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The reciprocal interaction between *Wolbachia* and host-plant specialization in spider mites

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Abstract

It is currently becoming increasingly clear that bacterial endosymbionts affect arthropod-plant interactions. For instance, they may act as nutritional mutualists, directly supplying their host with nutrients or enzymes insufficient in their plant diet or enabling them to manipulate plant physiology, such as anti-herbivore defenses, for their own benefit. Reciprocally, plants can influence the effects of symbionts on their hosts, specifically through the modification of their densities, a key factor for both symbiont maintenance and spread within natural populations.

Here, we studied the tripartite interaction between the two-spotted spider mite *Tetranychus urticae*, a polyphagous agricultural pest, five of its natural host plants and its endosymbionts *Wolbachia*, *Rickettsia* and *Cardinium*, which can manipulate the reproduction of their hosts, and/or be mutualists. The prevalence of these endosymbionts is highly variable (ranging from 0 to 100%) in natural populations of *T. urticae* worldwide, although the factors affecting their distribution are still largely unknown.

We first investigated whether *Wolbachia* affects the performance of *T. urticae* on different host plants. *Wolbachia* infection was found to be detrimental, beneficial, or neutral on eggs hatching rate depending on the host plant, and these results were unaffected by the mites' rearing history (i.e. laboratory maintenance on different plants). All other life history traits were affected only by the plant species, or by *Wolbachia* infection. Subsequently, we evaluated the effect of the same plants on endosymbiont prevalence in natural populations of *T. urticae* in Portugal (5 replicates per host plant). The prevalence of *Wolbachia* and *Rickettsia* varied with the host plants, but not that of *Cardinium*. Interestingly, the plants leading to the lower prevalence of *Wolbachia* in natural populations of *T. urticae* are also those in which *Wolbachia* infection results in lower eggs hatchability. These results suggest that host plants may play an important role in endosymbionts spread in *T. urticae* populations. Conversely, *T. urticae* host-plant colonization may hinge on their cortege of endosymbionts.

Keywords: *Tetranychus urticae*, *Wolbachia*, host-plant, tripartite interaction, endosymbiont

Resumo

Os seres vivos fazem parte de complexas redes de relações antagonistas e mutualistas. As consequências de uma destas interações para as espécies que interagem nestas redes não podem ser estudadas separadamente das outras, dado que uma interação pode afetar o resultado de outra. Este projeto foca-se numa interação interespecífica complexa, que envolve 3 espécies.

Há cada vez mais provas da importância de endossimbiontes bacterianos na relação artrópode-planta. Os organismos associados aos artrópodes herbívoros podem afetar as respostas das plantas à herbivoria tanto diretamente, interferindo com as defesas das plantas, como indiretamente, manipulando o comportamento e a fisiologia do seu hospedeiro, beneficiando-o.

Reciprocamente, as plantas podem influenciar os efeitos dos simbiontes nos seus hospedeiros: especificamente, dependendo do grau de adaptação do herbívoro à planta, assim pode variar a densidade dos simbiontes. Como consequência, um aumento na densidade dos simbiontes pode ter um efeito negativo na performance do herbívoro, já que os simbiontes podem ser prejudiciais para vários aspetos da vida do hospedeiro.

Este projeto investiga a interação entre o ácaro-aranha *Tetranychus urticae*, as bactérias endossimbiontes *Wolbachia*, *Cardinium* e *Rickettsia* e algumas das plantas que os *T. urticae* infetam na natureza.

O ácaro-aranha é uma praga agrícola e um dos artrópodes herbívoros mais polípagos, alimentando-se em mais de 1100 espécies de plantas. Alimentam-se perfurando células do parênquima das folhas com os seus estiletes e sugando os conteúdos celulares. Tem um ciclo de vida curto, descendência abundante e reprodução arrenotóica (as fêmeas são diploides e eclodem de ovos fertilizados; os machos são haploides e eclodem de ovos não fertilizados). Os ácaros-aranha podem abrigar várias espécies de bactérias endossimbiontes, como *Wolbachia*, *Cardinium*, *Rickettsia* e *Spiroplasma*; para as quais os efeitos nos seus artrópodes hospedeiros vão desde o parasitismo (incluindo o parasitismo reprodutivo; como descrito abaixo para *Wolbachia*) ao mutualismo. Nos ácaros-aranha, foi observado que tanto *Wolbachia* como *Cardinium* induzem incompatibilidade citoplasmática (IC; ver abaixo) que pode tanto ter custos como ser benéfico, enquanto que os efeitos de *Rickettsia* e de *Spiroplasma* nesta espécie ainda são desconhecidos.

Wolbachia (α -Proteobactérias) são bactérias intracelulares maternas transmitidas, conhecidas pelo seu parasitismo reprodutor, que lhes permite colonizar várias espécies de artrópodes. Têm também o potencial de ter relação mutualistas com os seus hospedeiros, fornecendo-lhes benefícios

facultativos ou sendo necessárias para a sobrevivência e/ou reprodução dos seus hospedeiros. A manipulação da reprodução de *Wolbachia* é feita através da indução de vários fenótipos: partenogênese, feminização, morte de machos e incompatibilidade citoplasmática (IC). IC corresponde a um fenómeno de esterilidade condicional que surge em cruzamentos entre machos infetados e fêmeas não infetadas ou fêmeas que contenham um tipo ou uma estirpe de *Wolbachia* diferente.

O efeito de plantas na quantidade relativa de simbiontes bacterianos já foi descrito previamente para outros artrópodes e dados obtidos no nosso grupo sugerem que as plantas hospedeiras podem afetar a prevalência de *Wolbachia* em populações naturais de *T. urticae*, sugerindo assim que estas plantas afetam a relação entre os endossimbiontes e os seus hospedeiros. Diferentes plantas infestadas naturalmente por *T. urticae* têm provavelmente uma diferente quantidade e qualidade nutricional, assim como diferentes mecanismos de defesa contra artrópodes herbívoros. É o que se observa na planta do feijão (*Phaseolus vulgaris*, Fabaceae), da beringela (*Solanum melongena*, Solenaceae), na corda-de-viola (*Ipomoea purpurea*, Convolvulaceae), planta do tomate (*Solanum lycopersicum*, Solenaceae) e da curgete (*Cucurbita pepo*, Cucurbitaceae). No entanto, o potencial papel de diferentes plantas nas interações *Wolbachia* – ácaro-aranha nunca foi investigado.

O estudo desta interação tripartida é pertinente por permitir-nos melhorar o nosso entendimento do efeito de *Wolbachia* na interação entre ácaros-aranha e plantas, a sua ecologia e evolução, assim como o efeito das plantas na dinâmica dos endossimbiontes nos ácaros-aranha.

Para abordar estas questões, foi testada a performance de *T. urticae* infectados (Wi) ou não por *Wolbachia* (Wu) em cinco plantas hospedeiras comuns (feijão, beringela, corda-de-viola, tomate e curgete).

Foram individualmente colocadas, em discos de folha com 2cm² de cada uma das 5 diferentes plantas hospedeiras, 100 fêmeas adultas fecundadas por tratamento. Foram medidos vários traços indicadores da história de vida dos ácaros-aranha: sobrevivência, proporção de fêmeas afogadas, fecundidade diária média, taxa de eclosão, sobrevivência de juvenis, rácio sexual e o número de descendência viável. No geral, o efeito de *Wolbachia* nos traços de performance do ácaro-aranha não variou de planta para planta: a proporção de fêmeas afogadas, a fecundidade diária, a sobrevivência dos juvenis e o número de descendência viável foram afetados diferentemente pelas diferentes plantas, enquanto que a presença de *Wolbachia* apenas teve efeito no rácio sexual. No entanto, foi encontrado um efeito significativo de *Wolbachia* associado a diferentes plantas na eclosão dos ovos de *T. urticae*: ácaros infetados com *Wolbachia* eclodiram menos em corda-de-viola e em curgete, enquanto que em beringela o contrário foi observado. Este efeito em diferentes

plantas pode ser explicado pelo facto de a *Wolbachia* pode depender de nutrientes que o seu hospedeiro obtém enquanto se alimenta. Uma dieta deficiente em nutrientes pode levar a um decréscimo na viabilidade dos ovos e consequentemente reduzir a dispersão de *Wolbachia* pela população.

O impacto de *Wolbachia* no rácio sexual (tendencioso em relação a fêmeas) pode ser explicado por várias razões não exclusivas, tal como: efeito do tratamento antibiótico usado para a remoção de simbiontes, usado na criação da população de ácaros-aranha Wu (não infetados com *Wolbachia*); efeito de *Wolbachia* no tempo de desenvolvimento dos juvenis, já que após quiescência, as fêmeas põem mais ovos que irão desenvolver-se em machos, e se populações Wi se desenvolverem mais depressa que as Wu, esses machos poderão não ter sido postos durante a experiência; *Wolbachia* poderá ter beneficiado as fêmeas de ácaro-aranha e assim ter enviesado o rácio sexual em direção a fêmeas, pois fêmeas *less fit* produzem mais machos; *Wolbachia*, por serem maternalmente transmitidas, irão sempre beneficiar de um rácio sexual tendencioso em relação a fêmeas.

Para determinar se o efeito de *Wolbachia* na *performance* dos ácaros-aranha se altera após várias gerações de ácaros mantidos em diferentes plantas, ácaros Wi e Wu foram mantidos em grandes números em plantas de beringela, corda-de-viola e feijão durante seis meses (cerca de 12 gerações). Esta escolha de plantas foi feita tendo com base os resultados obtidos no que diz respeito à eclosão dos ovos. Passados os seis meses, a *performance* de ácaros Wi e Wu, assim como dos respetivos controlos (i.e. ácaros mantidos em feijão, o seu hospedeiro original), foi testada nestas plantas com um procedimento idêntico ao descrito acima.

Os ácaros mantidos nas novas plantas (corda-de-viola e beringela) não mostraram sinais de adaptação quando comparadas com ácaros mantidos e testados em feijão (controlos) e chegam a desempenhar-se pior, em alguns traços, do que ácaros testados em plantas diferentes pela primeira vez. Estes resultados podem ser explicados por um número insuficiente de gerações de manutenção nestas plantas, da falta de replicados, mas também da baixa diversidade resultante de um efeito de gargalo (como resultado de dificuldades durante a manutenção). No geral, vemos uma congruência entre os resultados obtidos antes e após a manutenção em diferentes plantas durante várias gerações, mas a segunda experiência mostra mais interações entre *Wolbachia* e planta hospedeira do que a primeira. No entanto, tendo em conta as explicações anteriores, não podemos tirar conclusões sólidas destes resultados.

Finalmente, para determinar se as plantas hospedeiras afetam a prevalência de três do endossimbiontes mais comuns em populações naturais de *T. urticae*, foi realizado um estudo de campo de ácaros recolhidos de plantas de feijão, beringela, corda-de-viola, tomate e curgete. Devido

à baixa taxa de infestação de corda-de-viola por *T. urticae*, apenas 2 populações foram recolhidas (em contraste com as 5 recolhidas nas outras plantas). De cada população recolhida, 20 fêmeas foram testadas por PCR para a presença dos endossimbiontes em estudo. Foi descoberto que existe uma prevalência muito alta de *Wolbachia* em todas as plantas testadas. A prevalência de *Wolbachia* e *Rickettsia* variou com as plantas mas o mesmo não se observou com *Cardinium*. Caso as plantas afetem algum dos parâmetros que controlam a prevalência de endossimbiontes numa população, como o balanço custos/benefícios dos endossimbiontes nos seus hospedeiros, esta pode ser alterada. Curiosamente, as plantas que no campo continham ácaros com uma menor prevalência de *Wolbachia* (corda-de-viola e curgete) são as mesmas nas quais a infeção por *Wolbachia* resulta numa menor eclosão de ovos. Isto sugere que as plantas podem ter um papel importante na dispersão de endossimbiontes e/ou na sua manutenção em populações de ácaros.

Os nossos resultados não mostram um impacto importante da infeção por *Wolbachia* na performance de *T. urticae* em plantas diferentes. Por outro lado, tendo em conta os resultados relativos à prevalência de *Wolbachia* e à eclosão dos ovos, a colonização de plantas por ácaros pode beneficiar da presença de endossimbiontes. No entanto, este estudo é apenas um passo na compreensão desta interação, havendo ainda várias abordagens pertinentes para complementarem o nosso conhecimento.

Keywords: *Tetranychus urticae*, *Wolbachia*, planta-hospedeira, interação tripartida, endossimbionte

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Introduction

Species are usually immersed in complex antagonistic and mutualistic networks. The outcome of each interaction cannot be analysed in isolation, as it will depend on the fate of other interactions (Kissling & Schleuning, 2014). The tripartite interaction plant-herbivore-symbiont focused on this project is an example of a complex interspecific interaction. To disentangle this trio we first need to understand pairwise interactions.

Arthropods-plants interaction (herbivory)

Arthropods and plants have been coexisting for more than 400 million years (Labandeira, 1997). Since then, they developed refined interactions that affect organisms at different levels. These relationships range from mutualism, such as pollination, to antagonism, such as arthropod herbivory and plant defences against herbivores.

Plants have developed ways to defend themselves from herbivory. Some of their defences are constitutive while others are induced. The constitutive defences consist of morphological features like waxes (Riederer & Müller, 2007), trichomes (Myers & Bazely, 1991) and lattices (Agrawal & Konno, 2009) to make the feeding more difficult for arthropods. Regarding induced defences, arthropod herbivory induces the ethylene, jasmonate and salicylic cascade which results in the production of proteinase inhibitors (reviewed by Walling, 2000), which often interfere with arthropods' digestion (Duffey & Stout, 1996). Volatiles may also be released upon herbivory to repel herbivores (Kessler, 2001), attract predators (Dicke et al., 2003) or for communication between leaves or plants (Karban et al., 2000), which in turn may induce defence responses from the ethylene, jasmonate and salicylic cascade (Engelberth et al., 2001).

Due to this diversity of plants' defence strategies, along with their variability in size, shape and lifecycles (Schaller & Howe, 2008), it is expected that the strategies developed by herbivores to feed on those plants are also very diverse. For example, although some arthropod herbivores induce plant defences upon feeding (Awmack & Leather, 2002; Karban & Myers, 1989), others are able downregulate them to their benefit (Lawrence et al., 2008; Musser et al., 2002; Godinho et al., 2016; Sarmiento et al., 2011), to prefer less defended plants (e.g. Lamb et al., 2003; Travers-Martin & Müller, 2008), and in some cases to sequester the compounds and reuse them in their own benefit (Raffa & Berryman, 1983). Consequently, arthropod herbivores can be either generalists (polyphagous), tolerating a wide range of defences present in most plants while they cannot feed on certain plants that have evolved more unique defence mechanisms; or specialists, feeding on one (monophagous) or a few, (oligophagous) plant species (Fürstenberg-Hägg et al., 2013). In reality the

distribution of arthropods feeding on one to several plant species is a continuum (Ali & Agrawal, 2012).

Both plant defences and herbivore strategies to cope with these defences involve metabolic costs, so most plant-arthropod interactions reach a stand-off, where both host and herbivore survive although their development is costly.

Arthropods-symbionts interaction (symbiosis)

The term *symbiosis* was first used by Anton de Bary in 1879 as «the living together of unlike organisms» (Martin & Schwab, 2012). The term has been commonly restricted to mutualistic (when the interaction benefits both partners) and obligatory (symbionts that cannot survive without their hosts) associations. Nowadays, however, the term is mostly used following its original meaning: an intimate interaction between different species, independently of the outcome for the involved organisms. Thus, depending on the balance between the costs and benefits for the partners, their interaction may lie anywhere on a continuum between parasitism (when the symbiont benefits from the interaction at the expense of its host) and mutualism (where both benefit from the interaction; Combes, 2001).

Endosymbiotic bacteria are widely present in animal hosts, where they can affect their development (Braendle et al., 2003; Koropatnick et al., 2010), nutrition (Backhed, 2005; Baumann, 2005), reproduction and possibly speciation (Bandi et al., 2001; G. D. D. Hurst & Jiggins, 2000; Stouthamer et al., 1999), defence against natural enemies (Oliver et al., 2003; Piel, 2002; Scarborough et al., 2005) and immunity (MacDonald, 2005), including protection against viruses (Teixeira et al., 2008). Two fundamentally different modes of transmission can be distinguished (Bright & Bulgheresi, 2010): horizontal (that is, from an environmental, free-living symbiont source) and vertical (that is, inheritance of the symbiont from the mother or, more rarely, from both parents).

