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How does sympatric evolution shape reproductive isolation in spider mites?

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Resumo

A compreensão do processo contínuo da especiação tem uma relevância fundamental para a biologia da conservação, já que o grau de isolamento entre espécies ou populações determina a forma como estas interagem, assim como as consequências destas interações. Por exemplo, no caso de espécies incipientes ou populações divergentes possuírem apenas isolamento reprodutivo parcial entre si, tal pode levar a interações sexuais com custos de fitness para uma ou ambas as espécies/populações, podendo resultar na sua extinção. Este tipo de interações denomina-se interferência reprodutiva, e resulta do facto de barreiras reprodutivas entre espécies ou populações estarem incompletas. Tais barreiras podem atuar em qualquer fase no ciclo reprodutivo de um organismo, nomeadamente antes do acasalamento (barreiras pré-acasalamento), após o acasalamento mas antes da formação do zigoto (barreiras pós-acasalamento pré-zigóticas) e após a formação do zigoto (barreiras pós-zigóticas). As interações sexuais negativas entre espécies/populações poderem ter consequências ecológicas drásticas. No entanto, caso estas persistam em simpatria durante um período suficientemente longo, é expectável que ocorra evolução de mecanismos que permitem reduzir ou evitar custos de fitness. No caso da interferência reprodutiva, tal pode acontecer por duas vias. Por um lado, pode ocorrer a evolução de barreiras pré-zigóticas mais fortes, promovendo a especiação. Por outro, caso ocorra troca de genes entre as espécies ou populações, as barreiras pós-zigóticas podem tornar-se mais fracas, promovendo a fusão de ambas numa única espécie/população. Tendo em conta que o resultado destas vias evolutivas alternativas é bastante diferente, torna-se crucial perceber qual o tipo de barreiras reprodutivas, pré-zigóticas ou pós-zigóticas, que tende a evoluir em regime de simpatria e de que forma.

Para alcançar este objetivo, neste projeto a espécie de ácaro-aranha *Tetranychus urticae* foi utilizada como organismo modelo em experiências de evolução experimental. Este sistema é particularmente útil para o estudo da evolução de barreiras reprodutivas, pois possui um tempo de desenvolvimento curto, possibilitando assim a realização de várias gerações de evolução experimental. Para além disto *T. urticae* é haplodiploide, pelo que os machos resultam de ovos não fertilizados e as fêmeas de ovos fertilizados, permitindo analisar problemas de fertilização através da proporção de machos e fêmeas resultantes de cruzamentos entre indivíduos. Por fim, na maioria dos casos, apenas o esperma do primeiro macho a acasalar com uma fêmea é utilizado para fertilizar os seus ovos. Se este padrão de precedência espermática se mantiver quando fêmeas acasalam com machos de uma espécie parcialmente isolada ou de uma população divergente, tal deverá acarretar custos elevados constituindo assim uma forte pressão seletiva para a evolução de mecanismos que reduzam a interferência reprodutiva.

Ao longo do projecto, foram utilizadas duas populações geneticamente divergentes de *T. urticae*: uma população do morfotipo verde e outra do morfotipo vermelho. Um estudo prévio feito nestas mesmas populações revelou que cruzamentos entre ambas produzem híbridos maioritariamente estéreis cujos ovos não eclodem. Porém, sabe-se também que machos de ambas as populações preferem acasalar com fêmeas vermelhas. Assim, entre estas populações existe isolamento pós-zigótico completo, mas isolamento pré-zigótico incompleto, o que leva à ocorrência de custos de fitness associados a interferência reprodutiva. A evolução de barreiras reprodutivas face a estes custos foi testada submetendo ambas as populações a evolução experimental durante 69 gerações (cerca de 32 meses). Foram criados três regimes de seleção diferentes, cada um possuindo cinco réplicas populacionais: regime alopátrico com indivíduos da população do morfotipo verde; regime alopátrico com indivíduos da população do morfotipo vermelho; regime simpátrico com indivíduos de ambas as populações. No meu projeto realizei três experiências com indivíduos de cada população de modo a testar a evolução de barreiras pré-acasalamento, pós-acasalamento pré-zigóticas e pós-zigóticas em simpatria em comparação a alopatria.

A primeira experiência, com objetivo de testar a evolução de barreiras pré-acasalamento, envolveu observações de escolha de parceiro. Nesta experiência foram usados quatro tratamentos, nos quais machos verdes e vermelhos dos regimes de alopatria e simpatria puderam escolher entre duas fêmeas de populações diferentes para copular. Desta forma, foi possível medir a proporção de fêmeas de cada população escolhidas pelos machos, o que permitiu discernir a preferência destes por fêmeas da sua população. Também foi medido o tempo que os machos demoraram a iniciar a cópula, bem como a duração da mesma, fatores frequentemente associados à preferência por um determinado tipo de fêmea. Os resultados desta experiência revelaram ausência de evolução da preferência dos machos que evoluíram em simpatria em comparação aos que evoluíram em alopatria, já que tanto machos verdes como vermelhos continuaram a preferir fêmeas vermelhas. No caso da população do morfotipo vermelho, a ausência de evolução pode dever-se a um número insuficiente de gerações de seleção, devido a dificuldades na manutenção dos replicados de evolução experimental do regime de simpatria destas populações. No caso da população do morfotipo verde, os resultados obtidos podem ser explicados por restrições evolutivas, impossibilitando alterações de preferência de parceiro. O tempo que os machos demoraram a iniciar a cópula não aparenta estar relacionado com preferência por determinado parceiro, caso contrário os machos deveriam iniciá-la mais rápido com fêmeas vermelhas, o que não se verificou. Os resultados do tempo de duração das cópulas revelaram que os indivíduos não reconhecem se estão a copular com parceiros da mesma ou de uma população diferente após iniciarem a cópula, caso contrário seria expectável que cópulas com parceiros de populações diferentes se tornassem mais curtas, o que não foi observado.

A segunda experiência foi realizada de modo a testar a evolução de dois tipos de barreiras: pós-acasalamento pré-zigóticas e pós-zigóticas que ocorrem cedo no ciclo reprodutivo. Neste caso, foram realizados cruzamentos entre fêmeas verdes, dos regimes alopátrico e simpátrico, e machos verdes e vermelhos do regime equivalente. Estes cruzamentos permitiram assim a deteção de problemas na fertilização através da medição da proporção de filhos e filhas produzidos (barreira pós-acasalamento pré-zigótica). Para além disto, também possibilitaram testar barreiras pós-zigóticas através da medição da proporção de ovos não eclodidos e de juvenis mortos, ambos possíveis indicadores de inviabilidade dos híbridos resultantes dos cruzamentos. Adicionalmente, para testar a precedência espermática, parte das fêmeas verdes que acasalaram tiveram a possibilidade de acasalar uma segunda vez com um macho da outra população. Após estes segundos cruzamentos foi medida a proporção de descendentes de cada macho. Assim, foi possível detetar alterações na precedência espermática, possivelmente para os ovos serem maioritariamente fertilizados pelo macho da mesma população que a fêmea, independentemente da ordem de acasalamento (barreira pós-acasalamento pré-zigótica). Em termos da fertilização de ovos pelos dois machos que acasalaram com fêmeas verdes, verificou-se paternidade partilhada quando o primeiro macho a acasalar era vermelho, indicando que o padrão de uso do esperma do primeiro macho a acasalar é incompleto neste sistema. No entanto, nenhuma barreira evoluiu devido a interferência reprodutiva no regime de simpatria. O facto de não ter havido mudanças na proporção de zigotos e juvenis híbridos mortos após evolução experimental no regime de simpatria indica que a hibridação não confere vantagens. Porém, verificou-se que neste regime a proporção de juvenis mortos resultantes de cruzamentos entre indivíduos verdes aumentou. Para além disso, a proporção de filhas produzidas por fêmeas verdes também aumentou, revelando um aumento global da taxa de fertilização de ovos por parte destas fêmeas. Tal poderá ter sido uma resposta evolutiva para reduzir a competição por fêmeas entre machos verdes no regime de simpatria, porque neste regime cruzamentos entre fêmeas verdes e machos vermelhos levam a uma produção exacerbada de machos verdes.

A terceira experiência possibilitou testar a evolução de barreiras pós-zigóticas que ocorrem tarde no ciclo evolutivo, pela realização de cruzamentos entre machos e fêmeas da mesma população (morfotipo verde e vermelho) e cruzamentos bidirecionais entre populações, tanto com indivíduos do regime alopátrico como simpátrico. Destes cruzamentos foi possível obter fêmeas verdes, vermelhas e

dois tipos de híbridas, num total de oito tratamentos. A fertilidade destas fêmeas foi testada a partir da proporção que produziu ovos, bem como o número de ovos produzidos por dia. Adicionalmente, a proporção dos ovos que eclodiram foi usada para testar a mortalidade zigótica. Mais uma vez, nenhuma barreira evoluiu, neste caso no sentido de reduzir o isolamento entre as duas populações, o que sugere custos de hibridação que ocorrem após a geração F2. No entanto, verificou-se que este isolamento pós-zigótico não é completo, já que alguns ovos produzidos por híbridos eclodiram. Este resultado inesperado abre a possibilidade de existir troca de genes entre as populações, nomeadamente da população do morfotipo vermelho para a do verde. Tal poderia explicar alguns resultados obtidos relativamente às fêmeas verdes que evoluíram no regime de simpatria, como a redução do número de ovos produzidos por dia, o aumento da mortalidade dos juvenis resultantes e o aumento da proporção de filhas.

Em conclusão, o facto de não se ter verificado qualquer evolução das barreiras reprodutivas entre a população do morfotipo verde e vermelho de *T. urticae* devido a interferência reprodutiva, revela a necessidade de investigação adicional sobre respostas evolutivas alternativas face a interações sexuais negativas, bem como estudos acerca das consequências ecológicas da persistência das mesmas em populações naturais.

Palavras-chave: Isolamento reprodutivo; interferência reprodutiva; evolução experimental; simpatria; ácaros-aranha

Abstract

In many species, negative interspecific sexual interactions owing to incomplete species recognition and hybridization (*i.e.,* reproductive interference) can lead to the rapid extinction/displacement of populations. Nevertheless, if species persist long enough in sympatry, it may also drive the evolution of stronger pre-zygotic isolation (*e.g.,* reinforcement), thereby completing speciation, or, in the presence of gene flow, it may instead select for weaker post-zygotic isolation, thereby reversing speciation. Determining what reproductive barriers (pre-zygotic and/or post-zygotic) are more likely to evolve in sympatry may thus allow better understanding the fate of interacting species.

In this project, I used a pair of hybridizing genetically divergent colour form populations of the spider mite species *Tetranychus urticae* (green and red), which were previously shown to be fully post-zygotically isolated. Following up to 69 generations of experimental evolution in sympatry or allopatry, I performed three different experiments to assess, respectively, pre-mating, post-mating pre-zygotic and early post-zygotic, as well as late post-zygotic barriers.

I found that none of the tested reproductive barriers evolved during experimental evolution, but that green-form females evolved in sympatry differed from those evolved in allopatry. They had a lower fecundity, their offspring had a higher juvenile mortality, and their sex-ratio was more skewed towards females. Altogether, these results show that reproductive interference can quickly drive the evolution of a species' reproductive traits and sex allocation. However, I also found that, conversely to our previous knowledge, late reproductive barriers were incomplete between all populations of the two forms. Therefore, genetic analyses would be necessary to determine whether the observed evolutionary responses result from selection, or are the by-product of gene flow in this system. Finally, these results also call for further research on other possible evolutionary responses, as well as on the ecological consequences of reproductive interference in natural populations.

Keywords: Reproductive isolation; reproductive interference; experimental evolution; sympatry; spider mites

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1. Introduction

Understanding speciation, the process by which different species arise from common ancestry, is not only of academic interest but it also has important implications for conservation biology. Indeed, the fact that speciation is a continuous process by which "*the continuous sequence of genetically-based changes occur as two lineages diverge from one another on the pathway to reproductive isolation*" (*i.e.,* the "speciation continuum" as defined by Shaw & Mullen (2014); Figure 1.1a) poses a certain number of problems for conservation biology. For example, conventional captive breeding programs employ strategies to breed the most distantly related individuals to avoid inbreeding-associated problems (Meffert 1999), but such strategies may be compromised if genetically distant individuals have evolved some degrees of reproductive isolation (*e.g.,* outbreeding depression due to genetic incompatibilities; Fenster & Galloway 2000; Edmands & Timmerman 2003; Rollinson et al. 2014). In line with this, partial reproductive isolation between divergent populations or incipient species (*i.e.,* "semi-isolated species"; Roux et al. 2016; Stankowski & Ravinet 2021) in the wild, such as between native and closely-related invasive species, may lead to negative sexual interactions between them (*i.e.,* reproductive interference; *cf.* below), which in turn can result in their displacement or extinction (Rhymer & Simberloff 1996; Gröning & Hochkirch 2008). Finally, the conservation of biodiversity in natural populations may be threatened by "reverse speciation" (Figure 1.1b), a process by which gene flow leads to the substitution of the divergent populations by a single 'hybrid' population (*i.e.,* "genetic swamping" or "hybrid swarm"; Seehausen 2006).

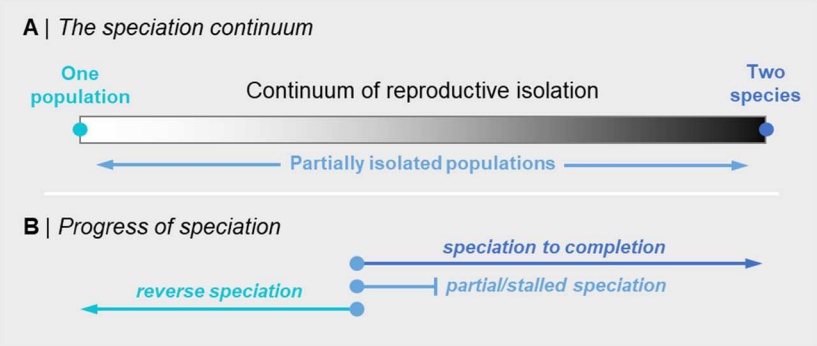

Figure 1.1. Graphical depiction of the speciation continuum and the progress of speciation. (A) The speciation continuum is usually depicted as a gradient with a single population at one end and two species at the other. States in between represent partially isolated populations. (B) During the progress of speciation, pairs of populations (represented by the arrows) may complete speciation, evolve partial reproductive isolation (which can be stable either as an optimum or due to a constraint), or mix when speciation reverses (*i.e.,* reproductive isolation breaks down). Figure adapted from Stankowski & Ravinet 2021.

Reproductive isolation hinges upon different reproductive barriers occurring sequentially at different stages of the reproductive cycle of organisms (Table 1.1): before mating (pre-mating isolation), after mating but before zygote formation (post-mating pre-zygotic isolation), or after zygote formation (post-zygotic isolation). If any of these barriers is even partially incomplete this may lead to reproductive interference; which is defined as any type of sexual interaction between different species/populations that results in fitness costs for at least one of them and that is caused by incomplete reproductive isolation (Gröning & Hochkirch 2008). Moreover, different types of reproductive interference result from different incomplete reproductive barriers (Table 1.1), and because reproductive barriers are set up sequentially along the reproductive cycle, all types of reproductive interference preceding a given type of reproductive interference will also occur. For example, if two species hybridize then at least one of them must have engaged in courtship behaviour towards the other, and should be suffering from misdirected courtship, erroneous mate choice, heterospecific/heterotypic mating, sperm/oocyte wastage and hybridization.

Table 1.1. Mechanisms of reproductive isolation (reproductive barriers involved and definitions) and associated type(s) of reproductive interference arising when a given barrier is incomplete (while at least one of the subsequent barriers is present). Note that when a given reproductive barrier is incomplete, all previous barriers are also necessarily incomplete, thus all previous types of reproductive interference can (but do not necessarily) occur.

