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Tese orientada por:
Professora Doutora Sara Magalhães

Documento especialmente elaborado para a obtenção do grau de doutor

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"Muito boa noite, senhoras e senhores
muito boa noite, meninos e meninas
muito boa noite, Manuéis e Joaquinas
enfim, boa noite, gente de todas as cores
e feitios e medidas
e perdoem-me as pessoas
que ficaram esquecidas
boa noite, amigos, companheiros, camaradas
A vida é feita de pequenos nadas!
A vida é feita de pequenos nadas!

Resumo

Na natureza, os organismos competem por diferentes de tipos de recursos, sendo os recursos mais estudados a comida, os hospedeiros e os parceiros sexuais. Infelizmente, o estudo de cada um destes recursos pertence a uma área de investigação diferente, o que dificulta a compreensão plena do papel da competição enquanto força selectiva. No trabalho que aqui se apresenta, começamos por demonstrar de que modo a evolução experimental pode ser aplicada transversalmente ao estudo da competição, de forma a permitir extrair padrões e processos comuns às várias áreas de investigação. Isto torna-se possível uma vez que, em todos os casos, os organismos competem por acesso a um recurso limitante, qualquer que seja esse recurso.

Os capítulos seguintes da tese focam-se na competição por parceiros sexuais, um tipo de competição moldado pela selecção sexual. O principal objectivo deste trabalho foi estudar que adaptações são favorecidas pela selecção sexual em diferentes fases da reprodução, nomeadamente antes e depois do acasalamento. Desta forma, esperamos contribuir para enriquecer o nosso conhecimento no que diz respeito à ocorrência de poliandria, isto é, ao acasalamento de vários machos com uma única fêmea, em espécies com precedência espermática do primeiro macho. Nestas espécies, o primeiro macho a acasalar com uma fêmea é aquele que fertiliza todos os seus ovócitos. Consequentemente, parece paradoxal que estas fêmeas acasalem mais do que uma vez, a não ser que este comportamento traga alguma vantagem, esteja geneticamente relacionado com outra característica que seja vantajosa, ou ocorra inadvertidamente. De modo a poder abordar esta aparente contradição, usámos o ácaro-aranha *Tetranychus urticae*, uma espécie haplodiplóide, com precedência espermática do primeiro macho e cujos indivíduos acasalam frequentemente com fêmeas já fecundadas.

Numa primeira abordagem, testámos se os machos desta espécie eram capazes de distinguir fêmeas virgens de fêmeas fecundadas e que tipo de pistas são usadas pelos machos para exercer a sua preferência. Graças a este estudo, pudemos confirmar que os machos usam pistas químicas, nomeadamente voláteis, e rastos químicos deixados no substrato, para distinguir fêmeas virgens de fêmeas fecundadas. Estes resultados permitiram-nos concluir que a poliandria não ocorre devido a uma incapacidade, por parte dos machos, de discriminar fêmeas fecundadas de fêmeas virgens, o que sugere que este comportamento é vantajoso para pelo menos um dos sexos. Na sequência desta constatação, procurámos encontrar potenciais benefícios provenientes da existência de poliandria em machos e fêmeas. Para esta experiência utilizámos a resistência a um pesticida como marcador genético, o que nos permitiu determinar a paternidade da descendência produzia pelas várias fêmeas testadas. Esta metodologia é essencial para determinar se a poliandria altera o padrão de precedência

espermática desta espécie, conferindo assim benefícios directos aos machos. Os nossos resultados indicam que nenhum dos sexos beneficia directamente com acasalamentos múltiplos. De facto, a precedência espermática do primeiro macho foi sempre mantida, independentemente do número e do intervalo entre acasalamentos, sugerindo que acasalar com fêmeas fecundadas não confere nenhum benefício directo aos machos. Para além disso, fêmeas que acasalaram múltiplas vezes apresentaram uma menor longevidade e fecundidade do que fêmeas que acasalaram só uma ou duas vezes, sugerindo que há custos em acasalar múltiplas vezes para as fêmeas. No entanto, os machos desta espécie, apesar de não terem obtido benefícios directos com a poliandria, não sofreram custos de longevidade quando foram colocados exclusivamente com fêmeas previamente fecundadas. Além do mais, o número total de descendência produzida pelo primeiro macho a acasalar com uma fêmea fecundada é menor quando essa fêmea acasala múltiplas vezes, do que quando essa fêmea não acasala novamente, ou acasala apenas mais uma vez. Esta diminuição do número de descendência quando a fêmea acasala múltiplas vezes, faz com que o sucesso reprodutor do primeiro macho diminua, levando a um aumento relativo do sucesso reprodutor dos machos seguintes. Este benefício indirecto pode explicar, ainda que em parte, a ocorrência de poliandria no ácaro-aranha. De qualquer forma, este resultado não exclui a possibilidade de existirem outros benefícios que possam explicar a existência de poliandria. Por exemplo, ao acasalarem múltiplas vezes, os indivíduos podem reduzir o risco de se envolverem apenas em acasalamentos inviáveis.

A existência de acasalamentos inviáveis pode ser atribuída à presença de bactérias endosimbiontes. Estes organismos empregam diversas tácticas como, por exemplo, alterar a reprodução dos seus hospedeiros, de forma a favorecerem a sua transmissão. Quando essas tácticas são custosas para o hospedeiro, prevê-se que este evolua estratégias de modo a poder evitar ou reduzir os custos da infecção. Frequentemente, as populações de ácaros encontram-se infectadas com *Wolbachia*, uma bactéria endossimbiótica que induz incompatibilidade citoplasmática. A incompatibilidade citoplasmática resulta na redução do número de descendência fertilizada proveniente de acasalamentos entre fêmeas não infectadas e machos infectados. Com as experiências seguintes, procurámos saber se fêmeas de ácaros-aranha não infectadas evoluem a capacidade de escolher parceiros sexuais compatíveis ou de acasalar múltiplas vezes, de modo a contornar os custos impostos pela incompatibilidade citoplasmática. De forma a testar esta possibilidade, realizámos uma experiência na qual populações de ácaros-aranha evoluíam nas seguintes condições: i) populações totalmente infectadas com *Wolbachia*, ii) populações totalmente livres de *Wolbachia* ou iii) populações com uma prevalência intermédia deste simbionte. No último caso, a cada geração, fêmeas não infectadas foram colocadas com machos infectados e não infectados, na mesma proporção, de modo a promover os acasalamentos entre indivíduos. Este último regime de evolução experimental

corresponde às condições ideais para a evolução de um mecanismo compensatório contra a *Wolbachia,* por parte do hospedeiro. Após doze gerações de selecção, indivíduos mantidos com prevalência intermédia de *Wolbachia* não alteraram a sua capacidade de escolha. Para além disso, não se verificaram modificações na latência à copula ou na duração de cópula em qualquer um dos regimes. Isto permite-nos concluir que o efeito da *Wolbachia* na evolução de isolamento reprodutor em ácaros, se existe, é residual. Este resultado é relevante para o estudo da especiação, uma vez que o isolamento reprodutor é considerado um comportamento com elevado impacto neste processo. Apesar de não termos encontrado diferenças no comportamento que antecede a cópula, fêmeas não infectadas que evoluíram em populações com prevalência intermédia de *Wolbachia* apresentaram uma redução no grau de incompatibilidade citoplasmática após acasalarem com machos infectados e machos não-infectados, sequencialmente. Este resultado permite-nos concluir que a poliandria pode ser vantajosa em ambientes em que existe o risco de acasalamentos inviáveis, uma vez que permite reduzir a incompatibilidade citoplasmática. Ao fazê-lo, os ácaros-aranha alteraram o seu padrão de precedência espermática a favor do segundo macho. No entanto, esta alteração só ocorreu numa direcção, uma vez que, nas situações em que o primeiro macho era compatível, i.e., não estava infectado com *Wolbachia*, a precedência espermática pelo primeiro macho foi mantida. A unidirecionalidade na alteração do padrão de precedência espermática é muito provavelmente um factor essencial à evolução de poliandria incitada pelos custos associados à incompatibilidade citoplasmática, em espécies com padrões de precedência espermática enviesados.

Em suma, o trabalho desenvolvido ao longo desta tese, ao endereçar questões importantes que tinham sido, até agora, negligenciadas, permite-nos compreender mais aprofundadamente qual o papel da poliandria em espécies com precedência espermática do primeiro macho. Para além disso, os resultados apresentados aqui contribuem substancialmente para o estudo das interações entre endosimbiontes e os seus hospedeiros, bem como para compreender de que forma essa interação afecta o processo de especiação.

Palavras-chave

poliandria; ácaro-aranha; precedência espermática; incompatibilidade citoplasmática; *Wolbachia*

Abstract

Organisms compete for several resource types, the most studied being food, hosts and mates. Regrettably, the study of competition for each of these resource types belongs to different research fields that rarely overlaps, which might hamper a comprehensive understanding of competition as pervasive selective force. In this work, we begin by showing how experimental evolution can be transversally applied to the study of competition across research fields and attempt to extract general patterns and processes, as in all cases individuals are competing for the use of a limiting resource.

The rest of this thesis is directed towards competition for mates, a type of competition that is shaped by sexual selection. The main goal of this work was to study the adaptations favoured by sexual selection at different stages of reproduction, namely prior and after mating, in order to better understand the occurrence of polyandry in species with first male sperm precedence. In these species, the first male that mates with a female will sire all her offspring. Consequently, it seems paradoxical that females mate multiply, except if this behaviour provides an advantage for females or males, if it correlates with other traits, or if it occurs inadvertently. In order to tackle this, we used the spider mite *Tetranychus urticae*, a haplodiploid species with first male sperm precedence in which polyandry is pervasive.

First, we tested whether males distinguish between virgin and mated females and which type of cues they use to exert their preference. In fact, spider mite males preferred virgin over mated females and used chemical cues, namely volatiles and chemical trails, to distinguish them. These results indicate that polyandry does not occur due to a lack of ability to discriminate females of different matings status, suggesting this behaviour might be advantageous for either sex. Consequently, the next step was to test the potential costs and benefits of polyandry for males and females. Neither males, nor females benefited directly with polyandry. In fact, females that mated multiple times survived less and laid fewer eggs, compared to females that mated once or twice only. Nevertheless, males did not suffer longevity costs when they mated with mated females and they were able to decrease the fitness of first males, gaining an indirect benefit with this behaviour. Polyandry can thus be, even if partially, explained by this indirect benefit. Still, these results do not rule out the existence of other, indirect benefits. For instance, by mating multiply, individuals might reduce the risk of only mating with incompatible mates.

Incompatible matings may be attributed to the presence of endosymbiotic bacteria. These organisms can employ various tactics, such as altering the reproduction of their hosts, in order to favour their own transmission. When these tactics are costly for the host, hosts are expected to evolve strategies to avoid or reduce such costs. Spider mite populations are often infected with *Wolbachia,* an endosymbiotic bacterium that induces cytoplasmic incompatibility (CI), whereby crosses between uninfected females and infected males yield reduced fertilized offspring. Here we tested whether *T. urticae* uninfected females evolve mate choice and multiple mating to circumvent the costs imposed by CI. To this aim, we performed experimental evolution on spider-mite populations with i) full *Wolbachia* infection, ii) no infection, or iii) mixed infection. In the latter, *Wolbachia*-uninfected females could copulate with both *Wolbachia*-infected and *Wolbachia*-uninfected males at each generation, which is expected to result in high costs for uninfected females, and hence promote the evolution of a compensatory mechanism. Evolving under mixed infection did not affect host mate choice, latency to copulation or copulation duration, after 12 generations of selection. Therefore, the role of *Wolbachia* in pre-copulatory reproductive isolation in spider mites, if present, is probably residual. However, after 20 generations of selection, uninfected females evolving under mixed infection that mated with *Wolbachia*-infected males presented a higher degree of CI than those mated first with *Wolbachia*-infected and then with *Wolbachia*-uninfected males evolving under mixed infection. Therefore, polyandry can be advantageous when there is the risk of incompatible matings, since it reduces the degree of CI. By doing so, spider mites break their sperm priority pattern in favour of the second male. However, this disruption of sperm precedence only occurred in one direction. Indeed, when the first mating was compatible, i.e., the first male was not infected with *Wolbachia,* individuals kept first male sperm precedence. The unidirectional disruption of the sperm precedence pattern might be a key factor for the evolution of CI-driven polyandry in species with skewed patterns of sperm precedence.

Overall, the results obtained here contribute to improve our understanding of mating strategies by addressing important questions that have been largely neglected so far, namely the putative drivers of multiple mating in species with first male sperm precedence.

Keywords

polyandry; spider mites; sperm precedence; cytoplasmic incompatibility; *Wolbachia*

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CHAPTER I

General Introduction

Competition shapes the evolution of organisms. Organisms compete for several resource types, the most studied being food, hosts and mates. The mechanisms underlying these processes are the same, but their study belongs to different research fields. In my thesis, I will focus on competition for mates, which is driven by sexual selection.

Sexual selection was first proposed by Darwin (1871, p.256) in *The descent of Man and Selection in Relations to sex* to explain why the evolution of some traits depended *" on the advantage which certain individuals have over other individuals of the same sex and species in exclusive relation to reproduction."* In this introductive chapter, I will focus on the adaptations favoured by sexual selection at different stages of the reproductive process (section 1). I will summarize what is known about pre- and post-copulatory mating strategies and how these strategies can benefit competition for mates. In addition, given that endosymbiotic infection can have important consequences on the matings strategies of hosts, I will review what is known about endosymbiotic-host interactions and their effect on host reproduction, focusing mainly on the interaction between the endosymbiotic bacteria *Wolbachia* and arthropods (section 2). All along these lines, I will highlight how haplodiploidy and different mating systems can impact on evolutionary predictions. Finally, in the last section, I will provide a brief overview of the biology of the model species used in my experiments, the two-spotted spider mite *Tetranychus urticae* (section 3), followed by an outline of my thesis (section 4).

1. Biology of mating strategies

Sexual selection is divided in intra-sexual competition and inter-sexual choice. Intra-sexual competition occurs when individuals of one sex compete to gain access to, or monopolize, mates and their gametes. Inter-sexual choice arises when a trait of the chosen sex leads to non-random mating success of the choosy sex (Shuker 2014).

In the classical view of sex roles, proposed by Darwin (1871) and reinstated by Bateman (1948), males compete for access to females and females select among males. However, throughout the years, it has become increasingly clear that the roles of males and females can be dynamic and interchangeable. Reverse sex roles, i.e., choosy males and female-female competition, were first observed in species where males contribute to parental care (Trivers 1972; Davies et al. 2012). For instance, in the pipefish *Nerophis ophidion*, embryo development occurs inside the body of the male. Pregnancy lasts several weeks during which females can produce several clutches of eggs. Consequently, males become the limiting resource and females compete for males, who prefer larger and more ornamented females (Rosenqvist 1990). Yet, male mate choice can occur even in species where males contribute with little more than sperm to females (Bonduriansky 2001; Edward & Chapman 2011). In fact, even in taxa where the ratio of sexually active males to females (operational sex ratio, OSR) is male-biased, in which available females are scarce and males are likely to be able to mate with all available females, male mate choice can evolve if the benefits of choice outweigh the costs of being choosy (Arnqvist 2014, section 1.3). Thus, in many species, both sexes are selective in their choice of mates, i.e., there is mutual mate choice (Bergstrom & Dugatkin 2012). Similarly, competition for mating opportunities in females is not necessarily dependent on the OSR. In fact, strong female mate choice itself might select for female-female competition, if the amount of females exhibiting preference for the most attractive males exceeds their mating capacity (Kvarnemo & Simmons 2013).

The intensity of intra-sexual competition will depend on the benefits obtained with mating (see section 1.3) and on the number of available mates (see section 1.4). Indeed, the more time females spend in a sexually-unreceptive state, the more male-biased will the OSR be, selecting for stronger male-male competition (Edward & Chapman 2011). Likewise, several key factors affect the evolution of mate choice. First, the reproductive strategies of the choosy sex (mating effort, i.e., the amount of resources invested in each mating, and investment in parental care) are expected to influence mate choice (Bonduriansky 2001; Edward & Chapman 2011; Davies et al. 2012). For instance, long courtship/copulations should favour mate choosiness. Indeed, increased mating effort and/or parental care increases the number of mates available to copulate with in the population, but reduces the capacity of mating of each individual (Edward & Chapman 2011). Second, there must be variance in the quality of the chosen sex for mate choice to evolve (Bonduriansky 2001; Edward & Chapman 2011; Gibson & Langen 1996). Finally, the evolution of mate choice will be contingent on the costs and benefits of being choosy (Bonduriansky 2001; Edward & Chapman 2011; Gibson & Langen 1996), as we will see in section 1.3.

In polyandrous species, i.e., in species where females have multiple mates, sexual selection can act prior to mating, via mate choice and competition for direct access to mates, or after mating, through sperm competition and cryptic choice. Consequently, sexual selection should favour adaptations that ensure male and female reproductive success both prior and after mating. However, adaptations for pre-copulatory and post-copulatory success are not necessarily synergistic (Kvarnemo & Simmons 2013) and male and female adaptations are often antagonistic. The net strength of selection acting on males or females will thus depend on the interactions between male and female strategies within and across episodes of selection (Figure 1).

In this section, I will summarize the reproductive strategies that can arise both prior to – (section 1.1) or after mating (section 1.2) and highlight their benefits for males and females. I will finish by considering the effect of sperm priority (section 1.4) and haplodiploidy (section 1.5) in some of these strategies.

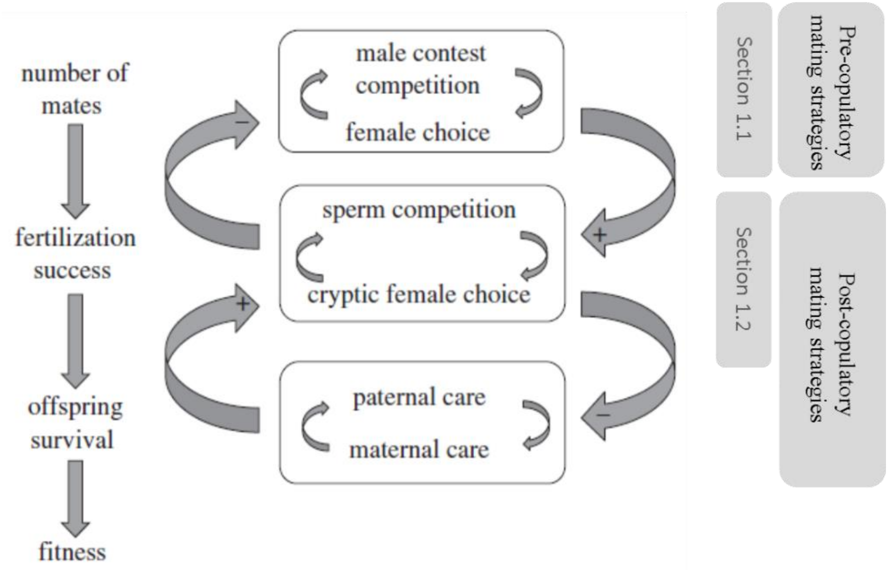


Figure 1. Schematic representation of episodes of sexual selection, their interaction and their impact on fitness. *White balloons represent stages during reproduction at which sexual selection can act. The first stage occurs before fertilization and will be discussed in section 1.1, the second takes place after mating and will be addressed in section 1.2. Arrows indicate synergistic (plus symbols) and antagonistic (minus symbols) selection. The net strength of selection acting on males or females will depend on the interactions between male and female processes within episodes of selection, and on the interactions across episodes of selection. (Adapted from Kvarnemo & Simmons 2013)*

1.1 <u>Pre-copulatory mating strategies</u>

1.1.1 Intra-sexual competition

As pointed out before, intra-sexual competition occurs when individuals of one sex compete to gain access to, or monopolize, mates and their gametes (Shuker 2014). Individuals can gain access to mates through several different pre-copulatory mechanisms, including endurance rivalry, scramble and physical contests (Shuker 2014). Endurance rivalry selects for traits that increase survival, assuming increased survival will result in increased reproductive success (Shuker 2014; Danchin & Cézilly 2008). Scramble competition, or exploitative competition, occurs whenever competitors exploit the same limiting resource, thereby reducing the quality of resources available (Chapter II, Danchin and Cézilly 2008). This type of competition should select for traits that improve searching and monopolization of mates. For instance, some species exhibit pre-copulatory mate guarding, a behaviour seen as means of monopolizing access to females until they become available for mating. In *Sepsis cynipsea*, for example, males guard gravid females while they oviposit, so copulation occurs as soon as females become receptive again, thus improving the male genetic representation in the subsequent eggs clutches (Simmons 2014). Finally, physical contests select for traits that improve the competitors' ability to win fights, including weaponry, increased aggression and large size. For instance, in several species of dung beetles, females build tunnels and fill them with dung in which they lay their eggs. Males have evolved horns (Figure 2) that they use in fights against other males to guard the tunnels and the winner male mates repeatedly with the resident female. In laboratory experiments, males with longer horns were more likely to win contests (Emlen & Oring 1977; Emlen 2014). These intense contests may also select for alternative mating tactics, in which individuals display strategies that circumvent the costs of direct contact. Indeed, in these dung beetles, small hornless males sneak in tunnels guarded by other males, by digging new tunnels that intercept the guarded ones belowground (Emlen 1997; Moczek and Emlen 2000; Emlen 2014 Figure 2). In this way, small males are able to avoid contests they were likely to lose and still fertilize some females.

Note that none of the mechanisms presented above are mutually exclusive and that they do co-occur in several species (Shuker 2014).

Figure 2. Examples of alternative male morphs in dung beetles. Onthophagus taurus *(top),* O. nigriventris *(bottom). Large horned males are shown on the left and small hornless males on the right. Females (not shown) are entirely hornless in both species. Adapted from Valena and Moczek 2012.*

1.1.2 Inter-sexual mate choice

Prior to mating, mate choice can take place in two phases: mate location and mate assessment (Brooks & Griffith 2010; Danchin & Cézilly 2008). Individuals can use different cues to exert their preference. These cues can be phenotypic traits, or resources defended or provided by the chosen sex, like nests or territories (Candolin 2003). Phenotypic traits include visual, chemical, acoustic, olfactory, tactile and behavioural traits (Candolin 2003; Johansson & Jones 2007). Some of these traits, such as body constitution, can arise and be maintained through natural selection but simultaneously be used as indicators of fitness. Others, like colourful ornaments or courtship behaviours, have evolved with the sole purpose of attracting mates and can be detrimental to survival (Candolin 2003).

Any trait that attracts and stimulates partners, or the monopolization of any high-quality resource necessary for reproduction (Danchin & Cézilly 2008) should be favoured in the competing sex. For instance, across insects, the most common traits preferred by males are female size or weight, which often act as proxies for female fecundity, and female mating status. Mating status can predict the risk - the probability that a female will mate with two males - or intensity - the number of competitors- of sperm competition, as we will see in section 1.2.2.2 (Kelly & Jennions 2011; Bonduriansky 2001).

Frequently, if not always, multiple cues are used in mate choice (Jennions & Petrie 1997; Candolin 2003). There are several explanations for the use of multiple cues. First, some of these cues might be unreliable indicators of mate quality solely maintained due to pre-existing sensory biases (Candolin 2003; Jennions & Petrie 1997). Second, they can provide different information regarding a prospective mate. In fact, there are some evidences that different signals are evaluated differently depending on which type of benefits the choosy individual is selecting (see section 1.3.1). For instance, in some species of birds, females use song repertoires, a proxy of male quality, to choose extra-pair mates, but use territory characteristics, like nest quality, to select cuckolded males (Candolin 2003). Third, multiple cues can be redundant. For instance, in *Bicyclus anynana*, females use both chemical and visual cues, even though only one type of cue is sufficient to elicit preference (Costanzo & Monteiro 2007). The redundancy of cues is associated with a decrease in mate-choice errors (Candolin 2003). Finally, different cues can be differentially useful in different environments (Jennions & Petrie 1997; Bro-Jørgensen 2010).

1.2 Post-copulatory mating strategies

Parker (1970) was the first to emphasize that sexual selection can persist after mate acquisition. If females mate multiply, then the sperm from two or more males can still compete for the same set of eggs, a term coined “sperm competition”. Thus, sperm competition can be seen as the post-copulatory equivalent of pre-copulatory male-male competition. Sperm competition is a widespread phenomenon, given that females mate multiple in most internally fertilized species (Simmons 2001). Sperm competition will generate sexual selection on males to improve their reproductive success, ultimately resulting in a pattern of sperm precedence, as we will see in section 1.4. However, before discussing the patterns of sperm precedence I will focus on the potential mechanisms of sperm competition (section 1.2.1).

Storage, maintenance and utilization of sperm occurs within the female. Hence, these traits are, ultimately, under female control (Simmons 2001). Thus, in the same way sperm competition can be seen as the post-copulatory equivalent of pre-copulatory male-male competition, pre-copulatory female choice can be extended to after copulation, a phenomenon called cryptic female choice (Thornhill & Alcock 1983; Arnqvist 2014). In addition, given that sperm production is costly, males can exert male choice after mating. These mechanisms of post-copulatory mate choice are briefly summarized in section 1.2.2. Note that strategic parental investment can also occur. However, I will not address it in this introduction.

Post-copulatory mechanisms can be essential to determine the intensity and direction of selection on sexual traits. Notwithstanding, there is a lot we do not know about them, mainly because of technical challenges related to direct observation of sperm within the female tract and of the limited ability to discriminate sperm from different males. For instance, until recently it was thought that in *Drosophila melanogaster* last male sperm precedence was achieved through chemical sperm removal (Harshman & Prout 1994). However, in 2010, when sperm observations were done in vivo, it was discovered that mechanical sperm displacement by rival males and sperm ejection by females were operating in this species, rather than chemical displacement (Manier et al. 2010).

1.2.1 Sperm competition

Sperm competition is usually viewed as an extension of male-male competition. Indeed, males are expected to evolve strategies that allow them to maximize their own reproductive success not only before but also after copulation, within the female. In fact, internal fertilization itself is thought to be an adaptation against sperm competition: the closer to the site of fertilization the sperm is delivered, the lower the intensity of sperm competition and thus the bigger the chance of successful fertilization (Simmons 2001). The existence of sperm competition can select for both defensive and offensive traits in males (Arnqvist & Rowe 2005). The outcome of a multiple mating will depend, in part, on how different male adaptations perform against each other (Danielsson 1998).

1.2.1.1 Defensive traits

Defensive traits reduce the risk of sperm competition – the probability that a female will mate with two males – by reducing or even preventing females from re-mating. These adaptations can be physiological, behavioural or mechanical.

Defensive reproductive behaviours include prolonged copulation and post-copulatory mate guarding. Prolonged copulations are described as a prolonged period of male-female association beyond the time necessary for insemination, functioning as a physical barrier against potential competitors. In post-copulatory mate guarding, in contrast, males stay attached to females without maintaining genital contact. Both behaviours prevent females from re-mating. However, frequently, during prolonged copulation and post-copulatory mate guarding, there are take-over attempts by other males. Selection is thus expected to favour males that are able to reduce or prevent those take overs. This can be achieved using special organs that serve to maintain the connection between male and female or displaying aggressive behaviour towards competitors (Danielsson 1998). Note that these strategies occur at the expense of mate searching, being selected only when the benefits of mate guarding are superior to the costs of lost mating opportunities. Consequently, selection is expected to favour phenotypic plasticity in mate guarding behaviour in response to the risk of sperm competition (Kvarnemo & Simmons 2013). For instance, in fire buds, *Pyrrhochoris apterus*, copulation duration is dependent on the OSR, with longer copulations, that can last up to seven days, occurring in more male-biased OSR (Figure 3). This happens even though sperm transfer is finished a few hours after pairing, suggesting that extended time of copulation serves to prevent females from re-mating (Simmons 2014).

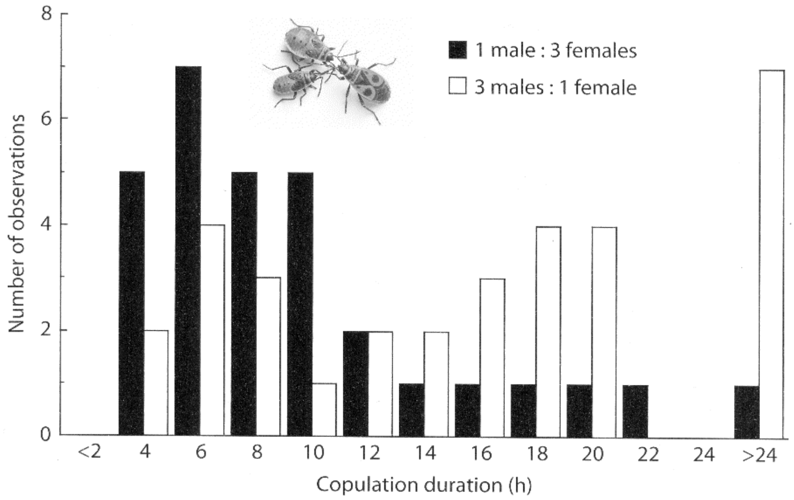


Figure 3. Duration of copulation in firebugs, Pyrrhochoris apterus*depends on the sex-ratio of the populations. Sperm transfer is complete after 4 hours of copulation but males adjust their time of association with the female depending on the risk of re-mating. Adapted from Simmons 2014. Picture firebugs: Alex Hide*

Males can also evolve defensive strategies that allow them to continue mate searching after copulation. Along with sperm, males transfer products from the accessory glands. These seminal products may have significant impacts on female physiology and behaviour. Males can reduce the attractiveness of females by decreasing their output of pheromones or by applying anti-aphrodisiac substances during copulation (Danielsson 1998). Alternately, seminal products can decrease or delay female receptivity to future matings and/or promote oviposition (Simmons 2001). For instance, an exhaustive study done on several mosquito species, namely ten *Aedes* species, *Culex pipiens* and *Anopheles quadrimaculatus,* has found that females with implanted male accessory glands on their thorax were never inseminated by males, while 75-100% of females with male gut on their thorax were successfully inseminated (Craig 1967). Another possible defensive strategy is to transfer with sperm masses of material secreted by the accessory glands that coagulate in the tract of the female, functioning as a physical barrier (Simmons 2001). These structures, called mating plugs serve the dual function of helping deliver the ejaculate (i.e., preventing passive loss of sperm; "leaking hypothesis") and of plugging the reproductive tract preventing subsequent matings (Simmons 2001). However, they can be short-lived barriers and thus not totally effective against females re-mating. Nevertheless, the mean proportion of effective second matings is lower in species with mating plugs than in species without such structure (Simmons 2001).

While these strategies leave the males free to search for additional mates, the production of seminal products can be a high-energy investment, limiting their ability to fertilize other females (Arnqvist 2014). Thus, as in mate guarding, we expect males to allocate their resources depending on the intensity of sperm competition (Arnqvist 2014). Consistent with this prediction, across species of butterflies, there is a negative correlation between female mating frequency and the investment of males in mating plugs (Figure 4).

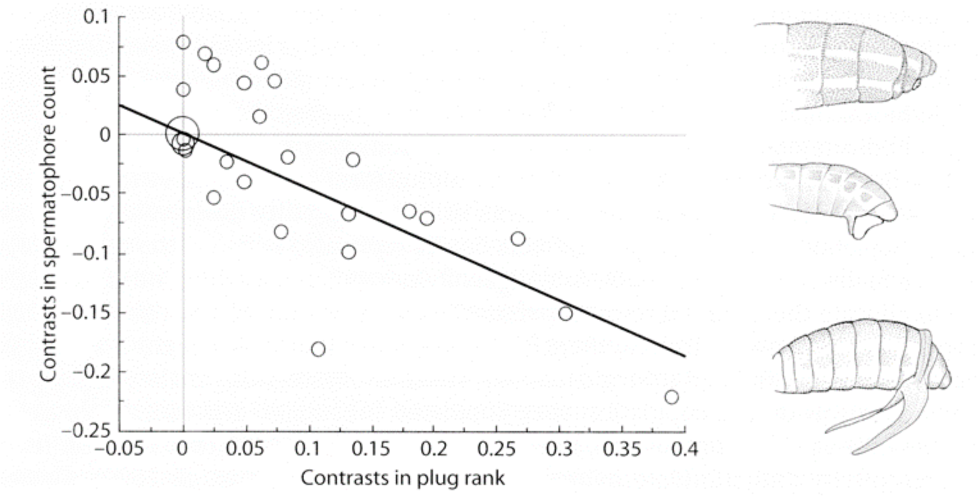


Figure 4. Correlated evolution of mating plugs and female re-mating frequency among butterflies. *Species with small mating plus (top right) have high mating rates, while those with bigger plugs have moderate to low female mating rates. From top to bottom:* Graphium sarpedon, Atrophaneura alcinous *and* Euryades corethrus. *Adapted from Simmons 2014.*

1.2.1.2 Offensive traits

Offensive traits increase the ability of males to outcompete the ejaculate of other males within the female. Several adaptations for sperm priority have been suggested so far. For instance, males can improve their chance of fertilization success by evolving behavioural adaptations, such as prolonged copulation. Indeed, the degree to which a male can displace rival sperm may depend on the time he spends copulating with a given female (Simmons 2001). Besides, prolonged copulation can facilitate repeated inseminations and consequently increase the reproductive success of males in species with sperm mixing (Simmons 2001). This behaviour can thus function as defensive trait, as see in the previous section, and as offensive trait. In fact, this is the case for several male adaptations (Simmons 2001).

In addition, to prolonged copulation, sexual selection driven by variation in sperm fertilization success can lead to rapid and divergent evolution of male genitalia, sperm production and sperm morphology and physiology.

Altered genitalia can act directly, by removing or displacing rival sperm, or by improving the chance of successful insemination. For instance, in seed beetles *Callosobruchus maculatus*, male's altered genitalia pierce the internal walls of the female genital tract, allowing seminal fluid compounds to enter the female's haemolymph. Males with longer spines have increased competitive fertilization success (Hotzy et al. 2012). These traumatic inseminations allow males to bypass the usual route of insemination, thus gaining a selective advantage over other males. Alternately, altered genitalia can act indirectly, stimulating the female reproductive tract in order to gain sperm priority (Simmons 2001; Danielsson 1998). Female stimulation can be achieved through chemical or behavioural processes, eliciting sperm transport, sperm removal and/or egg abortion. For example, in the fly *Dryomyza analis*, females expel a droplet of sperm before oviposition. By tapping repeatedly on the external genitalia of females, males succeed in increasing the proportion of sperm from previous males in the droplet expelled, thereby promoting their relative reproductive success (Danielsson 1998).

Sperm form and function can also play a large role in fertilization success. For instance, in *D. melanogaster*, mechanical sperm displacement by rival males and sperm ejection by females operate as mechanisms of sperm priority (Manier et al. 2010). Long and slow-swimming sperm are better able to enter the seminal receptacle of the female and less likely to be displaced than other sperm, conferring an advantage to the male that produces them (Lüpold et al. 2012).

1.2.2 Cryptic choice

Cryptic choice is defined as any pattern of non-random post-copulatory reproductive success across chosen individuals that is caused by the choosy individual (Thornhill & Alcock 1983; Arnqvist 2014; Bonduriansky 2001). As underlined before, cryptic choice can occur in both sexes.

1.2.2.1 Cryptic female choice

Cryptic female choice traits can be broadly categorized as morphological, physiological or behavioural (Arnqvist 2014). The shape of the female internal reproductive duct is an example of morphological cryptic female choice. For instance, long or fast sperm will gain an advantage over other sperm in a long and winding duct (Arnqvist 2014). Likewise, females might control the usage and storage of sperm. For instance, feral fowl females expel sperm following copulations with some males and yet accept sperm from others, biasing paternity (Simmons 2001; Pizzari & Birkhead 2000). Another example of cryptic female choice is found in the flour beetle *Tribolium castaneum.* In this species, a positive correlation is observed between the rate at which males rub with their tarsi on the lateral edges of the females' elytra during copulation and males' reproductive success in a double mating. However, when Edvardsson and Arnqvist (2000) ablated the tarsi of males, thus preventing the them from reaching the females' elytra, the positive correlation found in control males was lost (Figure 5). This occurred even though manipulated males kept "rubbing" in the air and the same amount of sperm was transferred during copulation by manipulated and control males. Their experimental manipulation thus show that female perception of male copulatory courtship behaviour, rather than the behaviour of the male itself, affects male competitive fertilization success in the flour beetle.

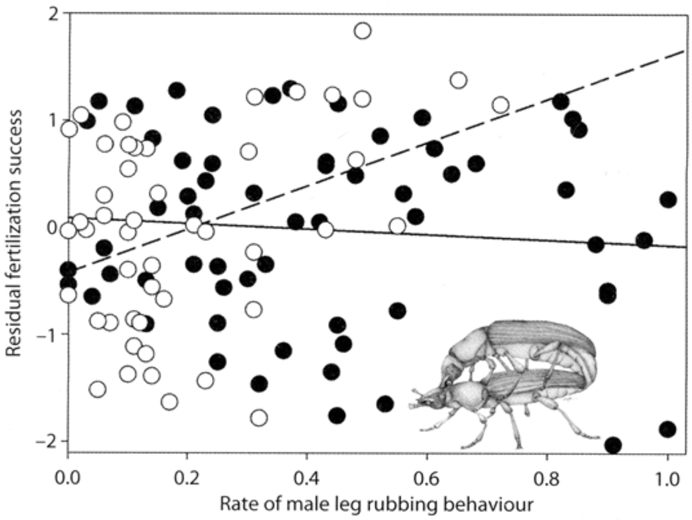


Figure 5. Cryptic female choice in the female flour beetle, **Tribolium castaneum.** *When males have ablated tarsi, they are prevented from reaching the females' elytra, which they normally rub during copulation. Consequently, the positive correlation found between the rate at which control males rub with their tarsi on the lateral edges of the females' elytra and male reproductive success in a double mating is lost. Ablated males: filled circles and solid line; control males: open circles and dashed line. Adapted from Arnqvist 2014.*

1.2.2.2 Cryptic male choice

As females, males can exert cryptic male choice, through strategic adjustment of ejaculates and differential allocation of parental care (Edward & Chapman 2011). Here, I will only focus on strategic ejaculation.

Strategic adjustment of ejaculates can involve changes in chemical composition, influencing sperm performance, in sperm morphology and in sperm number (Kelly & Jennions 2011). There are several main factors that can affect strategic ejaculation. Males should transfer more sperm in copulations with females of higher quality, assuming males can reliably assess female quality (Kelly & Jennions 2011; Wedell et al. 2002). Indeed, in some insects, males have been shown to transfer larger nutritious ejaculates to larger females, size being a proxy of fecundity (Kelly & Jennions 2011; Wedell et al. 2002). In addition, males should exhibit phenotypic plasticity in ejaculate properties in response to the risk and intensity of sperm competition (Kelly & Jennions 2011; Wedell et al. 2002; Edward & Chapman 2011). At low levels of sperm competition, when females may or may not mate with more than one male, males are expected to increase their ejaculate expenditure with increasing risk of competition, i.e., the probability that a female re-mates. Indeed, a meta-analysis done in 15 insect species has shown that males transfer more sperm in contact with rival males (Kelly & Jennions 2011). However, with increasing levels of sperm competition, which entails that all females mate more than once, male ejaculate expenditure should decrease with increased intensity of sperm competition, i.e., the number of male competitors (Kvarnemo and Simmons 2013; Simmons 2014; Figure 6). This is because the more polyandrous females are, the lower is their reproductive value for males (Kvarnemo & Simmons 2013). For instance, in the field cricket *Teleogryllus oceanicus*, males increase the proportion of viable sperm in their ejaculate when they perceive the odour of just one rival but reduce this proportion with cues from an increasing number of rivals (Thomas and Simmons 2009, Figure 6).

The risk and intensity of sperm competition are often predicted by males via the female mating status. That is because a virgin female might not mate again after mating with the focal male, while a mated female already contains sperm from another male. The risk of sperm competition is thus higher in mated females. In this sense, males should increase their ejaculate expenditure in matings with mated females, provided there are no additional competitors. The intensity of sperm competition, on the other hand, is predicted to be higher in mated females than in virgins and males should increase their ejaculate expenditure in matings with virgins. In fact, males often copulate during a longer period of time with virgins than with mated females (Kelly & Jennions 2011).

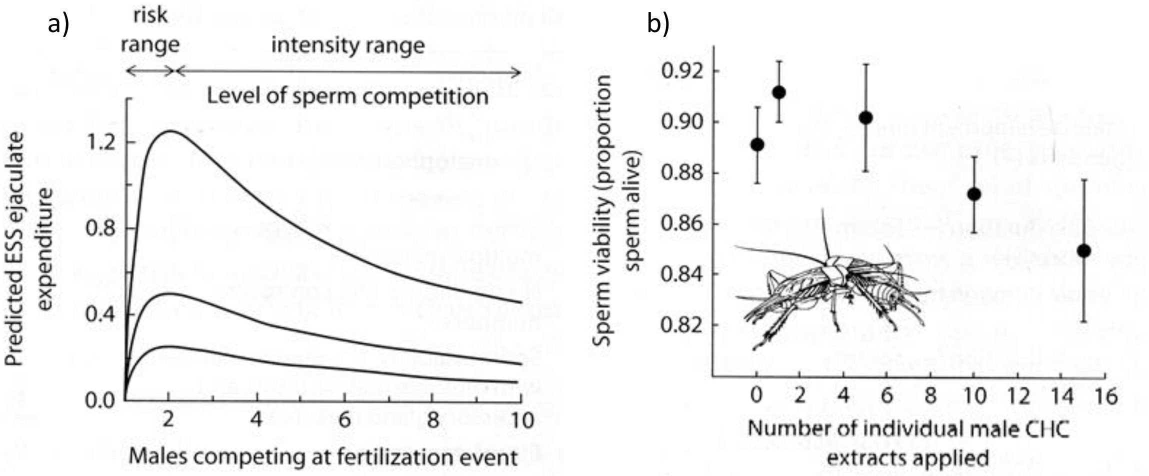


Figure 6. **The correlation between sperm ejaculate and sperm competition.** *a) Theoretical predictions from sperm competition game theory for ejaculate expenditure depending on the number of rivals. ESS: evolutionary stable strategy. After* Parker and Simmons 1996 *b) Empirical study using the field cricket* Teleogryllus oceanicus. *Females were impregnated with different amounts of extracted cuticular hydrocarbons (CHC), corresponding to a different number of rival males. Tested males transfer more viable sperm with increased risk of competition, when they perceive the odour of just one rival, but reduce the proportion of viable sperm transferred when mating with females to which increasing quantities of CHC were applied – increased intensity of competition. After Thomas and Simmons 2009. Adapted from Simmons 2014.*

1.3 Costs, benefits and sexual conflicts in reproduction

None of the strategies described so far is free of costs. Consequently, these mechanisms are expected to evolve when the reproductive advantage gained is superior to the costs incurred. However, as pointed out before, male and female adaptations do not need to be advantageous for the opposite sex. In fact, frequently the evolutionary interest of males and females do not coincide, giving rise to sexual conflicts, as we will see in the next section (1.3.2). In addition, I will summarize the potential costs and benefits of mate choice (1.3.1) and of mating (1.3.2).

1.3.1 Costs and benefits of mate choice

The costs of being choosy should be independent of the sex that chooses. Individuals risk decreasing their reproductive success with prolonged search time and loss of energy evaluating mates and/or wrong mate quality assessment. These costs are expected to be inversely proportional to the life expectancy, mating capacity and mate availability (Bonduriansky 2001). In addition, males and females risk increased exposure to predators, harassment, sexually-transmitted diseases and opportunity costs, as the best mates can be removed from the pool of available mates as time passes (Brooks & Griffith 2010). However, mate choice can be extremely advantageous as well.

During the last decades, many empirical and theoretical studies have tackled the problem of what benefits females might gain from being choosy. Although poorly addressed in the literature, it seems straightforward to assume that these benefits can be transversally applied to females and males.

Mate choice should be beneficial whenever it increases the number and/or quality of offspring for choosy individuals as compared to individuals that mate randomly (Johansson & Jones 2007). The benefits of mate choice are direct whenever choosy individuals acquire more resources that enhance their survival or reproductive success than non-selective ones. These resources include territories, food or protection (Hunt & Sakaluk 2014; Brooks & Griffith 2010; Davies et al. 2012). For instance, in hanging flies *Hylobittacus apicalis*, females mate with males that provide them with large insects, which they will eat while mating. This has direct implications for the reproductive success of the male, because the larger the insect, the longer he may copulate with the female and the more sperm he is able to transfer (Thornhill 1976). In some species, males supply females with more than one direct benefit. For instance, in flycatchers, males provide nest site, feeding territory, protection against harassment by other males, nest defence and parental care (Brooks & Griffith 2010).

