Distribution and reproductive effects of *Wolbachia* in stalk-eyed flies (Diptera: Diopsidae)

AHMAD R. HARIRI†§, JOHN H. WERREN‡ & GERALD S. WILKINSON*†

†*Department of Biology, University of Maryland, College Park, MD 20742, USA and ‡Department of Biology, University of Rochester, Rochester, NY 14627, USA*

Wolbachia are cytoplasmically inherited bacteria capable of altering the reproductive biology of their hosts in a manner which increases their spread within a population. These microbes can cause cytoplasmic incompatibility, parthenogenesis and feminization of genetic males. Because *Wolbachia* have been associated with female-biased sex ratio distortion, we used a PCR assay to examine 17 species of stalk-eyed flies (Diptera: Diopsidae), two of which exhibit female-biased sex ratios, for the presence of these microbes. Type A *Wolbachia* was detected in four diopsid species, three from the genus *Sphyracephala*, none of which exhibit biased progeny sex ratios. The reproductive effects of the microbe were examined in one of those species, *S. beccarii*, by conducting reciprocal crosses between infected and uninfected strains. In this species, *Wolbachia* do not cause detectable cytoplasmic incompatibility or reduce host fecundity. In contrast, our results are consistent with an association between the microbes and enhanced male fertility. Possible explanations for the pattern of distribution and effects on male fertility include a predisposition for acquiring Type A *Wolbachia* by these flies and accommodation by the host genome to bacterial presence.

Keywords: cytoplasmic bacteria, Diopsidae, *Sphyracephala*, stalk-eyed flies.

Introduction

Wolbachia are a monophyletic group of proteobacteria with two major divisions, Type A and B, which have been associated with a wide range of reproductive changes in arthropods (Werren *et al.*, 1995a). They are primarily found within the cells of the gonadal tissues of infected individuals (O'Neill, 1995) and are typically inherited by vertical transmission through the maternal cytoplasm (Hoffmann & Turelli, 1988). Horizontal transmission, especially of Type A *Wolbachia*, is believed to play an important role in spreading the infection between species and maintaining phylogenetically similar strains in diverse Orders of arthropods (Werren *et al.*, 1995b). *Wolbachia* have not been found extracellularly and their existence and proliferation appear to be intimately linked with that of their arthropod hosts (O'Neill, 1995).

Wolbachia typically alter the reproductive biology of their hosts in a manner which ultimately promotes the spread of the microorganisms. The effects of *Wolbachia* infection include unidirectional and bidirectional cytoplasmic incompatibility (CI), parthenogenesis, and feminization of genetic males (see Werren, 1997 for review). *Wolbachia* can have either positive effects on host fecundity (Girin & Bouletreau, 1995; Stolk & Stouthamer, 1996; Poinsot & Mercot, 1997) and fertility (Wade & Chang, 1995) or negative effects on host fecundity (Hoffmann *et al.*, 1990). The positive or negative consequences of infection may be related to differences in the mode of microbe transmission and maintenance in different groups of organisms. Models of symbiont transmission predict that negative effects on host reproduction will be prevalent in systems where symbiont populations are maintained by horizontal transmission, and positive effects will be more common when vertical transmission dominates (Frank, 1996).

Recently, female-biased sex ratio distortion has been reported in two species of stalk-eyed flies (Diptera: Diopsidae), *Cyrtodiopsis dalmanni* and *C. whitei* (Burkhardt & De La Motte, 1983), and ascribed to X chromosome meiotic drive (Presgraves *et al.*, 1997).

*Correspondence. E-mail: wilkinson@zool.umd.edu
§Present address: Brain Research Institute, UCLA, Los Angeles, CA 90095, USA.

Because *Wolbachia* have been associated with female-biased sex ratio distortion, either through parthenogenesis in haplodiploids (Stouthamer *et al.*, 1993) or feminization in diploids (Rousset *et al.*, 1992), we decided to determine whether or not these microbes are present in *C. dalmanni* and *C. whitei*. In order to determine the potential distribution of *Wolbachia* infection in stalk-eyed flies an additional 15 diopsid species from six genera were also examined. We also report on the reproductive effects of infection in one of those species, *Sphyracephala beccarii*, which harbours Type A *Wolbachia*.

