jefferson	M	NV, NG	-	11 NV	–	0
					–	1 NG	–	0
		Waynesburg	M	NV, NG	-	19 NV	–	2
					–	1 NG	–	0
	UT	Heber City	M	NV	–	42	–	23
	VA	Pembroke	M	NV, NG	-	3 NV	–	0
					–	33 NG	–	0
1990	UT	Logan	M	NV	–	11	–	5
		Ogden	M	NV	22	8	1	4
		Salt Lake City	M	NV	118	20	13	12
		Strawberry Reservoir	M	NV, NL	50 NV	28 NV	6	11
					1 NIL	5 NIL	1	3
1991	ID	Franklin	M	NV	66	–	8	
		Pocatello	M	NV, NL	18	–	0	
		Swan Valley	M	NV, NL	29 NV	–	1	
					1 NIL	–	0	
	NV	Wells	M	NV	2	–	0	
		Ruby Lake	M	NV, NL	12 NV	–	1	
					8 NL	–	3	
		Carlin	M	NV	7		0	
	UT	Logan	M	NV	77	–	4	
		Strawberry Reservoir	M	NV	16 NV	–	0	
					9 NL	–	1	
		Vernal	M	NV	27	–	3	
	WY	Alpine	M	NV	5	–	0	

Iso-female lines were established from both field-collected females and females emerging in the laboratory from field-collected fly pupae. Lines were tested using either the molecular (M) assay or both the molecular and bacterial (B) assays. CA, California; CO, Colorado; ID, Idaho; MN Minnesota;; MT, Montana; NV, Nevada; NY, New York; OH, Ohio; PA, Pennsylvania; UT, Utah; VA, Virginia; WY, Wyoming; NV, N. *vitrpennis;* NL, N. *longicornus;* NG, N. *girault.*

(21 out of *277)* in *1992.* These frequencies do not differ significantly (G-test, Gad; = *1.57, p > 0.1).* An analysis of infected female frequencies was also done for every patch from which five or more females were collected. For *1990,* none of 11 such individual patch frequencies differed significantly from *1990's* whole population frequency (binomial tests, p > *0.05* for all, data not shown). For *1991,* only one out of *15* individual patches differed significantly from *1991's* whole population frequency (binomial tests, p = *0.008* for the one significant difference; p > *0.05* for all others, data not shown). The probability that at least one significant difference of such magnitude would appear in *26* samples is *0.188* (binomial test), so there does not appear to be any evidence that there is any clumping of infected females in natural populations. It should be emphasized that the infection frequencies are based on adult females arriving at new patches, not from females dispersing from patches.

Estimates of relative firnesses of infected and uninfected females

Field-collected adult females. The head width frequency distributions of all of the infected and uninfected *N. vitripennis* females collected during *1990* and *1991* are shown in Fig. *2.* When all of the females collected in *1990* are considered together, *N. vitripennis* females that tested positive for the bacterium's presence had a significantly smaller median head width than those that tested negative (Table 3). In *1991,* no significant difference in head width between infected and uninfected females was found over the whole population for either *N. vitripennis* or *N. longicornis* (Table 3). These results are in contrast to the resource-release hypothesis, which predicts that infected females should be larger than uninfected females.

The above data were also compared on a patch by patch basis. In neither collection year was the median head width of infected females consistently larger than the uninfected females' median head width within each patch (Table 3). Combining the data from both sampling years also showed no difference between the infected and uninfected females.

Females emerging from field-collected nests. No significant difference in the mean head width of emerging infected and uninfected females was found for four of the five nests compared (Table 4). In the fifth nest, infected females had a significantly smaller mean head width than uninfected females.

Male killing in a New York strain of A. nasoniae

The results show that the strain of A. *nasoniae* collected from near Albany, New York, causes a reduction in the male family size similar to that found by Skinner *(1985).* Uninfected females of the ScDr strain produced a mean of 29.6 ± 7.2 (mean ± SD; $n = 23$) female offspring, while infected females produced 28.3 ± 7.6 ($n = 16$) females. This difference is not significant (Mann-Whitney U-test, U = *167.5,* $p = 0.637$). However, uninfected females produced a mean of 7.3 ± 2.3 ($n = 23$) male offspring, while infected females produced 2.2 ± 1.5 ($n = 16$) males. This difference is significant (Mann-Whitney U-test, U = *7.0,* $p < 0.001$). The estimated male lethality percentage was *68%,* similar to but lower than the value of *77%* that Skinner *(1985)* found for a Utah isolate of A. *nasoniae* in an ScDr strain.