Reproductive barriers		Definition of reproductive barriers	Reproductive interference	Examples of incomplete reproductive barriers and resulting reproductive interference
Pre-mating	Habitat isolation	Potential mates occupy different niches and do not meet.	Signal jamming[1]	Females of the eastern gray treefrog, *Hyla versicolor*, prefer conspecific calls to those of the sympatric Cope's gray treefrog, *H. chrysoscelis*, when signal overlap is low, but change their preference to heterospecific calls when both calls fully overlap (Marshall et al. 2006).
	Temporal isolation	Potential mates live in the same place, but the timing of mating differs.		
	Behavioural isolation	Potential rivals or mates meet but choose not to compete or mate due to heterospecific recognition.	Heterospecific rivalry[2]	Males of the Eastern amberwing dragonfly, *Perithemis tenera*, mistake heterospecific individuals of the horse fly *Tabanus* spp. and of the buttherfly *Ancyloxypha numitor* for conspecific competitors due to similarities in body size, colour, and flight heigh, thereby wasting time and energy pursuing them (Schultz & Switzer 2001).
			Misdirected courtship and/or attempts to copulate	In their moth stage, male bollworm, *Heliothis zea,* are attracted to and attempt to copulate with females of the tobacco budworm, *H. virescens* even though heterospecific copulations are fatal due to genitalia incompatibilities (Stadelbacher et al. 1983).
	Mechanical isolation	Differences in shape and size of the genitalia prevent successful copulations (*i.e.,* genitalia incompatibility).	Heterospecific copulation	Males of the nematode *Caenorhabditis elegans* copulate with *C. remanei* females, but do not transfer any sperm (Hill & L'Hernault 2001).
Post-mating pre-zygotic	No sperm transfer	Individuals copulate but male gametes are not transferred.	Sperm wastage	In the fruit fly, *Drosophila simulans,* species complex, *D. simulans* males transfer sperm to *D. mauritiana* females, who store it but do not use it as it is rapidly lost from the female's reproductive tract (Price et al. 2001).
	Conspecific/homotypic sperm precedence	Male gametes are transferred but not used when conspecific/homotypic male gametes are also present (cryptic choice).	Oocyte wastage	When males of the nematode *Caenorhabditis nigoni* mate with *C. briggsae* females, their sperm displaces conspecific sperm from the spermatheca and invade the ovaries, thereby inducing premature oocyte maturation and ectopic fertilization ultimately leading to oocyte degradation (Ting et al. 2014).
	Gametic isolation	Male gametes are transferred, but fertilization fails.	Hybridization[3]	Despite strong gametic isolation between females of the sand field cricket, *Gryllus firmus,* and males of the fall field cricket, *G. pennsylvanicus,* from allopatric populations, a few eggs are fertilized (but they subsequently fail to develop due to complete post-zygotic isolation; Larson et al. 2012).
Post-zygotic	Zygote mortality	Hybrid zygotes die during embryogenesis.	Hybridization[3]	In a recent contact zone in central Poland, embryos resulting from crosses between herring (*Larus argentatus*) and Caspian (*Larus cachinnans*) gulls survive and develop until adulthood (but adult hybrid females subsequently have a shorter lifespan; Neubauer et al. 2014).
	Hybrid inviability	F1 (juvenile or adult) hybrids are inviable.	Hybridization[3]	In *Heliconius* butterflies from Central and Andean South America, the sympatric sister species, cydno longwing butterfly (*H. cydno*) and postman butterfly (*H. melpomene*) produce viable hybrids in the wild (but hybrid females resulting from one of the cross directions are completely sterile; Naisbit et al. 2002).
	Hybrid sterility	F1 hybrids survive but are sterile.	Hybridization[3]	In the haplodiploid parasitic wasps of the genus *Nasonia*, crosses between *N. longicornis* males and *N. vitripennis* females produce fertile hybrid females (but the offspring of these females suffer from increased mortality and sterility; Koevoets et al. 2012).
	Hybrid breakdown	Backcross or F2 hybrids are inviable or sterile.	Hybridization[3]	Some crosses between the genetically differentiated green and red forms of the haplodiploid spider mite *Tetranychus urticae* lead to the production of viable and fertile hybrids even after the F2 (but continuous hybridization over several generations leads to a decrease in female production over time; Sugasawa et al. 2002).

[1]The signal emitted by a species for mate acquisition (for example, chemical cues, visual displays or calling songs) is suppressed by the signal of heterospecifics.

[2]Heterospecifics are mistaken for conspecific individuals of the same sex.

[3]Even though hybridization is the result of any of these barriers being incomplete, the cost of hybridization should increase from fertilization failure (as it leads to gamete wastage) to hybrid breakdown (as unfit hybrids will require resource investment, at least from their mother, and will compete with pure individuals).

Understanding the relevance of partial reproductive isolation for conservation biology does not only require knowledge on the type of reproductive interference it may trigger, but also the resulting fitness costs for each of the populations/species involved. Such costs will mainly depend on the biology of the species with, for instance, extreme costs in the case of semelparous organisms (*i.e.,* that reproduce only once during their lifetime) or species with parental care in the case of hybridization (Gröning & Hochkirch 2008). For a given population/species pair, however, the fitness costs of negative sexual interactions should depend on the type of reproductive interference (hence what reproductive barriers are incomplete), with increasing costs from signal-jamming to hybridization (hence from pre-mating to post-zygotic barriers being incomplete; *cf.* Table 1.1):

The types of reproductive interference resulting from incomplete pre-mating isolation between already well-formed species (*i.e.,* that are fully post-zygotically isolated), such as signal-jamming, heterospecific rivalry or erroneous mate choice, usually entail low fitness costs for one or both species as they imply wasting less time and energy than with the other types of reproductive interference (see below; Gröning & Hochkirch 2008). Nevertheless, these types of reproductive interference may have important ecological consequences, such as for the ground-hopper *Tetrix ceperoi*, where males invest strongly in heterospecific mating attempts with *T. subulata* females, thereby reducing the net reproductive success of their own species (Hochkirch et al. 2007). Depending on the biology of the species involved, this may even have dramatic consequences ultimately driving the replacement of one species by another. For instance, in New Zealand, males of the only native praying mantis species, *Orthodera novaezealandiae*, are more attracted to females of the invasive species *Miomantis caffra* than to conspecific females. This results in unidirectional sexual cannibalism that is displacing the native species (Fea et al. 2013).

The types of reproductive interference resulting from incomplete pre-zygotic isolation (both pre- and post-mating) between divergent populations or closely-related species usually entail higher costs, as they additionally lead to a waste of gametes (Gröning & Hochkirch 2008). In orchids, for example, crosses between *Anacamptis laxiflora* and *Orchis anthropophora* produce seeds, indicating that there is fertilization of ovules by heterospecific male gametes (*i.e.,* incomplete post-mating pre-zygotic barriers). However, all gametes invested in such crosses are wasted (and parental fitness is reduced), as none of the produced seeds are viable (*i.e.,* complete hybrid embryonic mortality; Scopece et al. 2007).

Finally, the types of reproductive interference resulting from incomplete post-zygotic isolation between incipient species may be the ones entailing the highest costs. Indeed, the production of hybrids will always require additional resources, even when these are inviable, have a shorter lifespan and/or are sterile. For instance, in eastern Japan, crosses between females of the native freshwater minnow, *Pseudorasbora pumila*, and males of the invasive *P. parva* produce hybrids that are infertile (Konishi & Takata 2004). This wasteful production of maladaptive hybrids directly affects the population growth of the endemic species, which is being replaced by the invasive one (*i.e.,* demographic swamping; Todesco et al. 2016). Even when all post-zygotic barriers are incomplete (*i.e.,* hybrids are fertile), outbreeding depression caused by hybridization can seriously threaten vulnerable populations by disrupting locally adaptive gene complexes (Lynch & Walsh 1998; Ralls et al. 2007). For example, wild and farm escaped salmon, *Salmo salar*, often hybridize and the offspring resulting from crosses between F1 hybrids (hence F2 hybrids) have lower lifetime success than wild salmon (McGinnity et al. 2003). Alternatively, hybridization may also lead to outbreeding enhancement (*i.e.,* increased fitness), also called 'heterosis' or 'hybrid vigour' (Lynch & Walsh 1998). In such case, it is no longer costly for the individuals involved, but it may have similar disastrous consequences for the conservation of endangered populations/species, as one or both parental population(s) can be replaced by a hybrid population (*i.e.,* genetic swamping; Todesco et al. 2016). For example, in the Lesser Antilles islands the endemic iguana, *Iguana delicatissima*, and the invasive common green iguana, *I. iguana*, mate with one

another and produce viable and fertile hybrids, which have completely replaced the endemic species in some of the islands (Vuillaume et al. 2015).

As ecological and evolutionary processes are intimately linked (Pelletier et al. 2009), understanding the ecological consequences of partial reproductive isolation also requires a good understanding of its evolution (Gómez-Llano et al. 2021). Whereas exclusion or replacement of partially isolated populations can take place very quickly when reproductive interference is strong and/or the size of the populations is small, their persistence in sympatry for a sufficient amount of time may enable the evolution of their interactions (Kyogoku & Wheatcroft 2020). This may happen, for instance, when the strength of reproductive interference is low, when the population of the lower competitor persists via immigrations, or in the case of hybrid zones. In general, negative interactions should be counter-selected and/or select for traits that reduce their costs. In the case of reproductive interference, this could be achieved in two ways: (1) by increasing pre-zygotic reproductive barriers (an example of this process is 'reinforcement', whereby pre-zygotic isolation increases as a result of selection against hybridization; Sætre 2012); (2) by decreasing post-zygotic reproductive barriers. Whereas the former involves divergent reproductive character displacement in sympatry (Sætre et al. 1997), thus speciation progressing towards completion (*cf.* Figure 1.1b), the latter may result from gene flow between populations that are not fully post-zygotically isolated, and may ultimately lead to reverse speciation (Seehausen 2006; Figure 1.1b). Alternatively, partial reproductive isolation may be stable, which means that there is no net force driving pairs of populations to diverge or converge (Servedio & Hermisson 2020). This may arise due to different reasons, such as: i) hybridization may produce individuals with higher fitness than the parental populations/species but only in a certain context; ii) the choice between conspecifics and heterospecifics may be balanced due to influence of both ecological and sexual selection; iii) there may be decreasing benefits and increasing costs to the strengthening of preference towards conspecifics (Servedio & Hermisson 2020).

Understanding how and in which order different forms of isolation evolve remains challenging (Stankowski & Ravinet 2021). In sympatry, pre-zygotic isolation is generally thought to evolve before post-zygotic isolation due to reinforcement (Coyne & Orr 2004). For instance, Coyne & Orr (1997) performed a comparative study using 171 *Drosophila* species pairs and found that sympatric pairs showed much higher pre-zygotic isolation than post-zygotic isolation, while allopatric pairs showed no difference between the two types of isolation. Williams & Mendelson (2014) quantified the strength of different reproductive barriers between sympatric species of darter fish and found much stronger pre-zygotic isolation, driven by pre-mating barriers, than post-zygotic isolation. Furthermore, several studies in *Littoraria* snails and *Drosophila* flies identified different pre-zygotic barriers that are stronger in sympatry than in allopatry, such as assortative mating (Hollander et al. 2018), gametic isolation (Matute 2010a), or conspecific sperm precedence (Castillo & Moyle 2019). However, the hypothesis that pre-zygotic isolation is more likely to evolve than post-zygotic isolation in sympatry is still controversial. Indeed, a positive feedback loop between pre-zygotic and post-zygotic barriers is theoretically possible (Servedio & Sætre 2003), and both have similar rates of evolution in some plant genera (reviewed in Widmer et al. 2009). Moreover, in some cases, post-zygotic isolation may evolve before pre-zygotic isolation in sympatry. This was found, for instance, when comparing two closely related oak species, *Quercus mongolica* and *Q. liaotungensis*, in zones of recent and ancient secondary contact (*i.e.,* when allopatric species are brought together geographically; Liao et al. 2019). The same conclusion was also inferred from genetic data for two cryptic species pairs of Amazonian birds, *Xiphorhynchus elegans* and *X. spixii*, and *Willisornis poecilinotus* and *W. vidua*, in a hybrid zone (Pulido-Santacruz et al. 2018). Overall, not only is it still unclear whether pre- or post-zygotic barriers are more likely to evolve in sympatry, but there is also a lack of knowledge on which specific barriers are more prone to evolve.

This latter issue is particularly relevant since different reproductive barriers lead to different types of reproductive interference (*cf.* Table 1.1).

Laboratory experimental evolution is an exceptional research tool to shed light on this issue, as it allows manipulating ecological conditions over multiple generations to assess their effect on the evolution of reproductive isolation (*i.e.,* experimental speciation; White et al. 2020). Among the various organisms used in such experimental evolution studies (*cf.* Kawecki et al. 2012), spider mites of the genus *Tetranychus* (Acari: Tetranychidae) are of particular interest. Indeed, reproductive barriers are often incomplete both within and between different species of this genus (*e.g.,* Clemente et al. 2016; Cruz et al. 2021), which provides an excellent opportunity to study the evolution of reproductive barriers at different points in the speciation continuum (Kulmuni et al. 2020). Moreover, these phytophagous arthropods have a very short developmental time (10-16 days at 25ºC; Riahi et al. 2013), which allows performing experimental evolution in a relatively short amount of time, and are haplodiploid (*i.e.,* males develop from unfertilized eggs while females develop from fertilized eggs; Oku 2014), which facilitates investigations on the evolution of reproductive isolation as fertilization failure can be easily assessed by measuring sex ratios. Furthermore, these mites exhibit sexual dimorphism, (Figure 1.2), and their virginity can be ensured by isolating individuals just before they become adults, either as deutonymphs or teleiochrysalis (*i.e.,* during their last larval or quiescent stage, respectively; Figure 1.3). Finally, another important characteristic of this system is that females mated with conspecific/homotypic males exhibit first-male sperm precedence, whereby only the sperm of the first male is used to fertilize all the eggs (Rodrigues et al. 2020). Thus, if this pattern is maintained when a female mates with a male from a strongly divergent population or semi-isolated species, the costs of reproductive interference should be particularly high, and should constitute a strong selective pressure for the evolution of reproductive traits.

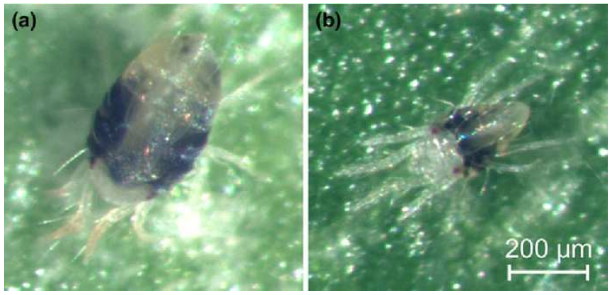

Figure 1.2. Sexual dimorphism in the spider mite *Tetranychus urticae*. (A) Adult female, (B) Adult male. Females are larger and have a rounder body shape than males which have a pointier abdomen. Photos adapted from Oku 2014.

