

**Background:**

Insects are hosts to a diverse assemblage of microorganisms, some of which are able to manipulate host reproduction to their own advantage. Perhaps the best studied of these reproductive parasites is the  $\alpha$ -proteobacteria *Wolbachia*. *Wolbachia* inhabit ~20% of insect species as well as some isopods and nematodes (Werren *et al.* 1995). They can cause such reproductive phenotypes as cytoplasmic incompatibility between infected and uninfected hosts, feminization of genetic males, parthenogenesis or embryonic male-killing (Stouthamer *et al.* 1999). *Wolbachia* are maternally transmitted and in each of these cases act to increase their own reproduction by favoring the reproduction of infected females. Of these, hosts of male-killers suffer the greatest fitness loss due to the embryonic death of half of their offspring (Charlat *et al.* 2003). Male hosts experience strong selection to suppress the male-killing phenotype and survive beyond the embryonic state, but evidence for suppression has been observed in only one male-killing infection (Hornett *et al.* 2006). In addition to the loss of male offspring, female hosts of male-killers may bear physiological effects of infection.

Theory suggests that maintenance of an equilibrium frequency of infection is possible only if there is an increase in fitness experienced by offspring of infected females. Benefits of having an infected mother are attributed to reductions in sibling competition, inbreeding, and egg cannibalism (Hurst 1991). In addition, parasite characteristics such as transmission efficiency affect the level of infection within a host population and are influenced by abiotic factors like temperature. It is clear that all of these parameters must reach a delicate balance in order to maintain infection without parasite fixation, complete loss of males and extinction of both host and endosymbiont.

Phylogenetic analysis of *Wolbachia* and their hosts shows repeated horizontal transmission, such that closely-related bacterial strains are present in very distantly-related hosts. Although the agent of transmission is unknown in most cases, parasitoid wasps are capable of transferring *Wolbachia* between distantly-related host species (Heath *et al.* 1999) and parasitoids often harbor *Wolbachia* infections that are closely related to those of their hosts (Vavre *et al.* 1999). While evidence of horizontal transmission of CI-inducing *Wolbachia* is common, only one male-killing strain of *Wolbachia* has been found in two distantly related hosts (Dyson *et al.* 2002), and no closely-related hosts share a strain of male-killing *Wolbachia*. The lack of evidence for or against interspecific transfer is a result of incomplete sampling of male-killing genotypes and the exacting requirements for equilibrium that result only rarely in stable infections. To date, only two strains of male-killing *Wolbachia* have been identified within the genus most widely used as an insect genetic model, *Drosophila*. Phylogenetic evidence shows that the strains have independent origins and that the hosts are also only distantly related (Dyer and Jaenike 2004).

Identification of closely related strains of male-killing *Wolbachia* in separate hosts would allow investigation of host and environmental correlaries for the ability of a host to maintain an infection at an equilibrium frequency. Within the genus *Drosophila*, male-killing *Wolbachia* are present in *D. bifasciata* and *D. innubila* (Hurst *et al.* 2000; Jaenike *et al.* 2003). Incidences of female-biased sex ratios have also been reported in several other species but have not yet been attributed to any causal agent. One of these infections was reported in *D. borealis* in 1956. A single female fly with an all-female brood was collected and a line was maintained for eight generations by crossing with male flies from another line each generation. Maternal inheritance of the trait and biparental inheritance of nuclear markers suggested that the causal agent was a cytoplasmic element that either killed or feminized male embryos (Carson 1956). During June

2005, wild female specimens of *D. borealis* were captured in Poygan Marsh, Wisconsin. Of two female flies that successfully produced offspring in the lab, one brood consisted entirely of females and the other had an approximately 1:1 sex ratio. Some offspring from the all-female brood were kept in isolation, but were unable to reproduce parthenogenetically in the lab. Other offspring were mated to males from a line with a normal sex ratio. All matings of this type resulted in large all-female broods indicative of either male-killing or feminization of genetic males. Flies from all-female broods tested positive for *Wolbachia* in a PCR-based screen.

Here I report the characterization of this strain as a male-killing *Wolbachia* infecting *D. borealis*, as well as evidence for its horizontal transmission between two distantly-related *Drosophila* species with non-overlapping ranges. Characterization included 1) a survey of ovarian bacterial flora to reveal any possible causal agents of the sex ratio trait 2) antibiotic curing of the trait 3) analysis of egg viability and Y-chromosome inheritance and 4) phylogenetic analysis of the *Wolbachia* with respect to other male killers.

