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**The interplay between *Wolbachia* and host-associated
reproductive barriers among populations of
*Tetranychus urticae***

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Abstract

Populations of a single species can display different degrees of reproductive isolation among them. This isolation can be due to reproductive barriers caused by genetic incompatibilities intrinsic to the organisms themselves, or induced by the symbionts they carry. Although it is probable that these two barriers interact, the study of this interaction is still incipient. Haplodiploid species are interesting systems to study incompatibilities, as fertilized eggs produce diploid females and unfertilized eggs produce haploid males. An experiment in which reciprocal crosses were performed between two colour morphs of the haplodiploid spider mite *Tetranychus urticae*, with or without the endosymbiotic bacterium *Wolbachia*, was previously carried out at my host institution. The results confirmed that both *Wolbachia*- and host-associated incompatibilities lead to partial and asymmetric reproductive isolation among populations. Moreover, they show that these barriers are differently expressed: while *Wolbachia* leads to an increase of female mortality in the brood, host-associated incompatibilities induce an overproduction of males (*i.e.*, unfertilized offspring) in crosses involving green females and red males. Furthermore, *Wolbachia*-induced incompatibility led to a reduction in the proportion of viable offspring in crosses between incompatible hosts. In this project, we built on these results and investigated the fertility of F1 hybrids resulting from the aforementioned crosses. We found that (i) *Wolbachia*-induced cytoplasmic incompatibility did not lead to hybrid breakdown (*i.e.* reduced viability of F2 offspring) and (ii) none of the inter-morph hybrid females laid eggs, regardless of *Wolbachia* infection, while (iii) all males resulting from F0 crosses were fertile, hence they probably result from unfertilized eggs, and not from the haploidization of fertilized offspring. Finally, mate choice experiments revealed that individuals did not discriminate between mates with different *Wolbachia* infection status, but males consistently chose females of the red morph, regardless of their own. Therefore, although populations are fully isolated due to hybrid sterility, we did not find any pre-copulatory isolation. Still, *Wolbachia* may interfere with population dynamics by reducing the proportion of F1 haploid sons resulting from inter-morph crosses. This warrants further investigations on possible consequences for population dynamics and invasive potential of both the host populations and *Wolbachia*.

Keywords: Reproductive incompatibility; host-parasite interaction; spider mites; endosymbionts

Resumo

O estabelecimento de barreiras reprodutivas entre populações encontra-se na base do processo de especiação. Estas barreiras podem ser pré-copulatórias, dificultando ou impedindo que indivíduos de diferentes populações se encontrem ou acasalem entre si; ou pós-copulatórias, reduzindo a viabilidade e/ou fertilidade de híbridos resultantes de cruzamentos entre as populações. Estas últimas estão frequentemente associadas a conflitos genômicos, que podem ser causados por fatores nucleares ou pela sua interação com fatores citoplasmáticos. Estas barreiras podem ainda ser parciais, permitindo a formação de uma proporção de híbridos viáveis, ou completas, quando não se formam quaisquer híbridos ou todos são inviáveis.

Além das incompatibilidades intrínsecas a uma dada espécie, certos microorganismos endossimbiontes têm a capacidade de manipular a reprodução dos seus hospedeiros, resultando também em incompatibilidades. Um dos mais comuns é a bactéria *Wolbachia*, capaz de manipular a reprodução do seu hospedeiro de forma a favorecer a sua transmissão através da linha materna de fêmeas infetadas. O fenótipo mais comum induzido pela bactéria é designado por incompatibilidade citoplasmática, ou **CI**, que resulta em mortalidade embriónica da descendência de um macho infetado. Esta mortalidade é resgatada se a fêmea estiver também infetada com a mesma estirpe (ou uma estirpe compatível) de *Wolbachia*. Foi proposto que esta incompatibilidade, se associada a populações em divergência, poderá contribuir para a especiação.

Embora artrópodes apresentem vários casos de incompatibilidade reprodutiva entre populações, e estejam frequentemente infetados por manipuladores reprodutivos, a interação entre os dois fatores tem recebido pouca atenção até recentemente. Os poucos estudos nesta área revelam resultados conflituosos, sugerindo uma grande variabilidade dependendo das espécies e populações usadas, bem como dos manipuladores envolvidos. Reunir mais conhecimento sobre estas interações é importante, pois endossimbiontes como *Wolbachia* podem ter implicações para processos de diferenciação entre populações de artrópodes.

Os ácaros-aranha são um sistema interessante para o estudo de incompatibilidades reprodutivas, dado que muitas espécies são haplodiplóides; ovos fertilizados produzem fêmeas diplóides, enquanto que ovos não fertilizados produzem machos haplóides. Problemas associados a fertilização são portanto facilmente identificáveis como um aumento na proporção de machos na descendência. O género *Tetranychus*, em particular, tem sido alvo de estudos sobre incompatibilidade entre populações, e várias espécies encontram-se infetadas com *Wolbachia*.

T. urticae é um bom modelo para estudar a interação entre incompatibilidades causadas pelo próprio organismo e pelos seus endossimbiontes. Esta espécie apresenta dois morfotipos, verde e vermelho, sendo que o vermelho foi originalmente considerado uma espécie diferente, *T. cinnabarinus*. A reprodução entre morfotipos foi estudada em várias ocasiões, e diferentes graus de incompatibilidade foram encontrados dependendo das populações usadas. Esta incompatibilidade é expressa como um excesso na produção de machos e esterilidade parcial de fêmeas híbridas F1. Esta espécie apresenta também uma elevada prevalência de *Wolbachia*, que induz padrões e níveis variados de **CI** dependendo dos genótipos do hospedeiro e do simbionte. Em espécies haplodiplóides, a mortalidade embriónica induzida por **CI** apenas afeta a proporção de descendência feminina, sendo que a proporção de machos não resulta de fertilização. Além disso, o efeito de **CI** pode estender-se até aos indivíduos F2, causando um fenómeno de colapso de híbridos (*i.e.* viabilidade reduzida da

descendência F2). Finalmente, poucas ou nenhuma evidências de preferência de parceiro foram encontradas neste sistema, quer para os morfotipos, quer para *Wolbachia*, facilitando a obtenção de híbridos. Sabemos de apenas um estudo que revela preferência para o estado de infecção, e nenhum que indique a existência de preferências entre morfotipos.

Recentemente, um estudo realizado na minha instituição hospedeira revelou que a proporção de ovos inviáveis devido a **CI** induzido por *Wolbachia* é menor em cruzamentos entre morfotipos, enquanto que cruzamentos entre morfotipos com fêmeas verdes produzem uma maior proporção de machos. A combinação de ambas as incompatibilidades resulta numa menor proporção de descendência viável. O objetivo deste projeto é aprofundar o estudo deste sistema, testando a presença de barreiras pré- e pós-copulatórias associadas ao hospedeiro, ao simbionte, ou a ambos, nomeadamente em termos de acasalamento seletivo e fertilidade dos híbridos.

De forma a completar a experiência realizada previamente, fêmeas infetadas de duas populações vermelhas e uma população verde foram cruzadas com machos não-infetados das três populações. Embora *Wolbachia* não induza **CI** neste tipo de cruzamento, o padrão descrito previamente para cruzamentos entre morfotipos é consistente com os resultados aqui apresentados; quando fêmeas verdes acasalam com machos vermelhos, há um aumento na produção de machos. Tal como anteriormente, fêmeas vermelhas são compatíveis com os três tipos de machos.

A descendência proveniente dos acasalamentos entre uma das populações vermelhas e a população verde, com ou sem *Wolbachia*, foi recolhida para testar a fertilidade e descendência de fêmeas e machos F1. As fêmeas foram mantidas virgens, de forma a apenas produzirem descendência masculina. Os resultados revelam que apesar da incompatibilidade entre morfotipos nos cruzamentos F0 ser apenas parcial, todas as filhas resultantes dos mesmos são estéreis, independentemente de infecção por *Wolbachia*. A bactéria não afetou a viabilidade da descendência. Assim, apesar da compatibilidade parcial na F0, as populações estão completamente isoladas. Quanto aos machos, estes foram cruzados com fêmeas provenientes da sua população materna. Se os filhos forem descendentes não fertilizados da sua mãe, espera-se total compatibilidade nestes cruzamentos. Os resultados mostram que as principais diferenças na fertilidade e na descendência dos filhos se devem ao morfotipo da fêmea com que acasalaram, tanto para filhos de cruzamentos intra-morfotipo como de cruzamentos inter-morfotipo. Como tal, os filhos de cruzamentos incompatíveis são férteis. Isto sugere que estes machos provavelmente resultam de ovos não fertilizados, e não da haploidização de ovos fertilizados. O rácio sexual obtido nos cruzamentos entre fêmeas verdes e machos vermelhos parece então resultar de problemas na fertilização. São necessários estudos do processo de fertilização neste sistema para confirmar esta conclusão.

Observações de escolha de parceiros foram realizadas para determinar se indivíduos destas populações têm a capacidade de evitar acasalamentos incompatíveis. Fêmeas e machos de diferentes morfotipos e estados de infecção por *Wolbachia* foram sujeitos a escolhas entre dois parceiros, diferindo também no morfotipo ou estado de infecção. Os testes para ambos os sexos mostram que fêmeas da população verde são menos recetivas ao acasalamento do que fêmeas da população vermelha. Como tal, os machos apresentam uma tendência a acasalar com fêmeas vermelhas, independentemente do seu próprio morfotipo. *Wolbachia* não teve efeito na escolha de parceiro. Estes resultados indicam que estas populações são incapazes de evitar acasalamentos incompatíveis. Não se encontraram diferenças na latência até à cópula para nenhum dos sexos. Quanto à duração da cópula, machos verdes acasalam durante mais tempo do que machos vermelhos. Esta diferença é potencialmente um mecanismo compensatório, devido à baixa recetividade e produção de descendência feminina que caracteriza as fêmeas verdes. Adicionalmente, os machos que escolhem fêmeas infetadas acasalam durante menos tempo do que com fêmeas não-infetadas, o que pode indicar um menor investimento de esperma

nestes acasalamentos. No entanto, a razão para esta diferença permanece pouco clara, pois a infecção por *Wolbachia* em fêmeas não parece ter custos para atributos associados à reprodução tal como fecundidade e rácio sexual da descendência.

Em conclusão, não se encontraram evidências de isolamento pre-copulatório entre as populações testadas, mas existe um forte isolamento pós-copulatório devido a um excesso na produção de machos nos cruzamentos inter-morfotipo com fêmeas verdes e esterilidade completa dos híbridos entre morfotipos. Embora *Wolbachia* aumente a incompatibilidade nestes cruzamentos, a esterilidade dos híbridos significa que a bactéria não desempenha um papel no isolamento reprodutivo entre estas populações. Ainda assim, é importante salientar que ambos os tipos de incompatibilidade aqui descritos, ao nível dos cruzamentos F0, atuam na mesma direção. Isto pode sugerir que a bactéria desempenhou um papel no desenvolvimento inicial desta barreira. Outra consideração importante é que estudos lidando com incompatibilidade entre morfotipos tendem a usar apenas uma população de cada. Por este motivo, ainda não é claro se este isolamento se deve aos morfotipos em si, ou apenas a distância genética entre populações.

Os resultados aqui descritos salientam a importância de sucessivos estudos sobre a natureza dos morfotipos, e a dinâmica da sua interação na natureza. *T. urticae* é uma praga agrícola problemática, e uma maior compreensão das consequências de invasões entre populações pode vir a ser útil para gerir o seu impacto.

Palavras-chave: Incompatibilidade reprodutiva; interação hospedeiro-parasita; ácaros-aranha; endossimbiontes

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1. Introduction

According to the biological concept of species (Mayr 1986), speciation events arise from the establishment of reproductive barriers that reduce (or block) gene flow between populations, leading to increased divergence and isolation over several generations (Funk et al. 2006). The mechanisms of reproductive isolation are diverse and can act at different stages of the life cycle of an organism.

Pre-zygotic isolation relies on any mechanisms hampering hybridization. It might result from ecological isolation, where differences of habitat preferences or temporal asynchrony between reproductive periods prevent individuals from different populations or species from encountering each other (Hawthorne and Via 2001). In absence of ecological isolation, recognition (due to visual, tactile, auditory, or chemical cues) of sexual partners and trait preferences can lead to assortative mating (Kondrashov and Shpak 1998), where individuals of the same population or species are more likely to mate among them than with others. Finally, two species may also be isolated due to incompatibilities in their reproductive organs or gametes (Alipaz et al. 2001; Masly 2012).

Post-zygotic isolation can occur when hybrid offspring has been created but suffers a reduction in viability (resulting in higher embryonic mortality), fertility (hampering the production of functional gametes), or fitness (due to genomic incompatibilities or disruption of local adaptations in the parental genotypes; Orr and Coyne 1989; Price and Bouvier 2002). This isolation can be partial (a proportion of viable hybrid offspring can be produced, depending on the level of isolation) or complete (no viable hybrid offspring is produced), depending on the time and level of divergence (Edmunds 2002). Phenomena of hybrid sterility and breakdown have often been attributed to intragenomic conflicts. These conflicts, leading to incompatibilities that severely hamper the fitness of hybrids (Dobzhansky-Muller model; Dobzhansky 1934), are thus expected to reinforce speciation (Crespi and Nosil 2013) and are not restricted to nuclear genes. Indeed, the mitochondrial genome (and its interaction with the nuclear genome) has been shown to play a large role in hybrid breakdown (Breeuwer and Werren 1995; Burton et al. 2013; Ellison and Burton 2008). Thus, both the nucleus and cytoplasm may play a role in raising the post-zygotic barriers potentially leading to speciation.

