Chapter 4

The prevalence of *Wolbachia* in populations of *Otiorhynchus sulcatus*, and its impact on offspring production

Introduction

The black vine weevil, *Otiorhynchus sulcatus* (Fabricius), is a serious economic pest in greenhouse and nursery ornamentals and small fruits throughout the world. Of European origin, it has now spread to the United States, Canada, Australia, Japan, and Chile (Moorhouse et al., 1992). Parthenogenetic females emerge from soil in spring and commence oviposition after 4-9 weeks of preovipositional feeding (Labuschagne, 1999). Hatched larvae dig into soil and feed on plant roots, which may result in reduced vigor or plant death in young or container-grown plants. As with other parthenogenetic weevils (Suomalainen et al., 1987), *O. sulcatus* is known to reproduce without males (Smith, 1932), i.e. thelyotoky, where all adults are females capable of laying viable eggs without fertilization (Seiler, 1947). Despite previous studies on *O. sulcatus* ecology, the basic questions about its parthenogenetic reproduction remain to be investigated.

While in many cases thelyotoky is a genetic mechanism, it is often associated with the presence of endosymbiotic bacteria of the genus *Wolbachia*. *Wolbachia pipientis* has been found in numerous arthropod hosts including insects, mites, and crustaceans (Jeyaprakash & Hoy, 2000; Werren & Windsor, 2000). However, thelyotoky induced by *Wolbachia* has been observed mostly in parasitic wasps (Stouthamer et al., 1990; Zchori-Fein et al., 1992; Cook and Butcher, 1999) and was recently reported in predatory thrips (Arakaki et al., 2001) and phytophagous mites (Weeks & Breeuwer, 2001). Most of the known thelyotokous females produce male offspring after ingestion of antibiotics or exposure to high temperatures

(Stouthamer et al., 1990), which kills or suppresses the activity of *Wolbachia*. Other reproductive phenotypes associated with *Wolbachia* infection include cytoplasmic incompatibility, the feminization of genetic males and male killing (for review, Werren, 1997; Stouthamer et al., 1999; Weeks et al., 2002). Because *Wolbachia* are vertically transmitted via the cytoplasm of the eggs in a host population, these reproductive alterations can be interpreted as a strategy to increase the frequency of *Wolbachia* in the host populations by improving transmission through females (O'Neill et al., 1997; Werren, 1997; Weeks & Breeuwer, 2001). Thelyotokous reproduction results in 100% vertical transmission, without any production of males.

So far, taxon-specific surveys have detected *Wolbachia* in over 16 % of Neotropical insect species using polymerase chain reaction (PCR)-based diagnostics (Werren et al., 1995), however, an improved PCR method revealed that 76 % of arthropod species sampled were *Wolbachia*-positive (Jeyaprakash & Hoy, 2000). Although *Wolbachia* infection has been found in numerous coleopteran species (see Werren & O'Neill, 1997), there are only a few reports on species in the Curculionidae (Hsiao & Hsiao, 1985; Werren et al., 1995; Jeyaprakash & Hoy, 2000; McClanan et al., 2004), with no report in the genus *Otiorhynchus*. Furthermore, prevalence of *Wolbachia* among geographical populations of coleopteran species was reported only in a few coleopteran species (Kondo et al., 1999; Clark et al., 2001; Vega et al., 2002). Interestingly, despite its possible implication in thelyotokous reproduction, there is no report of *Wolbachia* infection among parthenogenetic weevils. Since *O. sulcatus* harbors an endosymbiotic *Wolbachia*, I hypothesized that *O. sulcatus* may require the presence of this bacterium during at least some stages of their life cycle. This system may provide a good model to investigate the potential effects of *Wolbachia* on thelyotoky in parthenogenetic weevils. This

is the first report of *Wolbachia* infection in *O. sulcatus*. The objectives were to examine the prevalence of *Wolbachia* in geographically separate *O. sulcatus* field populations in the United States and to determine the effects of antibiotics on offspring production of *Wolbachia*-infected parthenogenetic *O. sulcatus*.

Materials and Methods

Insect collection and DNA extraction

A total of 96 *O. sulcatus* was obtained from five different geographical locations in the United States, including Virginia (Riner, VA), Maryland (Dr. P. M. Shrewsbury; Baldwin, MD), Ohio (Dr. M. Reding and Dr. A. Herm; Wooster, OH), Connecticut (Dr. R. Cowles; Windsor, CT), and Oregon (Dr. J. Fisher; Corvallis, OR). Live or ethanol-preserved weevils were shipped to Virginia Tech and stored at $-80\ ^{o}C$ in TE buffer (Tri-HCl, EDTA) until DNA was extracted.

Total genomic DNA was extracted from each adult using the protocol of Ashburner (1989). Weevils were placed individually in microcentrifuge tubes (1.5 ml) and homogenized with Kontes pestles in 300 µl of homogenization buffer (10 mM Tris-HCl, 60 mM NaCl, 10 mM EDTA, and 5 % sucrose). After homogenization, 300 µl of lysis buffer (300mM Tri-HCl, 100mM EDTA, 0.625% SDS, and 5% sucrose) was added to each sample and the suspensions were incubated for 15 min at $70\ ^{o}C$. After cooling to room temperature, 90 µl of 8 M potassium acetate was added to each sample and the samples were kept on ice for 30 min. The sample supernatants were collected by centrifuging at 20800 g at $4\ ^{o}C$ for 10 min and were extracted using 1:1 phenol/chloroform. An additional extraction with chloroform was used to remove residual phenol from each of the supernatants. The supernatants were transferred to clean microcentrifuge tubes and aliquots of 100% ethanol were added to each tube to precipitate

nucleotides. DNA pellets were obtained by centrifuging at 20800 g at room temperature for 5 min. After washing in 70% ethanol, the dried pellets were suspended in 50 µl of TE buffer. RNA in each sample was degraded with the addition of 1.5 µl of RNase cocktail (Ambion, Austin, TX) and incubating for 1 hr at 37 ^{o}C. The samples were purified with Microcon Centrifugal filter devices (Millipore Corporation, Billerica, MA) and the integrity of the DNA samples was visually examined with electrophoresis. High quality DNA was used for PCR analyses: these samples were characterized by the presence a single, high molecular weight band, with minimal smearing, following electrophoresis. DNA concentration was quantified using a SmartSpec 3000 spectrophotometer (Bio-Rad laboratory, Hercules, CA).

