UNIVERSIDADE DE LISBOA
FACULDADE DE CIÊNCIAS

The distribution and performance of two herbivorous spider-mites living in heterogeneous environments

" Documento Definitivo"

Doutoramento em Biodiversidade, Genética e Evolução

Diogo Alexandre Prino Martins Godinho

Tese orientada por:
Doutora Sara Magalhães e Doutora Cristina Branquinho

Documento especialmente elaborado para a obtenção do grau de doutor

2020

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- Doutora Paula Duque Magalhães Santos, Investigadora Principal Instituto Gulbenkian de Ciência da Fundação Calouste Gulbenkian.
- Doutora Sara Newbery Raposo de Magalhães, Professora Auxiliar com Agregação Faculdade de Ciências da Universidade de Lisboa (Orientadora).

Documento especialmente elaborado para a obtenção do grau de doutor

This study was funded by Fundação para a Ciência e a Tecnologia, through a PhD scholarship (PD/BD/114010/2015) and by a European Research Center (ERC) project granted to Sara Magalhães (COMPCON GA 725419).

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Acknowledgements

Agradecimentos

Gostaria de começar por agradecer às minhas orientadoras, Sara Magalhães e Cristina Branquinho, sem elas a minha aventura na ciência teria sido muito diferente. A Sara aceitou orientar um estranho rapaz barbudo (menos na altura) que nunca tinha sido seu aluno, com um particular interesse por espécies invasoras. Confesso que quando li a proposta do tema de mestrado não sabia nada sobre ácaros e pensei que "invasive spider-mite" era uma espécie de aranha invasora. "Que fixe, aranhas invasoras!!", pensei. Afinal eram ácaros e cá estou eu quase há 8 anos a trabalhar com estes bichos lindos e adoráveis. A Sara todos os dias me mostra como fazer ciência melhor e me incentiva a fazê-la. Como diz a Leonor, a Sara é a nossa mamã científica, e para grande sorte nossa, é uma excelente mamã. Ouve as nossas ideias, apoia as nossas decisões e aponta-nos o melhor caminho. Foi um enorme privilégio ver o grupo da Sara crescer e, nele, também eu crescer como cientista.

A Cristina é uma fonte de inspiração científica alternativa. Sempre com uma energia positiva e contagiante, uma palavra motivadora e um ponto de vista diferente mas estritamente necessário para o amadurecer das minhas ideias. A Cristina também me abriu as portas a seu grupo e nele tive oportunidade de aprender sobre diversos temas interessantes. Juntas, a Sara e a Cristina, mostraram-me como trabalhar em equipa e entre equipas, características muito importantes em ciência. Muito obrigado às duas por tudo o que me ensinaram e por toda a paciência para me aturar ao longo destes anos.

Por falar em equipa, tenho também muito a agradecer aos membros do Mite Squad, os passados, os presentes e também os futuros, quando finalmente me oferecerem um Delorean eu prometo que vou lá visitá-los. Queria agradecer em especial à Flore e à Inês, que estiveram lá do inicio ao fim desta aventura. Não só foram boas colegas como se tornaram boas amigas. Já passámos por muitos momentos, fáceis e difíceis, em trabalho e em lazer, espero que possamos partilha muitos mais. Queria também agradecer aos echangers por partilharem o seu trabalho comigo e dedicarem algum do seu tempo a discutir sobre o meu trabalho também. Queria estender os agradecimentos a todos os membros do cE3c, esta família alargada que me tem apoiado no meu percurso académico desde o mestrado. Agradeço também em particular à Helena Serrano e à Belucha por toda a ajuda no mundo da fisiologia das plantas.

I would also like to thank Alison Duncan for welcoming me in Montpellier and opening her lab to me. It was a great opportunity to learn, not only with her but with many people in ISEM. Thank you also to Sophie Lefèvre for the help in the lab during my stay in Montpellier. Finally, I would also like to thank the members of the Juri for kindly accepting to examine this thesis.

Para além de toda esta teia de pessoas maravilhosas na ciência, esta tese não teria sido possível sem o apoio dos meus amigos. Um obrigado especial ao meu duderude Zé, ao meu puto Luís, ao meu grande Carlos, ao meu lindo Tapia e ao meu primo Óscar, tanto bro-love e geekness faz-me bem à alma! Queria ainda deixar aqui um grande beijinho a toda a minha família, com um especial obrigado aos meus pais e à minha mana Marta por acreditarem sempre em mim mesmo sem saber muitas vezes o que eu ando a fazer.

Por fim claro, um enorme e gigantesco obrigado à Leonor, por partilhar esta relação de incesto científico comigo, mas, acima de tudo, por me mostrar onde é que dois mundos paralelos se cruzam, no infinito do nosso amor. Obrigado por me mostrares que vale a pena. E obrigado também por teres feito crescer a minha família e grupo de amigos, emprestando-me os teus. Eu sei que todos eles se sentem incluídos no parágrafo anterior.

Obrigado a todos, pelo amor e amizade, sem eles a vida e a ciência não seriam a mesma coisa.