Due to their transmission mode, the host-symbiont association involving vertically transmitted (VT) -symbionts is usually permanent (Bright & Bulgheresi, 2010). In some cases, the transmission of these symbionts occurs through both sexes (e.g. Moran & Dunbar, 2006; Usher et al., 2005), but it is maternal (i.e. through the female germ line) in most cases (Bright & Bulgheresi, 2010). In addition, although vertical acquisition of VT-symbiont, phylogenetic evidence often indicates that occasional horizontal transfer within or between species occur (Bright & Bulgheresi, 2010). These horizontal transfers are thought to arise through diverse types of interactions between organisms, such as cannibalism and predation (Le Clec'h et al., 2013), parasitism (e.g. Brown & Lloyd, 2015; Heath et al., 1999; Vavre et al., 1999), or herbivory (e.g. horizontal transfer of symbionts from one

arthropod host to another through plants; Caspi-Fluger et al., 2012).

Multilevel interaction

There is currently a growing evidence of the importance of bacterial endosymbionts in arthropod-plant interactions. For instance, arthropods' endosymbionts may act as nutritional mutualists, directly supplying their host with nutrients or enzymes, as in the aphid-*Buchnera* interaction where the aphid depends on the symbiont for essential amino acids that are scarce in their plant sap diet (Douglas, 1998). Endosymbionts may also enable arthropods to manipulate plant physiology, such as anti-herbivory defences for their own benefit (Douglas, 2009; Frago et al., 2012; Su et al., 2015). Herbivore-associated organisms can affect plant responses to herbivory both directly, by entering in contact with the plant's defences (via secreted fluids or excretions of the arthropod), and indirectly, by manipulating the behaviour and physiology of their host. Symbionts effect on host behaviour often leads to an increase of the symbiont fitness, for instance by inducing the host to feed on another place where the plant's responses to herbivory may differ (Rostás & Eggert, 2007), and thus where it can reproduce more and then increase the spread of symbionts (Hoover et al., 2011). Another strategy commonly used by herbivores to circumvent plant defences is avoiding detection by the plant, sometimes using bacteria to manipulate the biochemical pathways involved in induced plant defences. Larvae of the beetle *Leptinotarsa decemlineata*, for example, are able to use bacteria present in their oral secretions to deceive plants into incorrectly perceiving them as a microbial threat, which in turn lowers the plants' anti-herbivore defences (Chung et al., 2013).

Reciprocally, plants can also influence the effects of symbionts on their hosts (Wilkinson et al., 2001). In some cases, this can be achieved through the modification of their densities, depending on whether the herbivore is adapted or not to the plant (Pan et al., 2013). For instance, plant nutrients themselves may disturb the arthropods control over bacterial abundance (Chandler et al., 2008). In turn, an increase in symbiont densities may have a negative effect on the herbivore performance, as symbionts may be detrimental for several life history traits of the host (Brelsfoard & Dobson, 2011; Carrington et al., 2010; Suh et al., 2014).

The study system: the three players of a multilevel interaction

Tetranychus urticae

The two-spotted spider mite *Tetranychus urticae* is a cosmopolitan agricultural and horticultural pest and one of the most polyphagous arthropod herbivores, feeding on more than 1100 plant species (from more than 140 different plant families; Migeon & Dorkeld, 2015). Due to its short life cycle, abundant progeny and arrhenotokous reproduction (females are diploid and come from fertilized eggs, while males are haploid and develop from unfertilized eggs; Helle et al., 1970), it

is a major pest of at least 30 economically relevant greenhouse and field crops, feeding by perforating leaf parenchyma cells with their stylets and sucking out the cell contents. It has been suggested that no intrinsic limits to host-plant range hold in this generalist species (Magalhães et al., 2009), thus the occurrence of host races (i.e. genetically differentiated, sympatric populations of a species that are partially reproductively isolated as a consequence of adaptation to a specific host; Diehl & Bush, 1984; Drès & Mallet, 2002) cannot be explained by limited phenotypic plasticity or by strong trade-offs in adaptation to different plants (Magalhães et al., 2007).

Spider mites can harbour several endosymbiotic bacteria, such as *Wolbachia*, *Cardinium*, *Rickettsia* and *Spiroplasma* (Chaisiri et al., 2015; Hoy & Jeyaprakash, 2005); for which the effects on their arthropod hosts range from parasitism (including reproductive parasitism; see details below in the *Wolbachia* section) to mutualism. In spider mites, both *Wolbachia* and *Cardinium* have been shown to induce cytoplasmic incompatibility (CI; see below); and can be either costly or beneficial (Gotoh et al., 2007; Vala et al., 2002; Vala et al., 2000; Zhu et al., 2012) while the effects of *Rickettsia* and *Spiroplasma* in this species are, to our knowledge, still unknown.

Wolbachia

Wolbachia (α -Proteobacteria) are maternally transmitted intracellular bacteria, notorious for their reproductive parasitism (Stouthamer et al., 1999; Werren et al., 1995), which allow them to be extremely widespread among arthropods (c.a. 52% of terrestrial arthropod species; Weinert et al., 2015). They can also have the potential to engage in mutualistic relationships with their hosts, either providing facultative fitness benefits or being required for host survival and/or reproduction (Zug & Hammerstein, 2014).

Wolbachia manipulate host reproduction through the induction of several known phenotypes: parthenogenesis induction (induces an asexual daughter development), feminization (results in genetic males that develop as females), male killing (eliminates infected males during embryogenesis or late larval instar to the advantage of surviving infected female siblings) and most commonly cytoplasmic incompatibility (CI) (Vala et al., 2000; Werren et al., 2008). CI corresponds to a conditional sterility phenomenon that arises in crosses between infected males and uninfected females or females harbouring a different *Wolbachia* type or strain. Indeed, when uninfected eggs are fertilized by *Wolbachia*-imprinted sperm from infected males, it results in improper condensation and segregation of paternal chromosomes, resulting in the formation of aneuploid or haploid nuclei instead of diploid nuclei (Stouthamer et al., 1999). Thus, while in diploid species, CI results in the embryonic death of all offspring, in haplodiploid species such as *T. urticae*, it affects only the daughters (diploids). This will thus lead to a male-biased sex-ratio (Gotoh et al., 2003; Vala et al.,

2002), and in some cases to hybrid breakdown (i.e. when aneuploidy females survive; Vala et al., 2003; Vala et al., 2000). Because the reciprocal crosses (uninfected male and infected female) are compatible, CI benefits to infected females by decreasing number of uninfected females through generations (Werren et al., 2008). CI thus constitutes a very powerful mechanism for *Wolbachia* to spread among populations (Hurst & Mcvean, 1996) and, in some cases to reach very high prevalence in the field without being mutualists (Zug & Hammerstein, 2014). However, facultative mutualism still often arises through selection of *Wolbachia* that enhance the fitness of their female hosts. Indeed, given that *Wolbachia* is maternally inherited, its transmission is intimately linked to the reproduction of the female host (Smith & Dunn, 1991). Such benefits have been found in different *Wolbachia* infected arthropod species which include increases in fecundity (Brownlie et al., 2009; Dobson et al., 2002; Fry et al., 2004; Gotoh et al., 2007; Vala et al., 2002) and longevity (Almeida et al., 2011; Brelsfoard & Dobson, 2011), nutritional provisioning (Brownlie et al., 2009; Brownlie et al., 2007; Hosokawa et al., 2010), protection against pathogens (Glaser & Meola, 2010; Hedges et al., 2008; Osborne et al., 2009; Teixeira et al., 2008), and down-regulation of plant defences (e.g. Barr et al., 2010).

Host plant effects on symbionts

The effect of plants on the relative amount of bacterial symbionts has been described previously in whiteflies (Pan et al., 2013) and in aphids (Chandler et al., 2008; Leonardo & Muir, 2003; Tsuchida et al., 2004; Tsuchida et al., 2002; Wilkinson et al., 2001). Data previously collected in our group suggests that host plants may affect the prevalence of *Wolbachia* in *T. urticae* in the field, thus also suggesting that these plants affect the relationship between the endosymbiont and its hosts. Indeed, the host plants naturally infested by *T. urticae* in the field probably have different nutritional quality and nutrient composition, but also exhibit different defence mechanisms against herbivore arthropods. For instance, bean (*Phaseolus vulgaris*, Fabaceae) has jasmonate (JA) and salicylate (SA) dependent accumulation of defence proteins, often ineffective against spider mites (Tahmasebi et al., 2014); common morning glory or “purple” (*Ipomoea purpurea*, Convolvulaceae) releases indole alkaloid intoxicating compounds (Steward & Keeler, 1988); while eggplant (*Solanum melongena*, Solenaceae), tomato (*Solanum lycopersicum*, Solenaceae) and zucchini (*Cucurbita pepo*, Cucurbitaceae) all have trichomes on their leaves and have different digestion-affecting defensive proteins as well as JA and SA (Ament et al., 2004; Fürstenberg-Hägg et al., 2013; Habib & Fazili, 2007; Migeon & Dorkel, 2015). However, the potential role of plants in *Wolbachia*-spider mite interactions has, to date, never been investigated.

Objectives of my thesis

This project focuses on the interaction between *Wolbachia*, the two-spotted spider mite *Tetranychus urticae* and some of its natural host plants. I will study this particular three-way interaction to improve our understanding of the effect of *Wolbachia* on the interaction between spider mites and plants, their ecology and evolution, as well as the effect of the plants on the dynamics of endosymbionts in spider mites.

To tackle these questions, I will first test the performance of *Wolbachia*-infected (Wi) and -uninfected (Wu) *T. urticae* on five different common host plants. I will then test the performance of Wi and Wu spider mites after being maintained on different plants for several generations to disentangle whether the effects induced by *Wolbachia* vary through time. Finally, I will conduct a field survey of spider mites on the same plants to determine whether different host plants affect the prevalence of three of the most common endosymbionts of *T. urticae*: *Wolbachia*, *Cardinium* and *Rickettsia*.

Materials and methods

Spider mite populations, tetracycline treatment and Laboratory rearing

The spider-mite population used in this experiment was reared in large numbers (>5 000) on bean plants (*Phaseolus vulgaris*, Fabaceae, var. *Enana*), under controlled conditions (25°C, photoperiod of 16L:8D). A total of 65 females were originally collected on *Datura* sp plants at Aldeia da Mata Pequena, Portugal, in November 2013 and kept in a mass-rearing environment on bean at FCUL since then (25°C, photoperiod of 16L:8D). As this population was found fully infected by *Wolbachia* in the field (Zélé et al., *in prep*), it is called Wi hereafter. To obtain a *Wolbachia*-uninfected (Wu) population with a similar genetic background, the Wi population was treated with antibiotics roughly 3 months after collection. This was done by placing 30 adult females in petri dishes containing bean leaf fragments placed on cotton with a tetracycline solution (0.1 %, w/v). This treatment was applied continuously for three successive generations (Breeuwer, 1997), then the population was maintained in a mass-rearing environment without antibiotics for about twelve generations before the experiments, to avoid the potential side effects of the antibiotic treatment. Before use, up to 20 individual females and pools of 100 females were checked by PCR to confirm the absence and presence of *Wolbachia* infection in the Wu and Wi populations, respectively.

Effect of *Wolbachia*, on the host plant, and of their interaction on the performance of spider mites

To determine the effect of both *Wolbachia* infection and the host plant, as well as their possible interactions, on the performance of *T. urticae*, we measured several life history traits of both Wi and Wu mated females on bean (var. *Enana*), eggplant (var. *Larga Morada*), purple (var. *Vigorous*), tomato (var. *Money Maker*) and zucchini (var. *Bellezza Negra*).

This experiment was performed using spider mites from age cohorts produced from mass cultures of each of the Wi and Wu populations. Each cohort was produced by placing 100 females to lay eggs for one single day on detached bean leaves placed on water-soaked cotton. This procedure, performed during 5 consecutive days, allowed us to control the exact age of each offspring female used in the experiment. On the first day of the experiment, 50 adult mated female mites (10-13 days old) per treatment were haphazardly picked from either Wi or Wu cohorts and placed individually on a 2 cm² leaf disc from one of the 5 different host plants. The replicates were distributed along 5 days (10 replicates per treatment per day). Females that were alive after 3 days were transferred to new leaf discs where they could lay eggs for another 3 days. Their survival (**S**) and proportion of drowned females (**PD**) were followed daily during six days, while their fecundity was measured at days 3 and 6. In order to calculate **PD**, death of females trying to escape the leaf discs onto the water soaked

cotton was counted as censor data. Average female daily fecundity was estimated considering their survival ($DF = \text{total number of eggs laid per female} / \text{number of days the female was alive}$). The number of unhatched eggs was counted 5 days later to obtain the hatching rate ($HR = \text{hatched eggs} / \text{total number of eggs}$). Adult offspring (F_1 females + F_1 males) were counted after 6 additional days and used to calculate juvenile survival ($JS = [\text{total number of eggs} - \text{number of unhatched eggs} - \text{number of } F_1 \text{ adults}] / \text{total number of eggs}$), the F_1 sex ratio ($SR = \text{number of } F_1 \text{ males} / \text{number of } F_1 \text{ adults}$) and number of viable offspring ($VO = \text{total number of adult offspring per female per treatment}$). This experiment was repeated three months later (the experiments are thus called blocks 1 and 2) except for replicates involving tomato plants, as explained in the results section.

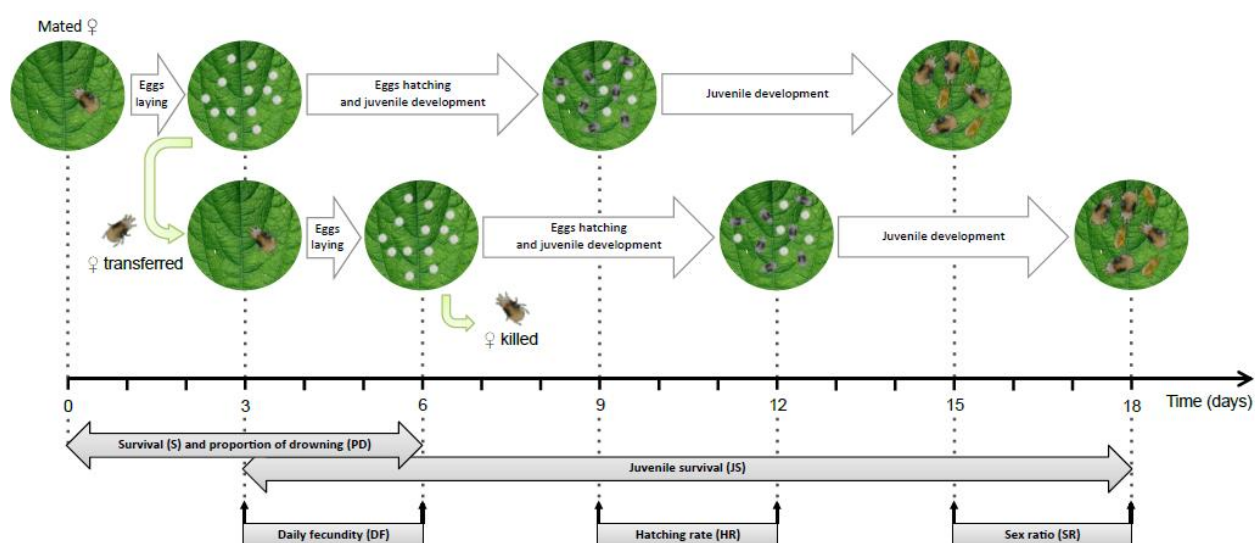


Figure 1. Experimental setup used to measure the performance of Wi and Wu *T. urticae* females on five different host plants. Grey boxes represent the variables measured during the course of the experiment. A total of 100 replicates per treatment were performed (except for the two treatments on tomato that were not included in the second block and for which 50 replicates have been done).