PCR diagnosis for *Wolbachia* detection

The presence of *Wolbachia* in *O. sulcatus* was assessed by polymerase chain reaction (PCR) amplification using *Wolbachia wsp* gene-specific primers. *Wsp* primer pairs used in this study amplify 590-632 bp of the *Wolbachia* surface protein (*wsp*) gene (Zhou et al., 1998): 81F (5'-TGG TCCAATAAGTGATGAAGAAAC-3') and 691R (5'-AAAAATTAAACGCTACTCCA-3'). Each reaction was performed in a total volume of 20 µl containing 200 µM dNTPs (PE Applied Biosystems, Norwalk, CT), 300 nM *wsp* primers, 0.5 unit Ampli*Taq* DNA polymerase (PE Applied Biosystems, Norwalk, CT), 2 µl PCR buffer (PE Applied Biosystems, Norwalk, CT), 1.5 mM MgCl$_2$, and 1 µl of DNA template. The thermal cycling profile used in the GeneAmp PCR system 9700 (PE Applied Biosystems, Norwalk, CT) was: 94 ^{o}C for 10 min, followed by 35 cycles of 94 ^{o}C for 1 min, 55 ^{o}C for 1 min, and 72 ^{o}C for 1 min with a final extension at 72 ^{o}C for 7 min. Ten microliter of amplified product was electrophoresed through 1.5% agarose and stained with ethidium bromide to visualize amplified DNA fragments under

UV illumination. Band masses, relative to molecular weight standards, were analyzed using 1D Image Analysis Software (Kodak, Rochester, NY). Negative controls containing DNA-free water and positive controls containing known *Wolbachia*-positive samples were included with each set of experimental samples to monitor PCR conditions and contamination.

Cloning and sequencing

For cloning, 2µl of PCR product was directly ligated into TOPO TA plasmid vector (Invitrogen, Carlsbad, CA), which was then introduced into *E. coli* TOP10F' bacterial cells. Transformants were selected after growth on ampicillin-containing plates, inoculated into Luria-Bertani broth and incubated overnight at 37 °C with shaking. Plasmid DNA was isolated from bacterial cultures using a standard alkaline lysis procedure and resuspended in 50 µl of TE buffer. Contaminating RNA was degraded with the addition of 1.5 µl of RNase cocktail (Ambion, Austin, TX) and incubating for 1 hr at 37 °C. The samples were purified with Microcon Centrifugal filter devices (Millipore Corporation, Billerica, MA) prior to submitting the plasmids for DNA sequencing at the University of California Davis. Sense and antisense strands of each plasmid insert were sequenced to resolve ambiguities. One plasmid clone per weevil was sequenced.

Antibiotic treatments on preovipositing adults

Mature *O. sulcatus* larvae were collected from a nursery in Riner, Virginia. Larvae were maintained individually in moist potting soil in clear plastic rearing cups (30 ml) under 21 °C and a photoperiod of 16L: 8D. Teneral adults were collected daily from the containers and they were transferred individually to a Petri dish (10 cm diameter) containing moist filter paper and a

cotton square (5 cm x 5 cm). Weevils after emergence (1 week old) were used for the experiment. We used two antibiotics, tetracycline and gentamicin, which have the same mode of action but differ in their effectiveness against *Wolbachia*. Tetracycline provides an effective cure for *Wolbachia* infection in many insects but gentamicin is known not to influence *Wolbachia* infection (Stouthamer et al., 1990; Dobson & Rattanadechakul, 2001; Dedeine et al., 2001). Both antibiotics are prokaryotic protein synthesis inhibitors, and kill bacteria by binding to the 30S bacterial ribosome (Wade, 1984). Twenty weevils were randomly assigned to one of four treatments: (1) water-treated control (2) low concentration (0.25 ppm) of tetracycline hydrochloride (3) high concentration (2.5 ppm) of tetracycline hydrochloride (4) high concentration (2.5 ppm) of gentamicin sulfate. Rhododendron leaves dipped in the solution of each treatment was provided to weevils weekly during the 3-week treatment period to allow them constant access to fresh foliage and antibiotics. The treated foliage was provided only during the preovipositional feeding period (i.e. 3 weeks at 21 °C), which is required for reproductive maturation. After the treatment period, non-treated fresh foliage was fed to each weevil every week until 7 weeks after treatments.

To determine the effects of antibiotics on *O. sulcatus* reproduction, egg production, egg viability, and the proportion of eggs hatching were monitored as follows. Once oviposition began, the eggs were collected weekly and surface-sterilized with 0.5 % solution of benzalkonium chloride to prevent fungal infection. The viability of eggs was determined by counting the melanized eggs one week after collection (Montgomery & Nielsen, 1979; Maier, 1981) and egg hatch was measured by counting the number of hatched eggs two weeks after collection. To determine possible detrimental effects of antibiotics, I measured leaf consumption (mm^2), the duration of the preoviposition period (i.e. days to first egg-laying), and survival. The

total duration of this test was three weeks of treatment with antibiotics and seven weeks of observation post treatment. Females surviving at the end of experiment were tested for the presence of *Wolbachia* to confirm their infection status using real-time PCR.