Choosy individuals do not always acquire material benefits. Indeed, they can distinguish among prospective mates on the basis of some phenotypic trait to improve the fitness of their offspring (Hunt & Sakaluk 2014). In this case, the benefits are indirect. Indirect, or genetic benefits, occur when certain genes (intrinsic male quality) or gene combinations (genetic compatibility) produce fitter offspring than others (Kvarnemo & Simmons 2013). In the first, discrimination is done based on the ability to produce sexier or better quality offspring. In this case, a certain genome always produces offspring of the same quality (Shuker 2014) In the second, genetic benefits arise as a consequence of an interaction between a given maternal and a given paternal genome and the quality of the offspring will depend on this interaction (genetic incompatibility hypothesis; (Zeh & Zeh 1997; Zeh & Zeh 1996; Tregenza & Wedell 2000; Wedell 2013). Genetic incompatibility can arise due to male-female relatedness (Tregenza & Wedell 2002) and selfish genetic elements, such as cellular endosymbionts, as we will see in section 2 (Zeh & Zeh 1996; Wedell 2013).

1.3.2 Costs and benefits of mating

Once individuals have been chosen, mating will take place. The act of mating encompasses several costs for both males and females as well (reviewed in Göran Arnqvist and Rowe 2005; Arnqvist and Nilsson 2000), the most common being energy expenditure, increased risk of predation due to decreased motility or greater visibility and loss of other mating opportunities. Besides the costs of mating themselves, male adaptations to sperm competition can also be costly for females. Females risk suffering from sexual harassment (McLain & Pratt 1999; Rossi et al. 2010), injury caused by male genitalia (Blanckenhorn et al. 2002; Rönn et al. 2007) and the effect of toxic ejaculate substances. For example, the seminal products transferred with sperm can have dramatic effects on female reproductive physiology, reducing its receptivity and longevity (Arnqvist & Rowe 2005). This was demonstrated in *Drosophila melanogaster*, in which an increasing exposure to products in the seminal fluid led to increased death rate in females (Chapman et al. 1995).

The differences in costs and benefits obtained with each mating by males and females will give rise to sexual conflicts (Arnqvist & Rowe 2005). Note that in sexual diploid species it is always beneficial for both males and females to mate at least once, as mating is necessary to produce offspring (Arnqvist & Rowe 2005). In this case, we do not expect sexual conflicts to arise over the first mating of an individual. However, this is not the case in haplodiploid species, as we will see in section 1.5.

Sexual conflicts occur whenever the fitness optimum of the sexes differs (Arnqvist & Rowe 2005). There are numerous examples that confirm the existence of sexual conflicts over mating rate and describe the reproductive tactics employed either to persuade or to resist matings (for review see Göran Arnqvist and Rowe 2005). For instance, in the water strider *Rheumatobates rileyi*, males have grasping antennal claspers to grab females during copulation (Figure 7). In response, females possess modified genital segments that appear to reduce the efficiency of male grasping devices (Arnqvist & Rowe 2005). However, rejecting male attempts can become too costly for females (Arnqvist & Rowe 2005). In these situations, females can mate at a rate higher than their optimum to avoid the costs of rejecting unnecessary mates, a phenomenon called convenience polyandry (Thornhill & Alcock 1983; Snook 2014). Note however, that females can harm males too during copulation, the most extreme cases being when males are eaten by females, as seen in several species of spiders (Arnqvist & Rowe 2005). Even though females are frequently the sex with the lowest optimum mating rate, multiple mating does not have to be always costly for females.

Polyandry is easily explained when females gain direct benefits by mating multiply. As in pre-copulatory mate choice, multiple mating is considered a direct benefit when it increases females reproductive output (Danielsson 1998). Multiply-mated females can directly benefit from nutrients in the ejaculate and nuptial gifts, which can increase egg production in females and/or their longevity. Arnqvist and Nilsson (2000) have found compelling evidence that direct benefits can explain polyandry in several species, especially when nuptial gifts are involved. In addition, females can also benefit with increased parental care and guarding services for herself and her offspring. Finally, polyandry can prevent against infertility when infertile or sperm depleted males are frequent in the populations (Snook 2014). In fact, the proportion of infertile matings in insects is quite high, varying between 0% and 63%, with a median of 22%, in a study with 30 insect species (Garcia-Gonzalez 2004). Ironically, polyandry can be the solution of sperm depletion but also the cause, when males mate with several females.

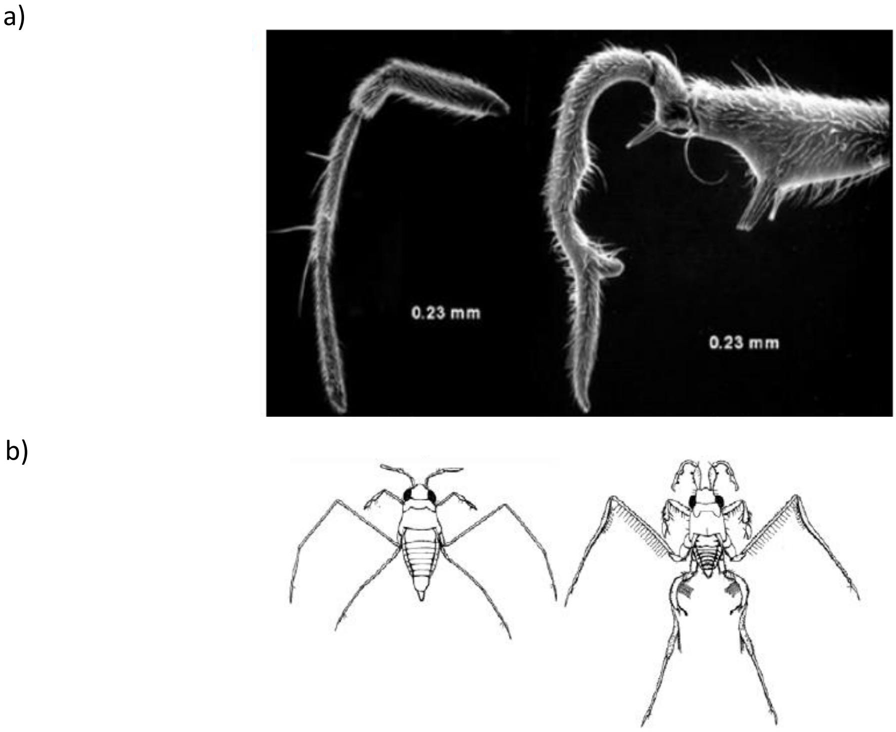


*Figure 7. **a) Male and female evolved structures in response to sexual conflicts.** Scanning electron microphotograph of the antennae of female (left) and male (right)* Rheumatobates rileyi*. The male uses his modified large antennae to grasp the female near the eye during the mating struggle in an attempt to subdue her (Image: Lock Rowe, university of Toronto) b) Female (left) and male (right)* Rheumatobates rileyi*. Adapted from Westlake, Rowe, and Currie 2000.*

Moreover, females might gain from polyandry indirectly, through increased performance of their offspring. As seen before, pre-copulatory mate choice can target genes that increase offspring performance. However, when gene quality cannot be assessed prior to mating, females may mate multiply to increase their chances of finding good quality sires. When there is variation in the genetic quality of males, multiple mating enables the female to exercise mate cryptic choice, thus improving offspring quality (the “good genes” hypothesis; reviewed in Andersson 1994; Davies, Krebs, and West 2012; Snook 2014). The same logic is applied when there is variation in insemination success. By mating repeatedly, the female is ensuring that the most competitive sperm will be the one fertilizing her eggs, thereby increasing the probability that her sons will also have competitive sperm ("sexy sperm" hypothesis; Parker 1970; Snook 2014). Moreover, polyandry can allow for increased genetic diversity of offspring which can be advantageous in unpredictable environments (Danielsson 1998). For instance, in the bumble bee *Bombus terrestris*, polyandry can increase colony resistance to parasites, thereby maximizing the chances that at least some individuals survive (Schmid-Hempel & Baer 1999). Finally, genetic benefits can be obtained through polyandry if females remate in order to avoid incompatible alleles (genetic incompatibility hypothesis; (Zeh & Zeh 1996; Zeh & Zeh 1997; Tregenza & Wedell 2000). By mating multiply, females will reduce the risk of mating with incompatible males only. In this case, polyandry is expected to increase the number of offspring, rather than improve offspring quality.

1.4 How mating systems and sperm priority patterns affect costs, benefits and conflicts over mating

Mating systems have long been recognized to influence the evolution of different mating strategies. For instance, in species where females are continuously receptive, monopolization is often impossible and pre-copulatory competition is, consequently, weak. However, competition among males can be extreme when females mate only once. For example, this may select for protandry - when males emerge or reach sexual maturity sooner than females- leading to increased monopolization of females (Kvarnemo and Simmons 2013, Simmons 2001). This phenomenon is observed in solitary bees, where males become adults several days before females and compete intensively for access to females as soon as they emerge, with larger males winning more matings (Paxton 2005).

Hosken et al. (2009) have reasoned that in monandrous species, where future matings do not exist, there may be conflicts of interest between males and females prior to mating, for instance over finding the best partner, but once mating has occurred the conflict between males and females disappears. The same rationale could be applied for mate competition or choice. Consequently, in monandrous species, we do not expect selection for post-copulatory strategies in either sex. Indeed, polyandrous species often present more complex male genitalia than monandrous species, suggesting that intra-sexual competition is more intense in the former (Hosken & Ward 2001; Crudgington et al. 2009; Simmons & García-González 2008). However, this is only true if there is no potential for future matings, e.i. if monogamy is not imposed by one sex on the other. Otherwise monogamy is actually a consequence of sexual conflict (Hosken et al. 2009). In fact, monandry can be imposed by males upon females via defensive strategies against sperm competition. In the house fly *Musca domestica*, for example, seminal fluids transferred with the sperm are responsible for permanently switching off the receptivity of females (Leopold et al. 1971).

In polyandrous species, sexual conflicts and competition among mates continues after mating. Insemination success should depend on the outcome of the male strategies summarized above, such as the ability to reach and fertilize eggs and to displace other sperm (Kvarnemo & Simmons 2013), and on cryptic choice. The combination of these strategies will produce a pattern of sperm priority (or sperm precedence). Sperm priority patterns are generally classified as the species mean value of P2 – the proportion of offspring sired by the second of two males (Simmons 2001). Among insect species, around 45% show mixed sperm utilization, 45% last male precedence and 10% first male sperm precedence (Simmons 2001). Note, however, that first male sperm precedence seems to be more frequent in arachnids, namely in spiders, than in insects (Austad 1984; Wedell et al. 2002).

Different patterns of sperm precedence reflect differences in mating opportunities. Accordingly, the patterns of sperm priority should influence mate choice. For instance, males are expected to adjust ejaculate investment differently, depending on the sperm precedence pattern (Engqvist & Reinhold 2006; Wedell et al. 2002). If the first male that mates with a female sires most of her offspring (first-male sperm precedence), males are expected to transfer more sperm when mating with virgins than with mated females, as mated females provide lower fertilization opportunities (Bonduriansky 2001). Indeed, in several species with first male sperm precedence, like the spiny orb weaver *Micrathena gracilis*, males provide fewer sperm to mated females than to virgins (Wedell et al. 2002; Bukowski & Christenson 1997; Yasui 1996). Alternately, males can choose unmated females prior to mating, as shown in the bushcricket *Requena verticalis.* In this species, males have been shown to prefer mating with younger females that are more likely to be virgin (Simmons et al. 1994). The opposite behaviour is expected in species with last male sperm precedence, where mating with mated females is expected to result in a higher proportion of offspring being fertilized.

Several hypotheses have been put forward to explain the variation in sperm priority patterns among species. In a comparative study with 57 insects, Ridley (1989) found support for the influence of mating frequency. In his hypothesis, he reasoned that in species with last male sperm precedence selection for increased mating rate should occur whereas if the first male was the most successful, then the species should evolve towards monandry. The evolutionary expectations for first male sperm precedence should thus be similar to the ones outlined for monandry. Indeed, strong selection pressure for the evolution of pre-copulatory strategies, such as protandry and pre-copulatory mate guarding, is found in species where the first male sires most of the offspring (Simmons 2001).

Besides variance in sperm priority observed among species, within species variance can be quite large as well (Simmons 2001). This variation can be due to differences in male competitive ability and in cryptic choice (Danielsson 1998). For instance, variation in sperm priority has been found to correlate with copulation duration, male size, sperm size, and seminal proteins. In addition, other factors such as the number of rematings, the interval between matings and sperm depletion may influence sperm priority (Amitin et al. 2014; Danielsson 1998; Zeh & Zeh 1994; Simmons 2001). For example, in the wasp *Dahlbominus fuscipennis*, when two successive matings occur within a few hours, there is sperm mixing. However, when the period between matings is longer, the sperm from the first male fertilizes the majority of the eggs (Wilkes 1966).

1.5 Haplodiploidy and sexual conflicts over mating rate

Unlike what happens in diploids, females in haplodiploid species can produce unfertilized offspring. Consequently, the conflict over mating can concern the act of mating itself, as females can potentially reproduce without paying the costs of mating. For example, in female-biased populations, producing sons is more advantageous than producing daughters. In haplodiploids this means that remaining a virgin in these conditions is beneficial (Charlat et al. 2003; Godfray 1990). This particularity of haplodiploids can be particularly useful in empirical studies, because it allows disentangling the cost of reproduction from the cost of mating.

2. Host-symbiont conflicts over mating strategies

Endosymbiotic bacteria are considered selfish genetic elements (SGE) because they employ various tactics, like altering their hosts behaviour or physiology, in order to favour their own transmission (reviewed in Wedell 2013; Price and Wedell 2008; Goodacre et al. 2006; Goodacre and Martin 2012). In the next section (2.1) I will review some of these tactics. As these bacteria are mainly transmitted through the female germ line, the tactics they employ to ensure their transmission may affect male and female mating strategies. Moreover, given the significant effects of these bacteria on the fitness of their hosts, hosts have evolved strategies to avoid or reduce infection costs, as we will see in section 2.2. In addition, the mode of reproduction of the host can affect the host-endosymbiont interaction. In section 2.3, I will discuss the impact of haplodiploidy in cytoplasmic incompatibility, one of the reproductive phenotypes induced by *Wolbachia*. Finally, I will highlight some of the evolutionary implications of the interaction between hosts and endosymbionts (Zuk and Wedell 2014, section 2.4).

2.1 Effects of endosymbionts on host biology

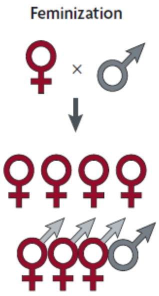
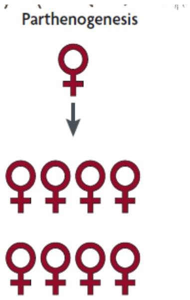
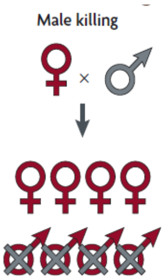
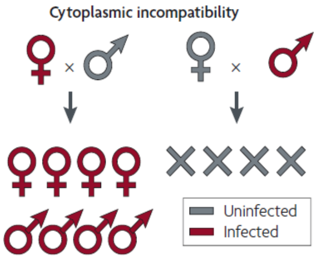
Endosymbiotic bacteria that act as SGEs are quite common in arthropods. Indeed, several intracellular bacterial lineages infect arthropods, like Ricketsiales, which include Wolbachia and Rickettsia, Bacteroidetes such as Cardinium, and Mollicutes such as Spiroplasma and Arsenophonus (Goodacre & Martin 2012). The most well studied bacterium in this context, Wolbachia, has been found in more than 65% species of insect (Werren et al. 2008).

Endosymbionts can promote their transmission through a variety of mechanisms. A summary of the four classical bacterial-induced phenotypes can be found in Table 1. These strategies include feminization, parthenogenesis induction, male killing and cytoplasmic incompatibility (CI) (Werren et al. 2008). Here, I will focus on cytoplasmic incompatibility (CI, henceforth), the most frequently found phenotype and the one present in *Tetranychus urticae*. I will also focus my attention on *Wolbachia*, the symbiont studied in my work.

Besides these phenotypes, symbionts have been shown to induce a wide range of additional effects on host physiology and behaviour (Goodacre & Martin 2012), the most common being directly related to reproductive processes, as SGE frequently target sex cells (Zuk & Wedell 2014). Symbionts manipulation can be achieved, for instance, by modifying sperm during development. Such modification can affect traits like male fertility or its competitive ability (Price & Wedell 2008). For example, in the sperm heteromorphic Mediterranean flour moth, *Ephestia kuehniella*, infected males transfer fewer fertile sperm than uninfected males (Lewis et al. 2011). Likewise, *Drosophila simulans* males infected with *Wolbachia*, have a decrease in fertility of 94% compared to uninfected males, due to a decrease in sperm production (Snook et al. 2000). In contrast, in *Tribolium confusum*, the fertility of infected males is higher than that of uninfected males (Wade & Chang 1995). However, infected females of this species produce fewer offspring than uninfected ones (Wade & Chang 1995), suggesting endosymbionts can also affect female physiology.

The effects of symbionts on the physiology and behaviour of the host can also be extended to traits unrelated to reproduction. For instance, symbionts can alter host susceptibility to viruses and fungi, change their susceptibility to pesticides or increase their survival (reviewed in (Haine 2008; Brownlie & Johnson 2009; Goodacre & Martin 2012). In *Drosophila neotestacea,* for example, *Spiroplasma* protects against the sterilizing effects of a parasitic nematode (Jaenike et al. 2010). Non-reproductive behaviours include changes in dispersal (Alexandrov et al. 2007) and larval competitive ability (Goodacre et al. 2009).

Table 1 Symbiont-induced phenotypes in arthropods. Adapted from Werren et al. 2008 and Engelstädter and Hurst 2009.

	**Feminization** is the process through which genetic males develop as females. Symbiont presence results in the hypertrophy of the androgenic gland of males inhibiting its function. Feminization has been described in the Hemiptera, Isopoda, Crustacea, Acari and Lepidoptera orders and can be induced by *Wolbachia* and *Cardinium*.
	**Parthenogenesis induction** has only been described in species with arrhenotokous development (haploid males result for unfertilized haploid eggs and diploid females stem from fertilized diploid eggs). Infected females produce daughters from unfertilized eggs which, unlike males, are able to transmit the symbiont to the following generations. Parthenogenesis is found in the Acari, Hymenoptera and Thysanoptera orders, induced by *Cardinium*, *Wolbachia* or *Rickettsia*.
	**Male killing** eliminates infected males mainly during embryogenesis, to the advantage of surviving infected female sibling. Male killing has been found in the Coleoptera, Diptera, Lepidoptera, Pseudoscorpiones, Hemiptera and Hymenoptera orders. *Wolbachia*, *Rickettsia*, *Spiroplasma* and *Arsenophonus* habe been found to induce this phenotype.
	**Cytoplasmic incompatibility** (CI) results in unsuccessful crosses between infected males and uninfected females or females that lack the same symbiont types. CI speeds the spread of the symbiont in host populations but it diminishes the reproductive success of these female hosts. CI is induced by *Wolbachia* and *Cardinium* and was described in the Acari, Coleoptera, Diptera, Hemiptera, Hymenoptera, Isopoda, Lepidoptera and Orthoptera orders. This phenomenon is detailed in section 2.1.1.

2.1.1 Cytoplasmic incompatibility

CI occurs when sperm from infected males is not compatible with the ovocyte of females, resulting in the disruption of embryonic development. In this process, *Wolbachia* acts in two ways: it induces the modification of sperm during spermatogenesis and it rescues embryos infected with the same *Wolbachia* strain, a process called “modification-rescue”. The mechanism of CI is currently unknown, although several propositions have been put forward (Poinsot et al. 2003; Lepage et al. 2017; Beckmann et al. 2017). Nevertheless, cytological work revealed that the incompatibility is due to an asynchrony of the male and female pro-nuclei in the beginning of mitosis. During metaphase, female’s chromatids become properly condensed while male’s chromosomes remain in a semi-condensed state, which hampers a proper segregation of paternal chromosomes during anaphase (Werren, Baldo, and Clark 2008; Tram and Sullivan 2002; Figure 8). The outcome of CI is frequently the haploid development of the egg. In diploid species, this normally results in embryonic mortality but in haplodiploids it can result in normal male development (section 2.3).

There are two types of CI, unidirectional and bidirectional (Werren et al. 2008). Unidirectional CI occurs in crosses between *Wolbachia*-uninfected females and *Wolbachia*-infected males. The opposite cross, between *Wolbachia*-infected females and *Wolbachia*-uninfected males, is compatible. Bidirectional CI arises when two different *Wolbachia* strains have different modification-rescue mechanisms, in which case, crosses are incompatible in both directions.

Furthermore, CI can be incomplete, whenever some fertilized offspring is produced from incompatible matings, and the level of CI induction varies from 0 to 99%. In addition, maternal transmission fidelity is not always complete, in which case a small portion of the offspring of infected females is uninfected. Note that the level and type of CI may not completely depend on the bacteria itself. For instance, the level of CI can be correlated with the host genotype, bacterial density in the testes, male’s age and mating history (Zhao et al. 2013; Goodacre & Martin 2012).

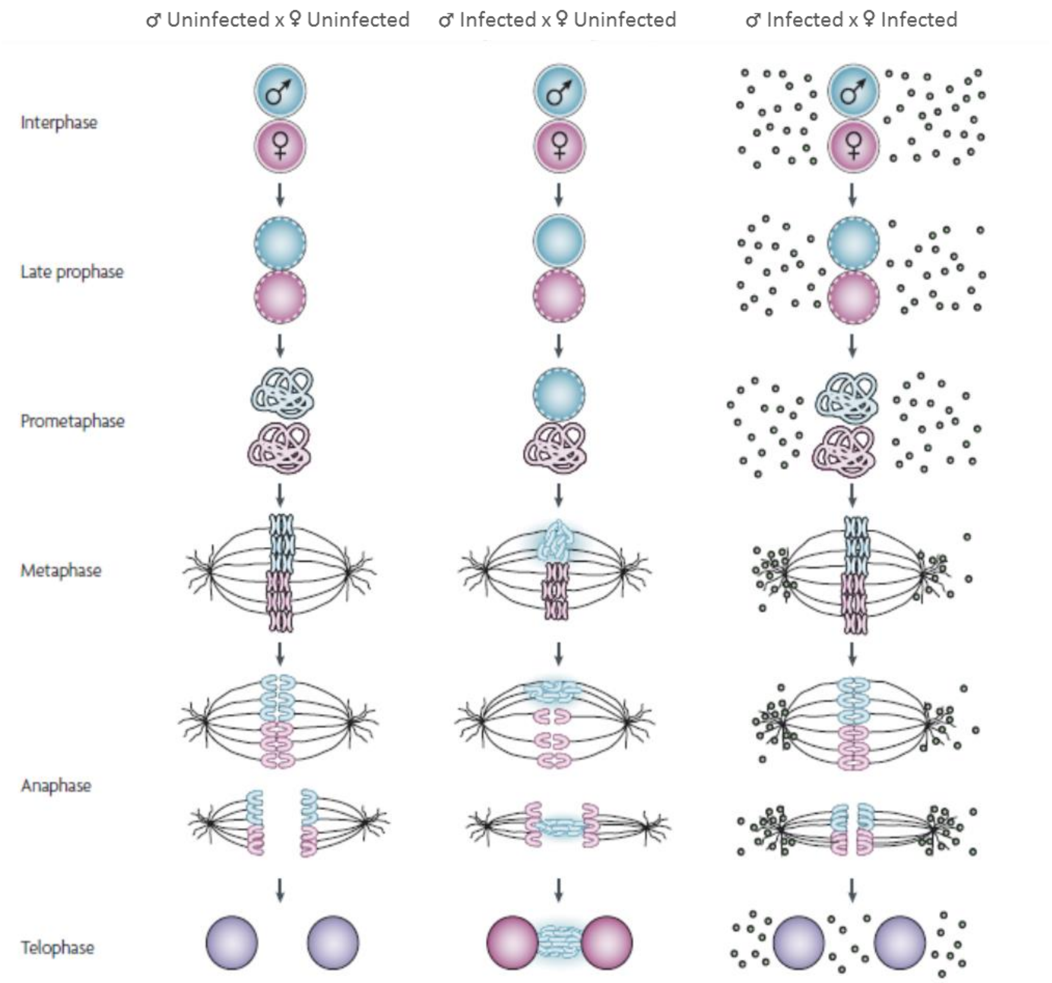


*Figure 8. **Cytological basis of symbiont-induced cytoplasmic incompatibility.** During metaphase, the chromatids of the female become properly condensed while male chromosomes remain in a semi-condensed state, which hampers a proper segregation of paternal chromosomes during anaphase. Adapted from Werren, Baldo, and Clark 2008.*

2.2 Host responses to symbiont effects on reproduction

Phenotypes induced by symbionts, such as reduced fertility or CI, have serious negative consequences on the reproductive success of the hosts. Uninfected hosts are thus expected to evolve strategies to avoid infected individuals or reduce the effect of infection. This avoidance can be achieved via pre-copulatory mate choice or through multiple mating.

2.2.1 Mate choice as strategy against endosymbionts effects on host reproduction

An obvious prediction resulting from the negative effects of selfish genetic elements is that uninfected individuals should avoid mating with infected ones (Vala et al. 2004; Zuk & Wedell 2014; Tregenza &

Wedell 2000). Indeed, different theoretical models have shown that mate preference for uninfected males can evolve in populations with uni- or bidirectional CI-inducing *Wolbachia* (Telschow et al. 2005; Telschow et al. 2007). Similarly, male preference for uninfected females is predicted to evolve in populations harbouring male-killing *Wolbachia* (Randerson et al. 2000). However, empirical studies provide evidence both in favour (Moreau et al. 2001; Vala et al. 2004; Sullivan & Jaenike 2006; Koukou et al. 2006) and against (Hoffmann et al. 1990; Wade & Chang 1995; Jiggins et al. 2002; Sullivan & Jaenike 2006; Champion de Crespigny et al. 2007; Duron et al. 2011; Arbuthnott et al. 2016) the existence of mate discrimination in species carrying endosymbionts. For instance, in the spider mite *T. urticae*, uninfected females prefer uninfected over males infected with CI-inducing *Wolbachia*, while infected females show no preference (Vala et al. 2004, Figure 13a). Likewise, in *D. melanogaster*, the removal of *Wolbachia* responsible for the induction of bi-directional CI, decreases the levels of mate discrimination between populations by around 50%, suggesting *Wolbachia* is responsible for the assortative mating observed (Koukou et al. 2006, Figure 9). In turn, in the African butterfly *Acrea encelon* and in *Drosophila innubila*, male-killing *Wolbachia* does not elicit preference for uninfected females (Jiggins et al. 2002; Sullivan & Jaenike 2006). Also, in *D. simulans* and *D. melanogaster* neither uninfected females nor CI-inducing *Wolbachia* infected males show preference for infected or uninfected mates (Champion de Crespigny et al. 2007).

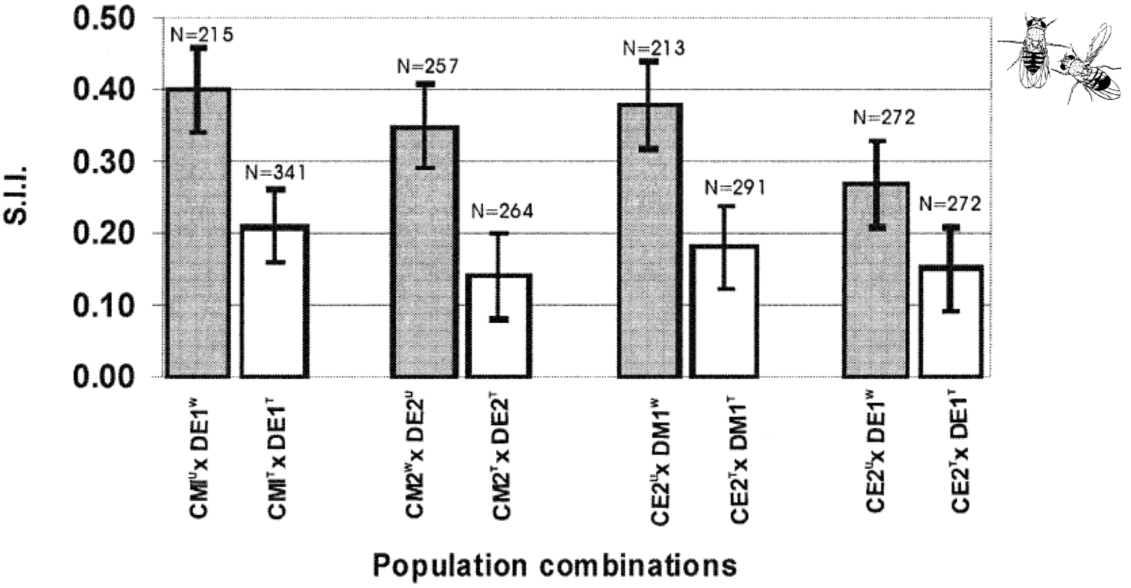


Figure 9. Effect of Wolbachia *on the reproductive isolation between population of Drosophila melanogaster. Treated populations show a significant reduction in the level of assortative mating compared to untreated populations. Dark blocks show original untreated infected populations and white blocks the treated uninfected duplicated populations. Population identity: CM1, CM2, DE1, DE2. W superscript:* Wolbachia*-infected individuals; U superscript:* Wolbachia*-uninfected individuals; T superscript: tetracycline treated individuals;* N: *total number of matings observed. Adapted from Koukou et al. 2006 Drawing from Jeffrey Hall, Brandeis University.*

The different results obtained across studies suggest that pre-copulatory mate preference is by no means universal, even if the effect on the host reproductive success is high. Most likely, the evolution of this behaviour depends on different factors, including the interaction between host and symbiont genomes. Indeed, different species of *Nasonia* exhibited different patterns of mate discrimination, being the ability to discriminate highly influenced, among other factors, by the natural distribution of the populations of each species (Buellesbach et al. 2014). In addition, another study in *D. melanogaster* has shown that assortative mating depends on the infection status as well as on the genotype of the host (Markov et al. 2009).

Another way by which the host might exert mate preference is through strategic sperm expenditure (Price & Wedell 2008). This was indeed found in some systems. For instance, in the terrestrial pill-bug *Armadillidium vulgare*, males become sperm depleted when they mate multiple times. However, sperm depletion affects the fertility of females differently according to their infection status. Indeed, males transfer fewer sperm to females infected with feminizing *Wolbachia*, this way only reducing the fertility of these type of females (Rigaud & Moreau 2004). This conditional decrease of fertility should reduce the spread of *Wolbachia* in host populations (Rigaud & Moreau 2004).

2.2.2 Polyandry as strategy against endosymbionts effects on host reproduction

As stated in section 1.3, polyandry can be beneficial against genetic incompatibilities resulting from crosses with endosymbiont-infected individuals (Zeh & Zeh 1997; Wedell 2013; Zeh & Zeh 1996). By mating multiply, individuals improve the probability of mating with a compatible mate. This can occur through two different non-exclusive mechanisms. On the one hand, females might evolve the ability to bias paternity in favour of sperm from uninfected males. In this case, polyandry allows for cryptic female choice depending on the infection status of the male sperm. On the other hand, mating multiple times may promote sperm competition, without the need for female cryptic choice (Price & Wedell 2008).

The success of both mechanisms might be contingent on the existence of costs that affect the mating success of infected males. Indeed, decreased sperm competitive ability of infected males is very frequent in species carrying SGE's (Price & Wedell 2008). For example, in *D. simulans* multiple mating reduces significantly the costs of CI-inducing *Wolbachia*, owing to the decreased competitive ability of infected males (Champion de Crespigny & Wedell 2006; Champion de Crespigny, Hurst, and Wedell 2007, Figure 10). This competitive disadvantage is thought to occur due to a decrease in the fertility of *D. simulans* infected males (Snook et al. 2000).

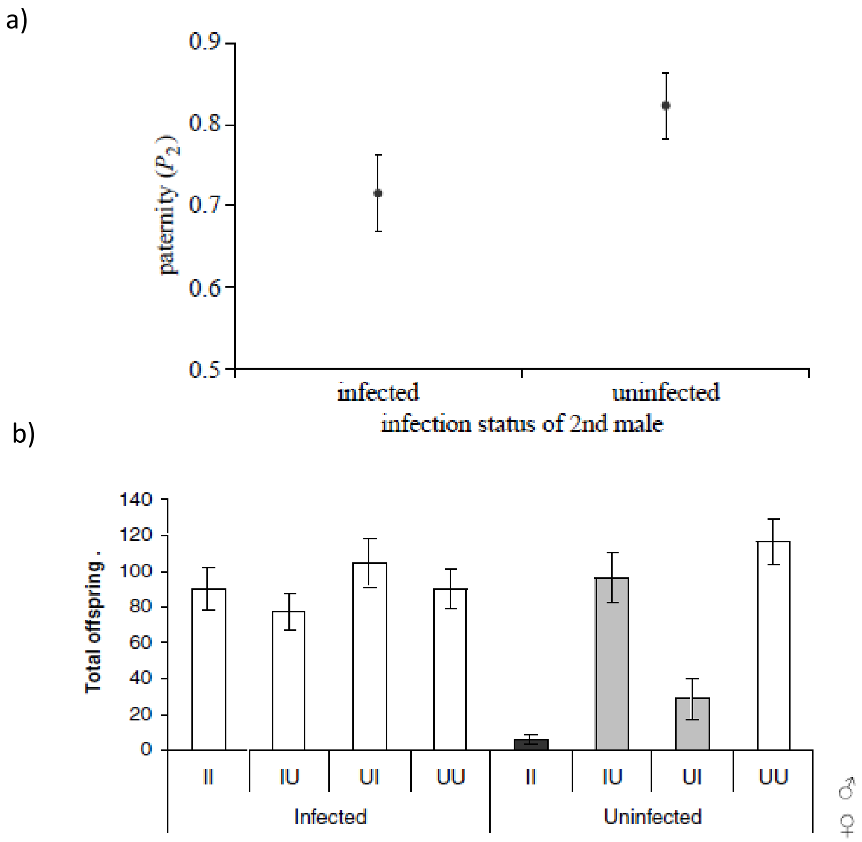


Figure 10. Paternity and offspring proportion in Drosophila simulans. *a) Mean paternity achieved by infected and uninfected males in crosses with infected females. Paternity is presented as P2, the proportion of offspring sired by the second male to mate. Infected males exhibit a lower P2 than uninfected males, suggesting a decrease in sperm competitive ability. Adapted from Champion de Crespigny & Wedell 2006 b) Mean offspring production of polyandrous females. Dark gray bars indicate crosses where all males are incompatible with females. Light gray bars indicate crosses where one of two males is incompatible. White bars indicate entirely compatible crosses. Female and male infection status are described in the x-axes. In male infection status, the first letter indicates the infection status of the first male and the second letter the infection status of the second male. I - infected male, U - uninfected male. Adapted from Champion de Crespigny et al. 2007*

There are two theoretical predictions concerning polyandry and reproductive manipulators (Zuk & Wedell 2014): First, endosymbionts may promote the spread of polyandry in the host. Second, polyandry may inhibit or prevent the spread of reproductive manipulators within populations. A model built upon the results obtained in *Drosophila simulans* tested both predictions (Champion de Crespigny et al. 2007). On the one hand, they found that polyandry could inhibit or prevent the spread of *Wolbachia* in this species. On the other hand, they could not find support for the hypothesis that *Wolbachia*-induced CI promotes the evolution of polyandry. This was due mainly to the low probability of double matings with infected and uninfected males with increased infection prevalence and owing to costs associated with multiple mating (Champion de Crespigny et al. 2007). Nonetheless, there are several lines of evidence supporting the hypothesis that the phenotypic effects of selfish genetic elements other than symbionts can promote polyandry (Zuk & Wedell 2014). For example, *D. pseudoobscura* uninfected females evolved increased mating rates when exposed for 10 generations to the risk of mating with males carrying a deleterious sex-ratio distorter (Price et al. 2008, Figure 11). In this case, mating rate was seen as a measure of the level of polyandry in the population, leading the authors to conclude that the risk of incompatible matings drove the evolution of polyandry.

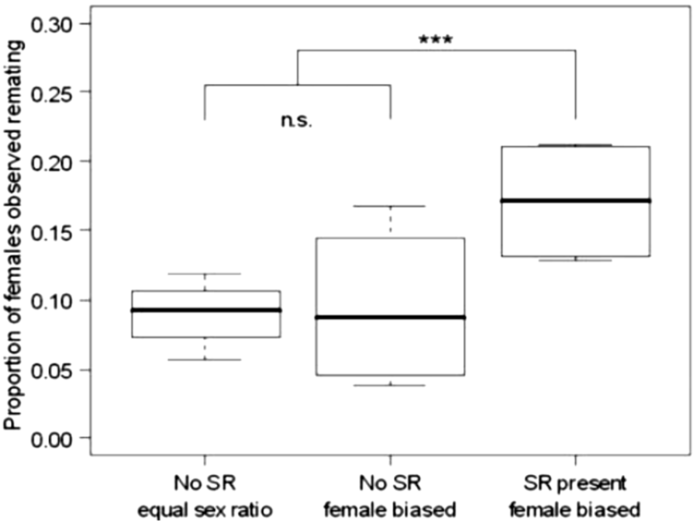


Figure 11. ***Proportion of*** D. pseudoobscura ***females observed re-mating after 10 generations of experimental evolution, for each selection regime.*** *Uninfected females exposed to the risk of mating with males carrying a deleterious sex-ratio distorter evolved increased mating rates. No SR equal sex ratio: lines lacking a sex-ratio distorter maintained at a 1:1 female-to-male sex-ratio. No SR female biased: lines lacking a sex-ratio distorter maintained at a 2:1 female-to-male sex-ratio. SR present female biased: lines carrying a sex-ratio distorter (sex-ratio) at an initial frequency of 30% maintained at a 2:1 female-to-male sex-ratio. *** P<0.001, n.s. - not significant. Adapted from Price et al. 2008*

The presence of endosymbionts is also associated with changes in male mating rate. For instance, *D. simulans* and *D. melanogaster Wolbachia*-infected males show higher mating rates than uninfected males (Champion de Crespigny et al. 2006). This behaviour can be *Wolbachia*-induced, as increased mating frequency will speed the endosymbiont spread in the population. Alternately, it can also be an adaptation of the host itself. In fact, matings with infected *D. simulans* males that have previously mated multiple times lead to reduced levels of CI, compared to matings with infected males that have mated only once (Karr et al. 1998). Increased mating rate could thus be a strategy of infected males to restore reproductive compatibility with uninfected females.

2.3 The consequences of haplodiploidy in host-symbiont conflicts

The mode of reproduction of the host can influence the interaction between the host and the symbiont. Here I focus on the effects of haplodiploidy in the interaction between hosts and CI-inducing *Wolbachia*.

In diploid species, CI results in an increase in F1 mortality. However, in haplodiploids, incompatible matings due to CI result in male-biased sex ratio. This occurs because in haplodiploids males come from unfertilized eggs and, consequently, they are not affected by CI. In haplodiploids there are two possible outcomes for fertilized eggs, resulting in two different modes of CI expression. In some species, like *Nasonia vitripennis*, CI causes complete loss of the paternal chromosomes in fertilized eggs, resulting in complete haploidization of the egg, thus increasing male proportion in the F1 at the expenses of the proportion of females (Breeuwer & Werren 1990). In other species, like the parasitoid wasp *Leptopilina heterotoma,* CI leads to the mortality of fertilized eggs, which decreases the number of females without affecting the number of males (Vavre et al. 2000). Note, however, that the mode of CI is not fixed within species. For instance, in *T. urticae*, both modes of CI have been documented (Breeuwer 1997; Perrot-Minnot et al. 2002). Regardless of the mode of CI, as male production is not affected in incompatible crosses, the conflict between haplodiploid host and *Wolbachia* is expected to be weaker than in diploid species.

Haplodiploidy can also impact on the predictions concerning the threshold of *Wolbachia* invasion, i.e., the frequency below which the infection will disappear. Vavre et al. (2000) found that haplodiploidy should increase the threshold of infection, even more so when fertilized eggs originate males. This is because an uninfected male-biased sex-ratio decreases the proportion of infected males in the population, thereby reducing the probability of incompatible matings.

Furthermore, an extra level of conflict should arise between *Wolbachia* and a haplodiploid host. In fact, females and *Wolbachia* do not have the same optimum sex-ratio. On the one hand, *Wolbachia* is expected to increase its transmission with increased number of infected females in the population. On the other hand, the female host benefits with the production of sons, as soon as the population sex-ratio becomes female-biased, and provided it is not under local mate competition. The existence of this conflict has been elegantly demonstrated in *T. urticae*. Vala et al. (2003) found that *Wolbachia*-infected females produced a normal sex-ratio (around 30% males), while cured females produced a male-biased sex-ratio. However, 42 generations after curing, the sex-ratio of cured females changed back, approaching the sex-ratio produced by infected females. This suggests that the host evolved a compensatory mechanism that allowed infected females to produce the sex-ratio favoured by nuclear genes (Vala et al. 2003), this way solving the conflict in favour of the host. A more extreme example of host compensatory mechanism was found in *Telenomus nawai*. In populations infected with parthenogenesis-inducing bacteria, the benefit of producing sons for the haplodiploid host appears to have driven "functional virginity" to fixation, thereby eliminating sexual reproduction (Huigens & Stouthamer 2003; Jeong & Stouthamer 2005).

2.4 Evolutionary implications of endosymbiotic-driven changes in host behaviour

Infection can cause a restriction of gene flow within populations, for instance when it leads to incompatible crosses between uninfected females and infected males. Even if spatially or temporal restricted, such a restriction can potentially help the process of reproductive isolation and thus influence speciation (Engelstädter & Hurst 2009; Brucker & Bordenstein 2012; Shropshire et al. 2016; Hurst & Werren 2001). Furthermore, the existence of CI-induced costs can select for pre-mating isolation in the host, i.e reinforcement, strengthening reproductive isolation (Shropshire et al. 2016; Brucker & Bordenstein 2012). There are several theoretical models that studied the effect of CI-inducing *Wolbachia* in speciation (Telschow et al. 2005; Telschow et al. 2007). In addition, empirical support for the existence of reinforcement was found in two sympatric species of *Drosophila*, *D. recens* fully infected by *Wolbachia*, and *D. recens* fully uninfected (Jaenike et al. 2006). Matings between uninfected *D. subquinaria* females and *D. recens* infected males result in a decrease of offspring viability due to interspecific CI. The opposite cross, between uninfected *D. recens* females and *D. subquinaria* infected males, on the contrary, is completely viable. In accordance to this, *D. subquinaria* females from the zone of sympatry discriminate against infected *D. recens* males, whereas females of the same species from allopatric populations do not. Furthermore*, D. recens* do no exhibit discrimination. This suggests that mate discrimination in *D. subquinaria* against *D. recens* in sympatry is promoted by selection against *Wolbachia* effects (Jaenike et al. 2006). Besides this study, behavioural isolation promoted by the presence of Ci-inducing *Wolbachia* has been found, for instance, in populations of *D. melanogaster* that had been selected for 30 years for tolerance to toxins in food (Koukou et al. 2006) (Figure 9) and in the *D. paulistorum* complex of sympatric semi-species (Miller et al. 2010). In the first, the curing of *Wolbachia* using tetracycline leads to a 50% decrease in mate discrimination between populations, suggesting *Wolbachia* was responsible for the assortative mating observed (Koukou et al. 2006). In the second, *Drosophila paulistorum* individuals infected with obligate mutualistic *Wolbachia* preferentially mate with individuals of their semi-species, which harbour the same type of *Wolbachia*. This ability is lost after mild rifampicin treatment, which leads to lower *Wolbachia* density (Miller et al. 2010).

Even when *Wolbachia*-induced costs do not lead to speciation, changes in the behaviour of the host, such as changes in dispersal or mating preference, are expected to influence the host genetic diversity. For instance, changes in the number of reproducing individuals, due to CI or male-killing, will reduce the effective population size (Engelstädter 2010), which will render populations more sensitive to the effect of drift. Decreased genetic variability will ultimately reduce the ability of populations to adapt and persist (Goodacre & Martin 2012). In addition, both reduced effective size and altered host behaviour are expected to affect the strength and shape of sexual selection and sexual conflicts (Martin & Hosken 2003; Hayashi et al. 2007). For instance, Martin and Hosken (2003) evolved for 44 generations populations of *Sepsis cynipsea* at different densities. They found that large populations have stronger sexual conflict than small populations. In addition, larger populations diverged to a greater extent that smaller populations, probably owing to the increased sexual conflicts with density. Endosymbiont-induced changes can also, on the contrary, favour population expansion, for instance by increasing females mating rate or by inducing parthenogenesis (Goodacre & Martin 2012).

