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Parasitic Hymenoptera: Their Mode of Action
and Utilization for Improvement of Biological
Control**

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Parthenogenesis-Inducing Microorganisms in Parasitic Hymenoptera: Their Mode of Action and Utilization for Improvement of Biological Control Agents

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Abstract

Wolbachia are intracellular bacteria known to cause reproductive and sex ratio disorders in many insects. In various parasitic Hymenoptera, *Wolbachia* induce thelytokous reproduction. The overall goal of this research was the improvement of biological control agents by reversion of their mode of reproduction. This was attempted from two directions: 1) studying the effect of naturally occurring *Wolbachia* on the thelytokous species *Muscidifurax uniraptor* and 2) trying to transmit thelytoky-inducing *Wolbachia* to *Nasonia vitripennis*. In *M. uniraptor*, gamete duplication was found to be the mode of diploidy restoration and *Wolbachia* density had a strong effect on sex ratio but not on host fitness. Studies on the natural horizontal transmission of *Wolbachia* between *Nasonia* wasps and their *Protocalliphora* hosts using the *Wolbachia* Outer Surface Protein (WOSP) gene revealed that (a) two *Nasonia* species (*N. giraulti* and *N. longicornis*) possess closely related strains of B-group *Wolbachia*, but *N. vitripennis* apparently acquired B *Wolbachia* by horizontal transmission from an unknown source, (b) *Nasonia* and its *Protocalliphora* host have similar *Wolbachia*, and (c) the *Protocalliphora* *Wolbachia* WOSP gene is a recombinant between the one found in *N. giraulti/longicornis* and *N. vitripennis*. Results show parasitoid-host insect transmission of *Wolbachia* and recombination among *Wolbachia* strains. Results from gynandromorph studies suggest a novel mechanism of sex determination in *Nasonia*.

II. Objectives of the original proposal

Microorganisms that cause female parthenogenesis have been discovered in several wasp species, and have been implicated in other insect groups (Stouthamer, 1997 for review). Antibiotic curing of infected strains causes them to revert to sexuality (Stouthamer *et al.*, 1990; Zchori-Fein *et al.*, 1992), and bacteria can be visualized in the eggs of parthenogenetic strains using standard methods (Stouthamer and Werren, 1993; Zchori-Fein *et al.*, 1998). This system has potentially broad implications for both applied and basic agricultural research. Parthenogenetic microorganisms may provide a mechanism for manipulating reproduction in a wide range of organisms, by introducing them into desirable species. In addition, studies of their mechanisms of action could provide basic and useful insights into early developmental processes in insects and other organisms.

Sequencing of 16S rDNA and protein coding genes (Stouthamer *et al.*, 1993; Werren *et al.*, 1995; Zchori-Fein *et al.*, 1995), shows that parthenogenesis microorganisms (PMs) present in parasitoid wasps are related to bacteria that are known to cause various reproductive and sex-ratio disorders in many insects and other arthropods (Werren 1997 for review). All of these symbionts compose a group of bacteria in the genus *Wolbachia* (Werren 1997).

When this research project was begun, the cytogenetic and biochemical mechanisms by which *Wolbachia* induce parthenogenesis were poorly understood. One cytogenetic study indicates that parthenogenesis bacteria in the parasitic wasp *Trichogramma* prevent segregation of chromosomes in the first mitotic division, thus restoring diploidy (Stouthamer and Kazmer, 1994). However, details of how the first mitosis is disrupted are not known. In addition, despite the diversity and probable polyphylectic origin of parthenogenesis-inducing *Wolbachia*, the cytogenetic mechanisms employed by these microorganisms have not been extensively investigated in species other than *Trichogramma*.

Although transfer of cytoplasmic incompatibility bacteria between species has been accomplished experimentally by microinjection (Summarized in Bourtzis and Braig, 1999), this had not yet been achieved with parthenogenesis bacteria when the project was planned. If parthenogenesis bacteria express their phenotype in novel hosts, then their transfer to new species could have broad implications for agricultural research.

The general research goal was the improvement of biological control agents by causing them to revert from a biparental to a uniparental mode of reproduction. To attain this overall objective, the following specific goals were set:

1) Clarification of the Cytogenetic Mechanism of *Wolbachia*-Induced Parthenogenesis.

