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BARD

FINAL REPORT

PROJECT NO. US-2062-91

**Understanding the Causes and Genetic Effects of
Thelytoky in the Aphelinidae: A Key to Improving
Biological Control**

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1996

Date: 10 June 1996

BARD Project Number: US-2062-91

Project Title:

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Aphelinidae: A Key to Improving Biological Control

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Project Start Date: July 1992

Type of Report: 1st Annual 2nd Annual Final XX

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Final Scientific Report

Understanding the Causes and Genetic Effects of Thelytoky in the Aphelinidae: A Key to Improving Biological Control

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Aphelinidae:**

A Key to Improving Biological Control

Richard T. Roush and David Rosen

ABSTRACT

Thelytoky is a type of parthenogenesis whereby females produce only female offspring without the involvement of males, even where males are occasionally produced. In the last few years, strong circumstantial evidence has implied that thelytoky can be caused by microorganisms called *Wolbachia* in at least some species of wasps. The thelytoky can be "cured" by treatment with antibiotics. Further, *Wolbachia*-like organisms can be found in microscopic examinations and genetically identified through their DNA.

The aphelinid wasps, and especially species in the genus *Aphytis*, are among the most important of all classical biological control agents. *Aphytis* species are critical in the biological control of scale insect pests in commercial orchards and ornamental plantings. About 30% of *Aphytis* species are thelytokous, of which we were able to study three in detail. In all three, thelytoky was curable by treatment with antibiotics and *Wolbachia* were identified morphologically and through their DNA. In contrast, *Wolbachia* were not detectable in biparental species of *Aphytis*. Studies of *Wolbachia* gene sequences obtained from *Aphytis* showed that they were most closely related to those from a very distantly related wasp, *Muscidifurax uniraptor*, strongly implying that the *Wolbachia* can be horizontally transferred. As revealed by electron microscopy, the *Wolbachia* show a strong association with the nurse and follicle cells of the female wasps.

Introduction

Much of the biological control of insect pests is due to parasitic wasps. Most wasps, ants, bees and their relatives (Hymenoptera) reproduce by arrhenotoky, a form of parthenogenesis in which unfertilized eggs give rise to haploid males and fertilized eggs give rise to diploid females. However, complete parthenogenesis, i.e. thelytoky, is also found in many hymenopterous lineages (Luck et al., 1992). In thelytoky, unfertilized eggs develop into daughters, and these females also produce daughters from unfertilized eggs. Males are occasionally produced, but are not observed to mate, and mating is not needed for reproduction by females. Consequently, thelytokous lines can persist indefinitely without sexual reproduction.

Thelytoky was, until recently, assumed to have an exclusively genetic cause in the Hymenoptera. Heat was long known to cause the production of males in thelytokous species, but Stouthamer et al. (1990) showed for the first time that high temperatures or antibiotic treatments could cause a reversion to arrhenotoky. Most species of parasitic wasps of the genus *Trichogramma* are arrhenotokous, but thelytokous reproduction also occurs. Stouthamer et al. (1990) exposed 9 lines of 4 species of uniparental *Trichogramma* to high temperatures and antibiotics. Three antibiotic treatments (rifampicin, tetracycline and sulfamethoxazole) caused a reversion from thelytoky to arrhenotoky, as did the high temperature treatment. These results led to the conclusion that a microorganism was associated with uniparental reproduction in *Trichogramma*, and this landmark paper inspired the search for such symbionts in other parasitic wasps.

Antibiotic treatments were soon shown to induce the production of males in *Encarsia formosa* (Zchori-Fein et al., 1992), and important parasite of whiteflies; other *Trichogramma* (see Rousset et al., 1992); *Muscidifurax raptor* (Stouthamer et al., 1993), a parasite of filth breeding flies; and other parasitic wasps. As will be discussed further in this report, subsequent work largely by R. Stouthamer and J.H. Werren showed a strong association of microbially-induced thelytoky and bacteria of the genus *Wolbachia*. *Wolbachia* species had previously been shown to be strongly associated with the failure of some strains of insects to produce offspring after successful pairings with other strains of the same species (see Rousset et al., 1992 for citations). Strictly speaking, however, because it has not yet been possible to transmit *Wolbachia* to uninfected hosts, it has not yet been proven conclusively that *Wolbachia* cause thelytoky.