Abstract

Plant quality for herbivores depends on plant characteristics (e.g. nutrients and defences), abiotic conditions (e.g. metal contamination) and the presence of competitors and antagonistic species. The aim of this thesis was to characterize how differences in plant quality affect plant-herbivore and herbivore-herbivore interactions in two spider-mite species, *Tetranychus urticae* and *T. evansi*. First, we describe the creation of outbred and inbred populations, important biological tools in laboratory studies, which we use throughout the thesis. Subsequently, we tested how interspecific competition affected within- and between-plant dispersal. We show that the performance of both spider mite species was higher on younger leaves. Accordingly, they chose this stratum on clean plants. However, on plants with heterospecifis, *T. urticae* avoided younger leaves, whereas the distribution of *T. evansi* was more even across strata. The dispersal of *T. urticae* away from host plants also increased in presence of *T. evansi*. However, we did not find evidence for a genetic correlation between the damage inflicted on plants and dispersal propensity. The performance of the two species was also similar on tomato plants exposed to different cadmium concentrations, revealing a hormetic response in both. However, *T. evansi* is more affected by cadmium accumulation than *T. urticae*. Also, induction/suppression of proteinase inhibitors did not interact with cadmium accumulation and the latter mechanism was not inducible by herbivory. Finally, we showed intraspecific variation for cadmium-induced hormesis in spider mites, being this metal the main driver of the patterns observed, rather than changes in the plant.

This thesis establishes the powerful cadmium-tomato-mite system in the study of ecological consequences of metal accumulation and provides novel insight into the interaction between *T. urticae* and *T. evansi* under different scenarios.

Keywords: plant-herbivore interactions; metal accumulation; hormesis; spider mites; herbivore-herbivore interactions.

Resumo

A qualidade das plantas para os herbívoros depende de características da planta (e.g. nutrientes e defesas), de fatores abióticos (e.g. metais) e da presença de outros herbívoros e antagonistas na planta. O intuito desta tese foi caracterizar o efeito de diferentes qualidades da planta nas interações planta-herbívoro e herbívoro-herbívoro nos ácaros-aranha, *Tetranychus urticae* e *T.evansi*. Primeiro descrevemos a criação de populações com elevada consanguinidade e com elevada diversidade, ferramentas com importantes aplicações em estudos laboratoriais, que utilizámos durante a tese. Depois, estudámos o efeito da competição interespecífica na distribuição dos ácaros na planta. Em plantas sem heterospecíficos ambas as espécies tiveram maior desempenho reprodutivo em folhas jovens, preferindo colonizar estas. Em plantas com heterospecíficos esta preferência perdeu-se, substituída por diferentes comportamentos em resposta aos heterospecíficos. *T. urticae* evitando folhas com o competidor e *T. evansi* distribuindo-se uniformemente entre as folhas. *T. urticae* também aumentou a dispersão de hospedeiros com *T. evansi*. Não encontrámos, no entanto, uma correlação genética entre o dano causado à planta e dispersão para outro hospedeiro. O desempenho reprodutivo das duas espécies também mostrou ser afetado da mesma forma pela acumulação de cádmio em tomateiros, correspondendo a uma resposta hormética. Durante exposições mais longas *T. evansi* mostrou ser mais negativamente afetado pela acumulação de cádmio do que *T. urticae*. Não houve interações entre a indução/supressão de defesas do tomateiro (inibidores de proteínas) e acumulação de cádmio, sendo este último um mecanismo passivo, não induzido por herbivoria. Finalmente, mostrámos variação intraespecífica nos ácaros, na resposta hormética à acumulação de cádmio em tomateiros, sendo a hormesis potenciada diretamente pelo metal e não por alterações na planta. Esta tese demonstra a importância do sistema planta-metal-ácaro no estudo das consequências ecológicas da acumulação de metais e revelou novas perspetivas sobre as interações entre *T. urticae* e *T. evansi* em diferentes contextos.

Palavras-chave: interação planta-herbívoro; acumulação de metais; hormesis; ácaro-aranha; interação herbívoro-herbívoro.

Resumo alargado

A qualidade das plantas enquanto hospedeiras de artrópodes herbívoros é muito variável. Depende, não só de características da própria planta, como a quantidade de nutrientes e defesas em cada tecido, como também de fatores abióticos, como a contaminação dos solos por metais e também da presença de competidores e outros antagonistas na planta. Embora estas diferentes fontes de variação na qualidade das plantas estejam bastante bem descritas na literatura, a informação sobre como é que a interação entre alguns destes fatores afeta o desempenho reprodutivo e a distribuição de artrópodes herbívoros é mais limitada. Aumentar o conhecimento nesta área não só permitirá entender melhor as relações entre plantas e herbívoros como também as relações entre diferentes herbívoros que partilham as mesmas plantas.

O objetivo desta tese foi compreender como é que diferentes fontes de variação na qualidade da planta afetam duas espécies de ácaro-aranha, *Tetranychus urticae* e *T. evansi*, considerando também as possíveis consequências desses efeitos nas interações entre os dois herbívoros. Estas espécies de ácaro-aranha são importantes pragas agrícolas, infetando diversas plantas como o tomateiro, a espécie utilizada na maioria dos capítulos desta tese. Embora exista algum conhecimento sobre as interações entre as plantas do tomate e os ácaros-aranha, este restringe-se maioritariamente à indução e supressão de metabolitos secundários do tomateiro como resultado da herbivoria e às consequências destes processos no desempenho reprodutivo e crescimento populacional destes herbívoros. Nesta tese estendemos o estudo das interações entre o tomateiro e os ácaros-aranha a outros fatores como a contaminação dos solos por metais. Também estudámos as interações entre as duas espécies de ácaros, para além das estabelecidas através da indução e supressão de metabolitos nos tomateiros.

