CHAPTER 2

# Parthenogenesis in *Venturia canescens* is genetically determined

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**Abstract**

*Venturia canescens* (Gravenhorst) (Hymenoptera: Ichneumonidae) is a haplodiploid solitary endoparasitoid wasp with two alternative modes of reproduction: thelytoky (parthenogenetic) and arrhenotoky (sexual). Thelytoky in Hymenoptera is often induced by intracellular microorganisms. Although it was previously shown that no *Wolbachia* bacteria are present in these wasps, it is still possible that thelytoky is caused by other endosymbionts. In this study we assessed the presence of microbial endosymbionts in the ovaries of *V. canescens* by antibiotic and high temperature curing and PCR amplification. Although specific RT-PCR amplification diagnostic for the small RNA containing virus (VcSRV) showed the presence of this virus in *Venturia*, it is not associated with thelytoky as it is present in both arrhenotokous and thelytokous strains from different geographic origins. No evidence was found for any microorganisms associated with thelytokous reproduction. We conclude that most likely thelytoky in *V. canescens* is not caused by endosymbionts but has a genetic basis.

## Introduction

All species within the Hymenopteran order have a haplodiploid mode of reproduction. Arrhenotoky is the most common mode of reproduction: males are produced parthenogenetically from unfertilized eggs and females develop from fertilized ones. Thelytoky is a less common mode of reproduction where diploid females develop parthenogenetically from unfertilized eggs after restoration of diploidy. Males do not occur or very rarely.

Thelytoky occurs in all major groups of Hymenoptera, but is especially present among sawflies (Symphyta) and some parasitoid families such as the Chalcidoidea and Cynipoidea (Cook, 1993; van Wilgenburg *et al.*, 2006). Thelytoky has been demonstrated to have a genetic basis in few species, but most cases of thelytoky concern species that are infected with parthenogenesis inducing microorganisms (Stouthamer, 1997; Braig *et al.*, 2002; van Wilgenburg *et al.*, 2006) where males, which are unable to transmit the bacteria, are not produced. *Wolbachia, Cardinium* and *Rickettsia* are groups of bacterial endosymbionts known to induce thelytoky (O'Neill *et al.*, 1997; Stouthamer *et al.*, 1999; Weeks & Breeuwer, 2001; Zchori-Fein *et al.*, 2001, 2004; Zchori-Fein & Perlman, 2004; Groot & Breeuwer, 2006; Hagimori *et al.*, 2006; Perlman *et al.*, 2006). They can also induce other reproductive alteration such as male killing, feminization and cytoplasmic incompatibility. *Wolbachia* is the best known of these reproductive parasites. In general, bacterial induction of thelytoky is restricted to host species with haplodiploid sex determination, although Weeks *et al.* (2001) found it in an entirely haploid mite species.

In insects, however, parthenogenesis can also originate by non-microbial induced processes such as hybridization between two related sexual species (Suomalainen, 1962; White, 1980). Hybridization events alter meiosis and create opportunities for new cytological processes to retain diploidy (Vrijenhoek, 1998). A well studied example is the grasshopper *Warramaba virgo* (Honeycutt & Wilkinson, 1989). Parthenogenesis can also originate through spontaneous mutations in the genes controlling meiosis that lead to the development of unfertilized eggs into female adults. A genetic basis for thelytokous parthenogenesis has been shown for *Trichogramma cacoeciae* (Stouthamer *et al.*, 1990b; Vavre *et al.*, 2004), several species of the genus *Lysiphlebus* (Belshaw *et al.*, 1999), the ant species *Plathythyrea puncata* (Schilder *et al.*, 1999), the ichneumonid *Venturia canescens* (Beukeboom & Pijnacker, 2000) and the cape honeybee *Apis mellifera capensis* (Tucker, 1958; Lattorff *et al.*, 2005). So far, the underlying genetics of thelytokous parthenogenesis is only known in the egg laying workers of the cape honeybee. In this species, thelytokous parthenogenesis is determined by a single recessive gene (Ruttner, 1988; Lattorff *et al.*, 2005) that acts as a transcription factor (Lattorff *et al.*, 2007).