1a. By looking at two A group *Wolbachia* systems (*Aphytis* and *Muscidifurax*) and two B group *Wolbachia* systems (*Encarsia* and *Leptopilina*) we hoped to determine whether different PM *Wolbachia* have similar or different cytogenetic mechanisms for inducing parthenogenesis.

1b. We hoped to determine whether PM *Wolbachia* disrupt or cause abnormal mitotic spindle formation, or whether other mechanisms are involved. If PM *Wolbachia* were found to prevent segregation of the chromosomes, we planned to address the question of how the spindle apparatus is disrupted, and whether later mitotic spindles are also abnormal.

1c. Manipulations by antibiotic treatments provided 1) a tool for determining the correlation between the segregation of chromosomes and the density of associated *Wolbachia*, and 2) a test for phenotype expression after elimination of *Wolbachia* in eggs.

2) Transfer of Parthenogenesis *Wolbachia* by Microinjection.

These studies were designed to determine whether PM *Wolbachia* can be transferred to the biparental parasitic wasp *Nasonia vitripennis* by microinjection, and to examine the phenotype they produce in that host. If uniparental reproduction had been successfully induced in that way, we planned to compare the fitness of the two lines.

Changes in Direction

Due to difficulties in rearing *Aphytis* and *Leptopilina*, we decided to focus our attentions on cytological studies of *Muscidifurax uniraptor*. This possibility was anticipated in the original proposal, where flexibility in study organisms was mentioned. Because our extensive efforts to transfer *Wolbachia* by microinjections have repeatedly failed, we investigated the related subjects of natural horizontal transmission of *Wolbachia* and parthenogenesis induction in *Nasonia*.

III. Methodology, Results and Discussion

A. Cytogenetic Mechanism of *Wolbachia*-Induced Parthenogenesis

Introduction:

Species of the genus *Muscidifurax* (Hymenoptera: Pteromalidae) are pupal parasitoids of synanthropic filth-breeding Diptera, and are among the principal natural enemies of the housefly (*Musca domestica*) and the stable fly (*Stomoxys calcitrans*). As discussed above,

Wolbachia is the cause of thelytoky in *M. uniraptor* (Stouthamer *et al.* 1993). Although over 23 species of thelytokous parasitic Hymenoptera are known to harbor *Wolbachia* (Stouthamer, 1997), the cytological mechanism of diploidy restoration has been described in only two cases: *Trichogramma* spp, and *Diplolepis rosea*, (Stouthamer and Kazmer, 1994, Stille and Davring, 1980). Since *Wolbachia* present in *Trichogramma* belong to the B group, and those in *Muscidifurax* to the A group, it was of interest to look at the mechanism of diploidy restoration in the latter. The fact that *M. uniraptor* is an ectoparasite, from which it is relatively easy to collect eggs, also made it a good candidate for studying the cytogenetic mechanism of diploidy restoration.

Experiments were conducted in order to study the mode and mechanism of diploidy restoration.

Strains

The uniparental species *M. uniraptor* was provided by J. Werren. The biparental species *M. raptorellus* was obtained from C. Geden (USDA, Gainesville, FL). The two *Muscidifurax* species were reared in the laboratory on *Musca domestica* pupae in a rearing chamber under standard conditions of $25\pm 2^{\circ}\text{C}$, and 50% RH.

A1. Mode of Diploidy Restoration

Material and Methods

0-2h-old eggs were collected from fresh pupae with a fine brush into a "basket" soaked in PBSx1, which was set on ice. Eggs were dechorionated by soaking the basket first in 50% commercial bleach for 10 minutes, and then for 5 minutes in a 0.7% NaCl/ 0.5 Triton-100 solution for egg recovery. The basket was then dropped into a scintillation vial filled with 3ml heptane (Scharlau)/ 3ml methanol (Frutarom), shaken to remove the eggs, and removed from the vial. After 10 minutes of fixation, all the dechorionated eggs sank to the methanol layer. The removal of the upper heptane layer together with all the chorionated eggs at the interface was followed by two methanol washes. The eggs were then transferred to a 1.5ml tube containing methanol, and refrigerated till staining.

