Oogenesis in the date stone beetle, *Coccotrypes dactyliperda*, depends on symbiotic bacteria

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**Abstract.** It has been suggested that sex ratio distorting symbionts are involved in the sex determination and female-biased sex ratios observed in strongly inbred scolytid beetles. *Coccotrypes dactyliperda* (Coleoptera: Scolytinae) is a species in which mother-son- and sib-mating occur inside the date seeds it inhabits, and the sex ratios produced are highly skewed toward females. In the present study, polymerase chain reaction (PCR) techniques and antibiotic treatments are applied to determine the possible role of Bacteria in this system. PCR with primers specifically designed to target the 16S rDNA gene in all Bacteria reveals the presence of *Wolbachia* and *Rickettsia* in control beetles, but not in antibiotic-treated individuals. Virgin females fed with antibiotics lay no eggs, and no sign of oogenesis is detected compared with all-male progeny of virgin control females. Mated females fed with antibiotics lay significantly fewer eggs than control females, with a strong effect of female age at the time of antibiotic feeding on the number of eggs laid. The study suggests that symbiotic bacteria are not involved in female-biased sex ratios but are required for oogenesis in *C. dactyliperda*. The specific role each of the bacteria (*Wolbachia* and *Rickettsia*) plays in the oogenesis remains to be determined.

**Key words.** *Coccotrypes dactyliperda*, oogenesis, *Rickettsia*, Scolytinae, symbionts, *Wolbachia*.

**Introduction**

It is now widely acknowledged that bacteria manipulating the mode of reproduction play an essential role in the biology of many arthropods. Among these symbionts are a number of male-killing bacteria, such as *Spiroplasma* and *Rickettsia* (Hurst & Jiggins, 2000), and two other bacteria, *Wolbachia* and *Cardinium*, which induce a number of reproduction-associated phenotypes (Stouthamer et al., 1999; Zchori-Fein et al., 2004). *Wolbachia*, an alpha-subdivision proteobacterium, has been the most extensively studied genus among bacteria that take control of host reproduction to increase their own numbers. This bacterium has been implicated in all types of reproductive manipulations discovered to date, including cytoplasmic incompatibility (in which uninfected females that mate with infected males fail to reproduce), male killing, feminization (in which genetic males develop as females) and thelytokous parthenogenesis induction (O’Neill et al., 1997; Werren, 1997; Stouthamer et al., 1999).

Recently, the symbiotic proteobacterium *Wolbachia* was found in the coffee berry borer, *Hypothenemus hampei* (Coleoptera: Curculionidae: Scolytinae) (Vega et al., 2002). This species is characterized by strong female-biased sex ratios, continuous inbreeding inside the berry and dwarfed males (Baker, 1999). The presence of *Wolbachia* in most of the *H. hampei* populations tested (11 out of 15) prompted Vega et al. (2002) to suggest that the symbiont may be involved in the skewed sex ratios exhibited by this species. To establish the role that *Wolbachia* may be playing in the sex determination of members of the Scolytinae, Vega et al. (2002) suggested comparing the occurrence of *Wolbachia* in other inbreeding and outbreeding members of this family.

The date stone beetle *Coccotrypes dactyliperda* F. (Curculionidae: Scolytinae) is a strongly inbreeding species. It is widespread in warm temperate regions, where it feeds on either green dates (Kehat et al., 1976) or date seeds,
which are also used as their breeding habitat (Hussein, 1990; Siverio & Montesdeoca, 1990). The newly emerged female colonizes a new date stone and the entire life cycle takes place inside that seed (Blumberg & Kehat, 1982). The sex determination mechanism of *C. dactyliperda* is haplodiploidy, where the haploid dwarf males are produced from unfertilized eggs and the diploid females result from sexual reproduction. The offspring sex ratio of mated females is strongly female-biased and inbreeding is the norm (Herfs, 1959).

The present study investigates whether *C. dactyliperda* beetles serve as hosts to bacterial symbionts, to test the hypothesis that symbionts are involved in the beetle's sex determination and to test the symbionts other effects on the beetles. Polymerase chain reaction (PCR) is used to detect the presence of *Wolbachia* and/or other bacteria and antibiotic treatments are used to study the influence of the symbionts on the reproduction of their beetle host.