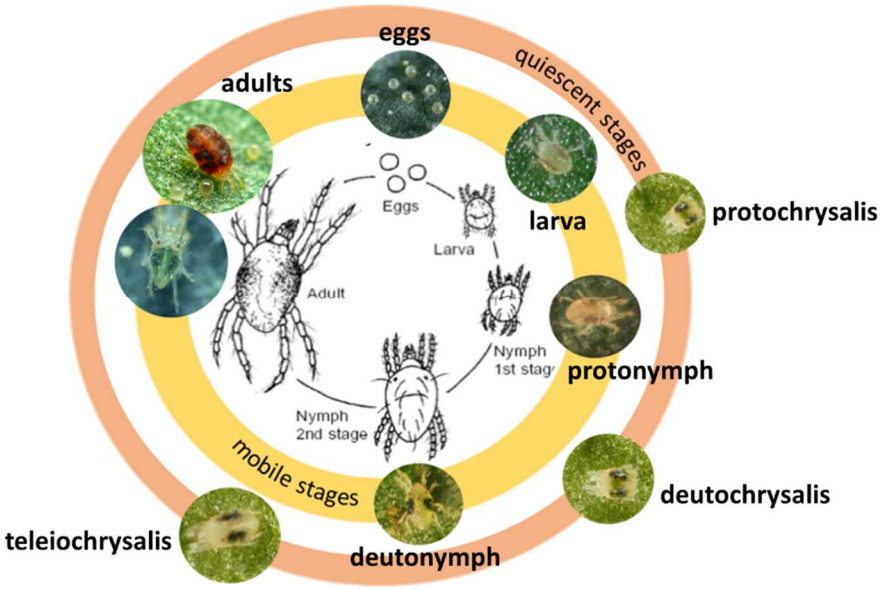

Figure 1.3. Life cycle of spider mites, depicting the 5 developmental stages from egg to adult. Between each mobile larval stage, individuals undergo a quiescent period, during which they are immobile and do not feed. Sexual dimorphism is visible starting from the third larval stage (deutonymph). Figure adapted from Rodrigues 2017.

In this project, I used a pair of populations from genetically differentiated colour forms of the spider mite species *Tetranychus urticae*: the green and red forms (Figure 1.4). These two forms have been described as separate species in the past (*T. urticae* and *T. cinnabarinus*, respectively) due to complete reproductive isolation among some populations (*e.g.,* Smith 1975). However, as other populations produce viable hybrids over multiple generations (*e.g.,* Gotoh & Tokioka 1996; Sugasawa et al. 2002), and both morphological and molecular data suggest synonymity, their taxonomic status has been revisited (Auger et al. 2013). Previous experiments performed in my host laboratory with the two populations used in this project showed that heterotypic crosses result in fully sterile hybrids (most hybrid females do not lay eggs and the few eggs laid do not hatch; Cruz et al. 2021), but that males of both colour forms prefer to mate with red females (*i.e.,* red males mate assortatively, while green males mate disassortatively; Cruz 2017). Moreover, preliminary results show that such incomplete pre-zygotic isolation coupled with strong post-zygotic isolation leads to strong reproductive interference when these two populations share the same environment (Cruz et al. unpublished). Therefore, to assess what reproductive barriers are more likely to evolve under the selection pressure caused by reproductive interference, these populations were allowed to evolve together (sympatry) or alone (allopatry) for up to 69 generations of experimental evolution. Subsequently, I performed three different experiments to measure the strength of all possible reproductive barriers occurring between these populations, from behavioural isolation to hybrid breakdown (*cf.* Table 1.1).

Figure 1.4. Body colour variation in *T. urticae* females. (A) Green form, (B) Red form, (C) F1 hybrids. Photos adapted from Auger et al. 2013.

2. Materials and methods

2.1 Spider mite populations

The spider mite populations were established in the laboratory by collecting green and red forms of *T. urticae* in central Portugal in May 2010 and November 2013, respectively. For the green-form population, 300 adult females were collected from tomato plants (*Solanum lycopersicum*) in Carregado, whereas the red-form population was founded by collecting 65 adult females from thorn apple plants (*Datura stramonium*) in Aldeia da Mata Pequena. Both were subsequently reared in the laboratory under the same conditions (*ca*. 500-1000 females; 24 ± 2ºC, 16/8h L/D) in mite-proof cages containing bean plants (*Phaseolus vulgaris*, cv. Contender seedlings obtained from Germisem, Oliveira do Hospital, Portugal). As both field populations were infected with *Wolbachia*, an endosymbiont capable of manipulating its host's reproduction (Werren et al. 2008), subsets of the green (*ca*. 270 adult females) and red (*ca*. 300 adult females) form populations were treated with rifampicin in March and May 2018, respectively, to obtain *Wolbachia*-free populations as described in Cruz et al. (2021). Briefly, adult females were installed in petri dishes containing bean leaf fragments placed on cotton soaked in a rifampicin solution (0.05%, w/v) for one generation (Gotoh et al. 2005; Zélé et al. 2020), and the absence of *Wolbachia* was confirmed by PCR diagnostic three generations later as described in Zélé et al. (2018). The treated populations were then reared under the standard conditions described above.

2.2 Experimental evolution

Three selection regimes were created in December 2018 using the *Wolbachia*-free green- and red-form populations described above, each with five independent experimental evolution replicates (Figure 2.1): (a) an allopatric regime consisting of green-form individuals only, (b) an allopatric regime consisting of red-form individuals only and (c) a sympatric regime consisting of green- and red-form individuals maintained together. For the allopatric regimes, each replicate population was created by transferring 200 young adult mated females from the *Wolbachia*-free populations to plastic boxes containing two bean plants (14 days old). Subsequently, each replicate was maintained by transferring 200 adult female offspring to new boxes with two fresh plants every two weeks (one discrete generation), and a fresh bean plant was added to each experimental box 7 days later to avoid resource depletion. A similar procedure was used for the replicate populations of the sympatric regime, except that a total of 400 young adult mated females, 200 of each colour form, were initially transferred to plastic boxes containing four bean plants (and two fresh bean plants were added 7 days after every transfer). This procedure ('Protocol 1') was used for the 16 first transfers, but was subsequently modified during the course of experimental evolution for the reasons explained below. For the entire duration of experimental evolution, all boxes were kept in a growth chamber with standard conditions (24 ± 2ºC, 60% RH, 16/8h L/D).

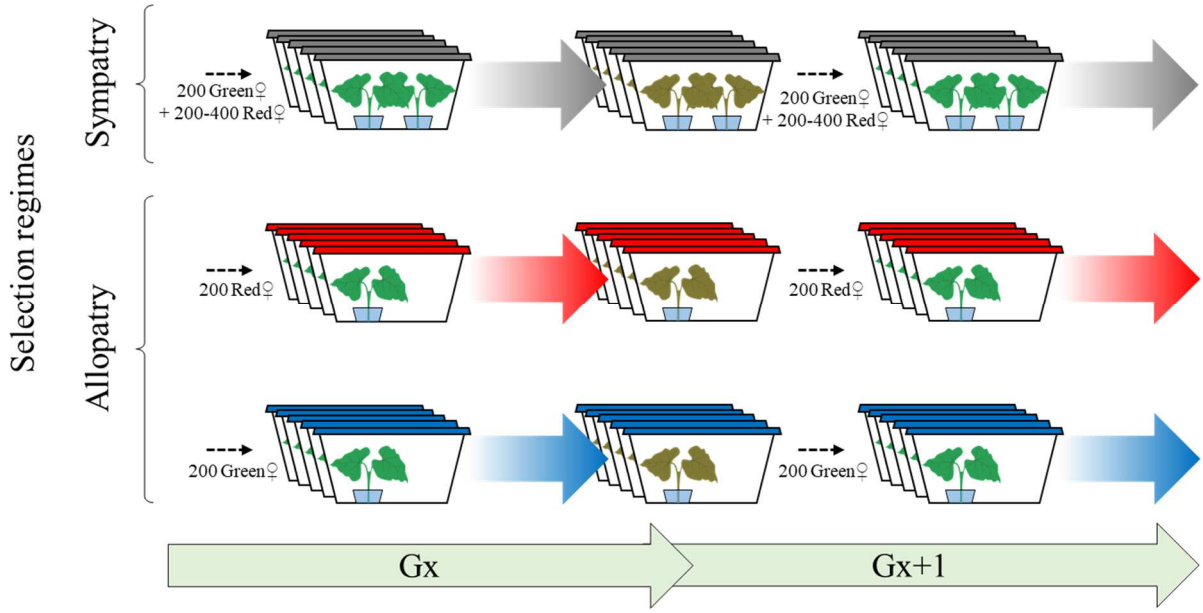

Figure 2.1. Selection regimes created with the two colour forms of *T. urticae*. In the sympatric regime (grey fill), the green- and red-form populations evolved together (coevolution), whereas populations of each form (blue and red fills, respectively) evolved separately in the allopatric regimes. Dashed arrows indicate the transfer of mated females (number indicated below) to each new box at each generation. For the sympatric regime, the number of red females transferred varied over the course of experimental evolution. Full arrows indicate offspring production and development. The entire procedure was repeated in 5 independent replicates.

2.2.1 Modifications of the experimental procedure during the course of experimental evolution

During the course of experimental evolution, it became increasingly difficult to maintain the red-form populations in the sympatric regime for two main possible reasons: (1) the green-form populations might be excluding the red-form populations through reproductive interference and/or resource competition, and (2) a proportion of the red females transferred at each generation could have been 'hybrids' (*i.e.,* females produced from heterotypic crosses) instead of 'pure' females (*i.e.,* females produced from homotypic crosses) due to their similarity in body colouration (*cf.* Figure 1.4). Therefore, to prevent the loss of replicates, missing red-form females were occasionally transferred from replicates of the allopatric regime (*e.g.,* females from replicate 1 of the red form allopatric regime were transferred into replicate 1 of the sympatric regime). However, this solution was problematic as it creates gene flow from the allopatric into the sympatric populations, thereby leading to non-complete independence of the selection regimes and reducing the effective number of generations of selection in the sympatric regime (see next section below).

As a first attempt to avoid this issue, the experimental evolution procedure for the sympatric regime was modified in July 2019 (after 17 transfers): 400 red females (instead of 200) were transferred per replicate population, along with the usual 200 green females to give an advantage to red-form females and/or compensate for the potential presence of hybrids in the transferred females ('Protocol 2'). However, this solution was not sufficient as red-form females were still missing and had to be transferred from the allopatric regime. As a result, one replicate was lost in December 2020 (after 53 transfers; *cf.* Figure 2.2).

As a second attempt to completely solve this issue, I implemented an additional modification of the experimental evolution procedure in January 2021 (after 55 transfers). This procedure ('Protocol 3'), adapted from Godinho et al. (2020a), consisted in the creation of a backup population for each replicate of the sympatric regime at each transfer. Briefly, in addition to the 400 red females that were transfered

along with 200 green females, an additional 200-400 red females were transfered to new boxes in abscence of green females (*i.e.,* relaxed selection) as a 'backup population'. If not enough red females could be found in the sympatric replicate populations at the next transfer, the missing ones were taken from the backup population. This last modification of the procedure finally allowed successful maintenance of the sympatric regime without any further gene flow from the allopatric regime.

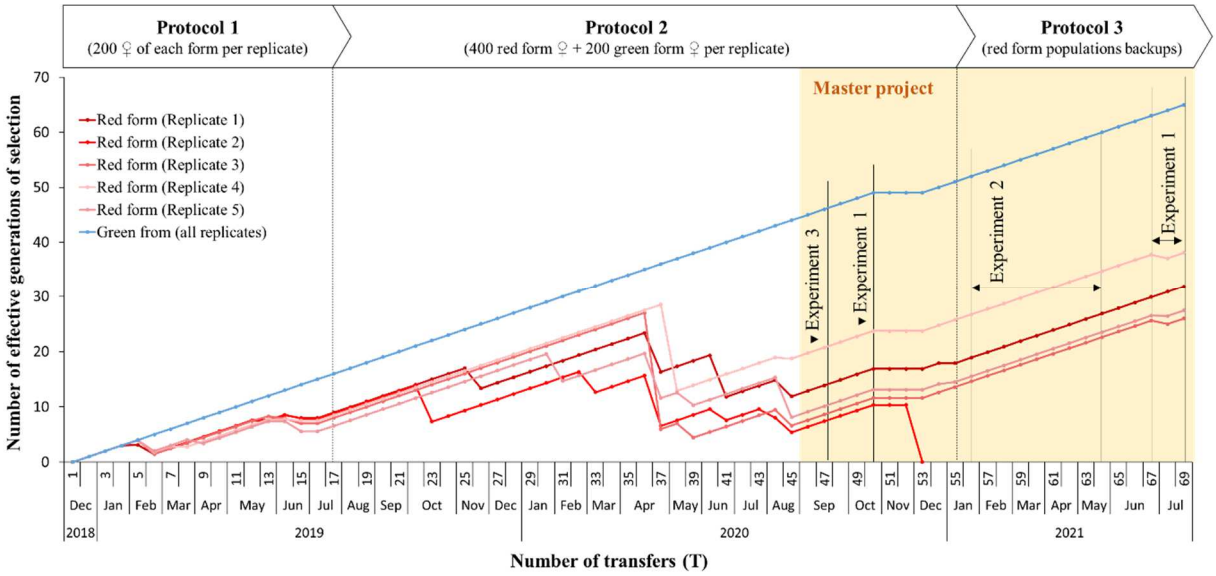

Figure 2.2. Number of effective generations of selection in the sympatric regime at each experimental evolution transfer (T) and events occurring prior and throughout my master's project. The number of effective generations of selection is consistent across population replicates of the green form (blue line) but varies among population replicates of the red form (red lines). From T4 to T44, drops in the number of generations of selection in the red-form population replicates were due to red females being transferred from the allopatric regime, and plateaus were due to relaxed selection (*i.e.,* when red-form populations temporarily evolved in absence of green-form individuals). Changes in the procedure occurred at T17 and T55 (the different procedures are indicated above the graph). Individuals used in the three experiments of this project were the offspring of females collected after different number of transfers: T47 to assess hybrid fertility and hybrid breakdown (Experiment 3); T50 and T67 to T69 to assess mate choice (Experiment 1); T56 to T64 to assess sperm precedence and hybridization (Experiment 2). My master's project lasted from T46 to T69 (shaded background).

2.2.2 Estimating the effective number of generations of selection

Because different replicate populations of the sympatric regime were replenished with variable numbers of red females from the replicate populations of the allopatric regime, or from the backup populations (before and after implementing the new procedure described above, respectively), the number of effective generations of selection in this regime (estimated according to Godinho et al. 2020a) does not always correspond to the number of transfers performed (Figure 2.2). Importantly, this number differs between (i) the sympatric and allopatric regimes, (ii) between the green- and red-form populations of the sympatric regime, and (iii) among red-from population replicates of the sympatric regime. For instance, after 47 transfers, the number of effective generations of selection was 47 in allopatric populations, 46 in the green-form populations of the sympatric regime, and between 7 to 21 in the red-form populations of the sympatric regime depending on the experimental evolution replicate. Being aware of this variability, I will refer to the number of experimental evolution transfers (T) for simplicity hereafter.

2.3 Experiments

A total of three experiments were conducted in this project to test for the evolution of different reproductive barriers (Table 2.1). The first experiment assessed pre-mating barriers (mate choice and genitalia incompatibilities); the second experiment assessed post-mating pre-zygotic barriers (barriers to sperm transfer/storage, gametic incompatibilities and homotypic sperm precedence) and early post-zygotic barriers (zygote mortality and hybrid inviability); the third experiment assessed late post-zygotic barriers (hybrid sterility and hybrid breakdown). All experiments described below were conducted in a growth chamber under standard conditions (24 ± 2ºC, 60% RH, 16/8h L/D).

Table 2.1. Type of reproductive barriers, tested traits, and corresponding measurements performed in each experiment (Exp.).

	Type of barriers	Tested traits	Measurements
Exp. 1	Pre-mating	Mate choice	Proportion of females of each colour form chosen by males
		Genitalia incompatibilities	Copulation latency[2]
			Copulation duration[2]
Exp. 2	Post-mating pre-zygotic	Fertilization failure	Proportion of F1 male/female offspring (*i.e.,* unfertilized/fertilized eggs)
		Sperm precedence	Proportion of F1 daughters sired by a first and a second male that mated with a female
	Post-zygotic	F1 Zygote mortality	Proportion of F1 unhatched eggs
		F1 Juvenile mortality	Proportion of dead F1 juveniles.
Exp. 3	Post-zygotic	F1 Fertility/Sterility	Proportion of ovipositing F1 females
			Number of eggs laid daily by F1 females
		F2 Zygote mortality[1]	Proportion of F2 unhatched eggs

[1] hybrid breakdown.

[2] note that copulation latency and duration may also be affected by mate choice.

