BEHAVIOURAL INTERPLAY AND PREPOTANCY COMPARISON BETWEEN *Wolbachia* AND HAPLOTYPE-ASSOCIATED REPRODUCTIVE BARRIERS BETWEEN TWO POPULATIONS OF *Tetranychus urticae* KOCH

by

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B.Sc. in Biology, İstanbul University, 2014

Submitted to the Institute of Environmental Sciences

in partial fulfillment of

the requirements for the degree of

Master of Science

in

Environmental Sciences

Boğaziçi University

2018

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DATE OF APPROVAL: 18/01/2018

In memory of Ekrem Bakırdöven,

ACKNOWLEDGEMENTS

I would like to express my gratitude to my supervisors Assoc. Prof. Dr. Raşit Bilgin and Assist. Prof. Dr. Sara Magalhães for their encouragement and valuable support throughout my studies, as well as their vital help on the bureaucratic affairs. I would also like to thank my committee members Prof. Dr. Andrzej Furman, Prof. Dr. Gülriz Bayçu Kahyaoğlu and Assist. Prof. Dr. Berat Haznedaroğlu for their supportive suggestions.

I would like to express my sincere gratitude to my lab advisor Flore Zélé for her continuous support for my Master's study and related research, for her patience, motivation, and immense knowledge. She has contributed to this work very significantly. Her guidance helped me through all, whether in Istanbul or Lisbon. I am happy to be her friend, colleague and lab partner.

I would like to thank my fellow lab colleagues at the Centre for Environmental Biology, University of Lisbon; Diogo Godinho, Inês Santos, Joaquim Santos, Leonor Rodrigues and Salomé Clemente for their friendship and their support.

I would like to thank Assoc. Prof. Dr. Burak Demirel, Prof. Dr. Nadim Copty, and Prof. Dr. Turgut T. Onay for their support during the planning of my journey to Portugal. I would also like to thank my friends at the Institute of Environmental Sciences, İlknur Temizel and Merve Şahan for their support.

I am also grateful to these special people; Aimilia Tsimini, Barış Erbil, German Kurapov, Neslihan Edeş, Okay Caner, Ruud Huntjens, Stephanie Olsohn, Şeyma Eren and Yvonne Gelissen for their persistent emotional support. Despite the distance or being in a foreign country, I have never felt that I was away from home. I am lucky and grateful for their precious presence in my life.

Finally, I would like to state my forever gratitude to my mother Anatürk Bakırdöven, the bravest woman I have ever known. This work would be incomplete without thanking her. I am very happy and proud for having been raised by her. I am also grateful to my brother Can Burak Bakırdöven for being my tutor and best friend throughout all the difficulties in my life.

ABSTRACT

BEHAVIOURAL INTERPLAY AND PREPOTANCY COMPARISON BETWEEN *Wolbachia* AND HAPLOTYPE-ASSOCIATED REPRODUCTIVE BARRIERS BETWEEN TWO POPULATIONS OF *Tetranychus urticae* KOCH

Tetranychus urticae Koch (two-spotted spider mite) is an arachnid species that infests and damages several economically valuable crops. *Wolbachia pipientis* is an intracellular endosymbiont alpha-proteobacterium. These bacteria cause a unidirectional reproductive mismatch between uninfected females and infected males, though *Tetranychus urticae* females are capable of producing male offspring via arrhenotokous parthenogenesis (laying haploid male bearing eggs without copulation). Also, another natural unidirectional reproductive barrier was found among two populations of *Tetranychus urticae*, namely green and red morphs. *Wolbachia* causes embryonic mortality, while population based incompatibility causes excessive male offspring production.

In this study, we investigated whether these population-based reproductive barriers were behavioural (*i.e.* due to prezygotic reasons) or not, through observations and tests on the frequency, latency and duration of matings. We found no differences before and during copulation, therefore we consider this incompatibility to be postzygotic. As our second experiment, we took records of all the crosses between the populations and compared our results using statistical techniques. We measured the fitness and sex ratio of the offsprings by including *Wolbachia* infected males in our experimental design. We also tested the cytoplasmic incompatibility that was either *Wolbachia*-based or population-based, and tested the incompatibility of the individuals that have the natural barrier *and* were infected by *Wolbachia*. Our results showed that when the copulation possesses two types of incompatibilities, though a slight effect of embryonic mortality can be observed, population-based excess in male production surpasses *Wolbachia*-based incompatibility.

ÖZET

İKİ *Tetranychus urticae* KOCH POPULASYONU ARASINDAKİ *Wolbachia* VE HAPLOTİP-TABANLI ÜREME BARİYERLERİNİN DAVRANIŞSAL ETKİLEŞİMİ VE BASKINLIK KARŞILAŞTIRMASI

Tetranychus urticae Koch (iki benekli örümcek akarı) ekonomik olarak değerli olan pek çok tarım ürününü istila ederek zarar veren bir örümceğimsi (araknid) türdür. *Wolbachia pipientis* ise hücre içi endosimbiyont olan bir alfa-proteobakteri türüdür. Bu bakteriler, her ne kadar *Tetranychus urticae* dişileri arhenotokik partenogenez (eşeysiz olarak haploid erkek verecek yumurta yumurtlamak) ile erkek döl elde edebilse de, sağlıklı bir dişi ve enfekte bir erkek arasında tek yönlü bir üreme uyuşmazlığına yol açmaktadır. Bununla birlikte yeşil ve kırmızı olarak adlandırabileceğimiz iki *Tetranychus urticae* popülasyonu arasında da tek taraflı doğal bir üreme bariyerine rastlanmıştır. *Wolbachia* embriyonik ölüme sebep olurken, popülasyon tabanlı uyuşmazlık aşırı miktarda erkek döl üretimine sebep olmaktadır.

Çalışmamızda bu popülasyon tabanlı üreme bariyerinin davranışsal olup olmadığını (yani prezigotik sebep) gözlemler ve çiftleşme sıklığı, çiftleşme gerçekleşene kadar geçen süre ve çiftleşme süresini test ederek araştırdık. Çiftleşme öncesi veya esnasında bir farklılık bulmadığımız için bu uyuşmazlığın sebebinin postzigotik olduğunu düşünüyoruz. İkinci deneyimizde popülasyonlar arası bütün çaprazlamaların kaydını tuttuk ve sonuçlarımızı istatistiki teknikler ile karşılaştırdık. Deney düzeneğimize *Wolbachia* ile enfekte erkekleri de ekleyerek dölün uyarlanma gücünü (yeti) ve cinsiyet oranını ölçtük. Ayrıca popülasyon tabanlı ve *Wolbachia* tabanlı sitoplazmik uyumsuzluk ile birlikte, hem doğal bariyer hem de *Wolbachia* ile enfekte bireylerin dölünü test ettik. Sonuçlarımız iki uyumsuzluğun aynı anda yaşandığı çiftleşmelerde, çok az miktarda embriyonik ölüm etkisi görülse de, popülasyon tabanlı aşırı miktarda erkek döl üretiminin, *Wolbachia* tabanlı uyumsuzluğun önüne geçtiğini gösterdi.

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LIST OF SYMBOLS/ABBREVIATION

[-]	Spider mite individual that is not infected by *Wolbachia*
[+]	Spider mite individual that is infected by *Wolbachia*
A	AMP (Red morph of *Tetranychus urticae*) in Appendix A
AMP	Red morph of *Tetranychus urticae*
Amtd+	If the male spider mite in Test A was successfully mated (values 0 or 1)
Bmtd+	If the male spider mite in Test A was successfully mated (values 0 or 1)
CBB	Coffee Berry Borer
CE3C	Centre for Environmental Biology, University of Lisbon
CI	Cytoplasmic Incompatibility
Dghtrs	Daughters (in Appendix)
Fec	Fecundity
h	Hour
Jvnls	Undistinguished offspring, juveniles (in Appendix)
L/D	Light/Dark
NA	Not Available
RH	Relative humidity
T	TOM (Green morph of *Tetranychus urticae*) in Appendix A
TestA♂	Morph of the male spider mite that was used in Test A (Day 3)
TestB♂	Gender of the male spider mite that was used in Test B (Day 4)
.tet	Individuals that were treated from *Wolbachia* by Tetracycline antibiotic
TOM	Green morph of *Tetranychus urticae*
Treat.	Mating combinations of samples (refers as Treatments)
Unhctd	Untached Eggs (in Appendix)
USDA	The United States Department of Agriculture

1. INTRODUCTION

Pesticides still represent the main weapon used to control crop pests. However, these chemicals are major threats for food safety and for the environment, in general. In order to keep the acaricidal chemicals as environmental friendly as possible (for the environment and for the consumer health), recent studies also focus on plant essential oils and oil vapour use (Aslan et al. 2004; Çalmaşur et al. 2006). However, the ubiquitous evolution of resistance to these compounds challenges the efficiency of such measures (Casida and Quistad 1998). Spider mites are among the most resistant groups to several pesticides, as they can rapidly develop resistance (Van Leeuwen et al. 2010). Therefore, alternative control measures are being investigated, with natural enemies giving some positive results, albeit to a limited extent.

In my thesis, we planned to use characteristics of the two-spotted spider mite (*Tetranychus urticae*) populations to gather knowledge on the mechanisms governing reproductive incompatibilities that naturally occur in this species. Indeed, sterilization-like phenomena like cytoplasmic incompatibility, can potentially be used as a novel environmentally friendly tool for the control of pest populations (Turelli and Hoffman, 1995; Engelstaedter and Telschow, 2009; Zabalou et al 2004). This knowledge could thus be used for pest control and reduce pesticide use in field crops.

Aside from its utilization in nature for manipulating reproductive barriers to develop a potential bioagent for agricultural purposes, understanding further steps and details about the natural unidirectional reproductive barriers within *Tetranychus urticae* might provide new ideas on the processes of speciation and evolution. Despite the high degree of polyphagy of *T. urticae*, host plant adaptation has been considered as a factor influencing population differentiation in spider mites, though not necessarily translating into speciation (Magalhães et al. 2007). Indeed, partial incompatibility on mating between conspecific populations that originate from different environments is a commonly occurring phenomenon in spider mites (Helle and Pieterse, 1965; de Boer, 1985). These reproductive incompatibilities have been attributed both to genetic factors (Navajas et al. 2000), and to infection by the bacterial reproductive manipulator *Wolbachia* (Gotoh et al. 1993, Navajas et al. 2000; Gotoh et al. 2003; Suh et al. 2015) – see details below.

However, the interaction between these two sources of incompatibility has not been studied until very recently (Zélé et al. in prep.). Zélé and colleagues studied two populations of *T. urticae*: one population, namely TOM was obtained from tomato (*Solanum lycopersicum*), in Corregado / Portugal

in 2010, and the other, namely AMP, was obtained in Aldeia da Mata Pequena / Portugal from datura plants (*Datura spp*.) in 2013. They found that uninfected AMP females (AMP is the assigned name for the red morph) are compatible with uninfected TOM males (TOM is the assigned name for the green morph), while uninfected TOM females are incompatible with AMP males. The same AMP and TOM population sources of *Tetranychus urticae* were used as the test subjects in this study. It is extremely important to understand the ongoing differentiation process that is a potential speciation initiator in spider mites, as well as its consequences for the population dynamics (i.e. a crucial parameter for pest control) of both (TOM and AMP) spider mite populations, and *Wolbachia* spread among populations. Yet, a good understanding of the mechanisms underlying this interaction are still lacking.

2. LITERATURE REVIEW

This experiment focuses on two main species and their interactions as a symbiotic relationship. To be able to apprehend and appreciate this topic; the Literature Review section will focus on the concept, definition and abilities of the host species *Tetranychus urticae* and the vector *Wolbachia pipientis*. Furthermore, their interactions with each other and other species will provide an understanding of the natural interplay of these species.

2.1. The Focus Species – Two Spotted Spider Mite (*Tetranychus urticae* Koch)

The two-spotted spider mite, *Tetranychus urticae* Koch, is a globally dispersed arachnid species. It is found to infest more than 1000 host species (Navajas, 1998), including several economically significant agricultural crops (Helle and Sabelis, 1985). In the presence of sperm –with a successful copulation- female offspring can be produced from fertilized eggs. If the female has not successfully mated with a male spider mite –or in some cases not at all-, unfertilized eggs produce only male offspring. This asexually reproduction phenomenon is called "arrhenotoky". Around 15% of arthropod species repopulate through haplodiploidy (de la Filia AG et al., 2015). Offspring produced through copulation (females) are diploids, while those that form through parthenogenesis (males) are haploids (Hill and O'Donell, 1991).

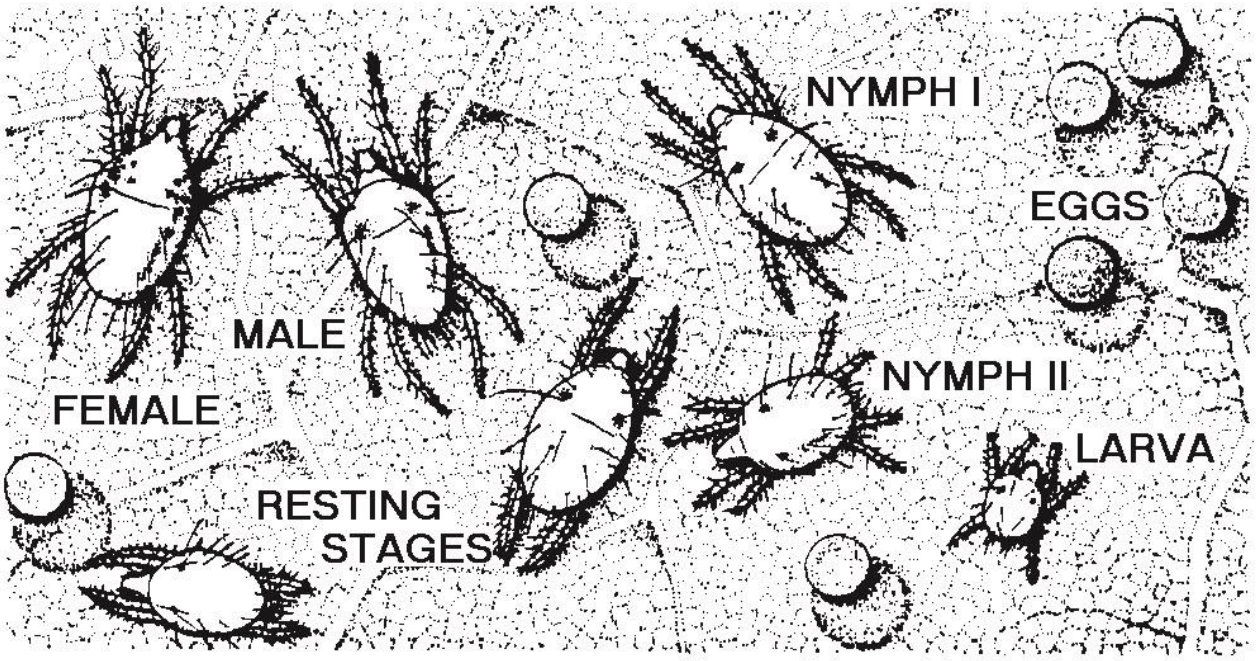


Figure 2.1. An illustration of *T. urticae* phases from binocular stereoscope display. Taken from The Ohio State University and the College of Food, Agricultural, and Environmental Sciences website.

Spider mite populations can rapidly increase in numbers, due to their haplodiploid nature. Consequently, the species can infect many valuable crop species such as tomato, zucchini, bean, cotton and eggplant (Helle and Sabelis, 1985). They can occur in green house crops, as well as on open fields. Its large number of host species, widespread distribution, and high fecundity makes it a major worldwide pest, destroying annual and perennial crops. For example, tomato and cotton, two major crop products of Turkey, according to the USDA Foreign Agricultural Service reports of 2009 and 2014 (TU9022 & TR5017) are frequently infested by spider mites in nature and greenhouses (Ay and Gürkan, 2005; Satar et al. 2013; Keskin and Kumral, 2015). The annual cost of chemical pest control of spider mites is estimated at 0.35 - 0.7 billion Euros worldwide, reflecting the significant economic impact of these pests. Computer models also suggest that with increasing global warming, the harmful effects of spider mites in agriculture will increase, as well (Migeon et al 2009).



Figure 2.2. Spider mite infestation that is observable with the naked eye. Photo credit: Nikolas R. Schiller.



Figure 2.3. Close up spider mite infestation with visible web production to keep the colony intact. Taken from Planet Natural website.

There are several studies on biological removal of *T. urticae* using other species. Howell & Daugovish (2016) mention that they used four commercially available predatory mites (Phytoseiidae): *Phytoseiulus persimilis*, *Neoseiulus californicus*, *N. fallacis*, and *Amblyseius andersoni* to be able to decrease or eliminate *T. urticae* and Lewis spider mite *Eotetranychus lewisi* infestation on strawberry plants in California, USA. The results showed that even though the pest numbers decreased initially, they were not able to keep the numbers below those for the economic thresholds.

On the study of Bugeme et al. (2014), the fungus species *Metarhizium anisopliae* was used on common bean to treat *T. urticae* infested leaves. Synthetic acaricide abamectin, the organosilicone surfactant Silwet L-77, oil and water were used for comparisons (water being the control). The experiments were conducted both in screenhouses, and on the field. They observed similar results on their field and screenhouse trials (Figures 2.4. and 2.5.). In their conclusion, Bugeme and colleagues (2014) stated that *M. anisopliae* treatments were as successful as abamectin, and suggested the *Metarhizium anisopliae* application as an alternative to the chemical compound.

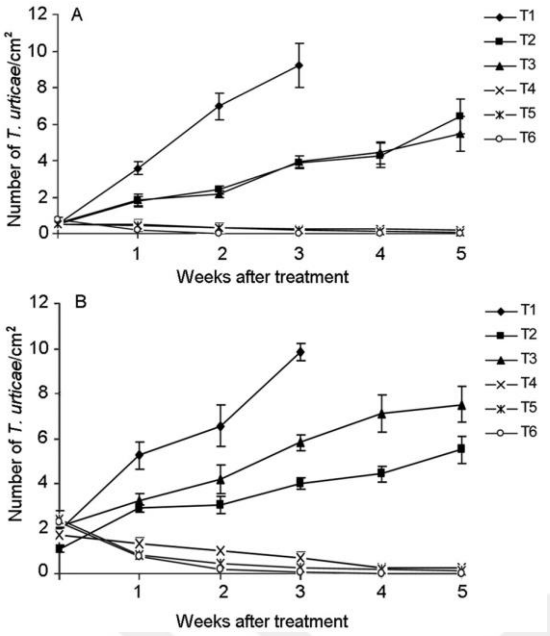


Figure 2.4. Efficacy of fungal applications on *T. urticae* population density on top (A) and middle (B) common bean leaves during the screenhouse trial I. T1: untreated control; T2: water + Silwet-L77; T3: water + Oil + Silwet-L77; T4: fungus in water formulation; T5: fungus in emulsifiable oil formulation; and T6: abamectin (Bugeme et al., 2014).