Effect of *Wolbachia* on spider mites performance after maintenance on different host plants

To determine whether the effect of *Wolbachia* on mite performance changes following several generations of maintenance on different host, mites from both Wi and Wu populations were reared in large numbers (100 mated females initially; around 24 generations after the tetracycline treatment) on entire plants of eggplant, purple and bean (same varieties as described above), under controlled conditions (25°C, photoperiod of 16h Light: 8h Dark) during 6 months (c.a. 12 generations). The choice of these plants was based on the results of the previous experiment. First, we did not include tomato since it was very difficult to maintain Wi and Wu mites on this plant due to the strong reduction it induces on female fecundity (cf. results section). Second, as both zucchini

and purple had similar effects for most of the studied traits (cf. results section) or the effects of *Wolbachia* on these two plants went in the same direction for other traits (cf. results section), we included only purple in this experiment. Following 6 months of maintenance on bean, eggplant and purple, the performance of Wi and Wu mites was tested on these plants, along with their respective controls (i.e. mites maintained on bean, the original host). The procedure to measure the performance of the females across the different treatments was similar to the one described above for the previous experiment (Fig. 1) with two minor differences. All replicates (50 per treatment) were done into two consecutive days instead of 5, and only one block was performed. The age cohorts were evenly created on bean (including the populations maintained on eggplant and purple) to equalize maternal effects across treatments (Magalhães et al., 2011)

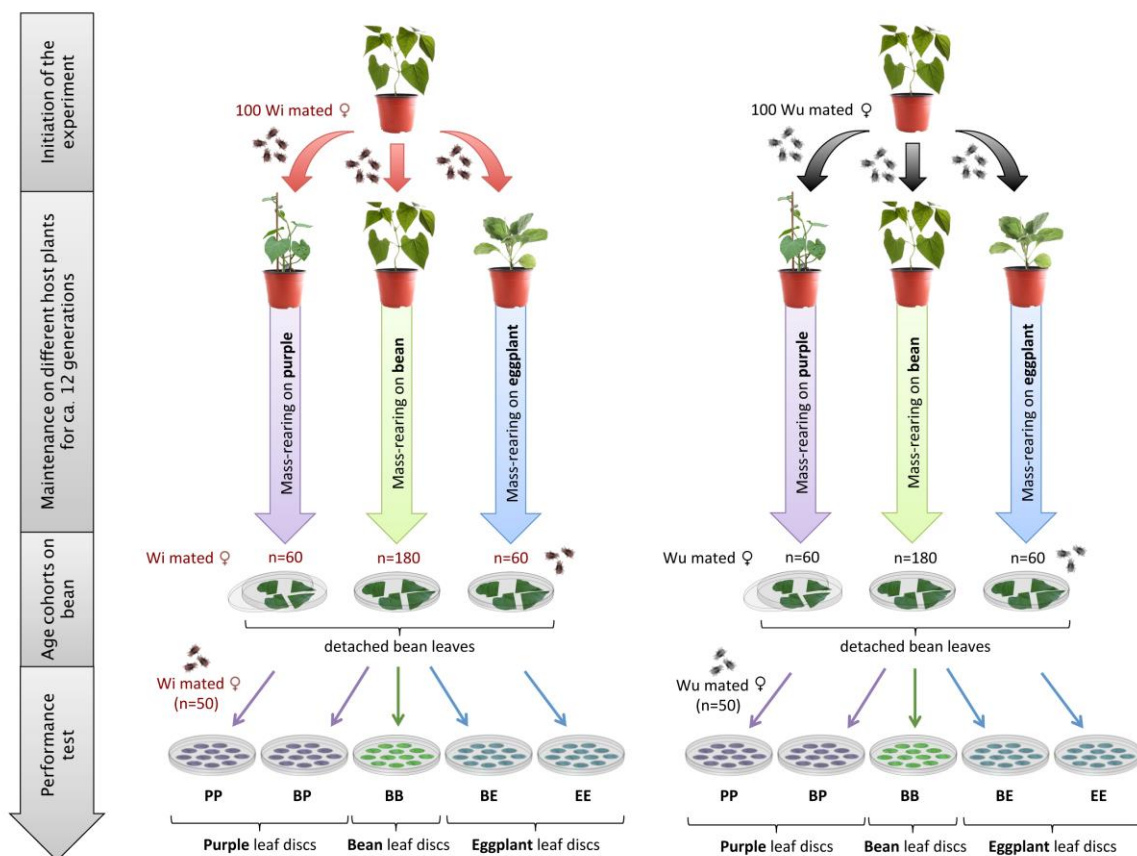


Figure 2. Experimental setup describing the different rearing conditions used to compare the performance of Wi and Wu *T. urticae* females after maintenance on bean, purple and eggplant. From two initial Wi and Wu populations reared on bean, 10 different treatments were created: BB, mites maintained on bean and tested on bean; BE, mites maintained on bean and tested on eggplant; EE, mites maintained on eggplant and tested on eggplant; BP, mites maintained on bean and tested on purple; PP, mites maintained on purple and tested on purple.

Effect of the host plant on endosymbiont prevalence in the field

Spider mite collections

To determine whether different host plants affected the prevalence of the three most common spider mites' endosymbionts in the field, namely *Wolbachia*, *Cardinium* and *Rickettsia*, a field survey of spider mites collected on bean, eggplant, tomato and zucchini across 12 different locations (Figure 3; see Annex 1 for more details) was conducted. These sampling sites consisted of open fields, greenhouses or small vegetable gardens of organic plantations, free of any insecticide or pesticide, avoiding this potential confounding effect on the prevalence of symbionts. Although an ideal orthogonal sampling design should involve all plants under study in each sampling location, this could not be performed despite a large sampling effort (Annex 2). Also, due to the weak infestation rate of purple by *T. urticae*, we could obtain only 2 populations over a total of 39 different locations where this plant was found (Annex 2). Mite collection consisted of detaching infested leaves and placing them in closed plastic boxes which were brought to the laboratory. Subsequently, 50 adult females were haphazardly picked from each population. These females' species was individually identified based on morphological characteristics under a binocular microscope, then placed on 2 cm² leaf discs of the same plant species where they were found, and allowed to lay eggs for 4 days. Four days later, 20 mites were randomly selected from each population and tested for the presence or absence of *Wolbachia*, *Cardinium* and *Rickettsia*. For spider mites species identification, the offspring of the 20 females screened for endosymbionts were allowed to develop until adulthood in order to extract the DNA of 1 daughter per female, pooled for each population, and to perform PCR-based species identification (see below).

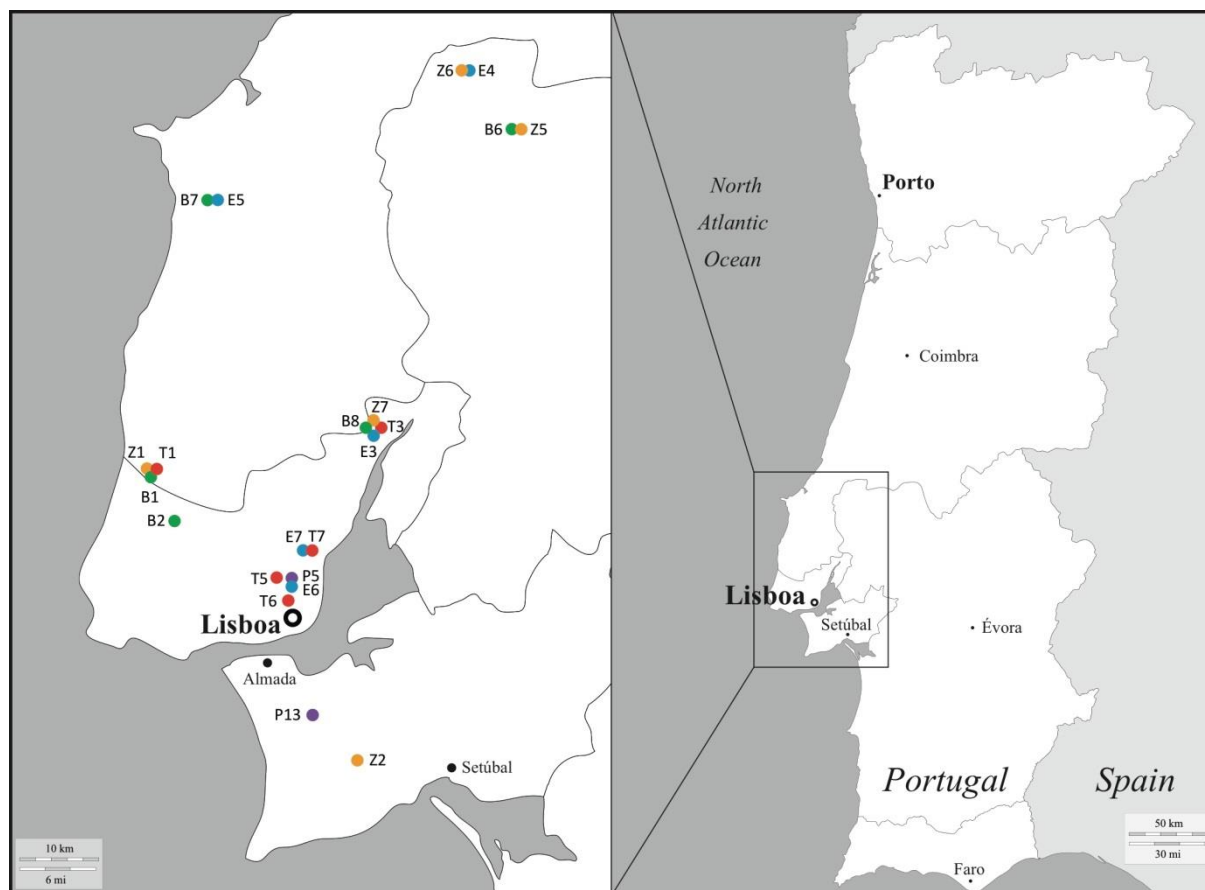


Figure 3. Map showing sampling sites where *Tetranychus urticae* were collected in the Tagus Valley region around Lisbon on different host plants: bean (green dots), eggplant (blue dots), purple (purple dots), tomato (red dots), and zucchini (orange dots)

Screening for the presence of endosymbionts and molecular species identification

The prevalence of *Wolbachia*, *Cardinium* and *Rickettsia* was tested on entire mites without DNA extraction (the females were placed individually directly in the PCR mix) by multiplex PCR using genus-specific primers (Zélé et al. *in prep*) given in Annex 3. PCR reactions were done in a 10µl final volume reaction containing 5µL of 2X QIAGEN Multiplex PCR Master Mix (Qiagen NV, Venlo, The Netherlands), 1µL of Q-solution (Qiagen NV, Venlo, The Netherlands), 2µL of RNase free water, and 2µL of a primer mix containing each primer at the concentration given in Annex 3. Amplification conditions were as follows: 15 minutes at 95°C, followed by 35 cycles of 94°C for 30s, 65°C for 1min30s, 72°C for 1 minute and a final step at 72°C for 10 minutes. Following the PCR, 5 µL of each PCR product was electrophoresed on a 2% agarose gel, stained with Envision™ DNA Dye as Loading Buffer (Amresco Inc., Solon, USA), and photographed under ultra-violet light. The amplification profile is given in figure 3.

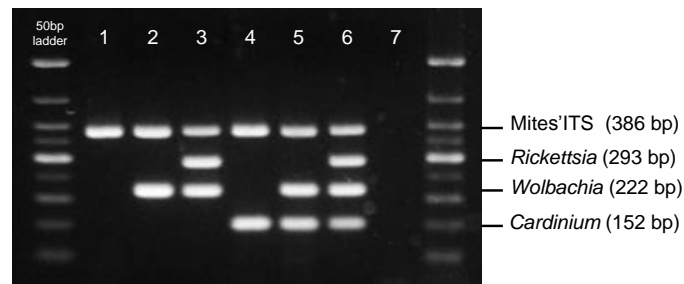


Figure 4. Molecular detection of *Wolbachia*, *Cardinium* and *Rickettsia* in *T. urticae* by multiplex PCR. The size of amplified fragment by each specific primer pair is given on the right part of the picture: a 293-bp fragment specific to *Rickettsia*, a 222-bp fragment specific to *Wolbachia*, and a 152-bp fragment specific to *Cardinium*. The presence of several of these bands indicates coinfections. DNA quality was controlled by amplifying a 386-bp fragment of the spider mite partial 5.8s rDNA and ITS2 gene. Lane 1, uninfected mite; Lane 2, *Wolbachia* single infection; Lane 3, *Rickettsia*-*Wolbachia* coinfection, Lane 4, *Cardinium* single infection; Lane 5, *Wolbachia*-*Cardinium* coinfection; Lane 6, *Rickettsia*-*Wolbachia*-*Cardinium* coinfection; Lane 7, without DNA template (Zélé *et al. in prep*).

For spider mites species identification, total genomic DNA was extracted from the pools described above using the Sigma-Aldrich protocol and materials (GenElute™ Mammalian Genomic DNA Miniprep Kit, Sigma-Aldrich, St. Louis, MO, United States). Total DNA was eluted in the final step with 30µL Elution Solution (Qiagen NV, Venlo, The Netherlands). Species were identified by multiplex PCR (Zélé *et al. in prep*) using species-specific primers given in Annex 4. PCR reaction were done in a 10µl final volume reaction containing 1µL of DNA template, 5µL of 2X QIAGEN Multiplex PCR Master Mix (Qiagen NV, Venlo, The Netherlands), 2µL of Q-solution (Qiagen NV, Venlo, The Netherlands), 1µL of RNase free water, and 2µL of a primer mix containing each primer at the concentration given in Annex 3. Amplification conditions were as follows: 15min at 95°C, followed by 35 cycles of 94°C for 30s, 58°C for 1min30s, 72°C for 1min and a final step at 72°C for 10min. Following the PCR, 5 µL of each PCR product was electrophoresed on a 2% agarose gel, stained with Envision™ DNA Dye as Loading Buffer (Amresco Inc., Solon, USA), and photographed under ultra-violet light. The amplification profile is given in figure 4.

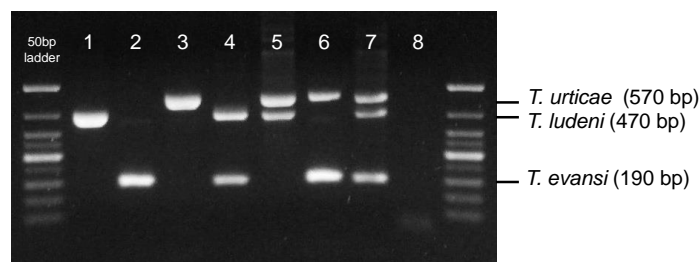


Figure 5. Simultaneous molecular identification of *Tetranychus urticae*, *T. ludeni* and *T. evansi* by multiplex PCR. The size fragment of the spider mite partial ITS1, 5.8s rDNA and ITS2 gene amplified by each specific

primer pair is given on the right part of the picture: a 570-bp fragment specific to *T. urticae*, a 470-bp fragment specific to *T. ludeni*, and a 190-bp fragment specific to *T. evansi*. Lane 1: *T. ludeni*; Lane 2: *T. evansi*; Lane 3: *T. urticae*, Lane 4: co-occurrence of *T. ludeni* and *T. evansi* in the template; Lane 5: co-occurrence *T. urticae* - *T. ludeni*; Lane 6: co-occurrence *T. urticae* - *T. evansi*; Lane 7: co-occurrence of the three species; Lane 8: without DNA template (Zélé *et al. in prep*).

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 fitness effects of *Wolbachia* on different host plants was as follows: the status of infection of the females by *Wolbachia* (i.e. I: infected or U: uninfected) and the host plants tested were fit as fixed explanatory variables, whereas block and day were fit as random explanatory variables. Survival data (**FS**) were analysed using Cox proportional hazards mixed-effect models (coxme, kinship package). The daily fecundity per female (**DF**) was log transformed to improve normality (Box-Cox transformation; Crawley, 2007) and subsequently analysed using linear mixed-effect models (lme, nlme package). All other parameters (**HR**; **SR**; **JS**; **PD**) were computed using the function cbind and analysed using generalized linear mixed models with a binomial error distribution (glmer, lme4 package). As also found in other systems (e.g. Drummond & Rodríguez, 2015; Simmons & Holley, 2011), number of viable offspring (**VO**) count data were greatly overdispersed. One way of handling this overdispersion is by using negative binomial pseudo distributions (Crawley, 2007). However, to our knowledge, it is not currently possible to account for negative binomial distributions within a mixed model *glmer* procedure. For this reason, we used instead a *glm* model with a negative binomial error distribution (*glm.nb*, MASS package) and we fitted block and day as fixed factors, next to our variables of interest (i.e. *Wolbachia*-infection status and host plant). 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 few levels (Bolker, 2008). When the interaction between the variable “*Wolbachia*” (I or U) and “plant” was found to be significant, we analysed each host plant separately for the effect of *Wolbachia*. When only the variable “plant” was found to be significant, *a posteriori* contrasts (Crawley, 2007) between plants were carried out by aggregating factor levels together and by testing the fit of the simplified model using ANOVA.

The statistical models used to analyse the effect of *Wolbachia* on the fitness of spider mites after being maintained on different host plants were similar to the ones described previously, with some minor differences. The variable “treatment” (BB, BE, EE, BP and BP) was fit as fixed explanatory variables instead of “plant”, and only the variable “day” was fit as random explanatory variables since only one block was performed for this experiment. The binary data juvenile survival (**JS**) were

greatly overdispersed, and it is not currently possible to use “quasi” families within a mixed model *glmer* procedure as described above for negative binomial distributions. For this reason, we used instead a *glm* model with a quasibinomial error distribution (*glm*, stats package) and we fitted day as fixed factors next to our variables of interest. Finally, contrasts between treatments were carried out *a priori* to compare the treatment involving bean and purple (BB; BP, PP) or those involving bean and eggplant (BB, BE, EE).

The analysis the binary data of endosymbiont prevalence were conducted separately for *Wolbachia*, *Rickettsia* and *Cardinium*, using a *glm* model with a quasibinomial error distribution (*glm*, stats package) to correct for overdispersion. Both “plant” and “location” were fit as fixed explanatory variables (as described above, it is not currently possible to use “quasi” families within a mixed model *glmer* procedure). When the variable “plant” was found to be significant, a stepwise *a posteriori* procedure was carried out for the contrasts between host plants (Crawley, 2007).