Começámos por desenvolver um protocolo para a criação de populações de laboratório com elevado grau de consanguinidade e com elevados níveis de variabilidade, dando especial enfase ao estudo de espécies com um sistema de reprodução haplodiplóide. Estas populações são ferramentas biológicas importantes porque possibilitam que as respostas obtidas em laboratório sejam mais representativas de populações naturais e permitem caracterizar a variação individual de diferentes caracteres (fisiológicos, comportamentais, etc.) e também testar correlações genéticas entre eles.

Em seguida estudámos o desempenho reprodutivo e a distribuição das duas espécies em folhas jovens e velhas de tomateiros, na presença e ausência de competidores heterospecíficos. Em plantas sem competidores, a distribuição das duas espécies reflete as diferenças no desempenho reprodutivo. Ambas preferem folhas jovens, onde a sua reprodução é maximizada. No entanto, em plantas infestadas por heterospecíficos a distribuição não reflete diferenças reprodutivas, sendo afetada pela localização dos heterospecíficos na planta. *T. urticae* evita o competidor mesmo que este ocupe as folhas preferidas, i.e. as jovens. Por outro lado, *T. evansi* distribui-se mais equitativamente entre folhas velhas e novas quando *T. urticae* está presente na planta, não evitando a localização do competidor. Estas diferenças podem estar relacionadas com a assimetria na competição entre as duas espécies e ajudam-nos a compreender a sua possível coexistência em escalas maiores.

Seguidamente, sendo os ácaros ectoparasitas de plantas, estudámos a relação entre o dano infligido à planta e a dispersão para outras plantas, à luz da hipótese do compromisso entre virulência e transmissão. Esta hipótese, central no estudo de interações parasita-hospedeiro, postula que um parasita, de forma a maximizar a sua transmissão para outros hospedeiros, multiplica-se, consumindo os recursos do seu hospedeiro. No entanto, este processo provoca danos no hospedeiro, i.e. virulência, podendo levar à morte do mesmo e desta forma diminuindo as oportunidades de transmissão. Assim, é esperado que a transmissão seja maximizada a níveis médios de virulência. No nosso estudo não encontrámos uma relação genética entre virulência e transmissão em *T. urticae*. No entanto, mostrámos que estes fatores são influenciados pela competição intraespecífica e interespecífica, com *T. evansi*. Isto sugere que, ainda que não haja uma relação genética entre virulência e transmissão, a relação entre estas características pode ser mediada pela competição entre parasitas que ocorre na planta hospedeiro.

Posteriormente, mostrámos uma resposta hormética em ambas as espécies de ácaro-aranha à acumulação de cádmio em tomateiros. O desempenho reprodutivo dos ácaros variou conforme a concentração de cádmio nos tomateiros a que foram expostos durante 4 dias: o desempenho reprodutivo aumentou a baixas concentrações de cádmio e diminuiu a altas concentrações deste metal, em comparação com o desempenho reprodutivo em plantas não expostas a cadmio No entanto, infestações de 14 dias em plantas expostas a baixas concentrações de cádmio, levaram a uma redução do crescimento populacional em comparação com o mesmo em plantas não expostas a cádmio. Estes resultados sugerem que a hormesis pode dever-se a uma compensação dos ácaros relativamente ao stress imposto pelo cádmio,

não levando a um aumento do crescimento populacional a longo prazo, mas podendo ser uma resposta suficiente para que o crescimento populacional não seja demasiado reduzido. Para além disso, mostrámos que a acumulação de cádmio não levou a custos aparentes no crescimento e fisiologia dos tomateiros e que é um processo passivo, não induzido por herbivoria. Também mostrámos que a indução / supressão de defesas do tomateiro (inibidores de proteínas) pelos ácaros não interfere com a acumulação de cádmio e vice-versa, revelando-se processos independentes e que podem ser utilizados em simultâneo pela planta.

Finalmente, mostrámos que existe variação intraespecífica nos ácaros para a resposta à acumulação de cádmio em tomateiros. De facto, algumas populações naturais de ambas as espécies mostraram uma resposta hormética à acumulação de cádmio, enquanto que outras não responderam da mesma forma, tendo apenas um decréscimo no desempenho reprodutivo com o aumento de cádmio nas plantas. Mostrámos também, através de testes com dieta artificial com diferentes concentrações de cádmio ou açucares, que estes padrões de resposta à acumulação de cádmio em tomateiros se devem maioritariamente a efeitos diretos do metal nos ácaros e não a alterações ocorridas na planta como resposta desta ao metal.