Beyond incompatibility phenomena intrinsic to any particular species (hereafter referred to as **HI** for “host incompatibility”), microorganisms such as reproductive manipulators have received increasing attention in the last decades for their potential role in speciation processes (Hughes et al. 2012). One of the most common of these parasites is the alpha-proteobacterium *Wolbachia*, which was recently estimated to infect up to 50% of arthropod species (Weinert et al. 2015). This success is thought to be due to efficient transmission through the female germline and through its manipulation of host reproduction to selectively favour infected females. This can be accomplished via induction of a number of host phenotypes, such as feminization (conversion of genetic male offspring into females), male killing (elimination of infected males during embryogenesis or late larval instars to the advantage of surviving infected female siblings), induced parthenogenesis (induced asexual development of daughters), and, most commonly, cytoplasmic incompatibility (**CI**; Werren et al. 2008). **CI** is a conditional sterility phenotype occurring when *Wolbachia*-infected males mate with uninfected females. It consists in paternal genome fragmentation in fertilized eggs, which results in partial to complete haploidization and is expressed as an increased or complete embryonic mortality. This incompatibility can only be rescued if the female is infected with the same (or a compatible) strain of *Wolbachia*. *Wolbachia* thus leads to unidirectional reproductive isolation between infected and

uninfected hosts and to either unidirectional or bidirectional reproductive isolation between hosts carrying different bacterial strains (Bordenstein and Werren 1998; Fig. 1.1). Thus, the induction of incompatibilities between diverging populations could drive the evolution of new species, and there is increasing empirical and theoretical evidence in support of this hypothesis, although this is still a matter of debate (Gebiola et al. 2012; Werren et al. 2008). For instance, one study concluded that two closely related parasitoid wasps, *Nasonia giraulti* and *N. longicornis*, were isolated only due to bidirectional **CI** induced by incompatible strains of *Wolbachia*; removing the endosymbiont led to a full rescue of hybrid offspring between the two species (Bordenstein et al. 2001). In addition, **CI**-inducing *Wolbachia* can readily select for pre-mating isolation, which reinforces genetic identity among populations (Telschow et al. 2005), as observed in natural populations of *Drosophila subquinaria* (Jaenike et al. 2006). Reciprocally, host-associated incompatibilities could hamper or facilitate the spread of *Wolbachia* within and among host populations, thus potentially affecting *Wolbachia*-based strategies to control vector-borne diseases (Hoffmann et al. 2011) and agricultural pests (Cheypppe-Buchmann et al. 2011; Zabalou et al. 2004).

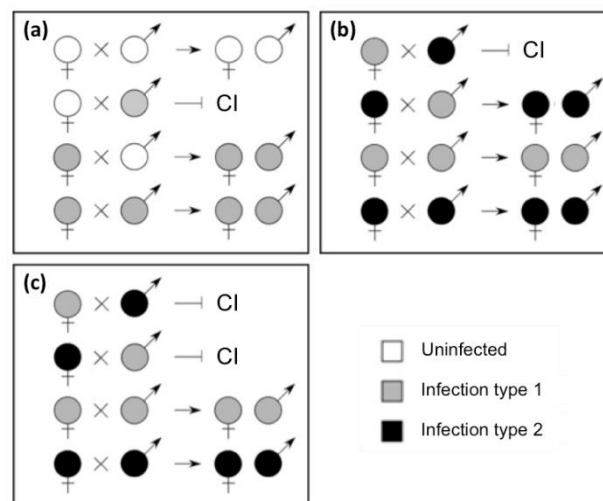


Figure 1.1. Patterns of *Wolbachia*-induced cytoplasmic incompatibility, or CI. Unidirectional **CI** can occur (a) between *Wolbachia* infected and uninfected individuals, or (b) between individuals infected with different *Wolbachia* infection types if only one of the *Wolbachia* infection types is able to rescue the **CI** induced by the other. Bidirectional **CI** can occur (c) between individuals infected with different *Wolbachia* infection types if neither of these types is able to rescue the **CI** induced by the other.

Even though reproductive incompatibilities between populations (**HI**) are commonly found in arthropods (Perrot-Minnot et al. 2002; Selivon et al. 1999; Wells and Henry 1992), and bacterial reproductive manipulators are widespread in nature (as mentioned above), the interaction between both factors has only recently been receiving attention. One such study investigated interspecific crosses between the fruit flies *D. melanogaster* and *D. simulans*, which are partially isolated with low frequency of hybridization and proportion of viable offspring. The infection by *Wolbachia*, while inducing **CI** in intraspecific crosses, does not induce **CI** in interspecific crosses. The bacterium thus does not contribute to reproductive isolation between these species (Gazla and Carracedo 2009). However, another study showed that in interspecific crosses between the mosquitoes *Aedes polynesiensis* and *Ae. riversi*, *Wolbachia* induces higher levels of **CI** than in intraspecific crosses, thus preventing hybridization between the two species (Dean and Dobson 2004). A recent study focusing on another **CI**-inducing symbiont, *Cardinium*, in two species of *Encarsia* wasps concluded that the unidirectional incompatibilities associated with either the host or the symbiont act in opposite directions, effectively complementing each other and leading to reproductive isolation (Gebiola et al.

2016). These conflicting results suggest that the interaction between host- and symbiont-mediated incompatibilities may vary depending on the host species and their degree of isolation, and/or the *Wolbachia* strain involved. Gathering more knowledge on this topic is, thus, a timely issue.

Spider mites present an interesting system to study reproductive incompatibilities due to the fact that many species are haplodiploid; unfertilized eggs develop into haploid males, whereas fertilized eggs develop into diploid females. Thus, if reproductive isolation leads to a failure in fertilization, this can be detected as an increase in the proportion of sons. The genus *Tetranychus*, in particular, has previously been studied concerning inter-population crosses, and different degrees of incompatibilities have been shown to occur for several species such as *T. quercivorus* (Gotoh et al. 1995), *T. evansi* (Knegt et al. 2016) and *T. urticae* (Navajas et al. 2000; Sugawara et al. 2002). Furthermore, *Tetranychus* spider mites may carry different reproductive manipulators, including *Wolbachia* (Gotoh et al. 2007; Xie et al. 2010; Zhang et al. 2013), and it has been proposed that haplodiploidy might have evolved in arthropods under the pressure of these endosymbionts (Engelstädter and Hurst 2006; Kuijper and Pen 2010; Normark and Ross 2014). Indeed, while in diploid species **CI** is expressed as an overall increased offspring mortality (*i.e.* both daughters and sons are affected), in haplodiploids, haploid embryos resulting from paternal genome elimination in fertilized eggs can develop into viable males. Consequently, fertilized eggs can either develop as haploid males or abort. This leads to two types of **CI** (Fig. 1.2): male production (MP; increased male production of an amount equal to the reduction in the number of females without reduction in offspring production; Vala et al. 2000), or female mortality (FM; reduction in female production through increased embryonic mortality; Gotoh et al. 2007).

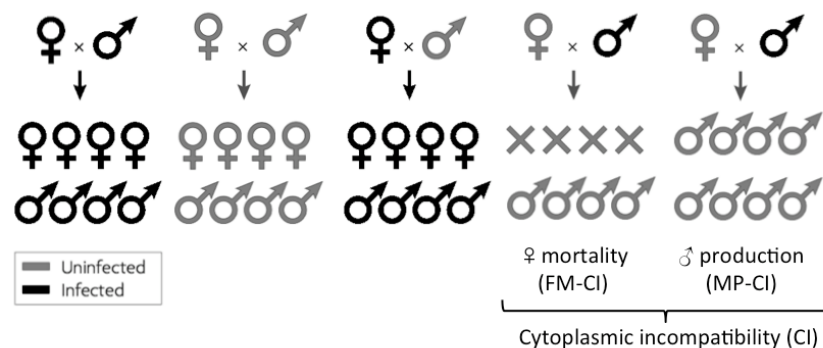


Figure 1.2. CI induction by *Wolbachia* in haplodiploids. Crosses between infected males and uninfected females can lead to either female mortality (FM), or male production (MP).

The haplodiploid two-spotted spider mite *T. urticae* is arguably the most well-studied spider-mite species, and a particularly good candidate to address the interaction between host- and endosymbiont-associated incompatibilities. Indeed, *T. urticae* can be found in two different colour morphs: green and red (Fig. 1.3). Historically, the red morph was first described as a separate species, *T. cinnabarinus*, but molecular and morphological data, as well as evidence for partial compatibility in inter-morph crosses, led to reconsidering its species status as synonymous with *T. urticae* (Auger et al. 2013). Reproduction between these two morphs has been studied at several occasions and different degrees of incompatibility have been found depending on the populations used; **HI** is expressed as an overproduction of male offspring and partial sterility of F1 hybrids (De Boer 1982; Lu et al. 2017; Sugawara et al. 2002). *T. urticae* is also commonly infected with *Wolbachia* (Gotoh et al. 1995; Perrot-Minnot et al. 2002; Vala et al. 2004), which induces markedly variable fitness effects. It

includes both increased (Xie et al. 2010) or decreased fecundity (Perrot-Minnot et al. 2002), as well as highly variable intensity and patterns of **CI** depending on the genotypes of both hosts and symbionts (Gotoh et al. 2007; Perrot-Minnot et al. 2002; Vala et al. 2000). In *T. urticae*, *Wolbachia*-induced **CI** may also extend to the F2 in spider mites: broods of virgin F1 females sometimes suffer increased mortality rates, a phenomenon called hybrid breakdown (Vala et al. 2000). Finally, in a polymorphic population (*i.e.* either for colour morphs or for infection by a **CI**-inducing *Wolbachia*), individuals may gain a reproductive advantage if they discriminate between compatible and incompatible mates and preferentially mate with the former (Koukou et al. 2006; Moreau et al. 2001). However, despite its crucial role in speciation, on the maintenance of species boundaries, and on the spread of *Wolbachia* among populations, the existence of assortative mating between morphs or infection statuses has been largely overlooked in *T. urticae*. Only one study, to my knowledge, reports avoidance of *Wolbachia*-infected males by uninfected females (Vala et al. 2004), with another two studies showing no assortative mating induced by *Wolbachia* (Rodrigues et al. *in prep*) or between morphs (Murtaugh and Wrench 1978). However, there are two important prerequisites for assortative mating to evolve: (i) that natural populations are polymorphic for either morphs or *Wolbachia* infection, and (ii) that this polymorphic state lasts for a sufficient amount of time (*i.e.* the invasion by *Wolbachia* or one of the morphs does not result in fixation or exclusion, respectively, before mate preference can evolve; Champion De Crespigny et al. 2005). This behaviour is thus expected to depend on populations' history and more studies in this direction are needed.

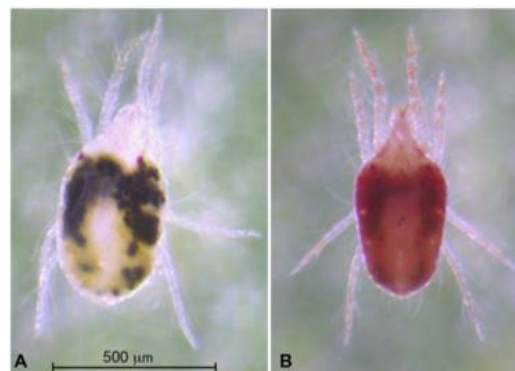


Figure 1.3. *Tetranychus urticae* colour morphs. A: Green morph; B: Red morph. Adapted from Bryon et al. 2013.

Recently, a study conducted in my host institution showed that the proportion of unhatched eggs resulting from *Wolbachia*-induced **CI** is lower in inter-morph crosses than in intra-morph crosses, whereas **HI** leads to a male-biased sex ratio. The combination of both incompatibilities results in a smaller proportion of viable offspring (lower proportion of both male and female offspring than in inter-morph crosses without *Wolbachia*; Z     et al. *in prep*). For clarity, the populations used will be referred to by their morph.

These findings raised two important questions: (i) how does *Wolbachia* affect host population dynamics and speciation processes; and (ii) how do host-associated reproductive incompatibilities affect the spread of *Wolbachia* between populations. With this project, I further investigate this system by testing for the presence of both pre- and post-copulatory barriers associated to either the host, the symbiont, or a combination of both, namely in terms of assortative mating, hybrid fertility, and hybrid breakdown.

2. Materials and Methods

Spider mite populations

Three different populations of *T. urticae* available in my host laboratory, and previously used to study the *Wolbachia*-morph interaction, were used for this project. The first, “Ri-1” (for “red infected 1”), belongs to the red morph of *T. urticae*, and was founded from 65 females collected in November 2013 on *Datura* sp. plants in Aldeia da Mata Pequena, Portugal. The second, “Ri-2” (for “red infected 2”), also belongs to the red morph of *T. urticae*, and was founded from 300 females collected in October 2013 on eggplant (*Solanum melongena*) in Lourinhã, Portugal. The third, “Gi” (for “green infected”), belongs to the green morph of *T. urticae*, and was founded from 300 females collected in August 2010 on tomato plants (*Solanum lycopersicum*) in Carregado, Portugal. All populations were fully infected by *Wolbachia*. The strains infecting the two red populations were identical based on the *wsp* gene and multilocus sequence typing (MLST; Baldo et al. 2006). They induce a high, but variable, level of **CI** (ca. 60% and 40% for Ri-1 and Ri-2, respectively), and are compatible with each other (Zélé et al. *in prep*). The *Wolbachia* strain infecting the green population does not induce **CI** and slightly differs from the former two strains based on the MLST (*fbpA* and *coxA* genes, in a single SNP for each; Zélé, pers. comm.).