Materials and methods

Collection

We collected flies from six of the 11 diopsid genera (Feijen, 1989): *Teleopsis*, *Cyrtodiopsis*, *Diasemopsis*, *Diopsis*, *Sphyracephala* and *Eurydiopsis*. These six genera contain over 96% of the described species and occupy the entire geographical range of diopsids (Steyskal, 1972). All *Teleopsis* species (*breviscopium*, *rubicunda*, *quadriguttata*) and *Cyrtodiopsis* species (*dalmanni*, *whitei*, *quinqueguttata*) were collected in January 1989 in peninsular Malaysia. All *Diasemopsis* (*aethiopica*, *dubia*, *munroi*, *silvatica*) and *Diopsis* (*apicalis* and *fumipennis*), as well as *Sphyracephala munroi*, were collected in December 1994 in the Natal Province, South Africa. *Sphyracephala brevicornis* were collected in October 1994 in Maryland and *S. detrahens* and *Eurydiopsis subnotata* in January 1996 in peninsular Malaysia.

Sphyracephala beccarii were collected on 22 November 1993 along a stream near Sudwala Caves, 40 km west of Nelspruit, Transvaal Province, South Africa. Approximately 20 flies were used to establish a laboratory population and were subsequently bred in the laboratory for 10 months prior to the start of this study. We estimate that the flies used in the first antibiotic treatment experiment (see below) were at least three generations removed from those collected in the field.

Screening for Wolbachia

The presence of *Wolbachia* was determined for each species by extracting DNA from pooled ovaries of two to four fecund females and then using the polymerase chain reaction (PCR) to amplify a *Wolbachia*-specific bacterial cell-cycle gene, *ftsZ*, sequence (Werren & Jaenike, 1995). All samples were screened with universal 28S primers as a positive control for amplification ability (see Werren *et al.*, 1995a, for details). Bacterial *ftsZ* DNA amplification

was confirmed by measuring product length from an agarose gel. All solutions were filter-sterilized (0.22 μm pore diameter) to reduce potential bacterial contamination. Control DNA samples were prepared from pupae of known infected and uninfected strains of *Nasonia vitripennis* and compared with amplified products from each stalk-eyed fly species to determine the presence of *Wolbachia*. Additional amplifications were performed using Type A and Type B specific primers for *ftsZ* (Werren *et al.*, 1995a).

Antibiotic curing

Flies were maintained in modified mouse cages with a 12 L:12D photoperiodic cycle and 30 min simulated dawn and dusk periods (Lorch *et al.*, 1993). Flies were allowed to feed *ad libitum* on a standard food medium consisting of puréed maize treated with a commercial mould inhibitor (Wilkinson, 1993). For breeding purposes, ≈ 35–40 mL of medium was presented in small plastic cups and changed biweekly. Newly eclosed flies were isolated by sex and maintained as virgins until they were reproductively mature and then utilized in reciprocal crosses.

In order to determine the optimum concentration of antibiotic, which would maximize elimination of the infection and minimize the mortality caused by the toxicity of the antibiotic, flies were bred on media treated with 0.5 mg mL^{-1}, 1.0 mg mL^{-1}, or 2.0 mg mL^{-1} concentrations of aqueous tetracycline, prepared by dissolving tetracycline hydrochloride (Sigma T-3383) in water over low heat. Fecund females (3 weeks post eclosion) from these treatment classes were then tested for the presence of infection using the PCR assay described above. These analyses, in addition to the number of pupae produced by five pairs of flies from each of the three tetracycline concentrations, were used to identify the optimum tetracycline concentration for curing.

These results indicated that the optimum concentration of tetracycline was 1.0 mg mL^{-1}. At this concentration, the infection was eliminated and the average daily pupal production was 8.0 ± 1.9 (SE) per pair. At 0.5 mg mL^{-1}, average daily pupal production was higher (10.0 ± 1.5), but the infection was not eliminated. At 2.0 mg mL^{-1}, the infection was eliminated, but average daily pupal production was much lower (2.0 ± 1.1).