*Venturia canescens* (Gravenhorst) (Hymenoptera: Ichneumonidae) is a solitary endoparasitoid wasp of lepidopteran larvae which has both arrhenotokous and thelytokous reproduction and females of either mode occur sympatrically

(Schneider *et al.*, 2002). As in all haplodiploid arrhenotokous species, females develop from fertilized eggs and males from unfertilized eggs (arrhenotoky). However, in thelytokously reproducing individuals, females produce haploid eggs meiotically that subsequently undergo diploidy restoration and develop into females (Speicher, 1937; Beukeboom & Pijnacker, 2000). In lineages capable of both arrhenotokous and thelytokous reproduction it is not yet clear why thelytokous individuals do not take over (Maynard Smith, 1971; Williams, 1975). To gain a better understanding of the evolutionary stability of thelytokous reproduction and how arrhenotokous and thelytokous individuals can coexist, it is important to know more about the origin and mechanisms that control and regulate thelytokous parthenogenesis in this species.

In an earlier study, Beukeboom & Pijnacker (2000) described a modification of central fusion automictic parthenogenesis as the mechanism for diploidy restoration in *V. canescens*. This mechanism enables heterozygosity to be maintained for loci close to the centromere, but loci distal from chiasmata will become homozygous over generations depending on the segregation pattern. In addition, they showed that *Wolbachia* is not likely the cause of thelytoky in *V. canescens*. Here, we refine this study and investigate the possibility that endosymbionts other than *Wolbachia* may cause thelytoky in *V. canescens*. To this end, we analysed both arrhenotokous and thelytokous strains of *V. canescens* for the presence of microbial endosymbionts using *Wolbachia* specific and general prokaryotic 16S ribosomal primers and we subjected thelytokous females to an antibiotic and high temperature treatment.

A small RNA virus (SRV) has recently been described in a long-standing thelytokous laboratory strain of *V. canescens* (VcSRV) (Reineke & Asgari, 2005). This virus, which belongs to the picorna-like group, is associated with the eggs during their production in the ovaries and is likely to spread by vertical transmission (Reineke & Asgari, 2005). The effect of the virus on its wasp host is not yet clear and no evidence of disease caused to wasps has been shown. Although there is only one study in the literature of a virus related with parthenogenetic reproduction (in turkey, Olsen & Poole, 1962) we also investigated the presence of VcSRV in arrhenotokous and thelytokous strains of *V. canescens* to test whether VcSRV may induce thelytoky in this species.

## Material and Methods

### *Venturia canescens* strains

Several thelytokous and arrhenotokous strains were collected from different places and during different years from southern and central France and southern Spain. Table 2.1 summarizes the origin of the strains, collection year and the experiment in which they were used. Each strain was named after the location and year that it was collected.

**Prokaryotic PCR analysis**

*V. canescens* DNA was extracted from three sympatric arrhenotokous and three thelytokous strains (see Table 2.1). Several organisms were used as a control to the experimental procedures for establishing presence and absence of *Wolbachia* and other prokaryotes. DNA was extracted from a *Wolbachia*-infected *Nasonia vitripennis* (Hymenoptera) STDR strain, as well as from a laboratory strain from which *Wolbachia* had been removed by antibiotic treatment more than 15 years ago (ASYM C). DNA was also extracted from a *Wolbachia*-infected *Ephestia kuehniella* adult. In addition, *Escherichia coli* (Gram negative, strain MC1061) and *Lactococcus lactis* (subsp. *cremoris*, Gram positive, strain MG 1363) DNA served as positive controls for the amplification of bacterial 16S ribosomal DNA fragments. To account for the possibility of inhibiting agents in the extracted DNA from *V. canescens*, a mixture of *V. canescens* and *L. lactis* DNA was included in the PCR analysis.