After collection, subsets of each population, “Ri-1”, “Ri-2” and “Gi”, were treated with antibiotics (see below) to obtain the *Wolbachia*-free populations “Ru-1”, “Ru-2” and “Gu”, respectively. “Ru-1” (for “red uninfected”) was obtained in February 2014 by installing 30 adult females from “Ri-1” in petri dishes containing bean leaf fragments, placed on cotton soaked in a tetracycline solution (0.1%, w/v) for three successive generations (Breeuwer 1997). After this treatment, the population was maintained in a mass-rearing environment without antibiotics for several generations (>50) before the experiments. “Gu” and “Ru-2” were created during this project in September 2016 and January 2017 by installing 300 adult females from “Ri-2” and “Gi”, respectively, in petri dishes containing fragments of bean leaves, placed on cotton soaked in a rifampicin solution (0.05%, w/v) for one generation (Gotoh et al. 2005). After this treatment, the populations were maintained in a mass-rearing environment without antibiotics for three generations and then tested for the absence of *Wolbachia* by multiplex PCR (Zélé et al. *in prep*) before performing experiments. All populations have been reared since then, and all the following experiments were performed, under the same standard laboratory conditions (24 ± 2°C, 16/8 h L/D) on a common host plant (bean, *Phaseolus vulgaris*, var. *Contender*).

Parental crosses

The interaction between *Wolbachia* and host-associated incompatibilities in F0 crosses involving Ru-1, Ri-1, Ru-2, Ri-2, Gu and Gi populations was previously investigated by assessing the hatching rate and sex ratio of offspring resulting from these crosses. In my thesis, I will present this data as it is needed to understand the outcome of my own experiments. In this experiment, the following crosses (Table 2.1) were performed: (1) crosses within populations (Ru♀xRu♂ and Gu♀xGu♂, and Ri♀xRi♂ and Gi♀xGi♂), which are controls for compatible intra-population crosses; (2) Crosses between populations of the same origin that were either infected or uninfected (Ru♀xRi♂ and Gu♀xGi♂), which are crosses to test for the effect of *Wolbachia* only (cytoplasmic incompatibility, **CI**); (3) crosses between populations of different morphs and in which *Wolbachia* is not expected to induce incompatibilities (Ru♀xGu♂ and Gu♀xRu♂), to test for morph-associated incompatibility (hybrid incompatibility, **HI**); finally (4) crosses between populations of different morphs and in which

Wolbachia incompatibilities are expected ($Ru_{\text{♀}} \times Gi_{\text{♂}}$ and $Gu_{\text{♀}} \times Ri_{\text{♂}}$, and $Ri_{\text{♀}} \times Gi_{\text{♂}}$ and $Gi_{\text{♀}} \times Ri_{\text{♂}}$), which allow testing the interaction between the two types of incompatibility. Crosses involving infected females and uninfected males ($Ri_{\text{♀}} \times Ru_{\text{♂}}$, $Ri_{\text{♀}} \times Gu_{\text{♂}}$, $Gi_{\text{♀}} \times Gu_{\text{♂}}$ and $Gi_{\text{♀}} \times Ru_{\text{♂}}$) were not included in this experiment due to logistic reasons and because *Wolbachia* is not expected to influence the offspring resulting from these crosses. To further investigate the interaction between *Wolbachia* and host-associated incompatibilities at the F1 generation (see below), here we complement this experiment by performing these missing F0 crosses. To this aim, we followed the protocol previously used, in which age cohorts were initially produced using spider mites from the cultures of each population. For each cohort, 100 adult females were placed for 5-7 days on bean leaves placed on water-soaked cotton to lay eggs. This procedure results in a large number of individuals of approximately the same age. 12 days after the creation of the cohorts, quiescent females from the infected populations (Ri-1, Ri-2 and Gi), and males from the uninfected populations (Ru-1, Ru-2 and Gu), were isolated and installed on bean leaf fragments. 2 days later (after quiescent females emerged as adults and males replenished their sperm reserves), which corresponds to the first day of the experiment (d1), 50 couples (1 male with 1 female) were installed on 2.5 cm² bean leaf discs for 3 days. Survival of both males and females was checked daily during this period. On the third day of the experiment (d3), surviving females and males were discarded, and fecundity registered. Offspring hatching rate, then juvenile survival and sex ratio were assessed 5 and 12 days later (d8 and d15), respectively. This experiment was carried out in two experimental blocks, performed one day apart.

Table 2.1. Table of reciprocal crosses performed between the red and green populations. Each cell indicates the expected effect to be tested in each cross. Shaded cells indicate crosses performed during this project. CC: compatible cross; HI: host-associated incompatibility; CI: *Wolbachia*-induced cytoplasmic incompatibility; W: potential effects of *Wolbachia* besides CI.

♀ \ ♂	Ru-1	Ri-1	Ru-2	Ri-2	Gu	Gi
Ru-1	CC	CI	CC	CI	HI	HI+W
Ri-1	CC	CC	CC	CC	HI	HI+W
Ru-2	CC	CI	CC	CI	HI	HI+W
Ri-2	CC	CC	CC	CC	HI	HI+W
Gu	HI	HI+CI	HI	HI+CI	CC	W
Gi	HI	HI+CI	HI	HI+CI	CC	CC

Hybrid fertility

Given that the inter-morph crosses in the F0 experiment produced hybrid female offspring, and because *Wolbachia* has been shown to induce hybrid breakdown in this system (Vala et al. 2000), the fertility of offspring resulting from F0 crosses was assessed. Since the two populations from the red morph produced similar results in the F0 crosses, but Ri-1 induced higher levels of CI, we excluded Ri-2 and Ru-2 and performed the following experiments using Ru-1, Ri-1, Gu and Gi only. For this experiment, all parental crosses described in the previous experiment were repeated (100 virgin females x 100 males) on an entire bean leaf. This procedure provides a large number of F1 offspring

of the same age. Two different tests were then performed to determine the fertility of (1) F1 hybrid females, and (2) F1 (potentially hybrid) males.

(1) For F1 females, a classical test for hybrid breakdown (Vala et al. 2000) was performed. Prior to the experiment, 25 quiescent daughters resulting from each cross were placed on bean leaf fragments for 2 days for them to emerge as adults while remaining virgin. These females were then isolated on 2.5 cm² bean leaf discs and allowed to lay eggs during 4 days. Since these females were not allowed to mate, they could only produce unfertilized eggs. These eggs are thus expected to either develop as viable haploid males, or to abort in case of hybrid breakdown. This experiment was repeated three times independently (test 1.a, 1.b and 1.c). 1.a was carried out in four experimental blocks: “B1f” and “B2f” focused on reciprocal crosses between uninfected females (Ru-1 and Gu) and all types of males (Ru-1, Ri-1, Gu and Gi), while “B3f” and “B4f” focused on reciprocal crosses between infected females (Ri-1 and Gi) and all types of males (Ru-1, Ri-1, Gu and Gi). For 1.b and 1.c, all reciprocal crosses involving uninfected and infected females were performed simultaneously, and each were performed in two experimental blocks (B5f and B6f, and B7f and B8f, respectively).

(2) To test the fertility of F1 males, they were backcrossed with females from their maternal population (to ensure cytoplasmic compatibility between the F1 males and the females they mate with). Prior to the experiment, 25 adult males resulting from each cross and 100 quiescent females of each maternal type were isolated on bean leaf fragments for 2 days. On the first experimental day (d1), each male was installed with one virgin female from the same population as its mother on a 2.5 cm² bean leaf disc, and allowed to mate during 4 days. If males result from re-haploidization of fertilized eggs in inter-morph crosses, we expect their ability to sire offspring (*i.e.* daughters only in haplodiploids) to be impaired. This test was carried out in five experimental blocks: “B1m” to “B3m” focused on reciprocal crosses between uninfected females (Ru-1 and Gu) and all types of males (Ru-1, Ri-1, Gu and Gi), while “B4m” and “B5m” focused on reciprocal crosses between infected females (Ri-1 and Gi) and all types of males (Ru-1, Ri-1, Gu and Gi).

For both tests, the daily survival of males and females was recorded during the 4-days laying period. On the fourth experimental day (d4), all remaining individuals were discarded and the fecundity of all females was assessed. Offspring hatching rate, juvenile survival and sex ratio, were assessed 5 and 12 days later (d9 and d16), respectively.

Mate choice experiment

To determine whether males and females were able to discriminate between mates and avoid incompatible crosses, both female and male choice were measured using the Ru-1, Ri-1, Gu and Gi populations. For both experiments, age cohorts to obtain virgin males and females were performed by placing either (1) 50 quiescent females or (2) 50 mated females, respectively, in petri dishes containing bean leaves placed on water-soaked cotton. 12 to 14 days later, quiescent males and females were isolated from these age cohorts on bean leaf fragments 2 days before the observations to ensure virginity. Both experiments were carried out over the course of 15 days, with each day being considered an experimental block. Four sessions of observations were conducted per day, each containing one replicate of each of the 8 treatments.

(1) For female choice, one focal female was provided with a choice between two types of males (male 1/male 2), thus forming the following treatments (Table 2.2): Ru♀xRu/Gu♂ and Gu♀xRu/Gu♂ (called “RuU” and “GuU” tests hereafter) for morph preference (*i.e.* avoidance of **HI**), Ru♀xRi/Ru♂ and Gu♀xGi/Gu♂ (“RuR” and “GuG” tests) for preference between infection statuses (*i.e.* avoidance of **CI**), Ru♀xRi/Gi♂ and Gu♀xRi/Gi♂ (“RuI” and “GuI” tests) to determine whether *Wolbachia*

infection in males affects morph preferences (*i.e.* avoidance of **CI**, **HI**, or both), and finally $Ri\text{♀} \times Ri/Gi\text{♂}$ and $Gi\text{♀} \times Ri/Gi\text{♂}$ (“**RiI**” and “**GiI**” tests) to determine whether *Wolbachia* infection in both males and females affects morph preferences (*i.e.* avoidance of **HI**). Adult females of the two morphs are easy to distinguish by naked eye, which is not the case for males. Thus, before the beginning of each session, males were painted with two different colours (blue or white, used in rotation) of water-based paint using a fine brush. Within the same population, males with different infection statuses were painted with different colours and assigned randomly to each experimental block. Previous experiments have shown no effect of paint on mating behaviour in this system (Rodrigues et al. 2017). Note that this experimental set-up can also be viewed as a test for competition between two males of different morphs or infection statuses.

(2) For male choice, one focal male was provided with a choice between two types of females, according to the following treatments (Table 2.2): $Ru-1\text{♂} \times Ru-1/Gu\text{♀}$ and $Gu\text{♂} \times Ru-1/Gu\text{♀}$ (“**RuU**” and “**GuU**” tests) for morph preference (*i.e.* avoidance of **HI**), $Ri-1\text{♂} \times Ri-1/Ru-1\text{♀}$ and $Gi\text{♂} \times Gi/Gu\text{♀}$ (“**RiR**” and “**GiG**” tests) for preference between infection statuses (*i.e.* avoidance of **CI**), $Ri-1\text{♂} \times Ru-1/Gu\text{♀}$ and $Gi\text{♂} \times Ru-1/Gu\text{♀}$ (“**RiU**” and “**GiU**” tests) to determine whether *Wolbachia* infection in males affects morph preferences due to the double incompatibility (*i.e.* avoidance of **CI**, **HI**, or both), and finally $Ri-1\text{♂} \times Ri-1/Gi\text{♀}$ and $Gi\text{♂} \times Ri-1/Gi\text{♀}$ (“**RiI**” and “**GiI**” tests) to determine whether *Wolbachia* infection in both males and females affects morph preferences (*i.e.* avoidance of **HI**). Females were distinguishable during observations based on their colour.

Table 2.2. Table of treatments performed in the behavioural observations. The table displays, for each combination of focal individuals and presented choice of mates, the types of incompatibility that could be avoided through mate preference. **HI**: host-associated incompatibility; **CI**: *Wolbachia*-induced cytoplasmic incompatibility.

Focal female (1)	Focal male (2)	Mate choice		Tested effect
Ru-1	Ru-1	Ru-1	Gu	Avoidance of HI (uninfected)
Gu	Gu			
	Ri-1	Ru-1	Gu	Avoidance of HI or of CI (males)
	Gi			
Ru-1	Ri-1	Ru-1	Ri-1	Avoidance of CI
Gu	Gi	Gu	Gi	
Ru-1		Ri	Gi	Avoidance of HI or of CI (females)
Gu				
Ri-1	Ri-1	Ri	Gi	Avoidance of HI (infected)
Gi	Gi			

For both experiments, the pairs of possible mates were installed on 0.40 cm² leaf discs prior to the beginning of the observations. The focal individuals were then installed on these leaf discs, and observations began. They ended when the focal individual finished copulating with its chosen mate, or after 30 minutes if no mating occurred. The type of mate chosen by each focal individual was assessed, and the latency to copulation and copulation duration were registered using an online chronometer (<http://online-stopwatch.chronme.com/>). At the end of the test, all mated females were

isolated individually on 2.5 cm² bean leaf discs for 3 days. On the third day, females were discarded and their fecundity was registered. Offspring hatching rate, and juvenile survival and sex ratio, were then assessed 5 and 12 days later, respectively, to confirm the results of the parental crosses experiment when matings were observed.

Statistical analyses

All analyses were carried out using the R statistical package (v. 3.2.5).

1. Parental crosses

The general procedure for building the statistical models used to analyse the outcomes of parental crosses was as follows: the interaction between types of father and mother was fit as fixed explanatory variable, whereas block was fit as a random explanatory variable. The results for the part of the experiment performed during this project were analysed separately from the ones obtained previously due to the considerable time gap between the two leading to a very large block effect, mainly concerning female fecundity (Annex 1).