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 X^2 values given in the text are for the minimal model (Crawley, 2007).

Results

Effect of *Wolbachia*, of the host plant, and of their interaction on the performance of spider mites

Female survival (S) over 6 days and the proportion of drowned females (PD)

Overall, there was no significant effect of *Wolbachia* ($X^2_1 = 1.25$, $p=0.26$), of the host plants ($X^2_4 = 6.68$, $p=0.15$), or of their interaction ($X^2_4 = 4.77$, $p=0.31$) on the survival of the females over the 6 first days of the experiment (Fig. 6A). However, although *Wolbachia* did not affect significantly the proportion of drowned females (i.e. accidental death of females trying to escape the leaf discs), neither in itself ($X^2_1 = 0.002$, $p=0.96$) nor through an interaction with the host plants ($X^2_4 = 1.87$, $p=0.76$), there was a significant effect of the host plants ($X^2_4 = 64.06$, $p<0.0001$; Fig. 6B). The contrast analyses revealed that no significant differences were found between eggplant, purple and zucchini (in average 47.1 ± 3.3 %; *Contrast between eggplant, purple, and zucchini*: $X^2_2 = 1.44$, $p=0.49$), while the highest proportion of drowned females was found on tomato (88 ± 3.3 %; *Contrast between tomato and eggplant-purple-zucchini*: $X^2_1 = 33.69$, $p<0.0001$) and the lowest on bean (26.1 ± 4.2 %; *Contrast between bean and purple*: $X^2_1 = 16.98$, $p<0.0001$).

Daily fecundity (DF)

Wolbachia did not significantly affect the average number of eggs laid per female per day ($X^2_1 = 0.20$, $p=0.65$; Fig. 6C) independently of the different host plants tested (*Wolbachia-plant interaction*: $X^2_4 = 1.84$, $p=0.77$). However, we found a significant effect of the host plant ($X^2_4 = 311.52$, $p<0.0001$). Contrast analyses revealed that the females laid a similar number of eggs on purple and zucchini (on average 3.37 ± 0.11 eggs; *Contrast between purple and zucchini*: $X^2_1 = 0.56$, $p=0.46$) but slightly less (c.a. 1 egg) than on bean, our control (*Contrast between purple-zucchini and bean*: $X^2_1 = 20.55$, $p<0.0001$). Eggplant reduced female fecundity by almost 3 eggs per days compared to bean and by more than 1 egg compared to purple and zucchini (*Contrast between eggplant and purple-zucchini*: $X^2_1 = 30.87$, $p<0.0001$). The strongest effect was found on tomato where both Wi and Wu mites laid on average less than 1 egg per day (*Contrast between tomato and eggplant*: $X^2_1 = 92.70$, $p<0.0001$). Given the high proportion of drowned females on tomato and the few number of eggs laid on this plant by the surviving females, the subsequent traits could not be measured on this plant, and thus replicates involving this plant were not performed on the second block.

Hatching rate (HR)

The analyses of the hatching rate revealed that the infection by *Wolbachia* affects differently the proportion of eggs hatched on the different host plant tested here (*Wolbachia-plant interaction*: $X^2_3=31.55$, $p<0.0001$; Fig. 6D). Further analyses conducted for each host plant separately revealed that the hatching rate of eggs laid by Wi mites was lower than those laid by Wu mites on purple and on zucchini ($X^2_1=10.51$, $p<0.01$ and $X^2_1=26.29$, $p<0.0001$, respectively), while the opposite was found on eggplant ($X^2_1=7.00$ $p<0.01$). On bean, however, there was no significant difference between the hatching rate of eggs laid by Wi and Wu mites ($X^2_1=1.04$, $p=0.31$).

Juvenile survival (JS)

The survival of offspring from both Wi and Wu females was not significantly different ($X^2_1=0.46$ $p=0.50$) whatever the host plant on which they developed (*Wolbachia-plant interaction*: $X^2_3=6.98$ $p=0.07$; Fig. 6E). However, the host plants contributed significantly to the average proportion of surviving juveniles ($X^2_3=281.41$ $p<0.0001$). Contrast analyses revealed no significant differences between bean and zucchini (*Contrast between bean and zucchini*: $X^2_1=0.46$ $p=0.50$), but juveniles survived significantly less on eggplant (*Contrast between eggplant and bean-zucchini*: $X^2_1=102.13$ $p<0.0001$) and significantly more on purple (*Contrast between purple and bean-zucchini*: $X^2_1=80.00$ $p<0.0001$).

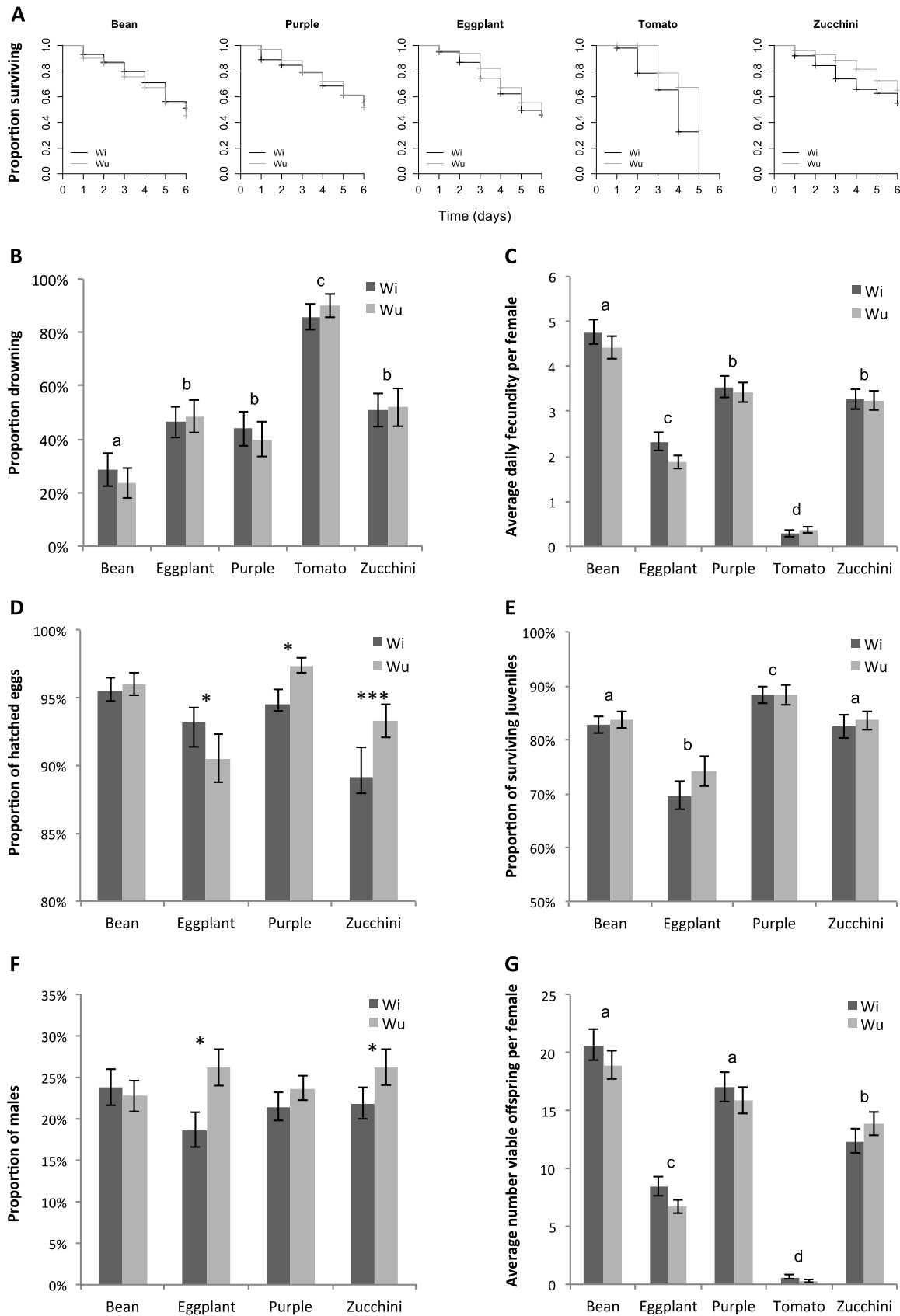


Figure 6. Effect of *Wolbachia* and of host plants on the performance of spider mites. A: survival (S); B: Proportion of drowned females (PD); C: daily fecundity (DF) over 6 days; D: proportion of hatched eggs (HR); E: juvenile survival (JS); F, sex ratio (SR); and G, number of viable offspring (VO). Bars represent means (\pm s.e.) for *Wolbachia*-infected (Wi; dark grey) and uninfected (Wu; light grey) females. Identical or absent superscripts (a, b, c, d) above bars indicate non-significant differences at the 5% level (contrast analyses). Stars represent the levels of significance: * ($p < 0.05$), ** ($p < 0.001$), *** ($p < 0.0001$)

Sex ratio (SR)

Our measure of the offspring sex ratio revealed that, overall, the proportion of males produced by Wu females was significantly higher than that produced by Wi females ($X^2_1=13.2$ $p<0.001$; Fig. 6F). However, although we did not detect any important role of the plants on this trait ($X^2_3=5.97$ $p=0.11$), the extent of the effect of *Wolbachia* differed marginally between plants (*Wolbachia-plant interaction*: $X^2_3=7.23$ $p=0.06$). The separate analyses of the effect of *Wolbachia* on each plant indeed revealed an increase of the proportion of females produced by Wi females on eggplant and zucchini ($X^2_1=8.53$ $p<0.01$ and $X^2_1=6.48$ $p=0.01$, respectively), but not on bean and purple ($X^2_1=0.78$ $p=0.38$ and $X^2_1=3.00$ $p=0.08$, respectively).

Number of viable offspring (VO)

The statistical analyses of offspring viability revealed that neither *Wolbachia* nor its interaction with the host plant significantly affected this trait ($X^2_1=0.95$ $p=0.33$ and $X^2_4=4.65$ $p=0.33$, respectively). However, host plants can significantly explain the results obtained ($X^2_4=254.81$ $p<0.0001$; Fig. 6G), with no difference between the effect of bean and purple (*Contrast between bean and purple*: $X^2_2=5.50$ $p=0.06$), but a significant decrease of about 5.02 ± 0.96 viable offspring on zucchini compared bean and purple (*Contrast between zucchini and bean-purple*: $X^2_2=6.62$ $p=0.04$), a decrease of 5.58 ± 0.89 viable offspring on eggplant compared to zucchini (*Contrast between eggplant and zucchini*: $X^2_2=46.4$ $p<0.0001$), and a decrease of 7.13 ± 0.52 viable offspring on tomato compared to eggplant (*Contrast between tomato and eggplant*: $X^2_2=97.74$ $p<0.0001$).

Effect of *Wolbachia* on spider mites performance after maintenance on different host plants

Females' survival (S) over 6 days and proportion of drowned females (PD)

To compare the performance of Wi and Wu *T. urticae* females after maintenance on bean, purple and eggplant 10 different treatments were created from two initial Wi and Wu populations reared on bean,: **BB**, mites maintained and tested on bean; **BE**, mites maintained on bean and tested on eggplant; **EE**, mites maintained and tested on eggplant; **BP**, mites maintained on bean and tested on purple; **PP**, mites maintained and tested on purple.

There was a significant effect of the interaction between *Wolbachia* and the different treatments on the survival of the females over the 6 first days of the experiment ($X^2_4 = 9.63$, $p < 0.05$; Fig. 7A), and further analyses within each treatments revealed that *Wolbachia* decreased mites survival in the PP treatment ($X^2_1 = 4.68$, $p = 0.03$), but had no effect in the other treatments: BB, BE, BP and EE ($X^2_1 = 3.43$, $p = 0.06$, $X^2_1 = 2.33$, $p = 0.13$, $X^2_1 = 0.25$, $p = 0.61$, $X^2_1 = 0.10$, $p = 0.75$, respectively). However, *Wolbachia* did not affect significantly the proportion of drowned females, neither in itself ($X^2_1 = 1.00$, $p = 0.32$) nor through an interaction with the treatments ($X^2_4 = 6.47$, $p = 0.17$), but we found a significant effect of the treatments ($X^2_4 = 40.9$, $p < 0.0001$; Fig. 7B). *A priori* contrast analyses revealed that the proportion of drowned females was not significantly different between treatments BB, BP and PP (on average $32.5 \pm 5.37\%$, *Contrast between BB, BP and PP*: $X^2_2 = 4.03$, $p = 0.13$), but an increase of c.a. 47% of drowned females in the treatment BE and EE compared to BB (*Contrast between BE-EE and BB*: $X^2_2 = 22.06$, $p < 0.001$), without difference between the treatments BE and EE (*Contrast between BE and EE*: $X^2_2 = 0.58$, $p = 0.75$).

Daily fecundity (DF)

The average number of eggs laid per female per day was significantly affected by the interaction between *Wolbachia* and the different treatments (*Wolbachia-treatment interaction*: $X^2_4 = 12.099$, $p = 0.02$; Fig. 7C) and the independent analyses of each treatment revealed that Wi females laid on average more eggs per day than Wu females within the treatments BB ($X^2_1 = 4.95$, $p = 0.03$), BP ($X^2_1 = 8.64$, $p < 0.01$) and PP ($X^2_1 = 6.62$, $p = 0.01$). Conversely, there was no difference between the number of eggs laid by Wi and Wu females for the treatments involving eggplant: BE and EE ($X^2_1 = 0.51$, $p = 0.47$ and $X^2_1 = 0.18$, $p = 0.67$, respectively).

Hatching rate (HR)

The analyses of the hatching rate revealed that the effect of *Wolbachia* infection on the proportion of eggs hatched differed between treatments (*Wolbachia-treatment interaction*: $X^2_4 = 113.26$, $p < 0.0001$; Fig. 7D). Further analyses conducted separately for each treatment revealed that eggs laid by Wi mites hatched more than those laid by Wu mites on treatments BE, BP and PP ($X^2_1 = 10.98$, $p < 0.001$; $X^2_1 = 13.14$, $p < 0.001$).

and $X^2_1=67.40$, $p<0.0001$, respectively), while the opposite was found on BB ($X^2_1=27.91$ $p<0.0001$), and no significant effect of *Wolbachia* was found on EE ($X^2_1<0.001$, $p=0.98$).

Juvenile survival (JS)

Overall, *Wolbachia* did not affect significantly the survival of females offspring independently of the treatments (*Wolbachia effect*: $X^2_1=6.52$ $p=0.13$; *Wolbachia-treatment interaction*: $X^2_3=6.80$ $p=0.67$), but the treatments themselves affected significantly the average proportion of surviving juveniles ($X^2_5=262.1$, $p<0.0001$; Fig. 7E). Contrast analyses revealed that juveniles from mothers reared on bean survived about 27% less when they developed on eggplant compared to when they developed on bean (*Contrast between BB and BE*: $X^2_1=98.24$, $p<0.0001$), and they survived even less (ca. 15%) on eggplant when their ancestors were reared on eggplant compared to those reared on bean ($X^2_1=12.13$, $p=0.04$). Similarly, juveniles survived ca. 5% less when they developed on purple compared to when they developed on bean (*Contrast between BB and BP-PP*: $X^2_1=37.2$, $p<0.001$), but this effect was independent of the rearing history of their ancestors (*Contrast between BP and PP*: $X^2_1=1.17$, $p=0.52$).