Bacteria were eliminated by allowing 20 females and 20 males to mate and oviposit *ad libitum* for 48 h on 1.0 mg mL^{-1} tetracycline-treated medium. Twenty pairs of progeny were then bred to produce

a second generation of flies which had developed in 1.0 mg mL^{-1} tetracycline-treated media. Fecund females obtained after both one and two generations of tetracycline exposure were tested with the PCR assay to determine if there were detectable levels of *Wolbachia*. Progeny derived from both levels of tetracycline exposure were then bred on standard, tetracycline-free maize medium for three generations (20 males and 20 females in each generation) in order to reduce the possibility of decreased fecundity resulting from maternal exposure to tetracycline.

Test for fertility effects

After three generations of maintenance on tetracycline-free medium, uninfected individuals from both levels of tetracycline exposure were used in reciprocal crosses with infected individuals. Crosses were conducted by pairing single males with single females. Mating and oviposition on the medium was allowed to occur for 120 h. For each of the four possible crosses between infected and uninfected individuals, 20 replicates were established for flies derived from one generation of tetracycline exposure, and 15 replicates for flies derived from two generations of exposure.

An effect of *Wolbachia* on fertility would be indicated by an association between the presence or absence of pupae and infection. Therefore, a multiway contingency table analysis was conducted on the number of fertile and infertile pairs from each treatment using SYSTAT v.5.2 (Wilkinson, 1989). Fertility (pupae or no pupae) was used as the response variable and the level of antibiotic exposure (one or two generations), female condition (infected or uninfected), and male condition (infected or uninfected) were used as explanatory variables in a four-dimensional analysis. The significance of each potential interaction was determined by testing the difference χ^2, calculated by finding the difference between likelihood-ratio χ^2 statistics of two models differing in only one term (Fienberg, 1981).

Reproductive performance between crosses may be influenced by several factors other than *Wolbachia*. First, tetracycline-treated media yielded relatively few offspring. Therefore, inbreeding may have occurred for four generations prior to the reciprocal crosses and could be responsible for fertility differences between infected and uninfected individuals. Secondly, the two treated lines may differ genetically because of stochastic effects of small population size following tetracycline exposure. Thirdly, antibiotic toxicity has an effect on the survival of eggs and

could potentially influence the reproductive biology of adults.

We explored the potential effects of these factors by allowing *Wolbachia* populations to recover (confirmed by PCR) in a subset of treated individuals which were then used in reciprocal crosses with individuals that were untreated and known to be infected. A multiway contingency table analysis was then used to determine if there was an association between fertility, presence of *Wolbachia* and infection status. If reproductive effects are the result of any of these factors, then reproductive performance should not change in this second set of crosses. If, however, the effects are associated with *Wolbachia*, then the two sets of crosses should exhibit a difference in fertility.

Test for fecundity effects

In fertile crosses between infected and uninfected individuals, an effect of *Wolbachia* on egg production or development would be indicated by inequalities in mean daily pupal production between reciprocal crosses. Analysis of variance was used to determine if heterogeneity existed between crosses in pupal production. Fisher's Paired Least Significant Difference test (PLSD) was used to identify significant differences between crosses in pupal production.

Results

Screening for Wolbachia

The results of the PCR screen for the *Wolbachia ftsZ* gene are presented in Table 1. Four positive amplifications were detected. Three species from the genus *Sphyracephala* and *Eurydiopsis subnotata* were found to be positive for Type A *Wolbachia*, but negative for Type B *Wolbachia*. Amplification products of the appropriate length, 1035–1047 bases, were present for all four species. Neither Type A nor Type B *Wolbachia* was detected in *C. dalmanni* or *C. whitei*; thus, the microbes do not contribute to the observed female-biased sex ratio distortion in these two species.

Fertility effects

Comparison of the fertility of flies from reciprocal crosses between infected and uninfected strains (Table 2) revealed a significant association between fertility and the presence of infection in males (diff. $\chi_1^2 = 11.53$, $P < 0.005$), with 43% of 70 uninfected males failing to produce pupae compared to only

Table 1 PCR amplification of *Wolbachia fts*Z gene in 17 species of stalk-eyed flies

Genus	Species	*Wolbachia**
Cyrtodiopsis	*dalmanni*	Absent
	whitei	Absent
	quinqueguttata	Absent
Teleopsis	*breviscopium*	Absent
	quadriguttata	Absent
	rubicunda	Absent
Eurydiopsis	*subnotata*	Present
Diasemopsis	*aethiopica*	Absent
	dubia	Absent
	munroi	Absent
	sylvatica	Absent
Diopsis	apicalis	Absent
	fumipennis	Absent
Sphyracephala	*detrahens*	Absent
	beccarii	Present
	brevicornis	Present
	munroi	Present

*Positive amplifications are Type A.