Female total fecundity (count data), offspring hatching rate and sex ratio (computed using the function `cbind`, which allows analysing proportion data without losing the information of sample size; Crawley 2007), were greatly over-dispersed. Several methods to deal with overdispersion are currently available such as using ‘quasi’ families in GLMs (Generalized Linear Models; Crawley 2007), or explicitly modelling the source of extra-variation in the data (e.g., β -binomial or negative-binomial models). However, to our knowledge, it is not currently possible to account for quasi-, negative-binomial, or β -binomial distribution within the usual mixed model `glmer` procedure. For this reason, we used instead a mixed model `glmmadmb` procedure (`glmmADMB` package) from AD Model Builder (Fournier et al. 2012) with a negative-binomial error structure for total fecundity, a β -binomial error structure for the proportions of unhatched eggs and sons, and a β -binomial error structure accounting for zero-inflation for the proportion of daughters.

When the interaction between populations (morph and *Wolbachia* infection status; e.g. “Ru”) of father and mother was found to be significant, the effect of father was analysed separately for each population of mother. Then, to understand differences between father types, *a posteriori* contrasts (Crawley 2007) were carried out by aggregating factor levels together and testing the fit of the simplified model using ANOVA.

2. Hybrid fertility

The general procedure for building the statistical models used to analyse hybrid fertility was as follows: here, the type of parental cross resulting in each tested individual (instead of the populations of father and mother as above) was fit as fixed explanatory variable, whereas block was fit as a random explanatory variable. Data obtained from blocks B1f-B4f of the hybrid females test was analysed separately from data from the remaining blocks, due to inconsistent results leading to a large block effect (cf. Fig.3.2, Fig.3.3).

Female daily fecundity was estimated by dividing the total number of eggs laid by each female by the number of days it was alive on the leaf disc. These values were log transformed to improve normality (Box-Cox transformation; Crawley 2007) and subsequently analysed using linear mixed-effect models (`lmer`, `lme4` package).

Fertility was computed as the proportion of females capable of laying at least 1 egg (for females) or as the proportion of males siring at least 1 daughter (for males). The proportion of fertile individuals, a binary response variable (an individual is either fertile or infertile), was analysed using general linear mixed models with a binomial error distribution (glmer, lme4 package).

Offspring hatching rate and sex ratio resulting from crosses involving hybrid males were computed using the function cbind and analysed using glmmadmb with either a β -binomial, or a zero-inflated β -binomial (for the proportion of daughters in the male fertility test) error structure to account for overdispersion (as explained above for the parental crosses). When the type of cross was found to be significant, *a posteriori* contrasts between crosses were carried out as previously described.

3. Mate choice experiment

For the mate choice analyses, the proportion of focal individuals that mated, a binary response variable (mated or not), was analysed using a generalized linear mixed model with a binomial error distribution (glmer, lme4 package). The population of the focal individual (“Ru-1”, “Ri-1”, “Gu”, “Gi”) and the type of choice they had (“Ru-1/Gu”, “Ri-1/Gi”, “Ri-1/Ru-1”, “Gi/Gu”) were fit as fixed explanatory variables, whereas time and date of the observations were fit as random explanatory variables.

The proportion of focal individuals choosing to mate with one of the individuals they were presented with (*i.e.*, thus excluding focal individuals that did not mate during the observation period), a binary response variable (either individual A or individual B), was analysed using a generalized linear mixed model with a binomial distribution (glmer, lme4 package). As above, the population of the focal individual and the type of choice were fit as fixed explanatory variables, whereas time and date of the observations were fit as random explanatory variables. 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).

Both latency to mating and mating duration 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, focal individuals not mated during the observation period were excluded. The type of focal individual, the type of choice they had, and the choice they made, were fit as fixed explanatory variables, whereas time and date of the observations were fit as random explanatory variables.

When the type of either focal, tested, or chosen individuals was found to be significant, *a posteriori* contrasts were carried out as previously described.

3. Results

Parental crosses

The following results refer only to the parental crosses performed during this project (infected females crossed with uninfected males). The results from the previously performed crosses are presented in Figure 3.1C.

Total fecundity

A significant interaction between type of mother and type of father was found ($X^2_4=20.09$, $p<0.001$) for the fecundity of mothers over 3 days (Fig. 3.1A). Indeed, analyses conducted for each mother type (combination of morph and infection status) separately revealed that Ri-2 mothers that mated with Ru-1 fathers laid fewer eggs (17.46 ± 1.45 on average) than those that mated with Ru-2 or Gu fathers (ranging from 21.02 to 22.78; $X^2_5=11.78$, $p<0.001$ and $X^2_5=6.53$, $p=0.01$ respectively). In contrast, the type of father that mated with Ri-1 or Gi mothers did not significantly affect their fecundity ($X^2_2=2.65$, $p=0.27$ and $X^2_2=0.55$, $p=0.76$ for each mother type, respectively).

Offspring hatching rate

There was no significant effect of the type of father ($X^2_2=5.32$, $p=0.07$) on the proportion of unhatched eggs (Fig. 3.1B). The type of mother, however, significantly affected this proportion ($X^2_2=14.67$, $p<0.001$), independently of the type of father with which they mated (mother-father interaction: $X^2_4=2.94$, $p=0.57$). Contrast analyses revealed no significant differences between Ri-1 and Ri-2 mothers (proportion of unhatched eggs ranging from 0.06 to 0.15; $X^2_5=2.27$, $p=0.13$), but significantly more eggs hatched from crosses involving Gi mothers (ranging from 0.03 to 0.1; $X^2_5=26.43$, $p<0.001$).

Proportion of daughters

A significant interaction between type of mother and type of father was found ($X^2_4=87.43$, $p<0.0001$) for the proportion of daughters (Fig. 3.1B). Indeed, independent analyses conducted for each mother type separately revealed no significant differences between Gi mothers mated with either Ru-1 or Ru-2 fathers (ranging from 0.10 to 0.11; $X^2_6=0.62$, $p=0.43$), but that these crosses produced a significantly lower proportion of daughters than Gi mothers mated with Gu fathers (0.42 ± 0.02 on average; $X^2_6=52.30$, $p<0.001$). There was no significant effect of the father types on the proportion of daughters for either Ri-1 or Ri-2 mothers ($X^2_2=1.93$, $p=0.38$ and $X^2_2=3.71$, $p=0.16$ respectively).

Proportion of sons

As for the proportion of daughters, a significant interaction between type of mother and type of father was found ($X^2_4=23.28$, $p=0.0001$) for the proportion of sons (Fig. 3.1B). Independent analyses conducted for each mother type revealed that for Ri-2 mothers, mating with Gu fathers results in a significantly higher proportion of sons (0.42 ± 0.03 on average) compared to mating with Ru-1 fathers (0.31 ± 0.03 on average; $X^2_2=7.89$, $p=0.02$), but no differences between mating with Ru-1 or Ru-2 fathers, nor between mating with Ru-2 or Gu fathers ($X^2_5=1.27$, $p=0.26$ and $X^2_5=2.26$, $p=0.13$ respectively). For Gi mothers, the analyses revealed no significant differences between mating with either Ru-1 or Ru-2 fathers (0.84 ± 0.02 and 0.84 ± 0.03 on average, respectively; $X^2_5=0.15$, $p=0.70$), but mating with Gu fathers reduced the proportion of sons (0.56 ± 0.04 on average; $X^2_5=22.84$, $p<0.0001$).

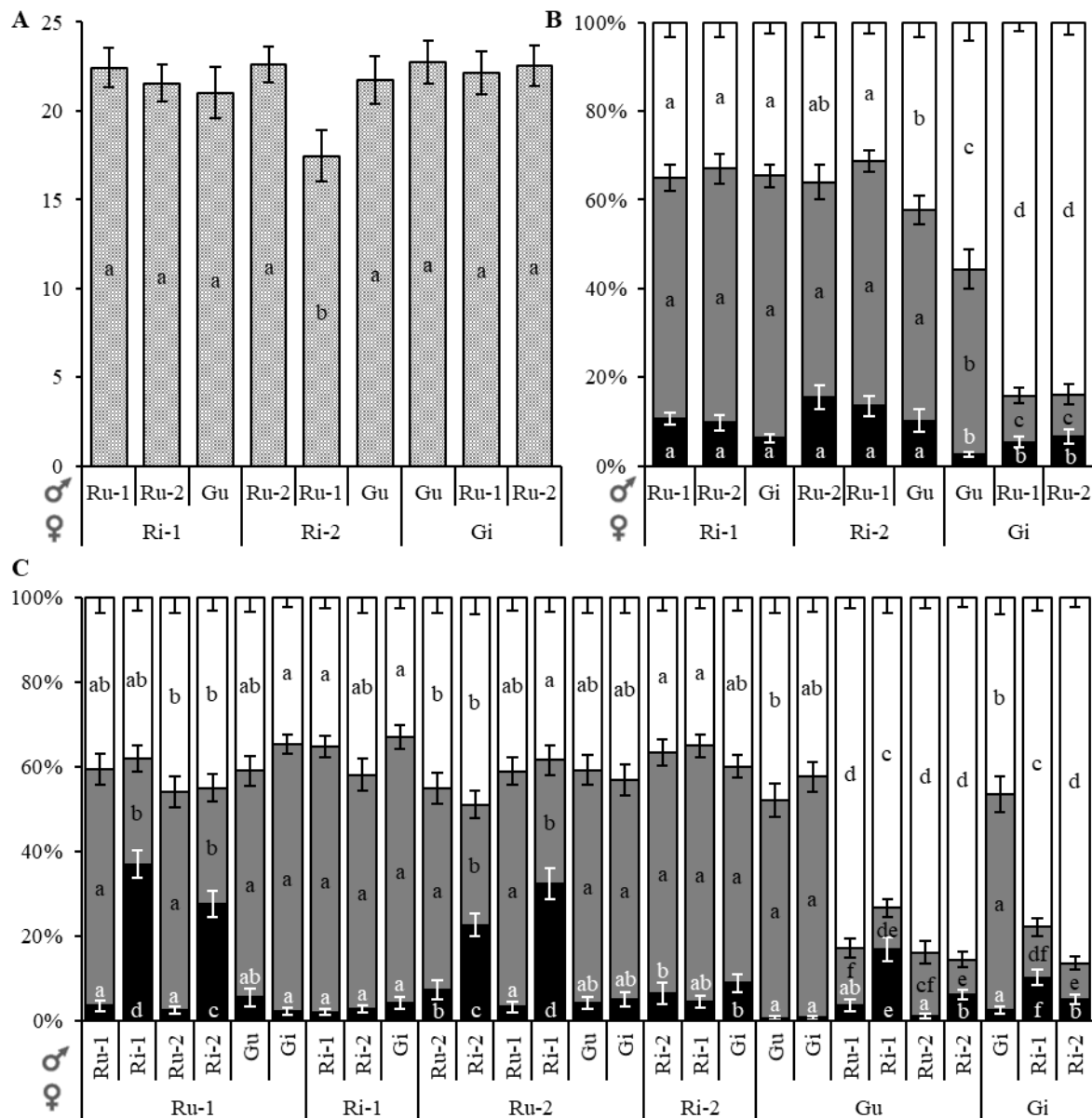


Figure 3.1. Offspring resulting from crosses between mothers (bottom level of the x-axis) and fathers (top level of the x-axis) of the different populations and infection statuses. A: Total fecundity (tested in this project); B: Offspring hatching rate and sex ratio (tested in this project); C: Offspring hatching rate and sex ratio (tested previously). Bars represent means (\pm s.e.) for the offspring of each cross. Identical superscripts (a, b, c, d) within each bar indicate non-significant differences at the 5% level (contrast analyses). Dotted grey: total fecundity; Black: proportion of unhatched eggs; Grey: proportion of daughters; White: proportion of sons.

Hybrid fertility (females, blocks B1f-B4f)

Daily fecundity

Significant differences were found between females resulting from crosses involving different morphs and infection statuses ($X^2_{4}=20.09$, $p<0.001$) for daily fecundity (Fig. 3.2A). Contrast analyses revealed that females resulting from intra-morph crosses between infected mothers and uninfected fathers laid significantly more eggs per day than those resulting from the other intra-morph crosses ($X^2_{7}=14.46$, $p=0.0001$). Females resulting from inter-morph crosses (excluding crosses between uninfected mothers and infected fathers) produced almost no eggs per day (ranging from 0 to 0.05), significantly less than those from intra-morph crosses (ranging from 3.9 to 6.5; $X^2_{7}=766.71$, $p>0.0001$). Females resulting from inter-morph crosses between uninfected mothers and infected fathers produced

significantly more eggs (Ru-1♀xGi♂: 0.74 ± 0.25 ; Gu♀xRi-1♂: 2.11 ± 0.57) than those from the other inter-morph crosses ($X^2_{7}=15.96$, $p<0.0001$), but significantly less than those from intra-morph crosses ($X^2_{7}=67.26$, $p<0.0001$)

Proportion of fertile females

A significantly different proportion of fertile females (Fig. 3.2B) resulted from crosses involving different morphs and infection statuses ($X^2_{15}=171.72$, $p<0.0001$). Contrast analyses revealed that inter-morph crosses between uninfected mothers and infected fathers produced significantly less fertile females (ranging from 0.24 to 0.44) than intra-morph crosses (ranging from 0.9 to 1; $X^2_{7}=112.69$, $p<0.0001$), but significantly more fertile females than other inter-morph crosses (ranging from 0 to 0.06; $X^2_{7}=47.37$, $p<0.0001$).