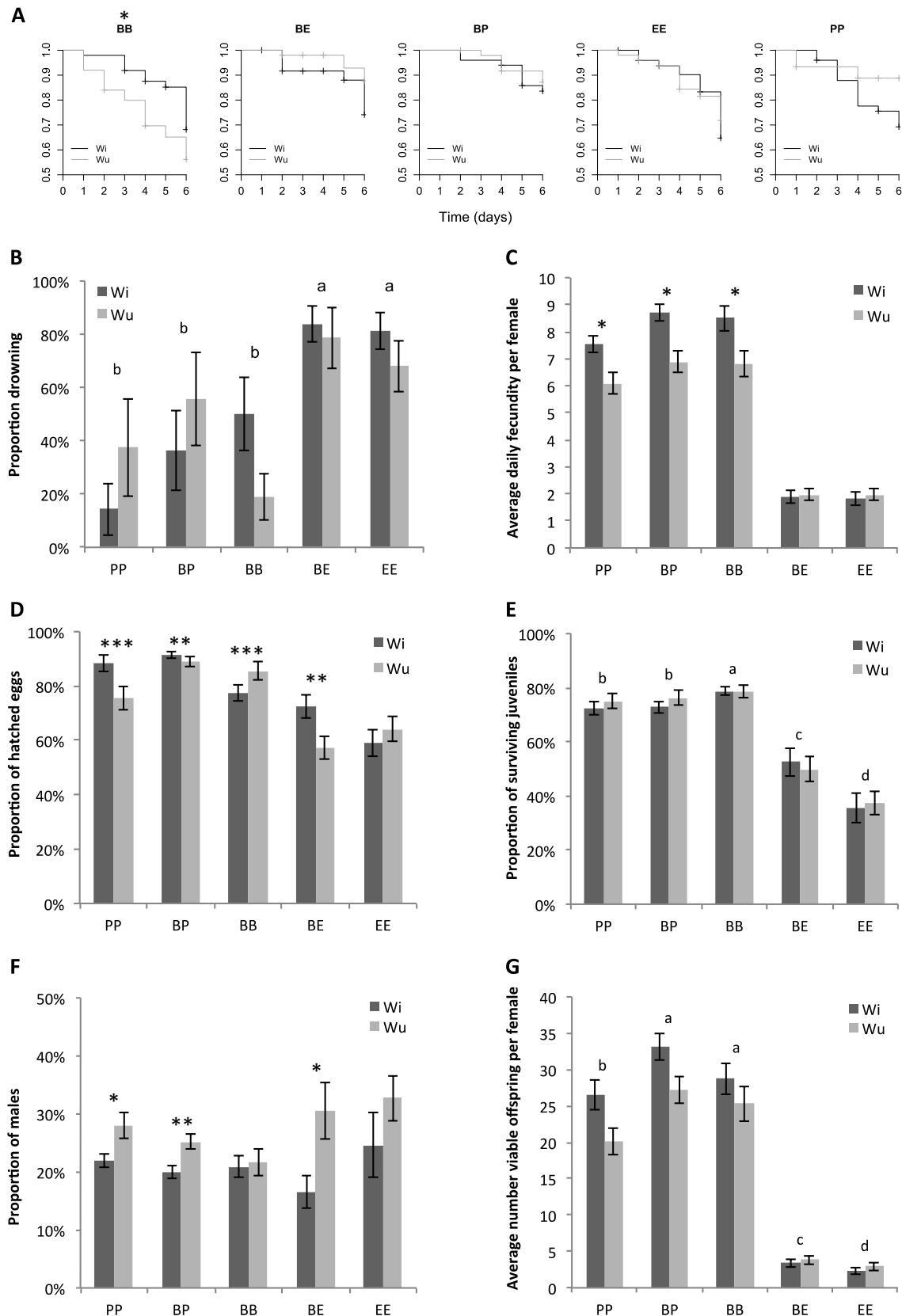


Figure 7. Effect of *Wolbachia* on spider mites performance after maintenance on different host plants. A: survival (S); B: Proportion of drowned females (PD), and C: daily fecundity (DF) over 6 days; D: proportion of hatched eggs (HR); E, juvenile survival (JS); F: sex ratio (SR); and G: number of viable offspring (VO). Bars represent means (\pm s.e.) for *Wolbachia*-infected (Wi; dark grey) and uninfected (Wu; light grey) females. Identical or absent superscripts (a, b, c, d) above bars indicate non-significant differences at the 5% level (contrasts analyses). BB, mites maintained and tested on bean; BE, mites maintained on bean and tested on eggplant; EE, mites maintained and tested on eggplant; BP, mites maintained on bean and tested on purple; PP, mites maintained and tested on purple. Stars represent the levels of significance: * ($p < 0.05$), ** ($p < 0.001$), *** ($p < 0.0001$)

Sex ratio (SR)

Measures of the offspring sex ratio showed that, overall, the proportion of males produced by Wi females was significantly lower than that produced by Wu females ($X^2_1=24.46$ $p<0.0001$; Fig. 7F), independently of the treatments (*Wolbachia-treatment interaction*: $X^2_4=6.08$ $p=0.19$; *Treatment effect*: $X^2_3=8.23$ $p=0.08$). However, the separate analyses of the effect of *Wolbachia* on each treatment revealed an increase of the proportion of females produced by Wi females on BE ($X^2_1=4.90$ $p<0.03$), BP ($X^2_1=12.44$ $p<0.001$) and PP ($X^2_1=8.48$ $p<0.01$), but not on BB and EE ($X^2_1=0.26$ $p=0.61$ and $X^2_1=3.82$ $p=0.05$, respectively).

Number of viable offspring (VO)

The statistical analyses of offspring viability revealed that neither *Wolbachia* nor its interaction with the treatments significantly affected the average number of viable offspring produced per female (*Wolbachia* effect: $X^2_1=1.58$ $p=0.21$; *Wolbachia-treatment interaction*: $X^2_4=6.26$ $p=0.18$). However, we found an important effect of the treatments ($X^2_4=383.4$ $p<0.0001$; Fig. 7G), since females from the treatments BB, BP and PP produced on average 27.0 ± 0.85 viable offspring over 6 days (*Contrast between BB, BP and PP*: $X^2_2=4.49$, $p=0.11$), while those from the treatments involving eggplant, BE and EE, produced on average 3.61 ± 0.42 and 2.59 ± 0.37 viable offspring, respectively (*Contrast between BB and BE*: $X^2_2=197.02$ $p<0.0001$; *Contrast between BE and EE*: $X^2_2=6.40$, $p=0.04$).

Effect of the host plant on endosymbiont prevalence in the field

The prevalence of *Wolbachia*, *Rickettsia* and *Cardinium*, endosymbionts of *T. urticae*, was studied on five different plants, namely bean, eggplant, purple, tomato and zucchini, over 12 different locations in Portugal. Overall, although the sampling site was found to be a major determinant of the infection frequency by *Wolbachia*, *Rickettsia* and *Cardinium* ($X^2_{11}=36.94$ $p=0.03$, $X^2_{11}=10.56$ $p<0.0001$, and $X^2_{11}=17.26$ $p<0.0001$, respectively), we also found a significant effect of the host plants on the endosymbiotic array carried by *T. urticae* females (Fig. 8 and Annex 5 for infection frequencies at the population level). More specifically, both the prevalence of *Wolbachia* ($X^2_4=20.44$ $p=0.02$) and *Rickettsia* ($X^2_4=8.69$ $p<0.0001$) were found to differ between host plants, but we failed to detect any significant effect of the host plants on the prevalence of *Cardinium* ($X^2_4=1.64$ $p=0.21$). The prevalence of *Wolbachia* was overall very high and did not differ between bean, eggplant and purple (on average 95.8 ± 1.29 %; $X^2_2=5.23$ $p=0.22$), or between purple, tomato and zucchini (on average 89.2 ± 2.01 %; $X^2_2=2.26$ $p=0.52$), but was about 8% higher on bean or eggplant compared to tomato or zucchini ($X^2_2=18.03$ $p<0.001$). Conversely, the prevalence of both *Cardinium* and *Rickettsia* were very low and none of them were found on bean. Similarly, *Rickettsia* was not found in any populations collected on zucchini. Importantly, further contrast analyses between plants for the prevalence of *Rickettsia* revealed that although the same average frequency of infection was found on eggplant and tomato ($2 \pm 1.4\%$), it differed significantly between these two plants ($X^2_1=1.41$, $p=0.01$), probably due to differences at the population level: only one population (one location) was found infected for eggplant, while two populations (two locations) were found infected for tomato (Annex 5). For the same reason (location effect), no difference in *Rickettsia* prevalence was found between purple and tomato ($X^2_1<0.0001$, $p=0.99$), despite an infection frequency ca. 11% higher on the former.

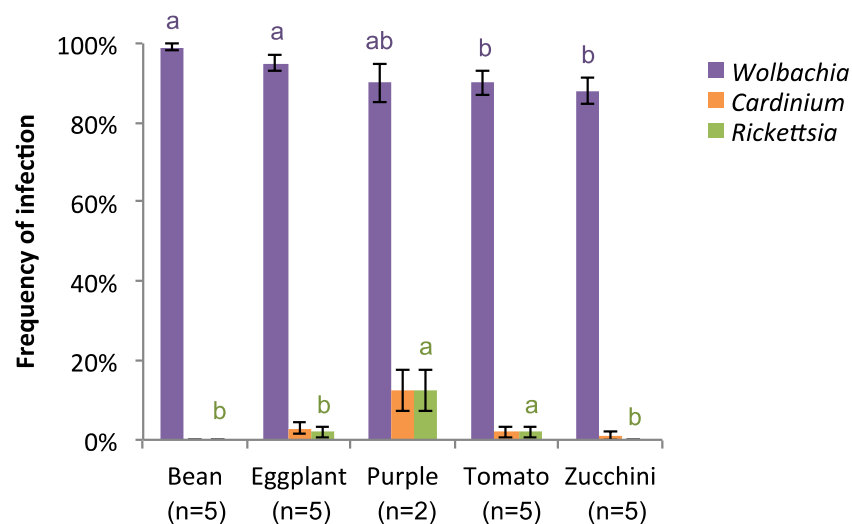


Figure 8. Host plant effect on the prevalence of *Wolbachia*, *Cardinium* and *Rickettsia* in *T. urticae* females. Bars represent means (\pm s.e.) infection frequencies by *Wolbachia* (purple), *Cardinium* (orange) and *Rickettsia* (green) for several *T. urticae* populations collected on bean, eggplant, purple, tomato, and zucchini. Numbers between brackets indicate the total number of mites populations sampled for each plant. Identical or absent superscripts (a, b, c, d) above bars for each endosymbiont indicate non-significant differences between plants at the 5% level (contrasts analyses).

Discussion

Our results show that *Wolbachia* infection may be detrimental, beneficial, or neutral on egg hatching rate depending on the host plant, and that these results were unaffected by the mites' rearing history (i.e. laboratory maintenance on different plants). All other life history traits were affected either by the plant species or by *Wolbachia* infection only, although they may change with different rearing methods. Our results also show that the prevalence of *Wolbachia* and *Rickettsia* in natural populations varied with the host plants, but not that of *Cardinium*.

Effect of *Wolbachia*, of the host plant, and of their interaction on the performance of spider mites

Overall, the effect of *Wolbachia* on spider-mites' life-history traits did not vary much across host plants: proportion of drowned, daily fecundity, juvenile survival and number of viable offspring were affected differently by the different host plants, while *Wolbachia*, had an effect on sex ratio only. However, we found significant costs or benefits of *Wolbachia* on the eggs hatchability of spider mites depending on the tested host plant. Such host plant-specific benefits of symbionts have been shown only very recently in another system involving the facultative endosymbiont *Arsenophonus* in a polyphagous herbivore, the cowpea aphid, *Aphis craccivora* (Wagner et al., 2015).

The variable effects of *Wolbachia* on eggs hatching rate, on different plants, may be explained by *Wolbachia*-dependence for nutrients obtained by the host while feeding, as it was demonstrated in other host-symbiont interactions (Chandler et al., 2008; Davy et al., 2012; Serbus et al., 2015). Analyses of *Wolbachia* genomes suggest that they lack many essential biosynthetic pathways, most notably those involved in amino acid production, and are therefore required to catabolize amino acids produced by their host (Foster et al., 2005; Wu et al., 2004). It is thus likely that *Wolbachia* impose a nutritional burden to their hosts, sequestering and using vital host nutrients for their own survival. *Wolbachia*-associated fitness burdens on hosts could be explained by host-symbiont competition for key resources, such as amino acids (Caragata et al., 2014), sugars (Markov & Zakharov, 2006), macronutrients (Ponton et al., 2014), or iron (Gill et al., 2014). More specifically for hatching rate, dietary amino acid supplementation in the mosquito *Aedes aegypti* improved egg viability of *Wolbachia*-infected strains (Caragata et al., 2014). Something similar may happen with spider mites: a nutrient deficient diet from some host plant species may lead to a decrease in egg viability, as spider mites may struggle to allocate enough nutrients to ensure their eggs' viability. In line with this, spider mites have to cope with different defence mechanisms of the plants; they may thus balance their resources allocation for counter-defences and reproduction in different manner. In our experiment, Wi mites hatched less on purple and on zucchini, while on eggplant we observe the opposite. However, we observe a lower juvenile survival for both Wi and Wu mites on eggplant when compared to mites on other plants, which may reflect the poor diet of their mothers on a highly defended plant.

The impact of *Wolbachia* on sex ratio may be explained by several non-exclusive explanations. First, it may be a side effect of tetracycline, the antibiotic used to cure our mites from *Wolbachia*. Indeed, in the pseudoscorpion, *Cordylocheres scorpoides*, tetracycline has been shown to decrease sperm viability through detrimental effects on mitochondria (Zeh et al., 2012). Further, consequences on sex ratio (male-biased) have been shown for more than nine generations post-treatment in *Drosophila melanogaster* (O'Shea & Singh, 2015). Since, in spider mites, only females develop from fertilized eggs, tetracycline may explain a decrease of daughters production by treated-females. In our experiments, however, *Wolbachia*-treated mites were used twelve generations after treatment and several consecutive experiments conducted in our laboratory using the same populations, between the tetracycline treatment and this study, did not reveal such effect of *Wolbachia* on sex ratio (Zélé et al., *in prep.*). For this latter reason, we cannot rule out the possibility of an impact of tetracycline on sex ratio, although we cannot totally exclude it. Second, it may also be explained by possible *Wolbachia* effect on juvenile development time as shown in other systems (Dong et al., 2007; Gavotte et al., 2010; Zélé et al., 2012). Indeed, the age of the females tested for each population belonged to a three-day time interval, but developmental time was not controlled. It is known that initially, after quiescence, females lay more eggs that will develop into adult males (Macke et al., 2012), so if Wi populations develop faster (or Wu develop slower), those males may have not have been laid during the experiment, which would lead to a female-biased observed sex ratio (i.e. but not necessarily life time sex ratio). One line of evidence favouring this hypothesis comes from the differences of protocols between this experiment and the previously cited ones conducted in our laboratory (Zélé et al., *in prep.*): in these previous experiments, the “absolute” age of the females (i.e. from the moment the eggs were laid) was not controlled but, instead, females were isolated from the “age cohorts” at the quiescent stage, thus compensating for any possible effect of *Wolbachia* on juvenile development. Finally, differential sex ratio produced by Wi and Wu females may also be a benefit conferred by *Wolbachia* to the females, since less fit (e.g. starved) predatory female mites tend to produce more males (Fries & Gilstrap, 1982). Indeed, *Wolbachia*, being maternally transmitted, it would always benefit from a female-biased the sex ratio. However, a female-biased sex-ratio does not always benefit to the female host, and it has been shown that spider mites may have difficulties to compensate the effect of *Wolbachia* on sex ratio in a new environment (Vala et al., 2003). Interestingly, although we did not find a significant interaction between the infection by *Wolbachia* and the host plant, the independent analyses of each plant showed no difference in the proportion of males produced by Wi and Wu populations on bean (control), while Wi populations produced significantly less males than Wu populations on zucchini and eggplant host plant. Note that the previous experiments conducted in our laboratory were performed on bean only (Zélé et al., *in prep.*). *Wolbachia* may thus affect the sex ratio on novel host plants only, although an experiment controlling the effect of *Wolbachia* on juvenile development time should be conducted to confirm (or infer) this hypothesis.

Effect of *Wolbachia* on spider mites performance after maintenance on different host plants

Mites maintained on novel host plants (purple and eggplant) did not show signs of adaptation when compared to mites reared on bean and tested on those plants (controls). The proportion of surviving juveniles of mites under maintenance on eggplant performed even worse than the ones performing on eggplant for the first time. These latter results, along with the lack of adaptation, may be explained by an insufficient number of generations of maintenance on these plants (c.a. 12 generations), the lack of replication, but also the possible low diversity resulting from bottlenecks (as a result of difficulties during maintenance) despite 100 mated females were initially used to start these populations. The responses observed on hatching rate, daily fecundity and survival, were probably caused by the genetic variance present in the original Wi and Wu populations, rather than by the occurrence of adaptation. Overall we can see congruence, at the plant level, between the results obtained before and after maintenance on different plants for several generations (without reaching adaptation), but the second experiment highlights more interactions than the first one. However, based on the explanations above, we cannot take any conclusions from these latter results.

Effect of the host plant on endosymbiont prevalence in the field

Recent estimations of endosymbionts incidence in arthropods report that 52% of species are infected with *Wolbachia*, 24% are infected with *Rickettsia* and 13% with *Cardinium* (Weinert et al., 2015). In various mite species, however, very contrasting results have been reported for the incidence of these endosymbionts. For instance, while some studies showed a higher incidence of *Cardinium* than that of *Wolbachia* (e.g. Enigl & Schausberger, 2007), most of them showed the opposite (e.g. Ros et al., 2012; Weeks et al., 2003; Zchori-Fein & Perlman, 2004). In Tetranychus spider mites (including *T. urticae*), several studies have previously reported infections by *Wolbachia* and *Cardinium*, although their prevalence may range from 0 to 100% depending on the tested populations (e.g. Gotoh et al., 2003; Gotoh et al., 2007; Liu et al., 2006; Ros et al., 2012; Su et al., 2011; Zhang et al., 2013). Conversely, to our knowledge, infection by *Rickettsia* has been reported only once in a laboratory colony of *T. urticae* in California (Hoy & Jeyaprakash, 2005). However, a very low incidence of *Rickettsia* in spider mites cannot be rule out from a lack of evidence in the literature, since almost all screening studies conducted so far did not check for this symbiont.