2.4 Common gardens prior to experiments

Prior to each experiment, a common garden was created for green and red females of each selection regime and replicate population. Indeed, common gardens for two to three generations allowed breeding individuals under the same conditions, thus eliminating the influence of environmental/maternal effects on the traits tested, prior to performing experiments (Huxman et al. 2022). In this project, it also ensured that none of the red females from the sympatric selection regime populations used in the experiments were F1 hybrids, and that the males were of the correct colour form. For experiments performed before the 55th transfer, common gardens were created by collecting 100 mated females from each experimental evolution population replicate (but 100 green and 200 red females separately in the case of the sympatric regime), and by placing them into a new plastic box containing two fresh bean plants. One fresh plant was added to each box 7-9 days later to avoid resource depletion, and mated female offspring were collected from each box 10-16 days after the establishment of the common gardens to create the experimental age-cohorts (*cf.* each experiment below). For experiments performed after the 55th transfer, the common garden procedure was substantially the same except that 200 mated females (instead of 100) were used for each common garden. This allowed using directly the allopatric regime populations from the experimental evolution, and the backup populations with 200-400 red mated females from the sympatric regime (*cf.* above), as common gardens. Only additional boxes with 200 mated green females

from each sympatric regime population and two fresh bean plants were created to obtain the missing common gardens. As the population size in these common gardens was twice as big as before, one or two fresh plants were added to each box 9 and 14 days later, while the older plants were removed if necessary. An additional fresh plant was added again 19 days and 22 days (when necessary) after the onset of the common gardens. Mated female offspring were collected from each of these common gardens to create the experimental age-cohorts 15 to 23 days after the establishment of the common gardens.

2.5 Experiment 1: Mate preference

To test how sympatric evolution affects pre-mating barriers between the green- and red-form populations, mate choice tests were conducted at T50 and T67-69 (*cf.* Figure 2.2 for effective generation of selection). In this experiment, a choice refers to the male inserting his genitalia inside the female. Four different treatments were established: green- and red-form males from the allopatric and sympatric regimes could choose between a green- and a red-form female from their own regime. Only the mate preference of males was tested here because, in spider mites, males usually guard quiescent deutonymph females (teleiochrysalis) and mate with them as soon as they emerge as adults, preventing females from refusing these matings (Oku 2014). Therefore, opportunities for females to choose a partner, as well as the window for selection to act on this trait, should be low in this system. In line with this, a previous experimental evolution study showed that female mate preference did not evolve in *T. urticae* to avoid *Wolbachia*-induced incompatible matings (Rodrigues et al 2022), and no preference for males of either form was found for females from the green- and red-form populations before evolution (Cruz 2017).

For the tests performed at T50, age-cohorts were created 11-12 days before the mating observations. This was done by transferring *ca*. 50 mated females from the common garden of each replicate of each selection regime onto a bean leaf placed on water-soaked cotton. In the case of red-form females from the sympatric regime, an additional 30 mated females were transferred from each replicate to compensate for the risk of transferring hybrid females. Females were allowed to lay eggs for two days and then they were transferred to a new fresh leaf to lay eggs for another two days before being discarded. Five to seven days later, quiescent females and males were isolated from each age-cohort and kept separately on 2.5 cm^2 leaf discs to ensure their virginity. The next day, quiescent individuals became virgin adults roughly of the same age. Mate choice tests were then organized into different sessions of observation. Before each session, the two possible females of each treatment were installed on a 0.5 cm^2 leaf disc (hereafter called 'arena'), and the test began as soon as a male was added to the arena. Each test lasted for 30 minutes and latency to copulation (time in seconds that males took to start copulation) and copulation duration (time in seconds males spent copulating), were recorded with a stopwatch ("Stopwatch: StopNow"), and the colour of the female copulating with the male (red or green) was noted. Two arenas per experimental evolution replicate (N=4 replicates) and per treatment were observed within each session (hence 32 arenas per session). A total of 8 sessions of observations (1 per day) were performed within 2 experimental blocks (4 sessions per block, with blocks corresponding to two independent age-cohorts created six days apart). Hence, a total of 256 arenas were observed at T50. Arenas where no matings occurred for 30 minutes were excluded from the statistical analyses.

The tests performed at T67-69 followed the same procedure with some minor differences: (1) age-cohorts with red-form females from the sympatric selection regime were created using 100 mated females per experimental evolution replicate (instead of 80 females); (2) these females could lay eggs for 3 days on each bean leaf (instead of 2 days); (3) quiescent females and males were isolated during 4 to 6 days (instead of 5 to 7 days) after their mothers were discarded; (4) mate choice tests took place 2 days after isolating the quiescent individuals (instead of 1 day), (5) 15 sessions of observations were

performed within 5 experimental blocks (3 sessions per block), with blocks (*i.e.,* independent age-cohorts) created 7 days apart (instead of 6 days apart), and (6) 3 arenas per replicate per treatment were observed in each session (hence 48 arenas per session, for a total of 720 arenas). Finally, these tests were performed by Bastien Aubry under my supervision, as part of an internship taking place in the host laboratory.

2.6 Experiment 2: Sperm precedence and hybridization

To test how sympatric evolution affects post-mating pre-zygotic and early post-zygotic barriers between the green- and red-form populations, I measured the production of viable hybrids, and assessed how it is affected by sperm precedence by performing a double mating experiment at T56-64 (*cf.* Figure 2.2 for effective generation of selection). Only green-form females were tested in this experiment for two main reasons. First, when red-form females mate twice, pure and hybrid female offspring (resulting from homotypic or heterotypic fertilization, respectively) cannot be easily distinguished solely based on their colours, whereas such distinction is easy to make in the case of green-form females (*cf.* Figure 1.4). Testing red-form females would thus require the use of genetic markers (expensive and time consuming). Second, it is less likely to detect an evolutionary response in red-form females from the sympatric regime, as they had undergone considerably fewer effective generations of selection than green-form females from the same regime (*cf.* Figure 2.2). Eight treatments were thus established in this experiment, corresponding to green-form females from the allopatric and sympatric regimes mating with a green- or a red-form male from their own regime, and subsequently remaining single mated ('single mated females' hereafter) or mating with a second male of the same regime but of the opposite colour form ('double mated females' hereafter; Table 2.2). Single mated females allowed detecting potential problems of sperm transfer and storage, and/or fertilization failure (*i.e.,* gametic incompatibilities), as well as hybrid inviability. Double mated females allowed testing whether the pattern of sperm precedence changed from first male sperm precedence to preferential use of sperm from a male of their own form (*i.e.,* homotypic sperm precedence).

Table 2.2. Treatments performed using green-form females in Experiment 2, to test for hybridization and hybrid inviability (single mated females), and for sperm precedence (double mated females). "-" indicates that no second mating was performed.

Selection regime	1st Male	2nd Male
Allopatry	Green	-
	Red	-
	Green	Red
	Red	Green
Sympatry	Green	-
	Red	-
	Green	Red
	Red	Green

Age-cohorts were created to obtain the individuals used in this experiment 11-12 days before the matings took place. To obtain both females and males of the green form, *ca.* 50 green-form mated females were transferred from the common garden of each replicate of each selection regime onto a fresh bean leaf placed on water-soaked cotton. To obtain red-form males, *ca.* 100 red-form mated females from each replicate of each selection regime were transferred onto a bean leaf under the same conditions. All females were allowed to lay eggs for two days, then transferred to a new fresh leaf to lay

eggs for another two days, then were discarded. After five to six days, quiescent females and males were taken from each age-cohort and kept separately on 4.9 cm^2 leaf discs to ensure their virginity.

Mating observation sessions took place two days later. For the 'first mating event' (*i.e.,* virgin females mating with a first male), 5 males were placed with 5 virgin females on 0.4 cm^2 leaf discs (the 'arenas'). Mating observations began as soon as a male was installed with the 5 females and lasted for a maximum of 1 hour. Immediately after a mating occurred during this period, the mated female was transferred to another 0.4 cm^2 disc (together with previously mated females) and the male was killed. Then, both were readily replaced by new virgin individuals in the original patch. At the end of the 1-hour mating observations, the females that did not mate were discarded, and half of those that mated was transferred altogether to an empty 0.8 cm^2 leaf disc. The remaining mated females were left on the 0.4 cm^2 leaf disc, which became the arena of the 'second mating event' (*i.e.,* females mating with a second male). In this case, the procedure was the same as in the first mating event except for a few details: (1) the number of males installed per arena was not necessarily 5, but depended on the number of mated females obtained from the first mating event (between 1 and 7) so the sex-ratio remained 50/50; (2) immediately after a mating occurred, all females were transferred to a 0.8 cm^2 leaf disc (instead of 0.4 cm^2). As before, females that did not mate after 1 hour were discarded.

One day after the mating observations, single and double mated females were individually isolated on 2.5 cm^2 leaf discs, where they laid eggs for 4 days and their daily survival was registered. On the fourth day, all females were removed and the eggs were counted. The egg hatching rate was assessed four days later by counting the number of unhatched eggs and juveniles. Finally, the number of F1 adult offspring (sons, green and hybrid daughters) and dead juveniles were counted both six and eight days later. These measures were used to compute different variables, each allowing to assess a different reproductive barrier:

- For single mated females: (1) The offspring sex-ratio (*i.e.,* number of adult daughters *vs.* adult sons) was used to assess fertilization failure potentially caused by inefficient sperm transfer/storage and/or gametic incompatibilities. Indeed, Cruz et al. (2021) previously showed that crosses between green females and red males lead to a decrease in the production of F1 females and a corresponding increase in the production of F1 males whose fitness did not differ from that of pure males. This led the authors to conclude that the observed 'male conversion' was unlikely due to paternal genome elimination following eggs fertilization (*i.e.,* pseudoarrhenotoky), but rather due to fertilization failure, as males are naturally produced from unfertilized eggs in spider mites (*i.e.,* true arrhenotoky; Helle & Bolland 1967). (2) The egg hatching rate (*i.e.,* number of hatched *vs.* unhatched eggs) was used to determine F1 hybrid zygote mortality. (3) The juvenile mortality (*i.e.,* number of dead juveniles *vs.* adult offspring) was used to assess F1 hybrid inviability.
- For double mated females: The paternity rate of each of the two males (*i.e.,* the proportion of offspring sired by each, assessed as the number of pure green *vs.* hybrid daughters) was used to detect changes in the sperm precedence pattern (possibly giving rise to homotypic sperm precedence in sympatry).

In this experiment, a total of 28 sessions of observations were performed within 7 experimental blocks (4 sessions per block), each block starting with the creation of an age-cohort and ending with the count of adult offspring. Blocks were performed 7 to 49 days apart (depending on the blocks), hence the entire experiment used mites from T56 to T64 of the experimental evolution. Within a given block, each of the 4 sessions of observations allowed testing a different experimental evolution replicate (all treatments being performed simultaneously), and two sessions were performed per day (all experimental evolution replicates were thus tested within two days). As not all females mated during the first and second mating events, the number of (single or double) mated females obtained per treatment per

experimental evolution replicate varied between 0 and 7 within each block, and between 17 and 33 for the entire experiment (for a grand total of 860 females).

2.7 Experiment 3: Hybrid fertility and hybrid breakdown

To test how sympatric evolution affects late post-zygotic barriers between the green- and red-form populations, the fitness of viable hybrids was assessed at T47 (*cf.* Figure 2.2 for effective generation of selection). In this experiment, 8 treatments were established, each corresponding to a different type of F1 female produced from the allopatric or sympatric regime: pure green form, pure red form, and hybrid resulting from crosses between red-form females and green-form males or the reverse.

2.7.1 Production of the F1 females tested

To obtain all the F1 females that were tested in this experiment, mass crosses ('F0 crosses') within and between the green- and red-form populations from the allopatric and sympatric selection regimes were performed. To this aim, age-cohorts were first created by transferring *ca.* 50 mated females from the common garden of each replicate of each selection regime onto a bean leaf placed on water-soaked cotton to lay eggs for three days before being discarded. Seven days later, 20 quiescent females and 20 adult males, of the same or a different colour form depending on the treatment, were installed together on 8 cm^2 leaf discs (except for crosses between green-form females and red-form males, for which 40 females and 40 males were used to increase the number of hybrid offspring obtained as these crosses result in a strong underproduction of hybrid females; Cruz et al. 2021). Individuals were allowed to mate and females to lay eggs for four days before being killed. Seven days later, deutonymph and/or quiescent daughters (hence F1 females) were transferred to 1.8 cm^2 leaf discs where they remained for two days to complete their last moult and emerge as adult virgins.

2.7.2 Hybrid fertility and hybrid breakdown

The young F1 virgin females obtained from the F0 crosses were individually installed on 2.5 cm^2 leaf discs to lay eggs, and their daily survival was registered. Three days later the number of eggs laid by each female was counted, and the pure green- and red-form females were killed. Hybrid females were killed two days later to circumvent the possibility that they may take more time to lay eggs (*i.e.,* possible fitness cost of hybridization), which could dramatically decrease the number of replicates to assess hybrid breakdown (*cf.* below). Still, the number of eggs laid by these females was also counted at day 3 to allow comparisons with pure females. Finally, the number of unhatched eggs and juveniles (dead or alive) was counted five to seven days after killing the females. The proportion of ovipositing F1 females (*i.e.,* females that laid at least one egg) and the number of eggs laid daily by ovipositing females (*i.e.,* total number of eggs at day 3 / number of days the female was alive) were used to assess hybrid fertility, while the hatching rate of F2 eggs (number of hatched eggs *versus* number of unhatched eggs) was used as a measure of hybrid breakdown.

The entire experiment was executed within 2 experimental blocks performed one day apart, each block starting with the creation of an age-cohort and ending with the measure of hatching rate. Within each block, all treatments were assessed simultaneously and, for each treatment, 12 F1 females were tested per experimental evolution replicate (N=5 replicates in this experiment), for a total of 60 F1 females per treatment (hence, 960 F1 females were tested in the entire experiment).

2.8 Statistical analyses

All statistical analyses were carried out using the R statistical package (v. 4.0.5; R Core Team 2021). The same general procedure was used to analyse the data from all experiments: a maximal model including all fixed effects and their interactions was built for each response variable. The type of model and error family used for each analysis was chosen based on error distribution, and when several models fit the data, the best model was selected based on the Akaike information criterion (AIC). Minimal models were then established by sequentially removing non-significant variables from the maximal models, and the significance of the explanatory variables was established using chi-squared tests (Crawley 2007) with the Anova function (car package; Fox & Weisberg 2019). The significant chi-squared values reported are for the minimal model while the non-significant values correspond to those obtained before removing the variable from the model. In Experiment 1, when the transfer from which originated the individuals tested (T50 or T67-67) was found to be significant, either alone or in an interaction, separate analyses were performed for each transfer. In all experiments, whenever fixed effects (alone or in interactions) were found to be significant, pairwise comparisons between the factor levels of interest were performed using t- or z-tests (with the functions 'emmeans' and 'contrast', emmeans package; Lenth 2021). Whenever a given set of data was used in more than one comparison, the p-values of the pairwise comparisons were corrected with the Bonferroni method.

2.8.1 Experiment 1: Mate preference

Mate preference data were computed as a binary response variable and analysed using a generalized linear mixed effect model with a binomial error distribution (glmmTMB, glmmTMB package; Brooks et al. 2017). Latency to copulation and copulation duration were both analysed using a cox proportional hazard mixed-effect model (coxme, coxme package; Therneau 2020), a non-parametric method to analyse time-to-event data (Crawley 2007). The selection regime (allopatry or sympatry), the colour form of the male and of the chosen female (only for latency to copulation and copulation duration), the transfer from which originated the individuals tested in the experiment (T50 or T67-69), as well as all interactions between these factors were fit as fixed explanatory variables, whereas the day at which each observation session took place and the experimental evolution replicate were fit as random explanatory variables.