### Materials and methods

#### Rearing Coccotrypes dactyliperda

Date seeds inhabited by *C. dactyliperda* were collected in a date plantation in Ein-Gev, in the eastern part of the Sea of Galilee, Northern Israel, in January, 2003. Voucher specimens were deposited with Lawrence R. Kirkendall (University of Bergen, Norway), who confirmed the identification using both morphology and DNA sequencing. More than 200 seeds were cut open and pupae, as well as adults, were collected and released on fresh date seeds placed in plastic bowls (10 × 5 cm) covered with a perforated plastic lid and kept at 28 ± 1 °C and an average relative humidity of 70 ± 2%. One month later, the seeds were cut open, young mated females were collected daily and transferred into small Petri dishes with sliced date seeds (< 2 mm thick) as food. To obtain virgin females, pupae were collected from cut seeds and kept individually in small tubes. Upon emergence, the beetles were transferred into a small Petri dish with sliced date seeds as above. The beetles do not oviposit on sliced-dates, but can be maintained on a final MgCl concentration of 1.5 mM in a total volume of 25 μL. PCR parameters were denaturation for 2 min at 95 °C, followed by 30 cycles of 30 s at 92 °C, 30 s at 57 °C, 30 s at 72 °C, and a 5-min final extension at 72 °C. Every reaction set included sterile water as a ‘negative’ control and *Aphytis* sp., which has been reported to carry *Wolbachia* (Zchori-Fein & Perlman, 2004), as a positive control. A primer set specifically designed to amplify a gene encoding for the *Wolbachia* surface protein (*wsp*) was used to obtain a better characterization of that symbiont. The PCR was performed on two randomly chosen females as described above, with primers and conditions as previously reported (Zhou et al., 1998), which yield an expected product of approximately 600 bp. The DNA produced by the PCR was extracted from the gel and cloned into *Escherichia coli* using pGEM-T Easy plasmid (Promega, Madison, Wisconsin) according to the manufacturer’s protocol. Clones were sequenced using an ABI 3700 DNA analyser (Macrogen Inc., Korea), and the sequence obtained was compared with sequences deposited in GenBank.

#### Detection of other symbionts

To determine whether the *Wolbachia* found in *C. dactyliperda* is the causative agent of the observed female-biased sex ratio, the 16S rDNA gene fragment (approximately 1500 bp) was amplified by PCR using primers and conditions known to amplify this gene from all known Bacteria (Weisburg et al., 1991). Reactions contained 5 μL of the template DNA lysate, 15 pmol of each primer, 1.5 mM dNTP and 0.5 units of REDTaq (Sigma, St Louis, Missouri) with a final MgCl concentration of 1.5 mM in a total volume of 25 μL. PCR products were visualized by ethidium bromide on an agarose gel.

### Vertical transmission

To determine vertical transmission of the symbionts via the eggs to the offspring, three 1-day-old eggs were ground in a lysis buffer and subjected to a PCR analysis using both the *Wolbachia*-and *Rickettsia*-specific primers, as described above, with an annealing temperature of 57 °C. The PCR products were visualized by ethidium bromide on an agarose gel.
Oviposition pattern

To be able to assess the influence of antibiotic treatments on C. dactyliperda reproduction, it was essential to determine the baseline reproductive potential of this species under our experimental conditions. Mated females were placed individually in a 50 × 15 mm Petri dish and offered an intact date seed (n = 170). During the next 17 days, seeds were cut open, females were removed and the number of oviposited eggs was counted (n = 10 seeds each day).

The effect of antibiotics on female survival

To determine the antibiotic concentration that affects the target symbionts, but not the insect longevity, various concentrations were tested in a preliminary experiment. Date seeds were chopped to pieces of approximately 60 mg each (< 2 mm width), soaked in 25 mL of water (control) or 1, 3 and 5% tetracycline (Sigma) solution in water and placed on a mechanical shaker for 24 h to ensure even absorption. The chopped seeds were then air dried for 12 h, and offered as food for 2-day-old mated females for 4 days. Groups of 10 females were then placed in a Petri dish (50 × 15 mm), together with an intact water-soaked date seed to feed on and oviposit in (n = 10 for each treatment). After 30 days, seeds were cut open and the number of live beetles was recorded. The data were analysed using one-way analysis of variance (ANOVA) followed by Tukey post hoc test.