Eggs were rehydrated through a methanol series, soaked in $1\mu\text{g/ml}$ DAPI (Sigma) for a few minutes, transferred to a microscope slide to which a drop of 80% glycerol/TBSTx1 was added, and then covered with a cover slip and sealed with clear nail polish. The slides were

carefully examined under a confocal microscope. Z-series and the amount of light (pixels/area) of each image were analyzed with IPLab software (Vaytek) tools.

Results and discussion:

In the normal course of events of haplo-diploid sex determination, the first mitosis in an unfertilized egg produces two haploid nuclei. Survey of the first mitosis in more than 150 unfertilized eggs of *M. uniraptor* revealed the scenario described in Figure 1: Meiosis is normal, and a diploid egg is seen undergoing reduction division, where the pronucleus separates from the polar nuclei (a-c). The first nucleus undergoes normal division until it reaches the anaphase stage. The sister chromatids are arranged on the metaphase plate (d) and then begin to migrate toward the poles (e,f). In the next observation the chromatids seem to separate but fail to enter telophase, and do not produce two nuclei (g). The two immature nuclei combine (h) to create one diploid nucleus that undergoes prophase of the second mitotic division (i). The two daughter nuclei will continue to divide into two diploid nuclei (j), and eventually create a cyncitial diploid blastoderm.

Although the *Wolbachia* strain from *Trichogramma* (B group) is different from that found in *M. uniraptor* (A group), they share the same mechanism of diploidy restoration.

Fig. 1: Course of events in the first mitotic division in *Wolbachia*-infected, unfertilized eggs of *M. uniraptor* (see details in text).

A2. Cytology of Diploidy Restoration

Material and Methods

Egg collection and fixation

0-2h-old eggs were collected from fresh pupae with a fine brush into a “basket” soaked in PBSx1, which was set on ice. Eggs were dechorionated by first soaking the basket in 50% commercial bleach for 10 minutes, and then for 5 minutes in a 0.7% NaCl/ 0.5 Triton-100 solution for egg recovery. Preliminary tests indicated difficulties in inserting antibodies into eggs (probably due to resistance of the vitelline membrane); we therefore tried several methods of devitellinization and fixation in order to make the eggs more permeable for antibody penetration. 1) Formaldehyde/paraformaldehyde (Sigma): Eggs were fixed in a 3.7% formaldehyde solution or 4% paraformaldehyde powder in TBS for 15min or overnight. The same volume of heptane was added and shaken for 5min, and the formaldehyde layer was removed. The same volume of cold methanol was added, and was washed three times with methanol. 2) Heptane/ methanol/ acetone: Eggs were soaked in heptane for 3min, then cold methanol was added and shaken for 5min. A drop of ddH₂O was added to burst the vitelline membrane, so it could be removed with a needle (the water, however, caused difficulty in viewing the eggs). The eggs were placed in acetone for 5min at -20°C. 3) Freeze fraction: Eggs were placed in a drop of PBS on a microscope slide and covered with cover slip. The slides were immersed in liquid nitrogen for a few seconds, and the cover slips were removed with a razor blade. Slides were immersed in methanol in a staining jar, and then in acetone and 0.5% acetic acid/ 1% triton X-100 in PBS for 10min in -20°C. The slides were washed three times in methanol and antibodies were applied directly to the slides. 4) Insoluble enzyme (Sigma): Eggs were soaked with protease beads for various lengths of time on a rotator. They were then washed three times in TBST, and the beads removed. The standard methanol/ heptane fixation was applied after this treatment. 5) Proteinase K (Sigma): Eggs were soaked with various concentrations of proteinase K (ranging from 100µg/ml to 1µg/ml), then washed with 0.05M EDTA to stop enzymatic activity, followed by three TBST washes. The standard methanol/ heptane fixation was applied following this treatment. 6) Mechanical breaking of the vitelline membrane: Eggs were placed on two-sided tape on a microscope slide. Using fine needles, attempts were made to peel off the vitelline membrane. The eggs proved too fragile for this method.

Antibody staining:

Fixed eggs were rehydrated in a serial methanol/ TBST solution, and were incubated with 1% BSA as a blocking solution before antibody treatment.