3. Biology of the two-spotted spider mite *Tetranychus urticae*

In this section I will provide a brief description of the model species used in all experiments, the two-spotted spider mite *Tetranychus urticae* (section 3.1). I will then focus on its reproduction (section 3.2) and what is known about *Wolbachia* infection in this species (section 3.3).

3.1 General description

The two-spotted spider mite *Tretanychus urticae* is a polyphagous mite that occurs on hundreds of different plants around the world, including crop plants of high economic importance such as tomato, bean and rose. Because it inflicts harm to their host plants, many of which of economic value, is it considered an important agriculture pest (Helle & Sabelis 1985).

Spider mites are characterized by an arrhenotokous genetic system, i.e., males are haploid, stemming from unfertilized eggs, whereas females are diploid, resulting from fertilized eggs (Helle and Sabelis 1985, Figure 12). Consequently, unfertilized females produce sons, whereas mated females produce sons and daughters. Haploid eggs are smaller than diploid eggs (Macke et al. 2011a) and males have approximately 15 to 30% of female’s body weight (Mitchell 1973). Typically, their adult sex ratio is female biased (around 70% females; Mitchell 1972) but it can change with several factors such as temperature, maternal age and different scales of mate competition (Roy et al. 2003; Macke et al. 2011b; Macke et al. 2012).

During their life cycle, individuals go from egg to adult, passing through a six-legged larva and two eight-legged nymph stages, called protonymph and deutonymph stages. Between each mobile stage, individuals undergo a quiescent period (Helle & Sabelis 1985) (Figure 12). Developmental time depends on factors such as temperature and humidity and is generally very fast (around 10 days at 25ºC). In addition, spider mites are protandrous, i.e., males reach sexual maturity before females (Helle & Sabelis 1985; Boudreaux 1963).

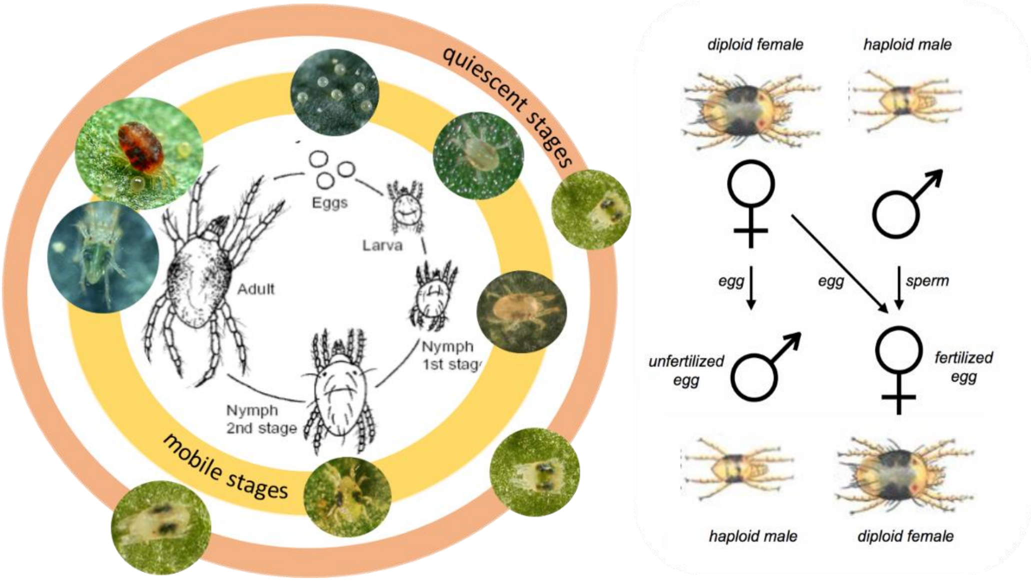


Figure 12. Life cycle and mode of reproduction of the spider mite Tetranhycus urticae. *Individuals go from egg to adult, passing through 3 juvenile stages. Between each mobile stage, individuals undergo a quiescent period. Spider mites are haplodiploids, i.e., males are haploid, stemming from unfertilized eggs, whereas females are diploid, resulting from fertilized eggs.*

Spider mites have poor eye sight and often use chemicals to perceive surrounding information, like the presence of predators (Grostal & Dicke 1999; Pallini et al. 1997) or of potential mates (Rodrigues et al. 2017; Penman & Cone 1974; Penman & Cone 1972; Royalty et al. 1992). In addition, all developmental stages, while walking, spin web that can be used in locomotion and dispersal, protection against predators and adverse conditions and facilitation of mating encounters (Gerson 1985; Clotuche et al. 2011). Individuals distribute in patches and reproduce locally (Mitchell 1973). Thus, at low density sib-mating is often the rule but with increased individual numbers, matings occur mainly between non-relatives. With population growth, the density of spider mites peaks and the leaf becomes overexploited. At this point, young mated females disperse to find a new host plant and establish a new colony. They achieve this by walking or by the wind attached to silk threads, a phenomenon called "ballooning" (Helle & Sabelis 1985).

3.2 Reproduction and mating behaviour in spider mites

In *T. urticae*, females can lay eggs up to 10 eggs a day for a period of 20-30 days (Wrensch & Young 1975). Fertilization occurs directly in the ovary, after sperm migration through the haemolymph towards the oocytes, suggesting that it occurs very early during egg development, just before the end of vitellogenesis (Pijnacker & Drenth-Diephuis 1973). Females produce all their fertilized eggs using the sperm of the first male they mate with, hence this species presents first male sperm precedence (in *T. urticae*, Helle 1967; in *T. kanzawai*, Oku 2008). Subsequent matings are ineffective, except if the first mating is incomplete, for example if it is interrupted (Satoh et al. 2001; Potter & Wrensch 1978). The mechanism underlying sperm priority remains unclear, although several propositions have been put forward (mating plug, Boudreaux 1963; threshold of sperm volume, Overmeer 1972).

Males form this species behave according to what is expected by theory for species with first male sperm precedence: Males actively guard quiescent, soon to be adult, females and mate with them as soon as they start emerging as adults (Boudreaux 1963; Potter et al. 1976). In addition, males prefer to mate with adult virgins over mated females (Oku 2010; Rodrigues et al. 2017), and they fight for their possession in male-biased conditions (Potter et al. 1976). Moreover, prolonged copulation has been documented in this species, so that sperm precedence is ensured (Satoh et al. 2001).

Surprisingly, copulations between mated females and males have been observed (Oku 2010; Rodrigues et al. 2017; Clemente et al. 2016). This behaviour is unexpected as males reproductive capacity is limited (Krainacker & Carey 1989) and females pay a cost of multiple matings, having lower fecundity and a smaller proportion of fertilized eggs, when compared with once-mated females (Macke et al. 2012). This puzzling mating behaviour has been the main subject of my thesis.

3.3 *Wolbachia* infection in spider mites

Spider mites are polymorphic for *Wolbachia* infection. Several studies have documented the distribution of *Wolbachia* and other symbionts in numerous species of spider mites (Enigl & Schausberger 2007; Gotoh et al. 2007; Breeuwer & Jacobs 1996; Gotoh et al. 2003). CI expression within *T. urticae* can range from none to complete CI and, as said before, it can result in the mortality or male conversion of fertilized eggs (Perrot-Minnot et al. 2002; Xie et al. 2011). Furthermore, considerable changes in *Wolbachia* density in males and females have been observed, depending on individuals age and mating history (Zhao et al. 2013). For instance, *Wolbachia* density in females increases with age, whereas in males it decreases (Zhao et al. 2013).

Besides CI, *Wolbachia* has been shown to lead to hybrid breakdown, with incompatible crosses resulting in a decrease in F2 viability (Vala et al. 2000). Furthermore, *Wolbachia* can influence *T. urticae* life-history traits differently across populations. For instance, in a study done by Xie, Chen, and Hong (2011), only one in three populations showed enhanced fecundity of infected females. In addition, both positive and negative effects of *Wolbachia* infection were found for survival and developmental time (Xie et al. 2011). These differences were found even though all populations had an identical *wsp* gene sequence, suggesting that there is an effect of the host genotype on CI expression.

Finally, only one study so far as explored the response of *T. urticae* to the costs induced by CI-inducing *Wolbachia*. In this study, uninfected females, cured by heat shock, prefer to mate with uninfected males, exhibiting assortative mating against males harbouring CI-inducing *Wolbachia* (Vala et al. 2004) (Figure 13a). In addition, both infected and uninfected females aggregate their eggs in the vicinity of eggs of the same infection status, thereby promoting sib-mating (Vala et al. 2004) (Figure 13b). These results suggest that spider mites can avoid the costs of CI. However, this was done using one and two isofemale lines, respectively for each test, possibly not being representative of the behaviour of this species.

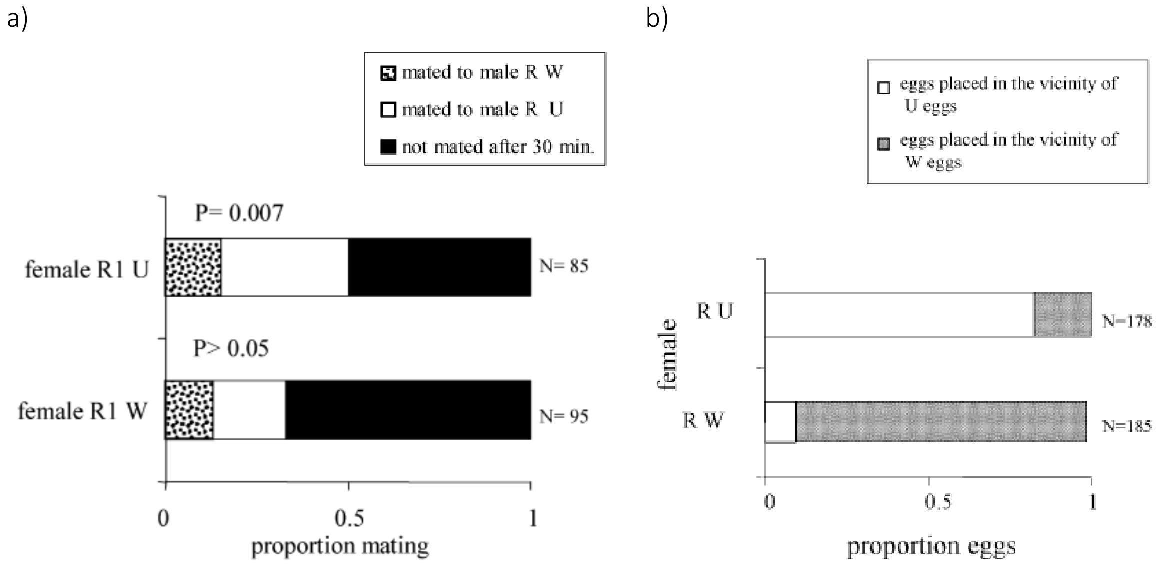


Figure 13. Tetranychus urticae ***adaptations against the costs induced by*** **Wolbachia.** *a) Proportion of uninfected females from the R1 strain mating with infected or uninfected males. Uninfected females, cured by heat shock, prefer to mate with uninfected males, exhibiting assortative mating against males harbouring CI-inducing* Wolbachia. *b) Distribution of eggs laid by R females. Infected and uninfected females aggregate their eggs in the vicinity of eggs of the same infection status, thereby promoting sib-mating. U:* Wolbachia*-uninfected; W:* Wolbachia*-infected; N: number of individuals tested. Adapted from Vala et al. 2004*

4. Outline of my thesis

In this thesis, I begin by reviewing the literature on competition, in an attempt to reconcile the knowledge from different research fields, namely competition for food, competition for hosts and competition for mates (Chapter II; Rodrigues et al. 2016). I then focus on competition for mates, studying both pre- and post-copulatory mating strategies in the haplodiploid *Tetranychus urticae*. I start by investigating which type of cues males use in mate choice to distinguish females from different mating status (Chapter III; Rodrigues et al. 2017) and studying the potential benefits of polyandry to males and females (Chapter IV). In the two following chapters I address the consequences of endosymbiotic infection on females mating strategies. I test whether *T. urticae* females evolve assortative mating in order to avoid incompatible *Wolbachia*-infected mates (Chapter V) and study the importance of polyandry as strategy against *Wolbachia* induced costs (Chapter VI).

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CHAPTER II

Integrating competition for food, hosts, or mates via Experimental Evolution

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Abstract

Competitive interactions shape the evolution of organisms. However, often it is not clear whether competition is the driving force behind the patterns observed. The recent use of experimental evolution in competitive environments can help establish such causality. Unfortunately, this literature is scattered, as competition for food, mates, and hosts are subject areas that belong to different research fields. Here, we group these bodies of literature, extract common processes and patterns concerning the role of competition in shaping evolutionary trajectories, and suggest perspectives stemming from an integrative view of competition across these research fields. This review reinstates the power of experimental evolution in addressing the evolutionary consequences of competition, but highlights potential pitfalls in the design of such experiments.

Keywords

Sexual selection; coinfection; character displacement; virulence; male-male competition; selection experiments

What does an experimental evolution approach add to the study of competition?

Competition has been extensively studied using mathematical models ever since the Lotka-Volterra equations (Krebs 2001). Following these classical models, numerous ecological experiments were conducted in the laboratory (Birch 1953; Crombie 1945; Gause 1932) and in natural populations (Schoener 1983) providing many examples of both exclusion and coexistence between species or phenotypes competing for resources. Since then, competition has been studied in many contexts, and three main types of competition have been described (Box 1).

Darwin identified competition as a source of evolutionary change (Krebs 2001). In fact, one can argue that all evolution results from a competitive advantage of one genotype over another. However, there are specific questions pertaining to the role of competition, as an ecological interaction, on the evolution of populations. For instance, do traits improving competitive ability evolve, especially in the face of trade-offs with other life-history traits (Hastings 1980; Levins & Culver 1971)? How does competition drives niche width, i.e., under which circumstances will individuals expand or contract the range of their resource use? When will character displacement evolve (Brown & Wilson 1956; Macarthur & Levins 1967)? How do eco-evolutionary feedbacks, arising from how competition impacts the resource itself, change evolutionary responses? Unfortunately, study systems in which a causal link between competition and these evolved responses can be inferred are scarce (Hastings 1980; Losos 2000; Pfennig & Pfennig 2012). Moreover, past competitive exclusion is nearly impossible to detect in the wild, a phenomenon coined as ‘the ghost of competition past’ (Connell 1980). Therefore, actual forces and mechanisms driving organisms’ distributions and traits, and their impact on ecosystems, are difficult to assess retrospectively, hampering a complete understanding of the reciprocal effects of competition on evolution.

One way to overcome this is using experimental evolution. This methodology consists of following real-time evolution of populations subjected to specific selection pressures under controlled conditions. Its explanatory power relies on: (i) knowledge of the ancestral (i.e., initial) state, (ii) easy manipulation of selection pressures, (iii) replication at the population level, and (iv) the ability to follow the dynamics of a process (i.e., evolutionary change over time), instead of measuring only the end product – the pattern (Kawecki et al. 2012; Magalhães & Matos 2012). Although this methodology has been used extensively to study other biotic interactions, such as antagonistic host-parasite interactions (Brockhurst & Koskella 2013) its use to address the evolutionary consequences of competition is relatively recent (Edward et al. 2010).

Type or resource	Type of competition		
	exploitative	**interference**	**Apparent**
	Within		
mates	increase sperm swimming ability	increase production of toxins against other sperm, or resist toxins of other sperm	increase evasion of or resistance to toxins produced by females
food	increase feeding rate	increase weapons or resist those of competitors	resist or evade antipredator (resource) traits
hosts	increase strain growth and virulence	increase production of toxins, or resistance to toxins produced by competitors	increase evasion of, or resistance to host immunity
	Among		
mates	increase females' searching and monopolising ability	increase weapons or resist those of competitors (e.g., horns)	Increase male persistence to overcome female's increase resistance to mating
food	increase home range/searching ability	increase traits that allow defence or attack of territories	Evade antipredator (resource) traits by exploiting a novel food source
host	increase strain transmission/searching ability/infectivity/survival outside the host	increase production of toxins, or resistance to toxins produced by competitors	Overcome host resistance to superinfection

Table 1 Examples of expected evolutionary outcomes for interference, exploitative, and apparent competition when competition is for mates, food, and hosts. *Expected outcomes are given for competition occurring at different hierarchical levels: either within or among resources. Competition within resources refers to sperm competition (in competition for mates), competition for nutrients within a territory (in competition for food), and for resources within a host (in competition for hosts). Competition among resources refers to male–male competition (in competition for mates), competition for territories (in competition for food), and for access to hosts (in competition for hosts).*

Organisms might compete for several resource types, the most studied (and probably biologically relevant) being food, mates, and hosts (Table 1). The mechanisms of competition for these resources are shared, yet their study belongs to different research fields, each with its own idiosyncrasies, including distinct vocabulary. Indeed, whereas competition for food is usually stated explicitly, competition for mates can be masked behind terms such as 'sexual selection' or 'sexual conflict', and competition for hosts is usually referred to as 'coinfection'. However, in all cases, individuals are competing for the use of a limiting resource. Experimental evolution, as a methodology that is transversally applied to studies of competition across resource types, opens a unique opportunity to (i) establish parallels across research fields, in view of establishing a common integrative framework, and (ii) highlight potential lessons from one field to the other. Those are the aims of this review.

Measuring evolutionary change: the importance of the ancestral state

One significant advantage of experimental evolution is knowing the ancestral state of populations before selection (Kawecki et al. 2012; Magalhães & Matos 2012). In studies of competition for food or hosts, the ancestral state is often no competition. Evolutionary change can be directly inferred, by comparing traits in their ancestral state to those following selection in a competitive environment. For example, experimental evolution clearly demonstrated character displacement for carbon source exploitation in *Escherichia coli* strains as a result of evolving in competition (Tyerman et al. 2008). Establishing such causality is difficult in natural populations (Losos 2000). By contrast, studies of competition for mates are often initiated from panmictic populations with many interacting males and females, suggesting that competition is the ancestral state. However, often the intensity of competition among same-sex individuals in the ancestral population is unknown. This uncertainty over the ancestral state hampers a clear inference of the direction of evolution (Figure 1 and Outstanding Questions) (Bell & Reboud 1997; Magalhães et al. 2009). These studies might assume that monandry is the derived state, but this assumption should be clearly stated and tested (e.g., Crudgington et al. 2005). In fact, it should be clear whether the operational sex ratio (OSR) imposed in 'control' lines corresponds to that of the ancestral state, in which case these lines can effectively be used as control. For example, in a study of experimental evolution of the bruchid beetle *Callosobruchus maculatus*, both the ancestral state and the control populations were monandrous (Gay et al. 2011) (see also Nandy et al. 2013).

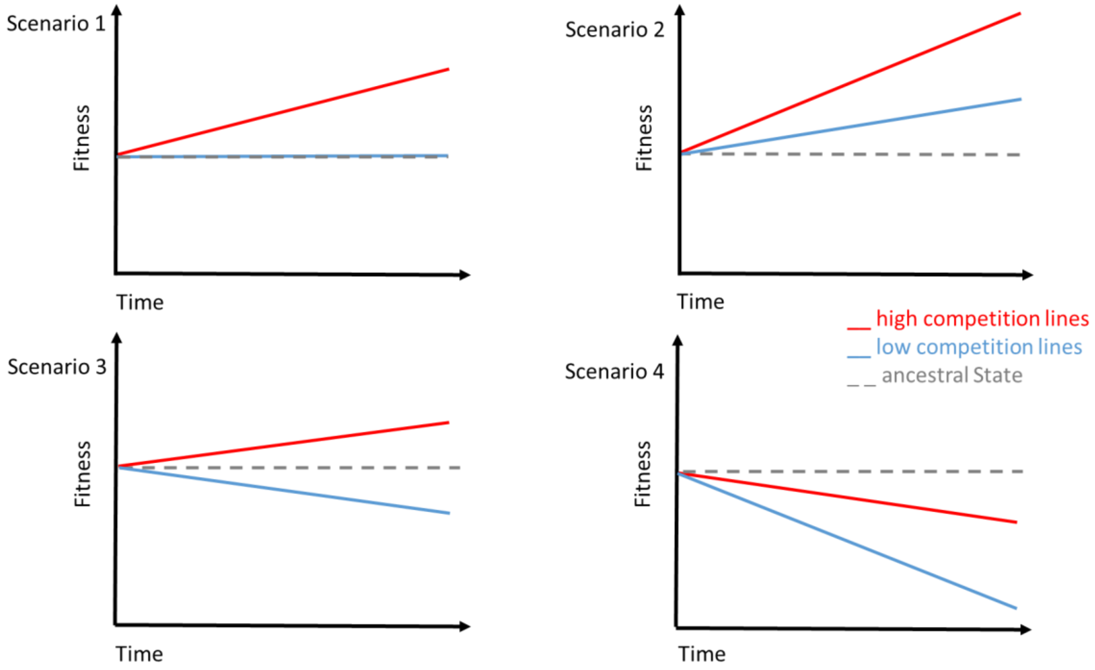

Figure 1: Measuring evolutionary change. *Four possible scenarios of evolutionary trajectories of populations undergoing experimental evolution. The red line represents populations in which evolutionary change is expected ("high competition" lines), whereas the blue line represents "low competition" lines, which are expected to reflect the ancestral state (dashed grey line). However, note that control lines might be lines in which competition is high (usually in competition for mates – where polyandrous lines are assumed to represent the ancestral state of the population). Because the distance between trajectories of the two selection regimes is the same in all graphs, the conclusion drawn from that comparison is always that selected lines have adapted to the regime they were exposed to (they increased in fitness). However, in Scenario 2 this fitness increase is underestimated, in Scenario 3 it is overestimated, and in Scenario 4 the conclusion of a fitness increase is incorrect. Therefore, it is important that control lines reflect the ancestral state.*

Experimental manipulations of competition and their (hidden) assumptions

Intraspecific competition for one resource

When competition concerns one species/strain and one resource, it is manipulated by either increasing the number of competitors or reducing the amount of resources per capita. Although these manipulations are equivalent from an ecological standpoint, they are not from an evolutionary perspective. Indeed, adding more competitors also potentially increases the amount of genetic variation, the raw material upon which evolution acts. This implies that responses to selection might differ among treatments, independently of the selection differential due to competition (Box 2). Therefore, manipulating resources leads to more straightforward evolutionary interpretations. Indeed, *Drosophila melanogaster* populations exposed to high competition increased their niche width (i.e., diet breadth with increasing cadmium) at a faster rate than populations under low competition. In this study, competition was manipulated by exposing the same number of flies to varying numbers of food vials containing different cadmium concentrations, while keeping the proportion of each concentration constant (Bolnick 2001).

Competition for mates is manipulated by varying the number of males (competitors) accessing females and/or the number of females (resource) offered to a male and/or density, sometimes resulting in changes in OSR. Stronger competition (i.e., more polyandry) is achieved when more males compete for fewer females (Edward et al. 2010). This implies that most treatments differ in genetic variability, since the effective population size is reduced with uneven sex ratios (Bergstrom & Dugatkin 2012). This can confound observed evolutionary responses (Reuter et al. 2008). Although, in some studies, treatments varying in sex ratio do not differ significantly in effective population size, as explicitly demonstrated in *Drosophila melanogaster* (Snook et al. 2009), this might not always be the case. By increasing the population size in parallel with the increase in female bias, it is possible to overcome this effect (Reuter et al. 2008), see also (Snook et al. 2009) for additional strategies).

Outstanding questions

The comparison of the literature regarding experimental evolution of competition for hosts, food or mates revealed a few questions that remain largely unexplored. Addressing these questions will contribute to a better understanding of the reciprocal effects of competition on evolution.

1. *Does competition lead to coevolutionary dynamics between competitors? If so, when is it characterised by arms race or fluctuating selection dynamics? And when does it allow for the maintenance of genetic variation in populations?*
2. *Can character displacement evolve as a result of competition for mates or hosts?*
3. *Does evolution toward monandry or polyandry evolve at different rates?*
4. *How does competition between resources (e.g., competition for territories) affect competition within resources (within-territory competition for food)?*
5. *How does the evolution of competition affect functional diversity in communities and ecosystems?*

Interspecific competition for one resource

In competition studies between species or strains, two main designs are implemented: the 'substitution design', where the overall density is maintained across treatments and, consequently, the competition treatment contains half as many individuals of each species/strain as the single species/strain treatments (2A, 1A:1B, 2B; *e.g.*, Lawrence et al. 2012), and the 'addition design', where the competition treatment contains the same number of each competitor as the single species/ strain controls, entailing an increased overall density in this treatment (2A, 2A:2B, 2B, *e.g.*, Terhorst 2011). This design is used in competition for hosts, although sometimes not explicitly stated (Dion et al. 2011; Garbutt et al. 2011). In competition for food, both designs are used ('substitution' = Lawrence et al. 2012, 'addition' = terhorst 2011, Wölfle et al. 2009). These designs are absent in competition for mates, as interspecific interactions were not addressed (but see Deere & Smallegange 2014).

Each of these designs has its flaws, as discussed in the ecological literature (Inouye 2001). Indeed, in the addition design, the competition regime differs from controls in that (i) two species/strains are present instead of one, and (ii) there is a twofold increase in the overall density. Therefore, which of these factors affects differential trait evolution between treatments is unclear. Equalising per capita resources across treatments might minimise this problem. This, however, requires knowledge on species functional responses, as doubling resources does not necessarily imply doubling feeding rates. The advantage of the addition design is that the intensity of intraspecific competition is maintained across treatments. Still, this only holds if we assume that interspecific individuals do not alter intraspecific interactions.

In the substitution design, all treatments have the same density hence, if intra- and interspecific/ strain competition have similar strengths, global competition levels are maintained across treatments. However, this implies lower intraspecific/strain density, hence lower intraspecific competition, in the 'competition' treatments. Thus, observed responses might be attributed to this rather than to interspecific/strain competition. Moreover, and importantly, differences in the number of individuals of each species result in differences in genetic variability. As in the single species case, this implies that responses to selection can differ among treatments, independently of the selection differential due to competition (Box 2). This can be overcome by seeding each of the intra- and interspecific/strain treatments with combinations of individuals from the same number of isogenic lines (Figure 2).

More than one resource

Although the addition and substitution designs have been used to describe the manipulation of competitors, they can also serve to describe manipulations of the resource. Indeed, competition can be manipulated by replacing half of the original resource by a novel one, or by adding a novel resource, while keeping the amount of the original constant. For example, Barret and colleagues manipulated competition by varying the number of carbon substrates involved in different *Pseudomonas* metabolic pathways by keeping the concentration of each substrate constant, thus increasing substrate availability in treatments with more substrate types (Barrett et al. 2005). It should be noted that only in the substitution design is competition increased (assuming that novel resources are initially of poorer quality than the original). The addition of novel resources can promote niche expansion or character displacement leading to a decrease in competition, at least transiently.

There are other, more subtle ways of addressing the role of competition, for example, by manipulating the spatial scale at which competition is operating (Griffin et al. 2004; Macke et al. 2011). For instance, Macke et al. (2011) varied the number of founders per patch to test whether local mate competition leads to a more female-biased offspring sex ratio (Hamilton 1967).

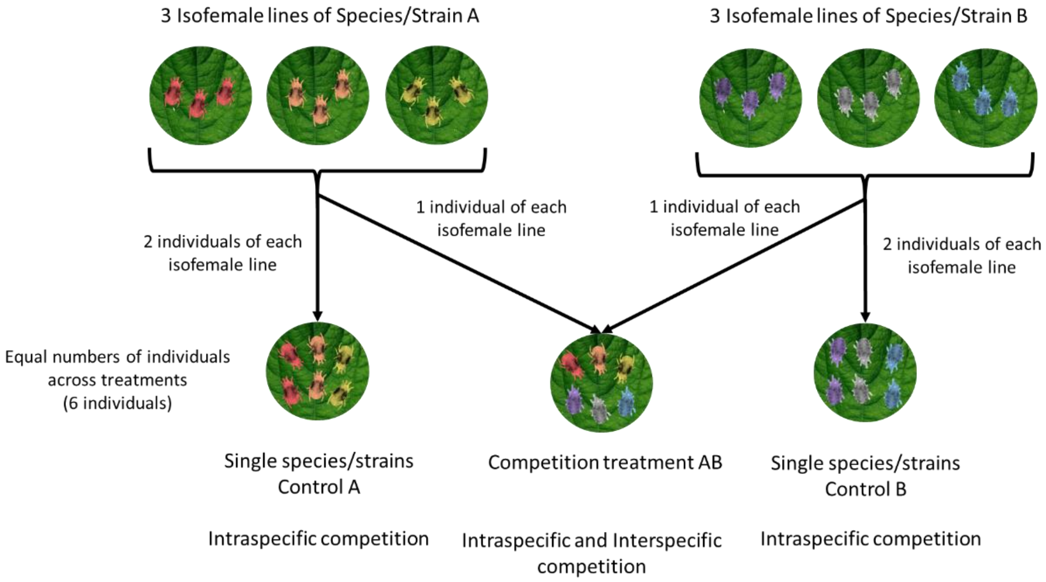

Figure 2. How to manipulate competition while keeping genetic diversity constant. *One possible way to keep overall densities the same across treatments (hence reducing the number of each species in competition) while maintaining similar levels of genetic variability is to use mixes of isogenic lines from each species in all treatments. In this way, all treatments could be drawn from the same number of isogenic lines (thus maintaining genetic diversity across treatments) but using a different number of individuals from each line (thus varying the intraspecific density as required in the substitution design). In the diagram, as an example: create three isofemale lines for species A and for species B; single species treatment A: use two individuals from each species A isofemale line, with a total of six individuals; single species treatment B: use two individuals from each species B isofemale line, with a total of six individuals; two species competition treatment A and B: use one individual from each isofemale of species A and species B, with a total of six individuals. Note: (i) a much higher number should be used; (ii) this solution is not valid if strong sib competition is an important concern in the system under study.*

Evaluating the effect of competition

In Tables 2 and S1.1, we compile representative examples of experimental evolution studies that manipulated competition for food, mates or hosts.

Measuring the evolutionary response to competition

In experimental evolution, adaptation is typically tested by comparing trait values in populations evolving under a given selection pressure (selection lines) with those evolving in conditions pertaining to the ancestral environment before the experiment (control lines). The general assumption is that control populations are at a steady state for the traits measured, hence differences observed can be attributed to evolutionary changes occurring in evolved populations (Kawecki et al. 2012) (Figure 1).

In lines selected under competition, a higher competitive ability is expected to evolve. This can be evaluated by comparing the performance of individuals evolving in competition with that of individuals evolving under controlled conditions, in a competition environment. This is a typical test of local adaptation (Kawecki & Ebert 2004) and this prediction is generally met (Table 2).

Evolving under competition might also affect resource utilisation. Testing this requires evolved and control lines to be tested in a competition-free environment. In some cases, such as in *Tribolium castaneum* feeding on wheat, evolution of resource utilisation in competitive and non-competitive environments is positively correlated (Agashe & Bolnick 2010). Other studies find trade-offs. For example, *Drosophila* evolving with competitive fungi perform better than control lines in the presence of the competitor, but this comes at a cost of performance in the ancestral, competitor-free environment (Wölfle et al. 2009). In another study, *Drosophila* evolving in crowded conditions increased resource acquisition, but had higher mortality when tested in uncrowded conditions (Joshi & Mueller 1996). Therefore, the direction in which resource utilisation evolves varies among studies.

Competition for or within resources

Competition can concern access to an entire resource or a portion of it (Table 1). Indeed, males can compete for access to females and/or their sperm might compete for egg fertilisation within the female – sperm competition; parasites can compete for access to hosts, and for resources within the host; individuals can compete for territories, or for food within territories. In studies of competition for food and hosts, however, evolutionary responses to competition for territories or among hosts remain to be investigated (Box 2; see Outstanding Questions).

The traits that respond to selection might differ depending on the level where competition is strongest. For example, mating rate is associated with competition for mates – between resources –

whereas fertilisation success is a measure of sperm competition – within a resource. Furthermore, trade-offs can occur between levels. For example, higher mating rates can trade off with sperm competitive ability. In the same way, defending a larger territory might be incompatible with the defence of particular resources within it, and within-host competition for resources might trade off with competition for new hosts. Hence, experiments should be designed such that the level at which selection is acting (within or between resources) is clear. For example, Fricke and Arnqvist (2007) exposed bruchid beetle females to several males sequentially, thus only allowing for competition within the female to occur (within resource). Alternatively, traits acting on each of these levels should be measured, so that responses to selection do not go undetected.

Correlated responses to selection: commonalities and specificities across resource types

Competition for mates: sexual conflict and secondary sexual traits

Sexual conflict is perhaps the most studied correlated response to selection using experimental evolution. When evolving in competitive environments, males typically become more harmful towards females. This can in turn select for female resistance. By contrast, under low male competition, male harassment and female resistance are no longer advantageous. For example, offspring of *D. melanogaster* females mated with males evolved in monandry, compared with polyandry, have lower mortality (Pitnick et al. 2001a). Moreover, ejaculates from males evolving in monandry are less effective at manipulating female behaviour towards being less receptive (Pitnick et al. 2001b). Conversely, females evolving in polyandry have higher mating rates, suggesting higher resistance to ejaculate manipulation (Pitnick et al. 2001b). Note that, when tested in a competitor-free environment, higher female fertility might be interpreted as relaxed sexual conflict (a correlated response to selection) or reduced resource exploitation by males (a direct response).

Another potential correlated response is the evolution of secondary sexual characters. For example, males evolving under relaxed sexual selection showed reduced cognitive abilities, considered a secondary sexual character because it permits discrimination of receptive and non-receptive females (Hollis & Kawecki 2014). To our knowledge, few experimental evolution studies have investigated this (Smallegange & Deere 2014).

Competition for host resources: evolution of virulence

Within-host competition among parasites is expected to select for higher growth rates to overcome competitors (Alizon et al. 2013). As more parasites supposedly induce more harm, theory assumes that within-host growth rates are positively correlated with virulence (Alizon et al. 2009). Alternatively, evolution of lower virulence is predicted if competing strains engage in interference competition (Gardner et al. 2004) (Box 1). Moreover, between-host competition can favour lower virulence if there is a trade-off with transmission (Alizon et al. 2009).

The link between virulence evolution and within-host competition (i.e., single versus multiple infections) has been the subject of many theoretical studies and an excellent recent review (Alizon et al. 2013). However, to date, only two empirical studies test these predictions using experimental evolution. The first shows that virulence decreases in coinfections with *Bacillus thuringiensis*, probably due to the production of bacteriocins that kill competitors (Garbutt et al. 2011). In the second, virus strains become more virulent when evolving with other strains compared with single infections, when tested in competition (Leggett et al. 2013). This result is consistent with exploitative competition between parasites, although this has not been explicitly tested.

Competition for food: niche width

Competition can select for traits allowing new regions of resource space to be explored, leading to niche expansion (Pfennig & Pfennig 2012). Alternatively, it can favour traits that decrease niche overlap, leading to niche contraction. If sufficiently intense, these processes can lead to disruptive selection, in which both tails of the phenotypic distribution are favoured, potentially leading to character displacement (Pfennig & Pfennig 2012). Whether traits evolve in one direction or the other depends on the relative availability of the different resource.

Results of experimental evolution studies on the role of competition on niche width are variable. For example, relaxing competition between two different *Escherichia coli* populations adapted to different carbon sources resulted in their phenotypic convergence; the reintroduction of competition led to character displacement, as predicted (Tyerman et al. 2008). However, this outcome might be contingent on the diversification ability of the competitor strain. Indeed, in *Pseudomonas fluorescens*, niche expansion of a focal strain is slowed when competing with generalist strains able to thrive in all environments. By contrast, competition with strains with reduced niche width promotes the diversification of the focal strain, facilitating the colonisation of a new environment (Bailey et al. 2013). Other studies show that strong intraspecific competition selects for niche expansion (Bolnick 2001; Agashe & Bolnick 2010; Bono et al. 2013).

Other correlated responses can also evolve, such as cannibalism in *Drosophila* as a result of competition for food (Vijendravarma et al. 2013) and reduced immunocompetence following competition for mates in the Indian mealmoth *Plodia interpunctella* (McNamara et al. 2013) and the beetle *Callosobruchus maculatus* (van Lieshout et al. 2014). Moreover, one can establish parallels between correlated responses: both virulence and sexual conflict, by-products of competition, induce harm to the resources (hosts and mates, respectively). Additional parallels are explored in Box 2. These could drive new research or reinterpretation of existing studies.

Perspectives for future research

Addressing all possible scenarios (coexistence vs exclusion)

In studies of interspecific/strain competition, experimental conditions are conceived such that species/strain exclusion is prevented, forcing coexistence to be maintained across generations. This means that only one of the Lotka–Volterra scenarios is considered, that in which intraspecific is stronger than interspecific competition. Hence, by narrowing down the conditions used, the experimenter is directing the evolutionary outcomes. To test for alternative scenarios (i.e., where competitive exclusion is expected), one could replenish the outcompeted player at regular intervals and measure evolution in the superior competitor.

This set-up would also allow to test how coevolution among competitors affects trait values as well as the maintenance of diversity (Pfennig & Pfennig 2012). Although this possibility has been theoretically proposed (Kisdi & Geritz 2001), it has never been tested in experimental evolution studies. Indeed, when competitors coexist, one can generate treatments in which both species coevolve and contrast them with treatments in which only one species evolves, the other being systematically replenished from the ancestral population at each generation, as in studies of host–parasite (Brockhurst & Koskella 2013) or male-female coevolution (Chapman et al. 2003). This set-up allows singling out the role of coevolution in shaping trait values, the rate of evolution and/or the maintenance of genetic diversity (Pfennig & Pfennig 2012) (cf. Outstanding questions).

Experimental Evolution studies should be strongly rooted upon ecological knowledge

Much theory has been developed to understand the ecological outcomes of competition. This knowledge might help interpret experimental evolution studies. For example, a clear definition of the type of competition helps refine predictions concerning experimental outcomes (Box 1). In fact, this approach has been taken to interpret the outcomes of coinfection scenarios (Graham 2008; Pedersen & Fenton 2007) and could also be applied to competition for mates or food. When the type of competition is unknown, it can later be inferred from the observed outcomes following evolution (i.e., which traits are affected and in which direction). In this case, evolution would inform ecology, providing knowledge on interactions among species or strains.

Experimental Evolution can shed light on functional traits and functional diversity

Competition is a major component of ecosystems. As such, it is likely to affect the functional diversity of such ecosystems. Unfortunately, knowledge on which functional traits are affected by competition within a species community is in its infancy (Reiss 2009). In this context, experimental evolution can be instrumental to identify which traits respond to selection imposed by competition. Those traits are likely to be ecologically relevant, as they will affect other competitors as well as resource levels. Hence, they are expected to act as effect traits (i.e., traits that have an effect on the functioning of the ecosystem; Suding et al. 2008). Moreover, the strength of competition is, by definition, linked to the amount of resource present. Thus, traits involved in competitive interactions are also expected to act as response traits (i.e., traits that change after environ-mental disturbance). Often, traits that respond to perturbations are also the same traits that have a relevant role in ecosystem functioning. In light of the recent evidence for widespread rapid evolution and eco-evolutionary dynamics in nature (Schoener 2011), it is clear that traits that respond to selection may be functional both in the evolutionary and in the ecological sense. Thus, knowledge stemming from experimental evolution might significantly contribute to strengthen the ongoing link between ecology and evolution, as it would contribute to merge ecosystem functioning to eco-evolutionary dynamics (cf. Outstanding Questions).

Concluding remarks

In summary, we aimed to highlight similarities in patterns and processes across diverse research fields that implicitly or explicitly study competitive interactions (parasitology, sexual selection, foraging ecology). We have shown how this integration helps generate novel predictions and clarify patterns observed in experimental evolution studies. Clearly, competition is a major biotic selection pressure imposed on organisms, the evolutionary outcomes of which are still largely unclear. Further experimental evolution studies addressing the rich diversity of competitive interactions across organisms will help clarify the commonalities among evolutionary responses to competition.

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Box1: Types of competition

Competition can be categorised according to whether individuals are competing against individuals of the same or a different species (intraspecific and interspecific competition, respectively). Moreover, three types of competition can be distinguished, according to the mechanism that leads to fitness reduction in other individuals (Krebs 2001):

1. Exploitative (or resource or scramble) competition is an indirect interaction, in which the consumption of a shared resource by some individuals entails a fitness cost for other individuals because these will be consuming a smaller share of the same resource. A classic example is competition between closely related beetle species (*Tribolium*) feeding on flour (Gause 1932).
2. Interference (or contest) competition is a direct, negative interaction, often via attack, between individuals that use a common resource. A classic example of this is males fighting for access to females, for instance, in dung beetles (Emlen 1997).
3. Apparent competition is a top-down indirect interaction leading to a fitness reduction in individuals that share an upper level resource (predator/host/female) (Holt 1977). For example, parasites can trigger the immune system of their host with negative consequences for other parasites sharing the same host (Mideo & al. 2009; Raberg et al. 2006).

Although in some cases the distinction among these three types of competition is straightforward, in others it can be a difficult task. For example, sperm competition can be exploitative if some sperm have better swimming ability, or interference competition when sperm produce toxins that destroy other sperm types (Table 1). For instance, in *D. melanogaster*, it was initially postulated that the ejaculate of the first male was destroyed by that of the second inside the female, suggesting interference competition (Harshman & Prout 1994), but later more detailed studies demonstrated that sperm displacement, hence exploitative competition, was the mechanism operating (Manier et al. 2010).]. Furthermore, the success of a particular sperm type might hinge on the female's genetic background, which parallels apparent competition (Clark 1999; Lüpold et al. 2013). In studies of experimental evolution, the type of competition might be known initially. For example, in Garbutt et al. (2011), bacteria produce toxins that destroy other bacteria, hence interference competition is operating. However, in many cases, the type of competition is initially unknown and is inferred only after experimental tests are done at the end of the evolutionary process (cf. main text). Note also that even the identification of the type of resource individuals are competing for might be a difficult task (Moya-Laraño et al. 2002).

Box 2: Learning from parallels involving different resource types

Female aggregation and evolution of mating rate

In polygynous systems, evolution of mating rates is a function of their variance (e.g., Jones 2009). Hence, if some males can monopolise females, selection intensity for mating rates will be stronger than in systems with no female aggregation (Klug et al. 2010; Wade & Shulter 2004; Wade 1995), leading to runaway sexual selection, stopping when males cannot defend more females. Hence, selection intensity is not simply dependent on the ratio between competitor number and resource availability.

Extrapolating to competition for food, it implies that the more resources (some) individuals can monopolise, the stronger selection for food acquisition. Hence, explicitly measuring standing genetic variance for resource acquisition might help to predict evolution. Moreover, if competition is experimentally manipulated through fewer resources, the opportunities for resource monopolisation can decrease, which might invalidate the prediction that more competition drives faster evolution. Similarly, parasites can evolve the ability to monopolise hosts, for example, by increasing host resistance to superinfection with other parasitoid wasps, as *Hamiltonella defensa* in aphids (Dion et al. 2011). This might modify the evolution of virulence in such populations.

Niche width

Evolution of niche width has been tackled mostly as a consequence of competition for food (cf. main text). A host is also an ecosystem within which parasites exploit different niches (Pedersen & Fenton 2007). Competition might thus select for parasites colonising novel hosts, or using different resources within a host (Mideo & al. 2009). For example, some trematodes specialise on different species or portions of the intestine (Karvonen et al. 2006). Experimental evolution could establish causal links between competition avoidance and within-host parasite distributions (e.g., evolution of niche expansion in viruses; Bono et al. 2013).

Similarly, competition for mates can select for alternative tactics to acquire mates (Pfennig & Pfennig 2012), such as monopolising females or sneaking (Emlen 1997). However, the evolution of such morphs does not necessarily result from character displacement. Experimental evolution on alternative morphs is scarce (Smallegange & Deere 2014; Deere & Smallegange 2014), and such studies are needed to establish causality (Cf. Outstanding Questions).

Virulence

How coinfections affect virulence evolution is discussed in the main text. Virulence can be paralleled with sexual conflict, and one can predict that exploitative or interference competition between sperm within females will lead to more or less male harm, respectively.

The competition/colonisation trade-off in competition for food (Amarasekare 2003) mirrors the virulence/transmission trade-off. Extending the parallel leads to predicting that under exploitative competition patches would be depleted at a faster rate in the presence of competitors, the reverse being true under interference competition (cf. Outstanding Questions).