The effect of antibiotics on female oviposition over time

Subsequent to the results of the preliminary experiment above, date seeds, chopped to pieces as above, were soaked in 25 mL of 3% tetracycline (Sigma) solution in water and placed on a mechanical shaker for 24 h. After air drying for 12 h, the chopped seeds were offered as food for 2-day-old mated females for 4 days (n = 80). The females were then placed individually in a Petri dish (50 × 15 mm), with half of the females offered an intact, water-soaked date seed to feed on and oviposit in, and the remaining half received an intact, antibiotic-soaked seed treated as above. Control females (n = 40) were presented with water-soaked chopped seeds for 4 days and then placed individually in a Petri dish with an intact, water-soaked seed to feed on and oviposit in. On days 1, 5, 9 and 13 after the female’s penetration into the new seed, 10 females from each of the three treatments were taken, the seeds were cut open, and the number of eggs in each seed was counted. The data were analysed using two-way factorial ANOVA.

The effect of female age on antibiotic treatment outcome

To test the hypothesis that symbionts are required in the early stages of oogenesis, C. dactyliperda females were treated at different ages. Chopped date seeds soaked in either 3% tetracycline solution or sterile distilled water as described above were offered as food for 7 consecutive days to 1, 7 or 21-day-old mated females (10 females in each treatment). After the treatment, the food was removed and the females were placed individually in a 50 × 15 mm Petri dish and offered an intact, untreated, date seed as an oviposition substrate. Ten days later, the seeds were cut open and the number of eggs of each female was counted and compared amongst age groups and treatments using two-way factorial ANOVA.

Parthenogenesis

The effect of antibiotics on eggs produced by virgin females was also tested. One-day-old virgin females were offered tetracycline (3%) (n = 15) or water-treated (n = 20) chopped seeds, as described above. The food was removed 10 days later and a whole seed was provided to each female in a Petri dish for an oviposition period of 2 weeks. The number of offspring in each treatment group was counted and compared using a t-test.

Results

Symbiont detection and identification

All samples of C. dactyliperda tested with the 16S rDNA Wolbachia-specific primers by means of PCR gave a product of the expected size. The use of wsp primers yielded two identical PCR products of 595 bp, which exhibit over 99% sequence similarity to the Wolbachia found in Drosophila melanogaster (AF020065). Using universal Bacteria primers, a second symbiont was detected in the beetle. The 16S rDNA sequence of this bacterium showed homology to sequences of various Rickettsia found in GenBank. This sequence is 1454 bp long, has a 52% GC content and exhibits over 98% homology to the Ixodes tick symbiont Rickettsia bellii (U11014). The Rickettsia sequence was deposited in GenBank under accession number AY961085. PCR analysis of C. dactyliperda eggs with both Wolbachia and Rickettsia primers showed that these two bacteria are present in the eggs produced by the control beetles, but not in the very few eggs produced by the antibiotic-treated ones.

Oviposition pattern

Mated females started to lay eggs 3 days after inhabiting a new seed. An average number of 3.4 ± 0.9 eggs (mean ± SE) was oviposited daily and these eggs were accumulated in the seed (Fig. 1). The maximum number of mature eggs found in the ovaries of females randomly taken from the laboratory colony was 1, with up to 45% of the tested females showing no mature eggs.
The effect of antibiotics on female survival

Thirty days after treatment with various antibiotics concentrations, a significant difference in female survival was detected ($F_{3,36} = 180.1, P < 0.001$). There was no difference in numbers of surviving females after feeding on seeds treated with either water, 1% and 3% antibiotic (mean ± SE: 8.5 ± 0.7, 7.8 ± 0.94 and 7.6 ± 0.67 females, respectively). However a significant difference was detected in females feeding on seeds treated with 5% antibiotic solution (2.0 ± 0.74 females) (Tukey post hoc, $P < 0.05$). Thus, the 3% tetracycline solution was used for testing the effect of antibiotics on female oviposition.