Among the Hymenoptera, parasites in the family Aphelinidae are especially important in biological control. Thelytokous reproduction occurs sporadically in different groups within the Aphelinidae, especially within the genus *Aphytis*, which are parasites of scale insect pests. Sixteen species, or about 30% of *Aphytis* whose mode of reproduction is known, are thelytokous. Most but not all of these species have related arrhenotokous species or forms

and the ramifications of thelytokous reproduction for genetic variation and growth of parasite populations, it was important to determine whether uniparental reproduction in *Aphytis* is associated with the presence of microorganisms, and to try to understand the fundamental effects of those organisms.

Objectives of the Project

As Stated in the Original Research Proposal

Primary: Improvement of biological control of armored scale insects by *Aphytis* species by investigating the causes and genetic effects of thelytoky.

To attain this overall objective, the following specific goals have been set:

- 1) Determine whether thelytokous *Aphytis* species can be cured from unisexuality by antibiotic treatments.
- 2) Investigate the mode of inheritance of genetic traits in the thelytokous species.
- 3) Assuming that thelytoky can be cured such that uniparental and biparental strains can be established from the same culture:
 - a) As measured by selection response, compare the adaptability of the arrhenotokous and thelytokous strains.
 - b) Compare their fitnesses, as measured by reproductive output, under different laboratory and field conditions to determine whether there is a reproductive cost or advantage to thelytoky.
- 4) In the event that thelytoky cannot be cured, conduct the experiments outlined above on arrhenotokous and thelytokous strains that are as taxonomically similar as possible.

Deviations and Modifications

Rapid progress was made in this field in 1991-92, largely due to the efforts of R. Stouthamer, J. H. Werren, F. Rousset and colleagues. Unknown to us at the time we submitted our grant proposal, there was already research under way that rendered obsolete some of the work we proposed. First, addressing the question of likely causes, bacteria from the genus *Wolbachia* were found in several wasp species where thelytoky had been shown to be cured by antibiotic treatment (Rousset et al. 1992 [submitted July 1992], Stouthamer et al., 1993 [submitted June 1992]). Second, we learned from a seminar given by Stouthamer that diploidy is restored in *Wolbachia*-associated thelytokous *Trichogramma* by gamete duplication (later published by Stouthamer and Kazmer in 1994). When we confirmed that thelytoky in *Aphytis* was also associated with *Wolbachia* (as discussed below), this left little doubt as to how genetic traits would be inherited in *Aphytis* (objective 2).

Based on the implications of these new developments, reviewers' suggestions, and our own early results, we modified some of our plans even within the first year. As described below, we were unable to establish biparental lines from thelytokous lines, in contrast to successes in *Trichogramma* (Stouthamer et al. 1990a). This rendered objective 3 infeasible,

and we were therefore unable to directly study the costs to fitness of microbially induced thelytoky. Further, we were unable to obtain from the field arrhenotokous and thelytokous lines that were sufficiently closely related taxonomically to justify valid comparisons of fitness (objective 4). Therefore, we focused our research efforts on the three most important remaining questions about *Wolbachia*-induced thelytoky: (1) describing how the microorganisms are associated with and may cause thelytoky, and (2) especially when our molecular analysis showed that these had been horizontally transmitted, testing possible routes of transmission, and (3) proving that *Wolbachia* caused thelytoky by artificial horizontal transmission (i.e., injection).

Relevant Data, Methodology, Results and Discussion

Wasps studied

Three species of *Aphytis* were successfully studied in detail; *Aphytis lingnanensis*, *Aphytis diaspidis* and *Aphytis yanonensis*. For *A. diaspidis* and *A. yanonensis*, we had only uniparental strains, whereas we had both uniparental and biparental "strains" of what were thought to be *A. lingnanensis*. Efforts to study yet another thelytokous species, *Aphytis maculicornis*, were unsuccessful due to rearing difficulties. In addition, another uniparental species of Aphelinidae, *Encarsia formosa*, was also used as a control in some experiments. The European spruce sawfly (*Gilpinia hercyniae*) and the parasitic wasp *Venturia* (= *Nemeritis*) *canescens*, two other uniparental species for which there was no evidence of micro-organismal involvement, were also used as a controls.