Em resumo, o trabalho desenvolvido nesta tese permitiu (a) estabelecer um novo sistema modelo para o estudo das interações entre herbívoros e plantas acumuladoras de metais e (b) aprofundar o estudo das interações entre os ácaros-aranha *T. urticae* e *T. evansi* e entre estes e as plantas. Curiosamente, as duas espécies mostraram não só respostas semelhantes à acumulação de cádmio, mas também variabilidade intraespecífica para esta resposta, abrindo espaço à especulação que fatores são responsáveis por estas diferenças. Também é notável o facto de i) a acumulação de cádmio não impor um compromisso com a produção de defesas do tomateiro como os inibidores de proteínas e ii) a resposta dos ácaros à acumulação de cádmio ser maioritariamente influenciada pelo próprio metal, em vez de ser influenciada por alterações da planta em resposta ao metal. Estes resultados sugerem que as duas estratégias de defesa da planta podem ser utilizadas em conjunto, criando duas pressões de seleção distintas sobre os herbívoros, visto que os metais, devido à sua natureza elementar, não podem ser destoxificados por enzimas utilizadas contra os metabolitos das plantas. Isto pode ter importantes consequências nas interações ecológicas e evolutivas entre plantas e herbívoros em ambientes contaminados por metais. Finalmente, os resultados obtidos nesta tese mostram que, na ausência de competição as duas espécies de ácaro aranha respondem de forma similar a diferenças na qualidade das plantas, mas, no entanto, a resposta de uma espécie à presença da outra difere entre as duas espécies. Estas diferenças e semelhanças entre as respostas das duas

espécies em escalas pequenas podem ter consequências importantes no resultado da competição entre elas em escalas maiores.

Em conclusão, os resultados apresentados nesta tese contribuem para um melhor entendimento das interações planta-herbívoro e herbívoro-herbívoro, em particular em ambientes contaminados por metais. Para além disso, os resultados proporcionaram o desenvolvimento de novas questões que podem impulsionar o conhecimento não só no contexto de ecologia de plantas e herbívoros em ambientes contaminados, mas também em contextos agroecológicos, dado os ácaros serem pragas agrícolas.

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Chapter I

General introduction

Herbivorous arthropods live and reproduce on plants. Therefore, the quality of a plant as a host has a major importance on the fitness of this group (Awmack & Leather 2002). Huge variety in quality can be found among the plant kingdom, among and within species and even within the same plant (Coley 1980; Raupp & Denno 1983; Walling 2000; Mithöfer & Boland 2012; Koricheva & Hayes 2018). The quality of a plant as a host rises not only from variability in traits that are intrinsic to the plant, such as the nutritional value of each tissue or the amount of physical and chemical defences, but also on abiotic and biotic factors that affect both the plant and /or the herbivores (Awmack & Leather 2002).

The niche width of a herbivorous arthropod, which is defined by the portion of a resource used by a species (Hutchinson 1957), is often measured as the host range of that herbivore, i.e. the number of plant species used as a host (Forister *et al.* 2015). Herbivores are generally divided according to their host range into generalists, if they use a wide range of host species, and specialists if they use a narrow range of host species, or in the extreme only one (MacArthur 1972; Jaenike 1990). However, because different tissues of a plant, e.g. leaves of different ages, may differ in quality, niche width may be smaller than host range. In any case, herbivorous arthropods are expected to choose their host, or different tissues within a host, according to differences in their performance (Jaenike 1978; Singer *et al.* 1988; Thompson 1988; Gripenberg *et al.* 2010). Given the importance of the quality of a plant for the fitness of herbivorous arthropods, all the existent variability has a crucial role in defining host choice. The differences in fitness and the patterns of host choice caused by variability in plant characteristics determines the fundamental niche of herbivores. However, the presence of antagonistic species in host plants may affect performance and choice of herbivores, determining their realized niche (Hutchinson 1957).

Herbivorous arthropods are a very variable group (Bernays 1998). Their interactions with different host plants are very diverse, even within the same herbivore species, different individuals or populations may have different performances on the same host plant species and / or chose different host plants species (Edwards 2001; Magowski *et al.* 2003; Ferrari *et al.* 2006; Kant *et al.* 2008; Zytynska & Preziosi 2011). However, some plant characteristics have generalized effects on herbivores. For instance, nitrogen is limiting for animals in general, since proteins are their main structural components and also because animals use nitrogen less efficiently than plants (Mattson 1980; Scriber & Slansky 1981). Therefore, the amount of nitrogen in plant tissues is commonly positively correlated with the performance of herbivores (Mattson 1980; Chen *et al.* 2008; Han *et al.* 2014, 2016; Kurze *et al.* 2017). The differences

and similarities in quality for different herbivores may have a strong influence on the overlap of the niche width of herbivore species and, thus, may also affect the degree of competition between them. In my thesis, I will focus on how different sources of plant quality affect the performance and preference of two species of spider mites, *Tetranychus urticae* and *T. evansi*. Additionally, I will discuss the consequences that the similarities and disparities in response between those two species may have for their niche overlap.

1. Sources of variation in the quality of a plant as a host

Differences in host quality for herbivores are ultimately quantified through variations in the fitness of the herbivores (Awmack & Leather 2002). Essentially, the factors that determine the fitness of herbivores are the nutritional quality of the plant tissue, the amount of defences produced and the presence of antagonistic species such as competitors, parasites or predators (Awmack & Leather 2002). Additionally, these characteristics, intrinsic to the plant or not, may vary according to abiotic conditions. In this section, I will outline the sources of variation in quality among and within plants and how those may affect the performance of herbivorous arthropods.