### **Materials and Methods:**

The line exhibiting the sex-ratio trait, PG05.16, was maintained by outcrossing with males from another line from the same collection, PG05.02, for ~9 generations prior to this study, resulting in a very similar nuclear genetic background in the two lines.

#### *Survey of ovarian bacterial flora*

The causal relationship of *Wolbachia* with the sex ratio trait was investigated by identifying microorganisms present in the ovaries of affected flies, followed by antibiotic curing of the infection. Ovarian DNA was isolated from sexually mature virgin female flies of PG05.02 and PG05.16 and previously published general primers were used to amplify eubacterial ribosomal DNA (Weisburg *et al.* 1991). Although the amplification was carried out on both lines, a PCR product was only observed for the all-female line, PG05.16. Amplified DNA was cloned and several clones were

sequenced. The resulting sequences were used to design a restriction digestion assay specific for the *Wolbachia* strain present, and 54 additional clones were typed. If the trait is caused by a *Wolbachia* infection, the *Wolbachia* strain responsible will be the only bacterium unique to PG05.16. With this in mind, any non-*Wolbachia* clones should be sequenced from both lines, but because no bacterial DNA was amplified from PG05.02 and because no non-*Wolbachia* clones were identified after restriction digestion of clones from PG05.16, this step was unnecessary.

#### *Antibiotic curing of sex ratio trait*

Female flies from each line were crossed with PG05.02 males and allowed to oviposit either on normal cornmeal media or media containing 0.025% tetracycline. Resulting female offspring were crossed twice more to PG05.02 males, and two more generations were reared for each line on each food type. After each generation, offspring were sexed and counted. DNA was extracted from ovaries of treated and untreated females from each line and *Wolbachia*-specific primers were used to determine *Wolbachia* infection status. Treated PG05.16 flies were used to establish a cured line, PG05.16C.

#### *Analysis of egg viability and Y chromosome inheritance*

In order to differentiate between feminization and male-killing as the cause of sex ratio distortion, a comparison of egg viability was carried out among PG05.16, PG05.02 and PG05.16C. Female flies from each line were mated *en masse* to PG05.02 males in population cages and allowed to oviposit on grape-juice agar supplemented with live yeast. Eggs were collected twice daily from each line and arrayed on additional grape-juice agar plates. After two days, hatch proportion was assayed for each collection by visually inspecting each egg. Egg hatch data was analyzed by carrying out a logistic regression to test the effects of line on egg hatch proportion while controlling for any effects of the blocked design created by collecting eggs over several days. Egg hatch proportion from PG05.16C was compared pairwise to that of PG05.16 and PG05.02. Male-killing is

expected to result in significantly different egg hatch proportion between PG05.16 and PG05.16C, and similar egg hatch proportion between PG05.16C and PG05.02. Feminization is expected to result in similar egg hatch proportions among all lines.

In addition to determining egg hatch proportion, primers specific for a Y chromosome gene were used to amplify DNA from two male and two female PG05.02 flies and ten female PG05.16 flies. Presence of Y chromosome genes in PG05.16 female flies would indicate feminization of genetic males, while absence is evidence for male-killing.

#### *Phylogenetic analysis of Wolbachia sex ratio distorters*

To determine the phylogenetic position of this *Wolbachia* strain, I sequenced the *Wolbachia surface protein (wsp)* gene from PG05.16 ovarian DNA. *Wsp* is the fastest evolving protein known in *Wolbachia*, and has been used extensively for phylogenetic analysis (Zhou *et al.* 1998). *Wolbachia* in insects belong to two deeply divergent clades, so the sequence was compared to other *wsp* sequences from those clades as well as two strains from another clade to root the phylogeny. All known male-killing strains were included in the phylogeny. Neighbor-joining was used to reconstruct a phylogeny with 472 bp of sequence from 32 strains of *Wolbachia*. One thousand neighbor-joining bootstrap replicates were also generated. If the investigated strain is most closely related to other male-killing *Wolbachia*, horizontal transmission is probable because all other hosts of male-killing *Wolbachia* are only distantly related to *D. borealis*.