Discussion

Evidence that *A. nasoniae* has been found in the northeastern, north central and western regions of the United States indicates that the bacterium is widespread in North American populations of *N. vitripennis. Arsenophonus nasoniae* has also been discovered not only in *N. vitripennis* but also in its western sibling species *N. longicornis.* The two species are sympatric in the western

United States and are often found parasitizing pupae in the same bird nest or carcass (Darling and Werren, 1990). As of yet it has not been determined that A. *nasoniae* kills males in *N. longicomis.* There is also no evidence that *A. nasoniae* infects *N.* girault*i*, the eastern sibling

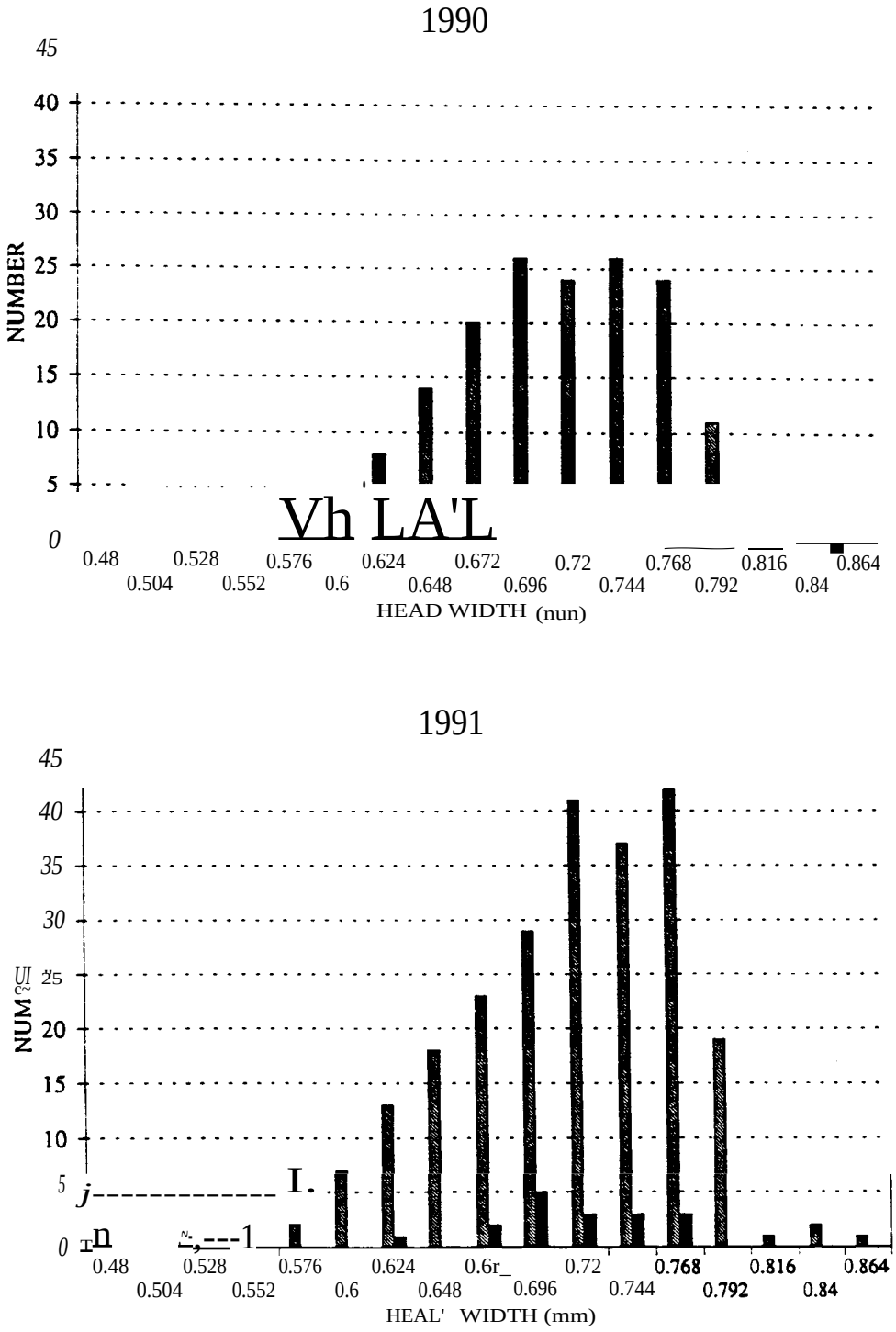

Figure 2. Frequency distributions of head widths of N. *vitripennis* females collected from the field during the summers of (a) 1990 and (b) 1991. Females that tested positive for the presence of A. *nasoniae* are indicated by solid bars; those that tested negative are indicated by hatched bars.