1.1. Variability intrinsic to the plant

1.1.1. Nutritional quality

Most of the nutritional differences among plant tissues stem from the amount of carbon and nitrogen (Awmack & Leather 2002). Carbon and nitrogen are key elements of essential amino acids, sugars and other nutrients, such as sterols and lipids (Mattson 1980; Bryant *et al.* 1983; Awmack & Leather 2002). As such, many studies used carbon and nitrogen as surrogates for digestible carbohydrates and essential proteins, respectively, and assessed the effects of those surrogates in the performance of herbivorous arthropods. However, carbon and nitrogen are also present in other non-nutritional molecules, which may undermine the power of such surrogates (Hamilton *et al.* 2001; Deans *et al.* 2016). Even though some studies have determined the optimal protein : carbohydrate for different herbivore species using artificial diets (Behmer 2009; Le Gall & Behmer 2014; Roeder & Behmer 2014), only recently has the performance of herbivores been correlated with amounts and ratios of proteins and digestible

carbohydrates quantified in plant tissues (Deans *et al.* 2016). Interestingly, even though nitrogen is generally limiting for herbivores, the optimal ratio between proteins and carbohydrates was shown to differ among herbivore species (Behmer 2009; Le Gall & Behmer 2014; Roeder & Behmer 2014). Moreover, other elements, macro and micronutrients, such as potassium, sodium, magnesium, among others, may also have an important nutritional value for herbivores (Joern *et al.* 2012). The amount of these elements present in plant tissues, as well as the amount of essential proteins and digestible carbohydrates is highly variable among and within plants (Joern *et al.* 2012; Deans *et al.* 2016; Wilson *et al.* 2019). For example, the protein : carbohydrates ratio and total amount of nutrients of younger leaves are normally higher than that of older leaves (Deans *et al.* 2016; Cao *et al.* 2018; Wilson *et al.* 2019), resulting in higher herbivore performances in the former.

1.1.2. Plant defences

Plants produce a wide array of defences against herbivores (Bennett & Wallsgrove 1994; Walling 2000; Kessler & Baldwin 2002; Howe & Jander 2008; Mithöfer & Boland 2012). These defences vary from physical structures, such as trichomes, wax, spines, to chemical compounds and volatiles (Walling 2000; Kessler & Baldwin 2002; Howe & Jander 2008; Rasmann & Agrawal 2009). Although the defensive pathways of different plant species, to different types of stresses rely mainly on the same phytohormones, such as jasmonic acid, salycilic acid and ethylene, the end products are extremely diverse (Walling 2000; Kessler & Baldwin 2002; Erb *et al.* 2012). Some groups of secondary metabolites are specific to certain plant orders or families and others are constrained to a given genus or even a single species. For example, the production glucosinolates, which are nitrogen-based secondary compounds, is restricted to plants of Brassicales order (Grubb & Abel 2006). An iconic defensive compound is nicotine that is mainly produced in plants of the genus *Nicotiana*, although present in vestigial amounts in other plants of Solanacea family (Baldwin 1999). Moreover, plants are able to recognize the identity of the herbivores, through the specificity of elicitors, and as a response, tailor their defence producing compounds that target a given herbivore taxon (Alborn *et al.* 1997; Bonaventure *et al.* 2011; Erb *et al.* 2012).

The production of defensive compounds is costly for the plants, requiring energy that otherwise would be used to growth or reproduction (Herms & Mattson 1992; Kessler & Baldwin 2002). Therefore, to reduce the cost of producing many different defences all the time, some plant defences are induced only upon exposure to a specific stressor (Karban & Myers 1989; Walling 2000; Kessler & Baldwin 2002; Howe & Jander 2008).There is also intraspecific

variation for the production of plant defences, some genotypes may produce more of one type of defences than other genotypes and some genotypes may be overall less defended than other genotypes (Hahn & Maron 2016). Moreover, there are shifts in the quality and quantity of defences during the ontogeny of plants and plant tissues (meta-analysied in Barton & Koricheva, 2010; Barton *et al.*, 2019). However, whereas these shifts in defences are variable between species for whole plant ontogeny, for leaf ontogeny the pattern is clearer (Barton & Boege 2017). In general, young leaves have higher amounts of chemical induced defences but lower amounts of constitutive defences (Barton *et al.* 2019).

Furthermore, the production of plant defences and the distribution of the elements within the plant may also be affected by the availability of those elements in the soil (Ballhorn *et al.* 2011; Gutbrodt *et al.* 2012; Ormeño & Fernandez 2012) and this, is expected to differ according to the geographical location and to several abiotic factors of the environment where plants are growing.