Proportion of unhatched eggs

Significant differences were found between females resulting from crosses involving different morphs and infection statuses ($X^2_{9}=49.05$, $p<0.0001$) for the proportion of unhatched eggs (Fig. 3.2C). A significant block effect was also found ($X^2_{12}=7.15$, $p<0.01$). Contrast analyses revealed that females resulting from the cross Ru-1♀xRi-1♂ produced significantly less hatched eggs (proportion of unhatched eggs: 0.21 ± 0.03) than females from the remaining intra-morph crosses involving red females (proportion of unhatched eggs ranging from 0.10 to 0.14; $X^2_{10}=5.67$, $p=0.02$). Females resulting from intra-morph crosses involving red females produced significantly less hatched eggs than those resulting from intra-morph crosses involving green females (proportion of unhatched eggs ranging from 0.06 to 0.17; $X^2_{8}=8.09$, $p<0.01$). There were no significant differences in the proportion of unhatched eggs between females resulting from the fertile inter-morph crosses (Ru-1♀xGi♂: 0.09 ± 0.04 ; Gu♀xRi-1♂: 0.05 ± 0.02) and those from intra-morph crosses involving green females ($X^2_{6}=2.89$, $p=0.09$).

Proportion of sons

As expected for virgin females in haplodiploids, all offspring were males. The results for the proportion of sons (Fig. 3.2C) are thus identical to those for the proportion of unhatched eggs.

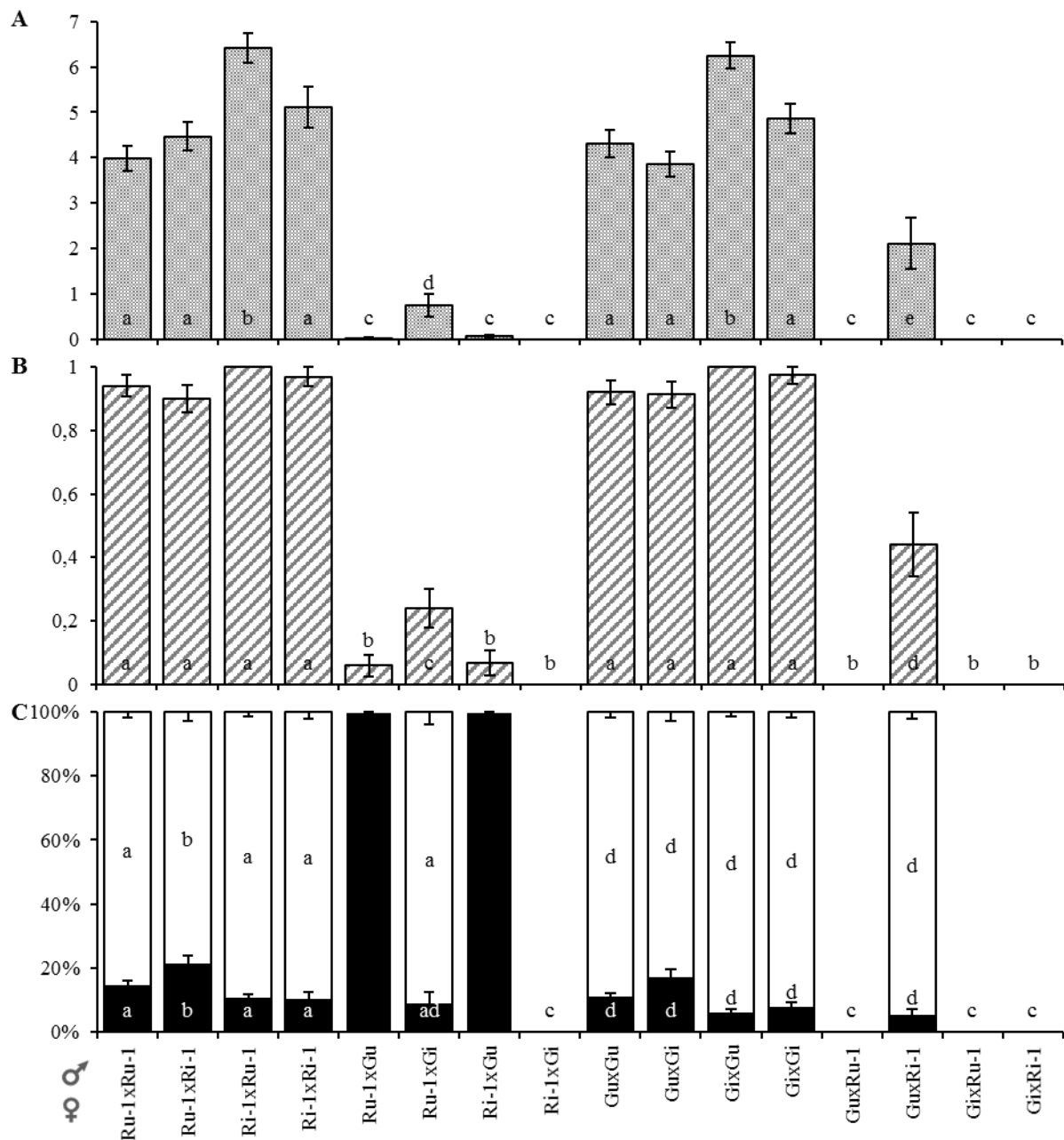


Figure 3.2. Fertility, offspring hatching rate and sex ratio (blocks B1f-B4f) of females produced by each parental cross (x-axis). A: Daily fecundity; B: Proportion of fertile females (females laying at least 1 egg); C: Offspring hatching rate and sex ratio. Bars represent means (\pm s.e.) for the offspring of each cross. Identical superscripts (a, b, c, d) within each bar indicate non-significant differences at the 5% level (contrast analyses). Dotted grey: daily fecundity; Dashed grey: proportion of fertile females; Black: proportion of unhatched eggs; White: proportion of sons.

Hybrid fertility (females, blocks B5f-B8f)

Daily fecundity

Significant differences were found between females resulting from crosses involving different morphs and infection statuses ($X^2_{15}=8367.4$, $p<0.0001$) for daily fecundity (Fig. 3.3A). A significant block effect was also found ($X^2_{18}=18.36$, $p<0.0001$). Contrast analyses revealed that females from intra-morph crosses involving red mothers and *Wolbachia* in either the father (Ru-1♀xRi-1♂) or the mother (Ri-1♀xRu-1♂) laid significantly more eggs (6.6 ± 0.29 and 7.30 ± 0.24 respectively) than other females from intra-morph crosses (ranging from 5.35 to 6.28; $X^2_{13}=5.21$, $p=0.02$ and $X^2_{13}=33.39$, $p<0.0001$).

respectively). Females from intra-morph crosses laid significantly more eggs than those from inter-morph crosses (ranging from 0 to 0.01; $X^2_6=2530.80$, $p<0.0001$).

Proportion of fertile females

Significant differences were found in the proportion of fertile females (Fig. 3.3B) hatching from crosses involving different morphs and infection statuses ($X^2_{15}=1730.66$, $p<0.0001$). Although no significant differences between all intra-morph crosses ($X^2_7=3.60$, $p=0.82$) and between all inter-morph crosses ($X^2_7=8.74$, $p=0.27$) were found, inter-morph crosses produced significantly lower proportions of fertile females (between 0 and 0.02) than intra-morph crosses (between 0.9 and 1; $X^2_7=465.78$, $p<0.0001$), regardless of *Wolbachia* infection status.

Proportion of unhatched eggs

Significant differences were found in the proportion of unhatched eggs (Fig. 3.3C) produced by females from different parental crosses ($X^2_7=84.26$, $p<0.0001$). A significant block effect was also found ($X^2_{10}=10.32$, $p=0.001$). Contrast analyses revealed that eggs produced by the females resulting from Ru-1♀xRi-1♂ and Ri-1♀xRu-1♂ crosses have similar proportions of unhatched eggs (ca. 0.13 ± 0.02 ; $X^2_{10}=0.28$, $p=0.60$), but hatched significantly less than those from Ri-1♀xRi-1♂, Gu♀xGu♂ and Gu♀xGi♂ ($X^2_7=61.36$, $p<0.0001$); these latter crosses led to a similar proportion of unhatched eggs (ranging from 0.04 to 0.07; $X^2_9=2.18$, $p=0.34$). Due to the very low proportion of fertile females resulting from inter-morph crosses (see above), they were not included in the analyses. Note, however, that none of the few eggs laid by these females hatched.

Proportion of sons

As expected for virgin females in haplodiploids, all offspring were males. The results for the proportion of sons (Fig. 3.3C) are thus identical to those for the proportion of unhatched eggs.

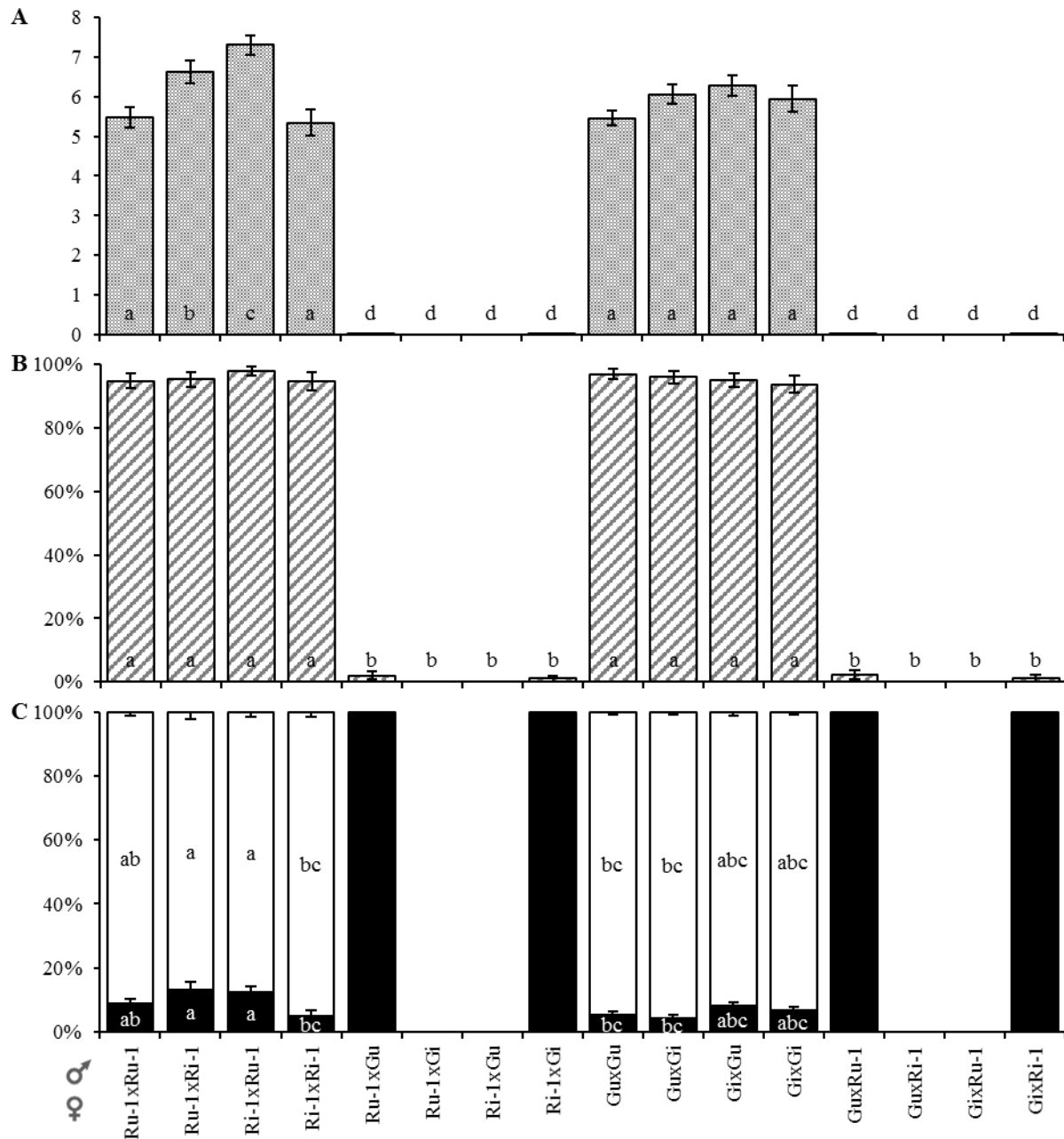


Figure 3.3. Fertility, offspring hatching rate and sex ratio (blocks B5f-B8f) of females produced by each parental cross (x-axis). A: Daily fecundity; B: Proportion of fertile females (females laying at least 1 egg); C: Offspring hatching rate and sex ratio. Bars represent means (\pm s.e.) for the offspring of each cross. Identical superscripts (a, b, c, d) within each bar indicate non-significant differences at the 5% level (contrast analyses). Dotted grey: daily fecundity; Dashed grey: proportion of fertile females; Black: proportion of unhatched eggs; White: proportion of sons.

Hybrid fertility (males)

Daily fecundity

A significant effect was found for the type of cross (males produced by each parental cross, crossed with a female from their mothers' populations; $X^2_{15}=33.52$, $p=0.004$) on female daily fecundity (Fig. 3.4A). However, contrast analyses revealed only near-significant differences among crosses involving red females ($X^2_{18}=13.74$, $p=0.06$), where treatments involving males from the Ru-1 ♀ xRu-1 ♂ and Ru-1 ♀ xRi-1 ♂ crosses led to a slightly lower production of eggs per day (5.40 ± 0.37 and 4.98 ± 0.27 respectively) than the remaining treatments with red females (ranging from 5.64 to 6.58).

Proportion of fertile males

A significant effect was found for the type of cross ($X^2_{15}=41.66$, $p<0.001$) on the proportion of fertile males (Fig. 3.4B). Contrast analyses revealed significant differences according the morph of the females the tested males were allowed to mate with ($X^2_5=45.39$, $p<0.0001$), with a higher proportion of green females that did not sire any female offspring (ranging from 0.59 to 0.79 for green females compared to 0.77 to 0.96 for red females). Minor differences were, however, found between some of the crosses involving Ru-1 or Ri-1 females, with males from parental crosses where the father was infected (Ru-1♀xRi-1♂ and Ri-1♀xRi-1♂) being overall less fertile (0.78 ± 0.04 and 0.77 ± 0.12 respectively) than the others (ca. 0.85 ± 0.1 ; $X^2_4=5.55$, $p=0.02$).