Real-time PCR was conducted using the ABI PRISM 7700 Sequence Detection System (PE Applied Biosystems, Norwalk, CT). Extraction of DNA was conducted using the protocol of Ashburner (1989) described above. Each amplification reaction was performed in a total volume of 25 μl containing 300 nM *wsp* primers (Zhou et al., 1998), 200 μM SYBR Green dNTPs (Roche Diagnostics, Indianapolis, IN), 2 μl SYBR PCR buffer (Roche Diagnostics, Roche Diagnostics, Indianapolis, IN), 0.5 unit of Ampli*Taq* DNA polymerase (PE Applied Biosystems, Norwalk, CT), 1.5 mM MgCl$_2$, and 1 μl of DNA sample. The thermal cycling profile used was 94 ^{o}C for 10 min, followed by 35 cycles of 94 ^{o}C for 1 min, 55 ^{o}C for 1 min, and 72 ^{o}C for 1 min. SYBR green binds double-stranded DNA and emits a fluorescent signal upon excitation. With each PCR cycle, SYBR green fluorescence increases in direct proportion to the amount of PCR product in a reaction. Samples are assayed for the presence of this signal during each PCR cycle and the specificity of amplification is confirmed by melting curve analysis, using a thermal ramp of 50 ^{o}C to 100 ^{o}C in 10 min. Single melting peaks are generated by depicting the negative derivative of the fluorescence versus the derivative of the temperature (*-dF/dT*) over the course of gradual melting of the PCR product.

Analysis of variance (ANOVA) was used to determine the statistical difference among treatments in leaf feeding and duration of the preoviposition period (SAS Institute, 1995). Significant differences among means were determined by the Student-Newman-Keuls (SNK) test. Repeated measures ANOVA was used to determine the statistical difference in production of total eggs, viable eggs, and egg hatch rate among treatments. Percentage data were subjected to

arcsine transformation prior to statistical analysis. Kaplan-Meier survival analysis was used to compare weevil death rates among treatments.

Tetracycline treatment on ovipositing adults.

To confirm the effect of tetracycline treatment on offspring production by *O. sulcatus*, a second experiment was conducted on actively ovipositing females. Nursery-collected *O. sulcatus* adults were maintained under constant 21 °C and a photoperiod of 16L: 8D for 2 months. Twenty-two ovipositing adults were randomly assigned to two groups, water-treated control and tetracycline-treatment (2.5 ppm). During the 3-week treatment, weevils in the treatment group were provided weekly with a rhododendron leaf dipped in tetracycline hydrochloride solution (2.5 ppm) and those in the control groups were given a leaf dipped in water (Lawson et al., 2001). After treatment, weevils in both treatment groups were fed non-treated fresh rhododendron leaves weekly. Egg production and egg viability were measured weekly following the same protocol as described above. Repeated measures ANOVA was used to determine the statistical difference in production of total eggs and viable eggs (SAS Institute, 1995). Means were separated by the SNK test. Weevil death rates at the end of the experiment were compared using χ^2 test between the control and tetracycline treatments.

Results

PCR assay and sequencing

Ninety-six individuals representing five geographical locations were screened by PCR assay using *Wolbachia wsp* gene-specific primers. All weevils tested, originating from VA, MD, OH, CT, and OR, were positive for *Wolbachia* infection (Table 4.1), as evidenced by amplification of

a product of ~600 bp from each individual (Fig. 4.1). *Wsp* gene fragments amplified from 11 weevils collected from the following locations were submitted for DNA sequencing: VA (n = 6), OR (n =2), OH (n = 2), MD (n = 1), and CT (n = 1). All amplimers were 593-bp nucleotides (Fig. 4.2) and there was 99% identity among all sequences with the exception of *wsp* amplimers from one OH specimen and one OR specimen, which were 97% identical to VA, MD, and CT *wsp* amplimers. BlastN alignment (Altschul et al., 1990) searches with *wsp* sequence data as queries resulted in high alignment scores with *wsp* gene sequences from *Wolbachia* strains in supergroup B. Based on BlastN alignment scores, consensus *wsp* gene sequences for amplimers from weevils from VA, MD, OH, CT, and OR weevils were 99% identical to *wsp* encoded by a reference *w*Calt2 strain (accession number AY56421) from *Chelymorpha alternans* (Coleoptera: Chrysomelidae) (Keller et al., 2004). Sequences of *wsp* amplimers from 1 OH and 1 OR weevil were 99% identical to *wsp* encoded by a *w*Nag1 strain (accession number AB094368) from *Orius nagaii* (Hemiptera: Anthocoridae) (Tagami & Miura, unpublished data). Despite these small sequence differences among *wsp* amplimers, all *O. sulcatus* examined appear to be infected by *Wolbachia* sp. supergroup B.

Antibiotic treatment on preovipositing adults

During the 3-week exposure to 0.25 and 2.5 ppm of tetracycline and 2.5 ppm of gentamicin, no significant difference was found in the amount (mm^2) of leaf feeding of preovipositing adults (df = 3, 79; F = 1.35; P > 0.05) (Fig. 4.3). Time to reproductive maturation (i.e. days to oviposit the first egg) was not significantly different among treatments (df = 3, 73; F = 2.33; P > 0.05) (Fig. 4.4). During the entire experimental period, adult mortality was not influenced by treatments (df

= 3; χ^2 =1.25; P > 0.05, Kaplan-Meier analysis) (Fig. 4.5). Therefore, the treatment did not influence feeding, mortality, or reproductive maturation.

Repeated measures ANOVA revealed that the total egg production per female during 7-weeks post treatment was not influenced by the treatments (df = 6, 78; F = 1.91; P > 0.05). However, there was a significant time effect (df = 6; F = 8.89; P < 0.01) and a significant interaction between time and treatments (P < 0.01). Significant differences among treatments in total egg production were observed only at 1 and 3 weeks post treatment (P < 0.05) (Fig. 4.6A). The total numbers of viable eggs per weevil during the 7 week-post treatment period were 280.6 ± 25.3, 186.1 ± 31.0, 94.9 ± 24.5, and 318.9 ± 18.7 for control, 0.25 ppm of tetracycline, 2.5 ppm of tetracycline, and 2.5 ppm of gentamicin, respectively. Repeated measures ANOVA revealed that viable egg production during the weeks after treatment was significantly influenced by the treatment (df = 3; F = 17.14; P < 0.01) and time (df = 6; F = 20.51; P < 0.01) (Fig. 4.6B). There was also significant interaction between time and treatment (df = 18; F = 5.59; P < 0.01). Weevils exposed to 2.5 ppm of tetracycline did not produce viable eggs up to 3 weeks after treatment (WAT) and significant reduction was found up to 4 WAT compared with eggs produced by non-treated weevils (P < 0.05). However, viable egg production increased as time passed for the tetracycline-treated weevils. Although weevils fed on the low dosage (0.25 ppm) of tetracycline recovered viable egg production more quickly, no difference was found between the treatments in viable egg production 4 WAT. However, treatment with gentamicin (2.5 ppm) did not affect viable egg production compared with the non-treated control at any time measured (P > 0.05). Repeated measure ANOVA on egg hatch showed significant effects of treatment (df = 3, 49; F = 133.32; P < 0.01), time (df =6; F = 63.39; P < 0.01), and interaction between time and treatment (df = 18; F = 63.39; P < 0.01) (Fig. 4.6C). There was no significant difference in