1.2. Abiotic effects on the quality of plants as hosts to herbivorous arthropods

Climate related abiotic factors, such as temperature, drought, carbon dioxide and UV exposure, may have a strong effect on the quality of plants for herbivores (Bidart-Bouzat & Imeh-Nathaniel 2008; Gutbrodt *et al.* 2011; Bauerfeind & Fischer 2013). Depending on the plant species, or genotype, the above-mentioned climatic variables may have stimulatory or inhibitory effects on the production of different secondary metabolites and, consequently, different outcomes on plant quality (Bidart-Bouzat & Imeh-Nathaniel 2008). In addition to the effects on secondary metabolites, carbon dioxide and drought also affect the plant nitrogen content (Koricheva *et al.* 1998; Coviella & Trumble 1999). In general, elevated carbon dioxide leads to lower amounts of herbivory, by increasing the amount of carbon on plants in relation to nitrogen (Coviella & Trumble 1999). In contrast, water deficit increases the amount of nitrogen per volume of plant tissue, increasing plant quality for herbivores (Koricheva *et al.* 1998). However, exceptions to these "rules" have also been shown, suggesting that these effects may depend on the species / genotypes involved (both of plant and of the herbivore), or on other overlooked co-variates (Huberty & Denno 2004; Bidart-Bouzat & Imeh-Nathaniel 2008; Mody *et al.* 2009; Robinson *et al.* 2012). Moreover, most of these abiotic factors are correlated with each other and, thus, their interactions may produce even more diverse patterns.

Furthermore, most of these abiotic factors have geographical and seasonal variations and these have major effects on the distribution of herbivores and, therefore, on community composition.

Within the same geographical region, plant quality for herbivores is often affected by the availability of nutrients in the soil. When essential nutrients for the plant, such as nitrogen and phosphorous, are scarce, plants often invest in slow growth, having less available biomass, and produce higher amounts of defensive compounds (Grime 1977; Mattson 1980). Indeed, several studies have shown that the performance of herbivores is lower when plants face a lack in macro and / or micronutrients (de Bruyn *et al.* 2002; Huberty & Denno 2006; Mundim *et al.* 2009; Visanuvimol & Bertram 2011). Other elements present in the soil, such as metals, may affect plant quality for herbivores (c.f. next section).

1.3. Metal accumulation: A plant defence derived from abiotic factors

Even though some heavy metals are essential at low concentrations, at high concentrations these elements are toxic to most organisms (Baker 1987). Still, some plants, termed metallophytes, are able to grow on metal polluted environments (Baker 1987). However, plants growing in these environments may be affected in several ways and, as a consequence, their quality as hosts for herbivorous arthropods. First, metal toxicity may hamper plant growth and biomass production (Foy *et al.* 1978; Maestri *et al.* 2010), restricting the amount of resources for herbivores. Secondly, the presence of metals in the soil may interfere with the acquisition of essential elements, affecting the plant nutritional value for herbivores (Larbi *et al.* 2002; Khan *et al.* 2016). Finally, metal toxicity may affect the production of plant defences. On the one hand, plants may require energy to deal with metal toxicity that could otherwise be used to produce defences against herbivores, creating a trade-off between the response to metal toxicity and to herbivory (Tolrà *et al.* 2001; Fones & Preston 2013; Fones *et al.* 2019). On the other hand, some compounds produced by the plant as a response to metal toxicity may also have adverse effects on herbivores, creating a synergistic effect between the response of plants to metal toxicity and to herbivory (Tolrà *et al.* 2006; Boyd 2007).

Furthermore, whereas some plants exclude metals on their roots, others are able to uptake them from the soil and accumulate them on their shoots, up to concentrations that are toxic to most organisms, a process termed metal accumulation (Baker 1987). Since metals are toxic to herbivores as well, accumulating them on their leaves may be used as an alternative defence mechanism against herbivores (Martens & Boyd 1994; Boyd 2004). This strategy, termed

elemental defence, differs from that relying on secondary metabolites in two main aspects. On the one hand, elemental defences are acquired from the soil, instead of being synthetized by the plant, which may be a less costly process (Boyd 2007). On the other hand, due to their elemental nature, metals cannot be chemically degraded by the detoxifying enzymes of herbivores, imposing a higher selective pressure on herbivores (Boyd 2007). Many studies have shown the effectiveness of metal accumulation against herbivores in laboratory conditions (Martens & Boyd 1994; Boyd & Moar 1999; Hanson *et al.* 2003; Jhee *et al.* 2005; Freeman *et al.* 2006, 2007; Rathinasabapathi *et al.* 2007; Quinn *et al.* 2010; Konopka *et al.* 2013), and in the field (Martens & Boyd 2002; Freeman *et al.* 2007; Galeas *et al.* 2008; Kazemi-Dinan *et al.* 2014).

1.4. Biotic effects on the quality of plants as hosts to herbivorous arthropods

Soil mediates the quality of plants to herbivores, not only through the elemental composition of the soil but also via soil organisms. On the one hand, the nutritional quality of the plant may be increased either by decomposers or by the presence of mycorrhizas, which enhance nitrogen absorption (Gehring & Whitham 1994; Kula *et al.* 2005; Haase *et al.* 2008). On the other hand, below ground biota may promote an increase in plant defences against above ground herbivores, decreasing the quality of the host plant (Bezemer & Van Dam 2005). Additionally, plants compete for nutrients in the soil, for light and for space. This may hamper plant growth, limiting the resources for herbivores (Rees & Brown 1992; Schädler *et al.* 2007).