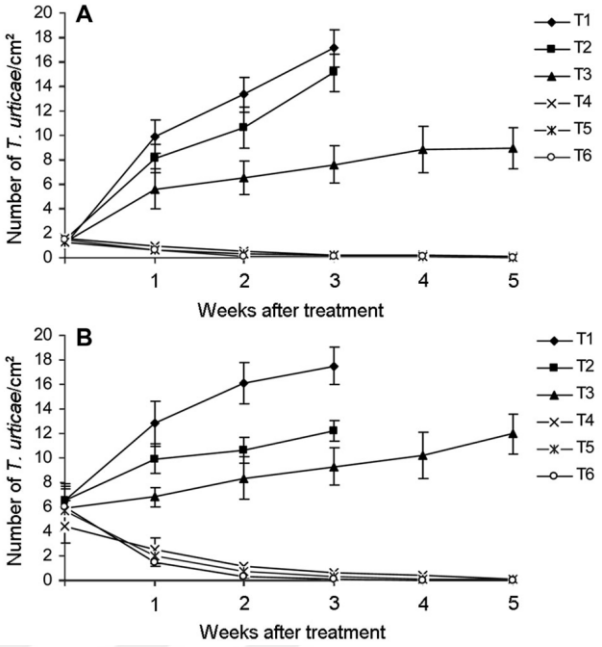


Figure 2.5. Efficacy of fungal applications on *T. urticae* population density on top (A) and middle (B) common bean leaves during the screenhouse trial II. T1: untreated control; T2: water + Silwet-L77; T3: water + Oil + Silwet-L77; T4: fungus in water formulation; T5: fungus in emulsifiable oil formulation; and T6: abamectin (Bugeme et al., 2014).

2.2. Haplodiploidy

For understanding the reproduction mechanism of *Tetranychus urticae*, it is important to analyse and comprehend the evolutionary costs and benefits of haplodiploidy. This has a crucial role on the life circle of the spider mite, and its symbiotic niche with *Wolbachia*.

Hartl & Brown's (1970) and Bull's (1979) models showed that when a haplodiploid mother produces haploid sons, the mother manages to pass on her full genetic composition, rather than passing on half to a diploid offspring. Therefore, she benefits fully from this occasion in terms of genetic succession. However, haploid sons are expected to live shorter than their diploid counterparts, which counterbalances their evolutionary demand. Consequently, haplodiploidy requires male offspring to survive a lifespan that is at least half as long as that of a diploid offspring, in order to continue to exist as a valid reproductive option. These models were confirmed by several studies that

were built upon Hartl & Brown's (1970) and Bull's (1979) work (Bull, 1983; Haig, 1993a,b; Nomark, 2004; Burt and Trivers 2006).

In a system where it seems that male offspring production leads to genetic success for mothers, a good question is why a fitness reduction in terms of female offspring is not observed. The reason is that in these species, sperm is produced by mitotic division (Bull, 1983) and the only possible reproductive and genetic succession for males is through producing daughters; hence these demands counterbalance each other (Kraaijeveld K., 2009).

Bull (1983) also suggested that haplodiploidy occurs more frequently in clades with fewer chromosomes. Bachtrog et al. (2014) stated that haplodiploidy seems to evolve from male heterogamety, based on Vicoso & Charlesworth's (2006) study that proposed X-linked deleterious recessive mutations being terminated in males (lower numbers of effective mutations lead to lower genetic loads). Bull also stated that the reason males are expected to live shorter than diploids is due to X-linked genomes being in majority among species with low chromosome numbers. Blackmon et al. (2016) tested this idea, and indeed found a correlation between low chromosome numbers and the likelihood of evolving into haplodiploidy, in mites. Furthermore, the lower genetic load in haplodiploid species, which is caused by parthenogenetic reproduction, is considered to lead to reduced inbreeding depression (Werren, 1993; Henter, 2003; Antolin 1999; Tien et al., 2015).

Finally, haplodiploidy also helped us determine the results of the fitness tests in this study. Since in arrhenotokous species, parthenogenesis results in male offspring, it is possible to monitor the success of the copulation. Male offspring can be the sign of reproductive incompatibilities due to prezygotic or postzygotic isolation, when mating is observed. The reproduction process of spider mites can be observed in the Figure 2.6., below.

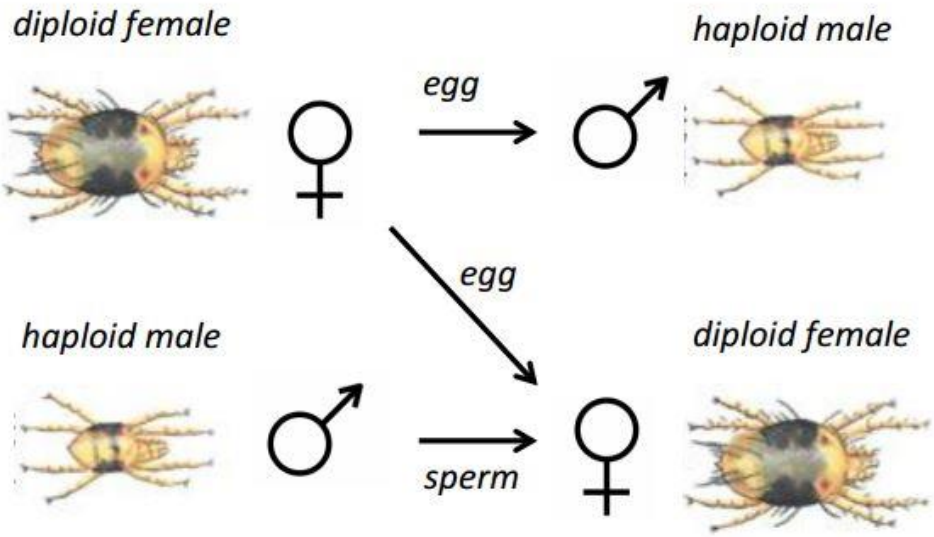


Figure 2.6. Reproduction process of spider mites. Used by courtesy of Flore Zélé (University of Lisbon, CE3C).

2.3. The Infecting Bacteria - *Wolbachia*

Bacteria that belong to genus called *Wolbachia* infect spider mites and change the course of their natural reproduction process. Generally, the type species is called *Wolbachia pipientis*, due to the species' first discovery in the mosquito *Culex pipiens* (Hertig and Wolbach, 1924). The alphaproteobacteria endosymbiont *Wolbachia* are transmitted vertically through the mites' eggs and supress the natural productive process. Manipulations in terms of feminization, parthenogenesis, male killing and egg-sperm incompatibility among insects were reported by Werren et al. (2008).

Breeuwer (1997) states that *Wolbachia* was studied thoroughly in insects until 1996 and cites Breeuwer and Jacobs (1996) as a surprising discovery of wide *Wolbachia* infestation among spider mites. A meta-analysis that was conducted in 2008 revealed that >65% of insect species are hosts of *Wolbachia*, which makes it one of the most abundant intracellular bacteria genus that affects at least 1,000,000 species (Hilgenboecker et al. 2008).

Wolbachia are highly adapted to living in invertebrate cells. It was shown that they can even use the spindle apparatus during the division of the cell leading to a possible mitosis disruption (Kose and Karr, 1995). A study suggests that *Wolbachia* modifies the sperm of the infected male during maturation, and if the infected individual mates with an uninfected female or a female that carries a different strain (another *Wolbachia* or a completely different endosymbiont), the results is embryonic

mortality in diploid species, and this drastically increases the number of male offspring in haplodiploid species (Tram, Feree & Sullivan, 2003). *Wolbachia* is transmitted maternally (through the cytoplasm of egg) and infects the offspring. Gotoh et al. (2003) stated that seven out of 42 spider mite species in Japan are infected by *Wolbachia*.

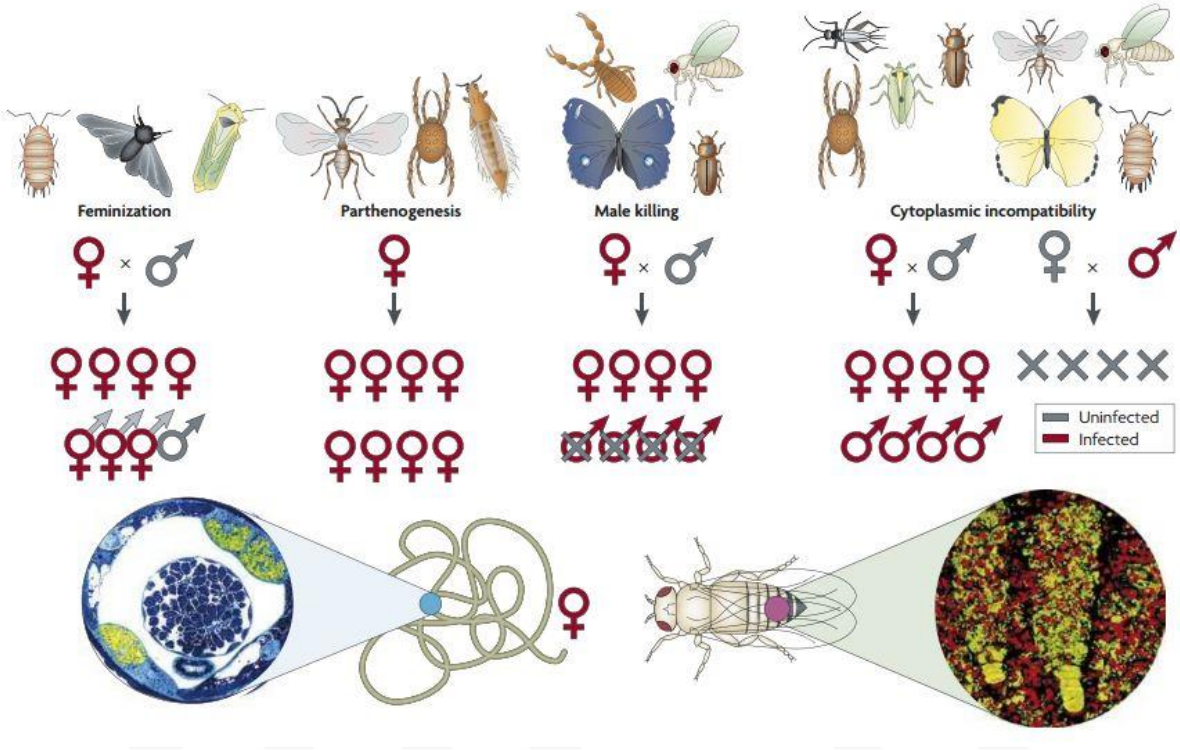


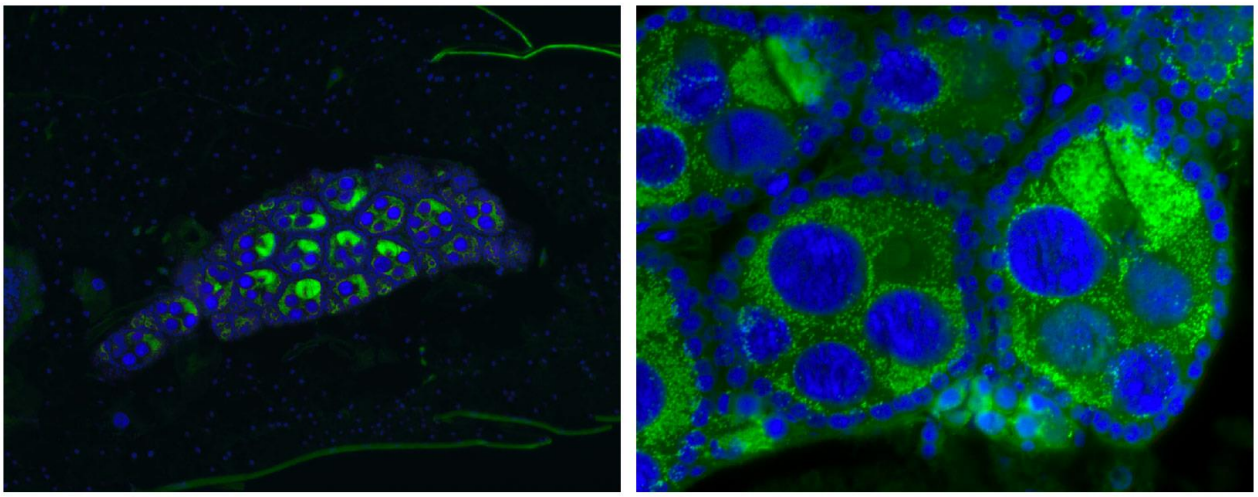
Figure 2.7. *Wolbachia* induced phenotypes (from Werren et al. 2008).

An important question is about the reason behind *Wolbachia* being so common in invertebrates. This question has more than one answer. *Wolbachia*'s strategy is wide and effectively applicable. It has evolved to target the gonads, which allows it to participate directly in the reproductive process and generation flow. In parthenogenic species, they are transmitted vertically, which allows them to even bypass the sexual reproduction phase. Moreover, they are also adapted to interact with its host species, even mutualistically. Its mutual interactions can be exemplified as positive immune responses in Nematodes (Simon et al., 2007), regulating the production of ovarian cells in the parasitic wasp species *Asobara tabida* by taking a role in the apoptosis mechanism (Pannebakker et al., 2007), and higher immune protection against RNA viruses in the fruit fly *Drosophila melanogaster* (Koukou et al., 2006). Additionally, according to the study of Dobson *et al.* (2004), *Wolbachia* can extend the fitness of its host by increasing the host's immunity against viral infections, and extending the female host's chances of survival and reproduction. It should also be noted that an infected female can mate with a male whether it is infected or not, thus slightly increasing her fitness.

In addition to its ubiquity, *Wolbachia*'s impressive survival and infection abilities should be mentioned as well. Studies have shown that *Wolbachia*, once assumed to die out after the host dies, actually survives long after the hosting cell dies according to culture experiments (Fallon, 2008). If it is taken out from the host, it can live up to one week at room temperature (Rasgon et al., 2006). Finally, when they are injected into the body of the host, e.g. *D. melanogaster*, they can find their way into the ovary at the somatic stem cell niche and reach into the reproductive cells, and ultimately into the developing eggs (Frydman et al., 2006).

Wolbachia has been used as a biological agent to control parasite populations on different species, previously. One of the most important approaches and applications is the vector control projects for the Dengue fever, which is caused by *Aedes* mosquitos. The early stages of the trials regarding their utilization as bioagents can be seen in the work of Brownstein et al. (2003). The topic has gained popularity over time, and recently Callaway (2016) suggested the technique of releasing *Wolbachia* to infect *Aedes aegypti* mosquitoes in order to decrease the prevalency of mosquitos carrying Zika, dengue fever and other viruses. In 2011, massive amounts of pre-infected mosquitos were released near the Northern Australian city of Cairns, and *Wolbachia* infected about 90% of the *Aedes* mosquitos in the designated area within weeks. An *in situ Wolbachia* that infested in mosquito ovarian tissue can be seen on Figure 2.8 (provided courtesy of Iñaki Iturbe-Ormaetxe from the Eliminate Dengue Program).

Trials were also conducted in Indonesia and Vietnam, with similar success. Small amounts were tested in Rio de Janeiro, Brazil and Medellín, Colombia to fight Zika, Dengue, chikungunya and other mosquito related viruses. The trials that were conducted in Rio de Janeiro (2014) and Medellín (2015) gave positive results with regards to halting the Zika and chikungunya viral replications, which had caused massive outbreaks in Latin America and the Caribbean. These two major projects were halted due to insufficient funds, but are being revived by the Eliminate Dengue Program, Brazilian government and some U.S. and U.K. based funders. Especially the Brazilian government being a funder is a good example for the importance of *Wolbachia* as a potential biocontrol agent, both today and in the future.



A. Localization of *Wolbachia* in mosquito ovarian tissue. **B.** Cellular localization of *Wolbachia*.

Figure 2.8. *Wolbachia* in mosquito ovarian tissue. DNA is stained with DAPI (blue) and *Wolbachia* (green) in *in situ* environment. Images belong to Iñaki Iturbe-Ormaetxe from the Eliminate Dengue Program.

The peer-reviewed book series Advances in Experimental Medicine and Biology, Issue 627 - Transgenesis and the Management of Vector-Borne Disease dedicated several chapters to *Wolbachia* and Biocontrol. In Chapter 9, Bourtzis (2008) gives the artificially infected European cherry fruit fly *Rhagoletis cerasi* as an example for the use of *Wolbachia* as a population control bioagent, and based on the study of Zabalou et al. (2004), points out that egg mortality rate was %100 (%16 - %32 egg mortality on reciprocal crosses) between uninfected females and infected males. Zabalou et al. suggest that *Wolbachia*-induced CI can be used; as an alternative population control mechanism to "Sterile Insect Technique", as a tool to spread desirable genotypes among the host, to control the age structure by the effect of virulency, and to dictate asexual reproduction to the host species as a biological control agent. Brelsfoard and Dobson's (2009) also listed important approaches concerning the release of a *Wolbachia* strain into the environment for biocontrol.

An agricultural approach can be exemplified by the study of Mariño et al. (2017) that focuses on the world's biggest coffee pest, the coffee berry borer (CBB), *Hypothenemus hampei*. After applying certain doses of tetracycline and experimenting with *Wolbachia* infected CBB individuals, Mariño et al. concluded that *Wolbachia*'s effect in reproductive regulation and overall manipulation is a promising population control method that could be executed as a biological control mechanism.

2.4. Cytoplasmic Incompatibility

Cytoplasmic incompatibility (CI) occurs when an infected male copulates with a female that is uninfected or is infected, but which possesses a different *Wolbachia* strain (Perlman et al. 2008). Sperm that was produced and supplied from an infected male cannot fertilize an uninfected female or an infected female that harbours another *Wolbachia* type (Werren et al. 2008). In contrast, when both male and female are infected with the same type of *Wolbachia*, they can copulate successfully, though there is the possibility for disruptions mentioned above and the long term outcome of infestation: passing on of *Wolbachia* to the offspring since it can be transmitted vertically through the egg's cytoplasm, which leads to rapid and widespread infestation among the populations.

Apart from the effects of *Wolbachia*, partial sterility has also been observed in different strains of *T. urticae* populations. Helle and Pieterse (1965) showed a high rate of failed egg hatchability among nine *T. urticae* populations. In a more recent study, Sun et al. (2016) also stated the significantly high ratio of unhatched eggs of uninfected female x infected male crosses of the same populations, when compared to other crosses (both uninfected, both infected, infected female x uninfected male) in all three *Tetranychus urticae* populations they examined. Fry (1988) suggested that the difference between nuclear genes were responsible for the high rate of unhatched eggs produced by hybrid spider mites.

CI comprises two processes. The first process is the modification of the sperms by *Wolbachia* during spermatogenesis. The second process is the "rescue" phenomenon, which can be explained as the possibility of development for the *Wolbachia*-infested embryos that have the same strain of infection in the eggs and sperms that produced them. This system is the result of the incompatibility of an infected male crossing with an uninfected female. If this sperm does not encounter the appropriate *Wolbachia* in the egg, embryonic development is interrupted (Werren, 1997). Results of several studies (Vala, Breeuwer & Sabelis, 2000, 2003; Perrot-Minnot et al., 2002; Vala et al., 2002; Gotoh, Noda & Hong, 2003; Gotoh et al., 2007; Xie, Chen & Hong, 2011) show that *Wolbachia* induced CI can be observed in different intensities in *T. urticae*, spanning the range from no expression to complete expression, and with different types of effects as well (e.g. female embryo termination, male transformation).