Table 2. Experimental evolution studies manipulating competition. *Examples of experimental evolution studies that have manipulated competition for food, mates or hosts. Our aim is to use representative examples, not to summarise all available literature. CA, Competitive ability; RU, resource use; M/P, monogamy and polygamy treatments; F-biased, female-biased. ↑, increase; ↓, decrease, ↔, no change. In Table S1.1 we provide more details and references.*

Type of resource	Organism	Manipulation	CA	RU	Correlated Response	References
Food	*Drosophila melanogaster*	number competitors	↑	↔ ↓↑	↔ parasitoid resistance	Joshi & Thompson 1996; Mueller & Ayala 1981; Mueller et al. 1991; Sanders et al. 2005
Food	*Drosophila melanogaster*	resource quality	↑	↔ ↓↑	↑ cannibalism ↓ motility	Vijendravarma et al. 2013; Vijendravarma et al. 2012; Kolss et al. 2009; Bochdanovits & de Jong 2003
Food	*Drosophila melanogaster*	resource quantity	-	↑	niche expansion	Bolnick 2001
Food	*Drosophila melanogaster*	type of competitors	↑	↓	-	Joshi & Thompson 1996; Wölfle et al. 2009
Food	*Escherichia coli*	resource diversity	-	-	character displacement	Tyerman et al. 2008; Friesen et al. 2004
Food	*Escherichia coli*	type of competitors	↑	-	character displacement	Forde et al. 2008
Food	*Fagus sylvatica*	type of competitors	-	↓↑	character displacement	Lawrence et al. 2012
Food	*Pseudomonas fluorescens*	resource diversity	↑	↑	-	Barrett et al. 2005
Food	*Pseudomonas fluorescens*	type of competitors	-	-	niche expansion, contraction	Bailey et al. 2013
Food	*Tribolium castaneum*	number & type competitors	-	-	niche expansion	Agashe & Bolnick 2010
Mates	*Caenorhabditis elegans*	Outcrossing/ Selfing	-	-	↓ genetic variability	LaMunyon et al. 2007
Mates	*Callosobruchus maculatus*	OSR, age at reproduction	-	-	sexual conflict affects lifespan	Maklakov et al. 2009
Mates	*Callosobruchus maculatus*	M/P	↔	↔	↑ sexual conflict	Gay et al. 2011

Table 2. continued

Type of resource	Organism	Manipulation	CA	RU	Correlated Response	References
Mates	*Drosophila melanogaster*	OSR	↑	↓?	↑ sexual conflict, body size	Crudgington et al. 2005; Pitnick, Miller, et al. 2001; Pitnick, Brown, et al. 2001
Mates	*Drosophila melanogaster*	OSR (female-biased)	↓	-	-	Reuter et al. 2008
Mates	*Drosophila melanogaster*	M/P	↑	↔	↑ male cognitive ability	Hollis & Kawecki 2014
Mates	*Drosophila pseudoobscura*	OSR	-	-	song elements, character displacement?	Snook et al. 2005
Mates	*Megabruchidius tonkineus*	OSR	-	↑	↔ sexual conflict	Booksmythe et al. 2014
Mates	*Mus domesticus*	OSR	↑	-	↓ sexual conflict	Firman 2011; Firman & Simmons 2010
Mates	*Onthophagus taurus*	M/P	↑	↑	↔ sexual conflict	Simmons & García-González 2008
Mates	*Rhizoglyphus robini*	M/P	↑	-	↑ sexual conflict	Tilszer et al. 2006
Mates	*Scathophaga stercoraria*	OSR	↑	-	↑ sexual conflict	Hosken et al. 2001
Mates	*Sepsis cynipsea*	M/P	-	-	↑ character displacement	Martin & Hosken 2003
Mates	*Tetranychus urticae*	local/global competition	-	-	sex ratio adjustment varies with scale of competition	Macke et al. 2011
Mates	*Tribolium castaneum*	OSR	↑	-	↑ sexual conflict	Michalczyk et al. 2011
Mates	*Tribolium castaneum*	OSR, resource quality	-	↓↑	↑ sexual conflict	Grazer et al. 2014
Hosts	*Bacillus thuringiensis*	type of competitors	↑	-	↓ virulence	Garbutt et al. 2011
Hosts	vesicular stomatitis Indiana virus	type of competitors	↑	-	-	Carrillo et al. 2007
Hosts	vesicular stomatitis Indiana virus	number & type competitors	↑↓	-	-	Miralles et al. 2001
Hosts	lytic phage ϕ2	type of competitors	↑	↓	↑ virulence	Leggett et al. 2013
Hosts	*Lysiphlebus fabarum*	type of competitors		↔		Rouchet & Vorburger 2014

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CHAPTER III

Male spider mites use chemical cues, but not the female mating interval, to choose between mates

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Abstract

The choice of the partner an individual will mate with is expected to strongly impact its fitness. Hence, natural selection has favoured the evolution of cues to distinguish among mates that will provide different fitness benefits to the individual that is choosing. In species with first-male sperm precedence, this is particularly important for males, as mating with mated females will result in no offspring. In the spider mite *Tetranychus urticae* only the first mating is effective, except if the interval between first and second copulations is shorter than 24 h. In line with this, males prefer to mate with virgin over mated females. They do not, however, choose between females that have mated at different time intervals. Here, we tested which type of cues males use to distinguish between females with different mating status (virgin versus mated). To do so, we firstly confirmed that males prefer virgins over mated females and that they do not select females on the basis of their age or mating interval. Next, we tested whether contact and volatile compounds or chemical trails affected male discrimination between mated and virgin females, by systematically varying the exposure of males to these cues. We found that volatile compounds and chemical trails were sufficient to induce discrimination between virgin and mated females in males. Direct contact with females, however, does not seem to play a role in this discrimination. The composition of such chemical cues (trails and volatiles) remains to be identified.

Keywords

First male sperm precedence; mating interval; mating behaviour; chemical cues

Introduction

Patterns of sperm precedence range from last-male to first-male sperm precedence (Danielsson 1998). Expected mating behaviours depend on these patterns. In species with last-male precedence, selection in males should favour increased mating frequency as a result of adaptation to sperm competition (Ridley 1989a). In species with first-male precedence, however, males do not benefit from mating with females that have already mated. Moreover, females cannot choose to use the sperm of subsequent males, which should favour monandry. Hence, both sexes are expected to invest all resources in matings that involve virgin females only, except if the pattern of first-male precedence is incomplete and some copulations with mated females are effective. In males, first-male precedence should also favour the ability to distinguish virgin from mated females, as well as pre-copulatory mate guarding, which sometimes extends to immature females (Ridley 1989a, b).

The existence of cues that enable a male to discriminate females with different mating status depends on the balance between costs and benefits conferred by this discrimination. In monogamous species, the benefits are clearly very high and thus such discrimination is likely to evolve. Indeed, several studies show that males from monogamous species prefer virgin over mated females (Thomas 2011). Moreover, even in species with multiple mating, males can evolve the ability to discriminate female mating status, depending on the species mating rate and sperm precedence patterns (Engqvist and Reinhold 2006). In particular, mated females of species with first-male sperm precedence, as well as of some species with mixed sperm precedence, provide low fertilization opportunities to males, if any. Accordingly, males frequently evolve the ability to discriminate female mating status, preferring virgins (e.g. Stoltz et al. 2007; Yasui 1994).

To distinguish females of different mating status, males can use several types of cues. In arthropods, these cues are usually chemical (Johansson & Jones 2007; Thomas 2011) and can be perceived by contact (e.g., cuticular hydrocarbons, CHCs), left in the substrate (i.e., chemical trails) or released into the environment (i.e., volatile compounds). For example, *Drosophila melanogaster* uses CHC's to distinguish mated from virgin females (Friberg 2006), whereas males of *Agelenopsis aperta*, a monogamous spider, use volatiles to identify virgin females (Riechert & Singer 1995). In the mealworm beetle *Tenebrio molitor*, males prefer virgin over mated females when only chemical trails are present in the arena (Carazo et al. 2004), indicating that such cues are sufficient for male discrimination. Identifying the type of cue used in mate choice adds to our understanding of mating behaviour, as it allows determining the ecological conditions in which they are perceived. For example, the minimal distance necessary for two individuals of the opposite sex to perceive each other's mating status will vary depending on which cues are used. This in turn may have consequences for species conservation and management.

In spider mites, the first male fertilizes most of the female's eggs (in *Tetranychus urticae*, Helle 1967; in *Tetranychus kanzawai*, Oku 2008), meaning that this species follows a first-male sperm precedence pattern. Consequently, it is expected that males only attempt to mate with virgin females to avoid unnecessary costs, such as loss of other mating opportunities or reduced survival. This requires that males have access to cues allowing the perception of the female mating status. Indeed, males actively guard quiescent females and mating occurs as soon as females emerge as virgin adults (Potter et al. 1976). In addition, both prolonged copulation and post-copulatory guarding behaviours are observed in these species, which ensures first-male sperm precedence (Satoh et al. 2001). Moreover, when males outnumber virgin females, they actively fight for their possession (Potter et al. 1976). In accordance with these observations, recent studies showed that males prefer to mate with virgin rather than mated females (Oku 2010). In addition, females pay a cost of multiple mating, having lower fecundity and a smaller proportion of fertilized eggs, when compared with once-mated females (Macke et al. 2012). Together, these results lead to the expectation that in *T. urticae* both sexes should invest all resources in the first mating event. However, surprisingly, copulations between males and mated females have been observed (Oku 2010; Clemente et al. 2016).

This puzzling behaviour could be adaptive if first-male sperm precedence in *T. urticae* is incomplete. In fact, the mating interval has been shown to affect the extent of first-male sperm precedence in this species, as found in other invertebrates (Danielsson 1998). In spider mites, if the interval between first and second copulations is shorter than 24 h, the second male can still sire some offspring (Helle 1967). However, a recent study has shown that, in an arena with females that have mated 6 or 30 h before the test, males first touch each female type indiscriminately (Oku 2013). Several possibilities may explain this result: (a) the female that is touched first does not fully represent mate choice, (b) the result is specific to the population/test details used, or (c) the result is a general feature of *T. urticae* populations, which seems to be maladaptive.

In this study, we set out to test which type of cues males use in their mate choices. We tested the role of tactile stimuli (contact compounds), volatile compounds and the presence of chemical trails, the three main chemical cues involved in female spider mite attraction (Penman & Cone 1974). To this aim, we first confirmed that males do distinguish virgin from mated females. Moreover, to limit the choices under test to these two types of females, we also verified that males do not distinguish between females mated at different time intervals in a population other than that used in Oku (2013).

Materials and Methods

Spider mite populations and rearing conditions

Spider mites (*T. urticae*) were reared in large numbers (>2000) on whole cucumber plants (varieties Ventura, provided by Rijkzwaan, France), for experiment (a) and on whole bean plants (varieties Enana, provided by Germisem, Portugal), for experiment (b) (see details of the experiments below), under controlled conditions (25°C; photoperiod of 16L:8D). The population of spider mites used for experiment (a) was originally collected from a cucumber greenhouse in Pijinacker, the Netherlands, in May 1994, and kept in the same rearing conditions at the University of Amsterdam (the Netherlands). This population was then established at the University of Montpellier (France) in 2007 from approximately 5000 individuals sampled from the Amsterdam stock. The population used for experiment (b) was originally collected from tomato plants in Carregado, Portugal, in August 2010 and has since then been kept on bean plants at the University of Lisbon (Portugal). Both experiments were done under controlled conditions (25 °C; photoperiod of 16L:8D).

Experimental Setup

Male discrimination ability: female status, age and mating interval

To address male discrimination between females with different mating status, males were given the choice between virgin and mated females, the latter being mated more or less than 24 h before the test. This interval has been shown to influence the extent of first-male precedence in *T. urticae* (Helle 1967). Moreover, male mating preferences in several species have been correlated with female age, with older females being less attractive than younger ones (Johansson & Jones 2007; Delisle & Royer 1994). In order to account for these differences, we controlled the age of the females tested, in addition to their mating interval.

In total, 300 quiescent females were randomly allocated to different conditions of age (‘’Y’’, young or ‘’O’’, old, i.e., 2 days older), mating status (‘’V’’, virgin or ‘’M’’, mated) and mating interval (‘’12’’ or ‘’60’’, with first mating having occurred 12 (±1) or 60 (±1) hours before the test, respectively). The combination of conditions resulted in five types of females, corresponding to five treatments (Figure 1). To establish such treatments, males and quiescent females were selected from the base population. Males and females were separately placed in groups of 10 on 2 cm^2 leaf squares on water-saturated cotton. Twenty-four hours later (day 1), adult virgin females, 1 day old since the last moult, were placed on 0.8 cm^2 leaf discs. One third of the leaf discs received one male (for the OM60 treatment), whereas in the other two thirds, females were left alone (OV and future OM12 treatments). New males and quiescent females were selected from the base population on day 2 and, on day 3, the females were either placed with males (YM12 treatment) or left alone (YV treatment). In addition, half the females kept as virgins in day 1 were allowed to mate on day 3, by adding one male to each leaf (OM12 treatment). Finally, on day 4, 12 hours after the establishment of the last three types of females, the preference of males was tested. Two females, each from a different treatment, were placed on a 0.8 cm^2 leaf square and randomly painted using pigment ink—blue or red—to allow for their discrimination by the human observer. These females were left alone for 10 minutes, after which one male was placed on the leaf disc and the first female the male touched and mated with was recorded as his mating choice. Simultaneously, time until the beginning of copulation (latency to copulation) and copulation duration were measured. If after 30 minutes the male had not touched any of the females, the group was discarded.

In total, 6 preference tests were done (OM60 x OM12, OM12 x YM12, OV x OM60, OV x OM12, OV x YV and YV x YM12), with 50, 51, 48, 51, 50 and 53 replicates (i.e., focal males), respectively. All males used throughout the experiment were isolated—kept without females—for more than 24 h before being used, so that sperm replenishment was guaranteed and mating was more likely to occur.

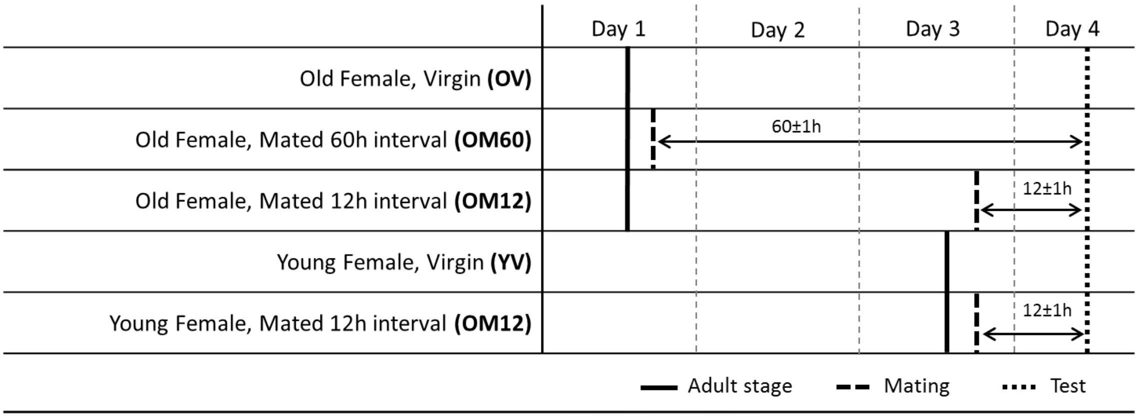
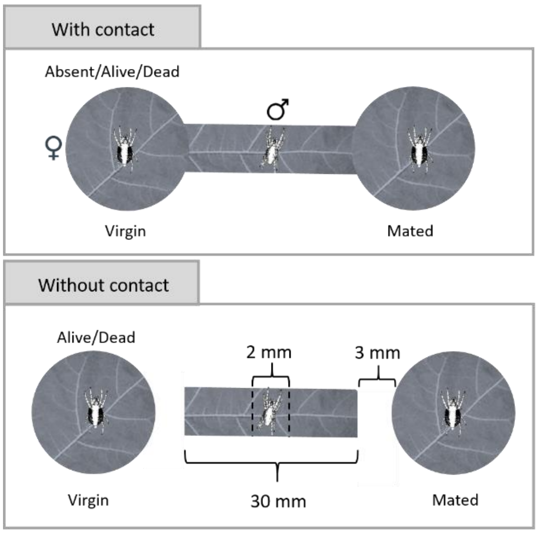


Figure 1. Experimental procedure to test **Tetranychus urticae** ***male choice.*** *To test whether males prefer females having mated at different intervals, females were randomly allocated to different conditions of age, mating status and mating interval. After emergence (solid line), virgin females were left alone on day 1 and tested (dotted line) on day 4 (OV); or they were placed with males (dashed line) on day 1 and tested with new males (dotted line) on day 4 (OM60); or they were left alone, placed with males (dashed line) on day 3 and tested with new males (dotted line) on day 4 (OM12). On day 3, after emergence (solid line), new virgin females were left alone until day 4 and tested (dotted line) on day 4 (YV); or they were placed with males (dashed line) and tested with new males (dotted line) on day 4 (YM12). Hence, the preference of males for each female type was tested always on day 4 (dotted line), but females had different ages and mating histories.*

Type of chemical cues used in male discrimination

To disentangle the role of tactile stimuli (contact compounds), volatile compounds and the presence of chemical trails in male discrimination ability, male choice between virgin and mated females was tested while varying the type of cues available to males (Figure 2). To this aim, quiescent females were collected from a subset of the base population (approximately 500 individuals) and they were placed in a patch of approximately 10 cm^2—isolation patch—, either with an average of two males per female—mated females—, or left alone, remaining virgins. The next day, males were removed. Two thirds of the females were isolated on the leaf discs used for the test the next day (treatments ‘’alive’’ and ‘’absent’’). One half of those females was removed from the patch immediately before the test, so that only chemical trails would remain (treatment ‘’absent’’), whereas the females of the other half were left intact (treatments ‘’alive’’). In addition, on the day of the test, the females remaining on the isolation patches were frozen at -4 °C for 1–2 hours prior to the beginning of the tests (treatments ‘’dead’’). These females were then added to empty leaf discs. Immediately before adding the focal male, a bridge (3 cm x 0.5 cm) was added, either connecting (‘’contact’’) or not (‘’no contact’’) the two leaf discs (0.64 cm^2). This setup, made entirely of bean leaf, was used to perform the choice experiment (Figure 2).



Treatment	Chemical trails	Volatile Compounds	Contact Compounds
Absent with Contact	✓	✗	✗
Alive with Contact	✓	✓	✓
Alive without Contact	✗	✓	✗
Dead with Contact	✗	✗	✓
Dead without Contact	✗	✗	✗

Figure 2. Experimental setup to address cues used by males to distinguish virgin from mated females. *To test which type of chemical cues males use to discriminate among females with different mating status, male choice between virgin and mated females was tested under various combinations of type of arena (Contact/No-Contact) and female condition (alive, absent, dead).* A dumbbell-shaped *arena, consisting of two bean leaf discs of 0.64 cm^2 connected to each other by a bridge (3 cm x 0.5 cm), was used to perform the choice experiment. In treatment “alive with contact”, males had access to all types of cues, and in treatment “dead without contact” no cue could be perceived. In treatments “alive without contact”, “absent with contact” and “dead with contact”, the focal male could only perceive volatile compounds, chemical trails or direct contact, respectively.*

Combinations of type of arena (contact/no-contact) and female condition (alive, absent, dead) were chosen in order to allow the focal males to perceive only one, all or none of the three types of cues. Treatments ‘’alive with contact’’, in which males had access to all types of cues, and ‘’dead without contact’’, in which no cue could be perceived, were used as positive and negative controls. In treatments ‘’alive without contact’’, ‘’absent with contact’’ and ‘’dead with contact’’, the focal male could only perceive volatile compounds, chemical trails or direct contact, respectively (Figure2). In every test, a virgin and a mated female from the same treatment were placed in opposite sides of the arena.

Focal males were collected at the quiescent stage and kept isolated for 1 day, in order to ensure their sperm was not depleted. The tests began with the placement of the focal male on a neutral zone (a 2 mm wide strip in the centre of the bridge). Each test lasted for 1 hour and total time spent by a male on either side of the neutral zone was recorded. A test was considered invalid if a male drowned or a female left her leaf disc. In total, 55 replicates of ‘’alive with contact’’ and ‘’alive without contact’’ and 54 replicates of ‘’absent with contact’’, ‘’dead with contact’’ and ‘’dead without contact’’, were analysed. We only performed this experiment for virgin vs mated females because in experiment (a) we found no difference in preference for recently-mated versus earlier-mated females (cf. Results).

To enhance the amount of volatile compounds, and of chemical trails in the females’ leaf discs, live females were placed in the discs 24 hours before the tests. Dead females, however, were placed on discs immediately before the beginning of the tests as, in that case, we wanted to minimize the loss of contact cues—due to tissue decomposition— following the females’ death. As such differences in the time females were placed on a patch could influence male behaviour, we first tested if male preference for live virgin over live mated females did not differ when females were placed in the leaf discs 1 hour before the test or 24 hours earlier, in a setup without contact.

Statistical analyses

Male discrimination ability: female status, age and mating interval

All statistical analyses were performed with the software R (version 3.0.3, R Development Core Team 2014). In order to test if touch and copulation were correlated, we did a Fisher exact test for each preference test. A strong correlation was found between first female touched and mated in every preference test (cf. Results). We thus conducted two different analyses of male choice: we considered that a male chose a female (a) when the female was touched and then mated, or (b) when the female was touched. As the results of the two analyses were similar, we only present the results of (a).

To test for a correlation between male choice and female pigment ink colour we used a Pearson's Chi squared test. This test was done to determine if there was an overall effect of colour on male choice behaviour. Since no correlation was found ($X^2_4 = 2.08$, P = 0.72), colour was not included in the subsequent statistical models. We then tested male choice within each preference test, again using a Pearson's Chi squared test.

To test for differences in mating behaviour between treatments (i.e., types of females), we used latency and duration of copulation as the response variables. To approximate both models to the assumptions of normality, we transformed the response variables using a boxcox transformation (package MASS, boxcox procedure). Treatment (of the chosen female), which was our variable of interest, was included as a fixed factor, and day and preference test as random factors. However, since none of the random factors was significant in both analyses (package lmerTest, rand() procedure), they were removed from the final models. We analysed both latency to copulation and copulation duration using a general linear model (lmer, lme4 package), with a gaussian distribution. A multiple comparison of means using a Tukey Contrast (multcomp package, glht procedure), test was done to compare duration and latency to copulation among female types.

Type of chemical cues used in male discrimination

To determine if the time spent by females on the leaf discs could influence male attraction, we used a generalized linear model with a quasibinomial error distribution (glm), as the response variable—the proportion of time spent with virgin females—was overdispersed. Time spent by females in the leaf discs—24 or 1 hours—and day were used as fixed factors. No differences were found between 24 hours and 1 hour in time spent with virgin females ($F_{1,54} = 0.0012$, P = 0.973).

A Wilcoxon signed rank test with continuity correction was used to discard preference for one side of the arena—left or right, and no preference was found (V = 17169.5, P = 0.328). The same test was used to analyse our variable of interest—the proportion of time spent by the males with a virgin female. Each treatment was analysed separately and compared to a proportion of 0.5, i.e., to a situation with no preference.

Results

Male discrimination ability: Female Status, Age and Mating Interval

A significant correlation was found between first female touched and first female mated in every preference test (OV x YV: $X^2_1 = 21.59$, $P<0.001$; YVYM12: $X^2_1 = 17.249$, $P<0.001$; OVO x M60: $X^2_1 = 14.196$, $P<0.001$; OVO x M12: $X^2_1 = 13.5807$, $P<0.001$; YVY x M12: $X^2_1 = 17.249$, $P<0.001$);

OM12OM60: X^2_1 = 17.897, *P*=<0.001). Every time a preference test was composed of females that were either both virgin or both mated, males showed no preference, irrespective of the female age or mating interval (OV x YV: X^2_1 = 2, *P* =0.157; OM60 x OM12: X^2_1 =0.08, *P* =0.777; OM12 x YM12: X^2_1 =0.49, *P* = 0.484; Figure 3). When males were offered the choice between a virgin and a mated female, they always preferred virgins, again, irrespective of the female age or mating interval (OV x OM60: X^2_1 = 18.75, *P* < 0.001; OV x OM12: X^2_1 = 18.843, *P* < 0.001; YV x YM12: X^2_1 = 23.113, *P* < 0.001; Figure 3).

Both the time until copulation (i.e., latency to copulation) and the time males spent copulating with a female (i.e., copulation duration) were significantly different between treatments (i.e., types of females) ($F_{4,296}$ = 5.37, *P* < 0.001 and $F_{4,292}$ = 24.3, *P* < 0.001, respectively). Pairwise comparisons (Tuckey Contrasts) revealed that treatments can be grouped into two groups: virgin females (OV and YV) and mated females (OM60, OM12 and YM12) (Table 1, Figure 4). The two groups do not overlap in time of copulation but differences between OV and YM12 were only marginally significant in latency to copulation (*t* =2.624, *P* =0.067). Overall, latency to copulation was lower and copulation duration higher when males mated with virgins, as compared to when they copulated with mated females. Moreover, and confirming the choice analysis, neither the mating interval nor the age of the female affected significantly latency and copulation duration.

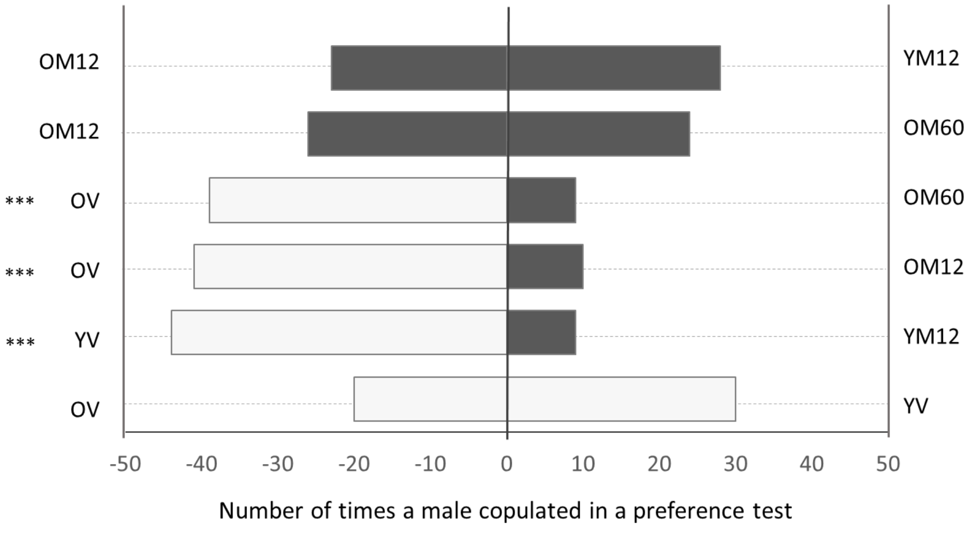


Figure 3. Male preference between different types of females. *Each bar represents the number of times a male mated first with a certain type of female (i.e., treatment) within a preference test. Each preference test was done by placing two females of two different treatments (e.g. Preference test: OV x YV) with a male. Treatments: Young virgins (YV); old virgins (OV); young females that mated 12 hours before the choice test (YM12); old females that mated 12 hours before the choice test (OM12); and old females that mated 60 hours before the choice test (OM60). Dark grey plots: mated females; light grey plots: virgin females.*

Type of chemical cues used in male discrimination

Males did not show a preference for virgins in treatments where females were dead, independently of the type of design used (contact or no contact) (treatment “dead without contact”: = 787.5, *P*= 0.527; treatment “dead with contact”: V= 915.5, *P*= 0.137; Figure 5). In contrast, males from all other preference tests spent more time with virgins than expected by chance (treatment “absent with contact”: V= 974.5, *P*= 0.045; treatment “alive with contact”: V= 1268, *P*= < 0.001; treatment “alive without contact”: V= 1288, *P*= < 0.001; Figure 5).

	comparison [a]	Estimate	Std. Error	t-value	*P-value* [b]
latency to copulation	OM12 x OM60	-0.250	0.682	-0.366	0.996
	OV x OM60	-1.714	0.624	-2.746	0.048*
	YM12 x OM60	-0.019	0.787	-0.024	1.000
	YV x OM60	-2.050	0.678	-3.023	0.022*
	OV x OM12	-1.465	0.513	-2.852	0.036*
	YM12 x OM12	0.231	0.702	0.328	0.997
	YV x OM12	-1.800	0.578	-3.115	0.017*
	YM12 x OV	1.695	0.646	2.624	0.067.
	YV x OV	-0.335	0.508	-0.660	0.964
	YV x YM12	-2.030	0.698	-2.908	0.031*
copulation duration	OM12 x OM60	-1.798	3.557	-0.505	0.986
	OV x OM60	19.141	3.255	5.880	<0.001**
	YM12 x OM60	4.638	4.137	1.121	0.790
	YV x OM60	19.742	3.567	5.534	<0.001**
	OV x OM12	20.938	2.678	7.820	<0.001**
	YM12 x OM12	6.435	3.700	1.740	0.404
	YV x OM12	21.540	3.050	7.063	<0.001**
	YM12 x OV	-14.503	3.411	-4.252	<0.001**
	YV x OV	0.601	2.692	0.223	0.999
	YV x YM12	15.104	3.710	4.071	<0.001**

Table 1. Multiple comparisons of latency to copulation and copulation duration between treatments using Tuckey contrasts. *[a] Treatments (i.e., type of female): Young virgins (YV), old virgins (OV), young females that mated 12 hours before the choice test (YM12), old females that mated 12 hours before the choice test (OM12) and old females that mated 60 hours before the choice test (OM60). [b] ** - P < 0.001; * P < 0.05;* **.** *P marginally significant*

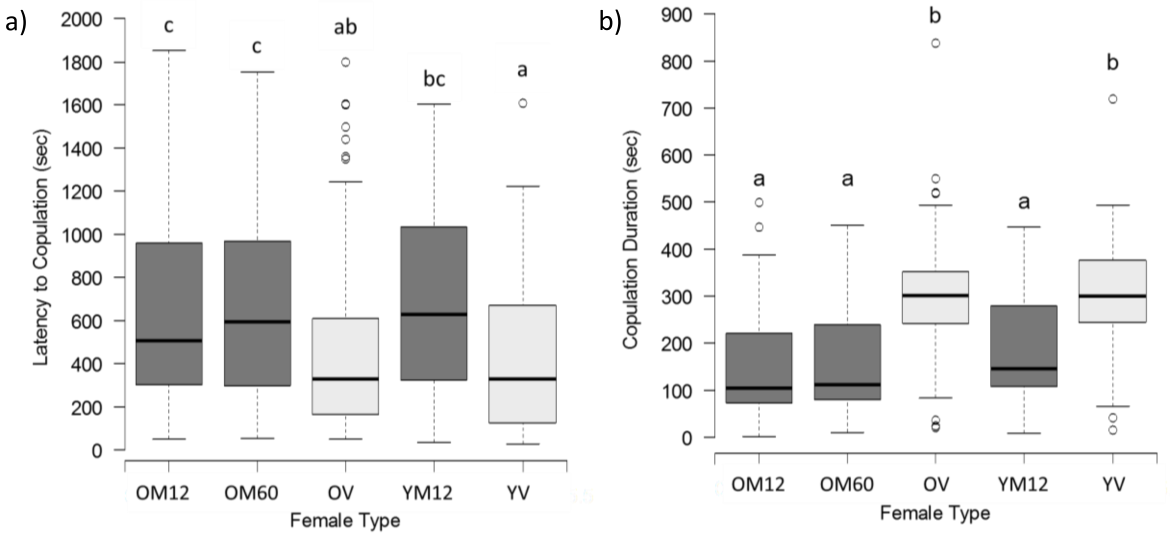


Figure 4. Mean a) latency to copulation and b) copulation duration in matings involving Tetranychus urticae *females of different ages and mating histories. Treatments (i.e., type of female): Young virgins (YV); old virgins (OV); young females that mated 12 hours before the choice test (YM12); old females that mated 12 hours before the choice test (OM12); and old females that mated 60 hours before the choice test (OM60). Different letters indicate significant differences in contrast analysis done using a Tukey test. Dark grey plots: mated females; light grey plots: virgin females.*

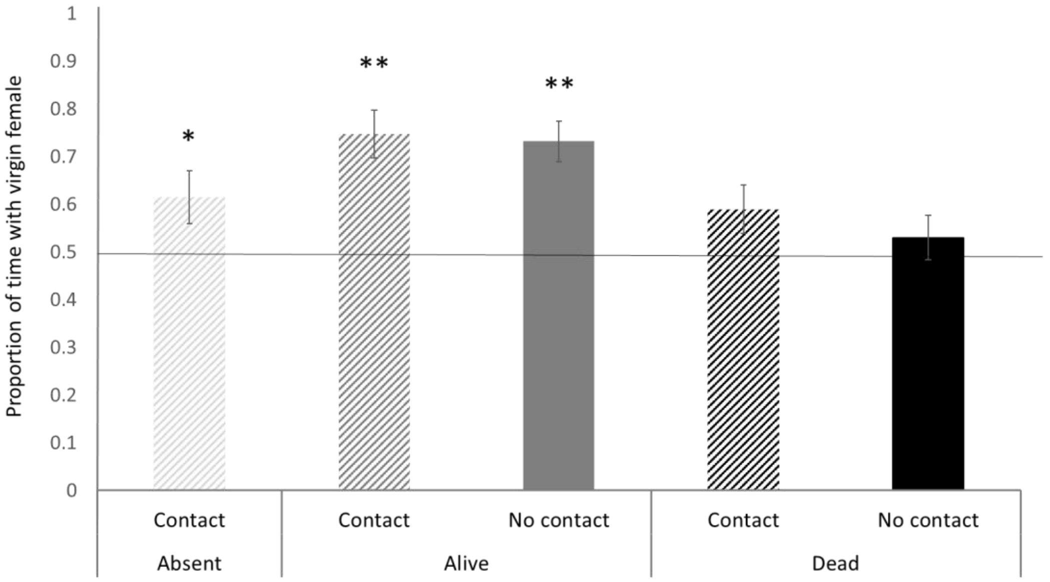


Figure 5. Proportion of time each focal male spent with virgin females during the choice test. *Virgin and mated females were allocated to different combinations of type of arena (Contact/No-Contact) and female condition (Alive, Absent, Dead). Dashed bars: contact; solid bars: no contact; light grey: absent females; dark grey: alive females; black bars: dead females. A Wilcoxon signed rank test with continuity correction was used to analyse the proportion of time spent by the males with a virgin female. Each treatment was analysed separately and compared to a proportion of 0.5, i.e., to a situation with no preference. * P < 0.05; ** P < 0.001*

Discussion

In this study, we set out to investigate which type of cues *T. urticae* males use to distinguish between females with different mating status (virgin or mated). To determine whether males' choice would also involve females with different mating intervals (mated 12 or 60 hours before the test), we first tested whether males distinguish between these female types, while controlling for their age. We found that males preferred virgins over mated females but did not discriminate between females that had mated at different time intervals, supporting earlier results (Oku 2010; Oku 2013). Latency to copulation was shorter and copulation duration longer when males mated with virgins relative to when they mated with mated females, which supports the hypothesis that they are more attracted to the former than to the latter. In addition, no differences were found in latency to copulation and copulation duration of mating events with mated females. Finally, no effect of female age on male choice was found.

In several species, older females were found to be less attractive than younger ones (Johansson & Jones 2007; Delisle & Royer 1994), most likely due to the correlation between age and female fertility. In *T. urticae*, Krainacker and Carey (1990) showed that female spider mites produce a smaller proportion of daughters when mated 5 or 10 days after emergence, compared to females mated immediately after emergence. As age differences used in our experiments were smaller than these, differences in fertility were unlikely to occur among females, and hence to affect male choice. In addition, as shown by Rasmy & Hussein (1994), the sex pheromone released by *T. urticae* females and its perception by males are both affected by age, but only when both sexes are 7 days old, whereas our females were never older than 4 days.

We also found that male choice is not affected by the female mating interval, a result that is consistent with those of Oku (2013), suggesting that this behaviour is not population-specific nor condition-dependent. Three alternative hypotheses may explain these discrimination patterns. A first possibility is that the mating interval does not play a role in determining the degree of first-male precedence. In this case, the fertilization success of males mating with recently-mated and earlier-mated females would be similar. This, however, is not in agreement with the results presented in Helle (1967). The second possibility is that the mating interval plays a role in sperm precedence, but males are not able to perceive differences between females mated at different time intervals. The third possibility is that mating with mated females occurs for a reason different than fertilizing offspring, for example, to limit the effectiveness of fertilization by the first male (Macke et al. 2012). Indeed, in certain species, males are able to increase their relative fitness by removing the sperm from a previous male, or transmitting seminal components that neutralize the sperm of competitors (Chapman et al. 1995). Even without neutralizing the sperm of another male, if the act of re-mating is costlier to females than to males, males are decreasing the fitness of females and, hence, indirectly, that of other males. The extent of such effects could still depend on the mating interval—being probably larger for recently mated females. However, the benefits of evolving a discrimination ability between recently-mated and earlier-mated females could be negligible for males. In order to disentangle these three hypotheses and to establish if the second mating results in viable offspring or reduced fertility of the first male, paternity and fertility tests should be performed in future studies, as well as analyses of female chemical profiles at different mating intervals.

Because males did not discriminate females that had mated at different time intervals, we excluded this choice from the experiment where chemical cues were manipulated. In this experiment, male discrimination was not found in treatments where females were dead. Contact cues, such as CHCs (cuticular hydrocarbons), of adult females have been implicated in male arrestment in *T. urticae* (Royalty et al. 1992), but no discrimination between mated and virgin females had been tested. Possibly, CHC's do not play a role in male discrimination of females with different mating status in this species. Alternatively, such compounds disappear at a fast rate in spider mites. As experiments with other arthropods show that dead females still contain enough CHCs to elicit male discrimination (e.g., Xue et al. 2016), the former hypothesis seems more likely. Nevertheless, we cannot rule out that female behaviour and contact compounds, though apparently not necessary, may contribute to male choice.

Our results clearly indicate that chemical trails and volatile compounds trigger male preference for virgins. Volatile pheromones have been implicated in male searching behaviour for quiescent females, in distances similar to the ones we tested here (Penman & Cone 1972). In addition, in the same study, when males had access to both pheromones and web, male searching behaviour was exacerbated (Penman & Cone 1972). Possibly, the chemical trails used by males in our experiment also include web. This suggests that virgin and quiescent female detection by males may rely on the same type of cues. Although volatile cues are usually considered to trigger mite dispersal from plants (e.g., Pallini et al. 1997), it is not very likely that males use such cues to distinguish virgins from mated females over long distances. This is because females are the most dispersing sex in *T. urticae* (Li & Margolies 1993), hence males will probably always have access to females within their closer environment.

Unlike in the case of attraction towards quiescent females where web alone is not sufficient to trigger a response (Penman & Cone 1972), volatile compounds and chemical trails alone are sufficient to elicit female mating status discrimination in males. The redundancy in mate choice cues is predicted to increase mate-choice costs but also to decrease mate-choice errors. If multiple cues increase the amount of information obtained and/or reduce the energy spent on mate assessment, such redundancy is expected to be selected (Candolin 2003). In addition, different cues may be differentially perceived depending on environmental conditions or distances, hence relying on multiple cues maximizes the probability of a correct mate choice in most environments (Bro-Jørgensen 2010). The fact that spider mite males are able to distinguish between females with different mating status, and that they rely on multiple cues to do so, reinstates that sperm precedence patterns affect the selection pressures on mating traits. Identifying the chemical composition of the cues involved in this behaviour will open the way to manipulative experiments allowing to go further in our understanding of the ecology and evolution of the mating behaviour of spider mites.

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CHAPTER IV

Do they do it for fun? The puzzle of polyandry in a species with first male precedence

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Abstract

In the spider mite *Tetranychus urticae*, most offspring is sired by the first male. However, males often attempt to copulate with mated females. As mating is frequently costly, this behaviour seems paradoxical.

Here, we test the potential costs and benefits of polyandry for *T. urticae* males and females. First, we measured sperm precedence, using a pesticide resistance marker. We found that first male precedence is most likely complete and independent of both mating interval and number of re-matings. Second, we measured the consequences of (re)mating in the fitness of males by measuring survival of males placed with different numbers of virgin or mated females and total offspring sired by first males, as indicator of potential indirect benefits. While being with 5 virgin females entailed survival costs, mating only with mated females did not. Furthermore, by mating multiple times with mated females, males were able to decrease the fitness of first males, indirectly benefiting with re-mating. Finally, we tested a potential benefit of polyandry in females, but found instead that multiple-mated females survived less and laid fewer eggs than females that mated once or twice.

Together, this suggests that opportunity for sexual conflicts in this species might be subtler than previously thought.

Keywords

Multiple mating; sperm competition; mating costs; spider mites

Introduction

Sperm competition plays a major role in the evolution of individual reproductive strategies. Indeed, patterns of sperm precedence, which range from last male to first male sperm precedence, give rise to different evolutionary predictions on mating rate, both in males and females (Danielsson 1998). In species with last male sperm precedence, selection in males should favour increased mating frequency as a result of adaptation to sperm competition (Ridley 1989b). However, the benefits obtained from multiple mating are not as straightforward for females, potentially leading to sexual conflict over mating rate (Bateman 1948; Arnqvist & Rowe 2005). In species with complete first male precedence, neither males nor females benefit from matings involving mated females. Hence, selection in these species should favour monandry, as both sexes are expected to invest all resources in copulations involving virgin females only (Thomas 2011). This pattern of sperm priority favours the evolution of pre-copulatory behaviours (Ridley 1989b; Ridley 1989a). However, even though males guard immature females and discriminate between virgin and mated females (Potter et al. 1976; Rodrigues et al. 2017; Stoltz et al. 2007; Rypstra et al. 2003; Simmons et al. 1994), female re-mating is not absent and may even be common (Allen et al. 1994; Helle 1967; Rodrigues et al. 2017; Bullini et al. 1976). Curiously, empirical studies addressing this paradox are remarkably scarce (Fisher et al 2013, Boulton et al. 2016).

Several possibilities could help explain this contradictory behaviour. One possibility is that males copulate with mated females because this enables them to sire offspring, i.e., if first male sperm precedence is incomplete. Different factors have been shown to affect the sperm precedence pattern in several invertebrates (Danielsson 1998; Simmons 2001). For example, the pseudoscorpion *Cordylochernes scorpioides* changes its precedence pattern, from last to mixed paternity, when females mated successively three times instead of twice (Zeh & Zeh 1994). Also, *Culex pipiens* second matings were only effective within the first 48 hours after the first mating, accounting for the siring of 10% of the offspring (Bullini et al. 1976). In addition, male traits and mating strategies, like male size and copulation duration, can also lead to intraspecific variation in male fertilization success. For instance, in the spider *Linyphia triangularis,* a complete mating results in first male sperm precedence, while an incomplete mating changes the precedence pattern to last male sperm precedence (Weldingh et al. 2011). In the butterfly *Pieris napi*, larger first males have higher fertilization rates, and this in turn influences the fertilization success the second male (Bissoondath & Wiklund 1997).

Even if males do not benefit directly (i.e., sire offspring) from mating with fertilized females, this behaviour may be selected if it provides indirect benefits to males. For example, by mating with mated females, males may be able to displace or kill the sperm inside the female and thereby indirectly increase their own reproductive success (Manier et al. 2010; Macke et al. 2012). Alternatively, males may mate indiscriminately early in their lives to gain experience in mating behaviour. This is the case for *Drosophila melanogaster* males, which initially mate indiscriminately with males and females (Gailey et al. 1982) but later in life learn to distinguish between sexes. A similar pattern could occur between virgin and mated females.

However the benefits, the cost of mating for males may also vary with the mating status of the female (i.e., virgin or mated), if males allocate sperm differently according to the reproductive value of females ("strategic ejaculates", Simmons 2001; Kelly & Jennions 2011). Females of increased value have been shown to receive more or better sperm than females of lower reproductive value (Kvarnemo & Simmons 2013; Kelly & Jennions 2011). For example, males of Australian field crickets, *Teleogryllus oceanicus*, can detect different intensities of sperm competition and allocate sperm of different quality according to the mating status of females (Thomas & Simmons 2009). Following the same logic, *D. melanogaster* males mate longer with mated females and transfer more sperm than with virgins, suffering more costs with longer copulations (Friberg 2006). Differences in male costs could also be due to differences in receptivity between the mating status of females. Indeed, mated females have been shown to reduce their receptivity after mating, due to the presence of seminal substances in the sperm of the male in several species (Simmons 2001). The costs of mating with mated females for males could then increase, due to increased female resistance (Simmons 2001).

Finally, female re-mating in species with first male precedence may occur if it is beneficial for females. For example, females may benefit from nutrients in seminal fluids (Arnqvist & Nilsson 2000). This benefit may translate into a direct benefit for females, for example by producing more offspring, as shown in the bruchid beetle *Callosobruchus maculatus* (Eady et al. 2000). Moreover, mating multiply may result in increased survival. For instance, in the wasp *Nasonia vitripennis*, females increase their fecundity and survival when mating multiply with virgin males (Boulton & Shuker 2015).