The effect of antibiotics on female oviposition over time

The effects of both treatment and day of oviposition were highly significant ($F_{2,108} = 73.2, P < 0.001$ and $F_{3,108} = 14.8, P < 0.001$, respectively). However, due to the significant interaction between them ($F_{6,108} = 8.9, P < 0.001$), the two effects were analysed separately, with the effect of the treatment analysed for each day. Females fed either on water-soaked or antibiotic-treated seeds oviposited a similar average of approximately 5–6 eggs on the first day after inhabiting the seed ($F_{2,27} = 0.265, P > 0.05$). However, the number of eggs oviposited by females fed with water-treated seeds increased over time, whereas the number of eggs oviposited by females fed with antibiotic-treated seeds did not increase throughout the experiment ($F_{2,27} = 36.5, P < 0.001$, $F_{2,27} = 19.8, P < 0.001$ and $F_{2,27} = 54.6, P < 0.001$ for 9, 13 and 17 days, respectively) (Fig. 2). An analysis of the influence of the treatments on the number of oviposited eggs revealed significant effect ($F_{2,117} = 415.47, P < 0.001$), with more eggs oviposited by females treated with water-soaked seeds than by females fed antibiotic-treated seeds, and no difference between the number of eggs oviposited during time between the 4 and 17 days of antibiotic treatments (Tukey HSD multiple comparisons, $P < 0.05$) (Fig. 2).

The effect of female age on antibiotic treatment outcome

Both antibiotic treatment and age of the female at the onset of the experiment significantly affected the number of offspring produced (ANOVA: $F_{1,54} = 103.40, P < 0.001$ and $F_{2,54} = 26.10, P < 0.001$, respectively). Because progeny numbers were also affected by the interaction between these two factors ($F_{2,54} = 31.75, P < 0.001$), they were analysed separately, using ANOVA followed by Tukey HSD multiple comparisons.

Females’ age at oviposition had no effect on the number of eggs laid by control females ($F_{2,27} = 0.346, P > 0.05$), but a significant effect of female age was detected when antibiotic-treated females were tested ($F_{2,27} = 150.74, P < 0.001$) (Fig. 3).

The antibiotic treatment had a significant effect on the number of eggs laid by females fed at 1-day and 7-days old ($F_{1,18} = 61.62, P < 0.001$ and $F_{1,18} = 98.09, P < 0.001$, respectively), but had no effect on females fed at 21-days-old ($F_{1,18} = 0.001, P > 0.05$). Almost no eggs were laid by 1-day-old females fed sliced date seeds treated with 3% tetracycline; significantly more eggs were laid by 7-day-old females fed antibiotics and still significantly more eggs were oviposited by females fed with antibiotics when they were 21-day-old (Tukey HSD multiple comparisons, $P < 0.05$) (Fig. 3). Dissections of control females revealed the presence of several oocytes in various maturation stages, with no more than one mature egg ready to be laid. By contrast, when antibiotic-treated females that had
not laid any eggs were dissected, no oocytes were detected in the ovaries.

*Parthenogenesis* Virgin, 1-day-old females fed on date seeds treated with 3% tetracycline solution for 10 days did not lay a single egg throughout the 15-day laying period, whereas virgin females at the same age which were fed on water-treated seeds oviposited 4.25 ± 0.52 (mean ± SE) eggs (One sample *t*-test with a mean of 0, *t* = 8.13, d.f. = 19, *P* < 0.001).

**Discussion**

It has been suggested that the maternally transmitted symbiotic bacterium *Wolbachia* affects the female-biased sex ratio observed in the coffee berry borer and other beetles within the subfamily Scolytinae (Vega *et al*., 2002). In one such species, the arrhenotokous date stone beetle *Coccytodes dactyliperda*, the elimination of *Wolbachia* was suspected to result in the production of a less female-skewed sex ratio. However, in the present study, when both mated and unmated *C. dactyliperda* females are treated with antibiotics, they stop producing eggs (Fig. 2).

The adverse effect of antibiotic treatment on female ovi-position is most evident in young females, whereas old females are not affected at all and females of intermediate age produce intermediate numbers of eggs (Fig. 3). It is also found that the beetles are synovigenic, and the gradually maturing eggs are laid at an average rate of 3.4 ± 0.9 a day (Fig. 1). Combined with the observation that no signs of developing oocytes are found in antibiotic-treated females that did not lay eggs, the results suggest a positive correlation between the presence of symbiotic bacteria and oocyte development. If this symbiont-dependent egg maturation hypothesis is correct, the prediction is that no new oocytes will develop in the absence of symbionts, whereas mature oocytes will not be affected. The results support this prediction and show that the number of eggs produced by older females is less affected by the antibiotic treatment (Fig. 3).