2.8.2 Experiment 2: Sperm precedence and hybridization

All proportion data analysed in this experiment were computed using the function cbind (*e.g.,* number of adult females *versus* number of adult males for sex-ratio) and analysed using a generalized linear mixed effect model (glmmTMB, glmmTMB package; Brooks et al. 2017). A binomial error distribution was used for the analysis of juvenile mortality, whereas a beta-binomial error distribution was used to account for overdispersion (testDispersion, DHARMa package; Hartig 2021) in the analyses of hatching rate, sex-ratio and paternity rate. The type of F0 cross (*i.e.,* homotypic or heterotypic crosses for single mated females, and first male green and second male red or the reverse for double mated females), the selection regime (allopatry or sympatry), and their interaction were fit as fixed explanatory variables, whereas the experimental evolution replicate, nested within experimental block, was fit as a random effect variable.

2.8.3 Experiment 3: Hybrid fertility and hybrid breakdown

All response variables were analysed using a generalized linear mixed effect model (glmmTMB, glmmTMB package; Brooks et al. 2017). The proportion of F1 ovipositing females was computed as a binary response variable and analysed using a binomial error distribution. The daily fecundity of these females, a continuous variable limited to positive values, was analysed using a normal error distribution and a log link function. The hatching rate of F2 eggs was computed using the function cbind and analysed using a beta-binomial error distribution due to overdispersion (testDispersion, DHARMa package; Hartig 2021). The type of F1 female (pure green form, pure red form, hybrid produced by a green-form mother, or hybrid produced by a red-form mother), the selection regime (allopatry or sympatry) and their interaction were fit as fixed explanatory variables, whereas the experimental evolution replicate, nested within experimental block, was fit as a random explanatory variable. Because pure and hybrid F1 females strongly differed in all traits tested in this experiment, they were analysed separately (*i.e.,* two data subsets were created) whenever a significant interaction was found between the type of F1 female and the selection regime.

3. Results

3.1 Experiment 1: Mate preference

In this experiment, most of the males mated with red (74 ± 2%) rather than green-form females (26 ± 2%; Figure 3.1), and this choice was independent of the males' colour form, and consistent across selection regimes and transfers (*i.e.,* the male form, as well as all interactions involving this factor were non-significant; *cf.* Table 3.1). Moreover, although the effect of the selection regime on male mate choice changed between transfers (X^2_1=4.07, p=0.04), as sympatric males of both colour forms seemed to decrease their preference for red females through time (Figure 3.1a *vs* 3.1b), analyses of each transfer dataset revealed no differences in the mate choice of sympatric and allopatric males both at T50 and T67-69 (Table 3.1). Hence, regardless of the selection regime or the colour form of males, they did not change their mating preference throughout experimental evolution.

Table 3.1. Statistical results obtained for the tested explanatory variables and their interactions on mate choice. The analysis was first performed on the complete dataset (incl. all transfers). Since the interaction between the transfer and the selection regime was found significant, separate analyses were then performed for the T50 and T67-69 datasets. Explanatory variables included in the minimal model are displayed in blue, and significant chi-squared values are in bold.

Datasets	Explanatory variables	Df	Chi-square	p-value
All data	Male form : Selection regime : Transfer	1	0.28	0.60
	Male form : Transfer	1	0.29	0.59
	Male form : Selection regime	1	3.31	0.07
	Male form	1	1.67	0.20
	Selection regime : Transfer	**1**	**4.07**	**0.04**
	Selection regime	1	3.38	0.07
	Transfer	1	0.11	0.74
T50	Selection regime	1	3.39	0.07
T67-69	Selection regime	1	0.70	0.40

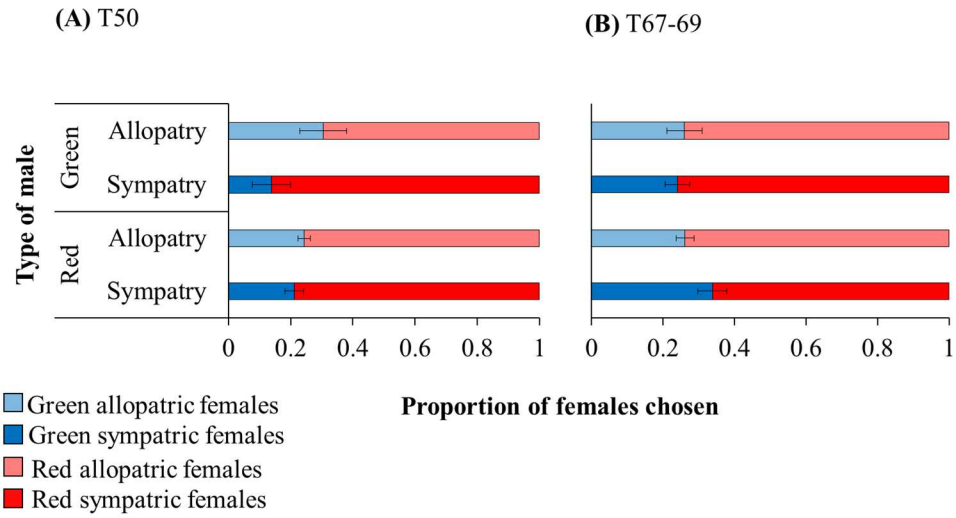

Figure 3.1. Proportion of females chosen by males depending on their colour form and selection regime after (A) 50 transfers and (B) 67-69 transfers. Bars represent means ± standard errors of the different experimental evolution replicates. Lighter and darker colour tones represent individuals from the allopatric and the sympatric regime, respectively. Blue: green-form females; Red: red-form females.

Overall, the latency to copulation measured for males changed through generations depending on their selection regime, their colour form, and the colour form of the female they chose (*i.e.,* significant four-way interaction; *cf.* Table 3.2). However, separate analyses for each transfer revealed that both green and red males from both selection regimes took the same amount of time to initiate a mating with both types of females at T50 (Table 3.2 and Figure 3.2a). Similarly, although the latency to copulation of males at T67-69 varied depending on their selection regime, their colour form, and the colour form of the female they chose (*i.e.,* significant three-way interaction; *cf.* Table 3.2), pairwise comparisons revealed that all males, independently of their selection regime and colour form, took the same amount of time to initiate a mating with both types of females (Table 3.3 and Figure 3.2b).

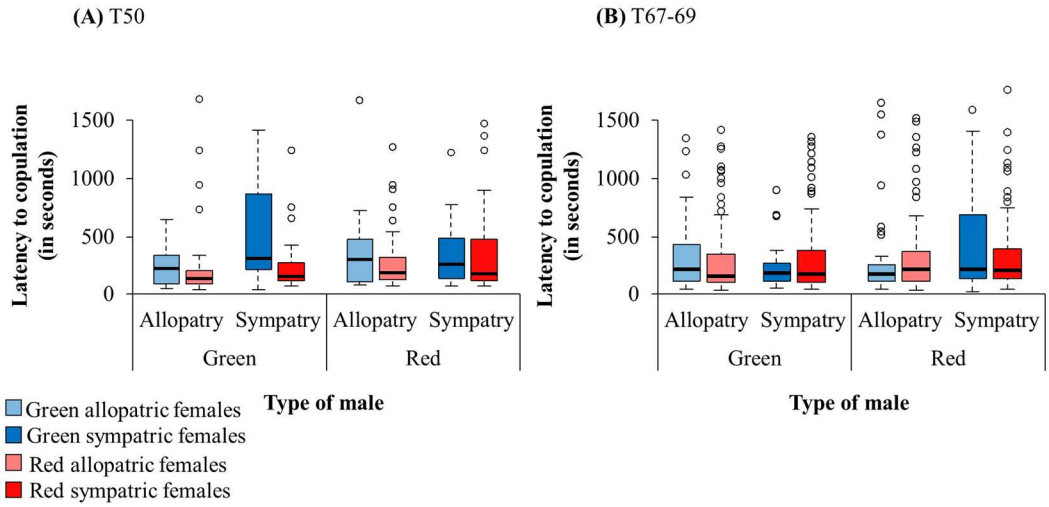

Figure 3.2. Boxplots of the latency to copulation of the different tested males when choosing to mate with either green or red females after (A) 50 transfers and (B) 67-69 transfers of experimental evolution. Lighter and darker colour tones represent individuals from the allopatric and the sympatric regime, respectively. Blue: green-form females; Red: red-form females.

Table 3.2. Statistical results obtained for the tested explanatory variables and their interactions on latency to copulation. The analysis was first performed on the dataset with transfers T50 and T67-69. Since a significant four-way interaction was found, the maximal model was not simplified and separate analyses were then performed with two different datasets, T50 and T67-69. Explanatory variables included in the minimal model are displayed in blue, and significant chi-squared values are in bold.

Datasets	Explanatory variable	Df	Chi-square	p-value
All data	**Male form : Female form : Selection regime : Transfer**	**1**	**5.33**	**0.02**
	Male form : Female form : Selection regime	1	2.27	0.13
	Male form : Female form : Transfer	1	1.30	0.25
	Male form : Selection regime : Transfer	**1**	**6.22**	**0.01**
	Female form : Selection regime : Transfer	**1**	**4.37**	**0.04**
	Male form : Female form	1	0.65	0.42
	Male form : Selection regime	1	2.41	0.12
	Male form : Transfer	1	2.49	0.11
	Female form : Transfer	1	0.11	0.73
	Female form : Selection regime	1	2.00	0.16
	Selection regime : Transfer	**1**	**5.47**	**0.02**
	Male form	1	2.19	0.14
	Female form	1	0.10	0.75
	Selection regime	1	2.82	0.09
	Transfer	1	1.12	0.29
T50	Male form : Female form : Selection regime	1	1.68	0.20
	Male form : Female form	1	0.13	0.72
	Male form : Selection regime	1	0.28	0.59
	Female form : Selection regime	1	0.31	0.58
	Male form	1	2.65	0.10
	Female form	1	3.48	0.06
	Selection regime	1	0.54	0.46
T67-69	**Male form : Female form : Selection regime**	**1**	**4.20**	**0.04**
	Male form : Female form	1	0.64	0.43
	Male form : Selection regime	**1**	**5.73**	**0.02**
	Female form : Selection regime	1	3.33	0.07
	Male form	1	0.32	0.57
	Female form	1	1.37	0.24
	Selection regime	1	3.55	0.06

Table 3.3. Results of the pairwise comparisons between **latencies to copulation** of **males mated with green-** ***versus*** **red-form females**, depending on the males' colour form and selection regime at T67-69.

Male form	Male regime	Estimate	SE	z value	p-value
Green	Allopatry	-0.22	0.19	-1.17	0.24
	Sympatry	0.26	0.19	1.41	0.16
Red	Allopatry	-0.01	0.19	-0.04	0.97
	Sympatry	-0.27	0.17	-1.62	0.11

The duration of copulations involving males and females of each colour form was consistent across transfers at which copulation duration was measured, but seemed to vary depending on the selection regime (*i.e.,* significant interactions between selection regime and male form, as well as between selection regime and female form; *cf.* Table 3.4). Nevertheless, pairwise comparisons revealed that both red and green males mated for the same amount of time when they evolved in allopatry or in sympatry, and that in each selection regime, matings with green and red females lasted the same amount of time (*cf.* Table 3.5 and Figure 3.3).

Table 3.4. Statistical results obtained for the tested explanatory variables and their interactions on copulation duration. Explanatory variables included in the minimal model are displayed in blue, and significant chi-squared values are in bold.

Explanatory variables	Df	Chi-square	p-value
Male form : Female form : Selection regime : Transfer	1	2.06	0.15
Male form : Female form : Selection regime	1	0.16	0.69
Male form : Selection regime : Transfer	1	0.80	0.37
Male form : Female form : Transfer	1	2.20	0.14
Female form : Selection regime : Transfer	1	0.63	0.43
Male form : Transfer	1	0.17	0.68
Male form : Female form	1	0.55	0.46
Female form : Transfer	1	0.80	0.37
Selection regime : Transfer	1	3.67	0.06
Transfer	1	2.31	0.13
Male form : Selection regime	**1**	**5.02**	**0.03**
Female form : Selection regime	**1**	**4.44**	**0.04**
Male form	**1**	**32.99**	**<0.001**
Female form	1	1.20	0.27
Selection regime	**1**	**5.48**	**0.02**

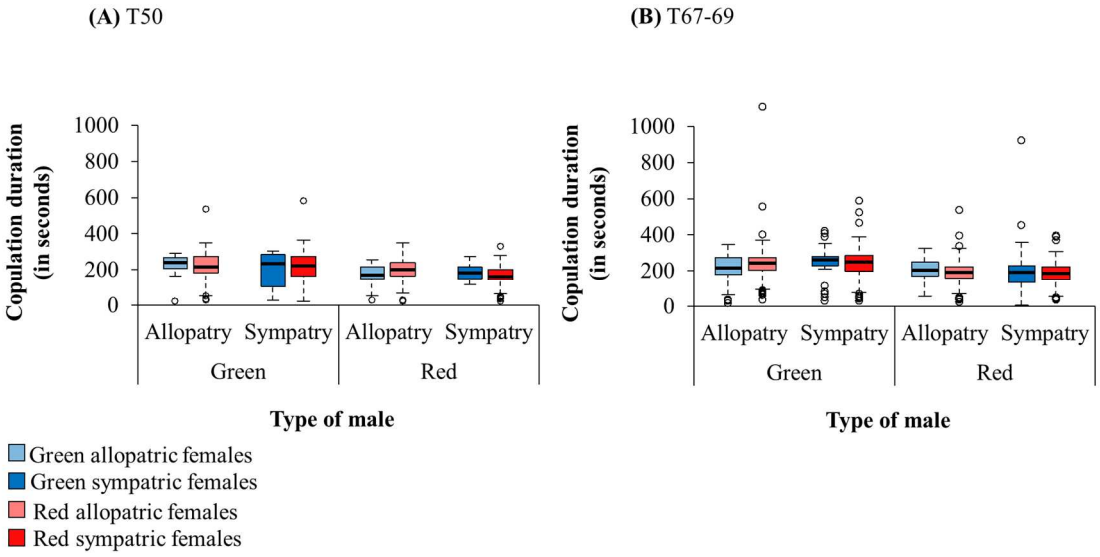

Figure 3.3. Boxplots of the copulation duration of the different tested males when mating with either green or red females after (A) 50 transfers and (B) 67-69 transfers. Lighter and darker colour tones represent individuals from the allopatric and the sympatric regime, respectively. Blue: green-form females; Red: red-form females.

Table 3.5. Results of the pairwise comparisons between **copulation durations of males** from different regimes or mated with different females, depending on their colour form or selection regime, respectively.

Comparison	Estimate	SE	z value	p-value
Allopatric *vs.* sympatric green males	0.20	0.11	1.87	0.24
Allopatric *vs.* sympatric red males	-0.11	0.10	-1.07	1.00
Allopatric green *vs.* allopatric red females	0.12	0.11	1.10	1.00
Sympatric green *vs.* sympatric red females	-0.21	0.11	-1.88	0.24

3.2 Experiment 2: Sperm precedence and hybridization experiment

3.2.1 F1 zygote mortality, F1 juvenile mortality and fertilization failure (Single mated females)

The hatching rate of eggs laid by green females mated with green males was, on average, higher than that of eggs laid by green females mated with red males (97 ± 0.2% and 95 ± 0.3%, respectively; X^2_1=10.37, p=0.001; Figure 3.4), but did not differ between selection regimes (*i.e.,* no significant effect of the selection regime or of its interaction with the type of F0 cross; Table 3.6).

Table 3.6. Statistical results obtained for all tested explanatory variables and their interactions on the hatching rate of F1 eggs. Explanatory variables included in the minimal model are displayed in blue, and significant chi-squared values are in bold.