We tried several different primary antibodies for staining. These included mouse anti *Wolbachia* Woll- E11 (supplied by Dr. T. Karr, The University of Chicago); monoclonal rat anti-tubulin IgG (Serotec); monoclonal mouse anti β -tubulin IgG (Boehringer-Mannheim); monoclonal mouse anti-*Drosophila* lamin; and polyclonal rabbit anti *Drosophila* lamin (provided by Dr. Paul Fisher, State University of New York, Stony Brook). The antibodies were used at both recommended and modified concentrations, and were applied for 1h at room temperature or overnight at 4°C for staining optimization. The primary antibodies were washed three times in TBST for 5min, followed by a fourth wash of 30min. The appropriate secondary fluorescence conjugated antibodies were used for labeling: Texas red donkey anti-mouse IgG; LRSC donkey anti rat IgG; LRSC goat anti rabbit IgG (Jackson). The antibodies were use at both recommended and modified concentrations, and were applied for 1hour at room temperature. The secondary antibodies were washed three times in TBST for 5min, followed by a fourth wash of 30min. The stained eggs were soaked in DAPI for a few minutes, transferred onto a microscope slide to which a drop of 80% glycerol/TBSTx1 was added, covered with a cover slip, and sealed with clear nail polish. Sealed slides were viewed with the appropriate fluorescence filter under a Ziess Axioplan II microscope.

Results and discussion

Although the proteinase K method enabled us to rupture the vitelline membrane in a few eggs, the antibodies did not yield sufficient staining. Of all the antibodies tested, anti-*Wolbachia* and anti-tubulin worked randomly, thus giving differential staining from which we could not draw any significant conclusions.

The vitelline membrane seems that the major obstacle preventing the insertion of a large molecule like an antibody into *M. uniraptor* eggs is. Other studies which showed antibody staining for parasitic wasps (e.g. Grbic and Strand, 1998) used the proteinase K method successfully. Optimization of this method might yield the desired results. Microinjection of antibodies is also an option, but requires special equipment as well as the development of new methods for optimizing penetration of fixed eggs. The detection, in some instances, of a weak signal from anti-*Wolbachia* and anti-tubulin antibodies, but not from the anti lamin antibody, may indicate that the latter is *Drosophila*-specific, and thus can not identify nuclear components of *M. uniraptor*.

B. Influence of *Wolbachia* Density

B1. *Wolbachia* Density and Thelytoky in *Muscidifurax uniraptor*

Introduction

Induction of cytoplasmic incompatibility by *Wolbachia* has been found to depend not only on the presence or absence of the symbiont, but on its density within the host, as well (Breeuwer and Werren, 1993). The following experiments were aimed at studying the effect of different *Wolbachia* densities on the sex ratio of *M. uniraptor*.

Methods

Antibiotic treatments

A stock concentration of 100mg rifampicin (Sigma) in 1 ml honey was prepared and diluted to concentrations of 0.1, 1.0 and 10 mg/ml. Pure honey with food coloring was fed to controls. 0-24h-old *M. uniraptor* females were starved overnight and then randomly assigned to the various treatments. The wasps were fed with a given antibiotic concentration for 24h as described by Zchori-Fein *et al.* (1995).

The effect of the antibiotic rifampicin on *Wolbachia* density was tested in the following way:

Egg collection and fixation

0-2h-old eggs were collected from fresh pupae with a fine brush into a "basket" soaked in PBSx1, which was set on ice. Eggs were dechorionated by soaking the basket first in 50% commercial bleach for 10 minutes, and then for 5 minutes in a 0.7% NaCl/ 0.5 Triton-100 solution for egg recovery. The basket was then dropped into a scintillation vial filled with 3ml heptane (Scharlau)/ 3ml methanol (Frutarom), shaken to remove the eggs, and removed from the vial. After 10 minutes of fixation, all the dechorionated eggs sank to the methanol layer. The removal of the upper heptane layer together with all the chorionated eggs at the interface was followed by two methanol washes. The eggs were then transferred to a 1.5 ml tube containing methanol, and refrigerated until staining.