Individuals can be infected by two different strains of *Wolbachia*, when these strains exist in the same habitat. Brelsfoard and Dobson (2009) explain this process in their study on multiple strains of *Wolbachia*. Figure 2.9. (from their work) explains the copulatory aspect of *Wolbachia*-induced CI

regarding one, two, or combined infections of insects with different strains. On part (a), *Wolbachia* causes a unidirectional reproductive failure when an uninfected female and an infected male copulate. The other combinations result in successful copulations. However, when there is an infection in female, it passes on to the offspring even if the male is uninfected. On the circumstance that two *Wolbachia* strains coexist and infect in the same environment (b), they cause a bidirectional reproductive failure if the counterpart sex carries the other strain. Copulation of the same strain carriers are successful and the respective infection passes on to the offspring. In this scenario, the major infector or stronger CI penetrator is expected to become dominant in the population. Finally, if there is a superinfection occurring in the population (c), the superinfected male? (infected by two strains) is incompatible with a single infected female. When females are superinfected, that superinfection is expected to spread through the population.

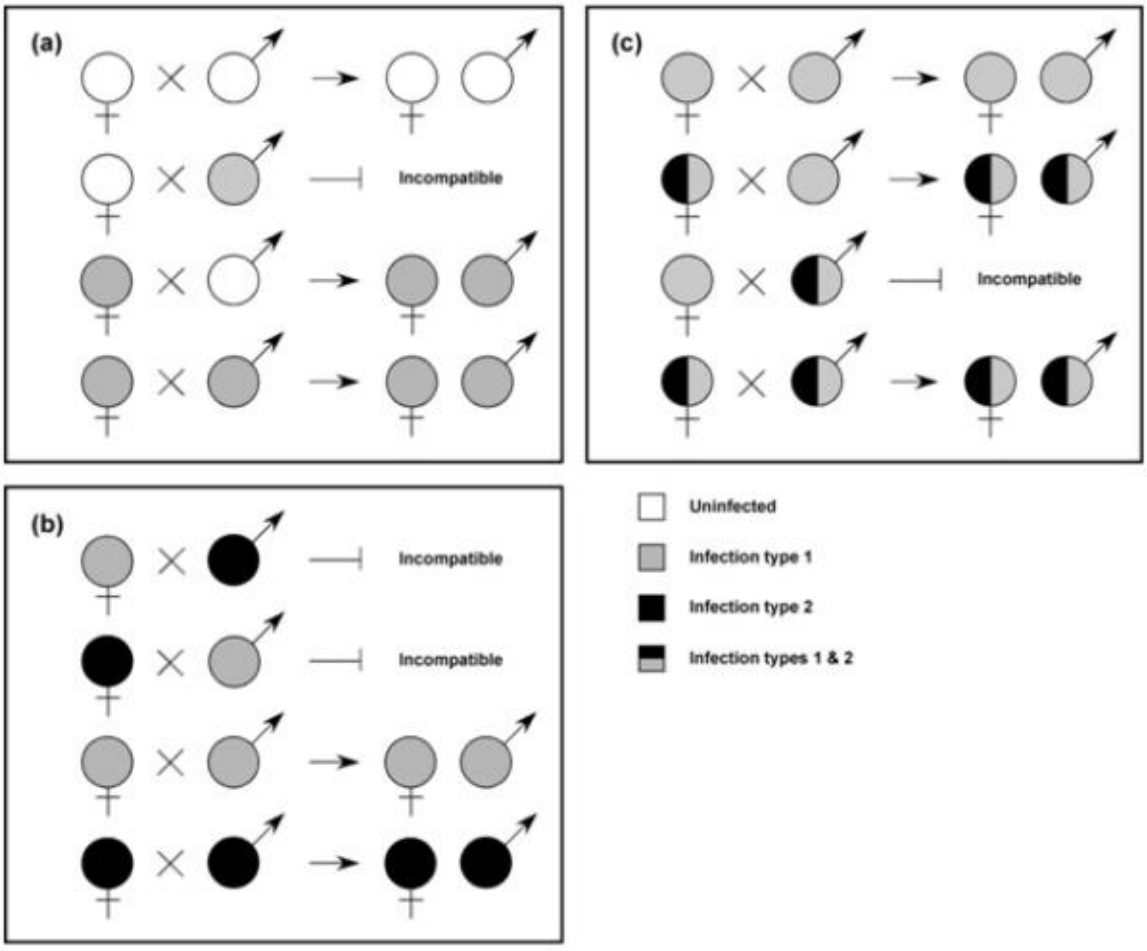


Figure 2.9. Examples of cytoplasmic incompatibility (Brelsfoard and Dobson, 2009).

Regarding the vectors' effect on *Tetranychus urticae*, Xie and colleagues' (2016) work can be a source, where they investigated the impacts of *Wolbachia*, *Cardinium* and their double infection on the two-spotted spider mites. They observed significantly increased fecundity among *Cardinium*

infected individuals, and lifetime longevity among all three types of infected *T. urtiae* females. Detailed *Cardinium* / *Wolbachia* interactions can be seen in their work.

At a cytological level, the reason for the incompatibility of crosses lies under the asynchrony of the nuclei of male and female gametes during their initial stage of mitosis (Reed and Werren, 1995). Intergenomic interactions leading to reproductive incompatibilities can also be adapted for population control by manipulation of sex ratio. Perrot-Minnot and colleagues (2004) state that introgression causes a change in female fecundity and increases the number of male spider mites and suggest that a nuclear gene might be controlling fecundity in *T. urticae*.

2.5. Aim of the Study

With this study, we want to contribute to a better understanding of mechanisms associated with *Wolbachia* related CI and host-associated CI, using a two-step experiment. Our main goals were to determine whether incompatibilities (due to genetic factors, to *Wolbachia*, and their interaction) that arise between populations are due to pre- (i.e. behavioural isolation) or post- (i.e. problems of sperm transfer or storage) copulatory mechanisms. Additionally, we wanted to see the effects of both types of reproductive barriers (*Wolbachia* related & host associated), when they occur at the same time. Finally, we wanted to understand how the results of copulation differ under the conditions of host associated (sex regulating) and *Wolbachia* related (offspring terminating) incompatibilities and determine which of these two barriers dominate when they coexist in an individual.

3. MATERIALS AND METHODS

3.1. Spider Mite Populations and Rearing

In this experiment, two populations of spider mites were studied: a green coloured population and a red coloured population of *Tetranychus urticae*. The first one, traditionally referred to as "TOM", belongs to the green morph of *T. urticae* and was sampled on tomato plants (*Solanum lycopersicum* L.) in Portugal in 2010. The second one, referred to as "AMP", belongs to the red morph of *T. urticae* and was sampled on datura plants (*Datura spp*.) in Portugal in 2013. Typical females belonging to these two populations can be seen in Figure 3.1., below.



Figure 3.1. Female individuals of AMP and TOM morphs. Used by the courtesy of Gilles San Martin.

These two populations are naturally infected by *Wolbachia* and will be called hereafter AMP[+] and TOM[+], respectively. In order to obtain their *Wolbachia*-free counterpart (AMP[-] and TOM[-], respectively), both have been cured of *Wolbachia* by antibiotic treatment (Tetracycline Hydrochloride; Li et al 2014) in a period of three generations in 2014 (Zélé et al. in prep). Previous experiments conducted in S. Magalhães' laboratory have shown that AMP[+] and TOM[+] are infected by similar *Wolbachia* strains (based on the *Wolbachia* MLST; Baldo et al., 2006) that induce either a high level (c.a. 60% of daughters' embryonic mortality in incompatible crosses; Zélé et al. in prep) or no cytoplasmic incompatibility (CI), respectively, indicating an effect of the host genotype at the CI level. During the course of the experiment, each population was maintained under standard conditions (25 ± 2°C, 60% RH, 16/8 h L/D) on bean (*Phaseolus vulgaris* L.) leaves, placed on wet cotton within petri dishes.

In order to study all the possible combinations of both mite and *Wolbachia* associated incompatibilities, as well as their interaction within a single experiment, we performed the crosses outlined in the section below.

3.2. Crosses

(1) Incompatible crosses:

- TOM[-] females x AMP[-] males (mite-associated incompatible crosses)
- AMP[-] females x AMP[+] males (*Wolbachia*-associated incompatible crosses)
- TOM[-] females x AMP[+] males (both types of incompatibility)

(2) Compatible controls:

- TOM[-] females x TOM[-] males
- AMP[-] females x TOM[-] males
- AMP[-] females x AMP[-] males

To obtain a sufficient sample size, the target number for the test subjects was set as 50 successfully mated females in each cross (300 in total). From a hypothetical mating success of 50% during the first step of the experiment, a total of 600 females (covering the six types of crosses) were used in this experiment. For Test A and Test B, 600 and 367 additional males were used, respectively. Each individual that was used in Test A and Test B were sampled as virgins to ensure their desire to mate. Since the females were selected through fitness tests due to their ability to lay eggs, their number in overall crosses represents the final sample size. The various combinations of crosses are presented in Table 3.1., below. The outcomes of these crosses can be seen in Figure 3.2.

Table 3.1. Sample size for each type of cross.

	TOM[-] ♂	AMP[-] ♂	AMP[+] ♂
TOM[-] ♀	n = 100	n = 100	n = 100
AMP[-] ♀	n = 100	n = 100	n = 100

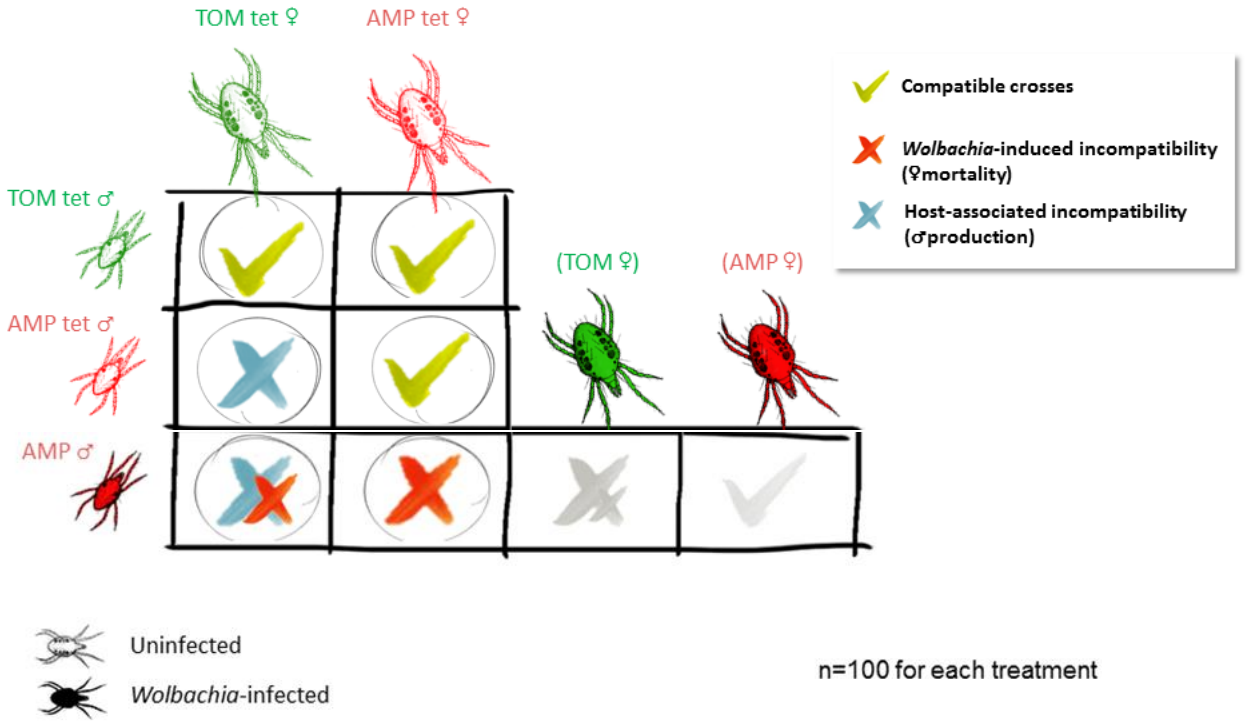


Figure 3.2. Every cross and outcome of AMP x TOM spider mite varieties. "tet" represents the mites that were treated by tetracycline antibiotics, which therefore were *Wolbachia*-free. Gray coloured symbols represent the expected copulatory success and failure for the respective crosses on the figure, but which were not studied in our experiments.

Due to the first male precedence (i.e. only the first mating of a female is successful; Helle 1967), and to avoid sperm depletion in males (males copulate 15 times the first day in average; Krainacker and Carey, 1989), we used both virgin males and females for these crosses. Virginity was also needed for a sufficient mating desire for both sexes, as mentioned previously. To ensure virginity, both males and females were isolated from our base colonies during their quiescent deutochrysalis stage one day prior to the mating event. Males and females of this species can be determined by their idiosomal width.

All individuals that were taken in their quiescent phase were grouped (TOM.tet females, TOM.tet males, AMP.tet females, AMP.tet males & AMP males), respectively and were put into separated petri dishes with wet cotton on their base, and parafilm around the dishes to prevent their escape. Therefore, all groups were isolated from each other until copulation.

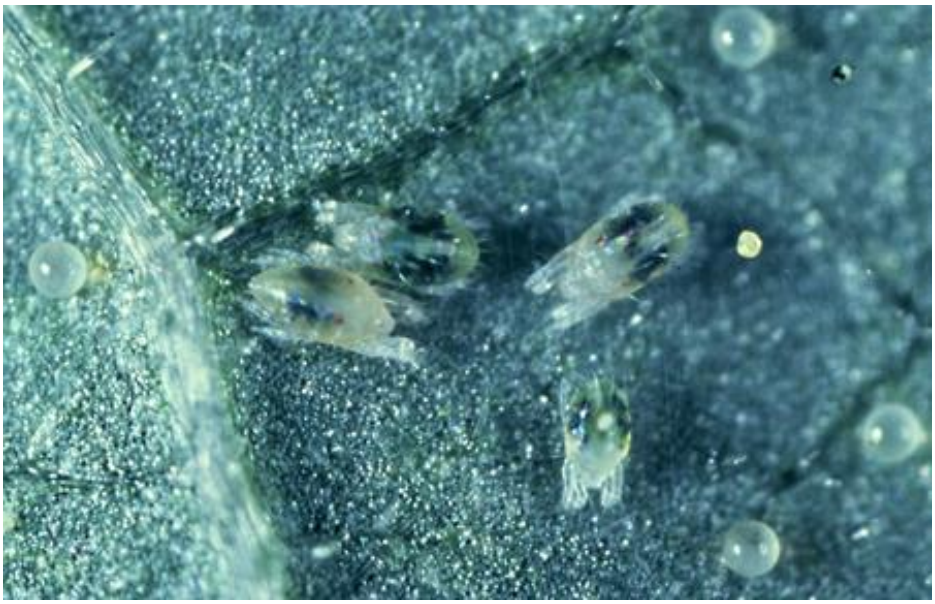


Figure 3.3. The quiescent stage (deutochrysalis) of *T. urticae*. Taken from Mid-Florida Research & Education Centre.

To determine whether the excess of males observed in crosses involving mite-associated incompatibility is due to pre-copulatory incompatibility, the experiment was designed in three steps. Indeed, since males are haploids in this species, these males may come either (i) from unfertilized eggs (as in compatible crosses) due to a decrease in mating proportions (i.e. behavioural avoidance of mating) or due to fertilization problems (i.e. problems of sperm transfer, storage and/or gamete recognition); or (ii) from post-zygotic haploidization of the eggs following fertilization (i.e. paternal genome elimination in fertilized eggs).

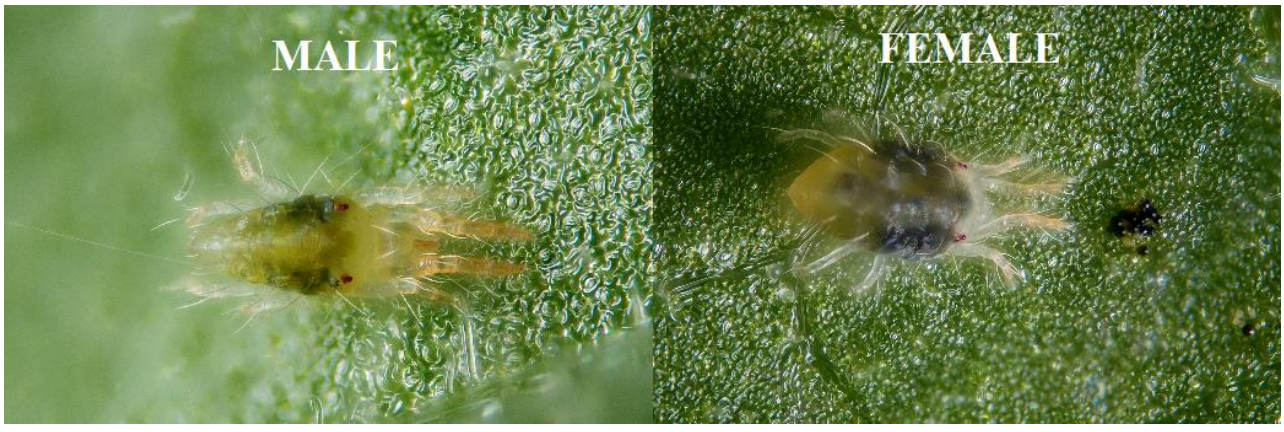


Figure 3.4. Size and Body Difference of Green Morph (TOM) of *T. urticae.* Used by the courtesy of Gilles San Martin.

3.3. Experimental Procedure

3.3.1. First Mating Event

To detect potential problems during mating (e.g. male-female recognition and/or copulatory organ dysfunction before and during sperm transfer), behavioural observations of the copulation events were conducted for all of the crosses mentioned above. Briefly, we installed both males and females of a given cross on a bean leaf disc (surface area ~0.5 cm^2) and recorded three important variables of the mite mating behaviour: (1) frequency of mating, (2) mating latency, and (3) mating duration. Mating frequency was then analysed in two ways: (i) the probability of mating, which corresponds to whether the mites mated or not, and (ii) the number of mating events during the time of observation. Mating latency corresponds to the time taken by the mites between the moment they were installed onto the leaf and the copulation event. Finally, mating duration is the (cumulative) time spent by the mites to copulate during the whole duration of the test. The entire test was conducted in blocs of nine simultaneous crosses observed continuously under the stereoscope for 60 minutes. Four blocs took place per day for a total of 19 blocs.

3.3.2. Second Mating Event

To determine whether the first mating was successful or not, we performed a double-mating test. This test assumes first-male sperm precedence in *T. urticae* and decreased female receptivity to a second mate if the first mating was successful: only when the first male has not delivered its sperm will the second copulation effectively contribute to fertilization (Helle, 1967). Thus 24 hours after the first mating event (Table 3.2.), females that had mated in the first mating event were paired with a compatible male (i.e. a *Wolbachia*-free male from their own population; TOM[-] and AMP[-] males for TOM[-] and AMP[-] females, respectively). Behavioural observations and measurements were conducted under the same criteria as those previously described for the first mating event.