*V. canescens* DNA was obtained from (1) whole adult wasps, (2) whole adult wasps that had been washed in digestion buffer (100 mM NaCl, 10 mM Tris-HCl pH 8.0 and 25 mM EDTA) to eliminate possible exosymbionts that could interfere with the experiment and (3) ovaries of 5 wasps of each strain. Wasps were dissected under sterile conditions in Phosphate Buffer Saline (PBS; 0.8M $Na_2HPO_4$, 0.14M $KH_2PO_4$, 0.26M KCl and 0.14M NaCl, pH 7.3). The ovaries were collected and pooled in digestion buffer and DNA was extracted using a standard proteinase K/salt-chloroform protocol. Presence and quality of DNA was checked on a 1% agarose gel.

The DNA samples were used in PCR reactions with specific primers to detect the presence of *Wolbachia* (wspF 5'-TGGTCCAATAAGTGATGAAGAAAC-3' and wspR 5'-AAAAATTAAACGCTACTCCA-3', named 81F/691R primers in Zhou *et al.*, 1998) or with 16S ribosomal DNA specific primers (B8F 5'-AGAGTTTGATCMTGGCTCAG-3' and U1406R 5'-ACGGGCGGTGTGTRC-3' according to Lane, 1991) to detect the presence of any prokaryotic endosymbionts. PCR reactions were performed in 1X PCR buffer magnesium free (Promega) with 2.5 mM $MgCl_2$, 0.2mM dNTPs (Roche), 0.2µM each primer, 0.4 units of Taq polymerase (Promega) and approximately 5ng of template DNA. The PCR profile was 1 cycle of 3 min at 94°C followed by 35 cycles of 1 min at 94°C, 30 sec at 55°C, 1 min at 72°C and a final cycle of 7 min at 72°C for wsp. For 16S amplification the PCR profile was similar except for the extension step in each cycle that was 4 min at 72°C.

**Antibiotic treatment**

Six thelytokous strains (see Table 2.1) were treated with antibiotics. One arrhenotokous strain (MV) served as a control to test for possible secondary effects of the treatment. For each population, one newly emerged female (P-generation) was fed a solution of 1% tetracycline and 10% sucrose every other day from their emergence onwards. Females were allowed to oviposit each day following tetracycline

feeding for five successive periods. Since eggs are in various developmental stages upon emergence and oogenesis continues during life, this feeding regime ascertained that eggs in different developmental stages were exposed to tetracycline. Wasp progenies were reared at 25°C for 4 weeks till the next generation emerged (F1). To increase the chance that the next generation originated from cured eggs, first and second clutches were not used. A maximum of 10 individuals from the third, fourth and fifth clutch were treated with tetracycline and allowed to lay eggs following the same regime as their mothers. This procedure was repeated for four generations because several generations of feeding may be required for complete curing of *Wolbachia* bacteria (Stouthamer *et al.*, 1990a). In parallel, a replicate of the experiment with the same thelytokous strains fed with a solution of 10% sucrose but no antibiotics was carried out as control. In addition, curing of mated females of the parasitic wasp *Nasonia vitripennis* infected with *Wolbachia* (LAB II strain) was carried out to ascertain that the curing procedure was effective in removing any bacterial endosymbionts. *N. vitripennis* were fed with a solution of 0.1% tetracycline and 10% sucrose.

**Temperature treatment**

High temperature can kill microorganisms and revert thelytoky to arrhenotoky (Stouthamer *et al.*, 1990a). Fifteen females from the thelytokous strain Antibes 2003 (Ant03) and thirty females from San Juan 2003 (SJ03) were subjected to three temperature regimes (20°C, 25°C and 30°C). Five wasps from Ant03 and ten from SJ03 were isolated in tubes, put at each temperature and fed with honey and water. Females were hosted three times and pupae were individually separated in tubes. Each generation, five females per isofemale line were hosted and the experiment lasted until the fourth generation. We checked for the presence of males among the offspring. When males were produced, they were screened together with their mother with seven microsatellites (*Vcan*002, *Vcan*066, *Vcan*070, *Vcan*099, *Vcan*112, *Vcan*113 and *Vcan*115) as described in Chapter III of this thesis.

**Virus RT-PCR**

*V. canescens* RNA was extracted from the abdomen of 5 wasps of each of the thelytokous strains and from a mix of 5 arrhenotokous wasps of different strains (see Table 2.1) with the RNeasy kit (Qiagen). RNA from the abdomen of 5 thelytokous virus-infected wasps from the laboratory strain RM (Reineke & Asgari, 2005) served as a positive control. The extracted RNA was checked on a 1% agarose gel for quality and on a spectrophotometer for quantity.