Egg staining and image analysis

Eggs were rehydrated through a methanol series, soaked in 1µg/ml DAPI (Sigma) for a few minutes and transferred to a microscope slide to which a drop of 80% glycerol/TBSTx1 was added. They were then covered with a cover slip and sealed with clear nail polish. The

slides were carefully examined under a confocal microscope. Z-series and the amount of light (pixels/area) of each image were analyzed with IPLab software (Vaytek) tools. The slides were examined under a Zeiss Axioplan II epi-fluorescence microscope equipped to capture z-series image files using a computer controlled z-stepper motor and a 12-bit research grade CCD camera (Princeton Instruments). Image files were processed with Power Microtome digital deconvolution software (Vaytek). Processed z-series were analyzed with VoxBlast software (Vaytek) designed to display 3-dimensional image sets. The amount of light (pixels/area) of each image was measured with the IPLab software tools, and gave an indication of the quantity of DNA in the egg. It was thus possible to determine the ploidy status of the figures.

Results:

***Wolbachia* density.** An increase in antibiotic dose resulted in descending estimated densities of *Wolbachia* in *M. uniraptor* eggs. A significant positive relationship was detected between antibiotic dose and the sex ratio of offspring produced by the treated females (Fig. 2). The offspring sex ratio was also strongly affected by the post-treatment time (Fig. 3).

Fig. 2. The total sex ratio produced by females treated with various antibiotic doses.

Fig. 3. The sex ratio produced by females treated with various antibiotic doses as a function of days post treatment. (sex-ratio was calculated as males/males+females).

B2. *Wolbachia* density and fitness of *Muscidifurax uniraptor*

Introduction

The effect of *Wolbachia* on the fitness of their arthropod hosts has been studied in only a few cases, with contradicting results (e.g. Zchori-Fein *et al.*, 1992; Stouthamer and Luck, 1993; Wade and Chang, 1995; Poinot and Mercot, 1997). In all reported cases, the influence of the symbiont, whether positive or negative, was relatively minor.

The most effective way to determine the fitness effect of a symbiont is to compare infected and uninfected hosts with identical genetic backgrounds. In the case of species for which uniparental reproduction is fixed in the population, and uninfected lines cannot be established, it is possible to measure the influence of *Wolbachia* on several life history traits of its host by comparing antibiotic- vs. honey-fed females (Stouthamer, 1997). Thus far, all the experiments conducted on parthenogenesis-inducing microorganisms correlate fitness traits with one dose of antibiotics that induces the production of males. Studies on cytoplasmic incompatibility-inducing symbionts have shown, however, that the influence of *Wolbachia* depends on its density within the host rather than simply on its presence or absence in the host (Breeuwer and Werren, 1993). The following experiments were aimed at studying the effect of various *Wolbachia* densities on selected fitness components of the host.

Methods

Following treatment with the various antibiotic concentrations, wasps were numbered and placed individually in 5ml glass tubes containing 20 hosts. Each wasp was transferred daily to a tube containing fresh hosts until she died. Upon emergence, the offspring (F1) of each mother were counted and sexed. For each treatment, the mother's longevity, number of offspring produced, sex ratio of offspring and F1 developmental time were recorded. To determine whether the antibiotic itself affects the fitness of the wasp, the same procedure was applied to the biparental species *M. raptor*, with the addition of 3 males during the antibiotic feeding.

Results

The density of the symbiont population had no significant effect on the host's longevity, fecundity or developmental time (Table 1).

Table 1. Fecundity and longevity of *M. uniraptor* females treated with various antibiotic doses

Antibiotic dose (mg/ml)	mean longevity ($\pm$ SE)	Mean ($\pm$ SE) offspring/female/day
0	14.70 ($\pm$ 2.61) ab	5.30 ($\pm$ 0.31) a
0.1	10.90 ($\pm$ 2.61) a	4.26 ($\pm$ 0.41) a
1	19.43 ($\pm$ 2.21) cb	4.87 ($\pm$ 0.20) b
10	12.55 ($\pm$ 2.49) ab	5.17 ($\pm$ 0.29) a
100	22.20 ($\pm$ 2.61) c	5.69 ($\pm$ 0.25) ab

C. Microinjection of *Wolbachia* into *Nasonia vitripennis*

Introduction

Objectives of this portion of the study were to transfer parthenogenesis-inducing *Wolbachia* into *Nasonia vitripennis* by microinjection, and to evaluate the effects of transferred bacteria on this novel haplodiploid host. These experiments were aimed at establishing the feasibility of moving these bacteria between host species for possible use in

biological control, and at determining whether parthenogenesis-inducing bacteria can cause parthenogenesis in parasitic wasps other than their standard hosts. Transfers of cytoplasmic incompatibility-inducing *Wolbachia*, which are known to be viable in this host, were used initially to establish methodologies.