17% of 70 infected males. No significant association was detected between fertility and presence of the infection in females (diff. $\chi_1^2 = 0.55$, $P>0.10$) or fertility and number of generations of tetracycline exposure (diff. $\chi_1^2 = 3.55$, $P>0.05$). Consequently, results from the two levels of tetracycline exposure were combined in subsequent analyses of fertility.

After recovery of *Wolbachia* in a subset of anti-biotic-treated individuals, the number of infertile males decreased from 43% to 18% (Table 3). A multiway contingency table analysis revealed a significant association between fertility and the presence of *Wolbachia* in males (diff. $\chi_1^2 = 6.66$, $P<0.01$), but not in females (diff. $\chi_1^2 = 4.09$, $P>0.10$). This analysis also indicated that the fertility effect observed in the first set of crosses was not present in the second set where there was recovery of *Wolbachia* (diff. $\chi_1^2 = 2.03$, $P>0.10$).

Fecundity effects

Analysis of variance failed to reveal any significant differences ($F_{3,87} = 1.7$, $P = 0.17$) in pupal production between fertile reciprocal crosses (Table 4). However, there was a significant difference

Table 2 Fertility of reciprocal crosses between strains of *Sphyracephala beccarii* infected and uninfected with *Wolbachia*

Tetracycline exposure	Fertility	Cross type (female × male)			
		I × U	U × I	I × I	U × U
One generation	Pupae present	9	16	16	10
	Pupae absent	11	4	4	10
Two generations	Pupae present	11	11	15	10
	Pupae absent	4	4	0	5

I, infected; U, uninfected.

Table 3 Comparison of the fertility of reciprocal crosses between strains of *Sphyracephala beccarii* infected and uninfected with *Wolbachia*. *Wolbachia* ($+$) strains treated with tetracycline had subsequent recovery of *Wolbachia*

Infection status of treated individuals	Fertility	Cross type (female × male)			
		I × T	T × I	I × I	T × T
Wolbachia ($-$)	Pupae present	20	27	31	20
	Pupae absent	15	8	4	15
Wolbachia ($+$)	Pupae present	15	17	13	18
	Pupae absent	5	3	7	2

Wolbachia ($-$), absent; ($+$), present. I, infected (not treated with tetracycline); T, treated with tetracycline. Values are totals from Table 2.

Table 4 Mean daily pupal production ($\pm$SE) per pair for all fertile reciprocal crosses between strains of *Sphyracephala beccarii* infected and uninfected with *Wolbachia*

Tetracycline exposure	Cross type (female $\times$ male)			
	I $\times$ U	U $\times$ I	I $\times$ I	U $\times$ U
One generation	$10.8 + 5.0$ ($n = 9$)	$8.3 + 1.8$ ($n = 15$)	$14.9 + 2.2$ ($n = 16$)	$10.7 + 1.9$ ($n = 10$)
Two generations	$8.4 + 0.6$ ($n = 11$)	$8.0 + 0.9$ ($n = 12$)	$9.0 + 1.0$ ($n = 15$)	$6.3 + 1.1$ ($n = 10$)

I, infected; U, uninfected.

($F_{1,89} = 5.0$, $P = 0.03$) in mean pupal production between lines receiving different levels of tetracycline exposure. Mean daily pupal production per pair was $11.3 + 1.3$ (SE) in lines with one generation of tetracycline exposure and $8.0 + 0.5$ in lines with two generations of exposure. There was no interaction between cross type and level of tetracycline exposure for pupal production ($F_{1,3} = 0.8$, $P = 0.47$). PCR analysis of uninfected females used in all crosses confirmed that these treated strains were free of *Wolbachia*.