Two-step reverse transcriptase PCR was performed for a VcSRV diagnostic test. For cDNA synthesis SuperScript III 1$^{st}$ strand synthesis SuperMix (Invitrogen) was used. 600 ng total RNA per sample was incubated during 5 min at 65$^{o}$C together with 50 μM oligo dT primer, annealing buffer and RNase inhibitor. First-strand reaction mix and the reverse transcriptase were subsequently added. PCR condi-

tions were: annealing at 37°C during 15 min, incubation at 50°C for 1h and heat-inactivation of RT by incubation at 85°C for 5 min. To detect the presence of viral RNA 10 µl of the reverse transcription mix was used as a template. PCR was carried out with PCR buffer, 0.2 mM dNTPs, 30 pmol of specific VcSRV primers (Vc-RdRp-F 5'-CCTAATCGGAAAGTCTCTGTC-3' and Vc-RdRp-R 5'-CATAAT-GTCGCCCTACTCTAAT-3', from Reineke & Asgari, 2005) and 2.5 units of Taq polymerase final concentration (Qiagen). The amplification conditions were 1 cycle of denaturation for 30 sec at 94°C, 35 cycles of annealing 1 min at 56°C, extension 2.30 min at 72°C and 1 cycle of final extension during 10 min at 72°C. 10 µl of the PCR reactions were checked for presence or absence of virus-specific bands on a 1% agarose gel.

**Table 2.1.** Summary of the different *V. canescens* strains used in the experiments of this study. Locality, collection year and reproductive mode are indicated. A: endosymbiont bacteria PCR; B: antibiotic treatment; C: virus RT-PCR; D: temperature treatment.

| Strain | Origin | Year | Mode | Experiment |
|---|---|---|---|---|
| Ant | Antibes, France | | Thelytokous | A, C |
| Ant03 | Antibes, France | 2003 | Thelytokous | B, C, D |
| MtB | Mont Boron, Nice, France | | Thelytokous | A, B, C |
| MtB03 | Mont Boron, Nice, France | 2003 | Thelytokous | B, C |
| Valb | Valence, France | | Thelytokous | B, C |
| SJ02 | San Juan de Alicante, Spain | 2002 | Thelytokous | A, B, C |
| SJ03 | San Juan de Alicante, Spain | 2003 | Thelytokous | B, C, D |
| RM | Laboratory culture, Germany | | Thelytokous | C |
| Ant | Antibes, France | | Arrhenotokous | A, C |
| Valb | Valence, France | | Arrhenotokous | C |
| Valb04 | Valence, France | 2004 | Arrhenotokous | C |
| MV | Mixed population from Mont Boron and Valbonne, France | | Arrhenotokous | A, B |
| SJ02 | San Juan de Alicante, Spain | 2002 | Arrhenotokous | A |

## Results

### Prokaryotic PCR analysis

Faint amplification products using 16S ribosomal DNA primers were observed in all *Venturia* samples (data not shown). This may be due to the presence of internal bacteria (e.g. gut or intestinal) and can possibly obscure the presence of endosymbionts. To avoid possible interference with such prokaryotes DNA was extracted from *Venturia* ovaries directly.

In both washed and non-washed *V. canescens* samples, both arrhenotokous and thelytokous, we observed no PCR products using *Wolbachia* specific primers. With these primers a diagnostic 600bp product was amplified only in the positive controls: *Wolbachia* infected *N. vitripennis* and *E. kuehniella* (Figure 2.1). The difference in intensity of the amplified DNA from *N. vitripennis* and *E. kuehniella* most likely reflects a lower number of endosymbionts in *N. vitripennis* as compared to *E. kuehniella* (see Figure 2.1). This confirms the absence of *Wolbachia* in both the arrhenotokous and thelytokous strains of *V. canescens* in concordance with the results of Beukeboom & Pijnacker (2000).