Here, and similarly to a previous study conducted by our group (Z  l   et al., in prep.), we found a very high (near 100%) although variable prevalence of *Wolbachia*, while *Cardinium* and *Rickettsia* infections were rare (from 0 to 12.5% for both symbionts). As suggested (but not formally tested) by the same study conducted by our group, we found that plants have a significant effect on the prevalence of *Wolbachia* and *Rickettsia* in *T. urticae*. These results thus suggest that some of these host plants may increase the prevalence of a symbiont within herbivorous populations, while other may decrease it.

Several different, but non-exclusive, hypotheses may explain our results. Indeed, the prevalence (and thus maintenance and spread) of endosymbionts in a population relies on several important

parameters, such as the balance costs/benefits for the host, the penetrance of reproductive manipulation (e.g. the level of CI), the efficiency of vertical transmission, and the probability of horizontal transfer of these symbionts from one host to another (Vavre et al., 2000). If the host plants affect any of these parameters, it will translate to changes of symbiont prevalence. Interestingly, plants harbouring mites with the lower prevalence of *Wolbachia* (purple and zucchini) in our study, were also those in which *Wolbachia* infection leads to lower eggs hatchability. By affecting the balance costs/benefits of *Wolbachia* on mites' eggs hatchability, host plants may thus affect the dynamics of Wi populations relatively to Wu ones, which in turn will change the proportion of Wi mites within populations. In addition, it was found in *Bemisia tabaci*, that *Rickettsia* horizontal transmission can be mediated by the host plants (Caspi-Fluger et al., 2012). Such horizontal transfer may be extremely important to explain the observed prevalence, especially if the intrinsic rate of vertical transmission of the endosymbiont is low (currently unknown in spider mites). Note, however, that only 2 populations of *T. urticae* were caught on *Ipomoea purpurea* (purple) which may have biased the prevalence of *Rickettsia* obtained on this plant.

A key mechanism involves in variations of all the above-cited parameters affecting symbiont prevalence in host populations is the density of these symbionts within individual hosts. Indeed, the fitness costs of the symbionts, the efficiency of symbiont transmission through the host germline, the penetrance of the reproductive phenotypes, and potential horizontal transfers are all usually correlated with bacterial density (Jaenike, 2009; Unckless et al., 2009). For instance, CI levels may depend on bacterial density within the reproductive tissues of the host (Breeuwer & Werren, 1993). Bacterial density is regulated by genetic factors of the host and the symbiont itself and is strongly influenced by environmental factors, such as temperature, host age, and nutrient availability (Bordenstein & Bordenstein, 2011; Jaenike, 2009). Plants may thus struggle symbionts and lower their density (Pan et al., 2013) directly via defences/toxins (Ryan, 1990), and/or indirectly, through resources availability. For instance, it has been shown by Chandler et al (2008) that low nitrogen content of *Lamium purpureum* phloem sap present in the diet of aphids may increase secondary symbiont density in aphids, which is then related with poor aphid performance. These authors suggest that high bacterial populations may consume nutrients and cause other physiological disturbances that collectively depress aphid growth. Iron, which is present in spider mites' diet (Chatterjee & Gupta, 1997; Rodriguez, 1951), may also be a good candidate as it is needed for *Wolbachia* survival (Gill et al., 2014). Although different concentrations of iron do not seem to affect the performance of *Tetranychus urticae* (Cannon Jr & Terriere, 1966), the comparison of its effect on Wi and Wu mites has, to our knowledge, never been investigated. In line with this, it would be interesting to study the role of different iron concentrations in different plants on the density of *Wolbachia* in spider mites.

Final remarks & perspectives

Although our results didn't show an important impact of infection by *Wolbachia* on the performance of *T. urticae* on different plants, plants leading to the lower prevalence of *Wolbachia* in the field were also those in which *Wolbachia* infection results in lower hatching rate. This suggests that host plants may play an important role in endosymbionts spread and/or maintenance in *T. urticae* populations. Conversely, *T. urticae* host-plant colonization may hinge on endosymbiont presence. However, several questions remain to complement the study on this tripartite interaction.

First, an extensive study of longevity, following the mites' survival until the death of all individuals, and lifetime fecundity should be performed, in order to investigate the long-term effects of new host plants on both Wi and Wu mites, and have a better estimate of the effect of these plants on the fitness costs/benefits of *Wolbachia*.

Second, experiments allowing disentangling how *Wolbachia* does affect sex ratio should be conducted. We should first determine whether *Wolbachia* affects the juvenile developmental time of *T. urticae*, and test whether it translates into biased sex ratio in the offspring. Then, we should repeat entirely the experiment while controlling for the age at quiescence for both males and females. We could also test the effect of tetracycline on naturally uninfected mites and follow these effects through time.

Third, the effects of both plants defences and resources on Wi and Wu spider mites should be addressed. Future studies could thus account for the effect of quantitative variation of plants' defensive compounds on the performance of Wi and Wu spider mites, and reciprocally, the effect of *Wolbachia* infection in mites on the quantity of defensive compounds in plants. The role of different availability of nutritive resources to spider mites, for example using different nutritive solutions to grow the plants, on the performance of Wi and Wu spider mites could also be investigated.

To complement the research on the effect of host plants on endosymbionts prevalence in *T. urticae*, a study on symbionts density (as a key mechanism affecting symbionts prevalence) before and after maintenance on new host plants should be done. For this purpose, a new maintenance experiment should be developed with several replicates and adequate conditions for the host plants. After numerous generations, the density of symbionts within individual females and males may be measured using qPCR in order to unravel potential correlation with fitness costs/benefits and CI levels induced by *Wolbachia*.

Finally, we could also test whether there is horizontal transmission through the host-plant of *Wolbachia* and *Rickettsia* in order to study the possible implications of it on the prevalence on these symbionts. It would be done by placing mites from both a population treated against endosymbionts and one infected with *Wolbachia* and *Rickettsia* on the same leaf disc during one week and then testing their offspring for

endosymbionts by PCR. Populations would be of different *T. urticae* forms, red and green, thus distinguishable. We could only test these two endosymbionts due to the fact that we do not have a population infected by *Cardinium* in the lab.

References

- Agrawal, A. A., & Konno, K. (2009). Latex: A Model for Understanding Mechanisms, Ecology, and Evolution of Plant Defense Against Herbivory. *Annual Review of Ecology, Evolution, and Systematics*, 40(1), 311–331.
- Ali, J. G., & Agrawal, A. A. (2012). Specialist versus generalist insect herbivores and plant defense. *Trends in Plant Science*, 17(5), 293–302.
- Almeida, F. de, Moura, A. S., Cardoso, A. F., Winter, C. E., Bijovsky, A. T., & Suesdek, L. (2011). Effects of Wolbachia on fitness of *Culex quinquefasciatus* (Diptera; Culicidae). *Infection, Genetics and Evolution*, 11(8), 2138–2143.
- Ament, K., Kant, M. R., Sabelis, M. W., Haring, M. A., & Schuurink, R. C. (2004). Jasmonic acid is a key regulator of spider mite-induced volatile terpenoid and methyl salicylate emission in tomato. *Plant Physiology*, 135(August), 1–13.
- Awmack, C. S., & Leather, S. R. (2002). Host Plant Quality and Fecundity in Herbivorous Insects. *Annual Review of Entomology*, 47(1), 817–844.
- Backhed, F. (2005). Host-Bacterial Mutualism in the Human Intestine. *Science*, 307(5717), 1915–1920.
- Bandi, C., Dunn, A. M., Hurst, G. D. D., & Rigaud, T. (2001). Inherited microorganisms, sex-specific virulence and reproductive parasitism. *Trends in Parasitology*, 17(2), 88–94.
- Barr, K. L., Hearne, L. B., Briesacher, S., Clark, T. L., & Davis, G. E. (2010). Microbial symbionts in insects influence down-regulation of defense genes in maize. *PLoS One*, 5(6)
- Baumann, P. (2005). Biology of Bacteriocyte-Associated Endosymbionts of Plant Sap-Sucking Insects. *Annual Review of Microbiology*, 59(1), 155–189.
- Bolker, B. M. (2008). *Ecological models and data in R*. Princeton: Princeton University Press.
- Bordenstein, S. R., & Bordenstein, S. R. (2011). Temperature affects the tripartite interactions between bacteriophage WO, Wolbachia, and cytoplasmic incompatibility. *PLoS One*, 6(12)
- Braendle, C., Miura, T., Bickel, R., Shingleton, A. W., Kambhampati, S., & Stern, D. L. (2003). Developmental Origin and Evolution of Bacteriocytes in the Aphid–Buchnera Symbiosis. *PLoS Biology*, 1(1)
- Breeuwer, J. A. J. (1997). Wolbachia and cytoplasmic incompatibility in the spider mites *Tetranychus urticae* and *T. turkestanii*. *Heredity*, 79(1), 41–47.
- Breeuwer, J. A. J., & Werren, J. H. (1993). Cytoplasmic incompatibility and bacterial density in *Nasonia vitripennis*. *Genetics*, 135(2), 565–574.
- Brelsfoard, C. L., & Dobson, S. L. (2011). Wolbachia Effects on Host Fitness and the Influence of Male Aging on Cytoplasmic Incompatibility in *Aedes polynesiensis* (Diptera: Culicidae). *Journal of Medical Entomology*, 48(5), 1008–1015.
- Bright, M., & Bulgheresi, S. (2010). A complex journey: transmission of microbial symbionts. *Nature Reviews Microbiology*, 8(3), 218–230.
- Brown, A. N., & Lloyd, V. K. (2015). Evidence for horizontal transfer of Wolbachia by a *Drosophila* mite. *Experimental and Applied Acarology*, 301–311.
- Brownlie, J. C., Adamski, M., Slatko, B., & McGraw, E. A. (2007). Diversifying selection and host adaptation in two endosymbiont genomes. *Bmc Evolutionary Biology*, 7, 68.
- Brownlie, J. C., Cass, B. N., Riegler, M., Witsenburg, J. J., Iturbe-Ormaetxe, I., McGraw, E. A., & O'Neill, S. L. (2009). Evidence for metabolic provisioning by a common invertebrate endosymbiont, wolbachia pipientis, during periods of nutritional stress. *PLoS Pathogens*, 5(4).
- Cannon Jr, W. N., & Terriere, L. C. (1966). Egg Production of the Two-Spotted Spider Mite on Bean Plants Supplied Nutrient Solutions Containing Various Concentrations of Iron , Manganese , Zinc , and

- Cobale. *Journal of Economic Entomology*, 59(1).
- Caragata, E. P., Rancès, E., O'Neill, S. L., & McGraw, E. a. (2014). Competition for Amino Acids Between Wolbachia and the Mosquito Host, *Aedes aegypti*. *Microbial Ecology*, 67(1), 205–218.
- Carrington, L. B., Hoffmann, A. A., & Weeks, A. R. (2010). Monitoring long-term evolutionary changes following Wolbachia introduction into a novel host: the Wolbachia popcorn infection in *Drosophila simulans*. *Proceedings. Biological Sciences / The Royal Society*, 277(1690), 2059–68.
- Caspi-Fluger, A., Inbar, M., Mozes-Daube, N., Katzir, N., Portnoy, V., Belausov, E., ... Zchori-Fein, E. (2012). Horizontal transmission of the insect symbiont Rickettsia is plant-mediated. *Proceedings. Biological Sciences / The Royal Society*, 279(1734), 1791–6.
- Chaisiri, K., McGarry, J. W., Morand, S., & Makepeace, B. L. (2015). Symbiosis in an overlooked microcosm: a systematic review of the bacterial flora of mites. *Parasitology*, 1–11.
- Chandler, S. M., Wilkinson, T. L., & Douglas, a E. (2008). Impact of plant nutrients on the relationship between a herbivorous insect and its symbiotic bacteria. *Proceedings. Biological Sciences / The Royal Society*, 275(1634), 565–70.
- Chatterjee, K., & Gupta, S. K. (1997). Depletion of minerals, inorganic and organic compounds in leaves of sponge gourd (*Luffa acutangula* Roxb.) due to feeding of mite, *Tetranychus ludeni* Zacher. *Journal of Entomologic Research*.
- Chung, S. H., Rosa, C., Scully, E. D., Peiffer, M., Tooker, J. F., Hoover, K., ... Felton, G. W. (2013). Herbivore exploits orally secreted bacteria to suppress plant defenses. *Proceedings of the National Academy of Sciences of the United States of America*, 110(39), 15728–33.
- Combes, C. (2001). *Parasitism: The ecology and evolution of intimate interactions*. Chicago: University of Chicago Press.
- Crawley, M. J. (2007). *The R book*. Chichester: John Wiley & Sons, Ltd.
- Davy, S. K., Allemand, D., & Weis, V. M. (2012). Cell Biology of Cnidarian-Dinoflagellate Symbiosis. *Microbiology and Molecular Biology Reviews*, 76(2), 229–261.
- Dicke, M., Poecke, R. M. P. Van, & Boer, J. G. De. (2003). Inducible indirect defence of plants: from mechanisms to ecological functions. *Basic and Applied Ecology*, 4, 27–42.
- Diehl, S. R., & Bush, G. L. (1984). An evolutionary and applied perspective of insect biotypes. *Annual Review of Entomology*, 29, 471–504.
- Dobson, S. L., Marsland, E. J., & Rattanadechakul, W. (2002). Mutualistic Wolbachia Infection in *Aedes albopictus* :, 1094(March), 1087–1094.
- Dong, P., Wang, J.-J., Hu, F., & Jia, F.-X. (2007). Influence of wolbachia infection on the fitness of the stored-product pest *Liposcelis tricolor* (Psocoptera: Liposcelididae). *Journal of Economic Entomology*, 100(4), 1476–1481.
- Douglas, A. E. (1998). Nutritional interactions in insect-microbial symbioses: aphids and their symbiotic bacteria Buchnera. *Annual Review of Entomology*, 43, 17–37.
- Douglas, A. E. (2009). The microbial dimension in insect nutritional ecology. *Functional Ecology*, 23(1), 38–47.
- Drès, M., & Mallet, J. (2002). Host races in plant-feeding insects and their importance in sympatric speciation. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, 357(1420), 471–492.
- Drummond, H., & Rodríguez, C. (2015). Viability of Booby Offspring is Maximized by Having One Young Parent and One Old Parent. *Plos One*, 10(7)
- Duffey, S. S., & Stout, M. J. (1996). Antinutritive and toxic components of plant defense against insects. *Archives of Insect Biochemistry and Physiology*, 32(1), 3–37.
- Engelberth, J., Koch, T., Schüler, G., Bachmann, N., Rechtenbach, J., & Boland, W. (2001). Ion channel-

forming alamethicin is a potent elicitor of volatile biosynthesis and tendril coiling. Cross talk between jasmonate and salicylate signaling in lima bean. *Plant Physiology*, 125(1), 369–377.