Proportion of unhatched eggs

A significant effect was found for the type of cross ($X^2_{15}=217.99$, $p<0.0001$) on the proportion of unhatched eggs (Fig 3.4C). A significant block effect was also found ($X^2_{18}=21.48$, $p<0.0001$). Contrast analyses revealed differences only due to the morph of the female involved in the crosses ($X^2_{11}=4.16$, $p=0.04$), with eggs laid by red females hatching slightly less (proportion of unhatched eggs ranging from 0.06 to 0.16) than those laid by green females (ranging from 0.04 to 0.09). Crosses with or without *Wolbachia* within each morph were grouped together without significant differences.

Proportion of daughters

A significant effect was found for the type of cross ($X^2_{15}=29.86$, $p=0.01$) on the proportion of daughters (Fig 3.4C). Contrast analyses revealed that males produced by the cross Gu♀xRi♂ sired less daughters on average (0.33 ± 0.04) than males from all other crosses (ranging from 0.35 to 0.67; $X^2_6=20.72$, $p<0.0001$), while the latter crosses did not differ from each other ($X^2_6=2.50$, $p=0.11$).

Proportion of sons

A significant effect was found for the type of cross ($X^2_{15}=124.11$, $p<0.0001$) on the proportion of sons (Fig 3.4C). Contrast analyses revealed no significant differences between males mated with red females ($X^2_{18}=2.90$, $p=0.89$). However, males produced by the Gu♀xGu♂, Gu♀xGi♂, Gu♀xRu-1♂, Gi♀xRu-1♂ and Gi♀xRi-1♂ crosses resulted in significantly lower proportions of sons (ranging from 0.44 to 0.54) than males produced by the Gi♀xGu♂, Gi♀xGi♂ and Gu♀xRi-1♂ crosses (ranging from 0.55 to 0.62; $X^2_5=18.72$, $p<0.0001$). Males produced by red females, independently of their fathers, produced even less sons than both these groups (ca. 0.27 ± 0.04 ; $X^2_5=42.50$, $p<0.0001$ and $X^2_5=86.00$, $p<0.0001$ respectively).

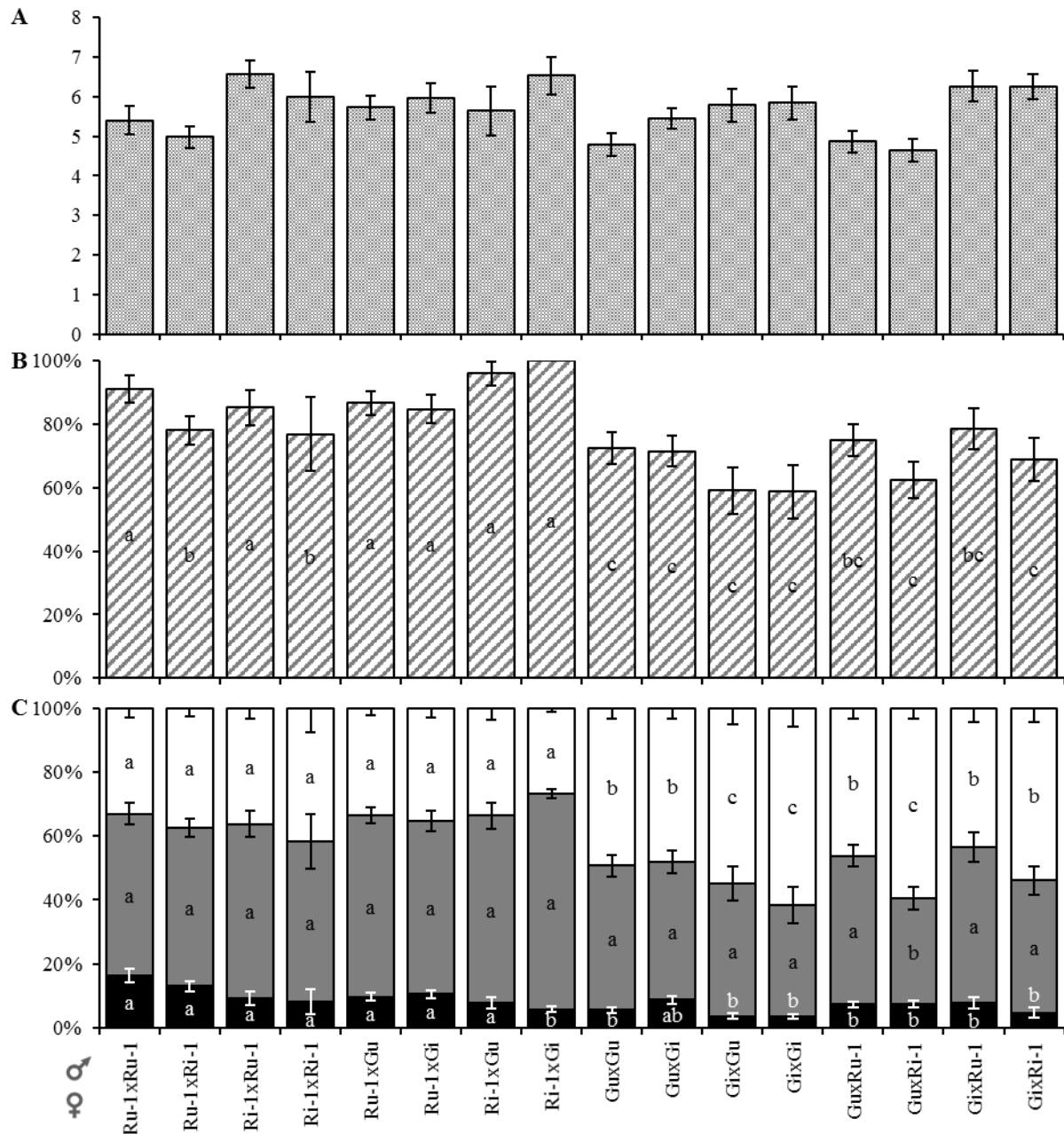


Figure 3.4. Fertility, offspring hatching rate and sex ratio of males produced by each parental cross and backcrossed with females from their maternal population (x-axis). A: Daily fecundity; B: Proportion of fertile males (males siring at least 1 daughter); C: Offspring hatching rate and sex ratio. Bars represent means (\pm s.e.) for the offspring of each cross. Identical or absent superscripts (a, b, c) within each bar indicate non-significant differences at the 5% level (contrast analyses). Dotted grey: daily fecundity; Dashed grey: proportion of fertile females; Black: proportion of unhatched eggs; Grey: proportion of daughters; White: proportion of sons.

Mate choice experiment

Female mating behaviour

The proportion of females that mated differed significantly between females that were given choices between two different types of males, ($X^2_3=8.03$, $p=0.04$; Fig. 3.5A), but was independent of the type of choice they had ($X^2_3=2.22$, $p=0.53$). A significant block effect was also found ($X^2_6=11.61$, $p<0.001$). Contrast analyses revealed that the only significant difference was between red and green females, regardless of their infection status by *Wolbachia* ($X^2_5=6.21$, $p=0.01$), with red females mating more often than green females. However, neither the type of focal female nor the type of presented

choice had a significant effect on the choice made by focal females (Fig. 3.5B; $X^2_3=1.96$, $p=0.58$ and $X^2_3=2.49$, $p=0.48$), but a significant block effect was found ($X^2_{10}=6.36$, $p=0.04$). Comparing each choice to a baseline of 50/50, it was found that when Ru-1 females are presented with either Ri-1 or Gi males, they prefer to mate with Gi males ($z=-2.14$, $p=0.03$). Similarly, no significant effect of the type of focal females, chosen mate, or presented choice was found on mating latency (Fig. 3.5C; $X^2_3=4.06$, $p=0.26$, $X^2_3=0.72$, $p=0.87$, and $X^2_3=2.30$, $p=0.51$ respectively), or mating duration (Fig. 3.5D; $X^2_3=6.61$, $p=0.09$, $X^2_3=7.57$, $p=0.06$, and $X^2_3=3.27$, $p=0.35$ respectively).

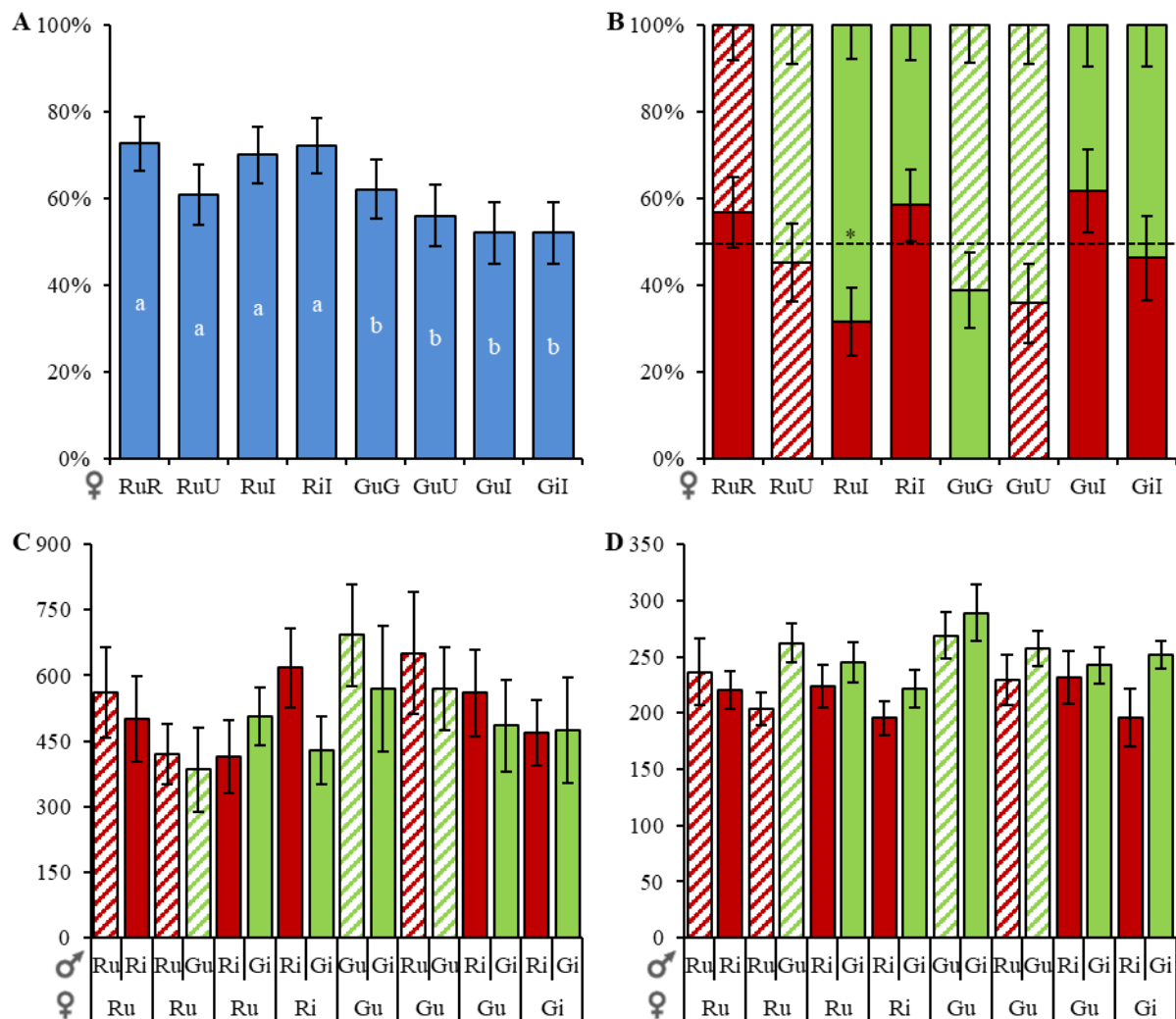


Figure 3.5. Female mating behaviour. Bars represent mean (\pm s.e.) proportion of females that mated (A), the proportion of females choosing each type of presented male (B), mating latency (C), and mating duration (D). The x-axis represents the type and provided choice for each female (RuR: Ru-1 female choosing between Ru-1 and Ri-1 males, RuU: Ru-1 female choosing between Ru-1 and Gu males, GiI: Gi female choosing between Ri-1 and Gi males, etc.; A and B), as well as each chosen male (C and D). Blue: proportion of mated females; Dashed red bars: Ru-1 males; Full red bars: Ri-1 males; Dashed green bars: Gu males; Full green bars: Gi males. Identical or absent superscripts (a, b) within each bar indicate non-significant differences at the 5% level (contrast analyses). An asterisk (*) represents a difference from 50% in proportions of choice.