Methods

Two approaches were undertaken for transfer of *Wolbachia* by microinjection (a) microinjection into pupae and (b) microinjection into eggs. *Wolbachia* used for injections originated from either eggs, pupae or adults.

Source for injections: By modifying procedures used in other laboratories, we have developed a rapid method for enriching pupal and adult hemolymph extracts for *Wolbachia*. These extracts are used for injection into either pupae or adults. Using a PCR-based assay, we can detect *Wolbachia* in serial dilutions of the extracts up to 10^{-4} per microliter. Viability of cells can only be ascertained by the bioassay, establishment of infections via microinjection.

Pupal injections: Recent reports suggest that pupal injection is a possible mechanisms for transmission of *Wolbachia* between species. This method has the advantage of being easier to accomplish and avoiding many of the difficulties of embryo survival associated with egg injections. The disadvantage is that successful establishment of the infection requires transmission to the germline. We do not currently know whether parthenogenesis-inducing bacteria are able to accomplish this. *Wolbachia*-enriched hemolymph extracts were injected into pupae. Surviving female pupae were then mated to uninfected males and given a constant supply of fresh hosts for up to two weeks, to allow bacteria to accumulate and increase potential for vertical transmission in subsequent oviposition. F1 and F2 progeny were tested for presence of *Wolbachia* using a PCR assay.

Egg injections: Undechorionated eggs were injected with *Wolbachia* under nujol oil or mineral oil. After 24 hours, embryos were transferred to parasitized hosts, using an eye-color mutant strain to distinguish injected from uninjected progeny. In some cases, vital dyes were used to determine the success of introducing the injection solution into eggs. Surviving injected progeny were serially transferred on hosts and assayed as describe above. Several methodological issues concerning survivability under different types of oil and details of transferring eggs to hosts had to be worked out.

Results

Research efforts were initially focused on pupal injections, as a possible rapid method for transfer of parthenogenesis *Wolbachia*. Focus later shifted to egg injections, due to our inability to obtain vertical transmission by the pupal method.

Pupal injections: Repeated attempts to establish *Wolbachia* infections by pupal injection have failed. *Wolbachia* were routinely detected in surviving adults following pupal injection, but transmission of the bacteria to the F1 progeny did not occur. Horizontal transfer with vertical transmission has been demonstrated in the isopod *Armadillidium vulgare*. It is possible that only certain strains of *Wolbachia* are capable of efficient germline incorporation following infection of somatic tissues. Alternatively, greater persistence may have led us to succeed with this method. We decided, however, to focus our attentions on egg injections, since this method greatly reduced the possible problems of germline incorporation of the transfected bacteria.

Egg injections: Currently, survival of injected eggs to the first larval instar stands at approximately 10-15%. Survival rates of eggs introduced into hosts are also approximately 10%. This gives an unacceptable survival rate of 1% from injection to adulthood. Previous experiments indicated that nujol oil was non-toxic to the *Nasonia* embryos. We have found, however, that toxic effects of oil on the embryos occur following hatching and introduction into the host. Our preliminary results indicate that this may be due to ingestion of the oil by the young larvae. We are now conducting experiments to see if rinsing of embryos prior to introduction into the host results in increased survival. Similarly, survival in the host can hinge on details of the introduction procedure. Our preliminary results (currently being confirmed) indicate greatly increased survival levels when the host is stung at the posterior end and when embryos are introduced into the mid section of the host, near the legs. Seemingly minor details in methodology can therefore greatly affect our yield in these experiments. A large-scale microinjection experiment will begin as soon as the survival criteria are optimized.

D. Natural horizontal transmission of *Wolbachia*

Introduction

The frequency of natural, interspecific transmission of *Wolbachia* is an important question for our study of transmission by microinjection. This question is particularly relevant to the parthenogenesis-inducing *Wolbachia* and CI *Wolbachia* used in these studies, and their close relatives. In previous studies, the most convincing phylogenetic evidence of specific horizontal transmission events is the evidence for such transfer between *Nasonia* wasps and their *Protocalliphora* fly hosts. These events are of additional interest because the B-group *Wolbachia* present in *N. vitripennis* are closely related to the parthenogenesis-inducing *Wolbachia* found in *Leptopilina clavipes* (a source bacterium which we planned to use). In addition, the A-group parthenogenesis *Wolbachia* of *Muscidifurax uniraptor* is a close relative of the A-group *Wolbachia* found in some *Nasonia*. We have therefore undertaken a study of natural horizontal transmission in these groups, to assess the frequency of horizontal transmission of these bacteria, and to determine whether such transfers have occurred recently.