egg hatch (%) between 2.5 ppm of gentamicin-treated weevils and non-treated weevils at any observation time (P > 0.05). In contrast, significant reduction in egg hatch was found at all observation times in weevils treated with 2.5 ppm of tetracycline. In tetracycline treatments, the recovery of egg hatch rate depended on the dosage and time post treatment. As reported by Montgomery & Nielsen (1979) and Maier (1981), only eggs whose chorion turned from white to brown hatched. Although not all the melanized eggs hatched, there was a strong correlation between numbers of melanized eggs and hatched eggs irrespective of treatments (r = 0.979; P < 0.01) (Fig. 4.7).

At 7 WAT, weevils were assayed for *Wolbachia* infection using a real-time assay for *wsp* sequence detection. Based on these data, the majority of weevils at 7 WAT appeared to be infected with *Wolbachia*. The percentage of weevils that tested positive for *Wolbachia* after the 7-week feeding period on non-treated foliage was 100, 100, 100, and 92.3% for control (n = 12), 2.5 ppm of gentamicin (n = 13), 0.25 ppm of tetracycline (n = 15), and 2.5 ppm of tetracycline (n = 11).

Antibiotic treatment on preovipositing adults

Repeated measures ANOVA revealed that there were significant effects of treatment (df = 1, 39; F = 19.74; P < 0.01), time (df = 7; F = 14.94; P < 0.01), and interaction between time and treatment (df = 7; F = 6.16; P < 0.01) in the total egg production throughout the periods of treatment and post treatment feeding (Fig. 4.8A). Viable egg production was also significantly influenced by treatment (df =1, 39; F = 62.26; P < 0.01), time (df = 7; F= 25.12; P < 0.01), and the interaction between time and treatment (df = 7; F = 14.32; P < 0.01) (Fig. 4.8B). Significant reduction in viable egg production, compared with control, was found at all observation times

beginning with the second week of tetracycline treatment (P < 0.05). Notably, weevils failed to produce viable eggs within 3 weeks of tetracycline treatment, and this complete loss of viability lasted up to 10 weeks post treatment, even when the weevils fed on non-treated leaves. However, viable egg production of tetracycline-treated weevils recovered to levels similar to untreated weevils 6 months after treatment with antibiotics ended. Survival rate of adults at 10 WAT was 68.2 and 63.6% for control and tetracycline-treated groups, respectively, showing no significant impact of tetracycline treatment on weevil survival (df = 1, 43; χ^2 = 0.101, P > 0.05).

Discussion

This is the first documentation of infection with *Wolbachia* not only in *O. sulcatus* but any parthenogenetic weevil. PCR assay in this study clearly demonstrated that *Wolbachia* is highly prevalent in *O. sulcatus* populations regardless of geographic origin. A 100% prevalence of *Wolbachia* infection suggests an obligatory symbiosis, which may result from co-adaptation and reciprocal dependence through long coevolution of a host and its symbiont (Yamamura, 1993). *Wsp* sequence data derived from amplimers from *O. sulcatus* indicate that these weevils are infected with *Wolbachia* in supergroup B. Werren et al. (1995) divided *Wolbachia* groups based on 13-16% divergence between the A- and B-*Wolbachia* groups using a phylogenetic analysis of *Wolbachia ftsZ* sequences. Zhou et al. (1998) proposed to subdivide the A-and B-*Wolbachia* groups into smaller groups, based on a sequence divergence of 2.5% of the *wsp* sequences. Phylogenetic analyses with more *wsp* sequences isolated from *O. sulcatus* populations may provide enough information to determine the geographic origin(s) of *O. sulcatus*, which was introduced from Europe.

Antibiotic treatment of *Wolbachia*-infected insects can cause two outcomes: toxic effects of antibiotics to the host and the curing effects of the antibiotics against the pathogen (Stouthamer & Mak, 2002). To demonstrate the possible toxicity of antibiotics, tetracycline (0.25 and 2.5 ppm) and gentamicin were used to treat preovipositional adults. Antibiotic treatment of preovipositional adults did not influence feeding, mortality, or the rate of reproductive maturation. However, tetracycline, which is known to remove or decrease *Wolbachia*, inhibited production of viable offspring. Gentamicin, which is known not to affect *Wolbachia,* had no effect on production of viable offspring. Tetracycline and gentamicin belong to the same antimicrobial class, and have the same target site. Given that two antibiotics differing only in their efficacy against *Wolbachia* were used to treat the infected weevils, the lack of egg hatch rate is more likely to result from the *Wolbachia*-curing effect than toxic or other pharmacological effects on the weevils.

Weevils treated with the low dosage (0.25 ppm) of tetracycline recovered earlier with regard to reproduction than those treated with the high dosage (2.5 ppm) of tetracycline. This dosage-dependent impact of tetracycline followed by recovery suggests that tetracycline may reduce the density of *Wolbachia* for a limited time post treatment but does not completely remove *Wolbachia* (Zchori-Fein et al., 2000; Yusuf & Turner, 2004). It has been reported that antibiotic treatments of *Wolbachia*-infected adults can fail to produce permanently aposymbiotic lines (Pintureau et al., 1993) and the influence of *Wolbachia* depends on its density within the host rather than being an all-or-none effect (Breeuwer & Werren, 1993; Clancy & Hoffmann, 1998; Hurst et al., 2000). For instance, Zchori-Fein et al. (2000) observed *Wolbachia* density in *Muscidifurax uniraptor* Kogan & Legner (Hymenoptera: Pteromalidae) recovered following treatments with low dosages of rifampicin.