In addition to these biotic effects, plant quality is also affected by the herbivores themselves. Indeed, by consuming the plant, herbivores reduce the amount of available resources, decreasing its quality over time (Awmack & Leather 2002). This may lead to density dependent effects of intraspecific competition on the performance of herbivores (Valladares & Lawton 1991; Rotem & Agrawal 2003). Additionally, plants respond to herbivory with inducible defences which affects their quality for herbivores (Karban & Myers 1989; Karban & Baldwin 1997). In most cases, induced defences decrease the performance of herbivores, making the quality of infested plants lower for subsequent infestations (Karban & Myers 1989; Karban & Baldwin 1997). However, plants may produce metabolites that are effective against one herbivore but non-effective against other herbivores (Felton *et al.* 1999; Thaler *et al.* 1999, 2002a). If the production of specific compounds is costly, a trade-off may arise, hampering the production of other compounds which could be used against other herbivores (Thaler *et al.*

2002a; Denno & Kaplan 2007). In this case, it may be beneficial to colonize infested plants. Additionally, some herbivores are known to increase the quality of the host plant and, in turn, the performance of herbivores on those plants (Musser *et al.* 2002; Sarmento *et al.* 2011a; Elzinga *et al.* 2014), a process termed facilitation (Stachowicz 2001). This can be achieved , either by stimulating the accumulation of nutrients in a given part of the plant or by suppressing the production of plant defences (Wool *et al.* 1999; Musser *et al.* 2002; Sarmento *et al.* 2011a).

Furthermore, the growth rate of herbivorous arthropods may be affected by other antagonistic species, such as predators or parasitoids, thus, their presence may influence the quality of a host plant as well (Murphy 2004; Singer *et al.* 2004).

2. Consequences of variability in plant quality

By affecting the performance of herbivores, all the sources of variation in host plant quality, detailed in the previous section, have the potential to mediate the distribution of herbivores among and within plants. In this section, I will review the effects of the several sources of variation in plant quality on host choice by herbivorous arthropods, as well as alternative mechanisms to cope with such variability quality, either determined by plant characteristics or by other biotic factors.

2.1. Consequences of variation intrinsic to the plant

2.1.1. Host choice driven by plant variability

The most limiting nutrient for herbivorous arthropods is nitrogen, as such, most herbivorous arthropods choose tissues with high contents of nitrogen (Minkenberg & Ottenheim 1990; Hoffland *et al.* 2000; Awmack & Leather 2002; Chen *et al.* 2008). Additionally, some herbivores, such as grasshoppers and caterpillars, have been shown to regulate their protein : carbohydrate ratio by differentially feeding on artificial diets with one or the other component or with different ratios of both (reviewed in Behmer, 2009). However, whether they move between and within plants to optimize that ratio is not known. Plant defences also affect the preference of herbivorous arthropods. Indeed, many species have been shown, in the laboratory and in natural settings, to avoid tissues or plants with higher amounts of constitutive and/or

inducible defences (Hägele & Rowell-Rahier 2000; Hoffland *et al.* 2000; Walling 2008; Alba *et al.* 2011). Still, some studies suggest that this behaviour differs according to the degree of specialization of the herbivore. On one hand generalists avoid defended tissues and, on the other hand, specialists, being able to sequester some metabolites which are toxic to predators, are attracted by tissues with high amounts of those metabolites (van Dam *et al.* 1995; van der Meijden 1996). This is in accordance with the fact that specialists are more tolerant to the defences of plants to which they are specialized than generalists (c.f. section 2.3.1 tolerance). Furthermore, the nutritional composition of different plants / plant tissues may interact with the defence composition in determining host choice, creating even more complex patterns (Behmer *et al.* 2002; Couture *et al.* 2016; Cao *et al.* 2018; Raubenheimer & Simpson 2018). Moreover, metal accumulation has also been suggested as a deterrent of herbivory. Laboratory studies have shown that herbivores avoid metal-accumulating plants (Hanson *et al.* 2003; Jhee *et al.* 2005; Gonçalves *et al.* 2007; Rathinasabapathi *et al.* 2007) and field surveys revealed that metal-accumulating plants harbour less herbivores than neighbouring non-accumulating plants, suggesting deterrence as well (Freeman *et al.* 2007; Galeas *et al.* 2008; Kazemi-Dinan *et al.* 2014).

2.1.2. Alternatives to deal with plant variability

Selection of "good" hosts and/or avoidance of "bad" hosts is not the only mechanism to deal with differences in plant quality. Alternative strategies to cope with the different sources of plant quality are present in herbivorous arthropods and may interact with host choice in determining the distribution of herbivores.

2.1.2.1. Nutritional rescue

Instead of losing time and energy foraging for high nutritional plant tissues, some herbivores may use strategies that allow them to feed on plant tissues with low nutritional value. For that, some species harbour on their digestive track microbes that enhance their digestion (Hansen & Moran 2014). One notorious example is that of aphids which feed on the phloem of plants (Douglas 1998). Phloem is rich on carbohydrates and has low amounts of nitrogen. Aphids compensate for this nutritional restriction by harbouring bacterial symbionts that enable them to modify non-essential amino acids to essential amino acids (Douglas 1998).

2.1.2.2. Dealing with plant defences

Instead of avoiding plants and plant tissues with high amounts of defences, herbivores may use alternative strategies to surpass the negative effects of such defensive compounds. In this section I will detail some of those strategies.