Methods

Using the rapidly evolving *Wolbachia* Outer Surface Protein (WOSP), we amplified, cloned and sequenced WOSP genes from *N. giraulti*, *N. longicornis*, *N. vitripennis* and *Protocalliphora*, and compared sequences (Zhou *et al.*, 1998)

Results

Based upon sequence analysis of the WOSP gene of B-group *Wolbachia*, we conclude that (a) the two sibling species *Nasonia giraulti* and *Nasonia longicornis* share nearly identical B-group *Wolbachia* strains, whereas *N. vitripennis* carries a different B *Wolbachia*, apparently acquired by horizontal transmission from an unknown source, (b) *Nasonia* and its blowfly host *Protocalliphora* have exchanged *Wolbachia* and (c) the B-*Wolbachia* present in *Protocalliphora* is a recombinant between the B-*Wolbachia* found in *N. vitripennis* and *N. giraulti/longicornis*. The *N. vitripennis* variant is closely related to the parthenogenesis-inducing *Wolbachia* of *Leptopilina clavipes* and *L. australis*, based on *ftsZ* protein sequencing. These results are significant in showing recombination between *Wolbachia* and in showing exchange of *Wolbachia* between parasitoid wasps and their hosts.

E. Parthenogenesis Induction in *Nasonia*

As part of our efforts to uncover the genetic basis of parthenogenesis and the role of cytoplasmic elements such as *Wolbachia*, we have investigated the genetics of a newly discovered strain in *Nasonia*, which originally produced gynandromorphic (part male - part female) and phenotypically female offspring at about 4%. The following was determined:

1. Gynandromorphs and phenotypic females develop parthenogenetically from unfertilized eggs.
2. *Wolbachia* do not cause the parthenogenetic development of females or gynandromorphs
3. The frequency of female/gynandromorphy production can be selected up to 20%.
4. Phenotypic females are incapable of reproduction – ovaries are rudimentary and females fail to orient to and sting hosts.
5. Crossing experiments and a ten-generation introgression experiment established that the phenotype is due to an interaction between a heritable cytoplasmic factor (presumably a mitochondrial variant) and one or more nuclear genes. Both factors are necessary for gynandromorphy production.
6. Crosses using visible markers indicate that the nuclear gene acts zygotically, and that it is on linkage group 4.
7. Gynandromorph production is associated with elevated mortality within the lines. This mortality occurs during the embryonic stages prior to hatching.

These results, combined with those of previous studies, suggest a possible mechanism of sex determination in *Nasonia*. According to this model, sex is determined in *Nasonia* by feminizing influences of mitochondria, possible masculinizing maternal nuclear products, and feminizing inputs from the paternal chromosomes. In the gynandromorph strain a nuclear gene defective in production of masculinizing product and a mitochondrial variant producing more feminizing product combine to increase the probability of somatic female development. The observation that fully phenotypic females are not capable of reproduction suggests that germline sex determination may have a different basis in *Nasonia* than somatic sex determination.

F. Other Projects

Male-Killing *Rickettsia* in a Buprestid Beetle: In collaboration with Dr. Tim Mousseau (U. S. Carolina), we have established that highly female-biased sex ratios in *Brachys*

tessellatus, a major pest of oak, is caused by a male-killing rickettsia. This was confirmed by sequencing of the 16S rDNA from the microorganism, and experiments showing its association with the male-killing phenotype. A low level of thelytoky is also found in these beetles. We have not yet established whether this is caused by a microorganism, or has a nuclear genetic basis. *Wolbachia* induce paternal genome loss in incompatible crosses. In addition, a supernumerary chromosome also causes paternal genome loss (except for itself) prior to the first mitosis following fertilization. Collaboration has been established with Tim Karr (University of Chicago) to investigate the protein profiles of *Wolbachia*-free and *Wolbachia*-bearing sperm (and PSR-free and PSR-bearing sperm). *Nasonia* sperm is suited to these studies because of ease of purification. Only preliminary studies have so far been conducted, but these indicate that *Wolbachia*-induced and PSR-induced differences are present. Future progress on this issue will depend upon obtaining additional funding.