Distributic

species o
population

A con;
using ger
bacterium
from wh:
phenotypf
would ex
characteri
sensitivit:

Table 3. i

Patch ID
1990
UTC30
UTC31
UTC31
UTC32
UTC3'
UTCK
UTC32
UTB3t
UTBK
UTB3:
Overai
1991
IDB41
IDB41
IDB42
IDB4:
IIDB4:
NVB4
UTB4
UTC4
UTC4
UTC4
Overa

Test on

Patch ID
the prese
Nevada;
indicate
individua
infected

co

species *of N. vitripennis.* but that may be due to inadequate sampling *of N. girault*i populations.

A comparison of the three assays used in this study clearly shows that the molecular assay using genomic DNA from the son-killer bacterium detects iso-female lines that carry the bacterium and express the son-killer phenotype. However, the molecular assay also detects lines from which bacteria were not isolated. Interestingly, these do not express the son-killer phenotype. It is possible that individuals from these lines had low bacterial infection levels, which would explain the failure to isolate the bacterium on agar plates. However, these lines were not characterized by unusually weak hybridization levels in the molecular assay, although the sensitivity *of* the assay for quantifying bacterial density is not known. An alternative is that these

Table 3. Median head widths of *Nasonia* females collected from the field during 1990 and 1991

Patch II)	Species	Median head width of uninfected females (in mm) (n)	Median head width of infected females (in mm) (n)	Difference	p-value
1990					
UTC306	NV	0.696 (33)	0.672 (3)	0.024	
UTC310	NV	0.744 (9)	0.708 (2)	0.036	
UTC318	NV	0.720 (19)	0.648 (3)	0.072	
UTC320	NV	0.720 (9)	0.600 (1)	0.120	
UTC326	NV	0.696 (21)	0.768 (1)	-0.072	
UTC335	NV	0.744 (23)	0.612 (2)	0.132	
UTC337	NV	0.744 (5)	0.600 (1)	0.144	
UTB301	NV	0.672 (10)	0.696 (1)	-0.024	
UTB323	NV	0.696 (5)	0.720 (1)	-0.024	
UTB324	NV	0.696 (5)	0.792 (1)	-0.096	0.28 (WSR)
Overall	NV	0.720 (170)	0.684 (20)		0.013 (MWU)
1991					
IDB415	NV	0.720 (6)	0.708 (2)	0.012	
IDB416	NV	0.732 (18)	0.720 (1)	0.012	
IDB430	NV	0.708 (6)	0.744 (2)	-0.036	
:)B433	NV	0.696 (13)	0.732 (2)	-0.036	
IDB435	NV, NL	0.720 (17)	0.696 (1)	0.024	
NVB401	NV, NL	0.672 (7)	0.624 (4)	0.048	
UTB416	NV	0.720 (13)	0.696 (2)	0.024	
UTC400	NV	0.768 (23)	0.744 (1)	0.024	
UTC402	NV	0.720 (47)	0.732 (2)	-0.012	
UTC406	NV, NL	0.708 (18)	0.720 (1)	- 0.012	0.57 (WSR)
Overall	NV	0.720 (242)	0.720 (17)		0.410 (MWU)
	NL	0.672 (14)	0.648 (4)		0.242 (M)VU)
Test on patch data for both years					0.29 (WSR)

Patch *IDs* indicate the locations from which at least five wasps were collected and at least one wasp tested positive for the presence ***of A. nasoniae.*** The first two letters indicate the state in which the patch was located (ID, Idaho; NV, Nevada; UT, Utah), the third letter indicates whether the patch type was a bait (B) or a carcass (C) and the numbers indicate individual patch sites. Tests for differences in the median head widths between infected and uninfected individuals were made using two-tailed Wilcoxon signed-ranks (WSR) tests. Differences in the head widths between infected and uninfected females for all wasps collected for each year were compared using the Mann-Whitney U-test *(MWU).* ***Nasonia vitripennis (NV)*** and ***N. longicornis*** (NL) were treated separately only for comparisons *of* all wasps collected. *No* test could be done on ***N. longicornis*** for 1990 because ***only*** one ***N. longicornis*** female was collected.