Induction of male production by antibiotic treatment.

Following the successes with *Trichogramma* (Stouthamer et al. 1990a) and *Encarsia formosa* (Zchori-Fein et al. 1992), a standard preliminary test for whether a case of thelytoky may be due to *Wolbachia* is to try to cure the infection by feeding a suitable antibiotic to (and/or heat treatment of) the thelytokous female wasps. If a significant increase in male production occurs in the otherwise essentially all female strain, it implies that microorganisms were causing the thelytoky.

For antibiotic treatments, the wasps were typically starved for 24 hours after emergence and then offered antibiotics for 1-2 days mixed with honey (which the wasps readily consume) and food coloring, the latter so that it was possible to observe feeding. With *Aphytis lingnanensis*, tetracycline and rifampicin were tested at four concentrations (25, 50, 75 and 100 mg/ml honey). The progeny (F₁) of *A. lingnanensis* that were fed antibiotics generally showed no higher proportion of males than controls, but these in turn produced about 70-93% male offspring (F₂) as opposed to only 10% in the controls (Zchori-Fein et al. 1995). Fifty mg/ml rifampicin was found to be the most effective treatment

Fifty mg/ml rifampicin was also used for *Aphytis diaspidis*. Here the trend was even sharper, with the antibiotic-treated *Aphytis diaspidis* females producing all female F₁

offspring, which in turn produced 100% male F₂ offspring. That the effect of antibiotic might not be expressed until the second generation was especially interesting because in all previously successful "curing" experiments, the effect was immediate, that is, males were produced in the F₁ by the treated females. This suggested that curing could be incomplete and yet the micro-organisms might continue to die off without further treatment, or that substances left by the micro-organisms could have a carry-over effect on eggs in production.

For *Aphytis yanonensis*, tests were done at 100 mg/ml rifampicin, and somewhat different results were obtained: 71% of the 38 F₁ offspring were males and all 50 F₂ offspring were males. In untreated controls, only 5 of 62 offspring were males. It is not clear why *A. yanonensis* responded more quickly and fully to the antibiotic treatment, although it could be due to differences in the concentration (and batch) of the antibiotic. In terms of gene sequences, the *Wolbachia* isolated from all three species seemed very similar, as will be discussed later in this report.

In contrast to the results with *Aphytis*, there was no significant increase of percentage of male sawflies in F₁ or F₂ offspring after extensive treatments with rifampicin, and no production of males whatsoever in *Venturia canescens*. For the spruce sawfly, where the adults would not feed, rifampicin was given to larvae by force feeding, painting on spruce foliage, and injection into larvae. These and subsequent PCR experiments (see below) confirmed that not all thelytoky in the Hymenoptera is associated with *Wolbachia*.

Visual confirmation of *Wolbachia*-like organisms.

With appropriate stains and under light microscopy, Stouthamer and Werren (1993) provided evidence of symbionts in 13 *Trichogramma* lines that exhibited revertible parthenogenesis (i.e., can be "cured" by antibiotic). Such symbionts were not found in the eggs of 6 field-collected arrhenotokous lines, 4 non-revertible parthenogenetic lines or in 3 arrhenotokous lines derived from thelytokous lines produced by antibiotic treatments. We were similarly able to confirm by both light and electron microscopy that *Wolbachia*-like microorganisms were present in thelytokous but not "cured" or arrhenotokous *A. lingnanensis* (Zchori-Fein et al. 1994). Ovaries of *Encarsia formosa* were also examined and found to contain microorganisms with the same ultrastructure. Further observations under microscopy will be described later in this report.

Use of males from cured females to develop new biparental lines for fitness studies

As one of the original objectives of our grant proposal was to see if cured wasps could contribute to an arrhenotokous (sexually reproducing) strain, males produced from antibiotic treatments were placed with females from antibiotic treated and untreated mothers, and the colony observed for sexual reproduction.