Amplification with 16S specific primers resulted in a fragment of the expected size (≈ 1400 bp) in all positive controls for prokaryotic DNA (*L. lactis*, infected *N. vitripennis* and *E. kuehniella*, *V. canescens*-*L. lactis* mixture and *E. coli*). Again, the band corresponding to *N. vitripennis* is faint and may be due to low number of bacteria. The lower intensity of the band in the *N. vitripennis* sample is consistent with the lower intensity of the *E. kuehniella* sample compared to Figure 2.1. Neither in antibiotic treated *N. vitripennis* individuals nor in any *V. canescens* sample an amplification product was detected (Figure 2.2). This indicates that neither the arrhenotokous nor the thelytokous strains of *V. canescens* carry a detectable level of prokaryotic endosymbionts in their ovaries.

**Antibiotic treatment**

A total of 645 *Venturia* wasps, all offspring from tetracycline treated females, were collected in the fourth generation of antibiotic treatment. As no males were produced in this generation nor in any of the previous generations, no reversal to sexual reproduction had occurred as a result of the antibiotic treatment. In the fourth generation, treated females and control females produced a mean of 12.90 ± 8.96 *SD* (N = 51) and 16.51 ± 10.17 SD (N = 41) female offspring respectively (2-tailed t-test, $t = 1.79$, $p = 0.075$). Similarity of variance was confirmed with Levene's test. These results indicate that the antibiotic treatment did not affect the number of *V. canescens* offspring.

A parallel curing of a *Wolbachia* infected *N. vitripennis* strain (LAB II) was carried out to control for efficiency of the treatment. In *N. vitripennis*, *Wolbachia* causes cytoplasmic incompatibility: crosses between uninfected females and infected males are incompatible and do not yield female offspring (Werren, 1997). Treated virgin females were crossed with (a) males from a cured laboratory strain and (b) non-cured males. Daughter production in the first but not in the second cross indicated that the bacterial infection was indeed successfully removed by the treatment.

**Temperature treatment**

From the two thelytokous strains used in the temperature treatment, SJ03 produced three males in the first generation at 30ºC and seven males in the second generation at 20°C. No males were produced at 25°C in any generation. From

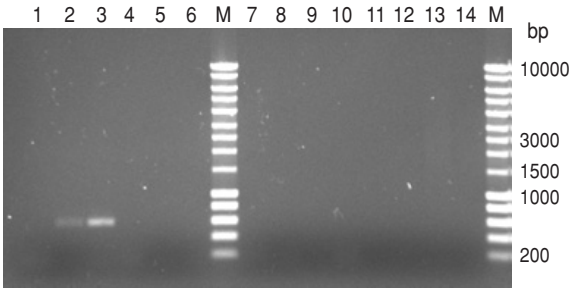


**Figure 2.1.** PCR amplification of a *Wolbachia* specific DNA fragment. 1. *L. lactis*; 2. *N. vitripennis* infected with *Wolbachia*; 3. *E. kuehniella* infected with *Wolbachia*; 4. *L. lactis-V. canescens* DNA mix; 5, 6, 7 arrhenotokous *V. canescens*; 8, 9, 10 thelytokous *V. canescens;* 11. N. *vitripennis* cured of *Wolbachia*; 12. Blank; 13. *E. coli*; 14. Blank; M. size marker.

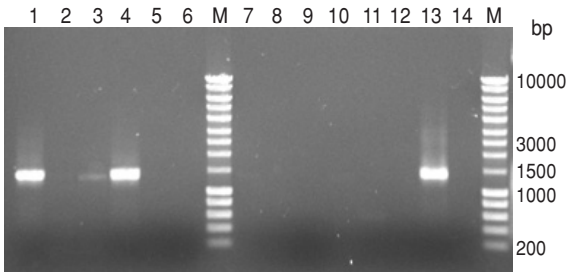


**Figure 2.2.** PCR amplification of a fragment specific to bacterial 16S gene. 1. *L. lactis*; 2. *N. vitripennis* infected with *Wolbachia*; 3. *E. kuehniella* infected with *Wolbachia*; 4. *L. lactis-V. canescens* DNA mix; 5, 6, 7 arrhenotokous *V. canescens*; 8, 9, 10 thelytokous *V. canescens*; 11. *N. vitripennis* cured of *Wolbachia*; 12. Blank; 13. *E. coli*; 14. Blank; M. size marker.