Table 4. Median head widths of emerging N. *vitripennis* females from bird nests containing fly pupae

Nest DD	Uninfected females' median head width (in mm) (n)	Infected females' median head width (in mm) (n)	Mann-Whitney U-score
MN202	0.588 (30)	0.600 (47)	649.5 (ns)
MN205	0.648 (40)	0.636 (10)	222.0 (ns)
MN206	0.648 (40)	0.672 (9)	171.0 (ns)
MN208	0.672 (44)	0.624 (36)	1280.0 ($p < 0.001$)
NY203	0.696 (29)	0.672 (21)	342.0 (ns)

Differences between infected and uninfected females' mean head widths were compared using the Mann-Whitney U-test. MN, Minnesota. NY, New York.

lines harbour a relative of *A. nasoniae* that does not express the male-killing phenotype and is unable to grow on the bacterial media used. No other bacteria were detected on the agar plates from these lines. Since weaker hybridizations were not observed, this organism would have to represent a very close relative with specifically different growth requirements.

These two alternatives were not resolved, although we suspect that a lower bacterial density in some iso-female lines is the more likely explanation. Thus, the molecular assay is able to detect son-killer lines, but surveys based solely on the molecular probe are likely to overestimate the frequency of lines expressing the son-killer phenotype.

The frequencies of infected wasps found in this survey, 11.0% for 1990 and 7.6% for 1991, are both significantly greater than Skinner's (1983) reported value of approximately 4% (proportions tests, $p < 0.001$ for 1990 versus 1983 and $p < 0.01$ for 1991 versus 1983). Skinner's (1983) value was based on a phenotypic assay. Since the frequencies found here are based solely on the molecular assay, they are likely to be overestimates of son-killer expression. However, we can estimate the frequency of wasps that express the son-killer phenotype if we use the proportion of wasps that score positive by the molecular assay but are phenotypically negative (four out of 14 iso-female lines, Table 1) as an estimate for natural populations. Based on this estimate, the population frequencies of females expressing the son-killer trait reduce to 7.8% for 1990 and 5.4% for 1991. These frequencies are much closer to Skinner's (1983) frequency. The son-killer trait has thus persisted in the western *N. vitripennis* population between the time of Skinner's (1983) study and this study.

The hypothesis that *A. nasoniae* male- killing has evolved because infected females have a greater fitness than uninfected females is not supported by these data. Infected females were never found to be significantly larger than uninfected females, in either samples of the whole population (Table 3), between individual patches of ovipositing females (Table 3) or in samples of emerging females from a patch (Table 4). Sometimes within these classes. infected females were actually significantly smaller than uninfected females. This suggests that the presence of *A. nasoniae* may be deleterious to growing females, although the data are equivocal on this point.

There are several alternative possibilities as to how *A. nasoniae* is being maintained in *Nasonia* populations. Firstly, there is the possibility that resource release is only beneficial to *Nasonia in* crowded populations. *Nasonia vitripennis* exhibits a facultative sex ratio production, which is dependent on the number of ovipositing females there are in one patch of pupae (Werren, 1983). Under uncrowded conditions, *N. vitripennis* females produce female-biased sex ratios. As conditions become more crowded, the sex ratio becomes increasingly male biased. Thus, male

lethality may not free enough resources for the remaining females to favour male killing in uncrowded patches.

Male lethality may thus only be favoured in crowded groups of ovipositing females, where superparasitization is more likely. Females that emerge from superparasitized pupae tend to be smaller because there is proportionately less food per wasp under crowded situations. A survey of an entire population of females, most of which had come from patches where females oviposited alone or in small groups, might not reflect an advantage gained by infected females solely under crowded conditions. If male lethality is favoured under crowded conditions, it should result in larger, infected females dispersing from a patch where superparasitization is frequent compared to uninfected females dispersing from the same patch.

Even a small increase in the fitness of infected females is sufficient to maintain the son-killer bacterium at the levels observed. Werren *(1987)* showed that the equilibrium frequency of a vertically transmitted male killer is $p^* = (Wcz - 1)/(Wa - a)$, where a is the transmission rate to progeny and W is the fitness of infected females relative to uninfected females. Given the observed transmission rate of *0.95* (Skinner, *1985)* and infection frequencies between 0.04 and *0.08* (Skinner, *1983;* this study), the equation can be used to predict the fitness increase of son-killer-infected females necessary to achieve the equilibrium. Infected females need only have a *5.5-5.7%* increase in fitness relative to uninfected females. It is not clear that the assay used in this study would detect such a small fitness difference.