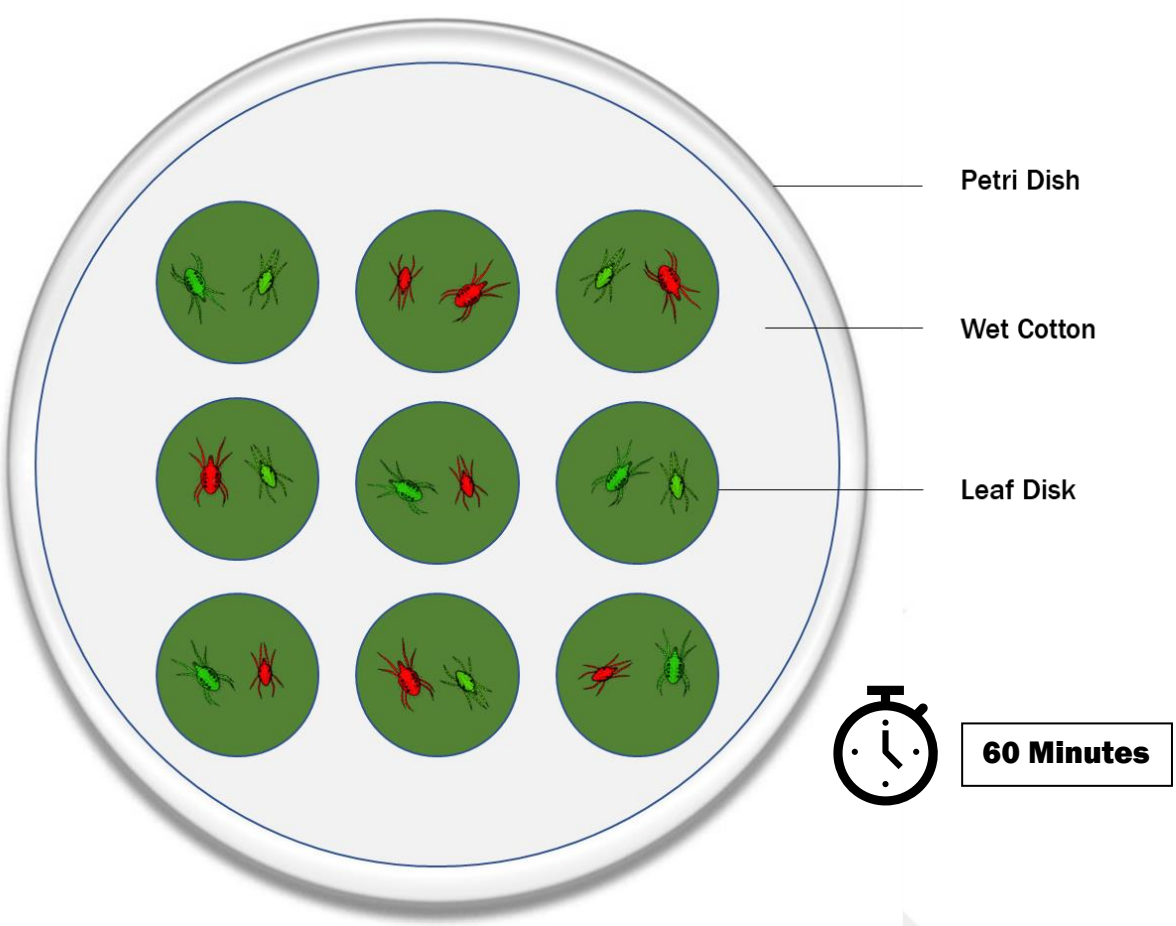


Figure 3.5. An illustrated bloc.

3.3.3. Fitness Measurements

To determine potential CI and/or any other incompatibilities between populations, we measured the fecundity of the females, the hatching rate of the eggs, and the sex-ratio of the offspring for each cross. All successfully mated females from the first step (independently of whether they mated or not during the second step) were placed under controlled conditions (25 ± 2°C, 60% RH, 16/8 h L/D) right after the second step, and were allowed to lay eggs on 2cm^2 bean leaf discs placed on wet cotton during the next three days. Every day, the females were controlled to record mortality. This information allowed to compute the daily fecundity. After three days, females were taken out from the petri dishes and the number of eggs were counted under the stereoscope (Fecunditiy (Fec) = total number of eggs divided by the total number of days the females are alive, in the first three days after the second mating event). Unhatched eggs and young adult males & females were counted 6 and 10 days later, respectively (Table 3.2.). Any *Wolbachia*-induced incompatible mating was spotted by a significant increase in embryonic mortality (i.e. an increase in the proportion of unhatched eggs), while host-associated incompatible matings resulted in a decrease in female offspring production (i.e. male-biased sex ratio). Differences between single- and multiple-mated females' offspring for these

traits revealed whether the first mating events involving incompatible males were successful. An overview of the procedure can be seen in Figure 3.6. The table of maintenance (Table 3.2.) is presented below, as well.

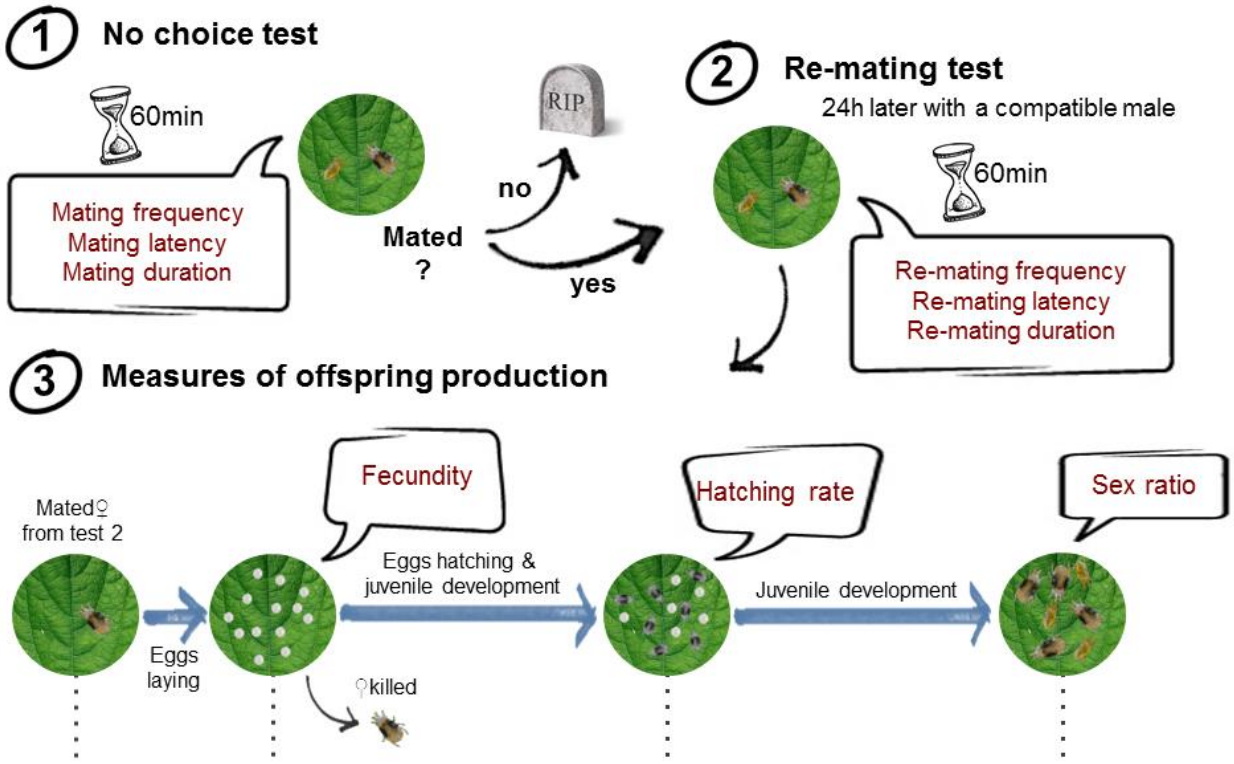


Figure 3.6. Overall experimental design.

Table 3.2. Summary of the experimental procedure.

1) Isolation of quiescent individuals	Day 1
2) First mating	Day 3
3) Second mating	Day 4
4) Counting total eggs (removing the female)	Day 7
5) Counting unhatched eggs	Day 13
6) Counting the offspring based on their sex	Day 15

3.4. Statistical Analyses

All analyses were carried out using the R statistical package (v. 3.2.0). The general procedure for building the statistical models used to analyse the interaction between the effects of *Wolbachia*-induced CI and the host-associated reproductive incompatibility was as follows: the type of males (TOM.tet, AMP.tet or AMP) and of females (TOM.tet or AMP.tet) tested were set as fixed explanatory variables, whereas "observation" was set as random explanatory variables.

The proportion of matings (i.e. mating probability) for the two tests (no choice and re-mating tests), the embryonic mortality (i.e. proportion of unhatched eggs), and the proportion of both daughters and sons in the brood produced from each cross, were analysed using a generalized linear mixed model with a binomial error distribution (glmer, lme4 package) (Bates et al., 2015). The number of mating, the latency to copulation, and the duration of copulation were observed to be greatly overdispersed. One way of handling this overdispersion is through the use of pseudo distributions (Crawley, 2007). However, to our knowledge, it is not currently possible to account for quasi distributions within a mixed model *glmer* procedure. For this reason, we instead used a *glm* model with a quasipoisson error distribution and we fitted "observation" as fixed factors, next to our variables of interest. Using fixed rather than mixed models results in some loss of statistical power, but the results are likely to be conservative, especially when the random factors consist of a few levels (Bolker, 2008).

When the variable "male" was found to be significant, a stepwise *a posteriori* procedure was carried out for the contrasts between male types (Crawley, 2007). Similarly, when a significant interaction was found between female and male, a new factor "type cross" including all the combinations tested here (six crosses) was created, and *a posteriori* contrasts between these crosses were carried out by aggregating factor levels together and by testing the fit of the simplified model using an ANOVA.

For all analyses described here, maximal models were simplified by sequentially eliminating non-significant terms to establish a minimal model (Crawley, 2007), and the significance of the explanatory variables was established using chi-squared tests (Bolker, 2008). The significant χ^2 values given in the text are based on the minimal model of Crawley (2007).

In our analyses of Offspring Production, we preferred to use offspring numbers rather than sex ratios of the offspring. This allowed us to keep the input category of "unhatched" for all six crosses,

since it is very important for comparing *Wolbachia*'s effect among the crosses. Our general average of unhatched eggs is 15%. However, this value goes up to 21% in individuals that laid one and two eggs. This significant difference would cause a bias in our statistical analyses. We have a normal distribution of produced eggs, with 14 eggs in average among nearly 300 mated females that produced at least one egg. In this regard, using the sex ratios in the cases of eggs laid in low numbers (for instance in our study there are 23 fertile test subjects that laid ≤2 eggs) would give us a biased distribution in terms of the sexes of the offspring. If the sex ratio of the offspring per test subject (mated female mites) was taken into account, the eggs that were laid in few numbers would cause biased changes in our results. For instance, in a scenario where two eggs were produced by one mite, two unhatched eggs would give us a ratio of 100% unhatched, 0% sons, 0% daughters. Likewise, two sons or two daughters hatched from a similar scenario would result in 100% sons or 100% daughters respectively, and decrease the overall unhatched egg ratio immensely with the 0% value. We tried to avoid these kind of biased percentages, in order to obtain more balanced results. Therefore, the sex ratio based calculation was not ideal in our study, as it would result in an uneven distribution, especially with fewer numbers of laid eggs. Since the overall sex/unhatched distribution guided us on the effects of *Wolbachia* and host-associated incompatibilities, individual based calculation was a better fit for our research for being more stable and dependable. Even though it is not a focus point for this study, the lower hatching percentage among fewer laid eggs might have been caused by malnutrition (Kliewer, 1961), the abnormal secretion of juvenile hormones (Cabrera et al., 2009) or inbreeding depression (Welle, 1965; Saito et al., 2000).

A larger sample size, obviously, is more representative for statistical analyses. We did not choose to exclude these mites that produced fewer eggs, but we chose to calculate the offspring averages based on actual numbers of offspring (i.e. the numbers of sons, daughters and unhatched eggs produced by each mating for all crosses), instead of a -sex ratio- in our Offspring Production calculation, which resulted in less-biased and consistent results. For instance, after calculating the numbers of offspring cumulatively in each cross, we calculated an overall percentile value as, for instance; 30% sons, 60% daughters, 10% unhatched for all six crosses we studied, in a consistent manner.

Finally, the high N/A data (roughly 50%) in our appendix is due to our prediction of 50% mating success, and our aim to collect data from 300 females, (50 for each cross), as explained above in section 3.2. Crosses, page 16. As we foresaw this issue, we ran the experiment with 600 females, about half of which provided the data we needed, and the other half was in the N/A category, as expected.

4. RESULTS

4.1. No Choice Test

Overall, we did not find any significant interaction between the types of females and males used on the proportion of mating ($\chi^2_2 = 1.31$, $p = 0.52$). However, TOM.tet females mated circa 7% less than AMP.tet females (females effect: $\chi^2_1 = 4.84$, $p = 0.03$). Males also affected the proportion of mating significantly ($\chi^2_2 = 6.33$, $p = 0.04$). Indeed, the contrast analyses conducted *a posteriori* between males revealed that both AMP and TOM.tet males, and TOM.tet and AMP.tet males mated equally ($\chi^2_1 = 1.56$, $p = 0.21$ and $\chi^2_1 = 1.63$, $p = 0.20$, respectively), however AMP males mated less than AMP.tet males ($\chi^2_1 = 6.33$, $p = 0.01$).

Similarly, we did not find a significant interaction between the types of females and males used, in terms of the number of matings ($\chi^2_2 = 0.35$, $p = 0.84$). Here we did not find any differences between the number of matings of TOM.tet and AMP.tet females ($\chi^2_1 = 0.62$, $p = 0.44$), however males exhibited significantly different numbers of mating ($\chi^2_2 = 9.32$, $p = 0.01$). Although both AMP and AMP.tet males, and TOM.tet and AMP.tet males mated equally (Contrast analyses: $\chi^2_1 = 2.03$, $p = 0.16$ and $\chi^2_1 = 3.00$, $p = 0.09$, respectively), AMP males mated less than TOM.tet males ($\chi^2_1 = 9.22$, $p = 0.003$).

Only females differed significantly in their latency to copulation, with AMP.tet females being faster to mate than TOM.tet females ($\chi^2_1 = 9701.2$, $p < 0.001$), independently of the male with which they mated (male effect: $\chi^2_2 = 119.47$, $p = 0.93$; interaction female-male: $\chi^2_2 = 247.38$, $p = 0.85$).

Conversely, only males significantly affected the duration of copulation ($\chi^2_2 = 585.44$, $p < 0.05$), independently of the female with which they mated (female effect: $\chi^2_1 = 182.9$, $p = 0.16$; interaction female-male: $\chi^2_2 = 68.63$, $p = 0.69$). Indeed, the contrast analyses revealed that TOM.tet males mated longer than AMP ones ($\chi^2_1 = 533.88$, $p = 0.02$), and that AMP.tet males mated with an intermediate duration (contrast between AMP.tet and TOM.tet males: $\chi^2_1 = 266.15$, $p = 0.10$; contrast between AMP.tet and AMP males: $\chi^2_1 = 68.00$, $p = 0.40$). The results can be seen on Figure 4.1. in four categories as Mating Proportion, Number of Matings, Latency to Copulation and Duration of Copulation.

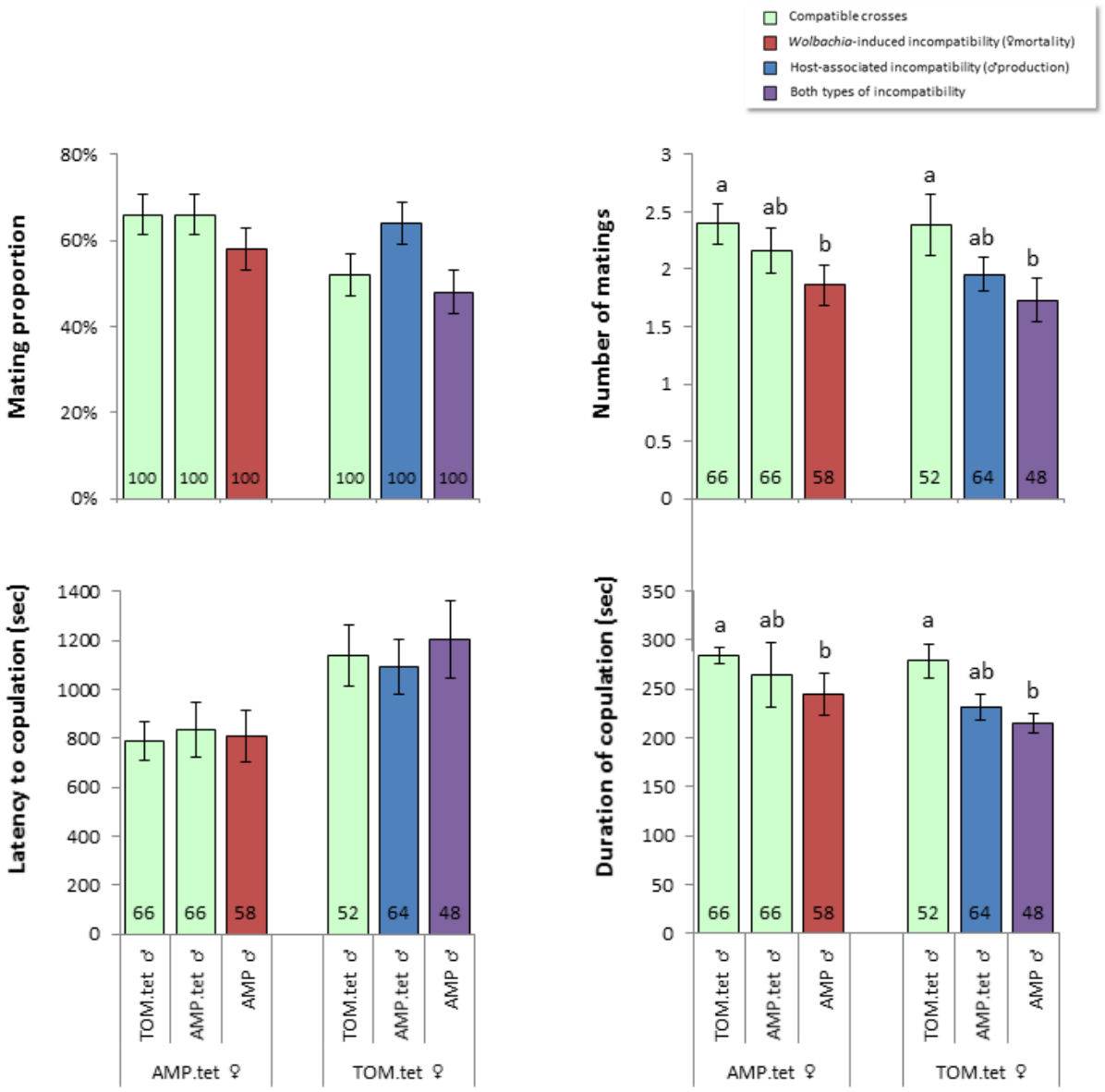


Figure 4.1. Bar graphs for the No Choice Test.