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V. Evaluation of Research Achievement

The original objectives of the research proposal were to study the influence of *Wolbachia* endosymbionts on their hosts. This was to be performed by 1) looking at the cytogenetic mechanism of *Wolbachia*-induced parthenogenesis and 2) transferring parthenogenesis *Wolbachia* by microinjections. We successfully determined the cytogenetic mechanism of diploidy restoration in *M. uniraptor* (paper in preparation). The use of antibiotic treatment facilitated experimenting with the influence of *Wolbachia* densities on both sex ratio and fitness of the host (paper submitted). However, in spite of immense efforts, we were unable to determine the cytogenetic mechanism by which the symbiont interferes

with normal embryonic development. Technical problems also prevented us from a satisfactory completion of the microinjection study. In spite of these difficulties, valuable progress was made in related areas, which will provide immediate benefits once the technical problems are overcome.

VI. Description of Cooperation

Throughout the entire research period, the Israeli and American groups maintained close collaboration. Yuval Gottlieb, a Ph.D. student, visited Dr. Werren's laboratory for two months to begin cytological studies of *M. uniraptor*, specifically to develop fixation procedures for eggs. She then went to Dr. Karr's lab (University of Chicago) to develop additional cytological techniques.

The useful collaborations with Dr. Tim Karr continued during the course of this project. Dr. Karr is an expert on confocal microscopy and fluorescent-antibody cytology of *Wolbachia*. He is cooperating on the fine-scale analysis of cytogenetic developments in *M. uniraptor*, and mechanisms of paternal genome loss in *Nasonia*.

The American and Israeli PI's corresponded regularly via e-mail and discussed the results and ways to solve problems. One of the papers prepared for publication is co-authored by the two teams.

VII. Conclusions

The overall goal of this research was the improvement of biological control agents by altering their mode of reproduction. This was attempted from two directions: 1) studying the effect of naturally occurring *Wolbachia* on the thelytokous species *Muscidifurax uniraptor* and 2) trying to transmit thelytoky-inducing *Wolbachia* to *Nasonia vitripennis*. In *M. uniraptor*, we found that *Wolbachia* density has a strong effect on the sex ratio but not on the fitness of the host. Since we could not establish an arrhenotokous line, it was impossible to determine whether such a line would have performed better as a natural enemy. However, the fact that microbe elimination had no effect on fitness parameters of the host, predicts no such improvement. Gamete duplication was found to be the mode of diploidy restoration in *M. uniraptor*, resulting in complete homozygosity. This knowledge may prove useful when introducing a beneficial gene through genetic engineering. In *Nasonia* our results, combined with those of previous studies, suggest a novel sex determination mechanism, in which sex is determined by feminizing influences of mitochondria, possible masculinizing maternal

nuclear products and feminizing inputs from the paternal chromosomes. A few side projects contribute to the accumulating knowledge of the importance of sex ratio distorters in biological systems.

VIII. List of Publications

One manuscript has been submitted and 4 are in preparation and near submission. These are listed below:

- Zchori-Fein, E., Y. Gottlieb and M. Coll. *Wolbachia* density and host fitness components in *Muscidifurax uniraptor* (Hymenoptera: Pteromalidae). (submitted to Journal of Invertebrate Pathology)
- Gottlieb, Y., E. Zchori-Fein, J. H. Werren and T. L. Karr. Diploidy restoration in *Muscidifurax uniraptor* (Hymenoptera: Pteromalidae). (for Developmental Biology)
- Werren, J.H. and J. Bartos. Phylogenetic evidence for horizontal transmission between parasitoid wasps and their dipteran hosts. (for Science)
- Ponce, A.R., V. Katju and J.H. Werren. Genetics of gynandromorph production in *Nasonia*: Implications for hymenopteran sex determination (for Heredity)
- Mousseau, T. and J.H. Werren. Evidence of a male-killing *Rickettsia* in a Leafmining Beetle (for Annals of the Entomological Society of America)

IX. Report on Patents

No patents have resulted from this research.