The effects of antibiotic treatment are very subtle and complicated, and may be influenced by factors such as duration of treatment, the dosages of antibiotics, and time post treatment elapsed before symptom measurement. The real-time PCR results in this study also showed that *Wolbachia* was detected in weevils tested with tetracycline at 7 weeks post treatments, the time point when egg hatch recovered. Thus, there may be a correlation between the presence (or density) of *Wolbachia* and the recovery of egg hatch rate in tetracycline-treated weevils. The inhibition of embryogenesis following tetracycline treatment was reported in the filarial nematode *Brugia pahangi* that is also infected with *Wolbachia* (Bandi et al., 1999), but no reports of similar results from *Wolbachia*-infected insects have been reported. Dedeine et al. (2001) found that antibiotic treatment curing *Wolbachia* infection specifically inhibited oogenesis in the parasitoid wasp *Asobara tabida* Nees (Hymenoptera: Braconidae). In contrast, *Wolbachia* infection was found to have no detectable effect on the fecundity of various *Drosophila* species (Stouthamer et al, 1994; Giordano et al., 1995; Bourtzis et al., 1996).

Tetracycline treatment on ovipositing *O. sulcatus* adults drastically reduced offspring production, but had no harmful effect on adult survival. This result is consistent with that of the preovipositing adult treatment, except that the loss of egg viability lasted longer post treatments and reduction of total egg production was also observed. This difference could be due to either variation in tetracycline ingestion by adult females or to differential tetracycline penetration within eggs at different stages of maturation in female ovary (Dedeine et al., 2001). Further studies in this area should include cytological analysis of eggs and ovaries to measure the effect of different dosages of antibiotics on the density of *Wolbachia* at different observation times post treatment.

Thelyotoky induced by *Wolbachia* has been observed in more than 30 species but it has been restricted to only haplodiploid species such as parasitic wasps (Stouthamer, 1997; Cook *&* Butcher, 1999), mites (Weeks & Breeuwer, 2001), and thrips (Arakaki et al., 2001). The genetic mechanism of *Wolbachia*-induced parthenogenesis in the haplodiploid parasitic wasp *Trichogramma* is known to be gamete duplication, where doubling the chromosome constitution of unfertilized eggs restores diploidy (Stouthamer & Kazmer, 1994). Polyploidy is commonly observed in parthenogenetic weevils (Suomalainen et al., 1987) and *O. sulcatus* is triploid (Seiler, 1947). The triploidy of *O. sulcatus* could not be explained by the theory of gamete duplication. Further investigation of *Wolbachia* infection on the F-1 generation from treated weevils would further clarify the impact of *Wolbachia* on *O. sulcatus* reproduction.

There is a growing amount of information describing the influence of *Wolbachia* infection on host fitness. The data acquired in this study show that *Wolbachia* in supergroup B are highly prevalent in *O. sulcatus* populations and tetracycline specifically inhibits egg hatching without negative impacts on other physiological traits of adults. This result strongly supports the hypothesis that the endosymbiont *Wolbachia* is required for normal egg development.

Table 4.1. The prevalence of *Wolbachia* in *O. sulcatus* populations in the United States

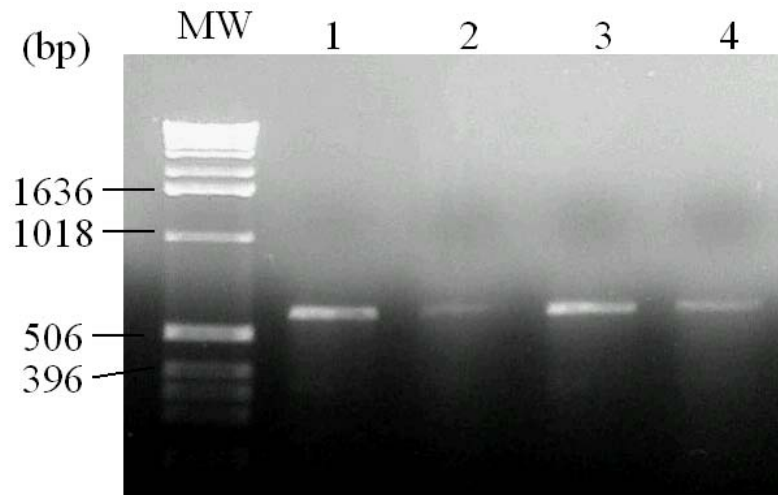| Source of weevil	Presence of *Wolbachia*	No. Tested	No. Positive
Blacksburg, VA	+	17	17
Windsor, CT	+	17	17
Wooster, OH	+	21	21
Baldwin, MD	+	14	14
Corvallis, OR	+	27	27

Fig. 4.1. Representative *wsp* gene amplified fragments (~ 600 bp) from *O. sulcatus* adults collected from VA (1), OR (2), CT (3), and OH (4). MW = molecular weight DNA standard in base-pairs (bp).