An alternative hypothesis is that male lethality might be advantageous to the bacterium because it reduces sibling mating and inbreeding depression. Werren *(1987)* showed that male lethal factors may be maintained in populations even if the mean fitness cost of inbreeding depression is very low, assuming a high rate of transmission of the trait. Since females oviposit male and female eggs in the same host pupa, there are usually ample opportunities for sibling mating that might be avoided if males are killed. It remains to be seen, however, whether or not inbreeding depression is a factor in *Nasonia*.

Since the bacterium can be contagiously transmitted (Skinner, *1983:* Huger *et al., 1985),* it is possible that horizontal transmission is very important in the maintenance of the son-killer trait in *Nasonia* populations. Male killing may play only a secondary role. A cytoplasmically inherited infection that reduces a carrier female's fitness cannot exist stably in a population unless it can be contagiously transmitted (Uyenoyama and Feldman. *1978).* Thus. horizontal transmission may have allowed the establishment of a bacterial infection in *Nasonia*. regardless of whether or not it killed males. If male-killing bacteria were subsequently derived from non-male-lethal bacteria, then the male-lethal bacteria may have spread at the expense of the more benign bacteria if the fitness of the male-lethal bacteria's female carriers were improved.

According to this model, which we call the `incremental gain' hypothesis, male killing is favoured because it increases the fitness of infected females relative to what their fitness would be if male killing did not occur. By this model. horizontal transmission can maintain the presence of any bacterium in the population so that male-lethal bacteria only have to improve their carriers' fitness to the point where they can out-compete carriers of non-male-lethal bacteria. The mechanism of fitness gain may be resource release or reduced inbreeding, but whatever the fitness gain, it need only be sufficient to allow females infected with male-killing bacteria to out-compete females infected with non-lethal bacteria, not with uninfected females. Formally testing this model may be difficult, particularly if selection has eliminated non-lethal bacterial strains from *Nasonia* populations. Surveys for non-lethal strains have not been conducted, although the results presented here suggest that they may occur.

It is formally possible that the only factor maintaining this bacterium in natural populations is its ability to be contagiously transmitted and that the positive effects of male lethality on female

offspring of *Nasonia* are non-existent. However, we consider the possibility that male-killing has no positive fitness consequences for the microorganisms that cause it to be unlikely, given that the trait has evolved independently a number of times in different systems (Werren *et al., 1986;* Hurst, *1991;* Ebbert, *1993).*

There is a possibility that co-evolution between bacterial virulence and wasp resistance to male killing may be occurring (Ebbert. *1993).* Compensatory shifts in primary sex ratio production in populations that include infected females (i.e. overproduction of males to compensate for male death) are not expected (Werren, *1987).* However, resistance to male lethality might develop. The somewhat lower male mortality percentage that we calculated for the New York strain of A. *nasoniae* suggests that there could be variation in the virulence of son-killer strains. A more comprehensive survey of the variation of both male lethality and resistance to it will be required before this question can be addressed adequately.

Finally, models that predict the possible benefits of male lethality have not included the possible interactions of male death with the other sex ratio distorters present in *Nasonia.* Both the paternal sex ratio (PSR) and maternal sex ratio (MSR) factors are found in the Utah population *of N. vitripennis* (Skinner, *1983).* These factors may interact with son killer and have population-level effects that have yet to be considered.

Acknowledgements

The authors thank members of the North American Bluebird Society for collections of nest materials, particularly P. Conklin. D. Ferris-McCarthy and M. Riley provided technical assistance. L. Beukeboom, J. Breeuwer and R. Stouthamer provided both assistance in processing field collections and helpful discussions on earlier versions of this paper. The research was supported by grants from the National Science Foundation to J.H.W.

References

Andreadis, T.G. (1990) Epizootiology of *Amblyospora connecticus* (Microsporida) in field populations of the saltmarsh mosquito, *Aedes cantator,* and the cyclopoid copepod, *Acanthocyclops vernalis. J. Protozool. 37,* 174-82.

Beukeboom, L.W. and Werren, J.H. (1992) Population genetics of a parasitic chromosome: experimental analysis of PSR in subdivided populations. *Evolution 46,* 1257-68.

Charlesworth, D. and Ganders, F.R. (1979) The population genetics of gynodioecy with Cyopasmic-genic male sterility. *Heredity 43,* 213-18.