The two-spotted spider mite – *Tetranychus urticae* – is an excellent species to test each of these hypotheses as it is characterized by an arrhenotokous genetic structure, producing haploid sons, which result from unfertilized eggs, and diploid daughters, stemming from fertilized eggs (Helle & Sabelis 1985). Therefore, male genetic contribution to the next generation is easily measured as the number of daughters produced by each female. In addition, a recessive mutation for resistance to a pesticide can be used in this species as a genetic marker to determine offspring paternity (see details below) and thus sperm precedence patterns. Finally, earlier studies suggest that in this species only the first copulation of a female is effective (Helle 1967). This, together with limited mating capacity in males (Krainacker & Carey 1989), leads to the expectation that males should only mate with virgin females to avoid unnecessary costs. Indeed, males actively guard juvenile quiescent females and mating occurs as soon as females become virgin adults (Potter et al. 1976), a behaviour that is consistent across species with first male sperm precedence (Ridley 1989a). Moreover, when males outnumber virgin females, they fight for their possession (Potter et al. 1976). In accordance with these observations, recent studies showed that males use volatiles and chemical trails to distinguish virgins from mated females (Rodrigues et al. 2017), and that they prefer to mate with virgins (Rodrigues et al. 2017; Oku 2010). Consequently, both sexes are expected to invest all resources in the female's first mating event. However, surprisingly, males often mate with mated females in the absence of virgin ones, and mated females accept copulations (Oku 2010; Clemente et al. 2016). Here, we provide a comprehensive account of potential costs and benefits of such behaviour for both males and females. Specifically, we (a) performed paternity tests to describe the sperm precedence pattern in this species, (b) compared the total number of daughters sired by first males mated to females with different mating status, to assess the potential benefits of mating with mated females for males, (c) analysed differences in male survival in the presence of different numbers of virgin or mated females, and (d) measured the fecundity, survival and offspring sex-ratio of females that re-mated at different time points.

Materials and Methods

Spider mite populations, rearing conditions

Three populations were used in this study. The TuTOM population was collected in Carregado, Portugal, in 2010 and was established at the University of Lisbon from approximately 300 individuals. The London strain, used as reference to sequence and annotate the spider-mite genome (Grbić et al. 2011) and a kind gift from M. Grbic, was originally collected in the Vineland region, Ontario, Canada and maintained at the University of Logroño. The EtoxR strain was originally collected in Japan and maintained for 5 years in the laboratory at Bayer CropScience before being transferred to Ghent's University, where it was maintained on potted bean plants and sprayed until runoff with 1,000 mg active ingredient per litre of etoxazole, a commonly used pesticide. This strain is fully resistant to etoxazole. The London strain, on the contrary, is completely susceptible to the same pesticide. Both strains were established at the University of Lisbon in 2013 from approximately 2000 individuals. EtoxR strain was infected by *Wolbachia* and *Rickettsia* and TuTOM was infected with *Wolbachia*. Although London strain was not infected, to account for potential side effects of antibiotic treatment, all strains were treated with antibiotics. This was done by placing groups of 30 adult females in petri dishes containing bean leaf fragments placed on cotton wet with 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 more than three generations. Before use, pools of 100 females were checked by PCR to confirm the absence of endosymbionts.

All spider-mite populations were reared in large numbers (>2000) on bean plants (*Phaseolus vulgaris*, Fabaceae, var. *Enana*; Germisem Sementes Lda, Oliveira do Hospital, Portugal), under controlled conditions (25°C, photoperiod of 16L: 8D).

Experimental Setup

Sperm precedence and potential benefits for male and female fertility

Some spider mite populations are resistant to etoxazole, a pesticide that interferes with chitin synthesis and deposition (Van Leeuwen et al. 2012), affecting spider mite embryos and juvenile stages at the time of hatching or ecdysis. In the EtoxR strain, resistance to this pesticide is recessive and conferred by a single chitin synthase 1 amino acid change (Van Leeuwen et al. 2012). In addition, in this strain resistance is fixed, which makes of resistance an excellent tool to be used as a marker for paternity tests (see protocol below). For this experiment, we used the EtoxR strain, resistant to etoxazole, and the London strain, fully susceptible to the same pesticide.

Virgin females from the EtoxR strain were randomly selected and allocated to 10 different treatments resulting from the combination of the following conditions: females could a) mate once, twice or multiply, b) in the latter two cases, mate either first with a resistant and then with susceptible males, or the opposite, and c) be given a mating interval between the first and subsequent matings of either 0 or 24 hours. The mating protocol was designed as follows (Figure 1): EtoxR quiescent females selected from a subset of the base population (approximately 500 individuals) were isolated for 24 hours on leaf discs on water-saturated cotton without males. Once they became adults (one day later), groups of 5 females were allowed to mate with 6 susceptible (S) - or resistant (R) - males on 0.8 cm^2 leaf circles. The patches were observed for 2 hours and every time there was a successful cross, the mated female was transferred to a new patch, either empty or with males of the other strain, in the same proportion. Half the females placed with males were observed for two more hours and isolated when mated (SR0/RS0). The other half was left unobserved in the same patch for 24 hours, thus allowing multiple matings (SmR0/RmS0) and, the next day, males were removed from the patch. The females left alone after the first mating on the first day were either left alone for one more day (S/R) or transferred to patches with males of the alternative strain. As in the previous day, half the females placed with males were observed for two more hours and isolated if mated (SR24/RS24) and the other half was left unobserved in the same patch for 24 hours (SmR24/RmS24). All females that did not mate and all matings that were interrupted were discarded. When females were two- to three-days old, they were isolated on a 2.55 cm^2 leaf disc placed on water-soaked cotton. Subsequently, females were transferred every three days to a new leaf and the eggs oviposited on the old leaves were counted. Female survival (FS) was followed daily. Average female daily fecundity was estimated considering their survival (DF = total number of eggs laid per female / number of days the female was alive). To assess paternity, eggs were allowed to develop until the juvenile stage (three days after female transfer), then leaf discs were transferred to water-soaked cotton in which we diluted etoxazole to get a new medium with 500 ppm of pesticide. Six days later, the number of adult daughters, adult sons and dead juveniles on each leaf disc was recorded. The number of alive daughters and dead juveniles indicate, respectively, the amount of offspring sired by resistant and by susceptible males. In this way, we expect all, none or some daughters to die after pesticide application, depending on whether there is complete first, complete last or incomplete first male sperm precedence and whether the first male mating with the female is resistant or susceptible to the pesticide. Note however, that natural death in the quiescent stage may be confounded with death by pesticide exposure.

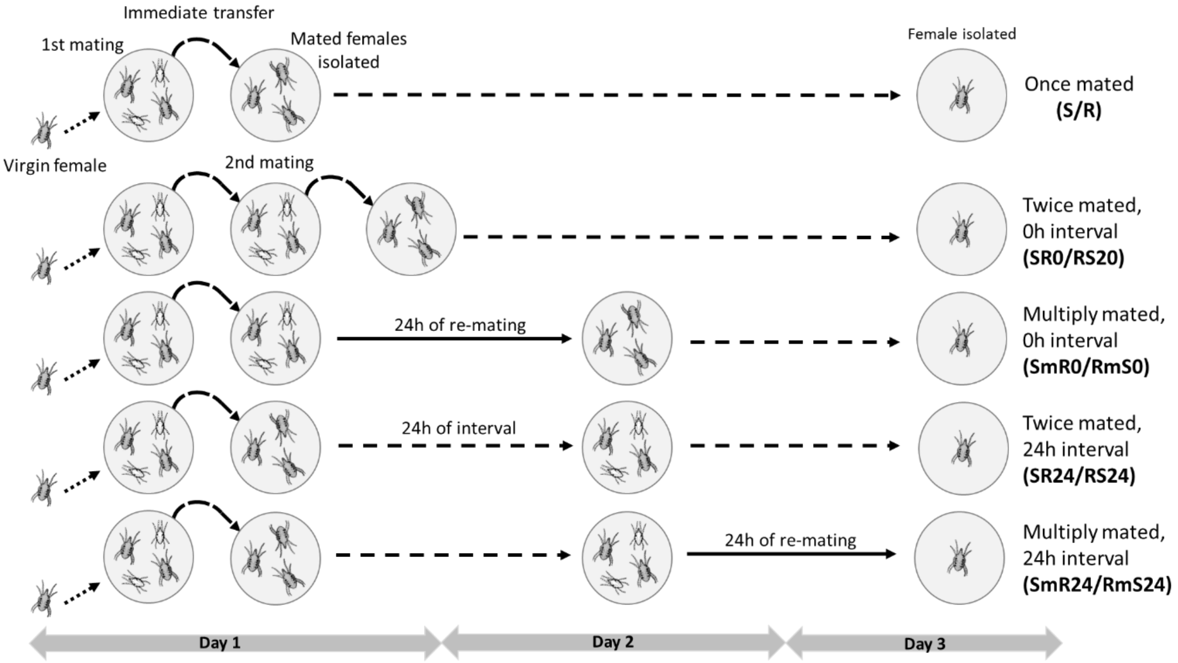

Figure 1. Protocol followed to assess sperm precedence. *Females could a) mate either first with a resistant and then with susceptible males, or the opposite, b) mate once, twice or multiply and c) mate with a mating interval between the first and subsequent matings of either 0 hours or 24 hours. Male type: R – resistant male from the EtoxR strain, S- susceptible male from the London strain. The first letter corresponds to the first male that copulated with a resistant female and the second letter to that of the second/subsequent male/s that mated with the same female. m – multiple matings; 0 – immediate second mating; 24 – twenty-four-hour interval between matings.*

Consequences of mating frequency and female identity for male survival

This experiment aimed to assess whether different numbers of females, and/or females with different mating status (i.e., virgin or mated) induced differential mating costs in males. Differences are expected as virgins are more valuable than mated females for *T. urticae* males, due to first male sperm precedence. Indeed, males prefer to mate with virgins rather than with mated females (Oku 2010; Rodrigues et al. 2017). In addition, males exhibit lower latency to copulation and mate longer with virgins than with mated females, suggesting these females have lower reproductive value (Rodrigues et al. 2017). To test this, males were individually placed in patches with different numbers of females of different mating status, replaced every day, and male survival was measured. Focal males were thus assigned to one of 6 treatments: they were placed with 1, 5 or 20 females and these females could be either mated (M1, M5, M20) or virgin (V1, V5, V20).

Spider mite males and females were isolated separately at the quiescent stage, to control their age and mating status (virgin) prior to the experiment. When these individuals became adults (*circa* 24 hours later), groups of ten females were either left isolated (virgin – V) or placed with 15 males (mated – M). The next day, focal virgin one-day old males were placed in a leaf circle with either 1, 5 or 20 mated or virgin females. To normalize densities across treatments, the size of the patch varied according to the number of individuals (0.38 cm^2, 2.55 cm^2 or 9.1 cm^2 for patches receiving 1, 5 or 20 females, respectively). The focal male was then transferred daily to a new patch with the same number of (mated or virgin) females in every treatment except for the one with 20 females. Since we know that male mating capacity decreases with age (Krainacker & Carey 1989), from the third day on, the focal male was placed with 12, instead of 20 females (size of the patch: 6.25cm^2). Every day, until death, male survival (MS) was recorded.

Statistical analyses

All analyses were carried out using the R statistical package (v. 3.0.3). 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, in the case of discrete distributions or F tests, in the case of continuous distributions (Bolker et al. 2008).

Sperm precedence and potential benefits for male and female fertility

The general procedure for building the statistical models used to analyse the effects of mating on female's fitness was as follows: the number of re-mating (i.e., 0: once-mated, 1: twice-mated and M: multiply-mated) and the mating interval (i.e., 0 and 24) were fit as fixed explanatory variables, whereas block and male type order (mated first with a resistant and then with susceptible males, or the opposite) were fit as random explanatory variables. Once-mated females were allocated randomly to the two mating intervals, so they could be analysed together with the female belonging to the other levels of number of re-mating.

To analyse the proportion of offspring sired by the first male, we created two variables called contribution of the 1st male (1M) and contribution of the 2nd male (2M) to offspring. The first consisted in the number of dead juveniles or the number of alive daughters, depending on whether the first male was susceptible or resistant, respectively. The second consisted in the number of alive daughters or the number of dead juveniles, depending on whether the first male was susceptible or resistant, respectively. These parameters were computed using the function cbind. As they were greatly over-dispersed, we used a generalized linear mixed model with a beta-binomial error distribution and added the term zeroInflation=TRUE to the models (glmmadmb, glmmADMB package) (Fournier et al. 2012; Skaug et al. 2013). We also analysed the total number of daughters sired by the first male, using the variable we named contribution of the 1st male (1M). This parameter was analysed using a model with negative binomial distribution and the term zeroInflation=TRUE, (glmmadmb, family="nbinom", glmmADMB package) to account for the overdispersion of the data (Θ= 14.624).

Survival (FS) was analysed using Cox proportional hazards mixed-effect models (coxme, coxme package). Since the interaction between the fixed variables was found to be significant, we analysed separately each "re-mating interval". *A posteriori* contrasts (Crawley 2007) between the number of re-matings were carried out by aggregating factor levels together and by testing the fit of the simplified model using ANOVA.

Daily fecundity per female (DF) was transformed to improve normality (λ=0.125) (Box-Cox transformation; Crawley 2007) and subsequently analysed using linear mixed-effect models (lmer, lme4 package). *A posteriori* contrasts with Bonferroni corrections, were done to interpret the significant interaction between the two fixed variables (testInteractions, phia package).

Consequences of mating frequency and female identity for male survival

To analyse the effects of mating on male survival, female status (i.e., M. mated, V: virgin) and the number of females in each patch (1, 5, 20) were fit as fixed explanatory variables, whereas block was fit as a random explanatory variable. Male survival (MS) was analysed using a Cox proportional hazards mixed-effect model (coxme, coxme package). When the interaction between the fixed factors was significant, we analysed separately each female number for the effect of female status.

Results

Sperm precedence and potential benefits for male and female fertility

Overall, there was no significant effect of the mating interval (X^2_1= 0.358, P=0.549), of the number of re-matings (X^2_2=0.341, P=0.843), or of their interaction (X^2_2=2.966, P=0.227) on the proportion of offspring sired by the first males (Figure 2). Since this analysis includes females that did not re-mate, this result indicates complete, or nearly complete, first male sperm precedence in this species. However, a significant effect of the interaction between mating interval and number of re-matings was found for the total number of daughters sired by the first male (mating interval: X^2_1=0.194, P=0.659, number of re-matings: X^2_2=2.024, P= 0.364 and mating interval-number of re-matings interaction: X^2_2= 20.457, P=<0.001). Indeed, multiply-mated females with an interval of 24 hours between first and subsequent matings produced fewer fertilized offspring, compared to once and double mated females of the same mating interval (0-1: X^2_1=0.006, P=1.00; 0-M: X^2_1=17.555, P=<0.001; 1-M: X^2_1=18.471, P<0.001; Figure 3). On the contrary, when the interval between matings was of 0 hours, no significant differences in total number of offspring were observed (0-1: X^2_1=0.007, P=1.00; 0-M: X^2_1= 1.516, P=1.00; 1-M: X^2_1= 1.565, P=1.00; Figure 3).

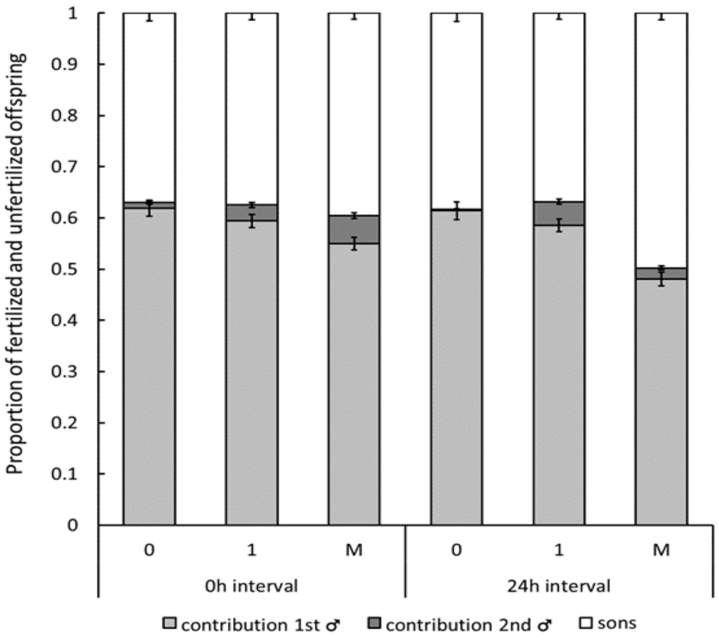

Figure 2. Proportion of fertilized and unfertilized offspring across treatments. *Fertilized offspring is divided in proportion sired by the first (contribution 1st* ***♂****) and the second male (contribution 2nd* ***♂****). Females re-mated 0, 1, or multiply (M). Re-mating was set immediately (0h interval) or 24 hours (24h interval) after the first mating. Vertical bars correspond to standard errors of the mean.*

The number of re-matings affected differentially the survival of females, depending on the interval between matings (number of re-mating - mating interval interaction: X^2_2=9.01, P=0.011). Further analyses conducted for each interval separately revealed that females survived the same amount of time when the interval between matings was of 0 hours (X^2_1=0.39, P=0.532; Figure 4). When the interval between matings was of 24 hours, however, females that mated multiply survived less than females that did not re-mate or that re-mated once (X^2_1=4.39, P=0.036 and X^2_1=6.82, P<0.001 respectively; Figure 4). The same pattern was found for daily fecundity. The interaction with number of re-matings and mating interval was significant ($F_{2,428}$=10.03, P<0.001): there were no significant differences in fecundity when the interval between matings was of 0 hours (0 vs M: X^2_1=3.28, P=0.421; 1 vs M: X^2_1=2.61, P=0.638; 1 vs 0: X^2_1=0,01, P=1; Figure 5) but when the interval between matings was of 24 hours, females that mated multiple times laid less eggs per day than females that did not re-mate or re-mated twice, (X^2_1=12.22, P<0.001 and X^2_1=17.06, P<0.001 respectively; Figure 5).

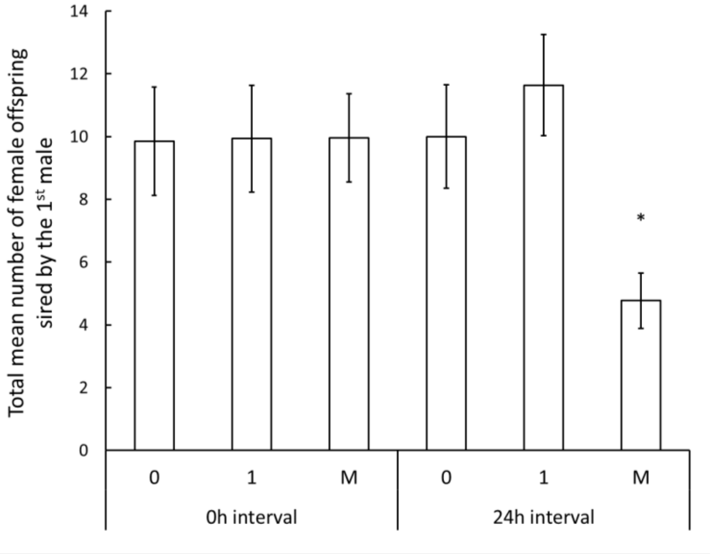

Figure 3. Total mean number of offspring sired by the first male. *Females re-mated 0, 1, or multiply (M). Re-mating was set immediately (0h interval) or 24 hours (24h interval) after the first mating. Vertical bars correspond to standard errors of the mean. * P <0.01*

Consequences of mating frequency and female identity for male survival

The analyses of male survival revealed a significant interaction between female status (i.e., M: mated, V: virgin) and the number of females on each patch (female status - number of females/patch*:* X^2_2=7.07, p=0.029). Further analyses conducted for each number of females separately showed no significant differences in survival when males were placed with virgin or mated females, when 1 or 20 females were added per patch (X^2_1=0.8, p=0.371 and X^2_1=8e-04, p=0.978, respectively; Figure 6). However, males survived longer in the presence of mated females, in patches with 5 females per day (X^2_2=7.085, p=0.008; Figure 6).

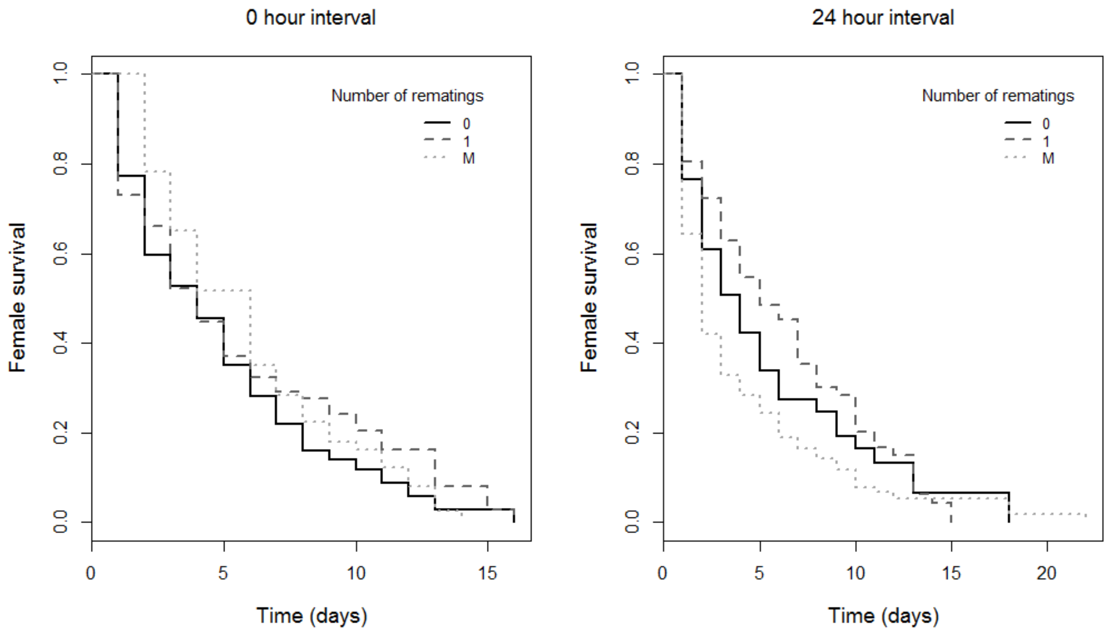

Figure 4. Survival curve of once, twice and multiply-mated females. Re-mating was set immediately (0 hours interval) or 24 hours (24 hours interval) after the first mating. 0 – not re-mated; 1– re-mated once, M – re-mated multiply.

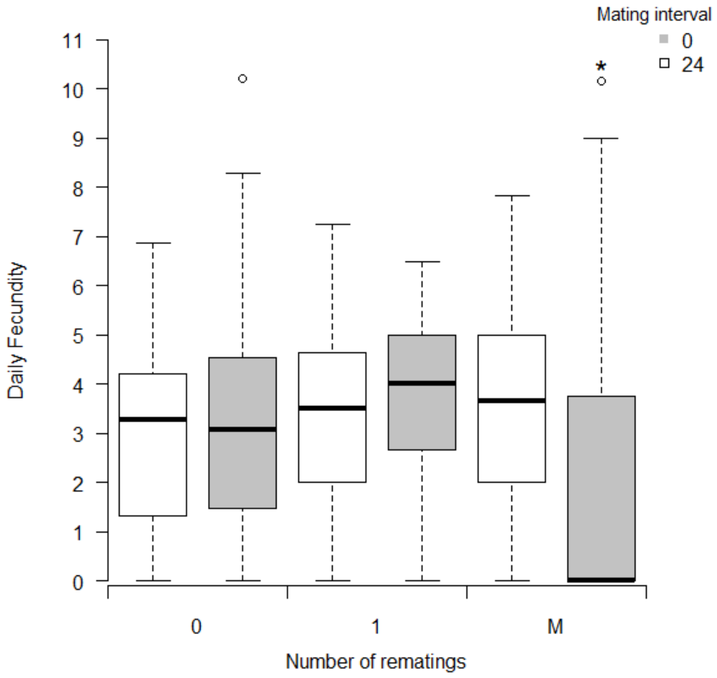

*Figure 5. Mean number of eggs laid daily by females. Females re-mated 0, 1, or multiply (M). Re-mating was set immediately (0h interval) or 24 hours (24h interval) after the first mating. Vertical bars correspond to standard errors of the mean. * P <0.01*

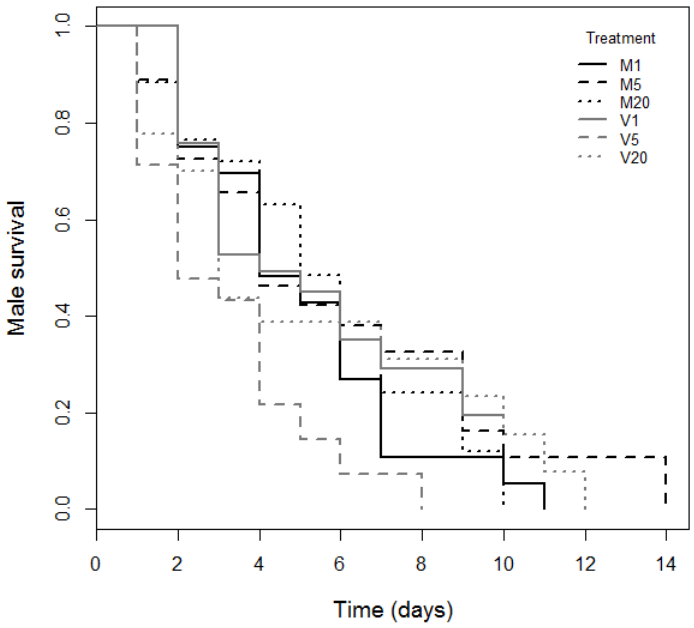

Figure 6. Survival curve of males placed daily with different numbers of virgin and mated females. *V/M1 – male in the presence of one virgin/mated female; V/M5 – male in the presence of five virgin/mated females; V/M20 – male in the presence of twenty virgin/mated females.*

Discussion

Our study revealed that all, or almost all, offspring of spider-mite females was sired by the first male, independently of the mating interval and the number of matings. In addition, the contribution of first males to the production of daughters did not change across treatments and they only suffered increased costs of mating when placed with 5 virgin females daily, and never when they mated with mated females. Finally, a decrease in fecundity and survival was found in females that had multiple mating opportunities after an interval of 24 hours between the first and subsequent matings.

We cannot disentangle complete, to nearly complete, first male sperm precedence, owing to the fact that natural death in the quiescent stage may be confounded with death by pesticide exposure. Even though this is not frequent, there is a non-null threshold of detection for fertilization by second males. In any case, the general conclusion taken from our results is that the contribution of second males is not significantly relevant and probably does not explain the existence of polyandry in this species. In addition, our findings indicate that that first male sperm precedence is independent of the number of matings and their interval contradicts early findings by Helle (1967). This author tested the effect of several mating intervals, from 20 minutes to 6 days, on sperm precedence in spider mites and showed that the second male can sire some offspring if the interval between copulations is shorter than 24 hours. However, he did not control for potential sperm depletion in males, as they were collected from the base population where they were in permanent contact with females right until the test. Indeed, Helle himself (1967) suggested that second males may stand a chance of siring some offspring if the first male has transferred an insufficient quantity of sperm. In our study, we controlled for this to a certain extent, by isolating males prior to testing them. In addition, males could never copulate more than five times, a value below their daily reproductive limit (Krainacker & Carey 1989). Therefore, differences between the study of Helle and ours suggest that sperm depletion may allow some paternity in second males, a hypothesis that deserves further testing.

Another factor proved to influence sperm precedence is mating interruption. Indeed, in *T. urticae*, the success of second males can increase with interrupted first matings (Potter & Wrensch 1978), even if the disruption occurs after complete sperm transfer, i.e., during post-copulatory mate guarding. In our experiment, all interrupted matings were discarded, so this factor is not expected to affect our conclusions. The fact that both interrupted and sperm-depleted matings influence sperm precedence patterns and that both mating interval and number do not, suggests that a physical barrier, such as a mating plug, is established after complete sperm transfer (Boudreaux 1963). It has also been proposed that upon completion of the first mating, a threshold of sperm volume is reached, after which no sperm transfer is possible (Potter & Wrensch 1978). However, old females, most likely partially depleted of sperm, continue not to use the sperm from subsequent males (Potter & Wrensch 1978; Macke et al. 2012), a result that does not support this last hypothesis. It seems likely that subsequent matings can only be effective when there is a problem with the female's first mating. Unfortunately, we have as yet no information on the frequency of both interrupted and sperm-depleted matings in natural populations, which is expected to determine their role in shaping the evolution of sperm priority.

Assuming a low frequency of sperm-depleted females in populations, and given that the mating interval or the number of matings does not affect the degree of sperm precedence in *T. urticae*, there is apparently no reason for post-copulatory strategies to be selected in males (Dougherty et al. 2016). However, another possibility accounting for males mating with mated females is that polyandry can also provide indirect benefits to males. In fact, a decrease in the fecundity of multiple mated females had already been observed in a previous study with spider mites, which led researchers to propose that males could decrease the relative fitness of competitors by mating multiply (Relative Fitness hypothesis, Macke 2012). However, they were not able to confirm this hypothesis, since in haplodiploids, it is the total number of daughters, rather than total fecundity, that determines the reproductive success of males. In our experiment, we could measure the contribution of males to offspring production. In addition, we were able to disentangle the contribution of the first male from that of the subsequent males that mated with the females, owing to the use of resistance as genetic marker. We found that females from all treatments produced the same number of daughters sired by the first male, except when females mated multiply with an interval of 24 hours between the first and the subsequent matings (M24), in which case there was a decrease in the number of fertilized offspring. Consequently, our experiment is the first to show that mating with mated females can indirectly increase male reproductive success. This result could explain, even if partially, why there is multiple mating in this species. Furthermore, we found that the benefit of re-mating is dependent on both the number of re-matings and the interval between matings. Indeed, it seems that the conditions for sperm competition change with these factors. For instance, numerous copulations may be necessary for displacement of the sperm of the first male to occur, as shown in *Trichogramma envanescens* (Damiens & Boivin 2006). In our case, however, sperm competition would be acting without changing sperm precedence. Possibly, the second male produces seminal fluids that reduce the efficiency of the sperm from the first male. Alternatively, the sperm from the first male may activate a barrier against the effect of subsequent sperm that loses its effect around 24 hours after its transfer, explaining why the effect on the number of female offspring is only observed in these mating interval. Again, this would suppose sperm competition does not disrupt sperm precedence.

Polyandry thus seems to be indirectly beneficial for males. However, mating can also entail costs and these costs may differ in matings with virgin and mated females. Here, the costs of polyandry for males were measured by analysing their survival when placed with different numbers of females from one of two different mating status (i.e., virgin or mated). In spider mites, matings with virgin females present a shorter latency to copulation and a longer copulation duration than matings with mated females (Clemente et al. 2016; Rodrigues et al. 2017). This suggests that, for a given amount of time, males are expected to spend more energy in matings with virgin females than in matings with mated females, given that they will mate more often and for longer periods. However, increased latency to copulation in matings with mated females can also reflect increased resistance to male mating attempts. If so, the pre-copulatory investment of males is likely to be higher in matings with mated females. In our experiment, by comparing the survival of males in patches with different numbers of mated females, we found that multiple mating exclusively with mated females does not result in costs of survival in males. However, a decrease in survival was observed when males were placed with 5, but not with 20, virgin females. This result is intriguing. Possibly, the resistance of virgin females is low, hence mating with virgins is not costly per se. In treatments with 20 virgin females, we do not expected an exhaustion of the pool of virgin females, as the number of females per patch is superior to the reproductive limit of spider mite males (Krainacker & Carey 1989), so there might be few costs on these patches. However, in patches with few virgin females, males are likely to mate with the same female repeatedly, suffering the costs of mated-female resistance. Because in those patches they are still perceiving cues from virgin females (Rodrigues et al. 2017), their mating propensity may be higher than in patches only with mated females. Hence, the costs of enduring female resistance may be higher in patches with a mix of virgin and mated females than in patches with mated (or virgin) females only. If that is true, males in patches with 5 virgin females are the ones that will attempt more re-matings and thus suffer higher costs of female resistance. Indeed, males exhibited higher mortality when placed with 5 virgin females than when placed with 5 mated females. Further experiments, for instance observations of locomotion patterns, should be done to determine if indeed males approach more mated females in patches where virgin females had been, compared to empty patches and if those females resist male attempts, increasing the investment of males in each copulation. Such patches, impregnated with pheromones from virgin females, are very likely common in the field, suggesting that costs of mating do exist for males in natural conditions.

Our results suggest that polyandry provides indirect benefit for males and costs can occur when males are placed in patches with a mix of virgin and mated females. It is as yet unclear whether benefits obtained with re-mating outweigh the costs of mating in mixed patches. Hence, we cannot as yet safely conclude that indirect benefits are sufficient to explain multiple mating. In fact, polyandry may have also been selected because it is beneficial for females. As spider mites display first male sperm precedence under the conditions tested, an increase in fecundity or survival of females was only expected if the seminal products from following males could provide the female with nutritious substances or stimulate their egg production (Ridley 1988). However, a stimulation of egg production by the second male without a change in sperm priority would be costly since it would increase the fitness of the first male or at least increase the number of sons produced by a female, leading to an increase in male-male competition. This trait should thus be counter-selected in males. In line with this, we found no benefits of multiple mating for females. On the contrary, females that mated multiply with an interval of 24 hours between the first and the subsequent matings (M24) showed a significant decrease in survival and fecundity compared to females that mated once or twice in any mating interval and females that were presented with multiple mating opportunities immediately after the first. Similar results have been found previously for multiply-mated females in *T. urticae*: Macke et al. (2012) found that virgin females display a higher life span than multiply-mated females, and that treatments where multiply-mated females had more mating opportunities showed decreased daily fecundity. In addition, females living with males produced fewer eggs than females alone (Oku 2010). It is possible that costs of mating are only apparent when females re-mate more than twice, if increased number of matings lead to increased costs.

Differences in fecundity and survival between females that mated multiply with different mating intervals could also be related with age. However, a recent study in this species has shown that males do not show any preference when having to choose between three- and one-day old mated females, nor did latency to copulation and copulation duration differ in crosses with those females

(Rodrigues et al. 2017). Since, all crosses in our experiment were done with females never older than 3 days and an effect of female age or male's loss of attraction is unlikely to explain our results. Another possibility is that the mating interval, instead of affecting the extent of sperm precedence, influences female receptivity. Females may become more resistant to re-mating a certain period after the first copulation, consequently suffering increased costs. In fact, females that mated 24 hours after the first mating, independently of the number of matings, took longer to mate and more easily interrupted matings than females who's matings followed each other immediately (authors personal observations, Clemente et al. 2016).

If females do not benefit from multiple matings, why then do they tolerate them? Possibly, females re-mate more than their optima to mitigate the costs incurred by persistent males attempting to copulate, a strategy called "convenience polyandry" (Thornhill & Alcock 1983). This might be the case if the re-mating rate in this species is driven by the indirect benefits observed for males. However, we cannot conclude that convenience polyandry is acting in our species, as we do not know if increased female resistance leads to higher costs for females.

Here we have explored how polyandry can influence females directly. However, multiple mating can also provide females with genetic benefits. For instance, if there is variation in the genetic quality of males ("good genes" hypothesis; Yasui 1997; Kuijper et al. 2012) or in insemination success ("sexy sperm" hypothesis; Kuijper et al. 2012), by mating with several males, females can improve offspring quality. Furthermore, multiple mating can increase the chance that females reproduce with males with whom they produce viable offspring (genetic incompatibility avoidance hypothesis (Zeh & Zeh 1996; Zeh & Zeh 1997; Wedell 2013). However, since subsequent copulations are not effective in species with first male sperm precedence, none of these hypotheses hold in such systems. Nevertheless, the existence of high genetic costs in these species can select for a disruption of sperm priority, provided polyandry has been already established (Boulton & Shuker 2015). This hypothesis has seldom been tested (but see Boulton & Shuker 2015).

With this study, we found that first male sperm precedence is complete, or nearly complete, in this species after a successful first mating and that mating can be costly for males. Furthermore, the decrease in offspring number and female survival suggests that females suffer costs by mating multiply. However, males can benefit from polyandry by increasing their relative fitness indirectly. The few benefits of re-mating for males and the costs for females help explaining why mating with mated females is less common than mating with virgins. Based on the results obtained here, it seems that in this species the opportunity for sexual conflicts is much subtler than previously thought. Nevertheless, more studies are needed to fully understand the occurrence of multiple mating in this species.

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CHAPTER V

Does mate choice in spider mites evolve in response to the presence of reproductive manipulators?

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Abstract

Spider mite populations are often infected with *Wolbachia* inducing cytoplasmic incompatibility (CI), whereby crosses between uninfected females and infected males yield reduced fertilized offspring. It is thus expected that uninfected females avoid mating with *Wolbachia*-infected males, potentially contributing to reproductive isolation between populations. To test the prevalence of such behaviour in the spider mite *Tetranychus urticae*, we measured the choice of uninfected females between *Wolbachia*-infected and uninfected males in 5 naturally infected populations. Females showed no preference, but copulations with *Wolbachia*-infected males started sooner and lasted longer than with *Wolbachia*-uninfected males, suggesting that *Wolbachia*-infected males are better competitors.

We then tested whether such behaviour evolved under conditions where it would yield most benefits. To this aim, we performed 20 generations of experimental evolution of spider-mite populations with i) full *Wolbachia* infection, ii) no infection, or iii) mixed infection. In the latter, we expect high benefits from choice, as females are continuously exposed to both infected and uninfected males. Evolving under mixed infection did not affect host mate choice, latency to copulation or copulation duration. Moreover, the previous advantage in *Wolbachia*-infected males disappeared. Therefore, the role of *Wolbachia* in pre-copulatory reproductive isolation in spider mites is probably residual.

Keywords

Wolbachia; spider mites; experimental evolution; cytoplasmic incompatibility; mate choice

Introduction

Wolbachia are widespread endosymbiotic bacteria commonly found in arthropods, which key feature is the capacity of manipulating its host cellular and reproductive processes (Werren et al. 2008). The most frequent *Wolbachia*-induced phenotype is cytoplasmic incompatibility (CI), a mechanism that results in the embryonic death of fertilized offspring from crosses between *Wolbachia*-uninfected females and *Wolbachia*-infected males. As all other crosses are compatible, CI promotes *Wolbachia* transmission by increasing the success of infected females. However, it also reduces drastically the fitness of both uninfected females and infected males, as the number of offspring resulting from incompatible crosses is reduced relative to those of compatible ones. Such an adverse effect of CI is expected to exert a strong selective pressure on hosts to evolve strategies that reduce the frequency and/or costs of such matings.

Discrimination of compatible mates prior to mating has been proposed as a potential strategy to avoid CI (Hoffmann et al. 1990; Vala et al. 2004; Champion de Crespigny & Wedell 2007). This, in turn may lead to reproductive isolation between infected and uninfected lineages. Indeed, different theoretical models predict that both bi- (Telschow et al. 2005) and unidirectional CI (Telschow et al. 2007) can select for premating isolation. *Wolbachia* may thus severely reduce gene flow between populations, both by reducing the viability of crosses between infected and uninfected individuals and by selecting for mate discrimination in uninfected females or infected males. This process, potentially contributing to speciation, has gained empirical support from a study involving two different *Drosophila* species (Jaenike et al. 2006). As *D. recens* is fully infected by *Wolbachia,* and *D. subquinaria* is fully uninfected, crosses between *D. subquinaria* females and *D. recens* males are incompatible, while the reverse is not true. Accordingly, sympatric *D. subquinaria* females exhibit mating discrimination against *D. recens* males, whereas allopatric *D. subquinaria* females do not (Jaenike et al. 2006).

Several studies tested mate discrimination in species manipulated by CI-inducing *Wolbachia.* Some of these could not find evidence for mate choice (Hoffmann & Turelli 1988; Hoffmann et al. 1990; Wade & Chang 1995; Champion de Crespigny & Wedell 2007; Arbuthnott et al. 2016; Duron et al. 2011), whereas several others did find that individuals discriminate between infected and uninfected mates (Vala et al. 2004; Jaenike et al. 2006; Koukou et al. 2006). Therefore, discrimination has not been universally selected across different species, and may similarly vary between populations within species. Indeed, most studies so far were done using strains instead of outbred populations, which may not reflect the full behavioural repertoire of the species. For example, four different *Nasonia* species exhibited different patterns of mate discrimination, being this ability to discriminate highly influenced, among other factors, by the natural distribution of the populations of each species (Buellesbach et al.

2014). Another study has shown that, in a single population of *D. melanogaster* and *D. simulans*, neither uninfected females nor infected males exhibited preference for infected or uninfected mates (Champion de Crespigny & Wedell 2007). However, a few years later, a different study in *D. melanogaster* has shown that assortative mating depends on *Wolbachia* infection status as well as on the genotype of the host (Markov et al. 2009).

This observed variation in the ability to discriminate between infected and uninfected individuals may hinge upon the benefits that such behaviour may provide. Indeed, the prevalence of *Wolbachia* and the intensity of CI vary across species and populations (Engelstädter & Telschow 2009; Werren et al. 2008). Concomitantly, the benefits of avoiding it differ. Indeed, discrimination is only expected to evolve if *Wolbachia* does induce a sufficient level of CI (i.e; cost of incompatible matings), and if individuals are often exposed to both infected and uninfected mates. The best conditions for CI avoidance to evolve are thus present in populations with intermediate infection levels, as choice is only possible in those circumstances. However, most studies addressing mate discrimination have been done in fully infected population and uninfected individuals were obtained after antibiotic treatment. Still, mate choice has been found under these settings (Koukou et al. 2006; Vala et al. 2004) and fully infected and uninfected populations that do not display mate choice may evolve to do so if they spend enough time under intermediate infection levels, assuming that preference is maintained at low frequency in the population.

In this study, we investigate whether there is intraspecific variation for mate choice in the spider mite *Tetranychus urticae* and whether this trait responds to selection. An earlier study found that uninfected *T. urticae* females not only preferred to mate with uninfected males, but also increased the chance of compatible matings by preferentially ovipositing near uninfected eggs (Vala et al. 2004). However, this study was done with a single isogenic line. We thus started by studying whether *Wolbachia*-uninfected females (*i.e.*, cured using antibiotics) could discriminate between *Wolbachia*-infected and *Wolbachia*-uninfected males in 5 populations naturally infected by *Wolbachia*. Next, to test if the evolution of mate preference in response to CI is contingent upon the prevalence of *Wolbachia* infection in the population, we performed experimental evolution under three selection regimes, corresponding to populations of spider mites that were either fully infected with *Wolbachia*, fully uninfected, or with intermediate infection frequency.

Materials and Methods

Spider mite populations, rearing conditions

Seven spider mite (*Tetranychus urticae*) populations were used for these experiments: AMP, CH, COL, DC, DF, LOU, RF. They were collected in 2013 in Portugal, around the Lisbon area (see Figure S2.1 and S2.2), from different plants: AMP on *Datura* spp.; CH and RF on tomato (*Solanum lycopersicum*); COL and DF on bean (*Phaseolus vulgaris*); DC on zucchini (*Cucurbita pepo*) and LOU on eggplant (*Solanum melongena*). These populations were then established at the University of Lisbon from 65 to 500 females. All populations were naturally infected with CI-inducing endosymbiotic bacteria *Wolbachia* (see Figure S2.3). To create uninfected homologue populations, roughly 3 months after collection, groups of 25 adult females were placed in petri dishes containing bean leaf fragments on cotton wet with tetracycline solution (0.1 %, w/v). This treatment was applied continuously for three successive generations (Breeuwer 1997), followed by at least 20 generations of mass-rearing in an antibiotic-free environment, to avoid (or limit) potential side effects of antibiotic treatment (Ballard & Melvin 2007; Zeh et al. 2012).

Before being used in all experiments, pools of 100 females were checked by PCR to confirm the *Wolbachia* infection status (Zélé et al. in prep). All populations were reared, since arrival to the laboratory, on bean plants (*Phaseolus vulgaris*, Fabaceae, var. *Enana*; Germisem Sementes Lda, Oliveira do Hospital, Portugal) under controlled conditions (25°C, photoperiod of 16L: 8D).