Explanatory variables	Df	Chi-square	p-value
Type of F0 cross : Selection regime	1	3.80	0.051
Selection regime	1	0.78	0.38
Type of F0 cross	**1**	**10.37**	**0.001**

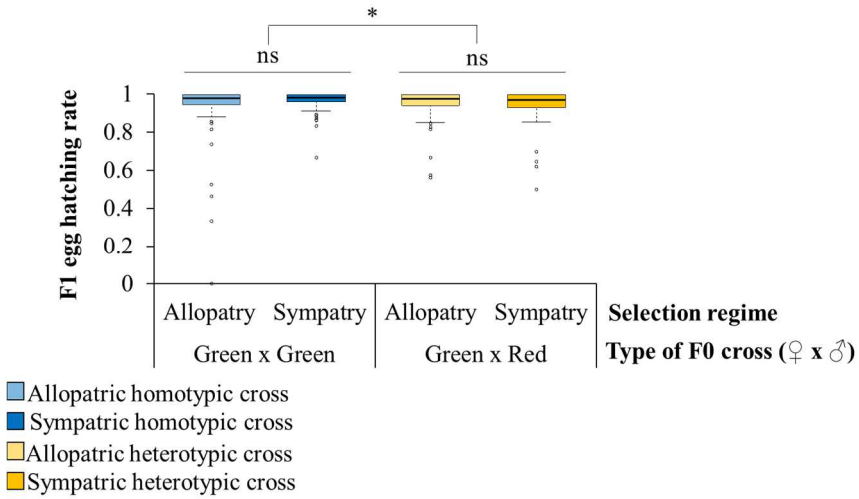

Figure 3.4. Boxplots of the proportion of hatched eggs resulting from homotypic and heterotypic crosses using green-form females from the allopatric and sympatric selection regimes. Lighter and darker colour tones represent crosses from the allopatric and from the sympatric regime, respectively. Blue: homotypic crosses; Yellow: heterotypic crosses. "*" indicates statistically significant differences, and "ns" non-significant differences, at the 5% level.

The mortality of F1 juveniles varied between selection regimes depending on the colour form of the male that mated with their mother (*i.e.,* significant interaction between the type of F0 cross and the selection regime; Table 3.7). Indeed, juveniles fathered by green males that evolved in sympatry had a higher mortality rate (18 ± 2%) than those fathered by green males that evolved in allopatry (13 ± 1%), whereas no differences were observed between juveniles fathered by red males evolved in both selection regimes (9 ± 1% on average; Table 3.8; Figure 3.5).

Table 3.7. Statistical results obtained for the tested explanatory variables and their interactions on F1 juvenile mortality rate. As a significant interaction was found in this analysis, the maximal model was not simplified. Explanatory variables included in the minimal model are displayed in blue, and significant chi-squared values are in bold.

Explanatory variables	Df	Chi-square	p-value
Type of F0 cross : Selection regime	**1**	**25.33**	**<0.001**
Type of F0 cross	**1**	**40.43**	**<0.001**
Selection regime	**1**	**40.40**	**<0.001**

Table 3.8. Results of the pairwise comparisons between the **mortality of F1 juveniles** observed in the **allopatric *versus* sympatric selection regimes**, depending on the colour form of their father. Significant values are displayed in bold.

Father	Estimate	SE	Df	t value	p-value
Green	**0.37**	**0.06**	**425**	**6.36**	**<0.001**
Red	-0.13	0.08	425	-1.62	0.11

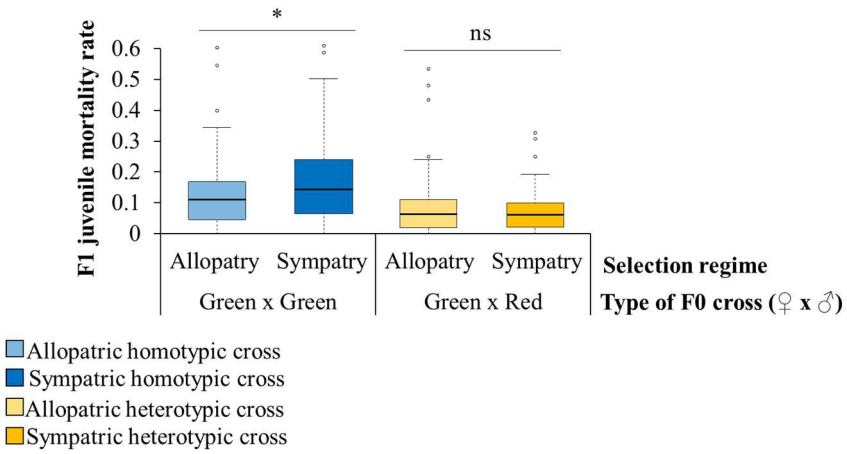

Figure 3.5. Boxplots of the proportion of dead juveniles depending on the type of F0 cross and selection regime. Lighter and darker colour tones represent crosses from the allopatric and from the sympatric regime, respectively. Blue: homotypic crosses; Yellow: heterotypic crosses. "*" indicates statistically significant differences, and "ns" non-significant differences, at the 5% level.

As previously observed by Cruz et al. (2021), a drastic reduction in the proportion of F1 females was found in the offspring of green females mated with red males as compared to control crosses (Table 3.9; Figure 3.6). This reduction, however, was the same for both the sympatric and the allopatric regime, as *ca.* 25% less F1 females were produced in heterotypic crosses from both selection regimes (no significant interaction between selection regime and type of F0 cross; Table 3.9). Nevertheless, regardless of the type of cross (homotypic or heterotypic), the proportion of F1 females produced in the sympatric regime (65 ± 3% pure and 16 ± 4% hybrid F1 females) was higher than that produced in the allopatric regime (52 ± 3% pure and 13 ± 3% hybrid F1 females).

Table 3.9. Statistical results obtained for all tested explanatory variables and their interactions on the proportion of F1 females in the adult offspring resulting from each cross in each selection regime. Explanatory variables included in the minimal model are displayed in blue, and significant chi-squared values are in bold.

Explanatory variables	Df	Chi-square	p-value
Type of F0 cross : Selection regime	1	1.44	0.23
Selection regime	**1**	**7.89**	**0.005**
Type of F0 cross	**1**	**168.40**	**<0.001**

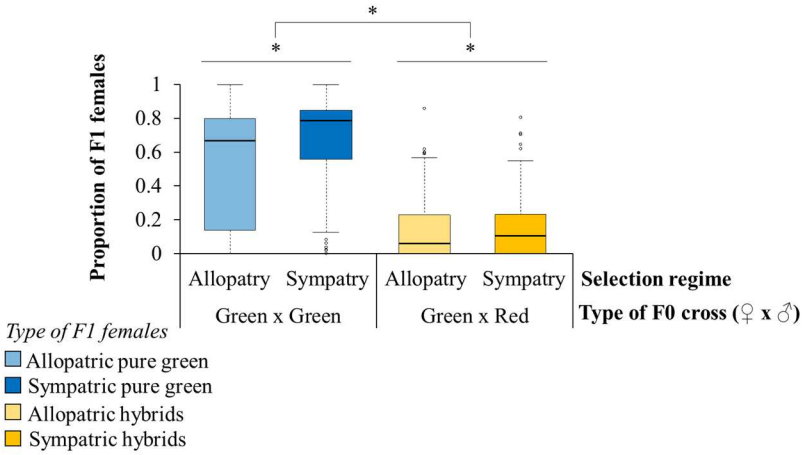

Figure 3.6. Boxplots of the proportion of females in the adult offspring resulting from homotypic and heterotypic crosses using green-form females from the allopatric and sympatric selection regimes. Lighter and darker colour tones represent crosses from the allopatric and from the sympatric regime, respectively. Blue: homotypic crosses; Yellow: heterotypic crosses. "*" indicates statistically significant differences at the 5% level.

3.2.2 Sperm precedence (Double mated females)

The proportion of F1 females sired by the first male that mated with green-form females was, on average, lower when the first male was of the red form than when it was of the green form (X^2_1=34.64, p<0.001; Table 3.10). Indeed, when the first male was of the red form 60 ± 10% of the F1 females were hybrids, whereas when the first male was of the green form 96 ± 1% of the females were pure green form (Figure 3.7). This pattern was not affected by the selection regime from which both males and females came from (*i.e.,* there was no significant effect of the selection regime or of its interaction with the type of F0 cross; Table 3.10).

Table 3.10. Statistical results obtained for all tested explanatory variables and their interactions on the proportion of F1 females sired by each male. Explanatory variables included in the minimal model are displayed in blue, and significant chi-squared values are in bold.

Explanatory variables	Df	Chi-square	p-value
Type of F0 cross : Selection regime	1	2.53	0.11
Selection regime	1	0.00	1.00
Type of F0 cross	**1**	**34.64**	**<0.001**

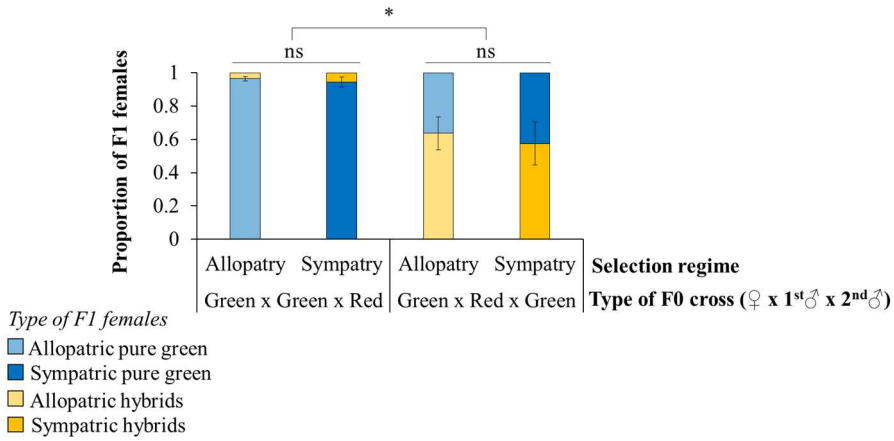

Figure 3.7. Proportion of pure green and hybrid F1 females resulting from green females double mated with red and green males in different orders. Bars represent means ± standard errors of the different experimental evolution replicates. The order by which the females mated with each type of male is given in the x axis. Daughters sired by the first male are displayed on the lower parts of the bars, and those sired by the second male are displayed on the upper parts of the bars. Lighter and darker colour tones represent crosses from the allopatric and from the sympatric regime, respectively. Blue: pure green offspring females (*i.e.,* daughters of green males); Yellow: red-coloured hybrid offspring females (*i.e.*, daughters of red males). "*" indicates statistically significant differences, and "ns" non-significant differences, at the 5% level.

3.3 Experiment 3: Hybrid fertility and hybrid breakdown

The proportion of F1 ovipositing females differed depending on their type (*i.e.,* pure green or red form, hybrid from a green mother and a red father or the reverse), independently of the selection regime (Table 3.11). Moreover, pairwise comparisons revealed that the proportion of ovipositing females only differed between pure and hybrid F1 females (with 97 ± 1% of the former, against 9 ± 1% of the latter, laying at least one egg; Figure 3.8a), but did not differ within pure (green *vs.* red form), nor within hybrid (from a green- *vs.* a red-form mother) F1 females (Table 3.12).

Table 3.11. Statistical results obtained for all tested explanatory variables and their interactions on the proportion of F1 ovipositing females. Explanatory variables included in the minimal model are displayed in blue, and significant chi-squared values are in bold.

Explanatory variables	Df	Chi-square	p-value
Type of F1 female : Selection regime	3	1.79	0.62
Selection regime	1	0.94	0.33
Type of F1 female	**3**	**331.32**	**<0.001**

Table 3.12. Results of the pairwise comparisons between the **proportion of ovipositing females** for different types of F1 females. Significant values are displayed in bold.

Comparison between F1 females	Estimate	SE	Df	t value	p-value
Pure *vs* hybrid	**5.94**	**0.34**	**954**	**17.44**	**<0.001**
Pure green *vs* pure red form	-0.95	0.60	954	-1.58	0.23
Hybrid with green *vs* red mothers	-0.36	0.32	954	-1.11	0.53

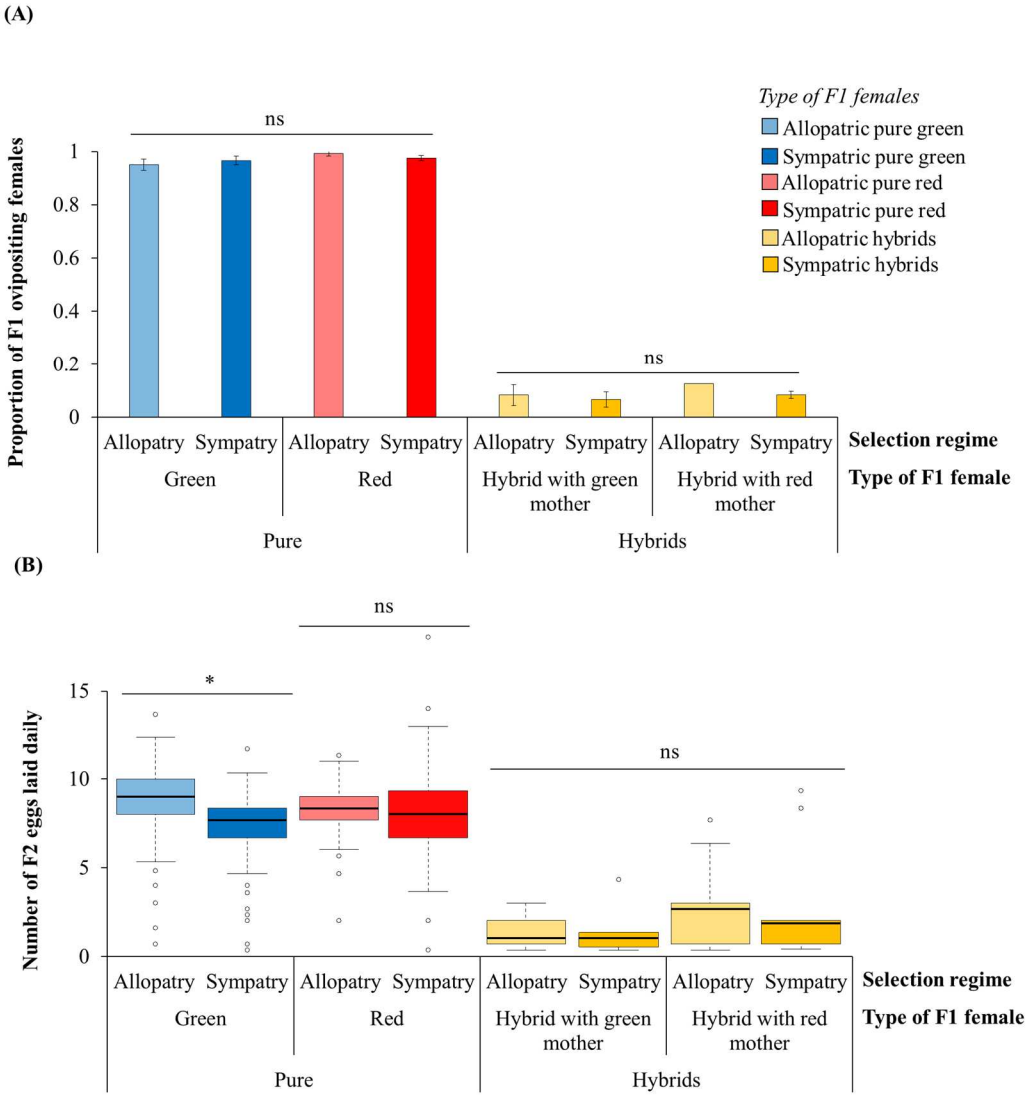

Figure 3.8. (A) Proportion of ovipositing F1 females and (B) boxplots of the number of eggs they laid daily. In (A), bars represent means ± standard errors of the different experimental evolution replicates. Lighter and darker colour tones represent crosses from the allopatric and from the sympatric regime, respectively. Blue: green females; Red: red females; Yellow: hybrid females. "*" indicates statistically significant differences, and "ns" non-significant differences, at the 5% level.