Darling, D.C. and Werren, J.H. (1990) Biosystematics of *Nasonia* (Hymenoptera: Pteromalidae): two new species reared from birds' nests in North America. *Ann. Entomol. Soc. Am. 83,* 352-70.

Ebbert, M.A. (1991) The interaction phenotype in the *Drosophila* willistoni-spiroplasma symbiosis. *Evolution 45,* 971-88.

Ebbert, M.A. (1993) Endosymbiotic sex ratio distorters in insects and mites. In *Evolution and Diversity of Sex Ratio in Insects and Mites (D.L.* Wrensch and M.A. Ebbert, eds), pp. 150-91. Chapman & Hall, New York.

Ebbert, M.A. (1995) Variable effects of crowding on *Drosophila* hosts of male-lethal and non-male-lethal spiroplasmas in laboratory populations. *Heredity 74,* 227-40.

Gherna, R.L., Werren, J.H., Weisburg, W., Cote, R., Woese, C.R., Mandelco, L. and Brenner, D.J. (1991) *Arsenophonus nasoniae* gen. nov., sp. nov., the causative agent of the son-killer trait in the parasitic wasp *Nasonia vitripennis. Int. J. Syst. Bacteriol. 41,* 563-5.

Huger, A.M., Skinner, S.W. and Werren, J.H. (1985) Bacterial infections associated with the son-killer trait in the parasitoid wasp ***Nasonia (= Mormoniella) vitripennis.*** *J. Invertebr Pathol. 46,* 272-80.

Hurst, G.D.D., Majerus, M.E.N. and Walker, L.E. (1992) Cytoplasmic male killing elements in *Adalia bipunctata* (Linnaeus) (Coleoptera: Coccinellidae). *Heredity 69,* 84-91.

Hurst, G.D.D., Majerus, M.E.N. and Walker, L.E. (1993) The importance of cytoplasmic male killing elements in natural populations of the two spot ladybird, *Adalia bipunctata* (Linnaeus) (Coleoptera: Coccinellidae). *Biol. J. Linn. Soc. 49,* 195-202.

Hurst, L.D. (1991) The incidences and evolution of cytoplasmic male killers. *Proc. R. Soc. Lond. B 244,* 91-9.

O'Neill, K.M. and Skinner, S.W. (1990) Ovarian egg size and number in relation to female size in five species of parasitoid wasps. J. Zool., *Lond. 220,* 115-22.

Saul, G.B., Saul. S.W. and Becker, S. (1967) Linkage in *Mormoniella. Genetics 57,* 369-84.

Shull, A.F. (1948) An all-female strain of lady beetles with reversions to normal sex ratios. *Am. Nat. 82,* 241-51.

Skinner, S.W. (1983) Extrachromosomal sex ratio factors in the parasitoid wasp, *Nasonia (= Mormoniella) vitripennis.* PhD dissertation, University of Utah, Salt Lake City, UT.

Skinner. S.W. (1985) Son-killer: a third extrachromosomal factor affecting the sex ratio in the parasitoid wasp, *Nasonia (= Mormoniella) vitripennis. Genetics 109,* 745-59.

Uyenovama, M.K. and Feldman, M.W. (1978) The genetics of sex ratio distortion by cytoplasmic infection under maternal and contagious transmission: an epidemiological study. *Theor Pop. Biol. 14,* 471-97.

Werren, J.H. (1983) Sex ratio evolution under local mate competition in a parasitic wasp. *Evolution 37,* 116-24.

Werren, J.H. (1987) The coevolution of autosomal and cytoplasmic sex ratio factors. *J. Theor Biol. 124,* 317-34.

Werren, J.H., Skinner, S.W. and Huger, A.M. (1986) Male-killing bacteria in a parasitic wasp. *Science 231,* 990-2.

Werren, J.H., Hurst, G.D.D., Zhang, W., Breeuwer, J.A.J., Stouthamer, R. and Majerus. M.E.N. (1994) Rickettsial relative associated with male killing in the ladybird beetle *(Adalia bipunctata). J. Bacteriol.* 176, 388-94.

Whiting, A.R. (1967) The biology of the parasitic wasp, *Mormoniella vitripennis. Q. Rev. Biol. 42,* 333-406.

Williamson, D.L. (1965) Kinetic studies of 'sex-ratio' spirochetes in *Drosophila melanogaster* Meigen females. *J. Invert. Pathol. 7,* 493-501.

William on, D.L. (1969) The sex ratio spirochete in *Drosophila robusta. Jap.* J. *Genet. 44,* 36-41.