Experimental setup

Mate choice in field-derived populations

5 naturally *Wolbachia*-infected *T. urticae* populations (AMP, CH, COL, DC, LOU) and their treated homologues were used to test if, within each population, uninfected females displayed a preference for uninfected or infected males. Uninfected and infected adult males and uninfected quiescent females were separately isolated onto 8 cm^2 leaf squares on water-saturated cotton from a subset of their base populations. The next day, quiescent females became virgin adults, roughly of the same age, whereas adult males had been isolated for around 24 hours, which guaranteed increased eagerness to mate (Krainacker & Carey 1990). Before the test, males were randomly painted with two different colours of water-based paint using a fine brush, so the observer could discriminate each male type. Within the same population, males from different infection status were painted with different colours. The tests were done on 0.5 cm^2 leaf discs (called “arena” hereafter). Two males, from the same

population but different infection status, were placed on the arena. The test started as soon as an uninfected virgin female from the same population was added to the arena. Each preference test lasted for thirty minutes and both the time until the beginning of mating - latency to copulation - and copulation duration were measured using a stopwatch. Simultaneously, the colour of the male that copulated with the female was registered, and later assigned to a male type. Trials where no mating occurred during 30 minutes were included in the final analysis, as uninfected females, besides exhibiting choice, could become less receptive to mating in the presence of infected males, i.e., could have a different propensity to mate. In total, around 40-70 replicates per population were performed (AMP: n=44; CH: n=53; COL: n=48; DC: n=60; LOU: n=68). Out of these replicates, in around 30 replicates per population mating was observed (AMP: n=34; CH: n=33; COL: n=38; DC: n=32; LOU: n=37).

Experimental Evolution

Two subsets with the same number of founding individuals were created for each population collected in the field (AMP, CH, COL, DC, DF, RF), one of them having been treated with antibiotics. This was done by placing groups of 25 adult females in petri dishes containing bean leaf fragments on cotton with a tetracycline solution (0.1 %, w/v). This treatment was applied continuously for three successive generations (Breeuwer 1997), after which the populations were maintained in a mass-rearing environment without antibiotics for three more generations. Before use, pools of 100 females from each population were checked by PCR to confirm the *Wolbachia* infection status. Two base populations, *Wolbachia*-infected and *Wolbachia*-uninfected, were then started by mixing 50 females from each of the 6 *Wolbachia*-infected populations and from their 6 *Wolbachia*-uninfected homologues, respectively.

Each population of experimental evolution was started by placing 200 infected (*Wolbachia*-infected control; hereafter “I” selection regime) and 200 uninfected mated females (*Wolbachia*-uninfected control; hereafter “C” selection regime) from the base populations in an experimental box (14x14x20cm) containing two bean plants (17 days old) at 23.5°C. Eggs laid by these females then hatched and developed to reach adulthood 14 days later (i.e., generation time; Figure 1). At each generation, 200 young mated daughters were randomly picked and placed anew on 2 bean plants. To allow for the evolution of host control over *Wolbachia*-induced CI, we created a third treatment (*Wolbachia*-uninfected mixed; hereafter “M” selection regime) consisting of *Wolbachia*-uninfected females and an even proportion of *Wolbachia*-infected and -uninfected males. In this regime, at each generation, 350 young adult virgin females were randomly picked-up from the offspring of the previous generation and placed on a patch where they could mate with 100 I and/or 100 M males. After mating, 200 of these females were transferred on fresh plants in a new box (Figure 1). For each selection regime, 5 independent replicates were maintained for 20 generation. Despite considerable care in the mixed regime, one of the replicates got contaminated by *Wolbachia* (i.e., infected females were found in the box) at generation 13. This replicate was thus excluded from the entire experiment. Consequently, only 4 replicates of all selection regimes were included in the experiment presented here. The experiment presented here was done from generations 12 to 15 of experimental evolution.

Mate choice after experimental evolution

Wolbachia-uninfected females belonging to the control (C) and mixed (M) regimes, were given the choice between males of the C and M regimes, of the I and M regimes or of the I and C regimes. To avoid an effect of preference due to differences in relatedness between and within replicates, females and males of each preference test belonged to different replicates: Replicate 1 females mated with replicate 2 males, replicate 2 females mated with replicate 3 males, replicate 3 females mated with replicate 4 males and replicate 4 females mated with replicate 1 males.

The protocol followed here was similar to that of the first experiment except for the age and mating history of the males. It has been shown that *Wolbachia* density changes with male age (Zhao et al. 2013) and that male mating history may influence their mating behaviour (Krainacker & Carey 1989). To control for these factors, males, like females, were isolated as quiescent from a subset of their base populations. This way, during the test, both males and females were virgin adults, roughly of the same age.

Statistical Analyses

All analyses were carried out using the R statistical package (v. 3.0.3). Maximal models were simplified by sequentially eliminating non-significant terms (Crawley 2007), and the significance of the explanatory variables was established using chi-squared tests, in the case of discrete distributions, or F tests, in the case of continuous distributions (Bolker et al. 2008).

Mate choice in field-derived populations

To test for an effect of colour on mate choice, we used a Pearson's Chi squared test. Since no colour effect was found (X^2_1=0.205, P=0.651), this factor was not included in the subsequent statistical models. We then began by testing if mating propensity (i.e., whether individuals mated or not during the time of the observations) and female mate choice were dependent on population identity (AMP, CH, COL, DC, LOU). To do so, in both analyses, we fit population identity as a fixed explanatory variable while block was fit as a random explanatory variable. Both mating propensity and mate choice were analysed using a generalized liner mixed effect model (glmer, lme4 package) with a binomial error distribution. To determine if mate choice was significantly different from 50/50, we changed the intercept of our model to zero. By doing so, the estimate of the fixed factor in a model with categorical factors and a binomial distribution is the difference to a probability of 0.5 (Crawley 2007).

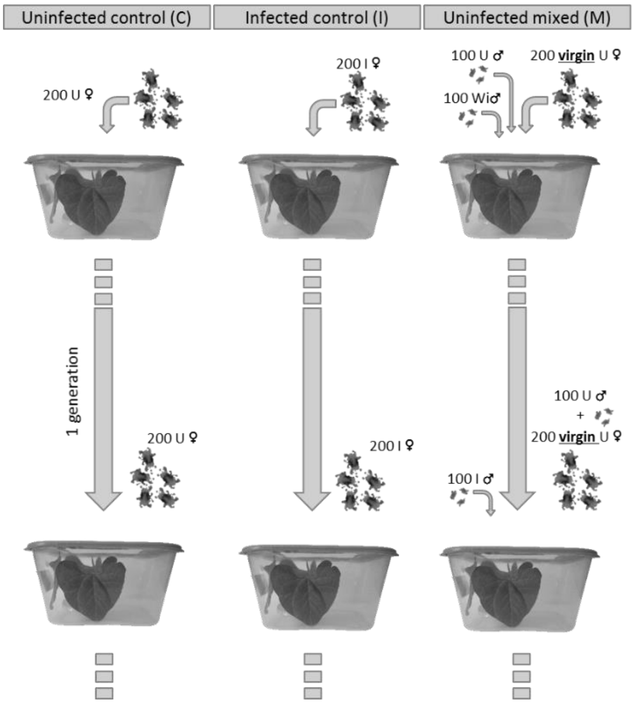

Figure 1. Protocol of Experimental Evolution. *Three regimes were created, one fully uninfected (C) one fully infected (I) and one where the prevalence of* Wolbachia *was intermediate (M). In treatment M, at every generation, 200 M virgin females were isolated and allowed to mate with I and M males, then transferred to new bean leaves to lay eggs. In treatments C and I, 200 mated females were transferred directly to a new box. We expect females from the M regime to evolve the ability to avoid I males, as crosses with this type of males are partially incompatible. For each treatment, 4 independent replicates evolved during 20 generations. U: uninfected; I: Wolbachia infected.*

Latency and duration of copulation were analysed using a cox proportional hazard mixed-effect model (coxme, package coxme), a non-parametric technique to analyse time-to-event data (e.g. time-to-death; Crawley 2007). In this analysis no censoring was used, as we only used analyzed data in which mating occurred during the observation time. Male type (infected or not with *Wolbachia*) and population identity were fitted as fixed explanatory variables, whereas day was fitted as random explanatory variable. Whenever there were significant differences across populations, a Tukey test with Bonferroni correction was done (glht, package multicomp).

Mate choice after experimental evolution

As in the previous analysis, colour was not included in the statistical models since no effect of this factor on choice behaviour was found using a Pearson's Chi squared test (X^2_3=0.60923, P= 0.894).

First, we tested whether mating propensity and female mate choice were dependent on the female selection regime (M or C) and/or on the type of preference test (choice between M and C, M and I or C and I). Both analyses were done using a generalized liner mixed-effect model (glmer, lme4 package) with a binomial error distribution. Female selection regime and type of preference test were fit as fixed factors, whereas day and replicate were fit as random factors. Again, to determine if mate preference of each population was significantly different from 50/50, we changed the intercept of our model to zero.

To test for differences in mating latency and duration of copulation between selection regimes, the male chosen (M, C or I) and the female selection regime (M or C) were fit as fixed explanatory variables, whereas type of preference test, day and replicate were fit as random explanatory variables. Latency and duration of copulation variables were analysed using a cox proportional hazard mixed-effect model (coxme, package coxme).

Results

Mate choice in field-derived populations

Different populations showed a significant difference in mating propensity (X^2_4=13.306 P=0.01; Figure 2a). However, the multiple comparisons analysis revealed that differences were only marginally significant between DC and COL (Z=-1.2015, P=0.063) and between LOU and COL (Z=-1.1581, P=0.071; Figure 2a; Table 1). Moreover, female preference did not vary between populations (X^2_4=1.0765 P=0.898) and it was not significantly different from random mating (X^2_5=3.1658, P=0.674; Figure 2b). Latency to mate with infected males tended to be shorter than that with uninfected males, although the difference was only marginally significant (male type effect: X^2_1=3.694, P=0.056; Figure 3). In addition, there was a significant difference in latency to copulation among populations (population identity effect: X^2_4=17.708; P=0.001; male type - population identity interaction: X^2_4=2.411; P=0.661; Figure 3). Contrasts between populations revealed that copulations started significantly sooner between males and females from the LOU population than between males and females from the DC population (Z=4.078; P<0.001; Table 2). As for copulation duration, it lasted longer with infected males than with uninfected males (male type - copulation duration interaction: X^2_1=4.917, P=0.027), independently of the population tested (population identity effect: X^2_4=6.998; P=0.1360; male type - population identity interaction: X^2_4=7.427; P=0.115; Figure 4).

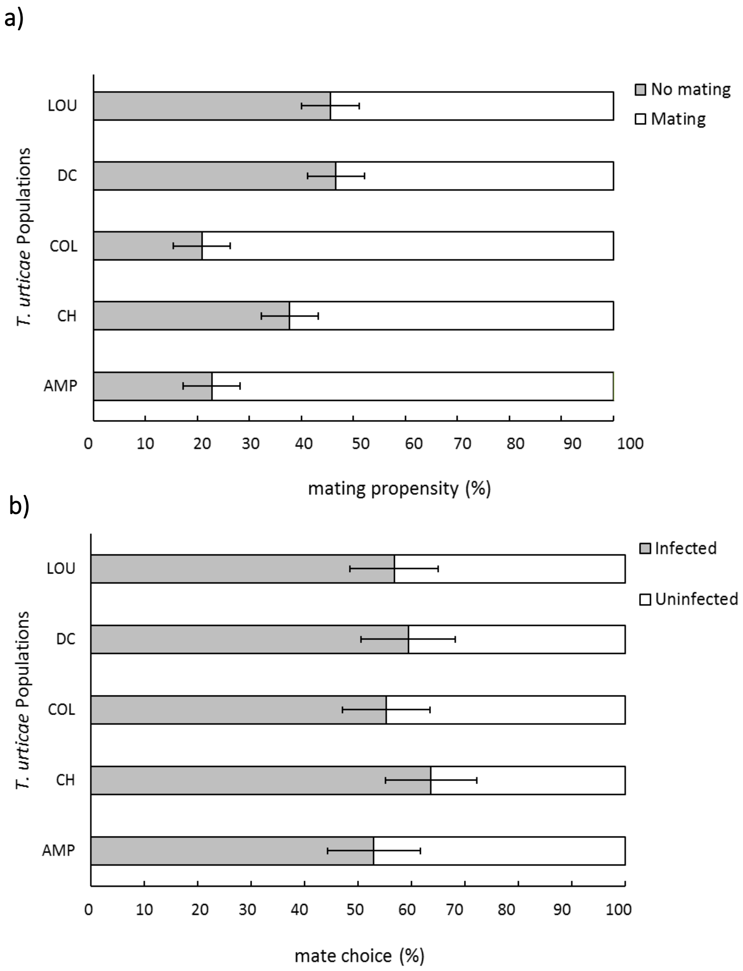

***Figure 2. Mating propensity (a) and mate choice (b) in crosses between uninfected females and infected or uninfected males in five populations of* T. urticae.** *a) Mating propensity across populations. Grey bars represent percentage trials where no mating occurred. White bars represent percentage of trials where uninfected females mated within the time of the observation. b) Mate choice across populations. Grey bars represent percentage of infected males chosen by uninfected females and white bars represent percentage of uninfected males chosen by uninfected females. Population identity: AMP, CH, COL, DC and LOU. Horizontal lines correspond to standard errors of the mean.*

Comparison			Estimate	SE	Z	P
AMP	x	CH	-0.723	0.458	-1.579	1
AMP	x	COL	0.1112	0.5057	0.22	1
AMP	x	DC	-1.0902	0.4431	-2.46	0.1389
AMP	x	LOU	-1.0468	0.4344	-2.41	0.1596
CH	x	COL	0.8342	0.4546	1.835	0.6647
CH	x	DC	-0.3672	0.3837	-0.957	1
CH	x	LOU	-0.3238	0.3736	-0.867	1
COL	x	DC	-1.2015	0.4396	-2.733	0.0628
COL	x	LOU	-1.1581	0.4308	-2.688	0.0719
DC	x	LOU	0.0434	0.3553	0.122	1

***Table 1. Multiple comparisons of mating propensity between five populations of* T. urticae**. *Population identity: AMP, CH, COL, DC and LOU. Multiple comparisons were done using Tukey contrasts with Bonferroni corrections.*

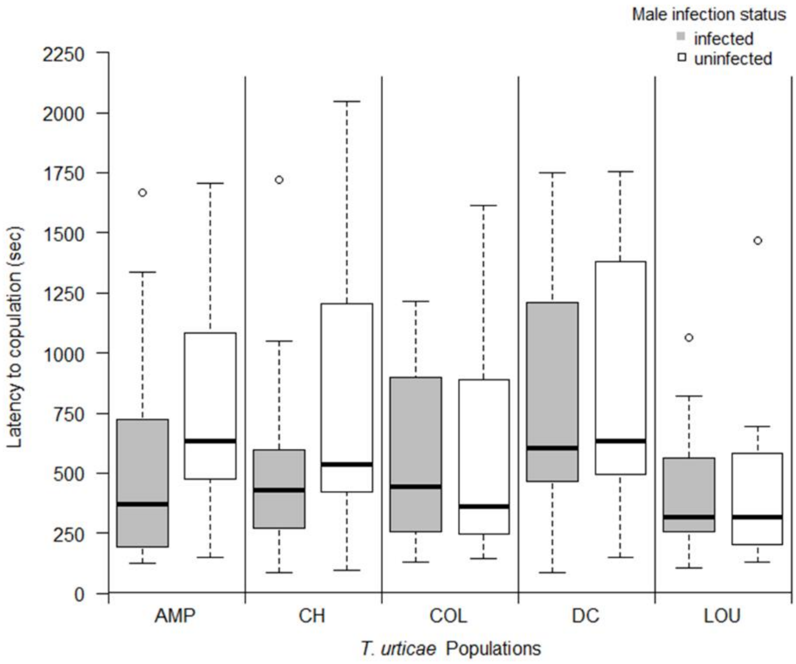

Figure 3. Latency to copulation (in seconds) involving matings with infected or uninfected males of five populations of **T. urticae.** *Population identity: AMP, CH, COL, DC and LOU. Infected males are represented by grey bars, uninfected males by white bars.*

Comparison	Estimate	SE	Z	P
AMP X CH	-0.004	0.249	-0.015	1.000
AMP X COL	0.232	0.238	0.973	1.000
AMP X DC	-0.421	0.252	-1.672	0.945
AMP X LOU	0.613	0.242	2.528	0.115
CH X COL	0.235	0.244	0.964	1.000
CH X DC	-0.417	0.252	-1.653	0.983
CH X LOU	0.616	0.248	2.490	0.128
COL X DC	-0.653	0.249	-2.625	0.087
COL X LOU	0.381	0.233	1.634	1.000
DC X LOU	1.034	0.254	4.078	<0.001 *

Table 2. Multiple comparisons of latency to copulation between five populations of T. urticae. *Population identity: AMP, CH, COL, DC and LOU. Multiple comparisons were done using Tukey contrasts with Bonferroni corrections. * indicate significant differences in contrast analysis*

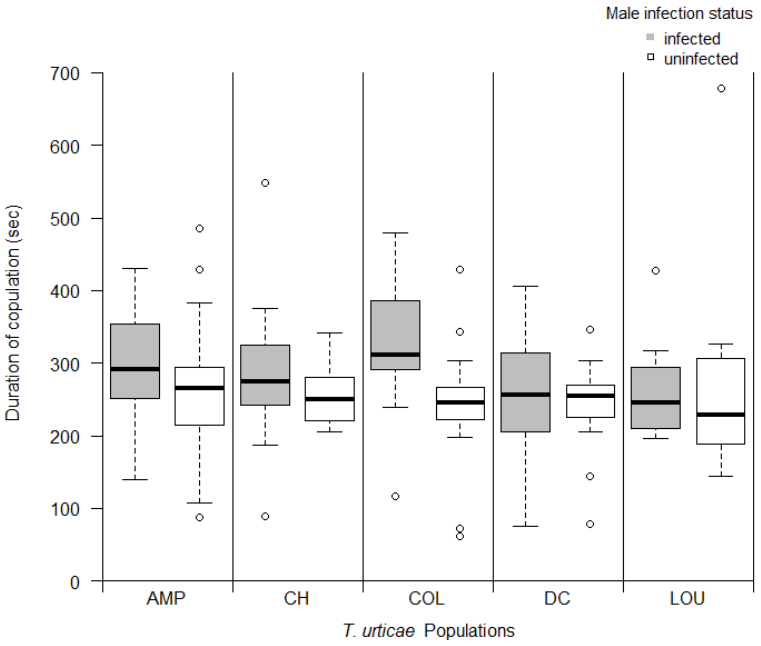

***Figure 4. Duration of copulation (in seconds) involving matings with infected or uninfected males of five populations of* T. urticae**. *Population identity: AMP, CH, COL, DC and LOU. Infected males are represented by grey bars, uninfected males by white bars.*

Mate choice after experimental evolution

Overall, there was no significant effect of the female selection regime, of the type of preference test, nor of the interaction between these two factors on both mating propensity (X21=0.464, P=0.496; X22=2.720, P=0.257; X22=1.53, P=0.465, respectively; Figure 5a) and mate choice (X21=0.002, P=0.967; X22=1.736, P=0.42; X21=1.073, P=0.585, respectively; Figure 5b). Furthermore, females did no show a preference for a specific type of male (X23=5.3305, P=0.149). As in mating propensity and mate choice, no effect of female selection regime, type of preference test or their interaction was found for latency to copulation (female selection regime: X21=3.01, P=0.078, type of preference test: X22=0.709, P=0.702; female selection regime - type of preference test interaction: X21= 4.774, P=0.092; Figures 6a) and copulation duration (female selection regime: X21=0.624, P=0.43; type of preference test: X22=2.324, P=0.313; female selection regime - type of preference test interaction: X21= 3.806, P=0.149, Figure 6b).

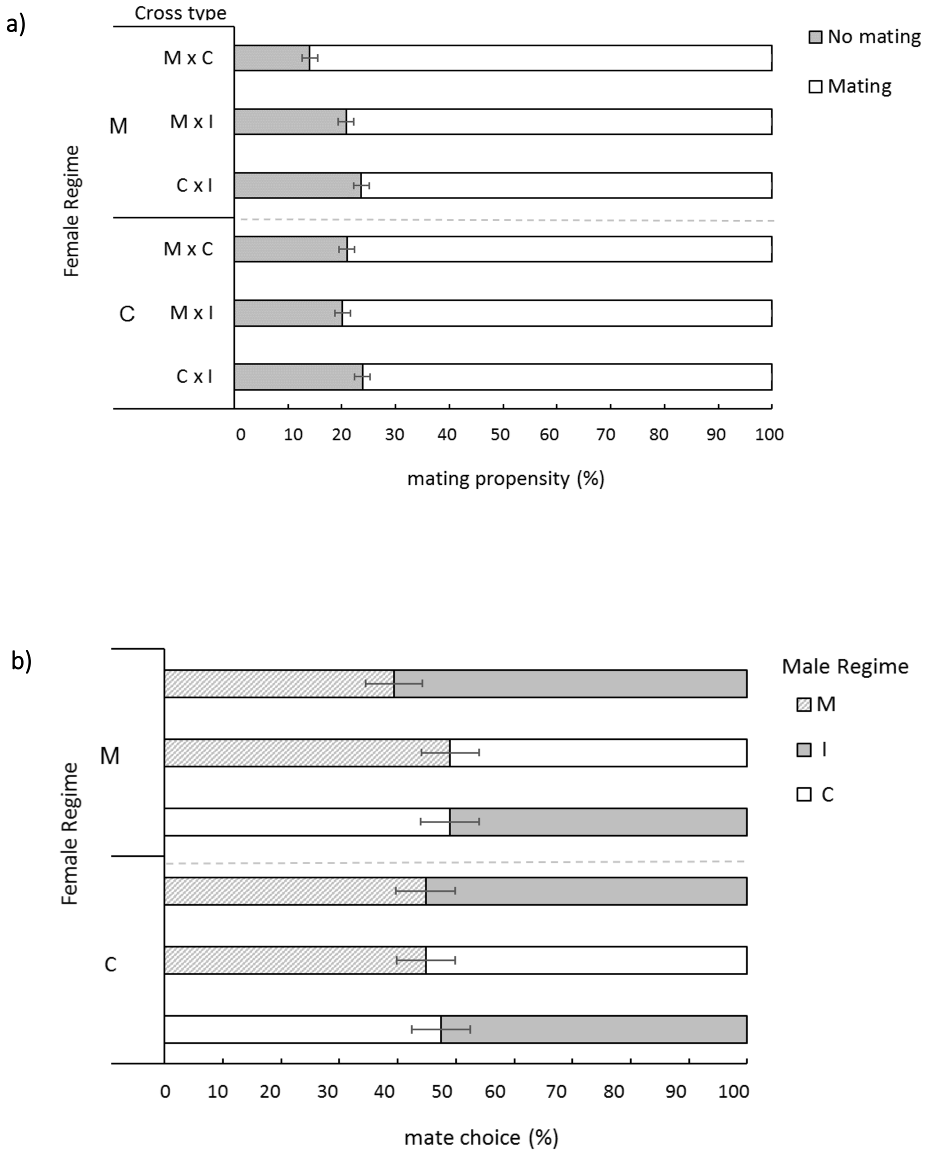

Figure 5. Mating propensity (a) and mate choice (b) in crosses with uninfected females and males from different selection regimes. *Uninfected females from the control and the mixed regimes (C and M, respectively) were given the choice between males from two different regimes (cross type). Males could be from the mixed regime (M), from the uninfected control regime (C) or the infected control regime (I). a) Grey bars represent percentage of trials where no mating occurred. White bars represent percentage of trials where uninfected females mated within the time of the observation. b) White bars represent males from the* Wolbachia*-uninfected control regime (C), grey bars represent males from the* Wolbachia*-infected control regime (I) and dashed bars represent males from* Wolbachia*-uninfected mixed regime (S). Horizontal lines correspond to standard errors of the mean.*

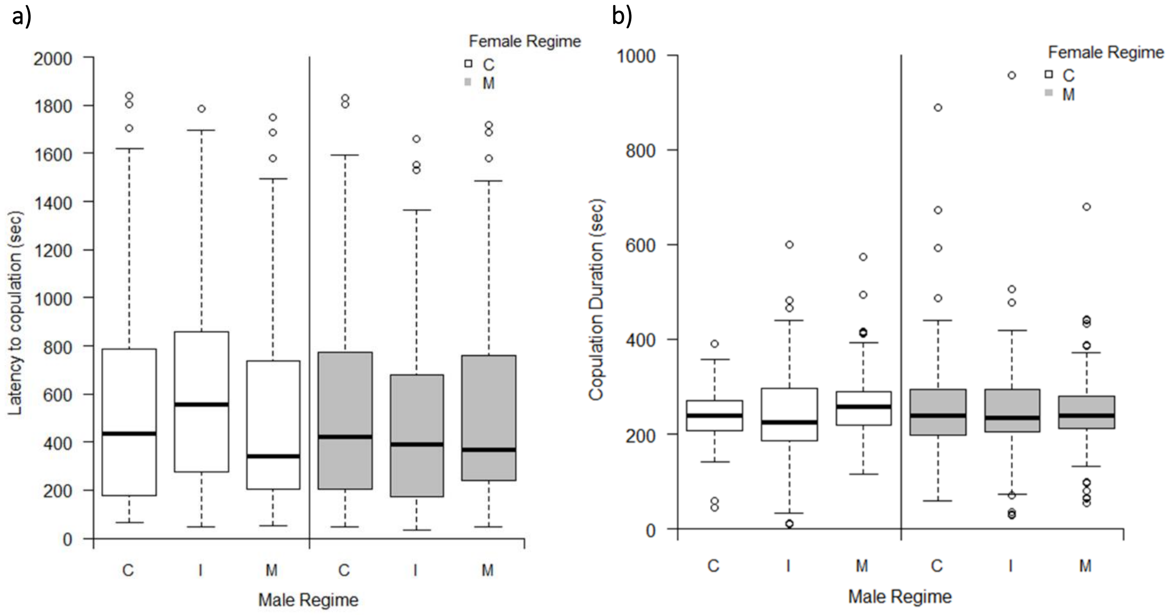

Figure 6. Latency to copulation (a) and duration of copulation (b) of matings between uninfected females and males ***from different selection regimes.*** *Grey bars: C females; white bars: M females. M –*Wolbachia*-uninfected no choice selection regime; C –*Wolbachia*-uninfected no choice regime; I –*Wolbachia*-infected no choice regime.*

Discussion

In this study, we tested whether spider mites avoided mating with incompatible males. Out of the 5 populations tested, none showed preference for *Wolbachia*-uninfected mates, indicating the absence of pre-copulatory strategies to avoid *Wolbachia*-induced incompatibilities. Even after 16 generations of evolution in a selection regime where choice between infected and uninfected males could be expressed, uninfected females continued not to show a preference for uninfected males, hence mate preference did not respond to selection. Moreover, in the experiment with field-derived populations, infected males started to mate sooner and mated longer than uninfected males. However, this difference was lost after experimental evolution

A decrease in latency to copulation increases the chance of being the first to copulate with a female. In addition, increased time of copulation has also been implied in the insurance of paternity by the first male (Satoh et al. 2001; Potter & Wrensch 1978). These behaviours are thus particularly important in species with first male sperm precedence, such as *T. urticae*, given that being the first male to mate with a female is crucial to ensure the production of offspring. The fact that latency to copulation was shorter and copulation duration was longer in infected males suggests that they are better competitors than uninfected males. Furthermore, prolonged copulation in infected males can be associated with an increase in the production of fertilized offspring (Simmons 2001). Thus, in a spider mite population with intermediate *Wolbachia* infection frequency, infected males (i) may produce more offspring than uninfected males when mated with infected females, and (ii) fertilize more eggs of uninfected females, thereby increasing the penetrance of CI. As a consequence, such behaviour is expected to enhance the spread of *Wolbachia* within the population. In fact, a behavioural advantage of infected males has been seen in other species infected with *Wolbachia* (Champion de Crespigny et al. 2006; Panteleev et al. 2007). However, the fecundity and sex-ratio in the field-derived populations studied here are overall similar in crosses between infected females and infected or uninfected males (Figure S2.4), which invalidates (i). In addition, a study in spider mites could not find any correlation between copulation duration and number of offspring (Satoh et al. 2001), suggesting that prolonged copulation may not be associated with increased fertility, which invalidates both (i) and (ii). Alternately, infected males may mate longer to compensate for a decrease in sperm quality or quantity, ending up with the same number of sired offspring as uninfected males. Indeed, *Wolbachia* has been shown to be associated with male fertility costs in several hosts (reviewed in Price and Wedell 2008). For example, in *D. simulans*, infected males sired fewer offspring than uninfected males due to a decrease in sperm production (Snook et al. 2000). Curiously, a similar pattern as the one presented here has been found for reproductive interference between two spider mite species. In *T. urticae*, incompatible crosses with *T. evansi* did not elicit strong mate choice but lasted less than conspecific crosses, suggesting changes in mating investment, rather than changes in preference (Clemente et al. 2016).

The absence of female choice, together with the shorter latency to copulation of infected males compared to uninfected males, should result in a higher proportion of copulations with infected males. However, we did not observe any difference in the number of copulations with both types of males. These two findings may be reconciled if uninfected females reject attempts of copulation by infected males more often than attempts by uninfected males. If this would be the case, it would imply a form of choice by uninfected females. Precise behavioural observations are needed to corroborate this hypothesis.

After experimental evolution, no differences in latency to copulation and copulation duration were observed between males from different selection regimes. Even if not selected directly, males from the mixed-infection selection regime could evolve increased competitive ability, as this would lead to more compatible crosses in a population with intermediate levels of infection, decelerating the spread of *Wolbachia*. The lack of differences in latency to copulation and copulation duration across selection regimes does not coincide with the results obtained with the field-derived populations, where infected males tended to start mating faster and copulated for a longer time period. The differences between the two experiments could be related to more than 30 generations that elapsed between the field test and the tests done after experimental evolution (see Figure S2.1). During this time, either infected males could have lost their competitive advantage against uninfected males in a scenario of full prevalence, or uninfected males could have become better competitors in a scenario free of infection. In fact, although unlikely, uninfected females could have been suffering a side effect of the tetracycline treatment that was lost through time. The differences between experiments observed here could also be due to different male mating histories: in the first experiment, males were experienced, whereas males used after experimental evolution were virgin. On the one hand, differences in male fertility, such as a decrease in sperm production, might only be evident after a few copulations. As said before, male mating behaviour might be adapted to its sperm supply, in which case we could expect to find differences when using experienced males only. On the other hand, by using experienced males, we do not know how many females they mated with prior to the experiment. The results obtained with the field-derived populations could also be simply due to differences in mating history between infected and uninfected males. However, that would be unlikely, having into account that this result was consistent across populations.

In contrast to differences in latency and copulation durations, we found no evidence for mate discrimination between infected and uninfected males across field-derived populations. This lack of mate discrimination suggests that the ability to choose between males with different infection status is not common in *T. urticae* populations. Hence, the results obtained by Vala et al. (2004) are probably not representative of the reproductive behaviour of this species. In fact, the variation found for mate discrimination in the literature (Vala et al. 2004; Markov et al. 2009; Hoffmann et al. 1990) suggests that the presence of discrimination may depend on a combination of several factors, such as the genotype, population structure and infection history of the host and the effect of the *Wolbachia* strain (Goodacre & Martin 2012; Engelstädter & Telschow 2009). Another factor that can condition the selection for mate discrimination in host populations is the prevalence of infection. To test this, we exposed uninfected females to a potential choice between infected and uninfected males during 12-15 generations. Unexpectedly, uninfected females evolving under this selection regime, as those evolving in the control regime, did not choose to mate preferentially with uninfected males.

The absence of mate preference in both experiments is surprising, given that the populations tested here present high CI levels (c.a. 30% for CH to 68% for DC; Figure S2.4, c.a. 48 % for the mixed population used for the experimental evolution; Figure S2.5), resulting in a strong decrease of the reproductive success of uninfected females. This lack of mate discrimination may take place, for instance, because males do not provide sufficient cues for females to distinguish them. However, infection has been shown to often alter molecular cues used for mate recognition (Beltran-Bech & Richard 2014). For instance, commensal bacteria have been shown to alter the cuticular hydrocarbon profile of *D. melanogaster*, influencing the mate preference of the host. Another possibility is that these cues exist but females are not able to perceive them, both before and after selection.

Females might not be able to discriminate males from different infection status for different reasons. For one, there may be no genetic variation for this trait in the starting populations, which would explain both the absence of choice in the field-derived populations and the fact that it did not evolve in the mixed regime. Alternatively, a preference allele could have been present in the starting populations at low frequency, but selection during experimental evolution was not strong enough for the allele to increase in frequency. Indeed, some factors in the biology of spider mites might have contributed to reduce the strength of selection for choice. For one, spider mites are haplodiploid, producing haploid sons and diploid daughters, stemming from unfertilized and fertilized eggs, respectively (Helle & Sabelis 1985). Consequently, females involved in incompatible crosses still pass on their genes by producing haploid males. In addition, CI is incomplete in this species (Figure S2.4, S2.5), which is expected to result in a weaker selection pressure for the evolution of preference, as part of the fertilized offspring resulting from incompatible crosses is still viable (Champion de Crespigny et al. 2005). The conflict between uninfected females and *Wolbachia* is thus weaker in spider mites than in diploid species and in species with complete CI. However, the phenotype induced by *Wolbachia* in these populations can still reduce the proportion of female offspring by up to 68% (Figures S2.4 and S2.5), which represents an important decrease in the reproductive success of uninfected females. Moreover, theoretical models predict that unidirectional CI is more likely to select for pre-mating isolation if the levels of incompatibility are intermediate, because under these circumstances polymorphism for infection is maintained for a longer period in the populations (Telschow et al. 2007). In addition, spider mites have first male sperm precedence, where only the first mating of a female is effective. We thus expect a strong selection pressure on pre-copulatory strategies in this species. Finally, mate choice may have not been observed because it trades-off with another beneficial trait. Indeed, this trait could have been already present in the population. For instance, if male quality is variable for other reasons, choosing better quality males can trade-off with choosing compatible males (Colegrave et al. 2002; Neff & Pitcher 2005). The ability to avoid incompatible crosses could then be too costly to be maintained in an environment where incompatible crosses do not occur, i.e., under complete prevalence or in an environment free of *Wolbachia.* This could explain our results since the populations studied here were kept in the laboratory, fully infected, for more than 1 year (around 24 generations) before being tested for mate choice, and 30 more generations passed between these measurements and the observations done after experimental evolution (Figure S2.1). However, we do not know if there is genetic variation in male quality in our populations. The selective pressure applied here may also have led to the evolution of another strategy that trades-off with mate choice or that renders mate-choice unnecessary. For instance, spider mites may have evolved cryptic female choice or improved sperm competitive ability to avoid incompatible matings, as seen in other species (Wedell 2013; Price & Wedell 2008). However, it might be harder for species with complete first male sperm precedence to evolve post-copulatory strategies than for species with other sperm precedence patterns, as cryptic female choice and sperm competition by definition, do not occur in these species.

Here we could not find evidences for the evolution for mate discrimination. Nevertheless, to our knowledge, this was the first attempt to test whether assortative mating evolves in response to genetic incompatibilities. This is surprising, given that this approach has the potential to significantly contribute to the understanding of a behaviour that underlies reproductive isolation and therefore speciation (Buellesbach et al. 2014; Jaenike et al. 2006). More studies on this subject using experimental evolution are thus needed, in order to determine under which conditions mate discrimination will evolve in response to CI-inducing *Wolbachia*.

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CHAPTER VI

Wolbachia modifies sperm precedence patterns in spider mites

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Abstract

In the spider mite *Tetranychus urticae*, the first male sires all the offspring of a female. Still, multiple matings with mated females take place frequently. Such behaviour may be adaptive if it decreases the costs of incompatible crosses that occur, for example, when uninfected spider mite females mate with males that are infected by the endosymbiotic bacterium *Wolbachia*. Indeed, such crosses lead to cytoplasmic incompatibility (CI), entailing the embryonic death of (some) fertilized offspring. Uninfected females are thus expected to evolve strategies to avoid the costs imposed by CI. For example, they may mate with multiple males to recover offspring viability. However, it is not clear whether such compensation occurs in species with first male precedence, such as spider mites, and if the evolution of this strategy is contingent upon the prevalence of infection.

To test this, we performed experimental evolution of spider-mite populations that were either fully infected by *Wolbachia*, fully uninfected or with an intermediate infection frequency. In the latter, to promote the evolution of a compensatory mechanism, uninfected females could copulate with both *Wolbachia*-infected and uninfected males at each generation.

After 20 generations of selection, uninfected females evolving under mixed infection frequency that mated with *Wolbachia*-infected males presented a higher degree of CI than those mated first with *Wolbachia*-infected and then with uninfected males evolving under mixed infection. These results suggest that spider mites are able to reduce CI by mating multiply. In addition, when the first male was uninfected and the second was infected, there was no induction of CI. This indicates that spider mites disrupt their pattern of sperm precedence pattern only when the first mating is incompatible, which might be a key factor for the evolution of CI-driven polyandry in species with skewed patterns of sperm precedence. These results constitute the first experimental evidence that evolution of CI-antagonist strategies is possible.

Keywords

Sperm precedence; cytoplasmic incompatibility; spider mites; *Wolbachia*; polyandry

Introduction

Sexual selection should favour the evolution of polyandry whenever it confers direct or indirect benefits for males or females. For instance, it has been hypothesized that multiple mating can evolve as a strategy to avoid genetic incompatibilities, arising as an effect of inbreeding or intra-genomic conflicts caused by selfish genetic elements (genetic incompatibility avoidance hypothesis; Tregenza and Wedell 2000; Wedell 2013; Zeh and Zeh 1996, 1997). Indeed, by mating with more than one male, females may increase the likelihood of using sperm from a compatible male and thereby improve their fitness (Zeh & Zeh 1997; Zeh & Zeh 1996; Tregenza & Wedell 2000; Wedell 2013). For instance, in the cricket *Grillus bimaculatus*, females that mate with siblings have decreased hatching rate, while those that mate with both a sibling and a non-sibling male are able to avoid the decrease in egg viability (Tregenza & Wedell 2002).

Genetic incompatibilities can occur due to endosymbionts. For example, the maternally inherited bacterium *Wolbachia* can induce cytoplasmic incompatibility (CI), thereby causing the embryonic death of fertilized offspring from crosses between uninfected females and *Wolbachia*-infected males. This mechanism ensures the spread of *Wolbachia*-infected hosts in mixed-infected populations, as it imposes an important cost to uninfected females. Uninfected females, in turn, are expected to evolve strategies to avoid or resist CI. Indeed, there are several studies on, for instance, mate discrimination in species manipulated by endosymbionts (Vala et al. 2004; Champion de Crespigny & Wedell 2007; Poinsot et al. 2003; Koukou et al. 2006). There are, however, remarkably little experimental studies on the benefits of polyandry against the negative effects of endosymbionts (but see reviews Tregenza and Wedell 2000; Wedell 2013). An exception is the work done by Champion de Crespigny and colleagues in *Drosophila simulans*. In this species, polyandrous uninfected females produce more viable offspring that monandrous uninfected females after mating with an infected male, which demonstrates that polyandry helps avoiding the costs of CI (Champion de Crespigny et al. 2007). In addition, infected males seem to be poor sperm competitors, which might inhibit or prevent the invasion of *Wolbachia* in the population (Champion de Crespigny et al. 2007).

Sperm precedence patterns can be contingent on the quality of the previous matings (Danielsson 1998; Garcia-Gonzalez 2004). In fact, changes in these patterns have been observed in crosses between species with last male sperm precedence, suggesting that genetic incompatibilities can impact on sperm precedence patterns themselves (conspecific sperm precedence, Price 1997; Price et al. 2000; Rugman-Jones and Eady 2007). For instance, in *Drosophila melanogaster*, sperm precedence is biased in favour of the last mate. However, in double matings with a conspecific and a heterospecific male, the sperm of conspecifics fertilizes most of the eggs, regardless of the order to the matings (Price 1997). Notwithstanding, overcoming the cost imposed by mating with an incompatible male is likely to be more difficult in species with first than in species with last male sperm precedence. Indeed, in the latter case, females can subsequently mate with a compatible male, thereby promoting sperm competition and potentially rescuing their offspring viability. This option is not available for species with first-male precedence, which, if mated with an incompatible male, cannot compensate later by mating with a compatible male. There should thus be strong selection for pre-copulatory mate choice in species with first male sperm precedence. Unfortunately, studies showing this are remarkably scarce (but see Vala et al. 2004). Alternately, tough not exclusively, such species may evolve the ability to distinguish sperm from infected and uninfected males, this way changing their pattern of sperm priority. Regardless, to our knowledge, no study has tested the effect of genetic incompatibilities on the evolution of sperm priority in species with first male sperm precedence.

In the spider mite *Tetranychus urticae* only the first mating of a female is effective (Helle 1967, chapter 3). Still, multiple mating occurs (Oku 2010). This behavior could be, even if partially, explained by the few indirect benefits polyandry provides to males (Chapter 3). In any case, there are several ways through which polyandry can be also beneficial for females, either directly, by providing nutrients or enabling sperm replenishment, or indirectly, by improving offspring quality or genetic variability (Danielsson 1998; Arnqvist & Nilsson 2000; Slatyer et al. 2012). However, none of the previous opportunities, except for nutritional provisioning, are expected to apply to species with complete first male sperm precedence, as there is no cryptic female choice or sperm competition. In addition, nutritional provisioning has been excluded as a mechanism to explain multiple mating in this species, as multiply-mated females produce fewer eggs and survive less than females with fewer mating opportunities (chapter 3, Macke et al. 2012). One possible explanation for the existence of polyandry in spider mites relies on the occurrence of reproductive manipulators, such as CI-inducing *Wolbachia*.

In *T. urticae*, *Wolbachia* prevalence varies across populations in the field (Figure S2.3), Breeuwer and Jacobs 1996; Enigl and Schausberger 2007; Gotoh et al. 2007), and these bacteria frequently induce intermediate to high level of CI, which will favour the evolution of antagonistic strategies against this phenotype. Due to the pattern of sperm precedence observed in this species, pre-copulatory mate choice is the most likely mechanism to avoid the costs of CI. Two different studies tested if there was mate discrimination against infected mates in this species. The first found that females from an isofemale line were capable of choosing between infected and uninfected males (Vala et al. 2004). However, a subsequent study, using 5 different outbred populations, showed that spider mite females were not able to distinguish males with different infection status, even after 15 generations of evolution in mixed infection (chapter V). This suggests that preference for males with different infection status is not common in *T. urticae*. Possibly, spider mites reduce the costs of CI via post-copulatory strategies. However, this possibility has never been tested in this species.

The evolution of host antagonistic strategies is expected to be contingent upon the prevalence of *Wolbachia* in a given population (Wedell 2013; Engelstädter & Telschow 2009). Indeed, we expect these strategies to evolve only when the costs of CI are present, i.e., at intermediate infection frequencies. To test this prediction, we exposed spider mites populations to three selection regimes corresponding to different *Wolbachia* prevalences: null (0%), full (100%) and mixed prevalence (where 0% prevalence in females but 50% prevalence in males).

Material and Methods

Spider mite populations, rearing conditions

The six populations used for this experiment (AMP, CH, COL, DC, DF, RF) were collected in Portugal, around the Lisbon area in 2013, on different plants: AMP on *Datura* spp.; CH and RF on tomato (*Solanum lycopersicum*); COL and DF on bean (*Phaseolus vulgaris*); DC on zucchini (*Cucurbita pepo*) and LOU on eggplant (*Solanum melongena*) (Figure S2.1, S2.2). These populations were then established at the University of Lisbon (from 65 to 500 founders) and maintained in a mass-rearing environment on bean plants (*Phaseolus vulgaris*, Fabaceae, var. *Enana*; Germisem Sementes Lda, Oliveira do Hospital, Portugal) under controlled conditions (25°C, photoperiod of 16L: 8D). All populations were naturally infected with CI-inducing endosymbiotic bacteria *Wolbachia* (see Figure S2.3 and S2.4).

Experimental Evolution

Two subsets with the same number of founding individuals were created for each population collected in the field, one of them having been treated with antibiotics. This was done by placing groups of 25 adult females in petri dishes containing bean leaf fragments on cotton with a tetracycline solution (0.1%, w/v). This treatment was applied continuously for three successive generations (Breeuwer 1997), after which the populations were maintained in a mass-rearing environment without antibiotics for three more generations. Before use, pools of 100 females from each population were checked by PCR to confirm the *Wolbachia* infection status. Two base populations, *Wolbachia*-infected and *Wolbachia*-uninfected, were then started by mixing 50 females from each of the 6 *Wolbachia*-infected populations and from their 6 *Wolbachia*-uninfected homologues, respectively.