Among ovipositing females, daily fecundity varied between the allopatric and sympatric regimes for pure F1 females, but not for any type of F1 hybrid females (Table 3.13). Pairwise comparisons between pure females further revealed no difference between selection regimes for red-form females, but a lower fecundity of green-form females evolved in sympatry than of those evolved in allopatry (7.22 ± 0.24 and 8.70 ± 0.18 eggs on average, respectively; Table 3.14; Figure 3.8b).

Table 3.13. Statistical results obtained for all tested explanatory variables and their interactions on the daily fecundity of F1 ovipositing females. The analysis was first performed on the dataset with all females. Since the interaction between the type of F1 female and the selection regime was significant, separate analyses were then performed with two different datasets, pure and hybrid females. Explanatory variables included in the minimal model are displayed in blue, and significant chi-squared values are in bold.

Datasets	Explanatory variables	Df	Chi-square	p-value
All females	**Type of F1 female : Selection regime**	**3**	**14.22**	**0.003**
	Type of F1 female	**3**	**58.60**	**<0.001**
	Selection regime	**1**	**33.22**	**<0.001**
Pure females	**Type of F1 female : Selection regime**	**1**	**13.86**	**<0.001**
	Type of F1 female	1	2.87	0.09
	Selection regime	**1**	**33.81**	**<0.001**
Hybrid females	Type of F1 female : Selection regime	1	0.01	0.91
	Selection regime	1	0.02	0.88
	Type of F1 female	1	3.19	0.07

Table 3.14. Results of the pairwise comparisons between the **daily fecundity of pure F1 females from the allopatric *versus* sympatric selection regimes**. Significant values are displayed in bold.

F1 female	Estimate	SE	Df	t value	p-value
Green	**0.18**	**0.03**	**459**	**5.82**	**<0.001**
Red	0.02	0.03	459	0.72	0.47

Subsequently, the hatching rate of F2 eggs differed between types of F1 females, but regardless of the selection regime (Table 3.15). Overall, the hatching rate of eggs laid by pure females was much higher than that of eggs laid by hybrids (96 ± 1% and 10 ± 4%, respectively), did not differ between pure females (*i.e.,* red or green form, 96 ± 1% on average), and was higher for hybrids with green mothers than for hybrids with red mothers (25 ± 8% and 4 ± 2%, respectively; Table 3.16; Figure 3.9).

Table 3.15. Statistical results obtained for all tested explanatory variables and their interactions on the hatching rate of F2 eggs. Explanatory variables included in the minimal model are displayed in blue, and significant chi-squared values are in bold.

Explanatory variables	Df	Chi-square	p-value
Type of F1 female : Selection regime	3	4.86	0.18
Selection regime	1	0.10	0.76
Type of F1 female	**3**	**340.11**	**<0.001**

Table 3.16. Results of the pairwise comparisons between the **hatching rate of eggs** laid by different types of F1 females. Significant values are displayed in bold.

Comparison between F1 females	Estimate	SE	Df	t value	p-value
Pure *vs* hybrid	**5.22**	**0.28**	**501**	**18.40**	**<0.001**
Pure green *vs* pure red form	-0.22	0.14	501	-1.56	0.24
Hybrid with green *vs* red mothers	**1.87**	**0.53**	**501**	**3.52**	**0.001**

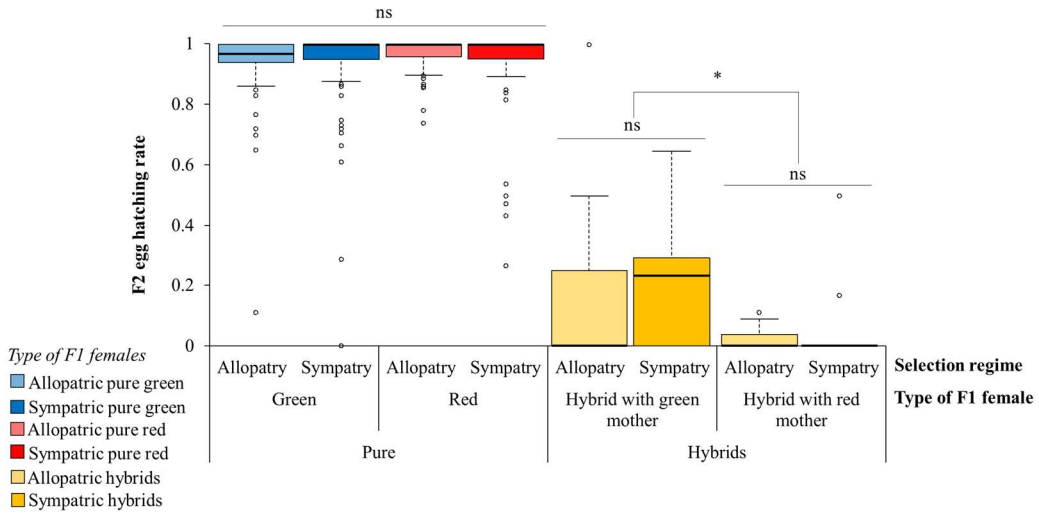

Figure 3.9. Boxplots of the proportion of hatched eggs laid by different types of F1 females. Lighter and darker colour tones represent crosses from the allopatric and from the sympatric regime, respectively. Blue: green females; Red: red females; Yellow: hybrid females. "*" indicates statistically significant differences, and "ns" non-significant differences, at the 5% level.

4. Discussion

The aim of this project was to investigate how sympatric evolution affects reproductive isolation between two genetically divergent colour form populations of the spider mite *T. urticae*. Specifically, I tested what reproductive barriers, either pre- or post-zygotic, were more likely to evolve under the selection pressure caused by reproductive interference. For this, I used two populations previously shown to be fully post-zygotically isolated due to strong hybrid sterility and full hybrid breakdown (Cruz et al. 2021). Also one population showed disassortative and the other assortative mating when the two populations co-occurred (Cruz 2017). Therefore, strong reproductive interference should occur between the two populations, with the potential to select for increased pre-zygotic isolation (*i.e.,* reinforcement; Sætre 2012) during experimental evolution in the sympatric selection regime. Although selection for reduced post-zygotic barriers in response to reproductive interference was not expected to occur in absence of gene flow, such barriers could still increase due to genetic linkage between pre- and post-zygotic isolation, and/or due to pleiotropic effects of genes involved in pre-zygotic isolation (Butlin & Smadja 2018).

One notable and unexpected result obtained in this project was that post-zygotic isolation between the two populations may, in fact, be incomplete. Indeed, I found that the proportion of ovipositing F1 hybrid females (*ca.* 9%), and their daily fecundity (> 2 eggs per day on average; Figure 3.8) was much higher than previously found (less than 1% of ovipositing F1 hybrid females and less than 1 egg laid daily; Cruz et al. 2021). Also, contrary to that previous study, *ca.* 10% of the F2 eggs hatched (Figure 3.9), showing that hybrid breakdown is incomplete, and that gene flow might be occurring between the two populations. These new results, which are likely due to the important sample size difference between the two studies (960 *vs.* 196 F1 hybrid females tested in Cruz et al. 2021), are extremely relevant for our understanding of this system and for the predictions we can make about its evolution. Indeed, if gene flow occurs between the two populations in sympatry, selection for compatible gene combinations is possible, which in turn may decrease post-zygotic isolation and the costs of hybridization. In such scenario, reinforcement might still occur if most of the hybrids are unfit (Matute 2010b), but continuous hybridization may also lead to speciation reversal (Seehausen 2006).

4.1 Did gene flow occur between the green- and red-form populations in the sympatric selection regime?

The results obtained for hybrid fertility and breakdown suggest that gene flow might be occurring between the two populations used in this study. Furthermore, unlike the study of Cruz et al. (2021), I found an asymmetric pattern for hybrid breakdown, with the hatching rate of eggs laid by hybrid daughters of green females being higher than that of hybrid daughters of red females (Figure 3.9). This suggests that besides nuclear-nuclear incompatibilities (*cf.* Cruz et al. 2021), cytonuclear incompatibilities may be involved in hybrid breakdown, as found between two genetically differentiated haplotypes of *T. evansi* (Knegt et al. 2017). Moreover, this result suggests that gene flow, if occurring, is probably asymmetrical, with more genes being introgressed from the red population into the nuclear genome and cytoplasm of the green population than the reverse. Note, however, that in terms of gene flow, this asymmetric pattern of post-zygotic isolation may be counter balanced by the asymmetric pattern of pre-zygotic isolation, since crosses between red males and green females should occur less often than crosses between green males and red females (*cf.* mate preference results).

The occurrence of gene flow from the red population into the green population may explain other results obtained in this project. Indeed, I found that (supposedly) pure green females from the sympatric regime differ from those of the allopatric regime for several traits: they showed decreased daily fecundity (Figure 3.8b), increased offspring juvenile mortality (Figure 3.5), and produced a more female-biased sex-ratio (Figure 3.6). Although at least two generations of common garden (including the 'age-cohort'; *cf.* methods) were performed prior to the experiments (thereby excluding the possibility of using F1 hybrid females or pure males from the 'wrong' form), this procedure did not exclude the possibility of using backcrossed 'green' females and hybrid males in case gene flow occurred. In other words, the "pure green" females used in the experiment might have carried genes from the red-form population as a result of hybridization over several generations. In such case, both the decreased daily fecundity of 'green' females mated with 'green' males, and the increased juvenile mortality in their offspring, may, in fact, be the expression of late hybrid breakdown. Similar costs of hybridization that accumulate over generations have been found in different taxa, such as plants (Cutler & Whitaker 1968), insects (Myers et al. 2013), birds (Wiley et al. 2009) and fish (Renaut & Bernatchez 2011). Previous work at my host laboratory has also revealed that the ratio of daughters to sons of red females is higher than that of green females when they oviposit in groups (pers. comm. Cruz and Zélé; unpublished data). Therefore, if genes of 'red' individuals that are responsible for an increased fertilization rate of eggs introgressed into the genome of 'green' individuals during experimental evolution, this may explain the higher proportion of females observed in the offspring of sympatric 'green' females.

Alternative explanations that do not involve gene flow could also be proposed for these results. In particular, the increased proportion of daughters in the offspring of green females in the sympatric regime may have evolved to compensate for the overproduction of males that result from reproductive interference. Indeed, because green males may mate more often with red females than with green females in sympatry (*cf.* mate preference results), green females might, on average, be fertilized later than red females and thus may lay a higher proportion of unfertilized eggs before copulation occurs. In addition, when these females mate with red males, fertilization failure leads to an important overproduction of green males, associated to a reduction in the proportion of green females, in the population (note that this is not the case when red females mate with green males). Therefore, the proportion of green males should be extremely high in sympatry, and so should be competition for mates among these males. This, in turn, may select for green females producing a higher proportion of daughters. Previous studies in *T. urticae* showed that strong competition for mates among males selects for a higher production of daughters (Macke et al. 2011a). This sex-ratio adjustment is achieved through an alteration of egg size (Macke et al. 2012a) with bigger eggs having a higher probability of being

fertilized (Macke et al. 2011b). Moreover, because egg size and egg number are negatively correlated in spider mites (Macke et al. 2012b), the shift in sex ratio observed for green females in the sympatric regime may also explain their reduced fecundity. However, this hypothesis does not explain the increased juvenile mortality in the brood of green females.

Overall, although the occurrence of gene flow in the sympatric regime may provide the best explanation to the above mentioned results, it is not possible to indisputably claim that the two populations exchanged genes. Indeed, gene flow could be prevented at life history stages and/or generations that were not tested here. For instance, I did not assess the survival of F2 males until adulthood and their fertility once adults, nor whether F1 hybrid females can be fertilized to produce viable and fertile F2 hybrid daughters. Moreover, hybrid breakdown was found to occur continuously after the F2 when some populations of these two colour forms hybridize (Sugasawa et al. 2002). Therefore, only genetic analyses would shed light on the amount and symmetry of gene flow between the two populations over multiple generations.

4.2 Did reproductive barriers evolve in the sympatric selection regime?

4.2.1 F1 hybrid mortality and sterility, and F2 hybrid breakdown (post-zygotic barriers)

Before experimental evolution, Cruz et al. (2021) found a cost of hybridization between green females and red males in terms of F1 zygote mortality (*i.e.,* hatching rate), but not for F1 juvenile mortality. Overall, these results were recapitulated after experimental evolution, but no differences were found between F1 zygotes and juveniles produced by 'green' females mated with 'red' males from the allopatric and sympatric regimes (Figure 3.4 and 3.5). In line with this, no differences were observed between the allopatric and sympatric selection regimes after experimental evolution in terms of F1 female fertility and F2 zygote mortality in heterotypic crosses (Figure 3.8 and 3.9). As only an advantage to hybridization could have selected for a decrease in post-zygotic barriers, these results confirm that hybridization is costly in this system. This was demonstrated by the high proportion of sterile F1 hybrids and reduced F2 eggs hatch rate, and fitness costs may have also occurred after F2 egg hatching (not tested here but see Sugasawa et al. 2002). Therefore, beneficial gene combinations in hybrids may be too rare for selection to be observed during the time frame of experimental evolution.

4.2.2 Fertilization failure (pre-zygotic post-mating barrier)

After experimental evolution, the production of female offspring increased by *ca.* 9% in the sympatric regime as compared to the allopatric regime (*ca.* 42% *vs.* 33%; Figure 3.6). However, the proportion of female offspring produced in heterotypic crosses suffered an equivalent reduction relative to the homotypic crosses in both selection regimes, revealing no change in reproductive isolation (*i.e.,* more hybrids are produced in heterotypic crosses as a by-product of sex-ratio shift in the sympatric selection regime).

The lack of selection for decreased fertilization failure in heterotypic crosses may suggest that gene flow does not occur between the two population forms, or that hybridization is too costly (and/or beneficial gene combinations in hybrids are too rare, *cf.* above) to be selected for in the presence of gene flow. Conversely, the lack of selection for an increased fertilization failure is less intuitive, as a strengthening of this barrier (*i.e.,* reinforcement of pre-zygotic barriers driven by costly hybridization; Sætre 2012) should decrease the strength of reproductive interference for green females (Cruz et al. 2021). Indeed, the overproduction of green males in crosses between green females and red males due to fertilization failure, while not in reciprocal crosses (*cf.* Cruz et al. 2021), should: (i) allow green

females to transmit more genes than red females; (ii) increase the probability of homotypic matings for green females at the next generation; and (iii) increase the probability of heterotypic matings for red females (which indirectly benefits green females). Finally, the fertilization failure of heterotypic matings should also be beneficial for green females if it (iv) increases the likelihood of homotypic sperm precedence (*cf.* next section). Given these advantages, the absence of selection of an increased fertilization failure may lie in the proximate mechanisms underlying it.

In a scenario where fertilization failure is caused by a reduction in sperm transfer in heterotypic crosses, a further decrease in the amount of sperm transferred would be beneficial for males as it would reduce sperm wastage. However, the selection pressure on such a male trait may be very weak (or even inexistent) given that spider mite males can mate with multiple females without being sperm depleted (Krainacker & Carey 1989; but see Kobayashi et al. 2022). Alternatively, if fertilization failure is caused by a defect of the reproductive process in the female reproductive tract (*e.g.,* reduction in sperm storage, sperm ejection/dumping, reduced sperm activation or attraction to the egg, and sperm-egg incompatibility; Zeh & Zeh 1997; see also Takafuji & Fujimoto 1985; Perrot-Minnot et al. 2004; Firman et al. 2017), any change in the strength of this barrier should be mostly neutral for males (*i.e.,* their sperm would be wasted for the production of unfit hybrids regardless), but should always be beneficial for females (*cf.* (i) to (iv) above). However, underlying female traits may be under strong stabilizing selection, thereby preventing their evolution. It would be the case if such traits affecting fertilization in heterotypic crosses also affect it in homotypic crosses. The fact that the fertilization rate of green females' eggs increased in both homotypic and heterotypic crosses in the sympatric regime seems to corroborate this latter hypothesis.