microsatellite screening performed on these males, we can conclude that the seven males produced at 20°C and their mother were arrhenotokous, which was most likely due to a contamination. We confirmed with microsatellites that the three males produced at 30°C had a thelytokous origin. We cannot say weather these males were haploid or diploid since they shared the same homozygous genotype as their mother. For both strains the total number of offspring produced at 25°C (794 in Ant03 and 676 in SJ03) was higher than at 20°C (53 and 300 respectively) and at 30°C (228 and 106 respectively). This indicates that 25°C is the optimal temperature for culturing *V. canescens*.

**Virus RT-PCR**

RT-PCR for the VcSRV was performed on the arrhenotokous and thelytokous strains listed in table 2.1. In all arrhenotokous and all but one (MtB03) thelytokous

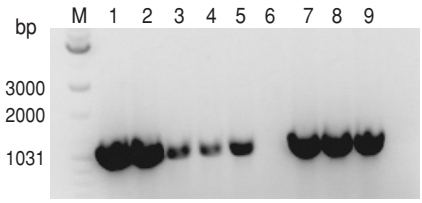


**Figure 2.3.** Detection of VcSRV in *V. canescens* wasps from different geographic origins by RT-PCR amplification. M. size marker; 1. Vc-RM; 2. Vc-Valb; 3. Vc-Ant; 4. Vc-Ant03; 5. Vc-MtB; 6. Vc-MtB03; 7. Vc-SJ; 8. Vc-SJ03; 9. Five Vc-sexual from different origins.

samples and in the positive control a diagnostic band of 1000 bp was obtained. This band corresponds with the expected size of the amplification of part of the RNA-dependent RNA-polymerase of the VcSRV (Figure 2.3). These results demonstrate the presence of this virus in *Venturia* individuals from different geographic origins but also show that there is no strict association of VcSRV with either reproductive mode.

## Discussion

The results of the tetracycline treatment and the combined prokaryote and *Wolbachia* diagnostic PCRs, indicate the absence of prokaryotic endosymbionts in the ovaries of both arrhenotokous and thelytokous *Venturia canescens* individuals. In addition, temperature treatment of two thelytokous strains supports this result.

In the PCR specific test (Figure 2.1), the band amplify in *Nasonia vitripennis* is more faint than the one showed for *Ephestia kuehniella*. Although we control for the total amount of DNA in the PCR reaction, we cannot determine how much *Wolbachia* DNA is amplified. It is possible that the amount of *Wolbachia* DNA is lower in *N. vitripennis* and thus a weaker band is amplified. Surprisingly, in Figure 2.2, we can hardly see the band amplify in *N. vitripennis* for the prokaryotic 16S ribosomal gene. One could even say that it is absent. But we can also argue that the band is present, but as the *E. kuehniella* band, it is weaker in Figure 2.2 than in Figure 2.1.

Antibiotic curing can revert asexuality to sexuality (Stouthamer *et al.* 1990a), but our antibiotic treatment did not yield any males. In the temperature treatment only three males were produced in the SJ03 strain that were confirmed to share the same alleles for eight microsatellites as their thelytokous mother. Unfortunately, the genotyping results do not allow us to determine the ploidy level of these males since they and their mother were homozygous for all the markers. We do not think that these males were the result of an obligate reversal to sexuality, as we only

found three males in the first generation and no males in the subsequent three generations. If thelytoky would be induced by endosymbionts causing thelytoky in *V. canescens*, one would expect that a high temperature treatment would induce a partial or total reversal to arrhenotokous reproduction with an increase of male production in each new generation. Alternative explanations include a mutation in the sex determination mechanism (e.g. homozygosity at the csd locus) or a temperature sensitive mutation in the diploidization process (in this case these males are haploid). Furthermore, the same strain was used for the antibiotic treatment and no production of males was observed. We tried to cross these males with arrhenotokous females, but no copulation took place.