Each population of experimental evolution was started by placing 200 infected (*Wolbachia*-infected control; hereafter “I” selection regime) and 200 uninfected mated females (*Wolbachia*-uninfected control; hereafter “C” selection regime) in an experimental box (14x14x20cm) containing two bean plants (17 days old) at 23.5°C. Eggs laid by these females then hatched and developed to reach adulthood 14 days later (i.e., generation time; Figure 1). At each generation, 200 young mated daughters were randomly picked and placed anew on 2 bean plants. To allow for the evolution of host control over *Wolbachia*-induced CI, we created a third treatment (*Wolbachia*-uninfected mixed; hereafter "M" selection regime) consisting of *Wolbachia*-uninfected females and an even proportion of *Wolbachia*-infected and -uninfected males. In this regime, at each generation, 350 young adult virgin females were randomly picked-up from the offspring of the previous generation and placed on a patch where they could mate with 100 I and/or 100 M males. After mating, 200 of these females were transferred on fresh plants in a new box (Figure 1). For each selection regime, 5 independent replicates were maintained for 20 generation. Despite considerable care in the mixed regime, one of the replicates got contaminated by *Wolbachia* (i.e., infected females were found in the box) at generation 13. This replicate was thus excluded from the entire experiment. Consequently, only 4 replicates of all selection regimes were included in the experiment presented here. The experiment presented here was done at generations 19 to 21.

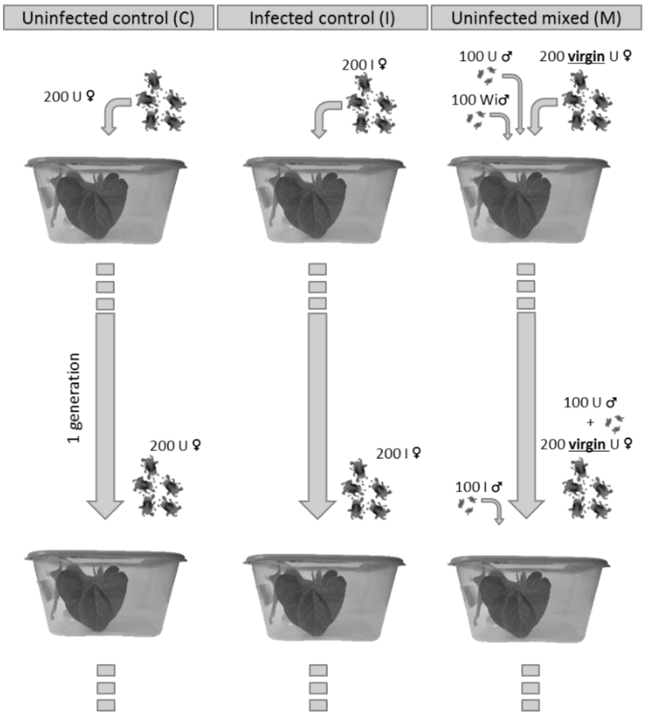

Figure 1. Protocol of Experimental Evolution. *Three regimes were created, one fully uninfected (C) one fully infected (I) and one where the prevalence of* Wolbachia *was intermediate (M). In treatment M, at every generation, 200 M virgin females were isolated and allowed to mate with I and M males, then transferred to new bean leaves to lay eggs. In treatments C and I, 200 mated females were transferred directly to a new box. We expect females from the M regime to evolve the ability to avoid I males, as crosses with this type of males are partially incompatible. For each treatment, 4 independent replicates evolved during 20 generations.* U: uninfected; I: Wolbachia infected.

Polyandry as a compensatory mechanism

To test if polyandry could reduce the effect of CI, and if this would be affected by the level of infection in the populations, we performed crosses between uninfected females, from either the control (C) or the mixed (M) regime, and infected (I) or uninfected males, which also stemmed from either the control (C) or the mixed (M) regime. We then assessed the level of CI by measuring the embryonic mortality and sex ratio of the offspring. In addition, to test whether the potential evolution of CI avoidance entails trade-offs, we also measured the survival and fecundity of the females. Finally, we recorded the latency to copulation of the second mating, which indicates the willingness to re-mate and is potentially correlated with the rate of polyandry.

Twelve days before the beginning of the experiment, 50 quiescent females from the 3 regimes were isolated from a subset of the base population in a petri dish with a quarter of a leaf. Quiescent females emerge into adults within 24 hours, after which they lay unfertilized eggs that will give rise to haploid males. A high number of virgin females with the same age were allowed to lay eggs for one day, resulting in a cohort of virgin adult males roughly with the same age 10 days later. This way, age effects on *Wolbachia* density were controlled for (Zhao et al. 2013). Quiescent uninfected females from the control and the mixed regimes were isolated from the same population subsets as before. 48 hours later, females were allocated to different treatments (day 0), in which they could mate once or twice with either infected or uninfected males. To ensure matings, approximately 5 males and 5 females were placed together 0.5 cm^2 leaf discs. Mated females were immediately transferred to a new patch without males. One hour later, new males were added to half of those patches, whereas in the other half, females remained alone. The identity of the second male varied with that of the first: if males on the first patch were uninfected, males on second patch were infected and vice-versa. Uninfected males belonged to the same regime as female (i.e., C females mated with C males, whereas M females mated with M males). Again, individuals were given 1 hour to mate and females were subsequently isolated onto a new leaf disc (0.95 cm^2) without males. During the observation of crosses on the second patch, the latency to copulation of each female was registered. In addition, females that did not mate after one hour, in both patches, were excluded from the experiment. The next day (day 1), females were individually isolated on new leaf discs (2.55 cm^2) where they laid eggs for 4 days. Female survival was registered daily and, at the fourth day females were removed, if alive (day 5). Eggs hatching rate was registered 4 days after female removal (day 9) and the number of sons and daughters per patch was recorded 6 days later (day 15). Note that spider mites are haplodiploid, whereby haploid sons stem from haploid eggs, and diploid daughters from fertilized eggs (Helle & Sabelis 1985). In this case, the phenotype of CI is a male-biased sex-ratio, as *Wolbachia* only affects the production of daughters, i.e., fertilized offspring. Thus, in *T. urticae*, the proportion of male offspring remains unchanged, while the effect of CI is measured as an increase in the number of unhatched fertilized eggs at the expense of a decrease in number of daughters (Breeuwer 1997). In our populations, however, CI is incomplete, since some daughters are produced from crosses between uninfected females and infected males (see Figure S2.4 and S2.5).

Finally, to avoid the effects of relatedness, females mated with males from a different replicate line of the same selection regime. Replicate combinations were assigned as follows: Replicate 1 females mated with replicate 2 males, replicate 2 females mated with replicate 3 males, replicate 3 females mated with replicate 4 males and replicate 4 females mated with replicate 1 males.

Statistical Analysis

All analyses were carried out using the R statistical package (v. 3.0.3). The general procedure for building the statistical models used to analyse the effects of re-mating on the offspring proportion of females and life history traits was as follows: selection regime (i.e., *Wolbachia*-uninfected from the control regime (C) and *Wolbachia*-uninfected from the mixed regime (M), the type of cross (i.e., cross with uninfected males (U), with *Wolbachia*-infected males (I), with uninfected followed by infected males (UI), or with infected followed by uninfected males (IU)), and their interaction were fit as fixed explanatory variables, whereas block and replicate were fit as random explanatory variables. To analyse the level of CI, the relative proportions of unhatched eggs, of daughters, and of sons, were computed using the function cbind and analysed using a generalized linear mixed model with a beta-binomial error distribution to account for overdispersion (glmmadmb, glmmADMB package) (Fournier et al. 2012; Skaug et al. 2013). Daily fecundity per female was Box-Cox transformed to improve normality (Crawley 2007) and subsequently analysed using linear mixed-effect models (lmer, lme4 package). Survival was analysed using a cox proportional hazards mixed-effect model (coxme, coxme package).To test the effect of the selection regime on the willingness to remate, latency to copulation was normalized using a Box-Cox transformation (Crawley 2007), then analysed using a linear mixed-effect models (lmer). In this model, the number of individuals per patch and the identity of the patch were added to the previous mentioned random explanatory variables.

Maximal models were simplified by sequentially eliminating non-significant terms to establish a minimal model (Crawley 2007). The significance of the explanatory variables was established using chi-squared tests, in the case of discrete distributions, or F tests, in the case of continuous distributions (Bolker et al. 2008). When an interaction was significant each selection regime was analysed separately. A posteriori contrasts were done between levels of each significant variable using a test for General Linear Hypothesis (glht, multcomp package).

Results

The analyses of the proportion of unhatched eggs revealed that the selection regime affected differentially the outcome of CI depending on the type of cross (regime-type of cross interaction: X^2_3=8.136, P=0.043; Figure 2). Further analyses conducted for each regime separately revealed no significant differences in the proportion of unhatched eggs between once and double mated females if females belonged to the control regime (I – IU: Z=0.951, P=0.565; U – UI: Z=0.822, P=0.652; Figure 2). However, if females had been evolving under mixed-infection, females that first mated with infected males and then re-mated with uninfected males presented a lower proportion of unhatched eggs than females only mated to infected males (I – IU: Z=3.788, P<0.001; Figure 2), while this proportion was the same for once and double mated females mated first with an uninfected male (U – UI: Z= 0.261, P=0.957; Figure 2). These results thus suggest a partial rescue of CI by double mated females evolved under mixed *Wolbachia*-infection frequency.

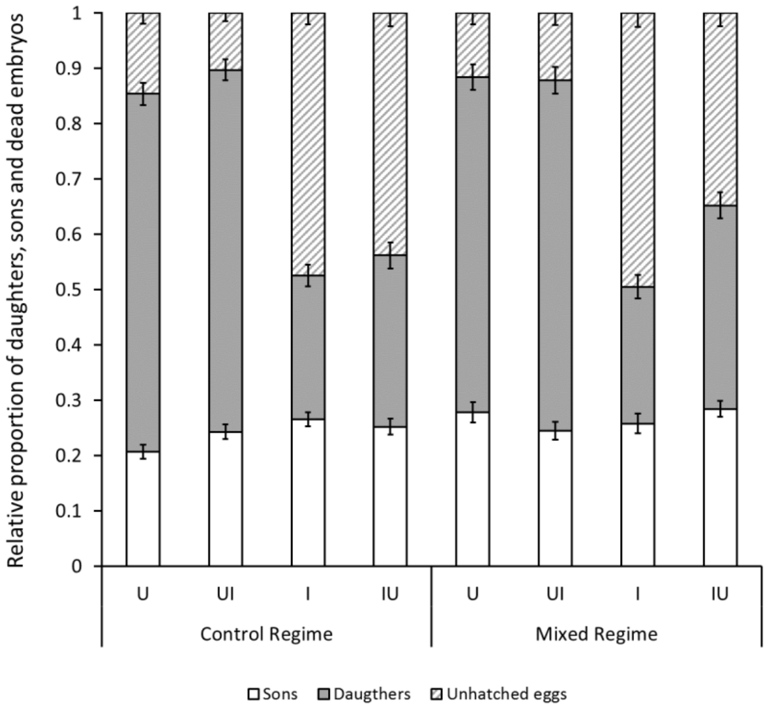

*Figure 2. **Effect of regime and cross type on the relative proportion of sons, daughters and unhatched eggs in spider-mite offspring.** Bars represent means (± s.e.) in the offspring of uninfected females from either the* Wolbachia *control regime (C) or the mixed regime (M) that mated with* Wolbachia*-infected (I) and/or* Wolbachia*-uninfected males (U). The order in which the letters appear in the x axis correspond to the order of matings: the first letter corresponds to the first male that copulated with the female, the second letter to the second male. Uninfected males belonged to the same regime as females (i.e., C females mated with C males; M females mated with M males).*

The proportion of sons was higher for females from the mixed regime than for females of the control regime (regime: X^2_1=6.032, P=0.014; cross type: X^2_3= 1.059, P=0.787; regime-cross type interaction: X^2_3=7.528, P=0.057; Figure 2), whereas the proportion of daughters was affected by the type of cross (X^2_3=556.631, P<0.001; Figure 2) but not by the selection regime (X^2_1=0.0018, P=0.966) or their interaction (X^2_3= 4.325, P=0.228). In this case, in both regimes, females that mated first with uninfected males had the same proportion of daughters, independently of the existence of a second male (U – UI: Z=-4.09, P=0.995; Figure 2), whereas females that mated first with infected and then with uninfected males, produced more daughters than females only mated to infected males (I–IU: Z= -0.09, P<0.001; Figure 2).

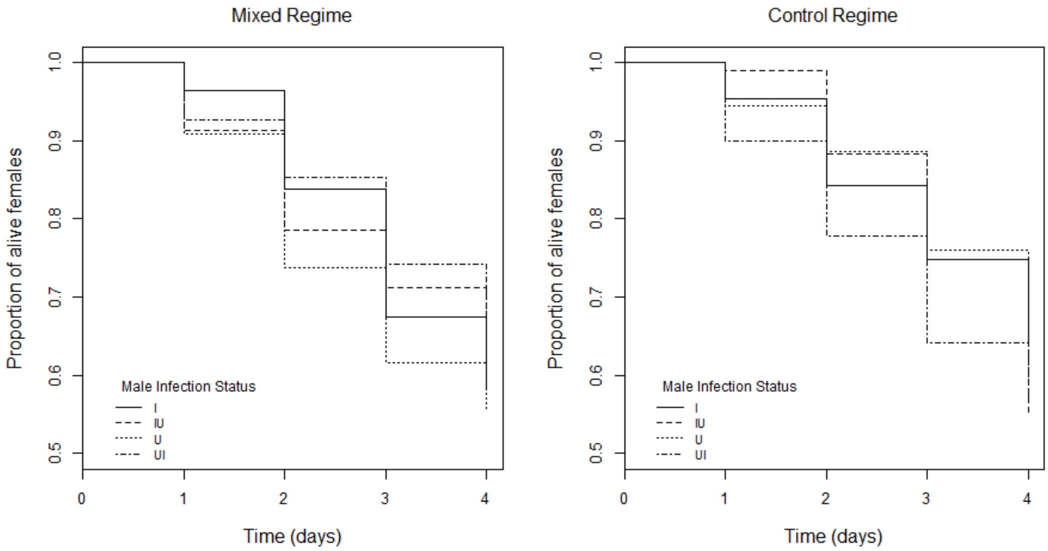

Figure 3. Effect of regime and cross type on female survival. *Survival curves for uninfected females form a) the mixed regime (M), and b) the control regime (C). Females were mated with* Wolbachia-*infected (I) and/or uninfected males (U). The order in which the letters appear in the x axis correspond to the order of matings. Uninfected males belong to the same regime as females (i.e., C females mated with C males; M females mated with M males).*

The survival of females was not affected by their selection regime (X^2_1=0.22, P=0.64), the type of cross (X^2_3=0.693, P=0.875) or the interaction between these two factors (regime-cross type interaction: X^2_3=7.679, P=0.053; Figure 3). However, the selection regime affected daily fecundity differentially, depending on the type of crosses (regime-cross type interaction: $F_{3,841.44}$=2.7166, P=0.044). Indeed, the separate analyses of each selection regime revealed that only females from the mixed-infection regime exhibited differences in fecundity (control regime: $F_{3,421.11}$=1.0605, P=0.366; mixed regime: $F_{3,408.28}$=3.400, P=0.018; Figure 4). In this regime, females that mated twice, first with an uninfected and then with an infected male, produced more offspring than females once mated to uninfected males or females first mated with infected and then uninfected males (UI -UU: Z=2.871,

P=0.025; UI - IU: Z=2.785, P= 0.032; UI - I: Z=2.475, P= 0.0799; Figure 4). Finally, no effect of regime ($F_{3,33.235}$=0.3811, P=0.5412), male type ($F_{3,35.199}$= 0.0256, P=0.8739) or their interaction ($F_{1,30.537}$=2.1998, P=0.1483) was found for latency to copulation (Figure 5).

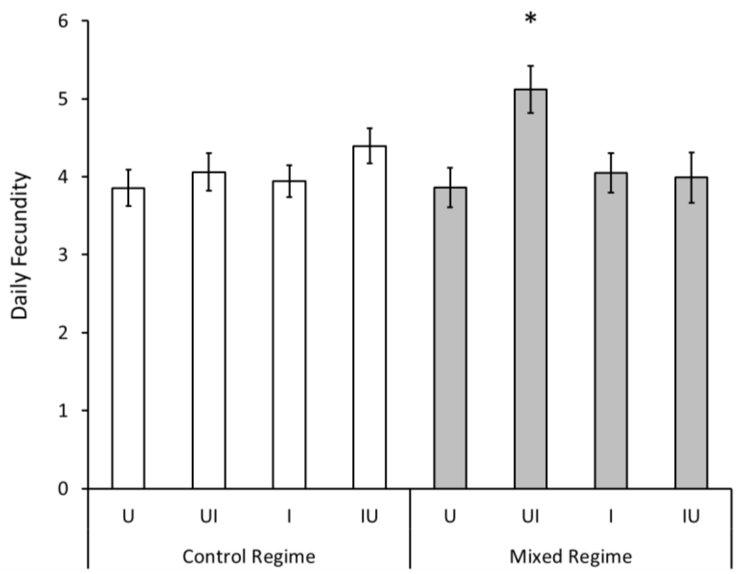

Figure 4. Effect of regime and cross type on the daily fecundity of females. *Average (±s.e.) daily fecundity per female (total number of eggs laid per female / number of days the female was alive) for* Wolbachia*-uninfected females from the control regime (C; light grey) and the mixed regime (M; dark grey). Females were mated with* Wolbachia*-infected (I) and/or uninfected males (U). The order in which the letters appear in the x axis correspond to the order of matings. Uninfected males belong to the same regime as the female (i.e., C females mated with C males; M females mated with M males). Absent superscripts (*) above bars indicate non-significant differences at the 5% level (contrasts analyses).*

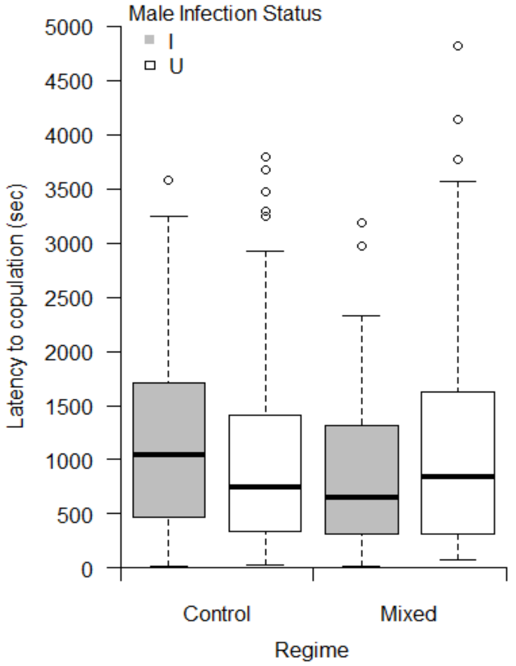

Figure 5. Effect of regime and infection status of the second male on the latency to copulation of females. *Boxplot of the latency to copulation for* Wolbachia*-uninfected females from the control (C) or the mixed (S) selection regimes. Second mating could be with* Wolbachia*-infected (I) or uninfected (U) males. Uninfected males belong to the same regime as the female (i.e., C females mated with C males; M females mated with M males).*

Discussion

In this study, we tested if spider mites adopted strategies to reduce the fitness costs imposed by mating with incompatible mates, and whether such strategies were selected when females evolved in presence of both compatible and incompatible males. We found that females evolving under mixed infection produced a lower proportion of unhatched eggs when mated to uninfected males after mating with infected males than when mated to infected males only. This suggests that spider mites, a species with first male sperm precedence, can evolve the ability to avoid the negative effect of *Wolbachia* by partially rescuing CI. Moreover, females from the mixed regime that mated first with an uninfected and then with an infected male produced more offspring than females from the same regime mated to a single uninfected male. This was not the case of females from the control selection regime. Together, these results suggest that evolving under mixed infections selects for strategies that reduce the cost of CI.

The unidirectional decrease of the proportion of unhatched eggs may be due to an inferior reproductive success of infected males. Indeed, decreased sperm competitive ability of infected males is very frequent in species carrying selfish genetic elements (Champion de Crespigny and Wedell 2006, in *Wolbachia*, and Price and Wedell 2008, for a review). This decrease may be due, for instance, to a decrease in quality or quantity of the sperm produced by infected males. In fact, an inferior fertility of infected males was found in *D. simulans* infected with *Wolbachia* (Snook et al. 2000), and in species carrying other SGE's (see Price and Wedell 2008 for review). The evolved ability of females to distinguish sperm from each type of male in the mixed regime may hinge on this difference in sperm property between infected and uninfected males. Alternatively, it is possible that the ability to rescue CI is not due to evolved cryptic female choice but to sperm competition promoted by multiple mating (Price & Wedell 2008). In this case, rescued CI in the mixed regime should be an evolved strategy of the uninfected males. Finally, another possibility would be that both mechanisms are occurring simultaneously. In any case, a lower competitive ability of infected males could also explain why females from both selection regimes produced more daughters when they mated first with infected and then with uninfected males than when they mated with infected male only. Indeed, it has been shown previously that female spider mites are capable of using sperm from subsequent matings when the first mating is not fully effective (Satoh et al. 2001).

Females from the mixed regime produced more sons than those from the uninfected control regime. This may represent a cost of evolving CI avoidance. Alternately, females from the mixed regime may produce more sons because sons are haploid and will be uninfected, independently of the infection status of the males the female mates with. In addition, uninfected males are never involved in incompatible crosses, as matings with males of this infection status and uninfected or infected females are compatible. By producing more sons, females can not only avoid the costs of incompatible matings, but also slow down *Wolbachia* spread in a polymorphic population. However, this strategy may be non-adaptive for females, for example under local mate competition, where a female-biased sex ratio is optimal (Hamilton 1967) This cost could help explain why in fully uninfected populations the ability to rescue CI is not maintained.

In populations where polyandry is advantageous, we expect high mating rate to evolve. Indeed, increased female mating rate has evolved in *Drosophila pseudoobscura* exposed for 10 generations to the risk of mating with males carrying a deleterious sex ratio-distorting gene (Price et al. 2008). However, here, even though individuals from the mixed regime could partially rescue CI, females from both selection regimes had a similar latency to re-mating. One possible explanation is that latency to second copulation is not a proper indicator of increased mating rate in our species. Since double matings are not costly for spider mite females (Chapter 3), females are likely to accept these matings independently of the presence of *Wolbachia*-induced costs. However, costs of re-mating in subsequent matings do exist (Chapter 3, Macke et al. 2012). Hence, differences in latency to re-mate between control and mixed regimes, may exist in such matings, a possibility not tested here. Another possibility is that females did not increase their re-mating rate under mixed infection because the intensity of polyandry already present in the control regimes is sufficient to elicit CI rescue. As re-mating can be costly for females (Chapter 3, Macke et al. 2012), an increase in willingness to re-mate would not be selected except if it was necessary to enable evolution of strategies against the effect of CI avoidance. Indeed, the prevalence of *Wolbachia* varies across spider mites populations in the field (Zélé et al. in prep, Breeuwer and Jacobs 1996; Enigl and Schausberger 2007; Gotoh et al. 2007), which might enable the maintenance of polyandry even in populations that go through periods of complete presence or absence of *Wolbachia*. In addition, polyandry can be maintained as strategy against other incompatible crosses. For instance, incompatibility between species, populations and morphs of spider mites has been reported in the literature (Navajas et al. 2000; Gotoh et al. 2005; Clemente et al. 2016). However, when tested, re-mating could not reduce the costs of incompatible crosses between populations (Navajas et al. 2000). Nevertheless, polyandry alone is not sufficient to avoid the effect of CI in species with skewed sperm precedence patterns. Indeed, for polyandry to be advantageous in species with first male sperm precedence, the host needs to evolve the ability to disrupt its sperm precedence pattern when the first mating is incompatible, i.e., the first male is infected. However, to disrupt first male sperm precedence when the first mating is compatible would be deleterious for the host. Consequently, it is the combination of polyandry and disruption of sperm precedence pattern in one direction only that will confer on the host an advantage against the spread of *Wolbachia*.

The ability to rescue CI could trade-off with other female traits. However, no costs of survival were observed and, on the contrary, females from the mixed regime that mated twice, first with an uninfected and then with an infected male had a higher fecundity than females once mated to uninfected males and females first mated with infected and then uninfected males. This may suggest that females are able to detect the presence of infected males and increase their egg production while keeping first male sperm precedence. By producing more uninfected offspring, uninfected females gain an advantage over *Wolbachia*-infected ones, which constitutes another way to decrease the prevalence of *Wolbachia* in the population. Other types of post-copulatory differential investment have been documented for several species (Neff & Pitcher 2005).

The fact that polyandry helps rescuing CI incompatibilities does not necessarily imply that polyandry evolved in response to the costs of CI. Indeed, theoretical work predicted that CI is not likely to explain the evolution of polyandry, owing to costs of multiple mating and a decrease in the number of incompatible matings with increased *Wolbachia* prevalence (Champion de Crespigny et al. 2007). However, in our experiment females from the mixed regime, could break their sperm priority pattern unidirectionally, keeping first male sperm precedence when the first mating was compatible (i.e., the first male was not infected with *Wolbachia*). If rescue was bidirectional the second mating would be partially effective independently of the infection status of the males. This means that polyandry may allow for rescue of CI, but also for partial induction of CI, if infected males mate with uninfected females following matings with uninfected males. This behaviour would thus only be advantageous for the host in some circumstances. In our species, however, first male sperm precedence is maintained in compatible crosses, while in incompatible ones CI is partially rescued. This behaviour is thus always beneficial for the host and costly for *Wolbachia*. Polyandry might thus have evolved in response to incompatible matings. Furthermore, theoretical work on the genetic incompatibility and the good genes hypotheses, has predicted that polyandry can evolve as a strategy against incompatible crosses (Colegrave et al. 2002). This is contingent upon a high cost of incompatibility and on the existence of some degree of post-copulatory selection for compatible matings (Colegrave et al. 2002). In our populations, the phenotype induced by *Wolbachia* greatly decreases the reproductive success of uninfected females as it reduces by up to 48% the proportion of their daughters (Figure S2.5), and post-copulatory strategy for compatible matings did evolve in our study. Hence, the system studied here meets the theoretical assumptions necessary for the evolution of polyandry as a compensatory mechanism against incompatible crosses.

Here, we have created the optimal conditions for the evolution of CI antagonistic strategies. However, whether this may be extrapolated to natural populations remains to be evaluated. In fact, host population structure is expected to have serious consequences on the infection dynamics of CI-inducing *Wolbachia* (Engelstädter & Telschow 2009). Spider mites populations are thought to follow a so-called ‘haystack model’ of population structure (Nagelkerke & Sabelis 1996), under which most selection occurs within patches. According to this model populations of spider are thus mainly exposed to soft selection, which is predicted to foster the spread of CI (Engelstädter & Telschow 2009). However, how this affects the probability that polyandry rescues CI is unclear. Indeed, on the one hand, CI-spread will select for CI-avoidance, on the other hand, too fast a spread of CI will hamper the effectiveness of this strategy.

The work presented here is the first experimental evidence of evolution of a post-copulatory strategy in response to the adverse effects of *Wolbachia*. Indeed, we have found that the pattern of sperm precedence can be modified, to the benefit of the host, under the selection pressure imposed by symbiont infection. Further studies should be done to verify if the CI antagonistic strategies evolved here allows the host to slower *Wolbachia* invasion, or even prevent its fixation.

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CHAPTER VII

Conclusion and Perspectives

This thesis contributes to improve our understanding of mating strategies by addressing important questions that have been largely neglected so far, namely what are the drivers of multiple mating in species with first male sperm precedence. Indeed, although patterns of sperm priority have been well documented, the underlying evolutionary processes remain poorly understood. Here, we show that spider mites, a species with first-male precedence, do not benefit directly with multiple mating. However, we uncover prospective indirect benefits in polyandry for males. Furthermore, we show how spider mites, and potentially other species, can adjust their strategies to different selection pressures. Using experimental evolution of host-symbiont interactions, we provide the first test concerning the evolution of assortative mating in response to genetic incompatibilities, and the first evidence that the pattern of sperm precedence can evolve in response to infection. Our finds are of key importance to understand the dynamics between host and endosymbionts

As these points have been extensively discussed in the previous chapters, I will refrain from discussing them here. I will rather summarize the key findings from this work and present some potential future directions.

1. Key results

1.1 Multiple mating is not explained by direct benefits

We found that neither males nor females benefit directly with matings that involved females that had already mated. Nevertheless, males benefited indirectly by mating with mated females. This benefit might help explain why multiple mating with mated females occurs frequently in this species. Still, these results do not rule out the existence of other, indirect benefits. In fact, polyandry is most likely to have evolved due to several types of benefits, rather than just one (Ivy 2007).

1.1.1 Spider mites use chemical cues to distinguish mated from virgin females

In species with first male sperm precedence, virgin females provide higher fertilization opportunities to males than mated females. Accordingly, males are expected to exhibit mate choice in favour of virgins (Kvarnemo & Simmons 2013; Kelly & Jennions 2011). Here, we confirmed that spider mites prefer virgins over mated females and that they do not distinguish between mated females that have mated at different mating intervals (Figure 1.1A). Furthermore, we found that spider mites use chemical cues to make the distinction between virgin and mated females. Indeed, they use both volatiles and chemical trails, which are redundant cues (Figure 1.1B). These results indicate that re-mating does not occur due to a lack of ability to discriminate females of different matings status.

1.1.2 Spider mites exhibit complete first male sperm precedence

Several factors have been implied in the variance observed in the patterns of sperm precedence within species, namely male size, number of re-matings and mating interval (Danielsson 1998; Simmons 2001). Here, we found that spider mites present complete, to nearly complete, first male sperm precedence and that this pattern is independent of the number of matings and the interval between them (Figure 1.1C). This suggests that once the first mating of a females is successful, it is very likely that the subsequent matings are ineffective. Nevertheless, re-mating may provide direct benefits for males when first matings are ineffective. Indeed, several bodies of evidence suggest that this might be the case (Satoh et al. 2001; Potter & Wrensch 1978; Helle 1967).

1.1.3 Re-mating provides males with indirect benefits

Given that spider mites are haplodiploid, male contribution to offspring is easily measured as the total number of female offspring produced by a female. Taking advantage of Etoxazole resistance as a paternity marker, we were able to disentangle the contribution of the first male from that of subsequent males. When comparing the total number of daughters sired by the first male across treatments, we found that multiple matings are detrimental for the fitness of first males, as multiply-mated females produced fewer daughters than once- and double-mated females (Figure 1.1D). This decrease in reproductive output of first males is indirectly advantageous for subsequent males. Polyandry can thus be, even if partially, explained by this indirect benefit.

1.1.4 Re-mating alone is not costly for males

By comparing the survival of males placed with different number of females of different mating status, we found that mating multiply exclusively with mated females does not result in costs of survival for males. However, the longevity of males decreased when they were placed with an intermediate number of virgin females (Figure 1.1E). This result was unexpected. We speculate that patches with an intermediate number of virgin females are impregnated with cues released by virgins even after all females become mated. Consequently, males might attempt the most re-matings in those patches, thus suffering more costs.

1.1.5 Multiple mating reduces both fecundity and female survival

Multiple mating can easily be explained when it provides direct benefits for females (Arnqvist & Nilsson 2000). However, by comparing fitness traits between females belonging to different treatments, we have found that multiple mated females produced fewer eggs and lived less than once- or twice-mated females, when the interval between matings was of 24 hours. These results are in accordance with previous studies in spider mites (Macke et al. 2012; Oku 2010) and indicate that females diminish their reproductive success when they mate multiple times. The degree of polyandry may thus be under male control, owing to the indirect benefits they obtain with this behaviour. In this case, females re-mate more than their optima to mitigate the costs incurred by persistent males attempting to copulate, a strategy called "convenience polyandry" (Thornhill & Alcock 1983; Snook 2014). However, it is unclear whether the benefits obtained by males with re-mating outweigh the costs of mating in mixed patches. Furthermore, there might be other benefits in polyandry for females (see General Introduction, section 1.3.2).

1.2 *Wolbachia* infection potentially shapes female reproductive strategies

Wolbachia can act as a selfish genetic element, by altering host biology, in order to favour their own transmission (Werren et al. 2008). This manipulation can be costly for the host, in which case the host is expected to evolve strategies to avoid or reduce the cost of infection. We began by investigating whether there was intraspecific variation for mate choice between infected and uninfected males in spider mite females. Then, spider mites evolved for 20 generations under different prevalences of *Wolbachia* that correspond to the presence (mixed regime) or absence (control regime) of risk of incompatible matings and tested whether mate choice and polyandry evolved in response to these selection regimes.

1.2.1 Assortative mating is not common across field-derived populations

Out of the five field-derived populations tested, none exhibited assortative mating in favour of compatible mates (Figure 1.2A). This suggests that the ability to discriminate individuals with different infection status is not common in populations of *Tetranychus urticae*. Our results are in line with other empirical studies (Hoffmann et al. 1990; Champion de Crespigny & Wedell 2007) that show that this behaviour is not common across different species in the field.

1.2.2 Assortative mating does not evolve under mixed infection

Given that the prevalence of *Wolbachia* can condition the selection for choice, we exposed uninfected females to a selection regime where choice between infected and uninfected males could be expressed (mixed regime). After 12 generations of selection, females from the mixed regime did not show preference for uninfected males, meaning that mate preference did not respond to selection (Figure 1.2B). The lack of mate discrimination could be explained by an absence of genetic variation for this trait in the ancestral, field-derived, populations or by weak selection pressure applied during experimental evolution. Alternately, host could have evolved other strategies against *Wolbachia* infection, rendering assortative mating unnecessary (see section below). To our knowledge, this was the first time someone tested whether assortative mating could evolve in response to genetic incompatibilities.

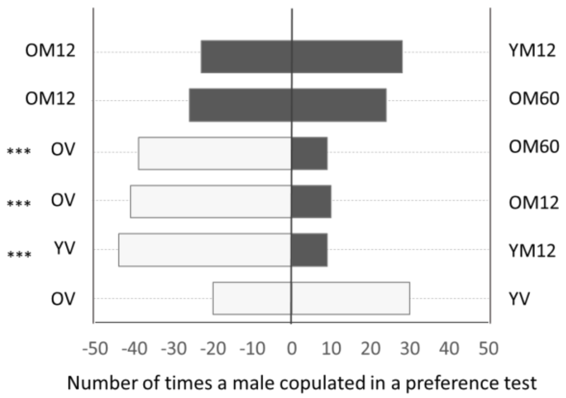
1.2.3 Polyandry helps avoiding the effects of CI

By comparing the hatching rate of eggs produced by females from the control and mixed regimes, we found that females evolving under mixed infection produced a lower proportion of unhatched eggs when mated to uninfected males after mating with infected males, than when mated to infected males only (Figure 1.2C). This result suggests that spider mites can evolve the ability to reduce the negative effect of *Wolbachia* by partially rescuing CI. By doing so, spider mites break their sperm priority pattern in favour of the second male. However, this disruption of sperm precedence only occurred in one direction. Indeed, when the first mating was compatible, i.e., the first male was not infected with *Wolbachia,* individuals from the mixed regime kept first male sperm precedence. The unidirectional disruption of the sperm precedence pattern might be a key factor for the evolution of CI-driven polyandry in species with skewed patterns of sperm precedence. Indeed, it is the unidirectionality that makes this strategy always costly for *Wolbachia* but beneficial for the female host. Moreover, females from the mixed regime that mated first with an uninfected and then with an infected male, produced more offspring than females from the same regime mated to a single uninfected male (Figure 1.2D). These results constitute the first experimental evidence that evolution of CI-antagonist strategies is possible.

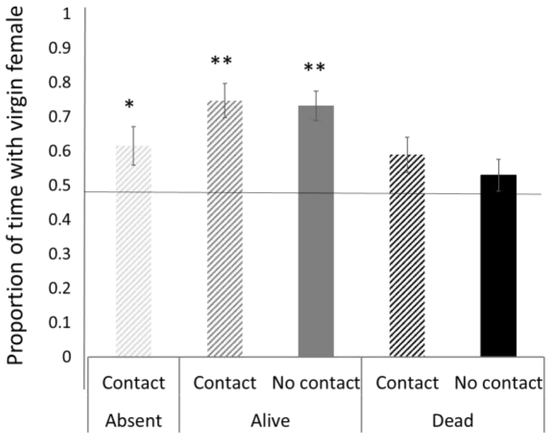
1.2.4 Mating rate does not increase at intermediate prevalence of *Wolbachia*

In a scenario where polyandry is advantageous, we expect a high mating rate. However, latency to second matings was not different between females of the control and the mixed regime, indicating that the mating rate did not increase at intermediate prevalence of *Wolbachia*, where the risk of incompatible matings exist and polyandry is advantageous. This result is not in accordance with what was found in D*rosophila pseudoobscura* by Price et al. (2008). In this species, females exposed to the risk of incompatible matings for 10 generations evolved increased mating rate. However, our result might be explained if the degree of polyandry necessary for the evolution of CI avoidance was already present in the ancestral population. The maintenance of polyandry might be due to variance in prevalence of *Wolbachia* across spider mites populations in the field (Zélé et al. in prep, Breeuwer and Jacobs 1996; Enigl and Schausberger 2007; Gotoh et al. 2007), and/or to the existence of other types of incompatible crosses, such as incompatibility between species, populations or morphs (Navajas et al. 2000; Gotoh et al. 2005; Clemente et al. 2016).

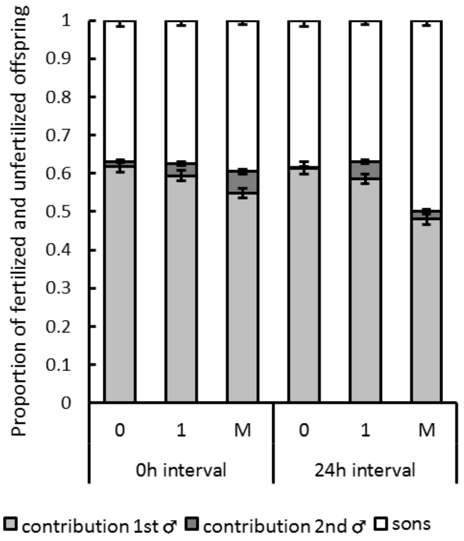
1.1 Multiple mating is not explained by direct benefits



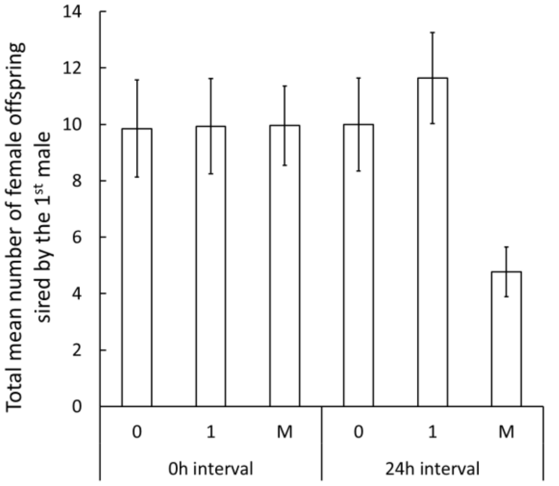
A. Male spider mites distinguish females according to their mating status and do not discriminate among mated females (Chapter III)



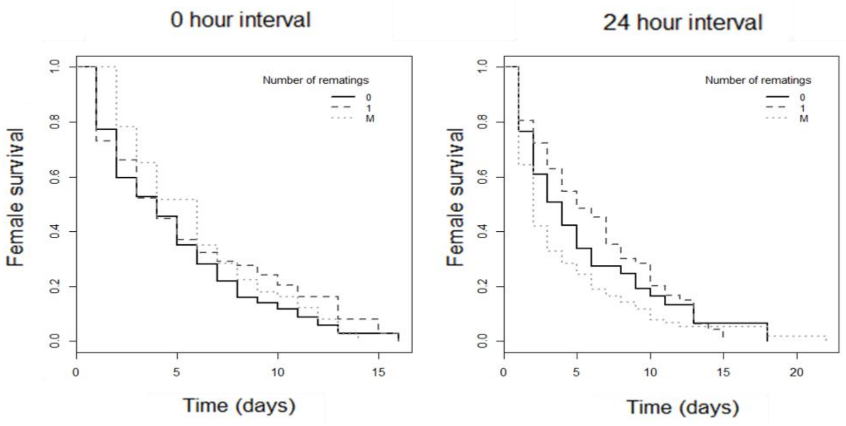
B. Spider mites use volatiles and chemical traits to distinguish virgins from mated females (Chapter III)



C. Spider mites have complete first male sperm precedence (chapter IV)

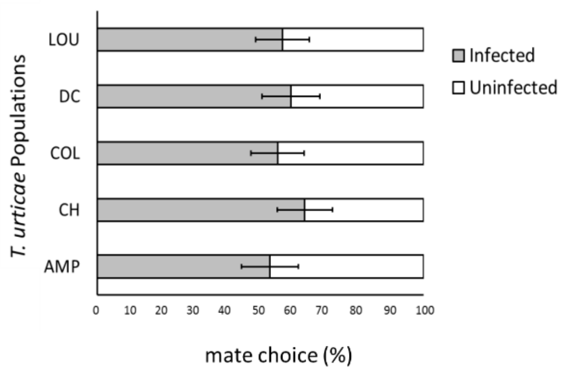


D. First males sire less offspring in multiple matings (chapter IV)

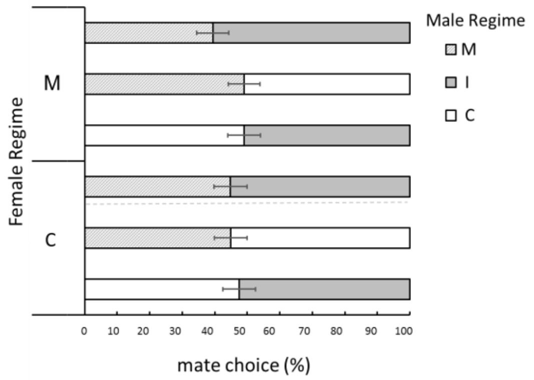


E. Males do not suffer costs of re-mating (chapter IV)

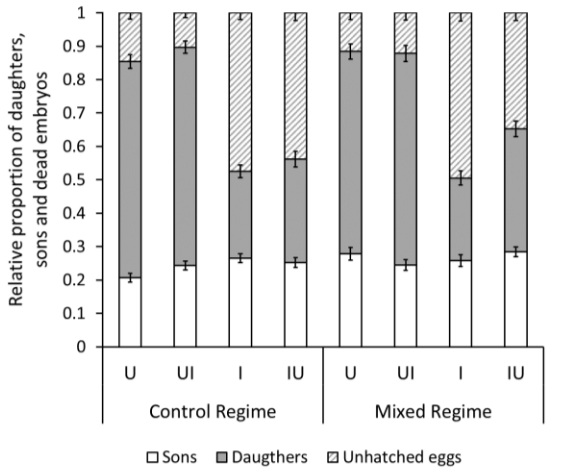
1.2 *Wolbachia* infection potentially shapes female reproductive strategies



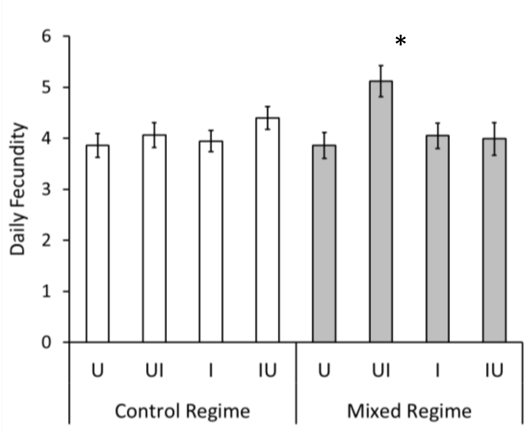
A. None of the 5 naturally infected populations exhibit mate discrimination (chapter V)



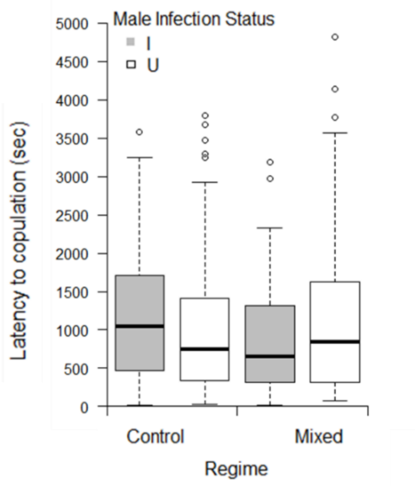
B. There is no assortative mating after experimental evolution (chapter V)



C. Rescue of CI is unidirectional in spider mites (chapter VI)



D. Females from the mixed regime increase fecundity after re-mating with infected males (chapter VI)



E. Individuals from the mixed regime do not evolve increased mating rate (chapter VI)

2. Main perspectives

This thesis suggests numerous exciting avenues for future research in mating strategies of polyandrous species, in particular with first male sperm precedence, such as spider mites. Here I propose potential perspectives that might provide additional significant information on the topics addressed here.