Using PCR, the proteobacteria *Wolbachia* and *Rickettsia* are found in whole-body extracts of untreated *C. dactyliperda*, but cannot be detected in antibiotic-treated females. Although *Wolbachia* is generally recognized as an arthropod's symbiont with a wide array of phenotypes, species of the genus *Rickettsia* are usually referred to as vertebrates' pathogens vectored by arthropods (Weiss & Moulder, 1984). However, *Rickettsia* has been previously reported to be associated with male-killing in the buprestid beetle *Brachys tessellatus* (Lawson *et al*., 2001), and the ladybird beetles *Adalia bipunctata* and *A. decempunctata* (Werren *et al*., 1994; von der Schenkenburg *et al*., 2001). *Rickettsia* has also been reported in insects such as the bruchid beetle *Ktyorhinus sharpius* (Fukatsu & Shimada, 1999) and even in plants (Davis *et al*., 1998), where its phenotype has not been established yet. Whatever the role *Rickettsia* plays in these systems, the above hosts are unlikely to serve as vectors to warm-blooded hosts.

Symbiotic bacteria associated with egg formation have been reported in several insects. The first correlation between egg formation and the presence of *Wolbachia* was discovered in the parasitoid *Asobara tabida* (Hymenoptera: Braconidae), where antibiotic treatments of *Wolbachia*-carrying females resulted in symbiont-free wasps that did not produce mature oocytes (Dedeine *et al*., 2001). Although no other physiological functions tested are affected by the removal of *Wolbachia*, the ovaries of apo-symbiotic females contain aborted egg chambers and there is no indication of vitellogenesis. Because egg production in *A. tabida* has also been shown to be strongly correlated with *Wolbachia* density in the females, it was suggested that this bacterium is essential for oogenesis. Starr & Cline (2002) suggested a mechanism by which the symbiont can affect oogenesis in its host. They observed that, in mutant *D. melanogaster* females that were prevented from producing eggs by protein-coding lesions in Sex-lethal (*Slx*) (i.e. the master regulator of sex determination), fertility is restored in the presence of *Wolbachia*. That study demonstrates the interaction between *Wolbachia* and a specific host regulator protein in a way that can rescue *Drosophila* oogenesis defects.

The present study shows that antibiotic-treated *C. dactyliperda* females do not produce mature oocytes and do not lay eggs. It is suggested that bacterial symbionts are essential for oogenesis in this species. Both the results and the interpretation are in agreement with data gathered from the beetle *Xyleborus ferrugineus*, another member of the tribe Scolytinae, in which an extensive study was conducted on the association of the beetle and a complex of symbiotic microorganisms (Peleg & Norris, 1972; Norris & Chu, 1980). One of these studies shows that surface-sterilized eggs contain both *Staphylococcus* and a nonidentified rod-shaped bacterium (Norris & Chu, 1980), but no molecular analysis is performed. Antibiotic treatments of *X. ferrugineus* females results in lower population levels of the two bacteria, accompanied by a significant reduction in the percentage of ovipositing females and the number of progeny per female (Peleg & Norris, 1972). These authors conclude that the symbiotic bacteria in *X. ferrugineus* are required for oogenesis, and speculate that they take the role of the sperm in activating the egg cell (Peleg & Norris, 1972). In light of the present findings and the electron micrograph picture provided by Norris & Chu (1980), it is believed that the symbiont responsible for egg formation induction in *X. ferrugineus* is the rod-shaped bacteroid, which may correspond to the *Rickettsia* or *Wolbachia* found here, rather than the suggested *Staphylococcus*.

Because the two bacteria found in *C. dactyliperda* appear to be transmitted from the mother to her offspring through the egg, it is difficult to determine which is the one (or the combination of the two) that affects the egg formation process. Moreover, the current data do not permit the exclusion of the possibility that other bacteria are present in the study insect, and may also influence its phenotype.
The study demonstrates, again, the importance of identifying the whole suit of symbionts associated with a given species or line because, even in arthropods where Wolbachia has been found, the possibility of double infections with an unrelated bacterium such as Rickettsia exists. Special care must therefore be taken in assigning particular effects on host reproduction to a particular bacterium.

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