- Enigl, M., & Schausberger, P. (2007). Incidence of the endosymbionts Wolbachia, Cardinium and Spiroplasma in phytoseiid mites and associated prey. *Experimental and Applied Acarology*, 42, 75–85.
- Foster, J., Ganatra, M., Kamal, I., Ware, J., Makarova, K., Ivanova, N., ... Slatko, B. (2005). The Wolbachia Genome of Brugia malayi: Endosymbiont Evolution within a Human Pathogenic Nematode. *PLoS Biology*, 3(4)
- Frago, E., Dicke, M., & Godfray, H. C. J. (2012). Insect symbionts as hidden players in insect-plant interactions. *Trends in Ecology & Evolution*, 27(12), 705–11.
- Friese, D. D., & Gilstrap, F. E. (1982). Influence of prey availability on reproduction and prey consumption of Phytoseiulus persimilis, Amblyseius californicus and Metaseiulus occidentalis (Acarina: Phytoseiidae). *International Journal of Acarology*, 8(2), 85–89.
- Fry, A. J., Palmer, M. R., & Rand, D. M. (2004). Variable fitness effects of Wolbachia infection in Drosophila melanogaster. *Heredity*, 93(4), 379–389.
- Fürstenberg-Hägg, J., Zagrobelny, M., & Bak, S. (2013). *Plant defense against insect herbivores. International Journal of Molecular Sciences* (Vol. 14).
- Gavotte, L., Mercer, D. R., Stoeckle, J. J., & Dobson, S. L. (2010). Costs and benefits of Wolbachia infection in immature Aedes albopictus depend upon sex and competition level. *Journal of Invertebrate Pathology*, 105(3), 341–346.
- Gill, A. C., Darby, A. C., & Makepeace, B. L. (2014). Iron Necessity: The Secret of Wolbachia's Success? *PLoS Neglected Tropical Diseases*, 8(10)
- Glaser, R. L., & Meola, M. A. (2010). The native Wolbachia endosymbionts of Drosophila melanogaster and Culex quinquefasciatus increase host resistance to west nile virus infection. *PLoS ONE*, 5(8).
- Godinho, D. P., Janssen, A., Dias, T., Cruz, C., & Magalhães, S. (2016). Down-regulation of plant defence in a resident spider mite species and its effect upon con- and heterospecifics. *Oecologia*, 180(1), 161–167.
- Gotoh, T., Noda, H., & Hong, X.-Y. (2003). Wolbachia distribution and cytoplasmic incompatibility based on a survey of 42 spider mite species (Acari: Tetranychidae) in Japan. *Heredity*, 91, 208–216.
- Gotoh, T., Sugawara, J., Noda, H., & Kitashima, Y. (2007). Wolbachia-induced cytoplasmic incompatibility in Japanese populations of Tetranychus urticae (Acari: Tetranychidae). *Experimental and Applied Acarology*, 42, 1–16.
- Habib, H., & Fazili, K. M. (2007). Plant protease inhibitors : a defense strategy in plants. *Biotechnology and Molecular Biology Review*, 2(3), 68–85.
- Heath, B. D., Butcher, R. D. J., Whitfield, W. G. F., & Hubbard, S. F. (1999). Horizontal transfer of Wolbachia between phylogenetically distant insect species by a naturally occurring mechanism. *Current Biology*, 9(6), 313–316.
- Hedges, L. M., Brownlie, J. C., O'Neill, S. L., & Johnson, K. N. (2008). Wolbachia and virus protection in insects. *Science*, 322(5902), 702.
- Helle, W., Gutierrez, J., & Bolland, H. R. (1970). A study on sex-determination and karyotypic evolution in Tetranychidae. *Genetica*, (41), 21–32.
- Hoover, K., Grove, M., Gardner, M., Hughes, D. P., McNeil, J., & Slavicek, J. (2011). A gene for an extended phenotype. *Science (New York, N.Y.)*, 333(6048), 1401.
- Hosokawa, T., Koga, R., Kikuchi, Y., Meng, X.-Y., & Fukatsu, T. (2010). Wolbachia as a bacteriocyte-associated nutritional mutualist. *Proceedings of the National Academy of Sciences*, 107(2), 769–774.
- Hoy, M. A., & Jeyaprakash, A. (2005). Microbial diversity in the predatory mite Metaseiulus occidentalis (Acari: Phytoseiidae) and its prey, Tetranychus urticae (Acari: Tetranychidae). *Biological Control*, 32, 427–441.

- Hurst, G. D. D., & Jiggins, F. M. (2000). Male-killing bacteria in insects: Mechanisms, incidence, and implications. *Emerging Infectious Diseases*, 6(4), 329–336.
- Hurst, L. D., & Mcvean, G. T. (1996). Clade Selection, Reversible Evolution and the Persistence of Selfish Elements: The Evolutionary Dynamics of Cytoplasmic Incompatibility. *Proceedings of the Royal Society B: Biological Sciences*, 263, 97–104.
- Jaenike, J. (2009). Coupled population dynamics of endosymbionts within and between hosts. *Oikos*, 118(3), 353–362.
- Karban, R., Baldwin, I. T., Baxter, K. J., Laue, G., & Felton, G. W. (2000). Communication between plants: induced resistance in wild tobacco plants following clipping of neighboring sagebrush. *Oecologia*, 125(1), 66–71.
- Karban, R., & Myers, J. (1989). Induced plant responses to herbivory. *Annual Review of Ecology and Systematics*, 20(1989), 331–348.
- Kessler, A., & Baldwin, I. T. (2001). Defensive Function of Herbivore-Induced Plant Volatile Emissions in Nature. *Science*, 291(5511), 2141–2144.
- Kissling, W. D., & Schleuning, M. (2014). Multispecies interactions across trophic levels at macroscales: Retrospective and future directions. *Ecography*, (March 2014), 346–357.
- Koropatnick, T. a, Engle, J. T., Apicella, M. a, Stabb, E. V, Goldman, W. E., & McFall-Ngai, M. J. (2010). Microbial Factor-Mediated Development in a Host-Bacterial Mutualism. *Science (New York, NY)*, 306(5699), 1186.
- Labandeira, C. C. (1997). INSECT MOUTHPARTS: Ascertaining the Paleobiology of Insect Feeding Strategies. *Annual Review of Ecology and Systematics*, 28(1), 153–193.
- Lamb, R. J., Sridhar, P., Smith, M. a H., & Wise, I. L. (2003). Oviposition Preference and Offspring Performance of a Wheat Midge *Sitodiplosis mosellana* (Gehin) (Diptera: Cecidomyiidae) on Defended and Less Defended Wheat Plants. *Environmental Entomology*, 32(2), 414–420.
- Lawrence, S. D., Novak, N. G., Ju, C. J.-T., & Cooke, J. E. K. (2008). Potato, *Solanum tuberosum*, defense against Colorado potato beetle, *Leptinotarsa decemlineata* (Say): microarray gene expression profiling of potato by Colorado potato beetle regurgitant treatment of wounded leaves. *Journal of Chemical Ecology*, 34(8), 1013–1025.
- Le Clec'h, W., Chevalier, F. D., Genty, L., Bertaux, J., Bouchon, D., & Sicard, M. (2013). Cannibalism and Predation as Paths for Horizontal Passage of *Wolbachia* between Terrestrial Isopods. *PLoS ONE*, 8(4).
- Leonardo, T. E., & Muir, G. T. (2003). Facultative symbionts are associated with host plant specialization in pea aphid populations. *Proceedings of the Royal Society B: Biological Sciences*, 270(Suppl_2), S209–S212. doi:10.1098/rsbl.2003.0064
- Liu, Y., Miao, H., & Hong, X. Y. (2006). Distribution of the endosymbiotic bacterium *Cardinium* in Chinese populations of the carmine spider mite *Tetranychus cinnabarinus* (Acari: Tetranychidae). *Journal of Applied Entomology*, 130(9-10), 523–529.
- MacDonald, T. T. (2005). Immunity, Inflammation, and Allergy in the Gut. *Science*, 307(5717), 1920–1925.
- Macke, E., Magalhães, S., Do-Thi Khanh, H., Frantz, A., Facon, B., & Olivieri, I. (2012). Mating Modifies Female Life History in a Haplodiploid Spider Mite. *The American Naturalist*, 179, E147–E162.
- Magalhães, S., Blanchet, E., Egas, M., & Olivieri, I. (2009). Are adaptation costs necessary to build up a local adaptation pattern? *BMC Evolutionary Biology*, 9(1), 182.
- Magalhães, S., Blanchet, E., Egas, M., & Olivieri, I. (2011). Environmental effects on the detection of adaptation. *Journal of Evolutionary Biology*, 24(12), 2653–62.
- Magalhães, S., Forbes, M. R., Skoracka, A., Osakabe, M., Chevillon, C., & McCoy, K. D. (2007). Host race formation in the Acari. *Experimental and Applied Acarology*, 42(4), 225–238.
- Markov, A. V., & Zakharov, I. A. (2006). The parasitic bacterium *Wolbachia* and the origin of the eukaryotic

cell. *Paleontological Journal*, 40(2), 115–124.

- Martin, B. D., & Schwab, E. (2012). Current Usage of Symbiosis and Associated Terminology. *International Journal of Biology*, 5(1), 32–45.
- Migeon, A., & Dorkeld, F. (2015). Spider Mites Web: A comprehensive database for the Tetranychidae. Retrieved January 1, 2015, from <http://www.montpellier.inra.fr/CBGP/spmweb>
- Moran, N. A., & Dunbar, H. E. (2006). Sexual acquisition of beneficial symbionts in aphids. *Proceedings of the National Academy of Sciences of the United States of America*, 103(34), 12803–6.
- Musser, R. O., Hum-Musser, S. M., Eichenseer, H., Peiffer, M., Ervin, G., Murphy, J. B., & Felton, G. W. (2002). Herbivory: caterpillar saliva beats plant defences. *Nature*, 416(6881), 599–600.
- Myers, J. H., & Bazely, D. (1991). Thorns, Spines, Prickles, and Hairs: Are They Stimulated by Herbivory and Do They Deter Herbivores? In D. W. Tallamy & M. J. Raupp (Eds.), *Phytochemical induction by herbivores*. New York: Wiley.
- O'Shea, K. L., & Singh, N. D. (2015). Tetracycline-exposed *Drosophila melanogaster* males produce fewer offspring but a relative excess of sons. *Ecology and Evolution*
- Oliver, K. M., Russell, J. a, Moran, N. a, & Hunter, M. S. (2003). Facultative bacterial symbionts in aphids confer resistance to parasitic wasps. *Proceedings of the National Academy of Sciences of the United States of America*, 100(4), 1803–1807.
- Osborne, S. E., Leong, Y. S., O'Neill, S. L., & Johnson, K. N. (2009). Variation in antiviral protection mediated by different Wolbachia strains in *Drosophila simulans*. *PLoS Pathogens*, 5(11).
- Pan, H. P., Chu, D., Liu, B. M., Xie, W., Wang, S. L., Wu, Q. J., ... Zhang, Y. J. (2013). Relative Amount of Symbionts in Insect Hosts Changes with Host-Plant Adaptation and Insecticide Resistance. *Environmental Entomology*, 42(1), 74–78.
- Pan, H., Su, Q., Jiao, X., Zhou, L., Liu, B., Xie, W., ... Zhang, Y. (2013). Relative amount of symbionts in *Bemisia tabaci* (Gennadius) Q changes with host plant and establishing the method of analyzing free amino acid in *B. tabaci*. *Communicative and Integrative Biology*, 6(May 2015), 37–41.
- Piel, J. (2002). A polyketide synthase-peptide synthetase gene cluster from an uncultured bacterial symbiont of *Paederus* beetles. *Proceedings of the National Academy of Sciences of the United States of America*, 99(22), 14002–14007.
- Ponton, F., Wilson, K., Holmes, A., Raubenheimer, D., Robinson, K. L., & Simpson, S. J. (2014). Macronutrients mediate the functional relationship between *Drosophila* and Wolbachia. *Proceedings of the Royal Society B: Biological Sciences*, 282(1800)
- Raffa, K. F., & Berryman, A. A. (1983). The Role of Host Plant Resistance in the Colonization Behavior and Ecology of Bark Beetles (Coleoptera: Scolytidae). *Ecological Monographs*, 53(1), 27–49.
- Riederer, M., & Müller, C. (2007). Plant-insect interactions on Cuticular Surfaces. In *Biology of the plant cuticle*. Oxford: Blackwell Pub.
- Rodriguez, J. G. (1951). Mineral nutrition of the two-spotted spider mite, *Tetranychus bimaculatus* Harvey. *Annals Entomological Society of America*, 44, 511–526.
- Ros, V. I. D., Fleming, V. M., Feil, E. J., & Breeuwer, J. a J. (2012). Diversity and recombination in Wolbachia and Cardinium from Bryobia spider mites. *BMC Microbiology*, 12(Suppl 1), S13.
- Rostás, M., & Eggert, K. (2007). Ontogenetic and spatio-temporal patterns of induced volatiles in *Glycine max* in the light of the optimal defence hypothesis. *Chemoecology*, 18(1), 29–38.
- Ryan, C. A. (1990). Protease Inhibitors in Plants: Genes for Improving Defenses Against Insects and Pathogens. *Annual Review of Phytopathology*, 28(1), 425–449.
- Sarmiento, R. A., Lemos, F., Dias, C. R., Kikuchi, W. T., Rodrigues, J. C. P., Pallini, A., ... Janssen, A. (2011). A herbivorous mite down-regulates plant defence and produces web to exclude competitors. *PLoS ONE*, 6(8), 8–14.

- Scarborough, C. L., Ferrari, J., & Godfray, H. C. J. (2005). Aphid protected from pathogen by endosymbiont. *Science (New York, N.Y.)*, 310(5755), 1781.
- Schaller, A., & Howe, G. (2008). Direct Defenses in Plants and their Induction by Wounding and Insect Herbivores. In *Induced plant resistance to herbivory*. Berlin: Springer.
- Serbus, L. R., White, P. M., Silva, J. P., Rabe, A., Teixeira, L., Albertson, R., & Sullivan, W. (2015). The Impact of Host Diet on Wolbachia Titer in *Drosophila*. *PLOS Pathogens*, 11
- Simmons, L. W., & Holley, R. (2011). Offspring viability benefits but no apparent costs of mating with high quality males. *Biology Letters*, 7(December 2010), 419–421.
- Smith, J. E., & Dunn, A. M. (1991). Transovarial transmission. *Parasitology Today*, 7(6), 146–148.
- Steward, J. L., & Keeler, K. H. (1988). Are There Trade-Offs among Antiherbivore Defenses in *Ipomoea* (Convolvulaceae)? *Oikos*, 53(1), 79–86.
- Stouthamer, R., Breeuwer, J. A. J., & Hurst, G. D. D. (1999). Wolbachia pipientis: Microbial Manipulator of Arthropod Reproduction. *Annual Reviews of Microbiology*, 71–102.
- Su, H., Jiang, F., Yu, M. Z., Zhang, K. J., Xue, X. F., & Hong, X. Y. (2011). Effects of Wolbachia on rDNA-ITS2 variation and evolution in natural populations of *Tetranychus urticae* Koch. *Insect Mol Biol*, 20(3), 311–321.
- Su, Q., Oliver, K. M., Xie, W., Wu, Q., Wang, S., & Zhang, Y. (2015). The whitefly-associated facultative symbiont *Hamiltonella defensa* suppresses induced plant defences in tomato. *Functional Ecology*, 29(8), 1007–1018.
- Suh, E., Sim, C., Park, J. J., & Cho, K. (2014). Inter-population variation for Wolbachia induced reproductive incompatibility in the haplodiploid mite *Tetranychus urticae*. *Experimental and Applied Acarology*, 55–71.
- Tahmasebi, Z., Mohammadi, H., Arimura, G. ichiro, Muroi, A., & Kant, M. R. (2014). Herbivore-induced indirect defense across bean cultivars is independent of their degree of direct resistance. *Experimental and Applied Acarology*, 63(2), 217–239.
- Teixeira, L., Ferreira, Á., & Ashburner, M. (2008). The bacterial symbiont Wolbachia induces resistance to RNA viral infections in *Drosophila melanogaster*. *PLoS Biology*, 6(12), 2753–2763.
- Travers-Martin, N., & Müller, C. (2008). Matching plant defence syndromes with performance and preference of a specialist herbivore. *Functional Ecology*, 22(6), 1033–1043.
- Tsuchida, T., Koga, R., & Fukatsu, T. (2004). Host plant specialization governed by facultative symbiont. *Science (New York, N.Y.)*, 303(5666), 1989.
- Tsuchida, T., Koga, R., Shibao, H., Matsumoto, T., & Fukatsu, T. (2002). Diversity and geographic distribution of secondary endosymbiotic bacteria in natural populations of the pea aphid, *Acyrtosiphon pisum*. *Molecular Ecology*, 11(10), 2123–2135.
- Unckless, R. L., Boelio, L. M., Herren, J. K., & Jaenike, J. (2009). Wolbachia as populations within individual insects: causes and consequences of density variation in natural populations. *Proceedings of the Royal Society B: Biological Sciences*, 276(1668), 2805–2811.
- Usher, K. M., Sutton, D. C., Toze, S., Kuo, J., & Fromont, J. (2005). Inter-generational transmission of microbial symbionts in the marine sponge *Chondrilla australiensis* (Demospongiae). *Marine and Freshwater Research*, 56(2), 125–131.
- Vala, F., Breeuwer, J. A. J., & Sabelis, M. W. (2003). No variation for Wolbachia-induced hybrid. *Experimental and Applied Acarology*, (1999), 1–12.
- Vala, F., Breeuwer, J. A., & Sabelis, M. W. (2000). Wolbachia-induced “hybrid breakdown” in the two-spotted spider mite *Tetranychus urticae* Koch. *Proceedings. Biological Sciences / The Royal Society*, 267(1456), 1931–7.
- Vala, F., Van Opijnen, T., Breeuwer, J. a J., & Sabelis, M. W. (2003). Genetic conflicts over sex ratio: mite-