### **Results:**

#### *Survey of ovarian bacterial flora*

Preliminary sequencing of eight bacterial ribosomal DNA clones from PG05.16 ovary DNA revealed mostly *Wolbachia* sequence (7 of 8 sequences). The remaining sequence showed significant BLAST hits to uncultured bacterial strains. The presence of this bacterium can be

attributed to contamination from gut flora during ovary dissection. A restriction digest assay was designed to differentiate the *Wolbachia* strain sequenced from ribosomal DNA of a variety of other microorganisms, including the uncultured bacterium and all known reproductive parasites. Of 54 additional clones screened, all tested positive as *Wolbachia*. All attempts to amplify bacterial DNA failed with template from PG05.02.

#### *Antibiotic curing of sex ratio trait*

After one generation, PG05.02 sex ratios were  $0.92 \pm 0.13$  and  $1.17 \pm 0.13$  for tetracycline treated and untreated flies and  $0 \pm 0$  for both treatments of PG05.16 flies. After two and three generations, tetracycline-treated PG05.16 females gave rise to broods with sex ratios of  $1.25 \pm 0.50$  and  $0.99 \pm 0.14$ , while non-tetracycline PG05.16 controls retained a sex ratio of zero. PG05.02 sex ratios remained at  $\sim 1$  regardless of treatment for the duration of the experiment (Table 1). Attempts to amplify *Wolbachia* DNA failed with template from the newly created cured line.

#### *Analysis of egg viability and Y chromosome inheritance*

Eight collections of 160 eggs per line were made (1280 eggs/line). Egg hatch proportions for PG05.02 and PG05.16C were  $0.77 \pm 0.02$  and  $0.78 \pm 0.02$ , while egg hatch proportion for PG05.16 was  $0.38 \pm 0.02$ . A logistic regression was fitted to the data and showed a significant effect of line on egg hatch ( $p < 0.0001$ ). Post hoc pairwise comparisons showed a significant difference between hatch proportion of PG05.16 and PG05.16C ( $p < 0.0001$ ), but not between PG05.02 and PG05.16C. The blocking design created by collecting over time did not have any discernable effect on hatch proportion.

#### *Phylogenetic analysis of Wolbachia sex ratio distorters*

Over the 472 bp of aligned *wsp* sequence, there were only two differences between the male-killing lineages in *D. borealis* and *D. innubila*. The neighbor-joining reconstruction

showed the *D. borealis* strain as sister to the male-killing strain of *Wolbachia* inhabiting *D. innubila*. Bootstrap support for the node connecting the *D. borealis* and *D. innubila* strains was of moderate strength (Figure 1). The third male-killing strain of *Wolbachia* in *D. bifasciata* grouped separately from that of *D. borealis* and *D. innubila*.

## **Discussion:**

### *Survey of ovarian bacterial flora*

Overall, 98% of all bacterial ribosomal DNA in ovaries of PG05.16C females appears to be *Wolbachia*. This observation, coupled with the absence of bacterial DNA in the ovaries of PG05.02 females strongly suggests that the *Wolbachia* strain present is responsible for the sex ratio differences between lines. Causal relationships between bacteria and disease are usually confirmed by fulfilling a set of criteria known as Koch's postulates. Koch's postulates state that the bacterium must be present in every case of the disease, that it can be isolated and grown in pure culture, that the pure culture can be used to inoculate a healthy host, and that you must be able to recover the bacterium from the newly-inoculated host. Unlike other microbial effectors, *Wolbachia* cannot be cultured independently of its host, so Koch's postulates cannot be applied. Instead, most studies of *Wolbachia* rely on an accumulation of circumstantial evidence for causation. This usually consists of a constant association between *Wolbachia* and the trait of interest, as well as an attempt to cure the trait to create uninfected hosts.

### *Antibiotic curing of sex ratio trait*

Three generations of tetracycline treatment were sufficient to restore a 1:1 sex ratio and reduce the level of bacterial DNA in the ovaries below levels detectable by PCR. Since the conclusion of this experiment, the cured line has been maintained for 7 generations, and the sex

ratio remains normal. These results strengthen the argument that the *Wolbachia* strain identified is the cause of the altered sex ratio in PG05.16.