Sexual reproduction was accomplished in four similarly treated species of *Trichogramma* (Stouthamer et al. 1990a), and in other species where heat was used to induce

the males (Legner 1985), but not in *Aphytis*. For all three species of *Aphytis*, males were mated to F₁ females from antibiotic treated mothers under various environmental conditions to encourage mating and occasionally were seen to mate. However, no sperm were found in the spermathecae of mated females, which all produced exclusively all-male broods, as is expected for unmated arrhenotokous females (which can lay only unfertilized eggs).

The most detailed and compelling results were obtained with *A. lingnanensis*, for which two morphologically indistinguishable lines are maintained at the Faculty of Agriculture in Rehovot. One strain is uniparental and the other is biparental, so in contrast to the other species examined, it was possible to mate males from antibiotic-treated uniparental females with females from the biparental line, following a procedure used by Stouthamer et al. (1990b).

Males of the uniparental line inseminated about 54% of their own females (Table 1, UXU cross), which did not differ significantly from the percent insemination seen in the control (biparental females with biparental males, BXB). However, although sperm from the uniparental line appeared normal (Figure 1), all of the females mated by these males produced exclusively male progeny, indicating that fertilization did not occur.

Table 1. Presence of sperm in the spermathecae of *Aphytis lingnanensis* females mated with males of the uniparental or biparental line. B=biparental; U=uniparental.

crosses female X male	# of spermatheca examined	# of full spermatheca	mean % of full spermathecae (SE)
B X B	29	16	67 (11)
U X U	28	15	54 (15)
B X U	38	1	2.8 (9.3)
U X B	35	0	0.0 (0)

In crosses between strains, only one out of 38 spermathecae examined was found to contain sperm (Table 1, BXU cross). The differences between within (UXU and BXB) and between (BXU and UXB) strain crosses was highly significant ($F=16$, $p<0.001$), suggesting that the two strains have significant pre-zygotic (or pre-mating) barriers and should be considered separate species.

The precise cause of the failure of antibiotic treated *Aphytis* to reproduce biparentally was not resolvable. It was as if the species have been uniparental so long that the females have lost the ability to use the sperm (presumably due to selection against a disused function). Perhaps the males have not lost the ability to produce sperm simply because they don't reproduce, such that selection is not operating on sperm production. However, it is also possible that something was wrong with the sperm. In any case, our results contrast with those with *Trichogramma* (Stouthamer et al. 1990a, 1990b), and suggest that the association of *Wolbachia* with *Aphytis* may be older than that for *Trichogramma*.

Figure 1. Squash of reproductive tract of an antibiotic-induced *Aphytis lingnanensis* male, showing sperm (x1000).

In summary, that it is possible to induce the production of males in a thelytokous *Aphytis* line by antibiotics suggests that microorganisms play a role in determining the mode of reproduction in these wasps. However, it was not possible to establish a biparental line of any *Aphytis* species. Similar results were obtained with *Encarsia formosa* (Zchori-Fein et al. 1992). Further, in contrast to the case with *Trichogramma*, the one morphologically identical biparental strain that was available proved to be reproductively incompatible. Thus, we were not able to execute comparative fitness studies.

In principle, a parasitic wasp that reproduces only daughters could produce twice as many offspring that can attack and kill hosts (which the males cannot) as a wasp that produces both sexes, depending on sex ratio and assuming that total number of offspring is the same. For that reason, and because uniparental strains are easier to colonize, thelytokous strains are attractive in biological control (Aeschlimann 1990). However, among *Encarsia formosa* treated with tetracycline, antibiotic-treated females produced about twice as many progeny than did the untreated control (Zchori-Fein et al. 1992), suggesting that antibiotic treatment was, in effect, controlling a disease organism. Other studies on strains with cured thelytoky have yielded results that may have been somewhat confounded by the antibiotic used. Antibiotic treatments of uniparental lines of *Trichogramma deion* and *T. pretiosum* resulted in an increase in the production of progeny, and in two of three cases arrhenotokous females produced more females than their thelytokous counterparts when host availability was not limited (Stouthamer and Luck 1993). In contrast, treatment of *E. formosa* with rifampicin significantly reduced

reproduction relative to the untreated control (Stouthamer et al. 1994). There were no significant differences in fecundity between rifampicin-treated and untreated *Muscidifurax uniraptor* (a uniparental species) (Stouthamer et al. 1994).