4.2. Re-Mating Test

As there is first-male sperm precedence in this species, on average, only a low proportion of the females (20% in average), which mated during the first test re-mated during this second test. In contrast with the first test, here TOM.tet females mated more than AMP.tet females (c.a. 25% vs 15%, respectively; $\chi^2_1 = 5.20$, $p = 0.02$), independently of the males (male effect: $\chi^2_2 = 0.3947$, $p = 0.82$; interaction female-male: $\chi^2_2 = 4.05$, $p = 0.13$).

However, the number of matings performed by these females during this test did not differ significantly ($\chi^2_1 = 0.05$, $p = 0.78$), and was not affected by the type of males with which they

previously mated during the first test (male effect: $\chi^2_2 = 0.79$, p = 0.53; interaction female-male: $\chi^2_2 = 0.79$, p = 0.52).

Similarly, during this re-mating test, neither the latency nor the duration of copulation was significantly different between females (latency: $\chi^2_1 = 25.47$, p = 0.83; duration: $\chi^2_1 = 2.24$, p = 0.89), independent of their first mate (male effect on latency: $\chi^2_2 = 1885.6$, p = 0.17; interaction female-male on latency: $\chi^2_2 = 2210$, p = 0.13; male effect on duration: $\chi^2_2 = 120.7$, p = 0.55; interaction female-male on duration: $\chi^2_2 = 327.05$, p = 0.15). Results of the Re-Mating Test can be seen in Figure 4.2., and are divided into the same four categories as in the No Choice Test.

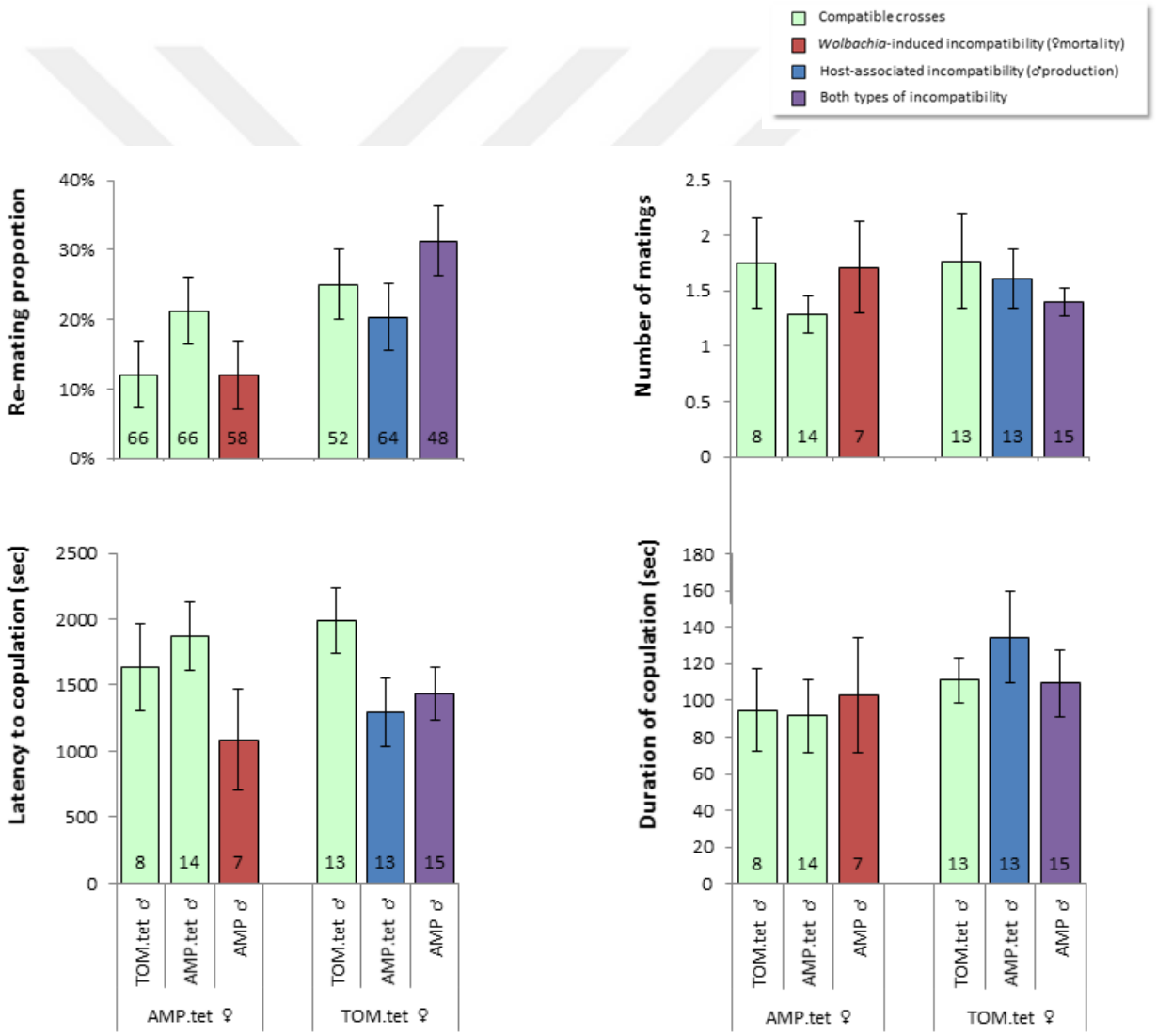


Figure 4.2. Bar graph for Re-Mating Test.

4.3. Measures of Offspring Production

The production of offspring in this experiment was strongly affected by the combinations of crosses. Results of our Offspring Production are visualised in Figure 4.3. below as Sons, Daughters and Unhatched for their respective crosses. Indeed, we found a highly significant interaction between the type of males and the type of females used for these crosses for all the variables measured here: embryonic mortality (i.e. hatching rate; $\chi^2_2 = 20.98$, $p < 0.0001$), proportion of sons ($\chi^2_2 = 36.5$, $p < 0.0001$) and proportion of daughters ($\chi^2_2 = 14.44$, $p < 0.001$).

This significant interaction comes from the two different types of incompatibility between spider mites: (1) induced by *Wolbachia* and (2) between morphs of spider mites independently of *Wolbachia*; as well as (3) from the combination of these two types of incompatibility. Contrasts analyses, performed between the different crosses on the different variables tested, revealed:

(1) An increase in embryonic mortality (c.a. 31%) of the offspring produced by red uninfected (AMP.tet) females when crossed with *Wolbachia*–infected red (AMP) males compared to any other type of male ($\chi^2_1 = 325.06$, $p < 0.0001$). Since in haplodiploid species only the females come from fertilized eggs (males are haploids), this increase of embryonic mortality severely affected only the proportion of daughters here ($\chi^2_1 = 179.29$, $p < 0.0001$).

(2) A drastic increase in the proportion of males (c.a. 24%) of the offspring produced by green *Wolbachia*–uninfected (TOM.tet) females when crossed with uninfected red (AMP.tet) males, compared to uninfected green (TOM.tet) males ($\chi^2_1 = 27.76$, $p < 0.0001$). This effect is obviously associated with a decrease in the proportion of females ($\chi^2_1 = 34.02$, $p < 0.0001$), but not with an increase in embryonic mortality ($\chi^2_1 = 0.52$, $p = 0.47$).

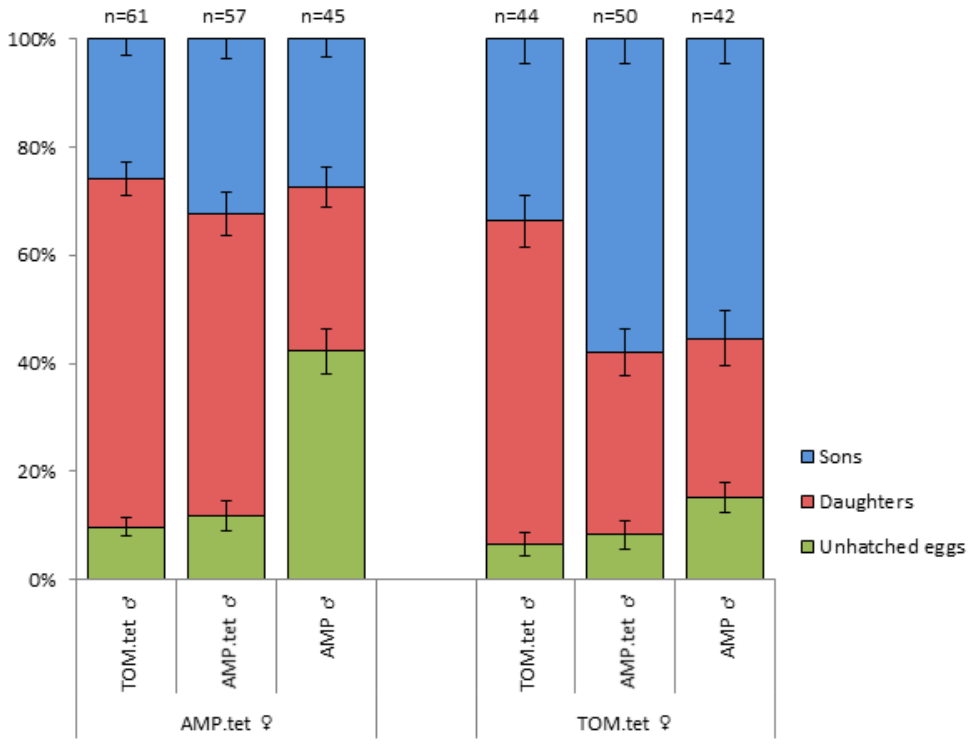


Figure 4.3. Bar graph for Offspring Production.

(3) When both types of incompatibility are combined in the cross between green *Wolbachia*–uninfected (TOM.tet) females and red *Wolbachia*–infected red (AMP) males, we can see a strong reduction of the effect of *Wolbachia* on embryonic mortality (contrast with AMP.tet females crossed with AMP males: $\chi^2_1 = 101.05$, $p < 0.0001$), although the embryonic mortality is slightly higher than in the absence of *Wolbachia* (contrast with TOM.tet females crossed with AMP.tet males: $\chi^2_1 = 39.89$, $p < 0.0001$). Conversely, the presence of *Wolbachia* in males did not significantly affect the proportion of sons produced in this host-associated incompatible kind of cross (contrast with TOM.tet females crossed with AMP.tet males: $\chi^2_1 = 3.54$, $p = 0.06$).

Finally, it should be noted that whether the females re-mated with a compatible male during the second test or not did not affect the production of offspring in this experiment. The mating pattern (mating only during the first test or during both tests) did not increase the fit of any of our statistical models built for hatching rate ($\chi^2_1 = 2.83$, $p = 0.09$), proportion of sons ($\chi^2_1 = 3.48$, $p = 0.06$) or proportion of daughters ($\chi^2_1 = 0.66$, $p = 0.42$). This latter result corroborates previous results showing first-male sperm precedence in *T. urticae*, and indicates that the second copulation event was ineffective even in compatible crosses.

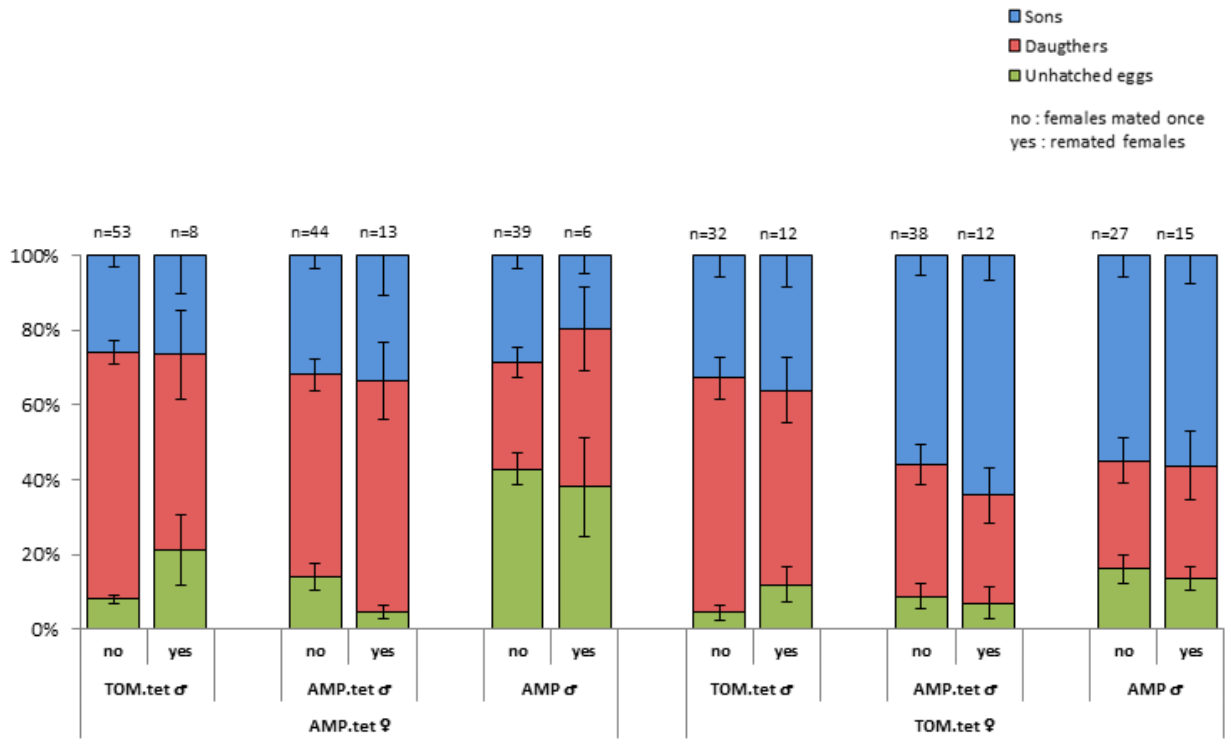


Figure 4.4. Bar graph for Offspring Production comparing the first and second matings.

On Figure 4.4. above, the results of the Offspring Production were divided in two sub-categories as “yes” and “no”, for whether they mated for the second time in the Re-Mating Test (yes) or not (no) in their respected crosses. Sum of the number of test subjects (n) for each cross, corresponds to the total number of female spider mites that were used in their respective cross.

5. DISCUSSION

As we can see from our No Choice Test, there are no significance of interactions between two sexes as a "preferable match" on the proportion of mating. Thus, these data suggest no behavioural repulse between TOM.tet ♀ x AMP.tet ♂, which can be categorized as host-associated incompatibility. We can interpret that AMP.tet individuals of both sexes mated more than TOM.tet individuals and AMP males. Fewer number of mated AMP males, when compared to AMP.tet males might be a sign of the healthy female identifying a *Wolbachia* infected male, and trying to avoid copulation. This can be investigated further by a new experimental design that compares the preferences of TOM and TOM.tet males for copulation with TOM.tet (uninfected) females, in a possible future study. Independently from the males' effect, only females showed significance in latency during copulation. AMP.tet females also approached males more quickly when compared to TOM.tet females, for copulation.

On the contrary to the latency, for duration of copulation only males showed their effect, independent of females. TOM.tet males mated longer than AMP males. AMP.tet males were observed as intermediate in terms of the duration of copulation, out of all three male types. This is a similar outcome to AMP males being preferred less than AMP.tet males for copulation, as indicated in our results on proportions. This might be an indication of females identifying the infected male and trying to avoid contact.

Our Re-Mating Test did not give significant results, which was parallel to the expectations based on Helle (1967). Since spider mites' reproductive processing is in favour of the first male precedence, we observed fewer copulations attempt (20% in average). We were also able to verify this with our experiment, by using control males (healthy [.tet] males for the respective population) for the second mating attempt. An interesting point about this experiment was the copulation interest being in the opposite direction when compared to the previous test, as TOM.tet females mated more than AMP.tet females, independently of the males.

Corroborating with previous knowledge, we also observed the first sperm precedence in our experiment. The second mating event did not affect the production of offspring. The mating pattern (mating only in the first or also in the second test) did not increase the fit of the hatching rate, proportion of sons or proportion of daughters. This is another indication that the second copulation was ineffective, even when the crosses were compatible. Also, the number of matings did not differ

significantly, and the latency of the copulation and the duration of copulation was not different between females.

Our post-copulatory maintenance and observations, summarized as “Measures of Offspring”, showed highly significant interactions for the following variables between the type of males and females that we used in our crossings:

1. Embryonic Mortality
2. Proportion of Sons
3. Proportion of Daughters

The reason for this significant interaction originates from two types of compatibility and their combination that is outlined in Figure 5.1. as follows:

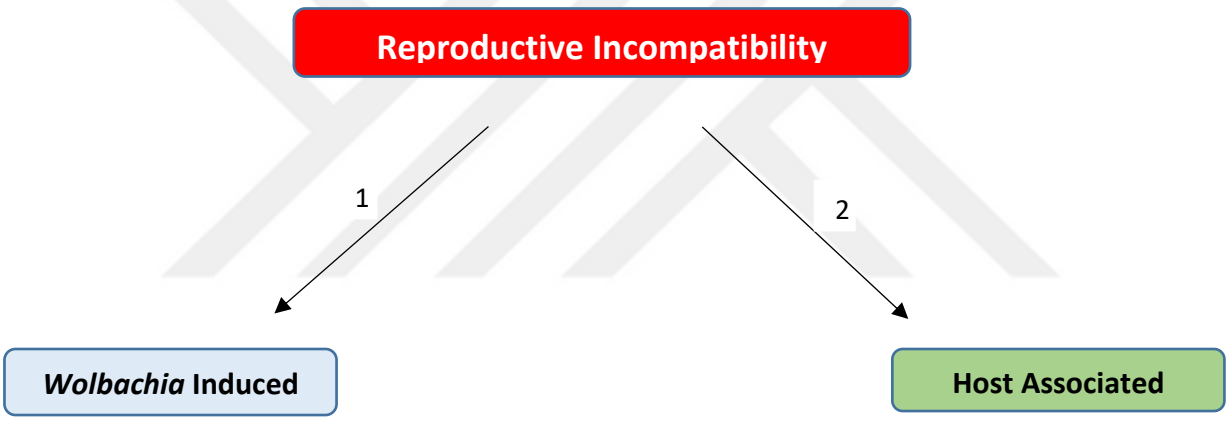


Figure 5.1. Reproductive incompatibility chart of spider mite.

Proportion of Daughters can be considered a proxy for successful mating. In our results, the largest proportion of daughters can be observed in AMP.tet ♀ x AMP.tet ♂ and AMP.tet ♀ x TOM.tet ♂ crosses (Figure 4.3.). Larger number of female offspring is the outcome of a compatible cross in *T. urticae*.

In our experiment, our hatching rate results provided us with data on *Wolbachia* induced incompatibility (third bar in Figure 4.3.). For the high embryonic mortality (defined as eggs which were successfully produced, yet remained unhatched), we expected our cross AMP.tet ♀ x AMP ♂ to provide us the highest values, due to the effect of *Wolbachia*. This cross produced circa 42% unhatched eggs with high significance, compared to the other male types ($p < 0.0001$). This result suggests us that the absence of offspring is not due to unfertilized eggs. Unfertilized eggs should have produced an increased number of male offspring. Instead, during the development of the embryo, the

offsprings of this cross were terminated by *Wolbachia'*s effect. Therefore, a high number of eggs remained unhatched. As stated in the literature review section above, *Wolbachia*-induced CI works as a correlative two-step mechanism. On the first step, *Wolbachia* modifies the sperm during spermatogenesis. On the second step, the same *Wolbachia* strain must be present in the developing embryo. Therefore, for a modified sperm, an infected egg (with the same strain) must be present for successful fertilization.