Discussion

The pattern of *Wolbachia* infection in the 17 species of stalk-eyed flies screened, with three of the four positive amplifications occurring in the genus *Sphyracephala*, suggests the existence of a unique relationship between the microbe and these species. Assuming the overall probability of infection in diopsids is 23% (4/17), the probability that at least three of four *Sphyracephala* harbour the infection by chance is only 0.04. There are two possible scenarios which can explain this nonrandom pattern of infection. First, the *Wolbachia* may have been acquired prior to the divergence of the *Sphyracephala* genus and then subsequently lost in *S. detrahens*. Secondly, given the high rate of horizontal transmission of Type A *Wolbachia*, the flies in this genus may have a predisposition for horizontal acquisition of the microbe and have been infected independently of each other. Although flies in the genus *Sphyracephala* can be found on several continents, all of the infected species are commonly found in dense aggregations near streams (Feijen, 1989). Possible sources of infection include predatory mites (Johanowicz & Hoy, 1995) and parasitoid wasps (Feijen & Schulten, 1981a,b). Determination of *Wolbachia ftsZ* sequence

divergence in these species is needed to differentiate between these two potential routes of infection.

Our results did not reveal any differences in pupal production between fertile crosses. Specifically, fertile crosses between uninfected females and infected males, which have significantly lower fecundity in systems exhibiting CI, did not produce significantly different levels of pupae from all other crosses. Thus, we can conclude that *Wolbachia* in this species do not cause CI or that the level of CI is sufficiently weak not to be detected by this assay. The absence of CI in this system is not surprising. For example, although some *Wolbachia*-infected populations of *Drosophila simulans* exhibit CI, others do not (Hoffmann *et al.*, 1996).

The observed reduction in fecundity in lines receiving two generations of tetracycline exposure in comparison to those receiving only one, is most likely an artifact of the crosses being conducted at two different times. Slight environmental differences, such as media quality or laboratory temperature, could translate into significant effects on both frequency of mating and survival of offspring.

Interestingly, the results of our crosses are consistent with an association between the microbes and enhanced male fertility. Approximately 43% of uninfected males exhibit infertility compared to only 17% of infected males. This pattern is also evident in treated flies that did not lose *Wolbachia* compared to those that did. Positive effects of *Wolbachia* on host productivity have been reported for *Trichogramma bourarachae* (Girin & Bouletreau, 1995), *Tribolium confusum* (Wade & Chang, 1995), *Nasonia vitripennis* (Stolk & Stouthamer, 1996) and *D. simulans* (Poinsot & Mercot, 1997).

The male fertility effect we found in *S. beccarii* is unlikely to be caused by inbreeding depression, line divergence or antibiotic toxicity. A multiway contin-

gency analysis revealed a significant association between fertility and the presence of *Wolbachia* in males, suggesting that the bacteria, rather than these other factors, are responsible for the observed fertility effect. Further evidence against inbreeding depression as the cause of the 26% reduction in male fertility comes from a study of artificial selection in another stalk-eyed fly, *Cyrtodiopsis dalmanni* (Wilkinson *et al.*, 1998). After 22 generations of selection with an effective population size of 29 (coefficient of inbreeding estimated to be 53%), male fertility declined 10% in comparison to an outbred stock population (Wilkinson, unpubl. data). In contrast, a comparable estimate of the inbreeding coefficient for the *S. beccarii* used in the reciprocal crosses reported here was 5%. Nevertheless, it remains possible that other genetic changes in the flies could have caused a paternal fertility effect. Introgression of the uninfected genotype into the infected background is needed to address this possibility further.

One possible explanation for the male fertility effect in *S. beccarii* is that the bacteria were originally CI producers which spread to near fixation, after which the host genome accommodated to their presence by adjusting spermatogenesis to reduce the negative effects of CI in males. By eliminating the microbes with antibiotics, spermatogenesis may have been altered causing reduced fertility. Turelli (1994) has suggested that the host genome can be selected to suppress microbial effects in males, whereas suppression in females is generally not favoured. In addition, recent molecular studies have illustrated that *Wolbachia* interact with centrosomal microtubules and host proteins, both important in gametogenesis (Kose & Karr, 1995). These interactions may provide a potential mechanism by which *Wolbachia* alter the reproductive biology of their hosts. Studies designed to determine how the bacteria influence spermatogenesis are needed to confirm this hypothesis.

Acknowledgements

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