#### *Analysis of egg viability and Y chromosome inheritance*

Egg viability was lower than expected for all treatments, but this can be attributed to the extensive handling of eggs when arraying them on agar to be counted. The effect of handling appears to be the same across all lines, resulting in a total inviability proportion of about 20%. Egg viability is reduced by an additional 50% in the infected lineage, PG05.16, compared to PG05.02 and PG05.16C. The loss of half of the offspring strongly indicates that the *Wolbachia* strain present acts to kill male embryos, rather than to feminize genetic males. This result is supported by the absence of Y chromosome genes in all PG05.16 females.

#### *Phylogenetic analysis of Wolbachia sex ratio distorters*

Phylogenetic analysis using the *wsp* gene shows that this strain of male-killing *Wolbachia* is very closely related to another male-killing strain in a distantly-related *Drosophila* species. This close relationship between the parasites of two distantly-related hosts is a hallmark of horizontal transmission. Interestingly, the two distantly-related hosts, *D. borealis* and *D. innubila*, do not share any portion of their natural ranges. *D. borealis* is found in the upper Midwest and Canada, while *D. innubila* is found solely on mountains known as sky islands in the desert Southwest and Mexico. Horizontal transmission between hosts with disparate ranges could have been accomplished via mites or parasitoid wasps, but our limited knowledge of the ecology of these species precludes identification of the intermediate species. Gaps in infection over geographic and phylogenetic space indicate that some species may be especially susceptible to sustained infection. Characterization of a third male-killing *Wolbachia* infection in *Drosophila* will allow investigation of correlaries for amenability to sustained infection and allow powerful interspecific comparisons for investigating the mechanism of male-killing.

## References:

- Carson, H.L. 1956. *Drosophila Information Service* 30: 109-110.
- Charlat, S., G. D. D. Hurst and H. Merçot. 2003. *TRENDS in Genetics*. 19:217-223.
- Dyer, K. A. and J. Jaenike. 2004. *Genetics* 168: 1443-1455
- Dyson, E. A., M. K. Klamath and G. D. D. Hurst. 2002. *Heredity* 88: 166-171.
- Heath, B. D., R. D. J. Butcher, W. G. F. Whitfield and S. F. Hubbard. 1999. *Current Biology* 9: 313-316.
- Hornett, E. A., S. Charlat, A. M. R. Duploux, N. Davies, G. K. Roderick, N. Wedell and G. D. D. Hurst. 2006. *PLoS Biology* 4: 1643-1648.
- Hurst, G. D. D., A. P. Johnson, J. H. G. v. D. Schulenburg and Y. Fuyama. 2000. *Genetics* 156: 699-709.
- Hurst, L. D. 1991. *Proc. R. Soc. Lond. B*. 244: 91-99.
- Jaenike, J., K. A. Dyer and L. K. Reed. 2003. *Evol. Ecol. Res.* 5:1023-1036.
- Stouthamer, R., J. A. J. Breeuwer and G. D. D. Hurst. 1999. *Annu. Rev. Microbiol.* 53: 71-102.
- Vavre, F., F. Fleury, D. Lepetit, P. Fouillet and M. Boulétreau. 1999. *Mol. Biol. Evol.* 16: 1711-1723.
- Weisburg, W. G., S. M. Barns, D. A. Pelletier and D. J. Lane. 1991. *J. Bacteriology* 173: 697-703.
- Werren, J. H., D. Windsor and L. Guo. 1995. *Proc. R. Soc. Lond. B*. 262: 197-204.
- Zhou, W., F. Rousset and S. O'Neill. 1998. *Proc. R. Soc. Lond. B*. 265: 529-515.

Table 1. Sex ratio (males/females) of offspring after tetracycline treatment.

Tetracycline	Generation 1		Generation 2		Generation 3	
	-	+	-	+	-	+
PG05.02	1.17 ± 0.13	0.92 ± 0.13	1.17 ± 0.40	1.09 ± 0.16	0.86 ± 0.17	1.04 ± 0.14
PG05.16	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	1.25 ± 0.50	0.00 ± 0.00	0.99 ± 0.14

Figure 1. Neighbor joining phylogeny of 32 *Wolbachia* strains with 1000 neighbor joining bootstrap replicates. Male-killing lineages are shown in red, and the major divisions of the *Wolbachia* clades are shown as ‘A,’ ‘B,’ and ‘C’.