As mentioned before, two-spotted spider mite is an arrhenotokous species. Unfertilized eggs produce haploid males, thus indicates the asexual reproduction in the species. Therefore, high frequencies of male offspring signal failed sexual reproduction. We can clearly see the highest male offspring frequencies in the cross TOM.tet ♀ x AMP.tet ♂. Alhough both test subjects were clean of *Wolbachia*, they produced significantly more males than any other cross by circa 42%. Their opposite cross AMP.tet ♀ x TOM.tet ♂ produced much higher levels of female offspring than males. The number of unhatched eggs was very similar in these two crosses. Another important result is the similarity of the female production of AMP.tet ♀ x TOM.tet ♂ cross, when compared to the ideal AMP.tet ♀ x AMP.tet ♂ cross. Finally, when we compare the number of unhatched eggs in the host-associated incompatible cross of TOM.tet ♀ x AMP.tet ♂ and the ideal cross of AMP.tet ♀ x AMP.tet ♂, we observed lower levels of embryonic mortality in the host-associated incompatible cross. These results suggest a strong possibility of a unidirectional reproductive incompatibility between TOM.tet females and AMP.tet males, confirming the previous study of Zélé et al. (in prep).

The last type of incompatibility we studied is the combined (*Wolbachia* induced and host associated) kind. We observed the results of this case through our cross TOM.tet ♀ x AMP ♂. Here, the female is a tetracycline treated TOM and the male is a *Wolbachia* infected AMP. In our results, we detected the significant reduction of embryonic mortality caused by *Wolbachia* that we observed on the intra-population cross of AMP.tet ♀ x AMP ♂ ($p < 0.0001$). When we compare the incompatible cross of TOM females with AMP males for both infected and treated varieties of *T. urticae*, we can see that the existence of *Wolbachia* slightly increased the embryonic mortality of the offspring. Even though the number of unhatched eggs differed slightly, son/daughter ratio seemed to remain similar as that in the host-associated incompatible cross.

In another study, Werren et al. (2008) also presumed that the interactions with *Wolbachia* can lead to the evolution of the host species, potentially even into a new species, stating the existence of supporting empirical and theoretical evidence. Gottlieb & Zchori-Fein (2001) state that parthenogenesis- inducing bacteria contributed to the evolution of parthenogenetic insect species due

to the loss of their sexually reproduction abilities, after being cured of bacteria. Thus, *Wolbachia* can indeed act as an evolutionary accelerator.

6. CONCLUSIONS AND RECOMMENDATIONS

In this study, we examined the behavioural display of two *Tetranychus urticae* populations, namely TOM.tet females x AMP.tet males in order to better understand if the unidirectional incompatibility originates before copulation, through repulsive behaviour. We also measured the fitness of the offsprings in all the crosses, in order to compare the different CI levels (host-associated CI vs. *Wolbachia* Related CI) when double cytoplasmic incompatibility occurs, and analyse which of these two CI types happen at a higher frequency.

Conclusions of this study are given below:

1. There were no significant differences between the interactions of healthy individuals of any population in terms of proportion, frequency latency and duration of the mating. Therefore, we think the host-associated incompatibility origins from postzygotic mechanisms.

2. Our results confirmed the existence of host-associated unidirectional incompatibility and the fertilization capability of the first sperm, as recorded in previous studies.

3. Host-associated incompatibility causes a very significant increase on proportion of males and *Wolbachia*- associated incompatibility causes a very significant increase on embryonic mortality.

4. Our results showed a highly significant interaction between the type of males and the type of females used for these crosses for all the variables measured as embryonic mortality (i.e. hatching rate; $\chi^2_2 = 20.98$, $p < 0.0001$), proportion of sons ($\chi^2_2 = 36.5$, $p < 0.0001$) and proportion of daughters ($\chi^2_2 = 14.44$, $p < 0.001$). When double cytoplasmic incompatibility occurs, host-associated CI surpasses *Wolbachia*-associated CI's effect, resulting in increased numbers of male offspring.

As a follow-up to this study, we suggest speciation related experiments, which can be executed on hybrid offspring. In this study, the AMP.tet ♀ x TOM.tet ♂ crosses provided the only compatible inter-population hybrids, which were somewhat disfigured, with longer extremities and idiosomal lengths. The hybrids' fertility capacity can be investigated, and if they are indeed capable of reproduction, further genetic analyses can be carried out for a better understanding of the speciation process.

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APPENDIX A - SUPPLEMENTARY DETAILED OBSERVATION DATA

Bloc	Name	Treat.	♀	TestA♂	Amtd+	TestB♂	Bmtd+	Eggs	Unhctd	Dghtrs	Sons	Jvnls
1.1	1	AT	A	T	1	A	0	10	1	3	2	4
1.1	2	AA+	A	A+	0	NA	NA	NA	NA	NA	NA	NA
1.1	3	AA-	A	A-	1	A	0	8	0	5	2	1
1.1	4	TA+	T	A+	1	T	0	14	0	0	11	3
1.1	5	TT	T	T	1	T	0	16	0	11	5	0
1.1	6	TA-	T	A-	0	NA	NA	NA	NA	NA	NA	NA
1.1	7	AA+	A	A+	0	NA	NA	NA	NA	NA	NA	NA
1.1	8	TA-	T	A-	1	T	0	33	0	22	11	0
1.1	9	TA+	T	A+	1	T	0	29	4	3	11	11
1.2	10	TA-	T	A-	0	NA	NA	NA	NA	NA	NA	NA
1.2	11	AA+	A	A+	1	A	0	10	6	1	2	1
1.2	12	TA+	T	A+	0	NA	NA	NA	NA	NA	NA	NA
1.2	13	TT	T	T	1	T	0	0	NA	NA	NA	NA
1.2	14	AA-	A	A-	0	NA	NA	NA	NA	NA	NA	NA
1.2	15	AT	A	T	1	A	0	7	0	4	3	0
1.2	16	TT	T	T	1	T	0	3	0	1	2	0
1.2	17	AA-	A	A-	0	NA	NA	NA	NA	NA	NA	NA
1.2	18	AT	A	T	1	A	0	2	2	NA	NA	NA
2.1	19	AA-	A	A-	1	A	0	2	1	0	1	0
2.1	20	TA-	T	A-	1	T	0	13	0	6	5	2
2.1	21	TT	T	T	0	NA	NA	NA	NA	NA	NA	NA
2.1	22	AT	A	T	1	A	0	3	0	0	2	1
2.1	23	AA+	A	A+	1	A	0	30	13	8	4	5
2.1	24	TA+	T	A+	1	T	0	19	0	0	19	0
2.1	25	TT	T	T	1	T	0	4	0	4	0	0
2.1	26	AA+	A	A+	0	NA	NA	NA	NA	NA	NA	NA
2.1	27	TA+	T	A+	1	T	1	15	2	0	13	0
2.2	28	TA+	T	A+	1	T	0	9	0	0	9	0
2.2	29	AA-	A	A-	0	NA	NA	NA	NA	NA	NA	NA
2.2	30	AT	A	T	1	A	0	19	2	10	4	3
2.2	31	TT	T	T	1	T	0	3	0	1	0	2
2.2	32	AA+	A	A+	1	NA	NA	NA	NA	NA	NA	NA
2.2	33	TA-	T	A-	1	NA	NA	NA	NA	NA	NA	NA
2.2	34	TA-	T	A-	1	T	0	14	0	6	5	3
2.2	35	AA-	A	A-	0	NA	NA	NA	NA	NA	NA	NA
2.2	36	AT	A	T	1	A	0	5	0	3	2	0
2.3	37	TA+	T	A+	0	NA	NA	NA	NA	NA	NA	NA
2.3	38	AA-	A	A-	1	A	0	12	0	3	5	4
2.3	39	AA+	A	A+	0	NA	NA	NA	NA	NA	NA	NA
2.3	40	TT	T	T	0	NA	NA	NA	NA	NA	NA	NA

2.3	41	AT	A	T	0	NA	NA	NA	NA	NA	NA	NA
2.3	42	TA-	T	A-	0	NA	NA	NA	NA	NA	NA	NA
2.3	43	AT	A	T	1	A	0	19	2	10	4	3
2.3	44	TT	T	T	0	NA	NA	NA	NA	NA	NA	NA
2.3	45	AA+	A	A+	1	A	0	22	14	4	4	0
2.4	46	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2.4	47	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2.4	48	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2.4	49	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2.4	50	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2.4	51	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2.4	52	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2.4	53	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2.4	54	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
3.1	55	TT	T	T	0	NA	NA	NA	NA	NA	NA	NA
3.1	56	AA-	A	A-	1	A	0	13	0	8	2	3
3.1	57	AA+	A	A+	1	A	0	19	9	5	5	0
3.1	58	AT	A	T	1	A	1	16	2	12	2	0
3.1	59	TA-	T	A-	1	T	0	8	1	0	7	0
3.1	60	TA+	T	A+	1	T	0	0	NA	NA	NA	NA
3.1	61	TA-	T	A-	1	T	0	22	0	6	15	1
3.1	62	AA-	A	A-	1	A	0	13	0	8	3	2
3.1	63	TT	T	T	1	T	0	13	0	10	3	0
3.2	64	TA-	T	A-	0	NA	NA	NA	NA	NA	NA	NA
3.2	65	AA-	A	A-	0	NA	NA	NA	NA	NA	NA	NA
3.2	66	AT	A	T	1	A	0	18	0	14	3	1
3.2	67	AA+	A	A+	1	A	0	2	0	2	0	0
3.2	68	TT	T	T	1	T	0	13	0	9	2	2
3.2	69	TA+	T	A+	0	NA	NA	NA	NA	NA	NA	NA
3.2	70	AT	A	T	0	NA	NA	NA	NA	NA	NA	NA
3.2	71	TA+	T	A+	1	T	0	0	NA	NA	NA	NA
3.2	72	AA+	A	A+	1	A	0	15	8	5	2	0
3.3	73	AA+	A	A+	0	NA	NA	NA	NA	NA	NA	NA
3.3	74	TA+	T	A+	0	NA	NA	NA	NA	NA	NA	NA
3.3	75	TT	T	T	0	NA	NA	NA	NA	NA	NA	NA
3.3	76	AA-	A	A-	1	A	0	1	0	0	1	0
3.3	77	AT	A	T	0	NA	NA	NA	NA	NA	NA	NA
3.3	78	TA-	T	A-	1	T	0	0	NA	NA	NA	NA
3.3	79	AA-	A	A-	0	NA	NA	NA	NA	NA	NA	NA
3.3	80	TA-	T	A-	0	NA	NA	NA	NA	NA	NA	NA
3.3	81	TA+	T	A+	0	NA	NA	NA	NA	NA	NA	NA
4.1	82	TT	T	T	0	NA	NA	NA	NA	NA	NA	NA
4.1	83	AA-	A	A-	1	A	1	11	1	9	1	0
4.1	84	AT	A	T	1	A	0	13	1	10	2	0
4.1	85	TA-	T	A-	1	T	0	16	0	13	2	1
4.1	86	AA+	A	A+	1	A	0	1	0	0	1	0

4.1	87	TA+	T	A+	1	T	0	14	4	5	4	1
4.1	88	TA+	T	A+	0	NA	NA	NA	NA	NA	NA	NA
4.1	89	TT	T	T	0	NA	NA	NA	NA	NA	NA	NA
4.1	90	AT	A	T	0	NA	NA	NA	NA	NA	NA	NA
4.2	91	AT	A	T	0	NA	NA	NA	NA	NA	NA	NA
4.2	92	TA-	T	A-	1	T	0	5	0	5	0	0
4.2	93	TA+	T	A+	1	T	1	10	3	0	7	0
4.2	94	AA+	A	A+	1	A	1	3	1	1	1	0
4.2	95	AA-	A	A-	1	A	0	14	3	6	4	1
4.2	96	TT	T	T	0	NA	NA	NA	NA	NA	NA	NA
4.2	97	AA+	A	A+	0	NA	NA	NA	NA	NA	NA	NA
4.2	98	TA-	T	A-	1	T	0	4	0	2	1	1
4.2	99	AA-	A	A-	1	A	0	15	1	10	4	0
4.3	100	AA+	A	A+	0	NA	NA	NA	NA	NA	NA	NA
4.3	101	TT	T	T	1	T	0	7	0	1	6	0
4.3	102	TA-	T	A-	1	T	0	14	0	6	6	2
4.3	103	AA-	A	A-	1	A	0	5	4	0	1	0
4.3	104	AT	A	T	1	A	0	3	0	3	0	0
4.3	105	TA+	T	A+	1	T	1	1	0	1	0	0
4.3	106	TT	T	T	1	T	0	14	4	2	8	0
4.3	107	AA+	A	A+	1	A	0	25	8	10	3	4
4.3	108	AT	A	T	1	A	0	0	NA	NA	NA	NA
4.4	109	AA-	A	A-	0	NA	NA	NA	NA	NA	NA	NA
4.4	110	TA-	T	A-	1	T	0	18	1	8	3	6
4.4	111	AA+	A	A+	1	A	0	4	3	0	1	0
4.4	112	TA+	T	A+	0	NA	NA	NA	NA	NA	NA	NA
4.4	113	AT	A	T	0	NA	NA	NA	NA	NA	NA	NA
4.4	114	TT	T	T	1	T	0	0	NA	NA	NA	NA
4.4	115	TA+	T	A+	1	T	0	6	0	3	2	1
4.4	116	TA-	T	A-	0	NA	NA	NA	NA	NA	NA	NA
4.4	117	AA-	A	A-	1	A	0	9	2	1	6	0
5.1	118	TA+	T	A+	0	NA	NA	NA	NA	NA	NA	NA
5.1	119	TT	T	T	0	NA	NA	NA	NA	NA	NA	NA
5.1	120	AA+	A	A+	1	A	0	16	9	2	5	0
5.1	121	AA-	A	A-	1	A	1	5	0	0	5	0
5.1	122	TA-	T	A-	1	T	1	22	0	15	7	0
5.1	123	AT	A	T	1	A	0	10	3	4	3	0
5.1	124	TT	T	T	1	T	1	21	5	0	16	0
5.1	125	AA-	A	A-	1	A	0	10	0	7	2	1
5.1	126	TA-	T	A-	0	NA	NA	NA	NA	NA	NA	NA
5.2	127	TA-	T	A-	1	T	0	16	3	0	13	0
5.2	128	AA-	A	A-	1	A	0	0	NA	NA	NA	NA
5.2	129	AA+	A	A+	1	A	1	9	6	1	2	0
5.2	130	TT	T	T	1	T	0	19	0	0	19	0
5.2	131	TA+	T	A+	0	NA	NA	NA	NA	NA	NA	NA
5.2	132	AT	A	T	0	NA	NA	NA	NA	NA	NA	NA

5.2	133	AA+	A	A+	1	A	1	13	1	8	4	0
5.2	134	TA+	T	A+	0	NA	NA	NA	NA	NA	NA	NA
5.2	135	AT	A	T	0	NA	NA	NA	NA	NA	NA	NA
5.3	136	AA+	A	A+	0	NA	NA	NA	NA	NA	NA	NA
5.3	137	AT	A	T	1	A	1	5	4	0	1	0
5.3	138	TA-	T	A-	1	T	0	3	2	1	0	0
5.3	139	AA-	A	A-	1	A	0	7	1	0	5	1
5.3	140	TA+	T	A+	1	T	1	19	3	1	13	2
5.3	141	TT	T	T	0	NA	NA	NA	NA	NA	NA	NA
5.3	142	TT	T	T	1	T	1	14	0	5	6	3
5.3	143	TA-	T	A-	1	T	0	10	9	0	1	0
5.3	144	TA+	T	A+	0	NA	NA	NA	NA	NA	NA	NA
5.4	145	TA-	T	A-	1	T	0	12	0	1	9	2
5.4	146	AA-	A	A-	1	A	0	0	NA	NA	NA	NA
5.4	147	TT	T	T	1	T	0	16	1	11	3	1
5.4	148	AT	A	T	1	A	0	9	1	5	2	1
5.4	149	AA+	A	A+	0	NA	NA	NA	NA	NA	NA	NA
5.4	150	TA+	T	A+	0	NA	NA	NA	NA	NA	NA	NA
5.4	151	AA-	A	A-	1	A	1	10	1	8	0	1
5.4	152	AA+	A	A+	1	A	0	2	2	0	0	0
5.4	153	AT	A	T	1	NA	NA	NA	NA	NA	NA	NA
6.1	154	AT	A	T	1	A	0	21	1	17	1	2
6.1	155	TT	T	T	1	T	1	14	1	9	1	3
6.1	156	AA+	A	A+	1	A	0	17	12	0	5	0
6.1	157	TA+	T	A+	0	NA	NA	NA	NA	NA	NA	NA
6.1	158	TA-	T	A-	1	T	0	13	0	0	12	1
6.1	159	AA-	A	A-	1	A	1	19	0	0	19	0
6.1	160	TA+	T	A+	1	T	0	20	5	0	13	2
6.1	161	TA-	T	A-	1	T	1	11	0	1	7	3
6.1	162	AA-	A	A-	1	A	0	6	4	1	1	0
6.2	163	AA+	A	A+	1	A	0	19	13	3	3	0
6.2	164	TA+	T	A+	1	T	1	7	1	0	5	1
6.2	165	AA-	A	A-	1	A	0	19	4	9	3	3
6.2	166	AT	A	T	1	A	0	0	NA	NA	NA	NA
6.2	167	TA-	T	A-	1	T	0	13	3	0	9	1
6.2	168	TT	T	T	0	NA	NA	NA	NA	NA	NA	NA
6.2	169	AA+	A	A+	1	A	0	1	1	0	0	0
6.2	170	AT	A	T	1	A	0	11	1	6	4	0
6.2	171	TT	T	T	0	T	0	0	NA	NA	NA	NA
6.3	172	AT	A	T	1	A	0	16	1	13	0	2
6.3	173	AA+	A	A+	1	A	0	12	7	2	2	1
6.3	174	TA+	T	A+	0	NA	NA	NA	NA	NA	NA	NA
6.3	175	AA-	A	A-	1	A	1	12	2	5	3	2
6.3	176	TT	T	T	1	T	0	14	0	8	1	5
6.3	177	TA-	T	A-	1	T	0	11	0	0	11	0
6.3	178	AT	A	T	1	A	0	2	0	0	2	0