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        G  P  I  S  D  E  E  T  S  Y  Y  V  R  L  Q  Y  N  G  E  I
  2   ggtccaataagtgatgaagaaactagctactacgttcgtttacaatacaacggtgaaatt   61
        L  P  L  Y  T  K  V  D  G  I  T  N  A  T  G  K  E  K  D  S
 62   ttacctctttacacaaaagttgatggtattacaaatgcaacaggtaaagaaaaggatagt   121
        P  L  T  R  S  F  I  A  G  G  A  F  G  Y  K  M  D  D  I
122   cccttaacaagatctttttatagctggtggtggtgcatttggctataaaatggacgacatt   181
        R  V  D  V  E  G  L  Y  S  Q  L  A  K  D  A  T  V  V  S  D
182   agagttgatgttgaagggctttactcacaattggctaaagatgcaactgtagtatctgat   241
        N  K  A  A  D  S  V  T  A  F  S  G  L  V  N  V  Y  Y  D  I
242   aacaaggctgcagatagtgtaacagcattttcgggattggttaacgtttattacgatata   301
        A  I  E  D  M  P  I  T  P  Y  I  G  V  G  V  G  A  A  Y  I
302   gcgattgaagatatgcctatcactccatacattggtgttggtgttggtgcagcatatatc   361
        S  N  P  S  K  A  D  A  V  K  G  Q  K  G  F  G  F  A  Y  Q
362   agcaatccttcaaaagctgatgcagttaaagggcaaaaaggatttggttttgcttatcaa   421