Confirmation and identification of *Wolbachia* via PCR amplification of eubacterial 16S rDNAs

Following the use of PCR to demonstrate via sequence analysis of bacterial 16S ribosomal DNA that *Wolbachia* were associated with thelytoky (Rousset et al. 1992, Stouthamer et al. 1993), PCR has become a primary diagnostic tool for investigating the probable cause of thelytoky in wasps. In addition, analysis of the DNA sequences obtained allows one to investigate the phylogenetic relationships of the *Wolbachia* involved, and investigate the possibility of horizontal transfer of the *Wolbachia* between wasp lineages. (We thank R. Stouthamer at the Agricultural University of the Netherlands at Wageningen for early discussions on this phase of the project.)

The details for the reaction procedures used for *Aphytis lingnanensis* and *A. diaspidis* are given in Zchori (1995) and Zchori et al. (1995) and were varied only slightly for *A. yanonensis*. In all cases, the primers were designed to be specific for *Wolbachia pipientis* in wasps; forward primer: 5'- TTGTAGCTTGCTATGGTATAACT; reverse primer: 5'- GAATAGGTATGATTTCCATGT. The primers were based on those of O'Neill et al. (1992), except that we modified our forward primer with a T instead of C in position 8, and the reverse primer with a C instead of T in position 17, based on the sequence published by Breeuwer et al. 1992 from *Nasonia*.

A 16S rDNA sequence of the proper length (1000 base pairs) was obtained from all three thelytokous species of *Aphytis* studied, but not from wasps from cured uniparental strains or two biparental lines (*Aphytis lingnanensis* and *Aphytis melinus*) used as controls (Zchori-Fein et al. 1995 and unpublished data). This confirmed the association of *Wolbachia* with thelytoky in *Aphytis*. Similarly, a fragment of the correct size was obtained from *E. formosa*, the other thelytokous aphelinid thought to be infected with *Wolbachia* (Zchori-Fein et al. 1992). In contrast, no fragments were obtained from the uniparental European spruce sawfly, *Venturia canescens* or two other uniparental strains, Cape honeybees (*Apis mellifera* from South Africa) and *Andricus quercuscalifornicus* (another bee). In fact, no signals could be obtained from the later four uniparental hymenopteran species even when the more general *Wolbachia*-specific primers were used.

Combined with the evidence that antibiotic treatment induced the production of males, the signal of about 1000 bases gives a strong indication that *Wolbachia* is responsible for parthenogenetic reproduction in all three thelytokous strains of *Aphytis* tested as well as the *Encarsia*.

The sequences of the 16S rDNA of *Wolbachia* from the uniparental *Aphytis lingnanensis*, *A. diaspidis* (deposited in Gene Bank under X87406 and X87407), and *A. yanonensis* were almost identical across the regions sequenced, yet considerably different than

Cytological observations of *Nasonia* revealed that eggs produced in incompatible crosses are normally fertilized, but syngamy of maternal and paternal pronuclei is aborted, resulting in haploid embryos (Breeuwer and Werren 1990). In *Drosophila*, where haploid embryos can't develop, developmental defects in cleavage nuclei result in sterility (O'Neill and Karr 1990). In contrast, the cytogenetic mechanism by which diploidy is restored in *Wolbachia*-associated thelytokous *Trichogramma* was shown to be gamete duplication (Stouthamer and Kazmer 1994). How a virtually identical microorganism can have these two completely different mechanisms for chromosomes manipulation is still an open question. One possibility is that the 16S rDNA sequence analysis does not provide a true phylogeny for these rickettsia-like symbionts. This possibility has been at least partially excluded by Rousset et al. (1992), who obtained essentially the same phylogeny based on 23S rDNA analysis. A second explanation is that different *Wolbachia* strains contain some virus or plasmid which is responsible for the chromosomal alteration in the host. No evidence for viruses was found in our electron microscopic studies (Zchori-Fein et al. 1994), but we cannot rule out that possibility.