6.3	179	TT	T	T	1	T	0	8	0	7	1	0
6.3	180	AA+	A	A+	1	A	0	0	NA	NA	NA	NA
6.4	181	TA+	T	A+	1	T	1	14	0	7	6	1
6.4	182	AA-	A	A-	1	A	0	14	0	12	1	1
6.4	183	AT	A	T	1	A	0	16	0	13	1	2
6.4	184	TA-	T	A-	1	T	0	0	NA	NA	NA	NA
6.4	185	AA+	A	A+	1	A	0	21	11	4	4	2
6.4	186	TT	T	T	0	NA	NA	NA	NA	NA	NA	NA
6.4	187	AA-	A	A-	1	A	0	5	0	2	3	0
6.4	188	TA-	T	A-	0	NA	NA	NA	NA	NA	NA	NA
6.4	189	TA+	T	A+	1	T	0	26	0	18	7	1
7.1	190	TA-	T	A-	1	T	0	1	0	0	1	0
7.1	191	TA+	T	A+	0	NA	NA	NA	NA	NA	NA	NA
7.1	192	AA-	A	A-	1	A	0	3	0	0	3	0
7.1	193	AA+	A	A+	1	A	1	0	NA	NA	NA	NA
7.1	194	AT	A	T	1	A	1	21	0	14	4	3
7.1	195	TT	T	T	1	T	1	14	2	10	0	2
7.1	196	AA-	A	A-	1	A	0	19	1	14	1	3
7.1	197	TA+	T	A+	0	NA	NA	NA	NA	NA	NA	NA
7.1	198	AT	A	T	0	NA	NA	NA	NA	NA	NA	NA
7.2	199	AT	A	T	1	A	0	22	0	17	4	1
7.2	200	TT	T	T	0	NA	NA	NA	NA	NA	NA	NA
7.2	201	AA+	A	A+	1	A	0	33	11	13	6	3
7.2	202	AA-	A	A-	1	A	0	11	0	6	3	2
7.2	203	TA-	T	A-	0	NA	NA	NA	NA	NA	NA	NA
7.2	204	TA+	T	A+	1	T	1	9	1	4	4	0
7.2	205	TT	T	T	0	NA	NA	NA	NA	NA	NA	NA
7.2	206	TA-	T	A-	1	NA	NA	NA	NA	NA	NA	NA
7.2	207	AA+	A	A+	1	NA	NA	NA	NA	NA	NA	NA
7.3	208	AT	A	T	1	A	0	17	0	14	3	0
7.3	209	AA+	A	A+	0	NA	NA	NA	NA	NA	NA	NA
7.3	210	TT	T	T	1	T	0	12	0	1	10	1
7.3	211	TA+	T	A+	1	NA	NA	NA	NA	NA	NA	NA
7.3	212	TA-	T	A-	1	T	0	24	0	0	15	9
7.3	213	AA-	A	A-	0	NA	NA	NA	NA	NA	NA	NA
7.3	214	TA-	T	A-	1	T	1	28	0	12	13	3
7.3	215	AA+	A	A+	0	NA	NA	NA	NA	NA	NA	NA
7.3	216	AT	A	T	1	A	0	1	0	1	0	0
7.4	217	TA-	T	A-	1	T	0	12	0	3	8	1
7.4	218	AA+	A	A+	1	A	0	0	NA	NA	NA	NA
7.4	219	TT	T	T	1	T	0	20	0	1	15	4
7.4	220	TA+	T	A+	1	T	0	3	0	1	1	1
7.4	221	AA-	A	A-	1	A	0	5	0	4	0	1
7.4	222	AT	A	T	1	A	0	20	1	12	4	3
7.4	223	TT	T	T	1	T	1	14	4	3	4	3
7.4	224	AA-	A	A-	1	A	0	7	2	2	2	1

7.4	225	TA+	T	A+	1	T	1	11	0	4	2	5
8.1	226	TT	T	T	0	NA	NA	NA	NA	NA	NA	NA
8.1	227	TA+	T	A+	1	T	0	2	0	1	1	0
8.1	228	TA-	T	A-	1	T	0	23	1	11	8	3
8.1	229	AT	A	T	1	A	0	11	1	8	2	0
8.1	230	AA+	A	A+	1	A	0	14	1	2	11	0
8.1	231	AA-	A	A-	1	A	0	10	0	6	3	1
8.1	232	AT	A	T	1	A	0	5	1	4	0	0
8.1	233	TA-	T	A-	1	T	0	11	6	3	2	0
8.1	234	AA-	A	A-	1	A	0	6	4	1	1	0
8.2	235	AT	A	T	0	NA	NA	NA	NA	NA	NA	NA
8.2	236	TA-	T	A-	1	T	1	5	0	2	3	0
8.2	237	AA+	A	A+	1	A	0	5	3	0	2	0
8.2	238	AA-	A	A-	1	A	0	7	0	5	2	0
8.2	239	TA+	T	A+	1	T	0	19	7	2	10	0
8.2	240	TT	T	T	1	T	0	10	1	4	2	3
8.2	241	AA+	A	A+	0	NA	NA	NA	NA	NA	NA	NA
8.2	242	TA+	T	A+	0	NA	NA	NA	NA	NA	NA	NA
8.2	243	TT	T	T	1	T	0	8	2	4	0	2
8.3	244	TA+	T	A+	1	T	0	20	1	0	16	3
8.3	245	AT	A	T	0	NA	NA	0	NA	NA	NA	NA
8.3	246	AA-	A	A-	1	A	0	7	4	1	1	1
8.3	247	AA+	A	A+	1	A	0	0	NA	NA	NA	NA
8.3	248	TA-	T	A-	1	T	0	16	0	5	9	2
8.3	249	TT	T	T	1	T	0	13	0	4	8	1
8.3	250	TA+	T	A+	1	NA	NA	NA	NA	NA	NA	NA
8.3	251	AA-	A	A-	1	A	0	0	NA	NA	NA	NA
8.3	252	AT	A	T	1	A	0	3	0	2	1	0
8.4	253	TT	T	T	1	NA	NA	NA	NA	NA	NA	NA
8.4	254	AA-	A	A-	1	NA	NA	NA	NA	NA	NA	NA
8.4	255	TA+	T	A+	1	T	0	12	7	2	2	1
8.4	256	AT	A	T	0	NA	NA	NA	NA	NA	NA	NA
8.4	257	TA-	T	A-	1	T	0	18	1	0	15	2
8.4	258	AA+	A	A+	0	NA	NA	NA	NA	NA	NA	NA
8.4	259	TA-	T	A-	0	NA	NA	NA	NA	NA	NA	NA
8.4	260	TT	T	T	0	NA	NA	NA	NA	NA	NA	NA
8.4	261	AA+	A	A+	1	A	0	6	6	0	0	0
9.1	262	AA+	A	A+	1	A	0	24	0	17	3	4
9.1	263	AT	A	T	1	A	1	29	0	3	23	3
9.1	264	TT	T	T	0	NA	NA	NA	NA	NA	NA	NA
9.1	265	TA-	T	A-	0	NA	NA	NA	NA	NA	NA	NA
9.1	266	TA+	T	A+	0	NA	NA	NA	NA	NA	NA	NA
9.1	267	AA-	A	A-	0	NA	NA	NA	NA	NA	NA	NA
9.1	268	AT	A	T	0	NA	NA	NA	NA	NA	NA	NA
9.1	269	TA+	T	A+	0	NA	NA	NA	NA	NA	NA	NA
9.1	270	AA+	A	A+	1	A	0	6	0	1	5	0

9.2	271	TA-	T	A-	1	T	1	16	0	1	12	3
9.2	272	AA+	A	A+	0	NA	NA	NA	NA	NA	NA	NA
9.2	273	TA+	T	A+	1	T	1	3	0	3	0	0
9.2	274	AT	A	T	0	NA	NA	NA	NA	NA	NA	NA
9.2	275	TT	T	T	0	NA	NA	NA	NA	NA	NA	NA
9.2	276	AA-	A	A-	0	NA	NA	NA	NA	NA	NA	NA
9.2	277	AA-	A	A-	0	NA	NA	NA	NA	NA	NA	NA
9.2	278	TA-	T	A-	0	NA	NA	NA	NA	NA	NA	NA
9.2	279	TT	T	T	1	T	0	2	1	1	0	0
9.3	280	AA-	A	A-	1	A	1	6	0	6	0	0
9.3	281	TT	T	T	1	NA	NA	NA	NA	NA	NA	NA
9.3	282	AT	A	T	0	NA	NA	NA	NA	NA	NA	NA
9.3	283	TA-	T	A-	0	NA	NA	NA	NA	NA	NA	NA
9.3	284	TA+	T	A+	0	NA	NA	NA	NA	NA	NA	NA
9.3	285	AA+	A	A+	1	A	1	22	1	13	3	5
9.3	286	AA-	A	A-	1	A	0	NA	NA	NA	NA	NA
9.3	287	TA-	T	A-	0	NA	NA	NA	NA	NA	NA	NA
9.3	288	AA+	A	A+	0	NA	NA	NA	NA	NA	NA	NA
9.4	289	TT	T	T	0	NA	NA	NA	NA	NA	NA	NA
9.4	290	AT	A	T	0	NA	NA	NA	NA	NA	NA	NA
9.4	291	TA+	T	A+	0	NA	NA	NA	NA	NA	NA	NA
9.4	292	AA-	A	A-	1	A	1	0	NA	NA	NA	NA
9.4	293	AA+	A	A+	1	A	0	21	0	10	8	3
9.4	294	TA-	T	A-	1	NA	NA	NA	NA	NA	NA	NA
9.4	295	TA+	T	A+	0	NA	NA	NA	NA	NA	NA	NA
9.4	296	TT	T	T	0	NA	NA	NA	NA	NA	NA	NA
9.4	297	AT	A	T	0	NA	NA	NA	NA	NA	NA	NA
10.1	298	TT	T	T	1	T	0	0	NA	NA	NA	NA
10.1	299	TA+	T	A+	0	NA	NA	NA	NA	NA	NA	NA
10.1	300	AA+	A	A+	1	A	0	11	7	2	2	0
10.1	301	AA-	A	A-	0	NA	NA	NA	NA	NA	NA	NA
10.1	302	TA-	T	A-	1	T	1	21	4	0	17	0
10.1	303	AT	A	T	1	A	0	20	5	13	2	0
10.1	304	AA+	A	A+	0	NA	NA	NA	NA	NA	NA	NA
10.1	305	TT	T	T	0	NA	NA	NA	NA	NA	NA	NA
10.1	306	TA-	T	A-	1	T	0	13	3	1	8	1
10.2	307	AT	A	T	0	NA	NA	NA	NA	NA	NA	NA
10.2	308	AA+	A	A+	0	NA	NA	NA	NA	NA	NA	NA
10.2	309	TA+	T	A+	1	NA	NA	NA	NA	NA	NA	NA
10.2	310	TA-	T	A-	0	NA	NA	NA	NA	NA	NA	NA
10.2	311	AA-	A	A-	1	A	1	20	1	15	4	0
10.2	312	TT	T	T	1	T	0	13	1	7	3	2
10.2	313	TA+	T	A+	0	NA	NA	NA	NA	NA	NA	NA
10.2	314	AT	A	T	0	NA	NA	NA	NA	NA	NA	NA
10.2	315	AA-	A	A-	1	A	0	19	5	4	6	4
10.3	316	TT	T	T	1	T	0	0	NA	NA	NA	NA

10.3	317	AA-	A	A-	1	A	0	16	0	5	6	5
10.3	318	TA-	T	A-	1	T	0	21	0	10	10	1
10.3	319	AT	A	T	1	A	0	21	4	14	2	1
10.3	320	TA+	T	A+	0	NA	NA	NA	NA	NA	NA	NA
10.3	321	AA+	A	A+	1	A	0	20	2	9	7	2
10.3	322	AT	A	T	1	A	0	5	1	2	1	1
10.3	323	TA-	T	A-	1	T	1	2	1	0	1	0
10.3	324	AA+	A	A+	0	NA	NA	NA	NA	NA	NA	NA
10.4	325	AT	A	T	0	NA	NA	NA	NA	NA	NA	NA
10.4	326	TA-	T	A-	1	NA	NA	NA	NA	NA	NA	NA
10.4	327	AA-	A	A-	1	A	0	0	NA	NA	NA	NA
10.4	328	TT	T	T	1	T	0	10	0	6	3	1
10.4	329	AA+	A	A+	0	NA	NA	NA	NA	NA	NA	NA
10.4	330	TA+	T	A+	1	T	0	9	2	0	6	1
10.4	331	TT	T	T	1	T	1	19	0	15	3	1
10.4	332	AA-	A	A-	0	NA	NA	NA	NA	NA	NA	NA
10.4	333	TA+	T	A+	0	NA	NA	NA	NA	NA	NA	NA
11.1	334	AA+	A	A+	0	NA	NA	NA	NA	NA	NA	NA
11.1	335	TA+	T	A+	0	NA	NA	NA	NA	NA	NA	NA
11.1	336	AA-	A	A-	1	A	1	11	0	6	2	3
11.1	337	TT	T	T	1	T	1	2	1	1	0	0
11.1	338	AT	A	T	1	A	1	13	3	7	0	3
11.1	339	TA-	T	A-	1	NA	NA	NA	NA	NA	NA	NA
11.1	340	TA-	T	A-	1	T	0	0	NA	NA	NA	NA
11.1	341	AA-	A	A-	1	A	0	3	1	2	0	0
11.1	342	TT	T	T	0	NA	NA	NA	NA	NA	NA	NA
11.2	343	TA-	T	A-	1	T	1	0	NA	NA	NA	NA
11.2	344	TA+	T	A+	1	T	0	19	13	2	4	0
11.2	345	TT	T	T	0	NA	NA	NA	NA	NA	NA	NA
11.2	346	AA-	A	A-	1	A	1	20	2	12	2	4
11.2	347	AA+	A	A+	0	NA	NA	NA	NA	NA	NA	NA
11.2	348	AT	A	T	1	A	1	18	0	6	1	11
11.2	349	TA+	T	A+	0	NA	NA	NA	NA	NA	NA	NA
11.2	350	AA+	A	A+	1	A	0	20	15	0	5	0
11.2	351	AT	A	T	1	A	1	22	5	6	10	1
11.3	352	TA-	T	A-	0	NA	NA	NA	NA	NA	NA	NA
11.3	353	AT	A	T	0	NA	NA	NA	NA	NA	NA	NA
11.3	354	TA+	T	A+	0	NA	NA	NA	NA	NA	NA	NA
11.3	355	TT	T	T	0	NA	NA	NA	NA	NA	NA	NA
11.3	356	AA+	A	A+	0	NA	NA	NA	NA	NA	NA	NA
11.3	357	AA-	A	A-	0	NA	NA	NA	NA	NA	NA	NA
11.3	358	TT	T	T	0	NA	NA	NA	NA	NA	NA	NA
11.3	359	TA+	T	A+	0	NA	NA	NA	NA	NA	NA	NA
11.3	360	AA+	A	A+	0	NA	NA	NA	NA	NA	NA	NA
11.4	361	TT	T	T	1	T	1	6	6	0	0	0
11.4	362	AA-	A	A-	0	NA	NA	NA	NA	NA	NA	NA

11.4	363	TA-	T	A-	0	NA	NA	NA	NA	NA	NA	NA
11.4	364	TA+	T	A+	0	NA	NA	NA	NA	NA	NA	NA
11.4	365	AT	A	T	1	A	0	20	0	8	7	5
11.4	366	AA+	A	A+	0	NA	NA	NA	NA	NA	NA	NA
11.4	367	AT	A	T	1	A	0	10	2	6	1	1
11.4	368	AA-	A	A-	0	NA	NA	NA	NA	NA	NA	NA
11.4	369	TA-	T	A-	1	T	0	0	NA	NA	NA	NA
12.1	370	AT	A	T	0	NA	NA	NA	NA	NA	NA	NA
12.1	371	TT	T	T	1	T	0	34	1	24	5	4
12.1	372	TA+	T	A+	0	NA	NA	NA	NA	NA	NA	NA
12.1	373	AA+	A	A+	0	NA	NA	NA	NA	NA	NA	NA
12.1	374	TA-	T	A-	1	T	0	34	0	2	27	5
12.1	375	AA-	A	A-	0	NA	NA	NA	NA	NA	NA	NA
12.1	376	AT	A	T	0	NA	NA	NA	NA	NA	NA	NA
12.1	377	TA+	T	A+	1	T	1	15	0	0	15	0
12.1	378	AA+	A	A+	1	A	0	33	26	0	7	0
12.2	379	AA+	A	A+	1	A	0	14	4	6	3	1
12.2	380	AA-	A	A-	0	NA	NA	NA	NA	NA	NA	NA
12.2	381	TT	T	T	0	NA	NA	NA	NA	NA	NA	NA
12.2	382	TA+	T	A+	1	T	0	1	0	1	0	0
12.2	383	TA-	T	A-	1	NA	NA	NA	NA	NA	NA	NA
12.2	384	AT	A	T	1	A	0	33	4	21	7	1
12.2	385	AA-	A	A-	1	A	0	29	4	17	6	2
12.2	386	TA-	T	A-	0	NA	NA	NA	NA	NA	NA	NA
12.2	387	TT	T	T	1	T	0	5	0	4	1	0
12.3	388	AA-	A	A-	0	NA	NA	NA	NA	NA	NA	NA
12.3	389	TA-	T	A-	1	T	0	36	0	25	6	5
12.3	390	TT	T	T	0	NA	NA	NA	NA	NA	NA	NA
12.3	391	AT	A	T	1	A	0	42	0	33	7	2
12.3	392	AA+	A	A+	1	A	0	8	5	2	1	0
12.3	393	TA+	T	A+	1	T	0	17	5	0	12	0
12.3	394	AT	A	T	1	A	0	28	5	19	4	0
12.3	395	TA+	T	A+	0	NA	NA	NA	NA	NA	NA	NA
12.3	396	AA+	A	A+	0	NA	NA	NA	NA	NA	NA	NA
12.4	397	TA-	T	A-	1	T	0	2	0	2	0	0
12.4	398	AA-	A	A-	0	NA	NA	NA	NA	NA	NA	NA
12.4	399	AA+	A	A+	1	NA	NA	NA	NA	NA	NA	NA
12.4	400	TT	T	T	0	NA	NA	NA	NA	NA	NA	NA
12.4	401	TA+	T	A+	1	T	0	4	1	0	2	1
12.4	402	AT	A	T	1	A	0	46	2	6	37	1
12.4	403	AA-	A	A-	0	NA	NA	NA	NA	NA	NA	NA
12.4	404	TT	T	T	0	NA	NA	NA	NA	NA	NA	NA
12.4	405	TA-	T	A-	1	T	0	15	0	12	1	2
13.1	406	TA+	T	A+	1	T	0	5	0	2	2	1
13.1	407	TA-	T	A-	1	T	1	46	1	28	8	9
13.1	408	AA-	A	A-	0	NA	NA	NA	NA	NA	NA	NA