4.2.3 Homotypic sperm precedence (pre-zygotic post-mating barrier)

In *T. urticae* and other arachnids, the sperm of the first male is used to fertilize the majority of the eggs of a female (*i.e.,* there is first-male sperm precedence; Helle 1967; Wedell et al. 2002; Rodrigues et al. 2020). However, this pattern of sperm precedence can be context-dependent, and can, for instance, change depending on the effectiveness of the first mating (Potter & Wrensch 1978; Satoh et al. 2001; Weldingh et al. 2011). Hence, when a female mates with both a conspecific (or homotypic) and a heterospecific (or heterotypic) male, the former may fertilize the vast majority of the eggs, regardless of the order of mating, simply because copulation with the latter is ineffective. Such pattern of conspecific (or homotypic) sperm precedence (Price 1997) was found in several taxa, ranging from insects (Price 1997) to marine invertebrates (Geyer & Palumbi 2005). In spider mites, when *T. urticae* (or *T. ludeni*) females mate with a conspecific male before or after mating with a heterospecific *T. evansi* (or *T. urticae*) male, they only produce daughters with the conspecific male (Clemente et al. 2018). However, this outcome cannot unambiguously be attributed to conspecific sperm precedence because heterospecific males possibly do not transfer any sperm (*i.e.,* single heterospecific matings do not yield any female offspring in both cases; Clemente et al. 2016, 2018). Conversely, fertilization does occur in single crosses between the green and red form of *T. urticae*, which allowed me to test whether first male sperm precedence is maintained in double mated females, or whether homotypic sperm precedence may evolve. My results show a mixed pattern, with green females that mated first with a green male and then with a red male producing nearly all green female offspring (hence daughters of the first, homotypic, male), and those that mated first with a red male and after with a green male producing a mixture of green and red-coloured hybrid female offspring (hence shared paternity between the first, heterotypic, male and the second, homotypic, male; Figure 3.7). Therefore, these results show that homotypic sperm precedence occurs in this system, but its strength depends on the mating order.

This pattern might be due to different, albeit not mutually exclusive, mechanisms. One possibility is that it is the by-product of fertilization failure in heterotypic crosses. Indeed, it is possible

that green females simply fertilize the eggs that were not fertilized with the sperm of a first heterotypic male, with that of a second homotypic male (regardless of the underlying mechanisms; *cf.* above). Alternatively, the observed pattern of sperm precedence could be the outcome of cryptic male or female choice, whereby individuals discriminate between mates once copulation begins (Firman et al. 2017; Aumont & Shuker 2018). For instance, green females might be interrupting their first mating with a red male if it increases their chance of fertilizing their eggs with the sperm of a second green male. Similarly, red males may be interrupting their first mating with a green female, as it would increase their opportunity to mate with more (incl. homotypic) females. Indeed, in this system, post-copulatory mate guarding behaviour is commonly used by males to ensure paternity (Oku 2014), and it is more likely for a second mating to be effective when the copulation duration of the first mating is short (Potter & Wrensch 1978). However, copulation duration data (Figure 3.3) shows that both types of males spent the same amount of time copulating with green and red females. Therefore, no discrimination ability between homotypic and heterotypic individuals seems to exist in this system once copulation starts. Nevertheless, nor cryptic male choice, nor cryptic female choice can be completely ruled out, and the observed pattern of homotypic sperm precedence may as well result from sperm competition (Wigby & Chapman 2004). Indeed, in the case of cryptic male choice, red males, but not green males, may transfer less sperm when mating with already mated female (regardless of their colour form). This effect may be independent of copulation duration, as no correlation between copulation duration and fertilization rate was found in this system (Satoh et al. 2001). In the case of sperm competition or cryptic female choice, the ejaculate of green males may outcompete that of red males or be preferred by green females to fertilize their eggs, but the sperm that arrives first in the female reproductive tract has a competitive advantage.

Regardless of the underlying mechanisms, homotypic sperm precedence should be selected when hybridizing species evolve in sympatry, as it reduces the likelihood of (costly) hybridization (Howard 1999). Such increase in post-mating pre-zygotic isolation after sympatric evolution (*i.e.,* reinforcement) has been shown between populations of *Drosophila pseudoobscura* and *D. persimilis* (Castillo & Moyle 2019). Here, however, I found that the proportion of daughters sired by the green and red males was the same in the sympatric and allopatric selection regimes. If homotypic sperm precedence, in this system, is only the by-product of fertilization failure, the observed lack of evolution may simply reflect the fact that fertilization failure itself did not evolve (*cf.* previous section). Alternatively, if homotypic sperm precedence results from sperm competition and/or cryptic male/female choice, one possible explanation to the fact that the pattern of sperm precedence did not evolve could be that the selective pressure acting upon the females and/or the males was too low. Indeed, given that the proportion of green males is expected to be particularly high in the sympatric selection regime (*cf.* above), the likelihood of a first mating between a green female and a red male should be low (and so should be the likelihood of a second mating with a green male after a red one).

4.2.4 Mate preference (pre-mating barrier)

Previous work showed that before experimental evolution males from both the green- and red-form populations preferred red females, thus that the green and red populations showed disassortative and assortative mating, respectively (Cruz 2017). This pattern likely results from the evolutionary history of the two populations before being brought to the laboratory. Indeed, it is possible that the red-form population had already been in contact with other incompatible populations before being collected from the field and evolved assortative mating to avoid outbreeding depression. In line with this, many examples in the literature (*e.g.,* Urbanelli & Porretta 2008; Pfennig & Rice 2014) show that populations that evolved in sympatry show a strong preference for conspecific mates due to reinforcement. Conversely, the green-form population may have never encountered individuals from red-form

populations, and the preference of green males for red over green females could have emerged as a by-product of selection to prefer more dissimilar females. Theoretically, the evolution of disassortative mating is favoured by stabilizing selection, as it can reduce the production of less fit phenotypic extremes, and, by increasing heterozygosity, it also decreases inbreeding depression (Jiang et al. 2013). Therefore, such behaviour can be adaptive in absence of incompatible mates or when the occurrence and/or the costs of incompatible matings are low. In line with this, inbreeding avoidance via female mate choice has been shown in another green-form population of *T. urticae* (Tien et al. 2011).

Other possible explanations for the preference of red females by both types of males could be that they have retained an ancestral preference for a trait that is present in red females but has been lost (or diverged) in green females, or that red females had evolved a new trait that is preferred by both types of males. The first scenario may occur, for example, if a trait becomes useless in environmental conditions that affect signal reception and perception (Endler & Basolo 1998), or diverged in response to sexual conflicts (Martin & Hosken 2003), local adaptation (Petegem et al. 2016), or by drift (Masel 2011), but the rate of evolution of male preference is slower than that of trait evolution (Endler & Basolo 1998). The second scenario may occur if a new trait that evolved in red females stimulates the same coding system as the ancestral trait, so that green males prefer it despite having no evolutionary history with it (*i.e.,* 'pre-existing bias'; Endler & Basolo 1998; Dawkins & Guilford 1996).

Although the evolutionary history of the green- and red-form populations was unknown in the wild, the costs associated with reproductive interference could have selected for an increase in pre-mating isolation between populations that evolved in the sympatric regime during experimental evolution. Two non-exclusive outcomes were expected: (i) red males increasing their preference for red females, potentially driving assortative mating to completion, and (ii) green males changing their preference from red to green females, thereby reversing disassortative to assortative mating. However, none of these outcomes came to fruition, as the same higher proportion of red females than green females was chosen by both types of males, in both selection regimes, and at both tested generation times (T50 and T67-69; Figure 3.1). Even though gene flow might be occurring between the two populations, the costs of hybridization are extremely high, and reinforcement may still be expected in these conditions (*e.g.,* Servedio & Kirkpatrick 1997; Matute 2010b; Roda et al. 2017). Moreover, the lack of mating preference evolution cannot be attributed to the absence of a target for selection (*i.e.,* a trait that could evolve), as the fact that both types of males prefer red females indicates that they utilize cues to discriminate between different females. In fact, *T. urticae* males can use chemical trails and volatile compounds to discriminate between virgin and mated females (Rodrigues et al. 2017), as well as contact chemicals to discriminate between conspecific and heterospecific (*T. evansi*) females (Sato & Alba 2020). Nevertheless, other (non-exclusive) hypotheses can be brought forward to explain these results.

Concerning the red-form population, it is possible that an insufficient number of generations of selection (*cf.* Figure 2.2), coupled with a weak selection pressure (because the red males already mated assortatively before experimental evolution), had prevented reinforcement. However, this does not hold true for the green-form population, which had experienced up to 50 and 66 effective generations of selection before being tested in this mate preference experiment (at T50 and T69, respectively; Experiment 1), with a strong selection pressure due to disassortative mating. For comparison, reinforcement of behavioural isolation was found within 10 generations of experimental evolution in *Drosophila* (Matute 2010a). Therefore, it is more likely that mate choice did not evolve here due to other reasons. One reason could be evolutionary constraints. For example, if red females produce a higher quantity signal than green females instead of a different type of signal (*e.g.,* amount of pheromones released instead of a different chemical composition), green males may simply not be able to evolve a preference for weaker signals, as such signals could be masked by the stronger signals. In line with this, red females may also not evolve to produce weaker signals to avoid heterotypic matings because, by doing so, they would also be less attractive to homotypic (red) males. Under such a scenario, however,

we could expect that males would take less time to initiate a copulation with females that release more pheromones (hence the red females), which is not what was observed. Thus, latency to copulation and mate preference are not correlated here, and latency to copulation may instead be an indicator of males' eagerness to mate and competitiveness. In line with this, the fact that this trait did not differ between the sympatric and the allopatric selection regime after experimental evolution (Figure 3.2) suggests that the males' competitive ability also did not evolve in response to reproductive interference.

As mentioned above for the red-form population, and regardless of the type of signals used by males to choose their mates, it is possible that the selection pressure on male preference is, in general, too weak in this system for it to evolve. Indeed, as males can mate multiple times (Krainacker & Carey 1989, 1990), they can still transmit their genes by mating with a female of their own form, even after mating with an incompatible one.

4.3 Alternative evolutionary responses to reproductive interference

In this project, I did not find evidence for the evolution of reproductive barriers between the green and red *T. urticae* populations despite reproductive interference. However, there are other means by which green- and red-form populations evolving in sympatry could reduce the costs of reproductive interference that were not tested. Indeed, I did not study all possible pre-mating barriers, such as temporal or habitat isolation (*cf.* Table 1.1). Indeed, species or populations experiencing high reproductive interference in the wild may evolve to reduce the occurrence of costly sexual interactions by decreasing their encounter rate. In spider mites, temporal isolation does not occur, even between distantly related species (*e.g.,* Hanna et al. 1996; Kishimoto 2002), but habitat isolation could be achieved, for example, via niche partitioning (Ferragut et al. 2013) or aggregation of conspecific individuals (Godinho et al. 2020b). Theoretical work suggests that even if two species (or genetically differentiated populations) are equally good at using a specific niche, hybridization avoidance alone can select for habitat specialization (Kyogoku & Kokko 2020). Furthermore, reinforcement evolves much more easily if hybridization is avoided based on habitat segregation than if the mechanism involves species/population recognition (mate choice traits) because the former also reduces resource competition (Kyogoku & Kokko 2020). Accordingly, many empirical examples show that resource/niche partitioning allow coexistence between reproductively interfering species (Gröning et al. 2007; Friberg et al. 2013; Kishi & Tsubaki 2014; Noriyuki & Osawa 2016). Here, the evolution of niche partitioning was not allowed during experimental evolution, as the two populations were placed in a homogeneous environment. Although aggregation of individuals from each population on separate sites within the plants could happen (*e.g.,* Sato et al. 2016; Godinho et al. 2020b; Fragata et al. 2022), observations performed during the transfers of females at each generation of experimental evolution did not reveal such a pattern (although precise quantification was not performed). Therefore, it is possible that only a heterogeneous environment and the evolution of niche partitioning could allow avoidance of reproductive interference in this system (but see Fry 1999). Alternatively, it is also possible that reproductive interference is evolutionary stable, *i.e.,* that partial reproductive isolation evolved as an adaptive optimum. This could be the case, for example, if there is a balance between natural selection (favouring stronger isolation) and sexual selection (constraining the evolution of several traits involved in sexual isolation; Servedio & Hermisson 2020). In such scenario, reproductive barriers between the two populations would remain unchanged, which matches the results obtained here.

4.4 Ecological consequences of persistent reproductive interference

Coexistence between species or populations can be considered at two different spatial scales. On one hand, it may take place locally within a community where interactions between species/populations are

limited to a portion of the landscape (*i.e.,* local coexistence). On the other hand, species/populations may coexist at a regional scale within a metacommunity, where various communities are linked by dispersal patterns (*i.e.,* regional coexistence; Amarasekare 2003). Both types of coexistence are expected to be hampered by persistent reproductive interference (Gröning & Hochkirch 2008; Kishi et al. 2009; Kishi & Nakazawa 2013; Sun et al. 2014; Iritani & Noriyuki 2021), which can strongly affect species/population dynamics and may lead to the displacement of at least one of them (Konishi & Takata 2004; Liu et al. 2007; Fea et al. 2013). Indeed, considering that the costs of reproductive interference are often asymmetrical (Ben-David et al. 2009; Kishi & Nakazawa 2013; Sato et al. 2014; Zhang et al. 2016), the inferior species/population may be forced to disperse into a new area (Ruokolainen & Hanski 2016). In the specific case of spider mites, colonization of a different host plant/crop field (local scale) or region (regional scale) as a result of reproductive interference could be highly problematic as these species are serious agricultural pests. Thus, in general, persistent reproductive interference can have negative impacts in the scope of conservation biology and pest control since native species or pests can be displaced. However, it may also be instrumental for the control of invasive species or pests through different strategies:

First, reproductive interference can be coupled with pest control techniques, namely the 'Sterile Insect Technique', which consists in mass release of sterile males of the target pest species. Indeed, the release of sterile males of a species closely-related to the invader, with which it would reproductively interfere, may help at supressing the invading population (Mitchell et al. 2022). Second, instead of just supressing the invading population, reproductive interference could be used to replace it with a population of a more benign closely-related interfering species. This could be achieved by releasing both males and females of the latter species (Mitchell et al. 2022). Finally, the control of invasive species could be promoted by employing signals (*e.g.,* pheromones, sounds, visual displays) that hamper communication between invasive individuals, thereby reducing their ability to find mates (*i.e.,* signal-jamming; Mitchell et al. 2022). This strategy has an additional advantage as it does not require the release of any other species that may have undesirable effects. Ultimately, if the invading species or population does not evolve resistance to reproductive interference (*e.g.,* reinforcement), persistent fitness costs should ensure the success of the above-mentioned control strategies.

4.5 Conclusions & Perspectives

Overall, reproductive isolation did not evolve after several generations of experimental evolution in sympatry despite costly sexual interactions resulting from incomplete pre-zygotic and strong post-zygotic isolation between the green- and red-form *T. urticae* populations. Nevertheless, research on how different reproductive barriers evolve in this system is still warranted, as more generations of experimental evolution might be necessary to detect reinforcement, especially in the red-form population. In line with this, it would be interesting to measure homotypic sperm precedence (Experiment 2) using red females, as they possibly suffer a higher selection pressure than green females (Cruz et al. 2021). Furthermore, this project showed that hybrid breakdown between these populations is not complete (contrary to previous evidence; Cruz et al. 2021) and that gene flow may be responsible for the decreased fecundity, higher juvenile mortality and sex-ratio shift observed in the green population that evolved in the sympatric selection regime. Alternatively, the observed change in oviposition rate and sex allocation may well be an evolutionary response to reproductive interference in this system. Thus, only genetic analysis and additional experimental tests would warrant the validation of these hypotheses. Finally, the fact that no evidence for the evolution of traits allowing to decrease the costs of heterotypic sexual interactions was found here, calls for further research on alternative evolutionary responses that were not tested in this project, as well as on the ecological consequences of reproductive interference in natural populations.

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