A closer examination of the relationships among the *Wolbachia* that cause thelytoky confirm the close relationship between those from *M. uniraptor* and *Aphytis* (lower clade, Figure 3). Given the very distant relationships of the two wasp genera (different insect families, with *Muscidifurax* in the Pteromalidae) and even their hosts and habitats (filth breeding fly pupae and scale insects on plants, respectively), it seems stunning that these two insect genera would share a very similar bacterium, and strongly suggests horizontal transfer. Assuming substitution rates similar to those estimated in other prokaryotes, the common ancestor of examined *Wolbachia* lived about 25-100 million years ago. This makes them much younger than any common ancestor of the hosts (>300 million years) (Moran and Baumann 1994). The investigation of horizontal transfer became another major thrust of our research effort.

How can the microorganisms cause thelytoky? Clues from antibiotic treatments

As noted above, males often appear only in the F₂ of antibiotic-treated uniparental *Aphytis*. However, no microorganisms can be seen in electron micrographs even of the F₁ of rifampicin-fed *A. lingnanensis* (Zchori-Fein et al. 1994). These results raise the question of correlation between the actual presence of *Wolbachia* and its influence on the host. *Wolbachia*-specific primers were used to test for the presence of the symbiont in the F₁ of rifampicin-fed females.

In the absence of hosts for oviposition, a strong PCR band for the *Wolbachia* 16S rDNA was detected in *A. lingnanensis* females even 7 days after antibiotic treatment (Zchori-Fein et al. 1995). A much weaker PCR product was obtained up to three days post-treatment when the wasps were permitted to oviposit. No detectable band was obtained in the daughters (F₁) of treated females, consistent with observations from electron microscopy.

Figure 3. A most parsimonious phylogenetic tree of parthenogenesis microorganisms based on 16S rDNA sequences. The tree was constructed using branch-and-bound method of PAUP, with *Ehrlichia platys* as an outgroup (A=*Aphytis*; M=*Muscidifurax*; T=*Trichogramma*; collection site abbreviations in parentheses are those of Stouthamer et al. [1993]).

Although *Wolbachia* were detectable in antibiotic-fed wasps that were allowed to lay eggs, that signal was much weaker than in wasps that were presumably forced to resorb their eggs. This difference in signals might best be explained by the presence of non-degraded DNA of dead *Wolbachia* in the ovaries of the wasps which were not allowed to oviposit, because the eggs when laid seem to produce daughters without *Wolbachia* (Zchori-Fein et al. 1995). However, the fact that at least in *A. lingnanensis* and *A. diaspidis* the F₁ offspring of antibiotic-treated females were mainly females suggests that thelytokous reproduction is induced by an unknown factor released by the symbiont.

How can the microorganisms cause thelytoky? Localisation of *Wolbachia* in ovaries and eggs

Summarizing the results of a great deal of transmission electron, confocal and two photon laser scanning microscopy, *Wolbachia* were found only in the gonads of females from the untreated uniparental lines, and mainly in nurse cells and eggs. Within the eggs, they were always found concentrated in the vicinity of the egg nuclei (similar to observations for *Wolbachia* involved in mating [cytoplasmic] incompatibilities [O'Neill and Karr 1990]), and usually on one side rather than evenly distributed around the nucleus. When two neighboring nuclei were observed, the symbionts were located between them (Figure 4). Some symbionts were found in close association with the nucleus, their membranes virtually touching that of the nucleus, and others were found closely crowded around the nuclear organizing center (Zchori-Fein 1995). This placement of the microorganisms would surely assist them in altering the function of chromosomes in their hosts.

Figure 4. *Wolbachia* in the vicinity of nuclei in eggs of uniparental *Aphytis lingnanensis*. (a) 0-2 h old egg, with two nuclei. The symbionts are concentrated between the two nuclei (X5,000). (b) Higher magnification of (a) (X6,600). n=nucleus; arrowheads point to *Wolbachia*.

How are *Wolbachia* transmitted between species? Tests of hosts

One of the most intriguing questions about *Wolbachia* is how it is acquired. Given the wide range of insects in which the *Wolbachia* have been found, including many where no effects had been previously noted (O'Neill et al. 1992), it seems possible that parasitic wasps acquire the *Wolbachia* through parasitism or feeding on other insects. For this reason, we used PCR to test large numbers of the hosts of *Aphytis* for the presence of the *Wolbachia* involved in thelytoky.