        A  K  A  G  V  S  Y  D  V  T  P  E  I  K  L  F  A  G  A  R
422   gcaaaagctggtgttagttatgatgtaactccagaaatcaaactctttgctggtgctcgt   481
        Y  F  G  S  Y  G  A  S  F  N  K  E  T  V  S  A  T  K  E  I
482   tattttggttcttatggtgctagttttaataaagaaacagtatcagctactaaagagatc   541
        N  V  L  Y  S  A  V  G  A  E  A  G  V  A  F  N  F
542   aacgtcctttacagcgctgttggtgcagaagctggagtagcgtttaattttt   593
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Fig. 4.2. Consensus nucleotide sequence (lower case) and translated amino acid (upper case) sequence of *Wolbachia wsp* gene fragment amplified from *O. sulcatus*.
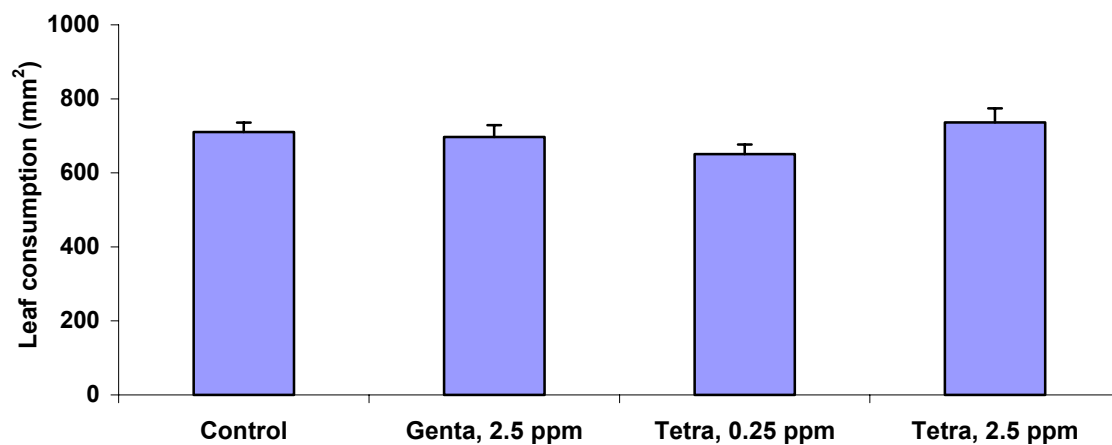
Fig. 4.3. Amount of leaf consumption (mean ± SEM) of preovipositional *O. sulcatus* adults on leaves treated with antibiotics. Genta: gentamicin, Tetra: tetracycline.
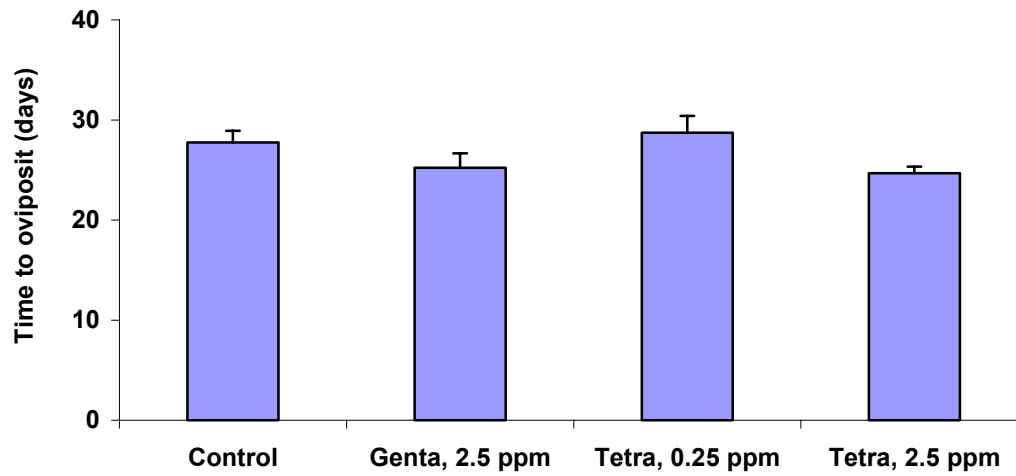
Fig. 4.4. Days (mean ± SEM) to reproductive maturation of preovipositional *O. sulcatus* adults when fed on leaves treated with antibiotics. Genta: gentamicin, Tetra: tetracycline.