13.1	409	TT	T	T	1	T	0	10	0	7	1	2
13.1	410	AA+	A	A+	1	A	0	20	1	11	5	3
13.1	411	AT	A	T	1	A	0	21	0	15	5	1
13.1	412	TT	T	T	0	NA	NA	NA	NA	NA	NA	NA
13.1	413	TA+	T	A+	0	NA	NA	NA	NA	NA	NA	NA
13.1	414	AA+	A	A+	0	NA	NA	NA	NA	NA	NA	NA
13.2	415	TT	T	T	0	NA	NA	NA	NA	NA	NA	NA
13.2	416	AA+	A	A+	0	NA	NA	NA	NA	NA	NA	NA
13.2	417	AT	A	T	1	A	0	42	10	3	26	3
13.2	418	TA-	T	A-	0	NA	NA	NA	NA	NA	NA	NA
13.2	419	AA-	A	A-	1	A	0	22	0	15	4	3
13.2	420	TA+	T	A+	0	NA	NA	NA	NA	NA	NA	NA
13.2	421	AT	A	T	0	NA	NA	NA	NA	NA	NA	NA
13.2	422	AA-	A	A-	1	A	0	22	0	16	5	1
13.2	423	TA-	T	A-	0	NA	NA	NA	NA	NA	NA	NA
13.3	424	AT	A	T	1	A	0	34	4	18	8	4
13.3	425	AA+	A	A+	1	NA	NA	NA	NA	NA	NA	NA
13.3	426	AA-	A	A-	0	NA	NA	NA	NA	NA	NA	NA
13.3	427	TA+	T	A+	1	T	0	34	13	9	9	3
13.3	428	TA-	T	A-	0	NA	NA	NA	NA	NA	NA	NA
13.3	429	TT	T	T	1	T	1	33	2	5	22	4
13.3	430	AT	A	T	0	NA	NA	NA	NA	NA	NA	NA
13.3	431	TA-	T	A-	0	NA	NA	NA	NA	NA	NA	NA
13.3	432	AA+	A	A+	0	NA	NA	NA	NA	NA	NA	NA
13.4	433	TA-	T	A-	0	NA	NA	NA	NA	NA	NA	NA
13.4	434	AT	A	T	1	A	0	6	0	3	2	1
13.4	435	TT	T	T	0	NA	NA	NA	NA	NA	NA	NA
13.4	436	AA+	A	A+	0	NA	NA	NA	NA	NA	NA	NA
13.4	437	TA+	T	A+	0	NA	NA	NA	NA	NA	NA	NA
13.4	438	AA-	A	A-	0	NA	NA	NA	NA	NA	NA	NA
13.4	439	AA-	A	A-	0	NA	NA	NA	NA	NA	NA	NA
13.4	440	TT	T	T	0	NA	NA	NA	NA	NA	NA	NA
13.4	441	TA+	T	A+	0	NA	NA	NA	NA	NA	NA	NA
14.1	442	AA-	A	A-	1	A	1	9	0	5	1	3
14.1	443	TA-	T	A-	1	T	1	12	0	5	7	0
14.1	444	TT	T	T	1	T	1	12	0	5	5	2
14.1	445	AT	A	T	1	A	0	10	0	2	6	2
14.1	446	TA+	T	A+	1	T	0	10	2	5	3	0
14.1	447	AA+	A	A+	0	NA	NA	NA	NA	NA	NA	NA
14.1	448	AT	A	T	1	A	0	8	2	4	2	0
14.1	449	TA-	T	A-	0	NA	NA	NA	NA	NA	NA	NA
14.1	450	TT	T	T	1	T	0	12	0	0	6	6
14.2	451	AA-	A	A-	0	NA	NA	NA	NA	NA	NA	NA
14.2	452	TT	T	T	1	T	1	12	0	9	3	0
14.2	453	AA+	A	A+	0	NA	NA	NA	NA	NA	NA	NA
14.2	454	TA-	T	A-	0	NA	NA	NA	NA	NA	NA	NA

14.2	455	AT	A	T	1	A	0	21	4	14	3	0
14.2	456	TA+	T	A+	1	T	0	8	0	0	8	0
14.2	457	TA+	T	A+	1	T	1	10	2	0	8	0
14.2	458	AA+	A	A+	1	A	0	17	12	2	3	0
14.2	459	AA-	A	A-	0	NA	NA	NA	NA	NA	NA	NA
14.3	460	TA+	T	A+	1	T	1	31	8	0	18	5
14.3	461	TT	T	T	1	T	0	11	0	6	1	4
14.3	462	AT	A	T	0	NA	NA	NA	NA	NA	NA	NA
14.3	463	AA-	A	A-	0	NA	NA	NA	NA	NA	NA	NA
14.3	464	AA+	A	A+	0	NA	NA	NA	NA	NA	NA	NA
14.3	465	TA-	T	A-	1	T	0	5	0	1	3	1
14.3	466	AT	A	T	1	A	0	13	0	8	1	4
14.3	467	TA-	T	A-	1	T	1	5	0	1	3	1
14.3	468	TA+	T	A+	0	NA	NA	NA	NA	NA	NA	NA
14.4	469	TT	T	T	0	NA	NA	NA	NA	NA	NA	NA
14.4	470	TA-	T	A-	1	T	0	12	0	9	3	0
14.4	471	AA-	A	A-	1	A	1	3	0	0	3	0
14.4	472	AT	A	T	0	NA	NA	NA	NA	NA	NA	NA
14.4	473	AA+	A	A+	0	NA	NA	NA	NA	NA	NA	NA
14.4	474	TA+	T	A+	0	NA	NA	NA	NA	NA	NA	NA
14.4	475	TT	T	T	1	T	1	18	0	3	9	6
14.4	476	AA+	A	A+	0	NA	NA	NA	NA	NA	NA	NA
14.4	477	AA-	A	A-	0	NA	NA	NA	NA	NA	NA	NA
15.1	478	AT	A	T	0	NA	NA	NA	NA	NA	NA	NA
15.1	479	TA+	T	A+	0	NA	NA	NA	NA	NA	NA	NA
15.1	480	AA-	A	A-	1	A	0	13	0	8	2	3
15.1	481	TA-	T	A-	1	T	1	22	2	4	13	3
15.1	482	AA+	A	A+	0	NA	NA	NA	NA	NA	NA	NA
15.1	483	TT	T	T	0	NA	NA	NA	NA	NA	NA	NA
15.1	484	AT	A	T	1	A	0	19	2	15	0	2
15.1	485	AA+	A	A+	1	A	0	15	11	1	3	0
15.1	486	TA+	T	A+	0	NA	NA	NA	NA	NA	NA	NA
15.2	487	AA-	A	A-	1	A	0	13	0	5	4	4
15.2	488	TT	T	T	1	T	0	9	0	6	0	3
15.2	489	TA-	T	A-	0	NA	NA	NA	NA	NA	NA	NA
15.2	490	AA+	A	A+	0	NA	NA	NA	NA	NA	NA	NA
15.2	491	TA+	T	A+	0	NA	NA	NA	NA	NA	NA	NA
15.2	492	AT	A	T	1	A	0	12	0	8	2	2
15.2	493	AA-	A	A-	0	NA	NA	NA	NA	NA	NA	NA
15.2	494	TT	T	T	1	T	0	3	0	2	0	1
15.2	495	TA-	T	A-	0	NA	NA	NA	NA	NA	NA	NA
15.3	496	AA+	A	A+	0	NA	NA	NA	NA	NA	NA	NA
15.3	497	AT	A	T	0	NA	NA	NA	NA	NA	NA	NA
15.3	498	TA+	T	A+	0	NA	NA	NA	NA	NA	NA	NA
15.3	499	TT	T	T	1	T	0	12	0	6	4	2
15.3	500	TA-	T	A-	0	NA	NA	NA	NA	NA	NA	NA

15.3	501	AA-	A	A-	1	A	1	7	0	5	1	1
15.3	502	TA+	T	A+	1	T	0	9	0	5	2	2
15.3	503	AT	A	T	1	A	0	15	2	12	1	0
15.3	504	AA+	A	A+	1	A	0	1	0	0	1	0
15.4	505	AA-	A	A-	0	NA	NA	NA	NA	NA	NA	NA
15.4	506	AA+	A	A+	1	A	0	2	0	0	0	2
15.4	507	TA-	T	A-	1	T	0	18	0	2	12	4
15.4	508	TT	T	T	0	NA	NA	NA	NA	NA	NA	NA
15.4	509	TA+	T	A+	0	NA	NA	NA	NA	NA	NA	NA
15.4	510	AT	A	T	1	A	0	16	0	12	2	2
15.4	511	TA-	T	A-	0	NA	NA	NA	NA	NA	NA	NA
15.4	512	AA-	A	A-	1	A	0	7	1	3	1	2
15.4	513	TT	T	T	0	NA	NA	NA	NA	NA	NA	NA
16.1	514	TA+	T	A+	0	NA	NA	NA	NA	NA	NA	NA
16.1	515	AA-	A	A-	1	A	0	2	1	1	0	0
16.1	516	AT	A	T	1	A	0	22	0	15	3	4
16.1	517	TA-	T	A-	1	T	0	NA	NA	NA	NA	NA
16.1	518	TT	T	T	0	NA	NA	NA	NA	NA	NA	NA
16.1	519	AA+	A	A+	1	A	0	16	5	8	2	1
16.1	520	AA+	A	A+	1	A	0	19	9	6	4	0
16.1	521	TA+	T	A+	1	T	0	22	0	0	22	0
16.1	522	AT	A	T	1	A	0	19	2	14	2	1
16.2	523	AT	A	T	0	NA	NA	NA	NA	NA	NA	NA
16.2	524	TA-	T	A-	0	NA	NA	NA	NA	NA	NA	NA
16.2	525	TA+	T	A+	1	T	0	22	2	3	15	2
16.2	526	TT	T	T	1	T	0	10	0	7	2	1
16.2	527	AA+	A	A+	0	NA	NA	NA	NA	NA	NA	NA
16.2	528	AA-	A	A-	1	A	0	17	0	9	3	5
16.2	529	AA-	A	A-	1	A	1	13	0	10	1	2
16.2	530	TT	T	T	0	NA	NA	NA	NA	NA	NA	NA
16.2	531	TA-	T	A-	1	T	0	21	3	4	11	3
16.3	532	TA-	T	A-	0	NA	NA	NA	NA	NA	NA	NA
16.3	533	AT	A	T	1	A	0	13	0	10	3	0
16.3	534	AA+	A	A+	0	NA	NA	NA	NA	NA	NA	NA
16.3	535	AA-	A	A-	1	A	0	2	0	1	1	0
16.3	536	TT	T	T	0	NA	NA	NA	NA	NA	NA	NA
16.3	537	TA+	T	A+	0	NA	NA	NA	NA	NA	NA	NA
16.3	538	TA-	T	A-	1	T	1	13	0	1	11	1
16.3	539	AT	A	T	1	A	1	13	3	9	1	0
16.3	540	TA+	T	A+	1	T	0	25	4	10	10	1
16.4	541	AA+	A	A+	1	A	1	8	7	1	0	0
16.4	542	TT	T	T	0	NA	NA	NA	NA	NA	NA	NA
16.4	543	TA-	T	A-	0	NA	NA	NA	NA	NA	NA	NA
16.4	544	TA+	T	A+	1	T	0	1	0	1	0	0
16.4	545	AA-	A	A-	0	NA	NA	NA	NA	NA	NA	NA
16.4	546	AT	A	T	1	A	0	13	0	9	3	1

16.4	547	TT	T	T	1	T	1	23	0	18	3	2
16.4	548	AA-	A	A-	0	NA	NA	NA	NA	NA	NA	NA
16.4	549	AA+	A	A+	0	NA	NA	NA	NA	NA	NA	NA
17.1	550	TA-	T	A-	1	T	0	19	1	12	5	1
17.1	551	AA-	A	A-	0	NA	NA	NA	NA	NA	NA	NA
17.1	552	TT	T	T	0	NA	NA	NA	NA	NA	NA	NA
17.1	553	AT	A	T	1	A	0	0	NA	NA	NA	NA
17.1	554	AA+	A	A+	1	A	0	11	5	4	2	0
17.1	555	TA+	T	A+	0	NA	NA	NA	NA	NA	NA	NA
17.1	556	TA-	T	A-	1	T	0	16	0	4	11	1
17.1	557	TA+	T	A+	0	NA	NA	NA	NA	NA	NA	NA
17.1	558	AA-	A	A-	1	A	0	8	0	5	2	1
17.2	559	TT	T	T	0	NA	NA	NA	NA	NA	NA	NA
17.2	560	AT	A	T	1	A	0	21	1	15	3	2
17.2	561	TA+	T	A+	0	NA	NA	NA	NA	NA	NA	NA
17.2	562	TA-	T	A-	0	NA	NA	NA	NA	NA	NA	NA
17.2	563	AA-	A	A-	1	A	0	12	0	9	3	0
17.2	564	AA+	A	A+	1	A	0	17	9	5	3	0
17.2	565	AA+	A	A+	1	A	0	21	11	5	4	1
17.2	566	AT	A	T	1	A	0	15	0	8	3	4
17.2	567	TT	T	T	1	T	0	10	0	7	2	1
17.3	568	AA-	A	A-	1	A	0	9	0	7	2	0
17.3	569	TT	T	T	1	T	0	10	0	8	2	0
17.3	570	TA-	T	A-	0	NA	NA	NA	NA	NA	NA	NA
17.3	571	AA+	A	A+	1	A	1	17	4	9	2	2
17.3	572	AT	A	T	0	NA	NA	NA	NA	NA	NA	NA
17.3	573	TA+	T	A+	1	NA	NA	NA	NA	NA	NA	NA
17.3	574	AA+	A	A+	1	A	0	10	6	3	1	0
17.3	575	TA-	T	A-	1	T	0	2	0	0	2	0
17.3	576	AT	A	T	0	NA	NA	NA	NA	NA	NA	NA
17.4	577	AT	A	T	1	A	0	17	3	11	2	1
17.4	578	TA+	T	A+	0	NA	NA	NA	NA	NA	NA	NA
17.4	579	TT	T	T	0	NA	NA	NA	NA	NA	NA	NA
17.4	580	AA-	A	A-	1	A	0	14	0	9	4	1
17.4	581	TA-	T	A-	0	NA	NA	NA	NA	NA	NA	NA
17.4	582	AA+	A	A+	0	NA	NA	NA	NA	NA	NA	NA
17.4	583	AA-	A	A-	1	A	0	0	NA	NA	NA	NA
17.4	584	TT	T	T	0	NA	NA	NA	NA	NA	NA	NA
17.4	585	TA+	T	A+	0	NA	NA	NA	NA	NA	NA	NA
18.1	586	AA+	A	A+	1	NA	NA	NA	NA	NA	NA	NA
18.1	587	TT	T	T	1	T	0	13	0	11	2	0
18.1	588	TA-	T	A-	1	NA	NA	NA	NA	NA	NA	NA
18.1	589	AT	A	T	1	A	0	7	0	5	2	0
18.1	590	AA-	A	A-	1	A	0	19	0	13	4	2
18.1	591	TA+	T	A+	1	T	1	35	11	12	11	1
18.1	592	TA-	T	A-	1	T	0	17	0	1	14	2

18.1	593	TA+	T	A+	1	T	1	24	4	5	14	1
18.1	594	AA+	A	A+	1	A	0	26	7	10	4	5
18.2	595	TT	T	T	0	NA	NA	NA	NA	NA	NA	NA
18.2	596	AT	A	T	1	A	0	29	1	18	8	2
18.2	597	AA-	A	A-	1	A	0	4	0	3	1	0
18.2	598	AA+	A	A+	1	A	0	29	14	5	9	1
18.2	599	TA-	T	A-	1	NA	NA	NA	NA	NA	NA	NA
18.2	600	TA+	T	A+	1	T	1	23	3	5	13	2
18.2	601	AA-	A	A-	1	A	0	0	NA	NA	NA	NA
18.2	602	AT	A	T	0	NA	NA	NA	NA	NA	NA	NA
18.2	603	TT	T	T	0	NA	NA	NA	NA	NA	NA	NA
18.3	604	AT	A	T	0	NA	NA	NA	NA	NA	NA	NA
18.3	605	TA-	T	A-	0	NA	NA	NA	NA	NA	NA	NA
18.3	606	TT	T	T	0	NA	NA	NA	NA	NA	NA	NA
18.3	607	AA+	A	A+	1	A	0	25	1	14	6	4
18.3	608	AA-	A	A-	1	A	0	34	0	23	5	6
18.3	609	TA+	T	A+	0	NA	NA	NA	NA	NA	NA	NA
18.3	610	AA-	A	A-	1	A	0	11	0	9	2	0
18.3	611	TA-	T	A-	0	NA	NA	NA	NA	NA	NA	NA
18.3	612	TT	T	T	0	NA	NA	NA	NA	NA	NA	NA
19.1	613	TT	T	T	1	T	0	7	0	6	1	0
19.1	614	AA-	A	A-	1	A	0	27	1	19	6	1
19.1	615	AA-	A	A-	0	NA	NA	NA	NA	NA	NA	NA
19.1	616	TT	T	T	0	NA	NA	NA	NA	NA	NA	NA
19.1	617	TT	T	T	0	NA	NA	NA	NA	NA	NA	NA
19.1	618	AA-	A	A-	1	A	0	18	0	15	3	0
19.1	619	TT	T	T	1	T	0	28	1	21	5	1
19.1	620	TT	T	T	0	NA	NA	NA	NA	NA	NA	NA
19.1	621	AA-	A	A-	1	A	0	18	2	11	5	0
19.2	622	AA-	A	A-	0	NA	NA	NA	NA	NA	NA	NA
19.2	623	AA-	A	A-	0	NA	NA	NA	NA	NA	NA	NA
19.2	624	TT	T	T	0	NA	NA	NA	NA	NA	NA	NA
19.2	625	TT	T	T	1	T	0	27	0	20	6	1
19.2	626	AA-	A	A-	0	NA	NA	NA	NA	NA	NA	NA
19.2	627	TT	T	T	0	NA	NA	NA	NA	NA	NA	NA
19.2	628	AA-	A	A-	1	A	0	21	12	1	0	8
19.2	629	TT	T	T	1	T	0	24	0	17	5	2
19.2	630	TT	T	T	0	NA	NA	NA	NA	NA	NA	NA
19.3	631	AA-	A	A-	0	NA	NA	NA	NA	NA	NA	NA
19.3	632	TT	T	T	1	T	0	2	2	0	0	0
19.3	633	AA-	A	A-	1	A	1	19	0	13	3	3
19.3	634	AA-	A	A-	0	NA	NA	NA	NA	NA	NA	NA
19.3	635	TT	T	T	0	NA	NA	NA	NA	NA	NA	NA
19.3	636	TT	T	T	1	T	1	30	0	25	4	1
19.3	637	TT	T	T	0	NA	NA	NA	NA	NA	NA	NA
19.3	638	AA-	A	A-	0	NA	NA	NA	NA	NA	NA	NA

19.3	639	AA-	A	A-	1	A	0	31	1	21	8	1
19.4	640	TT	T	T	0	NA	NA	NA	NA	NA	NA	NA
19.4	641	TA-	T	A-	1	T	0	18	0	7	11	0
19.4	642	TA-	T	A-	0	NA	NA	NA	NA	NA	NA	NA
19.4	643	AA+	A	A+	0	NA	NA	NA	NA	NA	NA	NA
19.4	644	TT	T	T	1	T	0	24	1	4	19	0
19.4	645	TT	T	T	0	NA	NA	NA	NA	NA	NA	NA
19.4	646	AA-	A	A-	0	NA	NA	NA	NA	NA	NA	NA
19.4	647	AA-	A	A-	0	NA	NA	NA	NA	NA	NA	NA
19.4	648	AA-	A	A-	0	NA	NA	NA	NA	NA	NA	NA