We tested all of the host insects used at both labs, including the seven armored scale insects, *Hemiberlesia lataniae*, *Aspidiotus nerii*, *Aonidiella aurantii*, *Parlatoria oleae* and *Diaspis echinocacti*, *Quadraspidotus perniciosus*, and a uniparental strain of *A. nerii*, plus two hosts of *Encarsia*, *Bemisia tabaci* (sweetpotato whitefly) and the greenhouse whitefly *Trialeurodes vaporariorum*. In addition we looked for *Wolbachia* among species that live or feed on the cactus scale *Diaspis echinocacti*. Cactus pads were collected from campuses of the Faculty of Agriculture and the Weizmann Institute, Rehovot, and from Bet-Elazari, near Rehovot, and placed in dark emergence cages. The arthropods that emerged were *Marietta javensis*, *Habrolepis* sp., *Rhizobius* sp., *Hemisarcophaga coccophagus*, *Aphytis* sp. and Psocoptera. Although we obtained the occasional and tantalizing positive band from some experiments, none of the results were repeatable, and we were forced to conclude that these hosts and associated insects were not a source of the same *Wolbachia* as found in *Aphytis*.

Proof of the causative agent: Culturing *Wolbachia*.

Due to their extremely fastidious nature, it has not been possible to isolate *Wolbachia* and grow them in pure culture or to convert an arrhenotokous strain to thelytoky through infection. With help from the Insect Biocontrol Lab, USDA, Beltsville, Maryland, we tried extensively to culture *Wolbachia* from the *Drosophila simulans* Riverside strain (a strain known to carry *Wolbachia*, O'Neill and Karr 1990) and *Aphytis yanonensis* by inoculating insect ovaries and homogenizations (filtered through 0.45µm Millex filters) into 12 different Lepidopteran, Coleopteran and Hymenopteran insect cell lines (Hackett et al. 1986). No *Wolbachia*-like organisms were detected in the cells of samples inoculated with insect ovaries by using fluorescent stain (Hoechst 33258) after three weeks.

Proof of the causative agent: Injection experiments

It has been possible to transfer cytoplasmic mating incompatibility between strains of *Nasonia* (Williams et al. 1993); although these authors did not identify the causative agent, other studies indicate that *Wolbachia* might be responsible (Breeuwer et al. 1992). It has also been possible to transfer the *Wolbachia* responsible for mating incompatibilities of *Drosophila* between strains and species (Boyle et al. 1993, Braig et

al. 1994). Given the small size of *Aphytis*, and the apparently close relationship of the *Aphytis* from *Wolbachia* to that from *Muscidifurax uniraptor*, we attempted injections into its sibling species *Muscidifurax raptor*. To give ourselves the maximum chance of a result, we used preparations from *A. yanonensis*, *Encarsia formosa*, *Muscidifurax uniraptor*, and *Drosophila simulans*, Riverside strain. Uninfected *Drosophila* were also used as controls.

Rather than try to inject directly into wasp pupae (where we had high control mortality) or their eggs (which had proven extremely difficult) we injected into the abdomens of adult females. This seemed reasonable since the *Wolbachia* appeared concentrated around the female reproductive tissues. Parasitized house fly pupae were held in individual gel capsules to isolate individuals so unmated female wasps would remain virgins. An electronic micro-injection apparatus with a joy stick for added control was used for the injections. Glass needles were pulled and then filled with *Wolbachia* preparations or control solutions. The needle was inserted between the 4th and 5th or 5th and 6th segments at a diagonal direction aiming for the nurse cells of the female. The solution was injected into the wasp by syringe.

For injections from *Drosophila simulans*, ovaries of the flies were dissected into a petri dish of TES Ringer solution. The ovaries were then macerated manually with a grinding rod and micro centrifuged for a few seconds to separate the larger particles from the supernatant, which was used in injecting the female wasp. A total of 99 wasps were injected, 50 with solutions from infected flies and 49 with solutions from uninfected flies. Of those, 41 and 49 wasps, respectively, survived injection.