- endosymbiont interactions. *The American Naturalist*, 161(2), 254–266.
- Vala, F., Weeks, A., Claessen, D., Breeuwer, J. A. J., & Sabelis, M. W. (2002). Within- and Between-Population Variation for Wolbachia-Induced Reproductive Incompatibility in a Haplodiploid Mite, 56(7), 1331–1339.
- Vavre, F., Fleury, F., Lepetit, D., Fouillet, P., & Boulétreau, M. (1999). Phylogenetic evidence for horizontal transmission of Wolbachia in host-parasitoid associations. *Molecular Biology and Evolution*, 16(12), 1711–1723.
- Vavre, F., Fleury, F., Varaldi, J., Fouillet, P., & Boulétreau, M. (2000). Evidence for female mortality in Wolbachia-mediated cytoplasmic incompatibility in haplodiploid insects: epidemiologic and evolutionary consequences. *Evolution; International Journal of Organic Evolution*, 54(1), 191–200.
- Wagner, S. M., Martinez, A. J., Ruan, Y.-M., Kim, K. L., Lenhart, P. A., Dehnel, A. C., ... White, J. A. (2015). Facultative endosymbionts mediate dietary breadth in a polyphagous herbivore. *Functional Ecology*
- Walling, L. (2000). The Myriad Plant Responses to Herbivores. *Journal of Plant Growth Regulation*, 19(2), 195–216.
- Weeks, A. R., Velten, R., & Stouthamer, R. (2003). Incidence of a new sex-ratio-distorting endosymbiotic bacterium among arthropods. *Proceedings. Biological Sciences / The Royal Society*, 270(1526), 1857–65.
- Weinert, L. A., Araujo-jnr, E. V., Ahmed, M. Z., & Welch, J. J. (2015). The incidence of bacterial endosymbionts in terrestrial arthropods. *Proceedings of the Royal Society B: Biological Sciences*, (282).
- Werren, J. H., Baldo, L., & Clark, M. E. (2008). Wolbachia: master manipulators of invertebrate biology. *Nature Reviews*, 6, 741–751.
- Werren, J. H., Zhang, W., & Guo, L. R. (1995). Evolution and phylogeny of Wolbachia: reproductive parasites of arthropods. *Proceedings of the Royal Society B: Biological Sciences*, 261, 55–71.
- Wilkinson, T. L., Adams, D., Minto, L. B., & Douglas, a E. (2001). The impact of host plant on the abundance and function of symbiotic bacteria in an aphid. *The Journal of Experimental Biology*, 204, 3027–3038.
- Wu, M., Sun, L. V., Vamathevan, J., Riegler, M., Deboy, R., Brownlie, J. C., ... Eisen, J. A. (2004). Phylogenomics of the Reproductive Parasite Wolbachia pipientis wMel: A Streamlined Genome Overrun by Mobile Genetic Elements. *PLoS Biology*, 2(3)
- Zchori-Fein, E., & Perlman, S. J. (2004). Distribution of the bacterial symbiont Cardinium in arthropods. *Molecular Ecology*, (13), 2009–2016.
- Zeh, J. a., Bonilla, M. M., Adrian, A. J., Mesfin, S., & Zeh, D. W. (2012). From father to son: transgenerational effect of tetracycline on sperm viability. *Scientific Reports*, 2, 1–5.
- Zélé, F., Nicot, A., Duron, O., & Rivero, A. (2012). Infection with Wolbachia protects mosquitoes against Plasmodium-induced mortality in a natural system. *Journal of Evolutionary Biology*, 25(7), 1243–1252.
- Zélé, F., Santos, I., Rodrigues, L., Godinho, D., Clemente, S., Olivieri, I., ... Magalhães, S. (in prep). Endosymbiont diversity in natural populations of Tetranychus mites is rapidly lost under laboratory conditions.
- Zhang, Y. K., Zhang, K. J., Sun, J. T., Yang, X. M., Ge, C., & Hong, X. Y. (2013). Diversity of Wolbachia in Natural Populations of Spider Mites (genus Tetranychus): Evidence for Complex Infection History and Disequilibrium Distribution. *Microbial Ecology*, 65, 731–739.
- Zhu, L. Y., Zhang, K. J., Zhang, Y. K., Ge, C., Gotoh, T., & Hong, X. Y. (2012). Wolbachia strengthens Cardinium-induced cytoplasmic incompatibility in the spider mite Tetranychus piercei McGregor. *Current Microbiology*, 65(5), 516–523.
- Zug, R., & Hammerstein, P. (2014). Bad guys turned nice? A critical assessment of Wolbachia mutualisms in arthropod hosts. *Biological Reviews*, 49.

Annexes

Annex 1. *Tetranychus urticae* populations collected on five different host plants from June to July 2015 and used to study the plant effect on the prevalence of *Wolbachia*, *Cardinium* and *Rickettsia*.

Host plant	Name	Collection date	Collection location	Coordinates
Zucchini (<i>Cucurbita pepo</i>)	Z1	08-06-2015	Hortas da Cortesia, São João das Lampas	38.865278, -9.384006
	Z2	09-06-2015	Quinta do Poial, Galeotas	38.536103, -9.000375
	Z5	10-06-2015	Correias	39.342914, -8.797936
	Z6	10-06-2015	Ribeira de Fráguas	39.366414, -8.851036
	Z7	10-06-2015	Aromas do Outeiro, Carregado	39.026500, -8.982278
Purple (<i>Ipomoea purpurea</i>)	P5	14-06-2015	Alvalade, Lisbon	38.755283, -9.147203
	P13	08-07-2015	Fernão Ferro	38.580006, -9.102147
Bean (<i>Phaseolus vulgaris</i>)	B1	08-06-2015	Hortas da Cortesia, São João das Lampas	38.865278, -9.384006
	B2	08-06-2015	Pêro Pinheiro	38.851900, -9.326903
	B6	10-06-2015	Correias	39.342914, -8.797936
	B7	10-06-2015	Biofrade, Lourinhã	39.258314, -9.294675
	B8	10-06-2015	Aromas do Outeiro, Carregado	39.026500, -8.982278
Tomato (<i>Solanum lycopersicum</i>)	T2	08-06-2015	Hortas da Cortesia, São João das Lampas	38.866183, -9.388956
	T4	10-06-2015	Aromas do Outeiro, Carregado	39.026500, -8.982278
	T6	13-06-2015	Campo Grande, Lisbon	38.755775, -9.156075
	T7	16-06-2015	Campo Pequeno, Lisbon	38.744336, -9.144289
	T8	16-06-2015	Quinta Pedagógica dos Olivais, Lisbon	38.762897, -9.112419
Eggplant (<i>Solanum melongena</i>)	E3	10-06-2015	Aromas do Outeiro, Carregado	39.026500, -8.982278
	E4	10-06-2015	Ribeira de Fráguas	39.366414, -8.851036
	E5	10-06-2015	Biofrade, Lourinhã	39.258314, -9.294675
	E6	15-06-2015	Alvalade, Lisbon	38.755283, -9.147203
	E7	16-06-2015	Quinta Pedagógica dos Olivais, Lisbon	38.762897, -9.112419

Annex 2. Sampling locations during the field survey of endosymbionts infecting *T. urticae* collected on bean (*Phaseolus vulgaris*), eggplant (*Solanum melongena*), tomato (*Solanum lycopersicum*), zucchini (*Cucurbita pepo*), and purple (*Ipomoea purpurea*) from June to July 2015. The table includes the information of whether plants were infested by spider mites or not (non-exhaustive list for uninfested plants) and, when infested, the corresponding mite species: *T. urticae* red form (TuR), *T. urticae* green form (TuG) and *T. ludeni* (TI). Conversely to previous sampling events conducted in Portugal (Zélé et al. *in prep*), none of the collected populations belonged to *T. evansi*. When no mites were found, the species is displayed as NA (not applicable). *: less than 10 individual mites found, so that population was excluded from further analyses.

Host Plant	Name	Collection date	Collection location	Coordinates	Infestation	Mite species
Bean	B1	08-06-2015	Hortas da Cortesia, São João das Lampas	38.865278, -9.384006	Yes	TuR
	B2	08-06-2015	Pêro Pinheiro	38.851900, -9.326903	Yes	TuR+TI
	B3	09-06-2015	Quinta de Santo António, Pegões	38.686669, -8.591297	Yes	TuG+TuR+TI
	B4	09-06-2015	Quinta do Poial, Galeotas	38.536103, -9.000375	Yes	TuR+TI
	B5	09-06-2015	Quinta das Margaridas, Pegões	38.687033, -8.591386	Yes	TI
	B6	10-06-2015	Correias	39.342914, -8.797936	Yes	TuG+TuR
	B7	10-06-2015	Biofrade, Lourinhã	39.258314, -9.294675	Yes	TuR
	B8	10-06-2015	Aromas do Outeiro, Carregado	39.026500, -8.982278	Yes	TuR
Eggplant	E1	09-06-2015	Quinta do Poial, Galeotas	38.536103, -9.000375	Yes	Tu+TI
	E2	09-06-2015	Quinta de Santo António, Pegões	38.686669, -8.591297	Yes	TuG+TuR+TI
	E3	10-06-2015	Aromas do Outeiro, Carregado	39.026500, -8.982278	Yes	TuR
	E4	10-06-2015	Ribeira de Fráguas	39.366414, -8.851036	Yes	TuR
	E5	10-06-2015	Biofrade, Lourinhã	39.258314, -9.294675	Yes	TuR+TI
	E6	15-06-2015	Alvalade, Lisbon	38.755283, -9.147203	Yes	TuR+TI
	E7	16-06-2015	Quinta Pedagógica dos Olivais, Lisbon	38.762897, -9.112419	Yes	TuR+TI

Host Plant	Name	Collection date	Collection location	Coordinates	Infestation	Mite species
Tomato	T1	08-06-2015	Hortas da Cortesia, São João das Lampas	38.865278, -9.384006	Yes	TuR
	T2	09-06-2015	Quinta de Santo António, Pegões	38.686669, -8.591297	Yes	TuR+TI
	T3	10-06-2015	Aromas do Outeiro, Carregado	39.026500, -8.982278	Yes	TuR+TI
	T4	10-06-2015	Correias	39.342914, -8.797936	Yes	TuR
	T5	13-06-2015	Campo Grande, Lisbon	38.755775, -9.156075	Yes	TuR
	T6	16-06-2015	Campo Pequeno	38.744336, -9.144289	Yes	TuR
	T7	16-06-2015	Quinta Pedagógica dos Olivais, Lisbon	38.762897, -9.112419	Yes	TuR
Zucchini	Z1	08-06-2015	Hortas da Cortesia, São João das Lampas	38.865278, -9.384006	Yes	TuR
	Z2	09-06-2015	Quinta do Poial, Galeotas	38.536103, -9.000375	Yes	TuR
	Z3	09-06-2015	Quinta de Santo António, Pegões	38.686669, -8.591297	Yes	TI
	Z4	09-06-2015	Quinta das Margaridas, Pegões	38.687033, -8.591386	Yes	TuR+TI
	Z5	10-06-2015	Correias	39.342914, -8.797936	Yes	TuR
	Z6	10-06-2015	Ribeira de Fráguas	39.366414, -8.851036	Yes	TuR
	Z7	10-06-2015	Aromas do Outeiro, Carregado	39.026500, -8.982278	Yes	TuR
Purple	P1	08-06-2015	Terrugem	38.840756, -9.374494	Yes	TI
	P2	08-06-2015	Pêro Pinheiro	38.850744, -9.327011	Yes	TI
	P3	08-06-2015	Alfouvar de Cima	38.868550, -9.284800	Yes	TI
	P4	10-06-2015	Casal Vale do Medo	39.248450, -9.294550	Yes	TI
	P5	14-06-2015	Alvalade, Lisbon	38.755283, -9.147203	Yes	Tu+TI
	P6	14-06-2015	Alvalade, Lisbon	38.753250, -9.146356	Yes	TI
	P7	25-06-2015	Sete Rios, Lisbon	38.740689, -9.166178	Yes	TI
	P8	25-06-2015	Campolide	38.730769, -9.167833	Yes	TuR*+TI
	P9	28-06-2015	Coimbra	40.211700, -8.402106	Yes	TI
	P10	02-07-2015	Entrecampos, Lisbon	38.740292, -9.157614	No	NA
	P11	02-07-2015	Jardim Botânico, Lisbon	38.717894, -9.149653	Yes	TI
	P12	02-07-2015	Alcântara	38.707181, -9.175942	Yes	TI
	P13	08-07-2015	Fernão Ferro	38.580006, -9.102147	Yes	TuR+TI
	P14	08-07-2015	Vale de Gatos	38.630586, -9.136025	Yes	TI
	P15	08-07-2015	Sesimbra	38.448725, -9.105550	Yes	TI
	P16	08-07-2015	Campo de Ourique	38.716978, -9.176314	No	NA
	P17	08-07-2015	Venda Nova	38.483467, -9.104325	Yes	TI
	P18	11-07-2015	Évora de Alcobaça	39.520042, -8.966897	Yes	TI
	P19	11-07-2015	Chiqueda	39.542350, -8.953192	Yes	TI
	P20	11-07-2015	Juncal	39.601828, -8.887736	Yes	TI
	P21	11-07-2015	Aljubarrota	39.570606, -8.920497	Yes	TI
	P22	11-07-2015	Cumeira	39.586336, -8.894306	Yes	TI
	P23	11-07-2015	Cascais	38.717122, -9.433808	Yes	TI
	P24	11-07-2015	Cascais	38.724636, -9.466950	Yes	TI
	P25	11-07-2015	Cascais	38.701583, -9.424286	Yes	TI
	P26	15-07-2015	Sintra	38.798969, -9.387533	Yes	TI
	P27	18-07-2015	Tituaria	38.948611, -9.210114	Yes	TI
	P28	18-07-2015	Roussada	38.944856, -9.219064	Yes	TI
	P29	19-07-2015	Pederneira	39.588375, -9.067222	Yes	TI
	P30	19-07-2015	Valado dos Frades	39.588642, -9.028781	Yes	TI
	P31	25-07-2015	São Romão	39.733819, -8.794236	Yes	TI
	P32	25-07-2015	Boa Vista	39.776908, -8.761403	Yes	TI
	P33	25-07-2015	Machados	39.783811, -8.750742	Yes	TI
	P34	25-07-2015	Planalto	39.757536, -8.786817	Yes	TI
	P35	25-07-2015	Maceira	39.684822, -8.889311	Yes	TI

Annex 3. List of primers used in multiplex for simultaneous detection of *Wolbachia*, *Cardinium* and *Rickettsia* infections. Spider mites generalist primers were used to control the DNA quality, and thus to discriminate uninfected individuals from PCR failure. The concentration of each primer in the primers mix used for the PCR reaction is also given in this table (Zélé *et al. in prep*).

Target gene	Primer name	Sequence (5'→3')	Concentration	Product size
Spider mite partial 5.8s and ITS2	5.8S_F	GGCTTTCGGGTCTTTTCCGAGGTCA	0.5μM	386-bp
	ITS2G_R	CGACTTTAGCGTCGTCAGATAGGCG	0.5μM	
<i>Rickettsia</i> gtIA	RICTG_F	AGGCTAATGGGCTTTGGTCATCGTGTAT	2μM	293-bp
	RICTG_R	TGTGCCATCCAGCCTACTGTTCTTGC	2μM	
<i>Wolbachia</i> wsp	WSPTG_F	GTTGGTGTGGTGCAGCGTATGTAAGC	2μM	222-bp
	WSPTG_R	AGTGCTGTAAAGAACTTTGATTCCGCCATC	2μM	
<i>Cardinium</i> 16S rRNA	CARDTG_F	GGCGGCTTATTAAGTCAGTTGTGAAATCCT	3μM	152-bp
	CARDTG_R	GCTGCCTACGCTATTGGTATTCTTATGAT	3μM	

Annex 4. List of primers used in multiplex to identify within a single PCR the 3 different spider mites species previously found in Portugal, namely *Tetranychus urticae*, *T. ludeni* and *T. evansi*. We used a *Tetranychus*-generalist forward primer but three different species-specific reverse primers (Zélé *et al. in prep*).

Target gene	Primer name	Sequence (5'→3')	Concentration	Product size
Tetranychus ITS1	ITS1G_F	AGGTGAACCTGCGGAAGGATCATTAACG	2μM	-
<i>T. urticae</i> ITS1	ITS1Tu_R	CCTTCTTTAAACCTTGCCGTCAGCATAAGC	2μM	570-bp
<i>T. ludeni</i> ITS1	ITS1Te_R	ACCAGAAGTATAGCAAGACAGGCTTACAAT	3μM	470-bp
<i>T. evansi</i> ITS1	ITS1TI_R	TGGATAACCCTCACTCTTGTTGCATTGGAT	3μM	190-bp

Annex 5. Status of infection by *Wolbachia*, *Cardinium* and *Rickettsia* of the 20 females tested from each *Tetranychus urticae* field population collected from the different plants. Each graph represent a population, in which the lines represent individual mites and the columns their infection status (empty cell: uninfected; filled cell: infected) by W: *Wolbachia* (purple), C: *Cardinium* (orange) and R: *Rickettsia* (green).