Specific RT-PCR amplification diagnostic for the small RNA containing virus in *V. canescens* (VcSRV) showed the presence of this virus in several arrhenotokous and thelytokous strains. The fact that the virus is found in individuals of both reproductive modes excludes the possibility that the virus is the cause for thelytoky in *V. canescens*. Although many picorna-like viruses are pathogens of insects, there are no evident signs for pathogenic effects of VcSRV on the host or wasp and our results rule out the possibility that the virus induces thelytoky in *V. canescens*. The fact that this virus is apparently present in laboratory as well as field populations of this wasp species from various geographic origins further strengthens the assumption that it may play an important role *i.e*. during development of the wasp within parasitised host caterpillars (Reineke & Asgari, 2005). On the other hand, a virus can just spread and maintain itself at high frequencies in the population without being involved in the host-parasitoid relationship and without being pathogenic for the wasp or the host (Varaldi *et al.*, 2003). It would be interesting to sequence the virus in both types of females to confirm that it is indeed the same virus infecting both reproductive modes.

Most microbe-mediated cases of parthenogenesis studied involve gamete duplication leading to complete homozygosity (Stouthamer & Kazmer, 1994; Gottlieb *et al.*, 2002; Pannebakker *et al.*, 2004). Two cases have been reported where heterozygosity is maintained through a functionally apomictic cloning mechanism (Weeks & Breeuwer, 2001; Adachi-Hagimori *et al.*, 2008). Diploidy restoration in the thelytokous strains of *V. canescens* is described as central fusion automictic parthenogenesis (Beukeboom & Pijnacker, 2000). A first meiotic division is followed by a mitosis of the restituted number of diploid chromosomes. One nucleus becomes a polar body and degenerates, whereas the other develops into the diploid embryo. A similar type of thelytokous reproduction occurs in the cape honey bee *Apis mellifera capensis*. This cytological mechanism of diploidy restoration does not instantly lead to complete homozygosity. The fact that thelytokous females can be heterozygous for genetic markers (Chapter III of this thesis) is consistent with this.

Taken together, our data combined with those of Beukeboom and Pijnacker (2000) indicate that it is very likely that parthenogenesis in *V. canescens* has a genetic basis. But studies like this one may present potential pitfalls (Weeks *et al.*

2002; Weeks *et al.*, 2003) and one has to be cautious when interpreting negative data. Other possible explanations for the absence of a PCR product can be inhibiting agents or PCR failure. By using a DNA mix of *V. canescens* and *L. lactis* we try to show that the absence of prokaryotic DNA amplification is not due to inhibiting agents. Other controls might be more appropriate to show that no inhibiting agents interfere in the PCR amplifications, such as the amplification of a different *V. canescens* gene. In future studies, more sensitive PCR methods should be used to discard the possibility of low endosymbiont numbers causing infections that cannot be detected by standard PCR. Even though we have searched for a wide range of possible infectious microorganisms, we did not check for non-prokaryotic endosymbionts. For example, microsporidia infections have been reported to cause male killing in mosquitoes (Kellen *et al.*, 1965; Hurst, 1991) and feminization in the crustacean *Gammarus duebeni*. We chose to use tetracycline for the antibiotic curing because of its broad spectrum of action, but one could argue that resistance to this antibiotic can easily be developed. Although the PCR and antibiotic curing results presented in this study are in concordance, a combination of at least two antibiotics with different mode of action (i.e. tetracycline and rifampicine) would have yeilded further assurance.

Very little is known about the underlying genetics of parthenogenesis in animals. In *A. mellifera capensis*, parthenogenesis in the egg-laying worker is a heritable trait probably determined by a single recessive locus (Latroffe *et al.*, 2005). In the case of *V. canescens* we do not know whether a single or multiple genes are responsible for thelytoky. We also cannot exclude that thelytoky is encoded by mitochondrial genes and is maternally inherited. Schneider *et al.* (2003) suggested that parthenogenetic populations frequently originate from arrhenotokous ones. In Chapter VI of this thesis, we have attempted to identify the genes underlying thelytoky in *V. canescens* in order to gain a better understanding of the evolutionary stability of asexual reproduction and how arrhenotokous and thelytokous individuals can coexist.

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