When injected with *Encarsia* and *Aphytis* preparations, the entire wasp pupa was used in the preparation at concentrations of 2 pupae of *Encarsia* /50 μ l of TES ringer and about 12-25 individuals of *Aphytis* /50 μ l TES ringer. The samples were then homogenized with a mechanical grinder, centrifuged and then the supernatant injected. A total of 53 mated female wasps were injected with *Encarsia*, with 48 wasps injected with TES ringer solution as controls. All control-injected wasps survived. Only 8 of 53 *Encarsia* -injected wasps survived and then for only 3 days, suggesting that there was something toxic in *Encarsia* preparations. A total of 33 mated females were injected with *Aphytis* and 39 injected with ringer solution.

Hundreds of wasps were reared in individual female lines from these injected females, with no differences observed in the sex ratios of the offspring or mating compatibilities over three generations.

Description of Cooperation

Through frequent communication via e-mail and FAX, both sides kept updated on the other's progress and developed a division of research emphases that was appropriate to the skills and interests of each side. The visit of Roush to Israel in August 1993 and Zchori-Fein to New York in August and September of 1994 was very important and effective for sharing skills and ideas. During her visit to Cornell University, Zchori-Fein was able to use a state-of-the-art experimental two photon laser scanning microscope in the Laboratory of Professor Watt

W. Webb in the Department of Applied Physics at Cornell for examination of *Wolbachia*. If only the organisms studied had been so cooperative!

Evaluation of Research Achievement

Consistent with the original research objectives, we documented that *Wolbachia* were responsible for thelytoky in *Aphytis* and investigated the causes of the thelytoky at the cellular level. We provided the first strong evidence for the horizontal transfer of thelytoky-inducing *Wolbachia* (see Figure 3). We described in detail the localization of *Wolbachia* within the wasp, and showed that the *Wolbachia* were found only in the reproductive tissues of the females and mainly in nurse cells and eggs. We also provided circumstantial evidence from microscopic and PCR studies of antibiotic treated females and their cured daughters that thelytokous reproduction is induced by an unknown factor released by the *Wolbachia*, rather than the physical presence of the *Wolbachia* itself. One student, Einat Zchori-Fein, completed a PhD, and another, Yuval Gottlieb, has completed much of the research for a masters degree. Finally, we showed that the uniparental line previously called *Aphytis lingnanensis* should be considered a new species and assigned a new name.

Conclusions

The exact means by which *Wolbachia* is able to cause thelytoky and can be transmitted from one species to the next remains a mystery. On the basis of our injection work and the survey of hosts, plus the observation that most *Aphytis* are still biparental, it is tempting to speculate that acquiring a *Wolbachia* infection is a rare event.

Because uniparental forms of parasitoids do not require mates, it has been argued that thelytokous strains should be sought out and used in biological control to insure rapid establishment (Aeschlimann 1990). It has now become clear, however, that many strains are thelytokous because of *Wolbachia*, which could restrict fitness and genetic recombination and therefore might be considered a pathogen. While we were not able to directly test whether *Wolbachia* cause fitness costs to the wasps that carry them, other work suggests that they do. Further, we did find that there is evidence for horizontal transfer of *Wolbachia*, which suggests that the deliberate importation of thelytokous forms not only runs the risk of loading fitness costs on the specific biocontrol effort, there is also the chance of transmission of these costs to other agents. Even if transmission is a rare event, the chances may be high once the parasites reach large population sizes. For these reasons, it seems prudent to avoid release of thelytokous forms, especially where arrhenotokous forms are available.

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al. 1994). Given the small size of *Aphytis*, and the apparently close relationship of the *Aphytis* from *Wolbachia* to that from *Muscidifurax uniraptor*, we attempted injections into its sibling species *Muscidifurax raptor*. To give ourselves the maximum chance of a result, we used preparations from *A. yanonensis*, *Encarsia formosa*, *Muscidifurax uniraptor*, and *Drosophila simulans*, Riverside strain. Uninfected *Drosophila* were also used as controls.

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List of Publications From This Research

- Argov, Y., E. Zchori-Fein and D. Rosen. Biosystematic studies in the *Aphytis lingnanensis* complex. Israel J. Entomol. (in press).
- Zchori-Fein, E. 1995. Association between parasitic wasps of the genus *Aphytis* (Hymenoptera: Aphelinidae) and parthenogenesis-inducing symbionts. PhD thesis, Hebrew University of Jerusalem.
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Patents: None