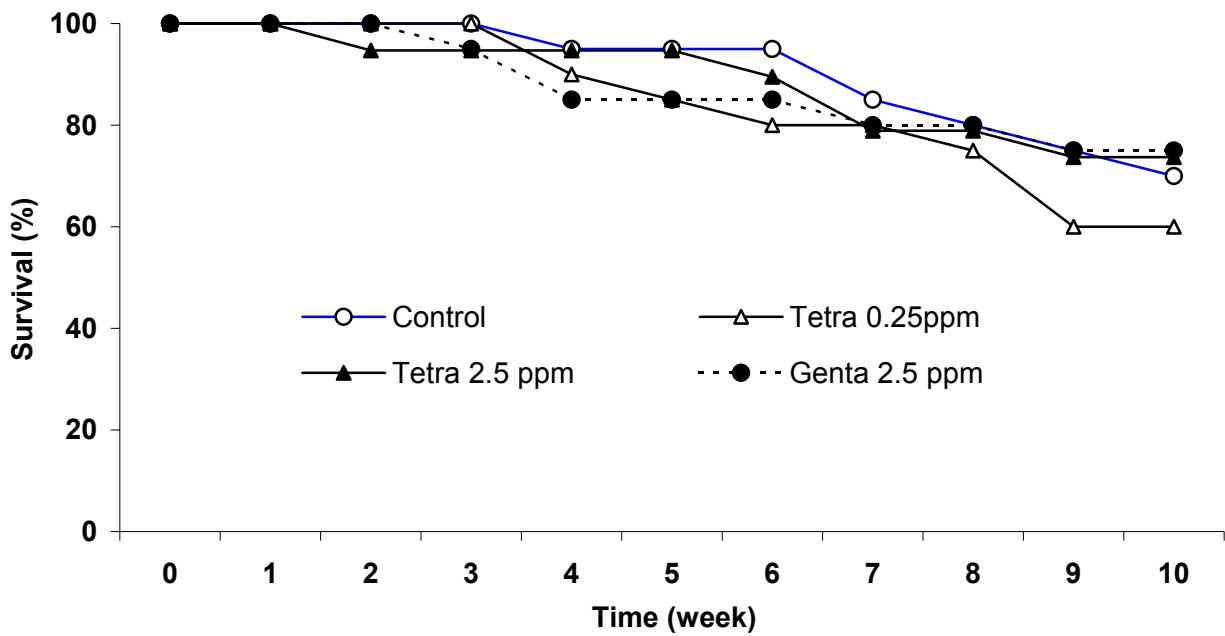
Fig. 4.5. Survival of weevils during 10-week experimental period composed of 7 weeks feeding on non-treated leaves following 3 weeks feeding on leaves treated with antibiotics. Tetra: tetracycline, Genta: gentamicin.
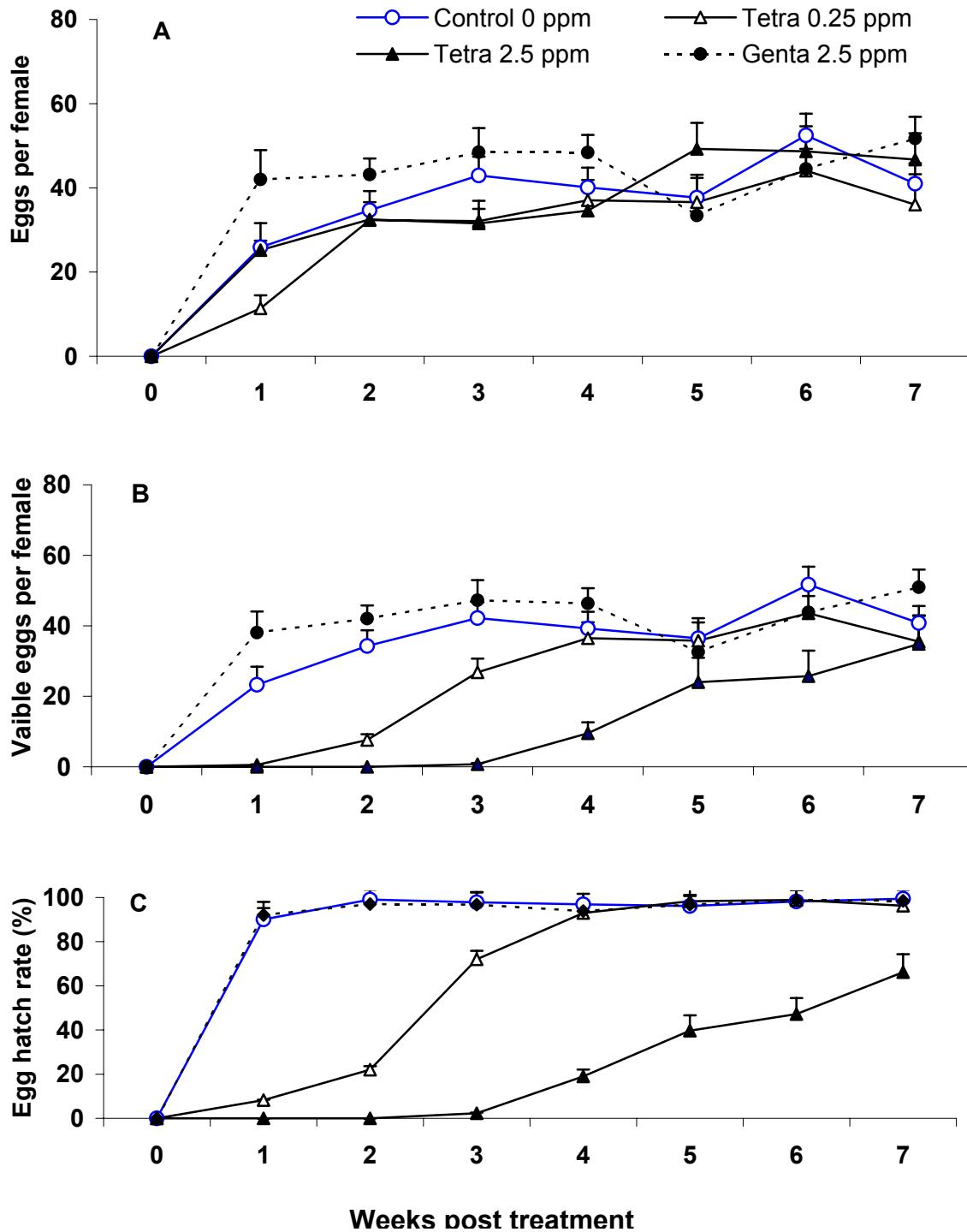
Fig. 4.6. Weekly reproduction of *O. sulcatus* during 7 week feeding on non-treated leaves following 3 week antibiotics treatments: (A) total egg production, (B) viable egg production, and (C) egg hatch rate (%). Tetra: tetracycline, Genta: gentamicin.
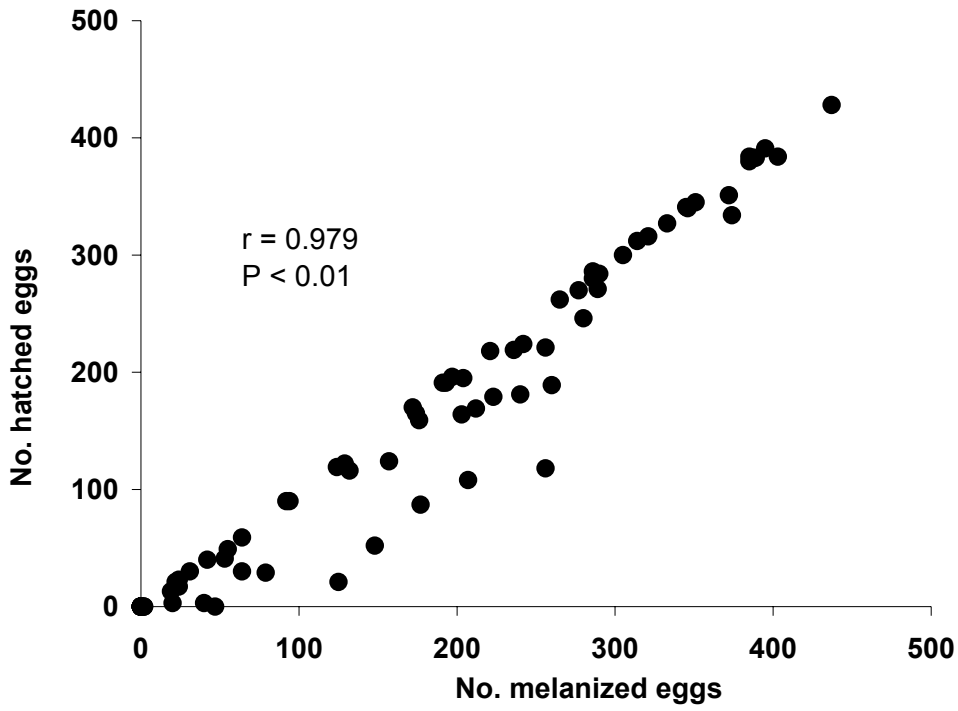
Fig. 4.7. Correlation between number of melanized viable eggs and number of hatched eggs in weevils from all treatments.
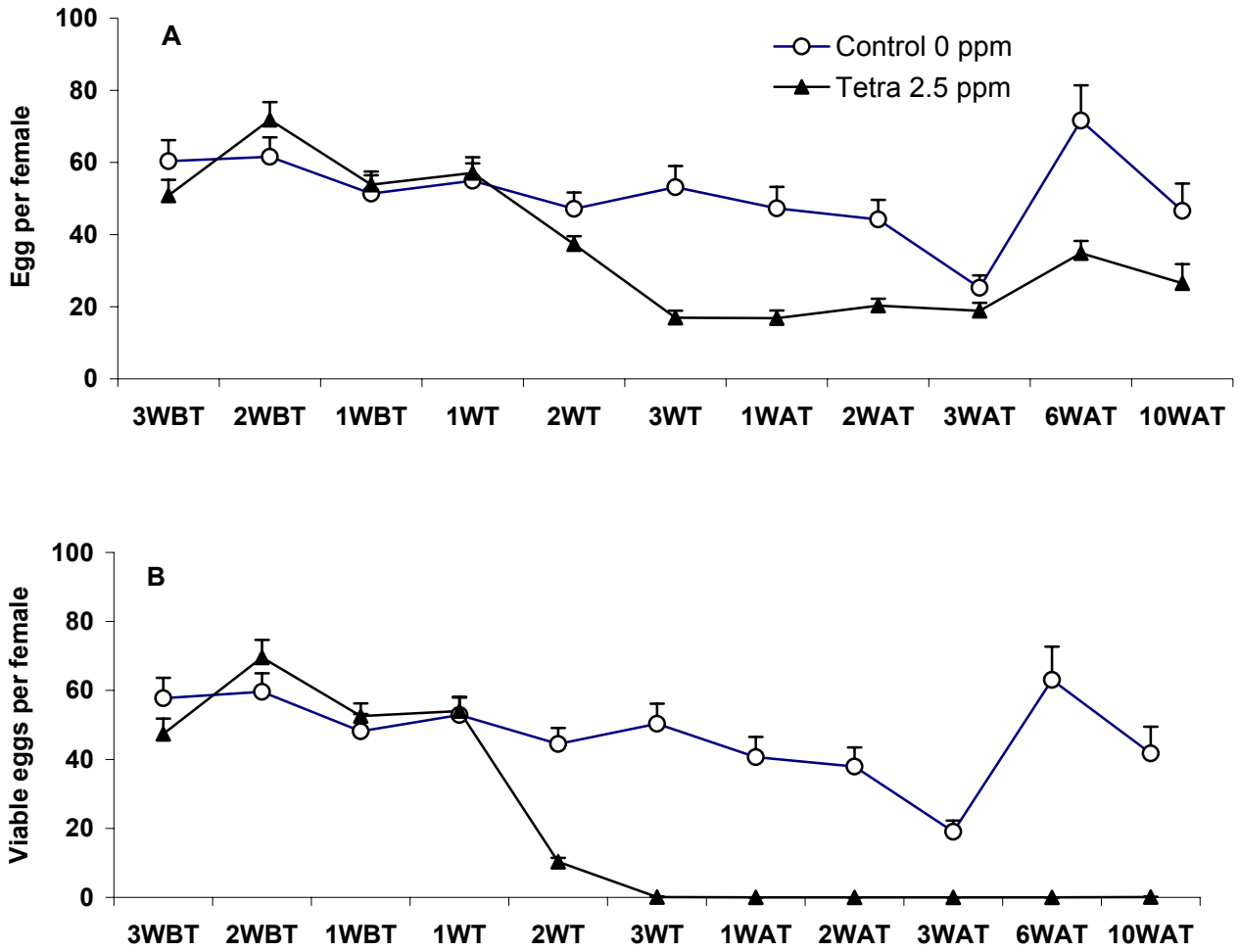
Fig. 4.8. Weekly reproduction of *O. sulcatus* during weeks before treatment (WBT), weeks of tetracycline (2.5 ppm) treatment (WT), and weeks after treatment: (A) total egg production, (B) viable egg production. Tetra: tetracycline.

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