Male mating behaviour

The proportion of males that mated did not differ between males that were given choices between two different types of females ($X^2_3=4.14$, $p=0.25$; Fig. 3.6A), but was significantly affected by the type of choice they were presented with ($X^2_3=10.06$, $p=0.02$), and a significant block effect was found ($X^2_6=4.20$, $p=0.04$). Contrast analyses revealed that a choice between Gu and Gi females resulted in significantly less matings than all the others ($X^2_5=15.06$, $p=0.0001$). Indeed, the type of focal male did not affect male mate choice ($X^2_3=4.14$, $p=0.25$; Fig. 3.6B), but the type of presented choice significantly affected the female chosen by all males ($X^2_3=9.94$, $p=0.02$). Contrast analyses revealed that male choice is more biased when they were presented with females differing only in morph than when they were presented with females differing only in *Wolbachia* infection statuses ($X^2_5=4.34$, $p=0.04$). Comparing each choice to a baseline of 50/50, it was found that males do not discriminate between different infection statuses, but prefer to mate with red females ($z=6.88$, $p<0.0001$). Although a significant block effect was found for mating latency ($X^2_2=4.28$, $p=0.04$; Fig. 3.6C), no significant effects were found for the types of focal male, chosen mate, or presented choice ($X^2_3=0.49$, $p=0.92$, $X^2_3=0.50$, $p=0.92$, and $X^2_3=1.34$, $p=0.72$ respectively). Mating duration, however, was significantly affected by the type of focal male ($X^2_3=26.90$, $p<0.0001$; Fig. 3.6D), but not by the type of presented choice ($X^2_3=2.03$, $p=0.57$) and only marginally by the type of chosen female ($X^2_3=7.07$, $p=0.07$). Nevertheless, further analyses performed by merging the focal males or the chosen females by either morph or infection status revealed that: for chosen females, *Wolbachia* infection leads to lower mating durations ($X^2_2=8.69$, $p=0.003$), but there is no effect of the morph ($X^2_2=4.43$, $p=0.11$); for focal males, *Wolbachia* infection had no significant effect ($X^2_2=2.96$, $p=0.09$), but red males display overall lower mating durations than green males ($X^2_2=23.41$, $p<0.0001$).

Offspring hatching rate and sex ratio

As the data referring to offspring hatching rate and sex ratio produced by the mated couples in the behavioural experiments presents an identical pattern to that obtained for the parental crosses, it is not presented here (see Annex 2).

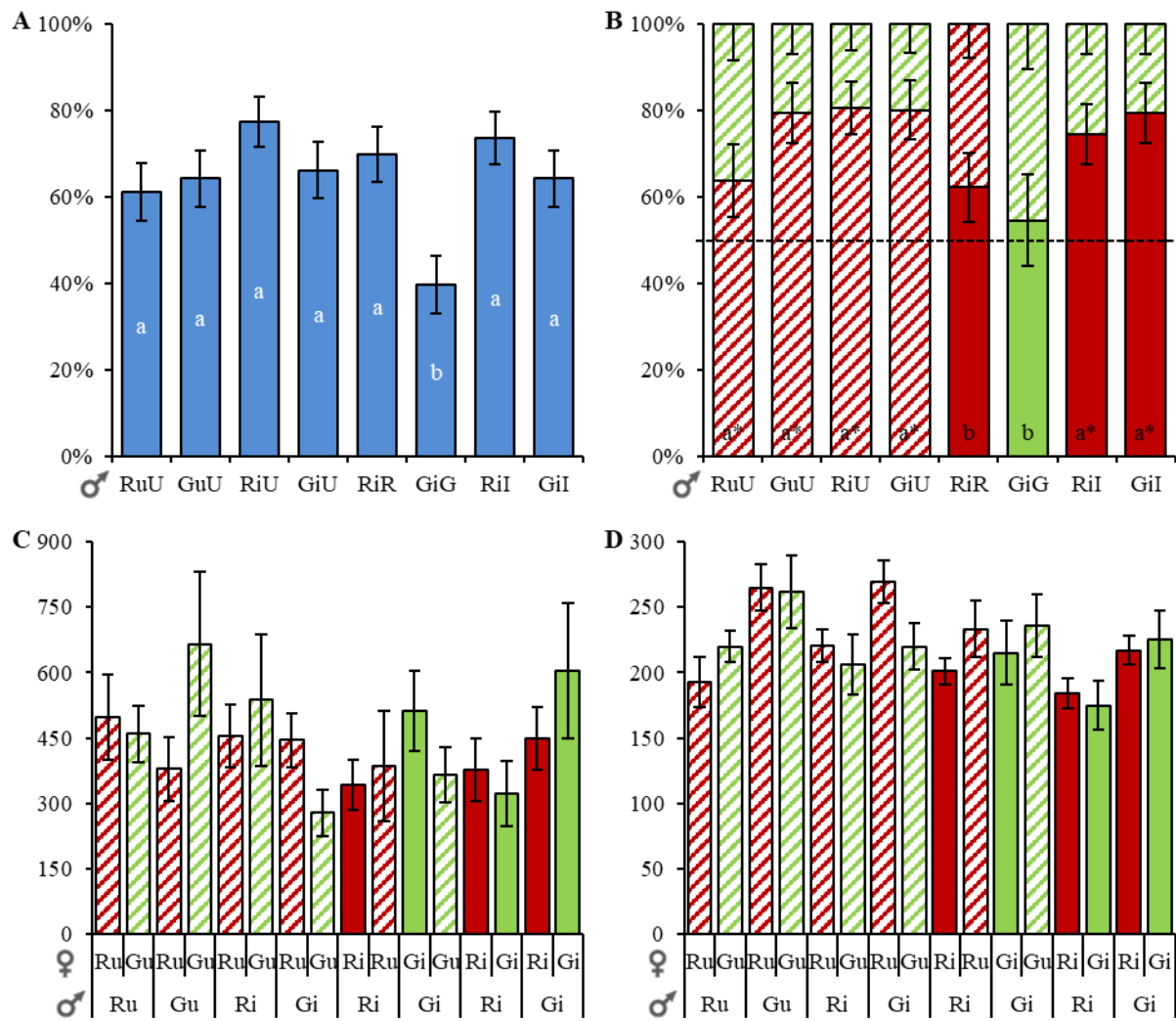


Figure 3.6. Male mating behaviour. Bars represent means (\pm s.e.) for the proportion of males that mated (A), the proportion of males choosing either type of presented male (B), the latency to mate with either female (C), and the mating duration with either mate (D). The x-axis represents the type and provided choice for each male (RuR: Ru-1 male choosing between Ru-1 and Ri-1 females, RuU: Ru-1 male choosing between Ru-1 and Gu females, GiI: Gi male choosing between Ri-1 and Gi females, etc; A and B), as well as each chosen female (C and D). Blue: proportion of mated males; Dashed red bars: Ru-1 females; Full red bars: Ri-1 females; Dashed green bars: Gu females; Full green bars: Gi females. Identical or absent superscripts (a, b) within each bar indicate non-significant differences at the 5% level (contrast analyses). An asterisk (*) represents a difference from 50% in proportions of choice.

4. Discussion

With this project, I set out to test for the presence of pre- and post-copulatory barriers associated to either the host, *Wolbachia*, or a combination of both, by investigating assortative mating, hybrid fertility, and hybrid breakdown. Overall, I found no evidence of pre-copulatory isolation between individuals of different morphs or *Wolbachia* infection statuses, but strong post-copulatory isolation leading to an overproduction of males in inter-morph crosses involving green females, and to full hybrid sterility in all inter-morph crosses. This work thus revealed that the two studied populations are fully isolated, independently of *Wolbachia*. Indeed, although *Wolbachia* was found to increase the incompatibility occurring in parental crosses, it did not affect hybrid fertility nor did it induce hybrid breakdown.

Parental crosses

Our results show only costly or neutral effects of crosses between different morphs or different *Wolbachia* infection statuses. Within morphs, the two red populations tested were fully compatible at F0. *Wolbachia* induced **CI** in both red populations, albeit at a lower level in the Ri-2 than in the Ri-1 population. However, the molecular markers obtained for the bacteria infecting both populations (see Materials and Methods), combined with the fact that reciprocal rescue occurred, suggest that both red populations are infected with the same strain. It has been proposed that the strength of **CI** may be influenced by the host genotype and by bacterial density (Werren 1997), which could account for the differences in **CI** found in this study. In the green population, *Wolbachia* did not induce **CI**. This result could be due to a host genotype effect, as previously shown to occur in *T. urticae* (Xie et al. 2010), or to a *Wolbachia* genotype effect, since the *Wolbachia* strain infecting the green morph differs slightly from the one infecting the red morph (see Materials and Methods). Although the genetic differences found between the bacteria in the green and red morphs were restricted to two SNPs on studied markers, the latter hypothesis is reinforced by the fact that there was only a low, partial rescue of **CI** in the inter-morph crosses involving infected green females ($\text{Gi}\text{♀}\times\text{Ri-1}\text{♂}$ and $\text{Gi}\text{♀}\times\text{Ri-2}\text{♂}$; Fig. 4.1; Annex 3).

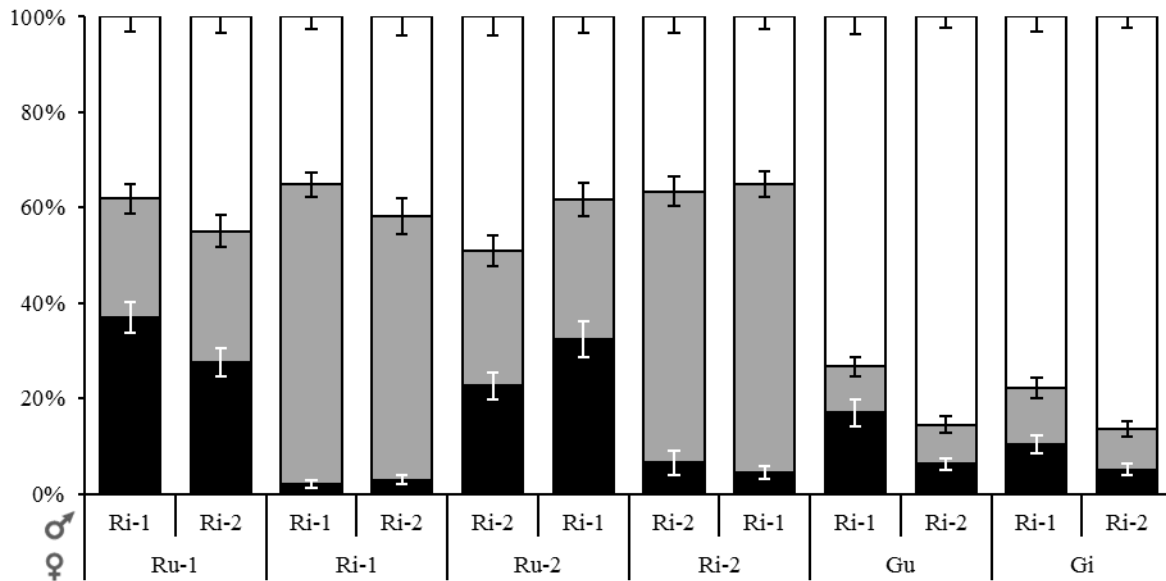


Figure 4.1. Pattern of CI induced by *Wolbachia* in parental crosses involving infected males from the red populations, and rescue of its effect in red infected females, but not in green infected females. Bars represent means (\pm s.e.) for the offspring of each cross. Black: proportion of unhatched eggs; Grey: proportion of daughters; White: proportion of sons.

Between morphs, reproductive incompatibility was found, at the first generation of crosses, only when green females mated with males from each of the red populations, and was expressed as a pronounced reduction in the proportion of daughters to the benefit of the sons (unlike in **CI** crosses, where the lower proportion of daughters corresponds to an increase in the proportion of unhatched eggs, the reduction in the proportion of daughters caused by **HI** corresponds instead to an increase in the proportion of sons). This biased sex ratio has been previously shown as a consequence of inter-morph crosses (Lu et al. 2017; Murtaugh and Wensch 1978; Sugawara et al. 2002). As behavioural observations revealed that inter-morph matings occur in normal proportions, this effect can be caused by either incomplete fertilization of the eggs or by re-haplodization of fertilized eggs due to paternal genome elimination (a phenomenon known to lead to functional haplodiploidy, or pseudoarrhenotoky, in phytoseiid mites and in some insects ; Hennig and Scheer 1999). **HI** in these populations is thus incomplete and unidirectional, acting in the same direction as **CI**. As *Wolbachia*-induced **CI** only affects daughters, its effects are less striking in these crosses; however, the level of **CI** induced does not differ from that of intra-morph crosses (*i.e.*, while the overall proportions of unhatched eggs and females are lower in inter-morph than in intra-morph crosses due to the overproduction of males, their relative proportions, without considering the proportion of males, do not differ; see Annex 3). This means that **CI** does not interact with **HI**: the two effects are additive. It should be noted that, while the results for the crosses performed in this project could not be compared to the ones performed previously due to an important block effect, the pattern shown by the former is consistent with expectations: crosses between infected females and uninfected males result in an identical pattern to that obtained in crosses between uninfected individuals.

Hybrid female fertility

The results from the first hybrid female fertility test (B1f-B4f) were analysed separately from the two following tests. This was due to an apparent rescue of fertility in the first test, where a proportion of females from inter-morph parental crosses involving infected fathers (Ru-1 ♀ x Gi ♂ and Gu ♀ x Ri-1 ♂) were fully fertile, as opposed to all other inter-morph crosses which failed to produce a single fertile female. This result was not found in the two subsequent tests, in which none of the inter-morph crosses produced fertile females. One possibility to explain this discrepancy is that this effect

disappeared through time, but it seems unlikely since current knowledge of the mechanism of **CI** does not provide a reasonable explanation for these results. Indeed, **CI** is caused by an asynchrony of the maternal and paternal pronuclei during the first embryonic mitotic division, leading to improper segregation of chromosomes and embryonic mortality (Tram and Sullivan 2002). This does not explain a modification on the germline of the offspring to restore fertility. Another, more likely, explanation, is that these results would be due to contaminations in the dishes where the parental crosses were prepared; due to the large number of females installed on the leaves, it is possible that a male of the same population was accidentally transferred at the same time and fertilized several of the females, resulting in compatible intra-morph crosses. In the two subsequent tests, the dishes were verified more carefully after installation of the females to prevent this issue. In these last two tests (B5f-B8f), the results show that while **HI** is unidirectional in parental crosses, all females resulting from inter-morph crosses are fully sterile. Only a very small proportion was found to occasionally lay a small amount of eggs, which always failed to hatch. Thus, the populations seem to be fully isolated. A similar pattern of F1 sterility has been shown for crosses between morphs, both as a lower proportion of females ovipositing and as an increased proportion of unhatched eggs (De Boer 1982; Sugawara et al. 2002). Finally, *Wolbachia* infection did not have a significant impact on either fertility or egg hatchability in females hailing from **CI** crosses, thus excluding any hybrid breakdown phenomenon here, although previously described using a different population of *T. urticae* (Vala et al. 2000).