2.1 Is the rate of mating attempts dependent on the type and intensity of cues present in a patch?

In this thesis, we have seen that male longevity decreased in patches with an intermediate number of virgin females (Figure 1.1E) and that males use chemical cues left by females to exert their mating preference in favour of virgins (Figure 1.1B). We propose that patches with an intermediate number of virgin females were impregnated with cues released by virgins even after all females become mated. Therefore, males might attempt more re-matings in those patches, thus suffering more costs due to increased resistance in mated females. This hypothesis deserves to be tested.

This could be tested by measuring the mating rate of males in patches previously occupied by different numbers of mated or virgin females. We could also observe the locomotion patterns of both sexes to determine if potential differences in mating rate with virgin and mated females are due to differences in resistance between females or to differences in mating propensity in males. To this aim, a MATLAB-based software appropriate for tracking spider mites in leaf discs has been developed in our lab, with some promising results (Figure 2).

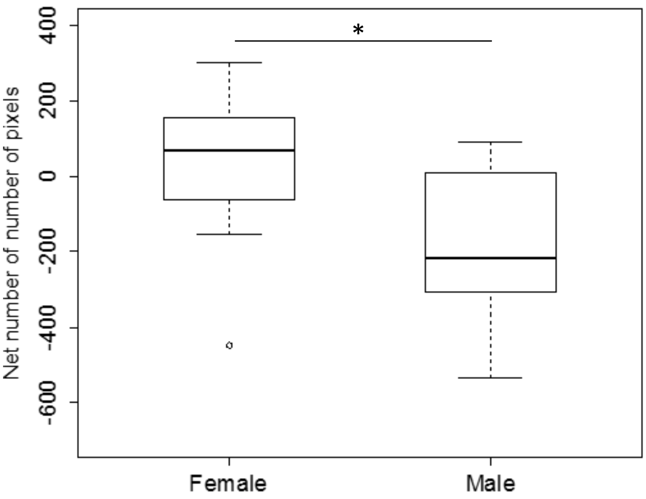


Figure 2. Net number of pixels travelled by each sex relative to the other. *Virgin males and females were placed together and filmed for 30 minutes. The net distance travelled relative to the other mite was calculated by the sum of the differences of the distances between one mite and the other on frame x and of the distance between one mite on frame x and the other on frame x+1. This quantity was used as a proxy for attraction: if the total number of pixels is close to 0, mites travelled the same distance towards and away from their prospective mate, whereas if it is significantly different from 0, it suggests the individual is either approaching (negative value) or moving away from (positive value) the other. Here, males moved significantly more towards females than the opposite (linear mixed effects model with a normal error distribution: F= 10.454; p = 0.0034). Asterisk (*) represents significance level (p < 0.05).*

2.2 Does relatedness influence male mating strategies?

In experiment IV, we have seen that males that mated with mated females can increase their relative fitness by decreasing the fitness of other males (Figure 1.1D). This could be achieved by removing sperm from the first male mechanically or by using seminal products that kill sperm from the first male, as found in several other species (Simmons 2001; Manier et al. 2010). Alternately, multiple matings can simply reduce female fecundity and survival, which will affect the fitness of the first male. One question arises from this result: Should males reduce their offensive mating strategies when competing with males are closely related? Indeed, individuals are predicted to behave more altruistically towards their relatives, with whom they share a relatively high proportion of genes (Hamilton 1963). Recently, it has been shown that females exposed to groups of three related males, unrelated to the female, have higher fecundity and reproductive lifespan compared to females exposed to groups of three unrelated males (Carazo et al. 2014), confirming this prediction. This could be tested in spider mites as well. Moreover, we could test whether evolving with related males would result in a decrease in the display of this strategy. We here propose an experimental evolution setting in which this could be tested (figure 3).

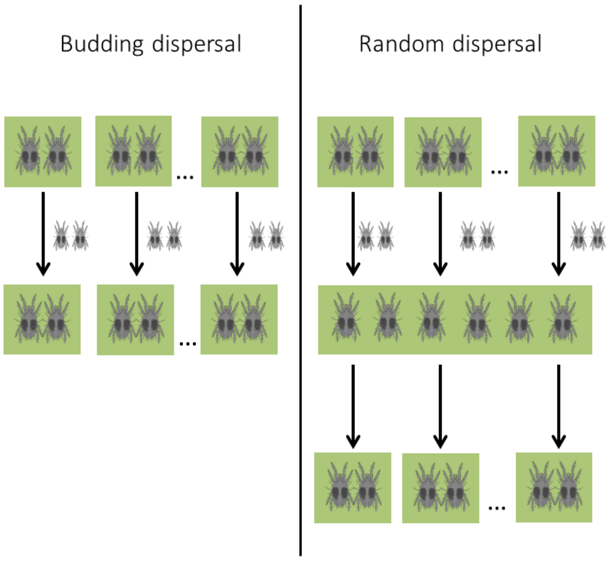


Figure 3. Schematic representation of the proposed treatments to test how evolving with related males may affect offensive male traits. *Budding dispersal: Every generation, 2 mated females from each patch are transferred together to a new patch. Random dispersal: Every generation, 2 mated females per patch are transferred to a common patch. Females are then haphazardly transferred in pairs to new patches.*

If males adjust their mating strategies according to the relatedness of their opponents, one could also ask how do males achieve this: Do they decrease the degree of polyandry and/or do they reduce the negative effect of ejaculates? As we have seen, polyandry does not confer a direct benefit for males, only being beneficial because it decreases the fitness of other males (Figure 1.1D), assuming there is no risk of incompatible matings. In addition, males are able to discriminate virgin from mated females (Figure 1.1A). In the absence of incompatible matings or any other type of benefit related to polyandry, decreasing the degree of polyandry in presence of related males is thus more likely to evolve than maintaining polyandry but reducing the negative effect of ejaculates, as this latter strategy would not benefit neither the male nor its relatives.

2.3 Is re-mating rate in males a by-product of selection for high mating frequency?

Along this thesis, we have gathered evidence on how polyandry can be beneficial for spider mites. We have found that males benefit indirectly by reducing the contribution to offspring of first males (Figure 1.1 D). In addition, males and females are able to reduce the negative effects of *Wolbachia* infection by mating multiply (Figure 1.2 C). However, we still do not know if polyandry evolved owing to, and/or is maintained in the populations by any of these benefits. Another possibility that could explain the existence of polyandry in this species is that female re-mating occurs because this trait is correlated with another trait in males. For example, males that re-mate often may also have a high mating rate. Because a high mating rate is probably advantageous for males, re-mating may be indirectly selected, even if it is costly for males and females (Macke et al. 2012). This could be tested by measuring the mating frequency of males and their propensity to mate with mated females in different isofemale lines. The genetic correlation between these traits can then be inferred.

2.4 Does polyandry prevent the fixation of *Wolbachia* in spider mites?

We have found that spider mites can evolve the ability to reduce the negative effect of *Wolbachia* by partially rescuing CI (Figure 1.2C). This was achieved by breaking the sperm priority pattern typical of *T. urticae*, in favour of the second male, only when it was advantageous for the host, i.e., when the first male mating with an uninfected female was infected and the second male was uninfected. A theoretical model has predicted that in diploid species with last male sperm precedence, the benefit provided by polyandry against the negatives effects of CI-inducing *Wolbachia* is sufficient to prevent *Wolbachia* fixation (Champion De Crespigny et al. 2007). However, to our knowledge this has never been empirically tested. In addition, the phenotype of CI differs between diploid and haplodiploid species (see General introduction, section 2.3). It would thus be interesting to test whether the strategies evolved by the host here are sufficient to slow *Wolbachia* invasion or even prevent its fixation in the host population. The results stemming from this experiment would contribute substantially to our knowledge concerning the dynamics of *Wolbachia* invasion in haplodiploid species.

2.5 Is functional fertility a key factor in the variance of sperm precedence patterns in *T. urticae*?

As we have seen along this thesis, in spider mites, once the first mating of a females is successful, the subsequent matings are ineffective (Figure 1.1C). However, we did not explore here what would happen if the first matings was not fully effective. Indeed, previous studies done in this species suggest that disruption of first matings and sperm depletion might influence the effectiveness of second matings (Helle 1967; Potter & Wrensch 1978; Satoh et al. 2001). Furthermore, our results concerning rescue of CI might be contingent on a decrease in quality or quantity of the sperm of infected males (chapter VI, Figure 1.2C). Consequently, several new questions can be posed, regarding the effect of functional fertility in intraspecific variation of sperm precedence. Indeed, functional fertility, i.e., the inability of a male to fertilize the egg of a female despite not being sterile, is predicted to be responsible for high intraspecific variation in sperm precedence patterns across species (Garcia-Gonzalez 2004).

By testing each of the following hypothesis, we will be able to better understand the conditions necessary for the disruption of sperm precedence in spider mites and ultimately, in other species with first male sperm precedence.

2.5.1 Does sperm depletion in the first male disrupt the pattern of sperm priority?

In future studies, it may be interesting to test whether females that mate with a sperm depleted male are able to use the sperm from a subsequent male, this way breaking sperm priority. This experiment can be easily done in our model species using resistance as paternity marker, as it has been shown in chapter IV.

This experiment will open numerous different avenues. For instance, if sperm depletion does disrupt the pattern of sperm priority, is this phenomenon significant enough to maintain re-mating in spider mite populations? Alternately, if the pattern of sperm priority is not altered, do females distinguish between sperm-depleted and sperm-replenished males in order to avoid the costs of mating without receiving sperm? Also, are sperm-depleted males less willing to mate than sperm-replenished males? In order to disentangle the last two questions, we can track the locomotion patterns of both sexes, taking advantage of the software previously described in section 2.1, using males containing different amounts of sperm.

2.5.2 Does sperm depletion alter female receptivity to future matings?

As we have seen in chapter II, matings with mated females take longer to begin and are shorter than matings with virgins. However, we do not know whether this change in behaviour is elicited by the use of sperm in fertilization or by-products in the seminal fluid of males. If the first hypothesis is true, females might behave as virgins after copulating with sperm-depleted males. Alternately, if the latter hypothesis holds, sperm-depleted males might be able to decrease female receptivity to subsequent matings, improving their offspring representation in the population. This can be easily tested by comparing the latency to copulation and copulation duration of virgins, females mated with sperm-depleted males and females mated to sperm-replenished males.

2.5.3 Are *Wolbachia*-infected males worse competitors than *Wolbachia*-uninfected males?

As we have seen in chapter VI, spider mites evolved the ability to reduce the effect of CI-inducing *Wolbachia* (Figure 1.2C). This ability might be contingent on a decrease in the quality or quantity of the sperm of infected males, as has been observed in several other species carrying selfish genetic elements (Price & Wedell 2008; Wedell 2013). If the sperm from infected males is of inferior quality, we would expect infected males to lose in competition against uninfected males in an environment neutral for both type of females. We could test this by measuring the pattern of sperm precedence in double crosses with infected females. If the proportion of offspring sired by the second male changes according to the infection status of the first male, then the disruption of sperm precedence can be attributed to a decrease in competitive ability of the first male. Another possibility is that a decrease in sperm quantity influences the mating frequency of males. Infected males with inferior sperm may mate with fewer females that uninfected males or be more selective and avoid mated females, with whom they are unable to sire offspring.

2.6 Which sex controls CI rescue?

The rescue of CI in the mixed regime might have taken place due to an evolved ability of females to exert cryptic choice or to an improvement in the sperm competitive ability of males. These two mechanisms can be disentangled by crossing females from the control regime mated with infected males with males from the mixed regime and vice versa. We would then be able to demonstrate which sex is adjusting the pattern of sperm precedence in spider mites. This would bring us one step closer to determine the mechanisms acting in the pattern of sperm precedence, which is crucial to determine the intensity and direction of sexual selection.

2.7 Can evolved mechanisms be generalized to a *Wolbachia*-free environment?

If it is proven that the mechanism acting against *Wolbachia* in the mixed regime is due to an improvement of sperm competitive ability in uninfected males, it would be interesting to further explore whether this advantage is conditional, i.e., if males only have a competitive advantage against sperm from infected males, or whether this advantage is transversal to any type of sperm. This could be achieved by measuring, in uninfected females from the control regime, the degree of sperm precedence in double crosses with uninfected males from the mixed and the control regime.

A similar reasoning could be applied for cryptic female choice. That is, assuming that the evolved mechanism against *Wolbachia* in the mixed regime is cryptic female choice, can females from the mixed regime express their preference for any sperm, or are they only capable of distinguishing infected sperm from uninfected sperm?

A transversal improvement of competitive ability or cryptic female choice would indicate that disruption of sperm precedence is possible even in populations free of *Wolbachia*. Therefore, it might be interesting to test whether there is variability in the pattern of sperm precedence across individuals in a population. A screening for variability in sperm precedence could be done using isofemale lines of individuals resistant to the pesticide used here. By crossing males from different lines with females from a single line that have mated with susceptible males, we could determine whether there is genetic variation for competitive ability. Conversely, by crossing males from a single line with females from several lines that have mated with susceptible males, we could determine whether there is genetic variation for cryptic female choice. This protocol would be very similar to the one used in chapter IV.

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Appendix 1

Supplementary Material

Chapter II

Table S1 Experimental evolution studies manipulating competition. *Examples of experimental evolution studies that manipulated competition for food, mates or hosts with some details on the experimental system, the traits measured and their responses to selection. Our aim here is not to be exhaustive but to merge different bodies of literature. Note that "resource diversity" refers to manipulations that use more than one type of resources. It may correspond to resource substitution, or to resource addition (cf. main text).*

Type of resource	Ref	Selection regime created in	species/strain A	species/strain B	change in competition levels	nr generations	Response to selection					
							competitive ability	resource utilization	Correlated Responses			
									sexual conflict	*evolution of virulence*	*niche width/ character displacement*	other
Food	Agashe and Bolnick 2010	Agashe and Bolnick 2010	*Tribolium Castaneum*	-	varying the number of competitors (intraspecific), varying the type of competitors (interstrain)	8	-	-	-	-	niche expansion	-
Food	Bailey et al. 2013	Bailey et al. 2013	*Pseudomonas fluorescens*	*Pseudomonas fluorescens*	varying the number of competitors (intrastrain), varying the type of competitors (interstrain)	7 days	-	-	-	-	niche contraction or expansion (depends on type of competitor)	-
Food	Barrett and Bell 2006	Barrett et al. 2005	*Pseudomonas fluorescens*	-	varying resource diversity	900	-	-	-	-	character displacement	-
Food	Barrett et al. 2005	Barrett et al. 2005	*Pseudomonas fluorescens*	-	varying resource diversity	900	increase	increase	-	-	-	-
Food	Bierbaum et al. 1989	Mueller and Ayala 1981	*Drosophila melanogaster*	-	varying the number of competitors (intraspecific)	20-36	increase	no change	-	-	-	-
Food	Bochdanovits and de Jong 2003	Bochdanovits and de Jong 2003	*Drosophila melanogaster*	-	varying resource quality	10	-	decrease acquisition, increase utilization	-	-	-	-
Food	Bolnick 2001	Bolnick 2001	*Drosophila melanogaster*	-	varying resource quantity	4	-	increase	-	-	niche expansion	-
Food	Borash et al. 2000	Borash et al. 2000	*Drosophila melanogaster*	-	varying resource quality	21	increase	no change	-	-	-	-
Food	Forde et al. 2008	Forde et al. 2008	*Escherischia coli*	T7	varying the type of competitors (interspecific),	150	increase	-	-	-	character displacement	-

					varying resource quality							
Food	Freisen et al. 2004	Freisen et al. 2004	*Escherischia coli*	-	varying resource diversity	1000	-	-	-	-	character displacement (in 3 out of 12 populations)	-
Food	Futuyma 1970	Futuyma 1970	*Drosophila melanogaster*	*Drosophila simulans*	varying the type of competitors (interspecific), varying the levels of genetic variation (intraspecific)	10	increase (in 1 out of 28) and decrease (in 2 out of 28)	-	-	-	-	-
Food	Greig and Travisano 2008	Greig and Travisano 2008	*Saccharomyces cerevisiae(toxin-producing strain)*	*Saccharomyces cerevisiae(toxin-sensitive strain)*	varying the type of competitors (interstrain), varying the number of competitors (intrastrain and interstrain)	unknown	increase with toxin production	-	-	-	-	-
Food	Griffin et al. 2004	Griffin et al 2004	*Pseudomonas aeruginosa*	-	varying scale of competition (local vs global)	7	local competition selects for lower levels of siderophore production	-	-	-	-	-
Food	Guo et al. 1991	Mueller and Ayala 1981, Mueller et al. 1991	*Drosophila melanogaster*	-	varying the number of competitors (intraspecific)	25	increase	-	-	-	-	-
Food	Hollis 2012	Hollis 2012	*Dictyostelium discoideum*	*Dictyostelium discoideum*	varying the type of competitors (interstrain)	10	increase	-	-	-	-	-
Food	Joshi et al. 1998	Mueller et al. 1993	*Drosophila melanogaster*	-	varying the number of competitors (intraspecific)	50	increase (adult/adult), trade-off between adult and juvenil crowding	-	-	-	-	-
Food	Joshi and Mueller 1988	Mueller and Ayala 1981	*Drosophila melanogaster*	-	varying the number of competitors (intraspecific)	140	-	increase	-	-	-	-
Food	Joshi and Mueller 1996	Mueller et al. 1993	*Drosophila melanogaster*	-	varying the number of competitors (intraspecific)	50	-	increase acquisition, decrease utilization	-	-	-	-

Food	Joshi and Mueller 1997	Mueller et al. 1993	*Drosophila melanogaster*	-	varying the number of competitors (intraspecific)	50	no changes	-	-	-	-	-
Food	Joshi and Thompson 1995	Joshi and Thompson 1995	*Drosophila melanogaster*	*Drosophila simulans*	varying the type of competitors (interspecific), varying resource diversity	11	increase (in 8 out of 9)	-	-	-	-	-
Food	Joshi and Thompson 1996	Joshi and Thompson 1995	*Drosophila melanogaster*	*Drosophila simulans*	varying the type of competitors (interspecific), varying resource diversity	11	increase	-	-	-	-	-
Food	Kloss et al. 2009	Kloss et al. 2009	*Drosophila melanogaster*	-	varying resource quality	29- 64	increase	no change	-	-	-	-
Food	Lawrence et al. 2012	Lawrence et al. 2012	*Fagus sylvatica*	*5 different species*	varying the type of competitors (interspecific)	70	-	increase + decrease (depends on spp)	-	-	character displacement	-
Food	MacLean et al. 2005	MacLean et al. 2005	*Pseudomonas fluorescens*	*Pseudomonas fluorescens*	varying the type of competitors (interstrain)	unknown	-	-	-	-	character displacement	-
Food	Mueller and Ayala 1981	Mueller and Ayala 1981	*Drosophila melanogaster*	-	varying the number of competitors (intraspecific)	8	increase	decrease	-	-	-	-
Food	Mueller 1988	Mueller and Ayala 1981	*Drosophila melanogaster*	-	varying the number of competitors (intraspecific)	128	increase	-	-	-	-	-
Food	Mueller 1990	Mueller and Ayala 1981	*Drosophila melanogaster*	-	varying the number of competitors (intraspecific)	125	-	decrease	-	-	-	-
Food	Mueller et al. 1991	Mueller et al. 1991	*Drosophila melanogaster*	-	varying the number of competitors (intraspecific)	25	increase	decrease	-	-	-	-
Food	Mueller and Sweet 1986	Mueller and Ayala 1981	*Drosophila melanogaster*	-	varying the number of competitors (intraspecific)	130	increase(pupation height)	-	-	-	-	-
Food	Mueller et al. 1993	Mueller et al. 1993, Mueller and Ayala 1981, Rose 1984	*Drosophila melanogaster*	-	varying the number of competitors (intraspecific), selection for starvation	unknown	increase resistance to starvation	-	-	-	-	-

Food	Pekkonen et al. 2011	Pekkonen et al. 2011	*Novosphingobium capsulatum*	*Serratia marcescens*	varying the type of competitors (interspecific), varying resource availability	7 days	increase	-	-	-	-	-
Food	Sanders et al. 2005	Sanders et al. 2005	*Drosophila melanogaster*	-	varying the number of competitors (intraspecific)	8	increase	-	-	-	-	parasitoid resistance not correlated
Food	Santos et al. 1997	Mueller et al. 1993	*Drosophila melanogaster*	-	varying the number of competitors (intraspecific)	unknown	-	increase	-	-	-	-
Food	Sokolowsky et al. 1997	Mueller and Ayala 1981, Sokolowsky et al. 1997	*Drosophila melanogaster*	-	varying the number of competitors (intraspecific)	286	-	-	-	-	-	rover/sitter (density-dependent selection)
Food	Stanton et al. 2004	Stanton et al. 2004	Sinapsis arvensis	-	varying the number of competitors (intraspecific), varying abiotic factors	4	variable	-	-	-	-	-
Food	Taper 1990	Taper 1990	*Callosobruchus maculatus*	*Callosobruchus chinensis*	varying the type of competitors (interspecific), varying resource diversity?	9	-	-	-	-	character displacement	-
Food	TerHorst 2011	TerHorst 2011	*Colpoda*	*Pseudocyrtolophosis alpestris*	varying the number of competitors (intraspecific), varying the type of competitors (interspecific)	60-120	-	-	-	-	some traits converged and some diverged	-
Food	Trienens and Rohlfs 2011	Wölfle et al. 2009	*Drosophila melanogaster*	*Aspergillus nidulans*	varying the type of competitors (interspecific)	26	-	-	-	-	-	tolerance vs survival
Food	Tyerman et al. 2008	Tyerman et al. 2008	*Escherischia coli*	-	varying resource diversity?	1000	-	-	-	-	character displacement	-
Food	Vijendravarma et al. 2012	Kloss et al. 2009	*Drosophila melanogaster*	-	varying resource quality	80	-	-	-	-	-	rover phenotype more frequent in high density
Food	Vijendravarma et al. 2013	Kloss et al. 2009	*Drosophila melanogaster*	-	varying resource quality	118	-	-	-	-	-	cannibalism
Food	Wölfle et al. 2009	Wölfle et al. 2009	*Drosophila melanogaster*	*Aspergillus nidulans*	varying the type of competitors (interspecific)	3 and 6	increase	decrease	-	-	-	-

Mates	Crudington et al. 2005	Crudington et al. 2005	*Drosophila melanogaster*	-	varying the number of competitors (intraspecific)	21, 23-25, 27, 29, 30, 31, 35	-	-	increase	-	-	-
Mates	Demont et al. 2014	Demont et al. 2014	*Tribolium castaneum*	-	varying the number of competitors (intraspecific)	36	increase	no change	-	-	-	-
Mates	Firman and Simmons 2010	Firman and Simmons 2010	*Mus domesticus*	-	varying resource quantity	8	increase (indirect in males)	-	no	-	-	-
Mates	Firman et al. 2011a	Firman and Simmons 2010	*Mus domesticus*	-	varying resource quantity	16	increase	-	-	-	-	-
Mates	Firman et al. 2011b	Firman and Simmons 2010	*Mus domesticus*	-	varying resource quantity	12	increase	-	-	-	-	-
Mates	Firman et al. 2011c	Firman and Simmons 2010	*Mus domesticus*	-	varying resource quantity	16	no change (indirect)	-	-	-	-	-
Mates	Firman et al. 2012	Firman and Simmons 2010	*Mus domesticus*	-	varying resource quantity	14	-	increase?	-	-	-	-
Mates	Fricke and Arnqvist 2007	Fricke and Arnqvist 2007	*Callosobruchus maculatus*	-	varying the number of competitors (intraspecific), varying food quality	35	-	-	-	-	niche expansion	-
Mates	Gay et al. 2010	Gay et al. 2010	*Callosobruchus maculatus*	-	varying the number of competitors (intraspecific), varying resource quantity, varying density, varying the type of competitors (intraspecific)	30	no change	no change	increase	-	-	-
Mates	Grazer et al. 2014	Demont et al. 2014	*Tribolium castaneum*	-	varying the number of competitors (intraspecific), food quality	39	-	depends on environment tested	depends on environment tested	-	-	-
Mates	Holland and Rice 1999	Holland and Rice 1999	*Drosophila melanogaster*	-	varying the number of competitors (intraspecific)	47	-	-	increase	-	-	-
Mates	Hollis and Kawecki 2014	Hollis et al. 2011	*Drosophila melanogaster*	-	varying the number of competitors (intraspecific), varying resource	100	increase	no change	-	-	-	learning ability

					quantity, varying density							
Mates	Hosken et al. 2001	Hosken et al. 2001	*Scathophaga stercoraria*	-	varying the number of competitors (intraspecific)	10	increase (direct and direct)	-	increase	-	-	-
Mates	Hosken and Ward 2001	Hosken and Ward 2001	*Scathophaga stercoraria*	-	varying the number of competitors (intraspecific)	10	increase (indirect)	-	-	-	-	-
Mates	LaMunyon and Ward 2002	LaMunyon and Ward 2002	*Caenorhabditis elegans*	-	outcrossing (sperm competition present) and selfing (no sperm competition)	60	increase (indirect)	-	-	-	-	-
Mates	LaMunyon et al. 2007	LaMunyon and Ward 2002	*Caenorhabditis elegans*	-	outcrossing (sperm competition present) and selfing (no sperm competition)	60	-	-	-	-	-	decreased genetic diversity
Mates	Macke et al. 2011	Macke et al. 2011	*Tetranychus urticae*	-	varying scale of competition (local vs global)	54	-	-	-	-	-	sex ratio adjustment varies with scale of competition
Mates	Lumley et al. 2015	Lumley et al. 2015	*Tribolium castaneum*	-	varying the number of competitors (intraspecific), varying resource quantity, varying density	54, 45	-	-	-	-	-	protects from inbreeding
Mates	Maklakov et al. 2009	Maklakov et al. 2009	*Callosobruchus maculatus*	-	varying the number of competitors (intraspecific), varying resource quantity, varying density, varying age at reproduction	13,11	-	-	sexual conflict?	-	-	-
Mates	Martin and Hosken 2003	Martin and Hosken 2003	*Sepsis cynipsea*	-	varying the number of competitors (intraspecific), varying resource quantity, varying density	35	-	-	-	-	-	character displacement
Mates	Michalczyk et al. 2011	Michalczyk et al. 2011	*Tribolium castaneum*	-	varying the number of competitors (intraspecific), varying resource quantity	20	increase	-	increase	-	-	-

Mates	Nandy et al. 2013	Nandy et al. 2013	*Drosophila melanogaster*	-	varying the number of competitors (intraspecific), varying resource quantity	45-47, 50 -55	-	-	increase	-	-	-
Mates	Pitnick et al. 2001a	Holland and Rice 1999	*Drosophila melanogaster*	-	varying the number of competitors (intraspecific)	38-81	increase (indirect, direct)	decrease?	increase	-	-	body size
Mates	Pitnick et al. 2001b	Holland and Rice 1999	*Drosophila melanogaster*	-	varying the number of competitors (intraspecific)	84	-	-	increase	-	-	-
Mates	Reuter et al. 2008	Reuter et al. 2008	*Drosophila melanogaster*	-	varying resource quantity	24, 28	varies with trait	-	-	-	-	-
Mates	Simmons and Garcia-Gonzalez 2008	Simmons and Garcia-Gonzalez 2008	*Onthophagus taurus*	-	varying the number of competitors (intraspecific), varying resource quantity, varying density	21	increase	increase	decrease	-	-	no evolution of horns
Mates	Snook et al. 2005	Crudington et al. 05	*Drosophila pseudoobscura*	-	varying the number of competitors (intraspecific)	25, 27-29, 30-33	-	-	-	-	character displacement?	song elements
Mates	Tilszer et al. 2006	Tilszer et al. 2006	*Rhizoglyphus robini*	-	varying the number of competitors (intraspecific), varying resource quantity, varying density	37	increase	-	increase	-	-	increased body size
Mates	Wigby and Chapman 2004	Wigby and Chapman 2004	*Drosophila melanogaster*	-	varying the number of competitors (intraspecific), varying resource quantity	18, 22, 26, 32, 31, 33	-	-	increase	-	-	-
Mates	Booksmythe et al. 2014	Booksmythe et al. 2014	*Megabruchidius tonkineus*	-	varying the number of competitors (intraspecific) varying resource quantity	20	-	increase	no change	-	-	-
Mates	Carter et al. 2015	Carter et al. 2015	*Nicrophorus vespilloides*	-	artificial selection for mating rates	12	-	-	-	-	-	social plasticity
Host	Carrillo et al. 2007	Carrillo et al. 2007	vesicular stomatitis Indiana virus	vesicular stomatitis Indiana virus	varying the type of competitors (interstrain), varying periodicity	100 virus	increase	-	-	-	-	-

Host	Miralles et al. 2001	Miralles et al. 2001	vesicular stomatitis Indiana virus	vesicular stomatitis Indiana virus	varying the number of competitors (intrastrain), varying the type of competitors (interstrain), varying periodicity	100 virus	yes (coinfection), no (superinfection)	-	-	-	-	-
Host	Garbutt et al. 2011	Garbutt et al. 2011	*Bacillus thuringiensis*	*Bacillus thuringiensis*	varying the type of competitors (interstrain)	4 host	increase	-	-	decrease	-	-
Host	Hall et al. 2011	Hall et al. 2011	lytic phage ϕ2	-	varying resource quantity	80 host	-	no change	-	-	-	-
Host	Leggett et al. 2013	Leggett et al. 2013	lytic phage ϕ2	-	varying the number of competitors (intrastrain)	400 host	increase	decrease	-	increase	-	-
Host	Rouchet et al. 2014	Rouchet et al. 2014	*Lysiphlebus fabarum*	*Hamiltonella defensa*	varying the type of competitors (interspecific)	11 parasitoid	-	no change	-	increase	-	-
Host	Dion et al. 2011	Dion et al. 2011	*Aphidius ervi*	*Hamiltonella defensa*	varying the type of competitors (interspecific)	10 parasitoid	-	-	-	increase	-	-
Host	Sachs and Bull 2006	Sachs and Bull 2006	Bacteriophages f1	Bacteriophage Ike	varying the number of competitors (intraspecies), varying the type of competitors (interspecies)	50 transfers	increase	-	-	-	-	-

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Appendix 2

Supplementary Material

Chapters V and VI

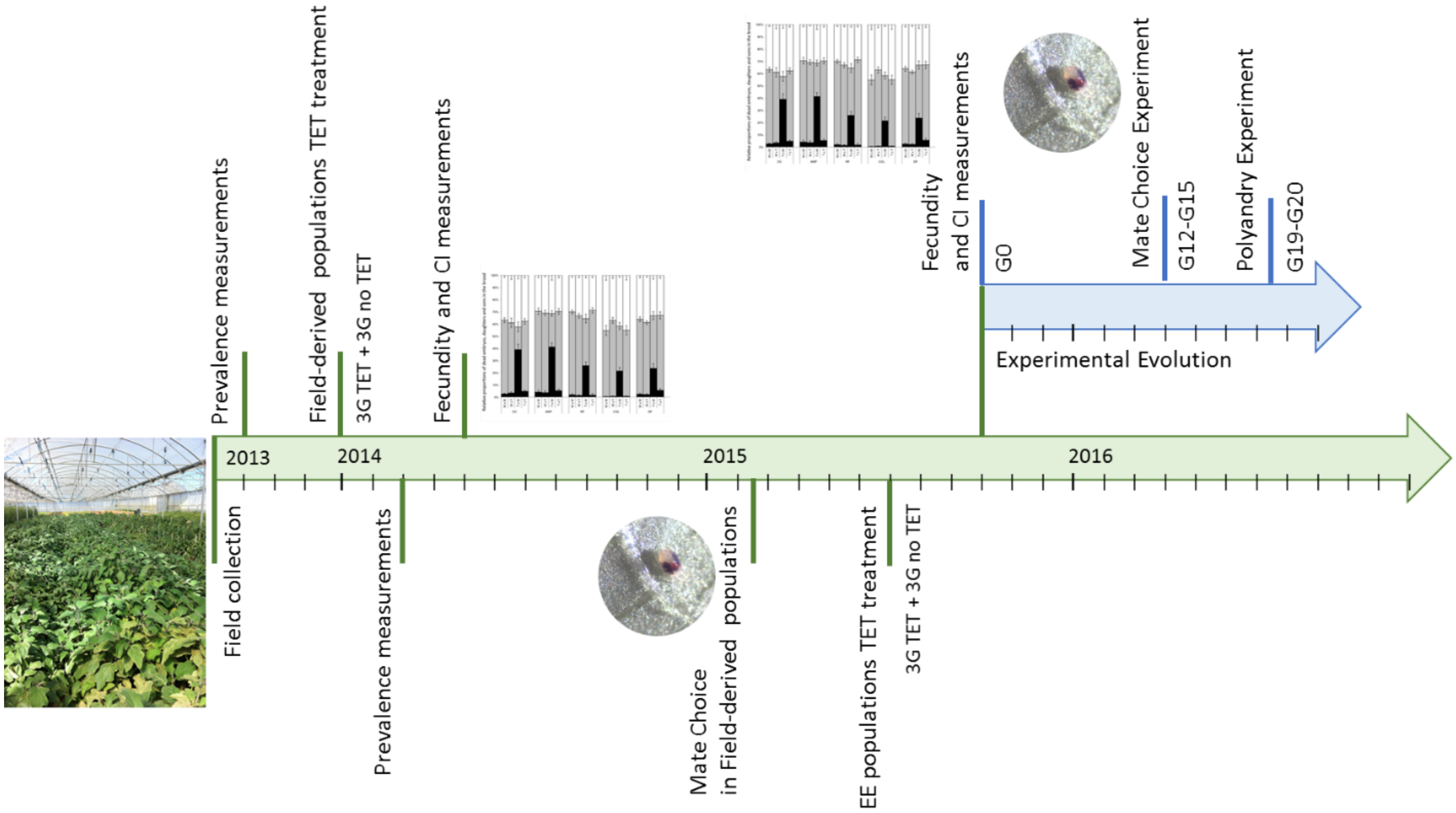
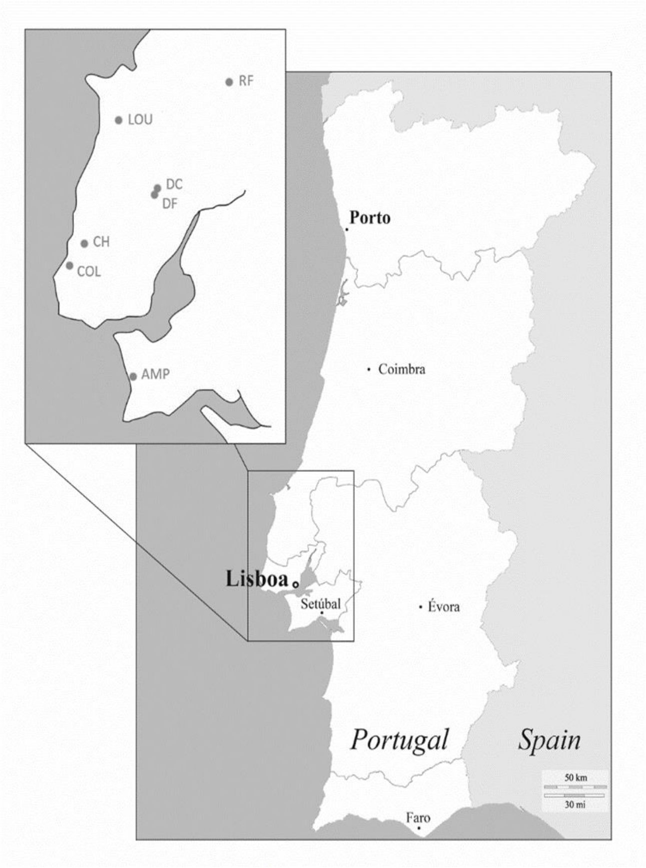


Figure S2.1. Timetable of measurements and procedures performed in Tetranychus urticae *populations, from the collection in the field to the end of the experimental evolution.*

TET: tetracycline treatment; G: generation (around two weeks). Each tick mark corresponds to one month.

The supplementary material S2.2-S2.6 is part of different manuscripts (Zélé et al. in prep).



Name	*Collection date*	*Collection Location*	*Coordinates*	*Host plant*
DC	*10/09/2013*	*S. Domingos*	*39.058742, -9.135427*	Cucurbita pepo
DF	*10/09/2013*	*S. Domingos*	*39.058742, -9.135427*	Phaseolus vulgaris
COL	*08/09/2013*	*Colares*	*38.799517, -9.448335*	Phaseolus vulgaris
LOU	*03/10/2013*	*Lourinhã*	*39.248145, -9.276321*	Solanum melongena
CH	*31/10/2013*	*Casal Hortelão*	*38.851962, -9.393918*	Solanum lycopersicum
RF	*04/11/2013*	*Ribeira de Fráguas*	*39.366415, -8.851037*	Solanum lycopersicum
AMP	*18/11/2013*	*Aldeia da Mata Pequena*	*38.534363, -9.191163*	Datura stramonium.

Figure S2.2. T. urticae *populations collected in Portugal from September to November 2013.*

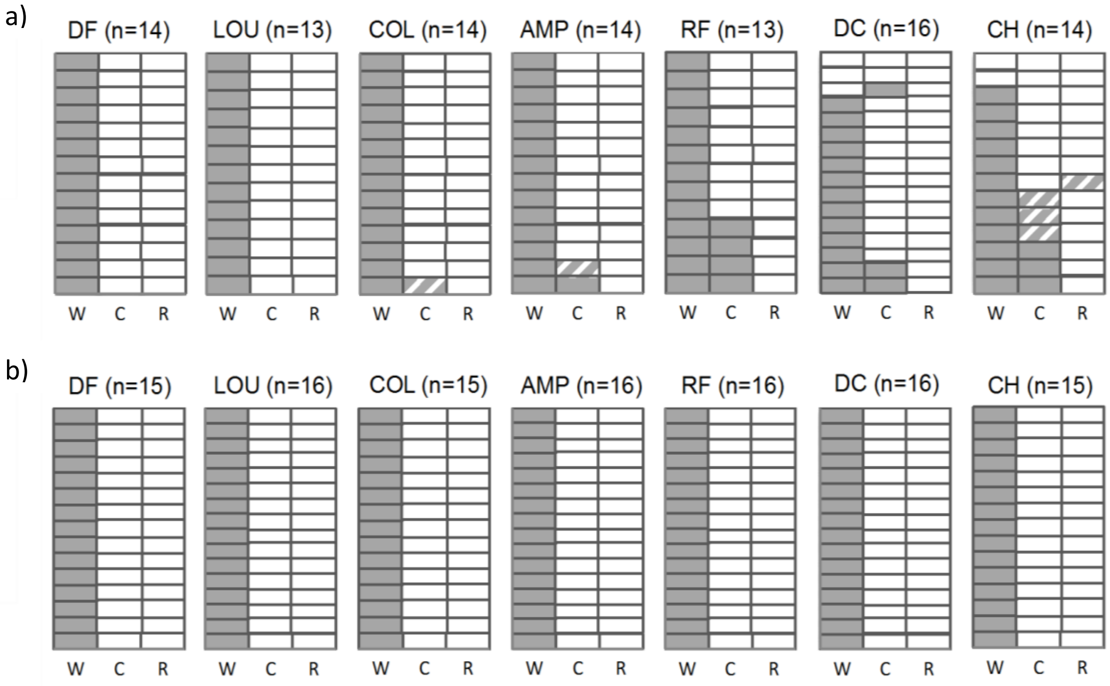


Figure S2.3. Prevalence of endosymbionts in the laboratory for each **T. urticae** ***population 0-3 months (a), and 6 months (b) after collection in the field.*** *Each graph represents a population, in which the lines represent individual mites and the columns their infection status by W:* Wolbachia*; C:* Cardinium*; and R:* Rickettsia*. White cell: uninfected; Grey cell: infected; Hatched cells:* Cardinium *and* Rickettsia *failed sequences but positive through PCR. None of the tested colonies were infected by* Spiroplasma *or* Arsenophonus*.*

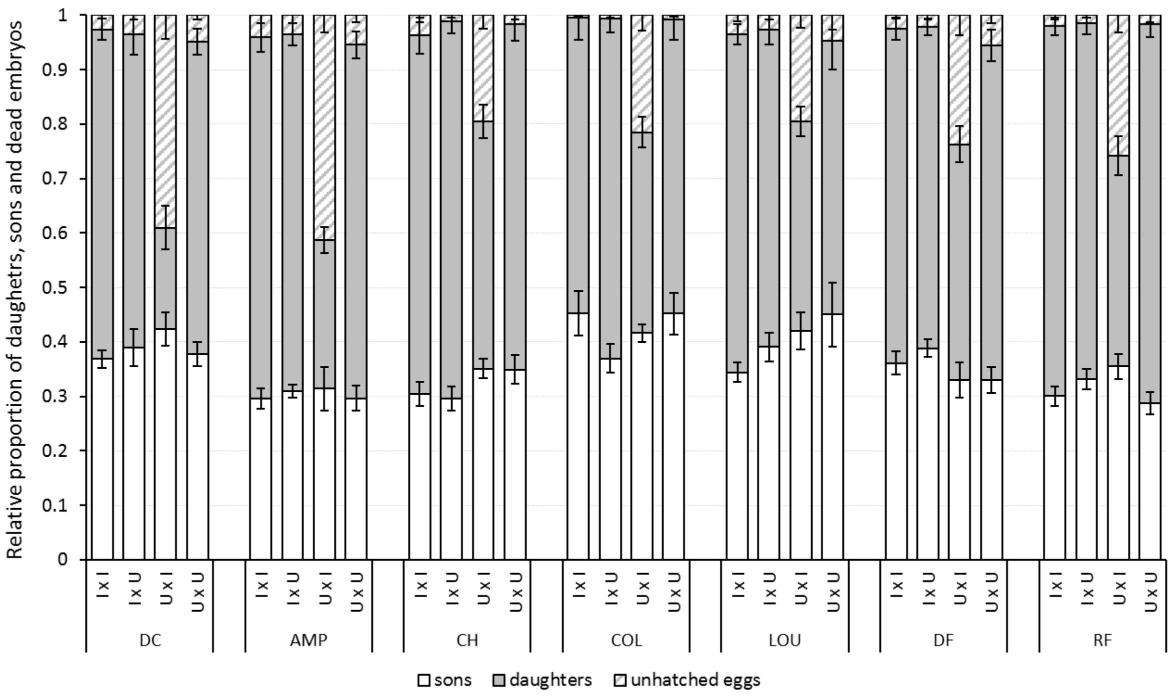


Figure S2.4. Summary of the development of **T. urticae** ***eggs for intra-population crosses between*** **Wolbachia*-infected and -uninfected mites.*** *Relative proportions of unhatched eggs (stripped bars), adult males (light grey bars) and females (dark grey bars) are given for each possible cross. U: tetracycline-treated* Wolbachia*-uninfected; I:* Wolbachia*-infected. Vertical lines correspond to standard errors of the mean.*

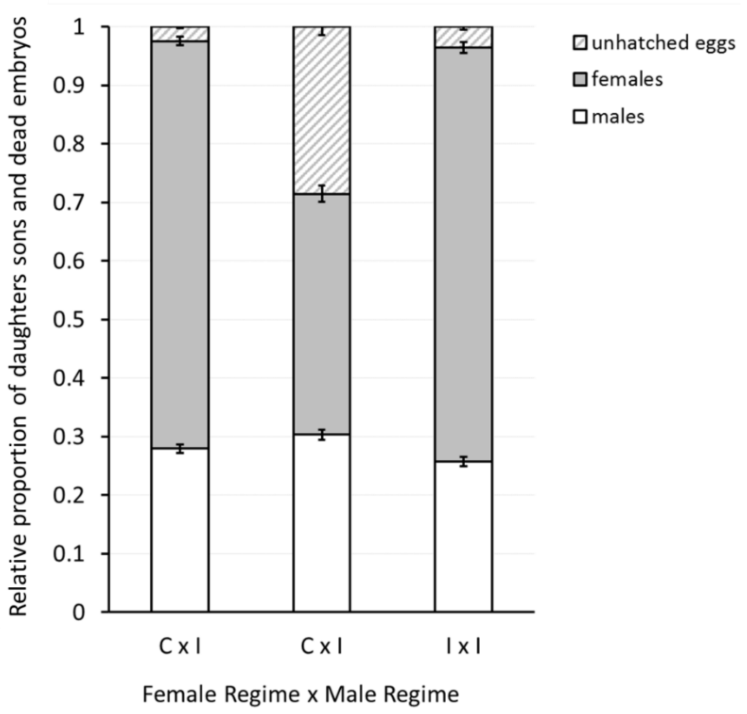


Figure S2.5. Summary of the development of **T. urticae** ***eggs for intra- and inter-regime crosses at generation 0 of experimental evolution.*** *Relative proportions of unhatched eggs (stripped bars), adult males (light grey bars) and females (dark grey bars) are given for each cross. C:* Wolbachia*-uninfected control regime; I:* Wolbachia*-infected control regime. Vertical lines correspond to standard errors of the mean.*