(Hybrid) male fertility

As no significant differences were found between males from intra- and inter-morph parental crosses, we can conclude that the males tested were able to reproduce normally. These results thus suggest that the overproduction of males found for inter-morph parental crosses involving green females is caused by incomplete fertilization of the eggs, and not to any form of aneuploidy due to re-haploidization. In line with this, the lower proportion of fertile males found in crosses involving green morph mothers can be explained by an overall lower receptivity to mating of the green females used for the backcross (see below). Indeed, males and females used for the backcrosses were installed directly on the leaf discs and matings were not observed.

Mate choice

Both tests show that females from the green morph are less receptive to mating than females from the red morph, and therefore, that males (regardless of their morph) are more likely to choose red over green females. *Wolbachia*, conversely, did not affect mate choice. These results thus reveal an absence of assortative mating in these populations (*i.e.* individuals are incapable of avoiding either **CI** or **HI**), which is in accordance with the other study that has investigated mate choice between morphs (Murtaugh and Wrensch 1978), but in discrepancy with the study that investigated the effect of *Wolbachia* on *T. urticae* mating preferences and showed avoidance of *Wolbachia*-infected males by uninfected females (Vala et al. 2004). In other systems, however, several studies have often failed to detect such effect of *Wolbachia* on host mating behaviour (Atyame et al. 2011; Champion De Crespigny and Wedell 2007; Jiggins et al. 2002). It is thus possible that traits enabling individuals to display mating preferences between morphs or *Wolbachia* infection statuses are rare in nature. Another possible explanation for the absence of choice is that the studied populations did not remain in a polymorphic state (different morphs existing in sympatry or a mix of infected and uninfected individuals) long enough for these traits to be selected. Indeed, the red and green populations used here were collected at different times, on different locations, and we have no information on whether they had contact with populations of the opposite morph prior to this study. As for *Wolbachia*, when Ri-1 was collected from the field, the endosymbiont was found to be fixed in the population (Zélé et

al. *in prep.*). The red morph population was thus not polymorphic for *Wolbachia* in the field, and as such, it is possible that a preference trait was never selected. No information was available for *Wolbachia* field prevalence in Gi.

There were no overall differences between latency to mating in either test. It could be expected that the lower receptivity of green females would lead to higher values of latency for the observations where they successfully mated, yet this was not the case. Possibly, the provided time limit of thirty minutes was not enough for the males to choose their mate when only green females were present. However, given the lower proportion of males that produced daughters in crosses involving green females during the fertility experiment (see above), this explanation is unlikely (*i.e.*, they spent multiple days with a green female and may still choose not to mate).

Green males mated longer with both red and green females, which does not, *a priori*, explain the lower proportion of daughters produced by these males when mated with green females. An alternative explanation for this longer mating duration could be that it is a mechanism to compensate for potentially lower sperm quality, or for a possible sexual conflict leading to the more male-biased sex ratio found in green females. However, a previous study performed in *T. urticae* found no evidence for correlation between mating duration and offspring sex ratio (Satoh et al. 2001), making these hypotheses unlikely. The same study does, however, suggest that higher copulation durations may be a strategy of post-copulatory guarding, which could provide an explanation for this result: as green females are less receptive, the males that successfully mate with them benefit from investing more in post-copulatory guarding behaviours, to prevent them from remating until first male sperm precedence (Helle 1967) is assured. Further, *Wolbachia* infection in females, regardless of the strain, resulted in lower mating durations. This might suggest that males invest less sperm (or spend less time in post-copulatory guarding) in mating with infected females. This behaviour could potentially be explained if *Wolbachia* exerts a detrimental effect upon fecundity of the host (*i.e.*, infected females would be perceived as worse mates during copulation and thus worth less investment), and indeed other studies have shown a great variability in the potential effects of *Wolbachia* on fecundity, potentially increasing, reducing, or not affecting host fecundity (Gotoh et al. 2007; Perrot-Minnot et al. 2002; Vala et al. 2000). Nonetheless, *Wolbachia* infection in the females studied here did not prove to be costly for fecundity. As such, the reason for this lesser investment by males remains unclear.

Conclusion and perspectives

Overall this study shows that both **CI** and **HI** acted in the same direction, which could suggest that the bacterium has played a role in the initial development of this barrier. Nonetheless, the fact that hybrids are fully sterile means that *Wolbachia* no longer plays a role in reproductive isolation between these populations. Thus, to determine whether the bacterium can contribute to reproductive isolation in this species, it would be necessary to test its effect on crosses between populations displaying lower intrinsic **HI** (*i.e.*, partially isolated).

Importantly, although previous studies have shown incompatibility between the two *T. urticae* morphs studied here, these works have primarily dealt with only one population for each morph. It can thus be argued that it is as yet unclear whether this isolation is due to the morphs themselves (which would suggest that the morphs are currently undergoing a process of speciation), or to genetic distance between populations, independently of the morph to which they belong. Identifying the cause of this incompatibility would require many different populations, varying by their morph and displaying variable genetic distances. Indeed, it is unclear whether the results obtained in this project are due to an effect of the morphs or of differentiated populations. It is still necessary to genetically analyse the populations used here and determine whether they are particularly distant relative to other populations

collected in the vicinity. While the results obtained here are, to an extent, consistent with those of previous studies, this lack of information prevents us from concluding with any degree of certainty that they are indeed due to the morphs.

T. urticae is a species with first male sperm precedence (as mentioned above), and double mating experiments performed in my host institution showed that sperm transfer in these incompatible crosses is effective, as subsequent matings do not rescue the proportion of daughters resulting from them (Zélé et al. *in prep*). As the fertility and sex ratio produced by males in inter-morph crosses involving green females did not differ from that of intra-morph crosses, egg re-haploidization was ruled out as the cause for the overproduction of males and the most likely scenario is that the eggs were not fully fertilized. Confirming this conclusion would, however, require further studies on the process of fertilization in spider mites.

The results described here warrant further investigations into the nature of the morphs, and the dynamics of their interaction in the wild. *T. urticae* is a problematic agricultural pest, and understanding the consequences of invasions among populations could be particularly relevant to help manage its impact. Based on the results described here, green males preferentially mate with red females, resulting in a steady production of infertile hybrids. Conversely, red and green males will mate with green females with equal frequency. The less female-biased sex ratio produced by green females, combined with the overproduction of males in incompatible crosses, is expected to lead to the production of large numbers of green sons when green females mate with males from either morph, but especially with those from the red morph. These interactions could thus potentially result in one of the populations excluding the other, depending on the initial conditions of the invasion. This highlights the need for further studies on this system, such as investigating whether males avoid mating with hybrid females. Additionally, *Wolbachia* infection may interfere with the outcome of these invasions, by reducing the proportion of fertile haploid males produced in incompatible inter-morph crosses. However, knowledge on the consequences for the invasive potential of *Wolbachia* itself is limited, as the lack of fertile hybrids prevents the bacterium from being transferred between morphs. Thus, if one of the morphs excludes the other, *Wolbachia* in that morph would be excluded alongside it, and the bacterium can only invade if it is carried by a successful invading host. Recently in my host institution, a model has been developed for reproductive interference between two species of spider mites (Clemente et al. *in prep*). This model could be adapted for the interaction between morphs and used to generate important predictions for population dynamics and their invasive potential, which could then be tested experimentally.

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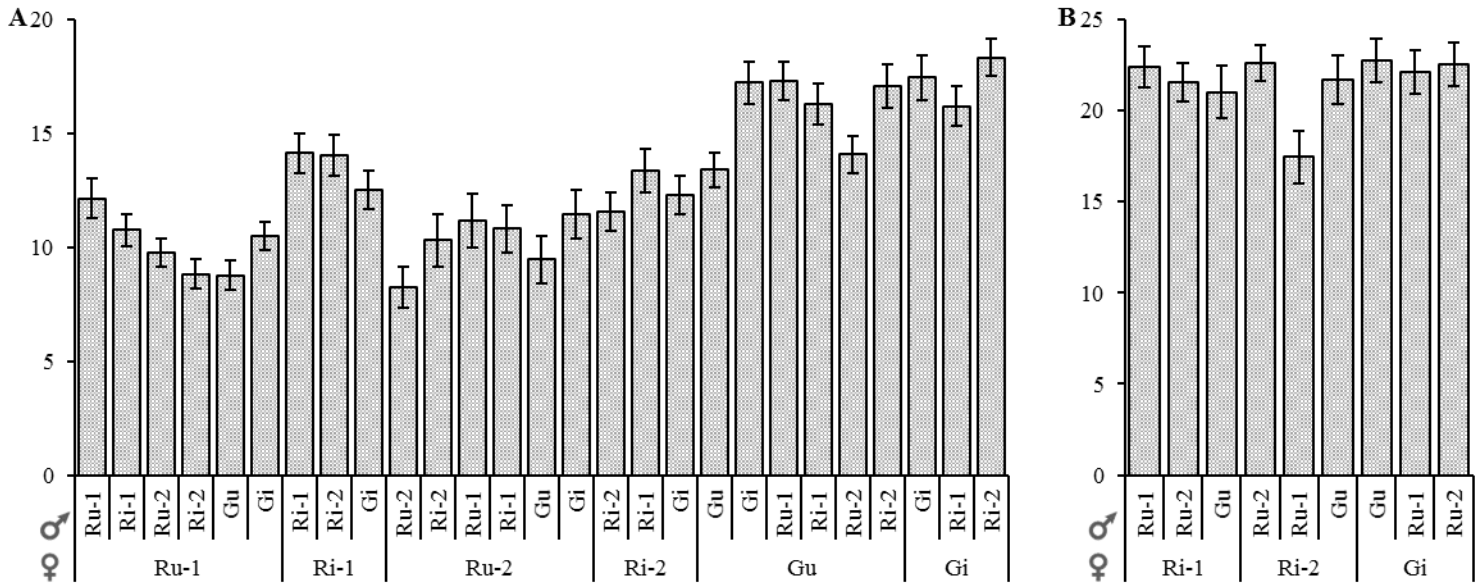
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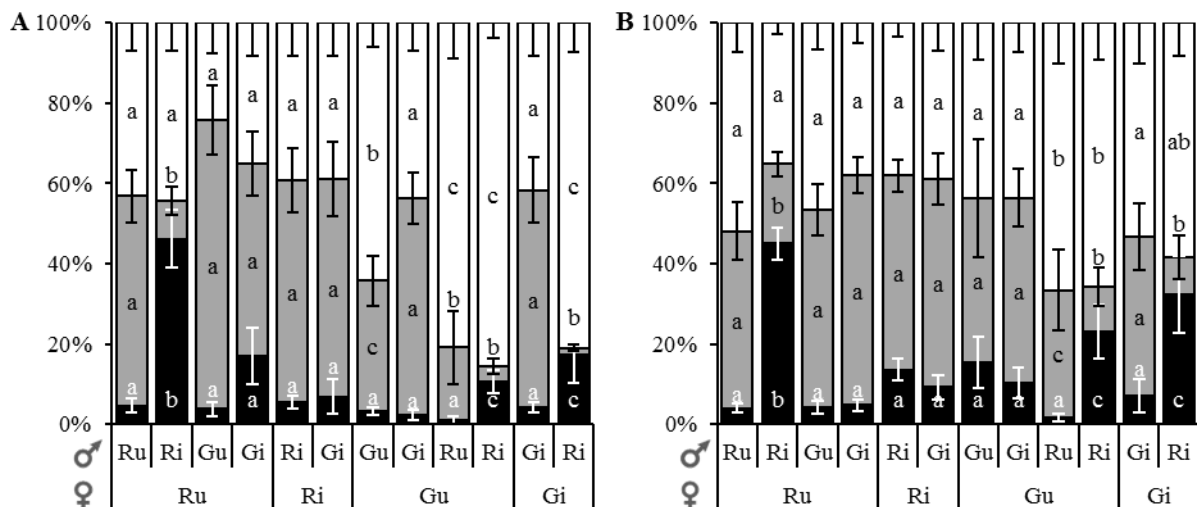
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Annexes

Annex 1. Female total fecundity of the parental crosses performed in the previous experiment (A) and during this project (B). Bars represent mean (\pm s.e.) total fecundity of each cross. Over the time between experiments, total fecundity of all females, but particularly the red ones, increased significantly, leading to a large block effect between them.



Annex 2. Offspring resulting from crosses between mothers (bottom level of the x-axis) and fathers (top level of the x-axis) of the different populations and infection statuses tested during behavioural observations. A: Offspring hatching rate and sex ratio from focal female crosses; B: Offspring hatching rate and sex ratio from focal male crosses. Bars represent means (\pm s.e.) for the offspring of each cross. Identical superscripts (a, b, c) within each bar indicate non-significant differences at the 5% level (contrast analyses). Black: proportion of unhatched eggs; Grey: proportion of daughters; White: proportion of sons.



Annex 3. Level of *Wolbachia*-induced CI in intra- and inter-morph parental crosses (x-axis).
 Bars represent means (\pm s.e.) of the level of **CI** for each appropriate cross, calculated as the proportion of unhatched eggs relative to the proportion of female offspring.