2.1.2.2.1. Tolerance

As previously referred to, plants produce a myriad of different defences against herbivores (Walling 2000; Kessler & Baldwin 2002; Howe & Jander 2008; Mithöfer & Boland 2012). As these defences exert a strong selective force upon herbivores, it is expected that some herbivores adapt to specific compounds they are exposed to and become tolerant (Bernays 1998; Rosenthal & Berenbaum 2012). Indeed, some herbivores have metabolic adaptations that allow them to detoxify and digest plant secondary metabolites (Després *et al.* 2007; Barbehenn & Peter Constabel 2011). Others harbour bacterial symbionts that provide that function (Douglas 2013; Hansen & Moran 2014). Most of the detoxifying enzymes target specific compounds and are more common on specialist herbivores that use hosts from a narrow plant taxa where such compounds are present (Després *et al.* 2007; Ramsey *et al.* 2010). However, some exceptions are known for generalist species that are able to detoxify and metabolize several plant secondary metabolites (Ramsey *et al.* 2010; Grbić *et al.* 2011; Acuña *et al.* 2012). These herbivores contain in their genome genes that code for a wider variety of detoxifying enzymes than that of specialists, or express detoxifying enzymes with a broader spectrum of subtracts but are less efficient against particular plant metabolites (Li *et al.* 2003; Després *et al.* 2007; Ramsey *et al.* 2010).

Similarly, the magnitude and prevalence of the negative effects of metal accumulation on herbivory varies according to the herbivore feeding guild and the degree of specialism (Jhee *et al.* 2006; Wall & Boyd 2006; Konopka *et al.* 2013; Kazemi-Dinan *et al.* 2014). Cadmium and nickel accumulation by different Brassicaceae plants was shown to affect chewing herbivores, whereas phloem sucking aphids were not affected (Jhee *et al.* 2005; Konopka *et al.* 2013). In addition, generalist herbivores were shown to be more affected by cadmium and zinc accumulation than specialists (Wall & Boyd 2006; Kazemi-Dinan *et al.* 2014). This suggests that some herbivores are more tolerant to metal defences than others. Indeed, some herbivores are able to circumvent metal toxicity by producing metallothionine enzymes or by accumulating non-toxic methylated forms of the metal (Cobbett & Goldsbrough 2002; Freeman *et al.* 2006). Given these evidences for interspecific variability in the response of

herbivores to metal accumulation, intraspecific variation is also expected to occur. However, little is known regarding this topic (but see Freeman *et al*., 2006)

2.1.2.2.2. Suppression

One particular way to circumvent plant defences is by suppressing their production. This ability has been shown for several species, including viruses (Burgyán & Havelda 2011), bacteria (Zhao *et al.* 2003; Abramovitch *et al.* 2006), nematodes (Haegeman *et al.* 2012; Goverse & Smant 2014) and several arthropod species (Musser *et al.* 2002; Sarmento *et al.* 2011a; Chung *et al.* 2013; Elzinga *et al.* 2014; Godinho *et al.* 2016). This strategy consists in inhibiting the production of plant secondary metabolites, at least to some extent, avoiding their negative effects on the performance of herbivores (Blaazer *et al.* 2018). Some organisms are able to manipulate the hormone crosstalk of their hosts, triggering responses that are inefficient against their herbivory while hampering the hormone pathway that signals the compounds to which they are most susceptible (Stahl *et al.* 2018). Other organisms sabotage the molecular machinery of their hosts (Khan *et al.* 2018). Suppression of plant defences increases the quality of the host plant not only for the suppressor but also for all herbivores that would be negatively affected by the suppressed compounds (Sarmento *et al.* 2011a; Alba *et al.* 2015; Godinho *et al.* 2016). Therefore, suppressed plant tissues may be more appealing to heterospecifics leading to higher rates of exploitation of the host plant in the long term. Thus, suppression may only be fruitful if the suppressing species is able to exploit the resources better than the species with whom it may share them.

2.1.2.2.3. Hormesis

Most toxic compounds have a linear negative effect on organisms or no effect if the organism is resistant. However, the response to toxic compounds may have different patterns, including non-linear ones. For instance, some toxic compounds may produce hormetic responses, in which mild doses have a stimulatory effect and higher doses produce negative effects (Calabrese *et al.* 1999; Calabrese 2013). A hormetic response to several toxic compounds, including pesticides and metals, have been shown for different species of arthropods (Zhang *et al.* 2009; Cordeiro *et al.* 2013; Guedes & Cutler 2014). Thus, it is possible that some herbivores have a hormetic response to plant defences as well.

2.2. Consequences of the presence of competitors (and other antagonistic species)

A major source of variation in the quality of a plant as a host is the presence of competitors and other antagonistic species that may hamper the performance of herbivorous arthropods. Competition occurs when several individuals share the same limiting resource(s) and it can be scramble, when the individuals deplete a given resource by using it, or contest, when individuals interfere with one another to gain access to the resource (Birch 1957). Additionally, competition can be apparent, when is mediated through another trophic level (normally the shared resource), for example the induction of plant defences, which hampers the performance of any herbivore consuming that plant (c.f. section 1.4). Competition is divided in intraspecific, when the individuals competing belong to the same species, or interspecific, when the individuals belong to different species (Connell 1983). The balance between these two types of competition may have important consequences in determining species coexistence or exclusion (Connell 1983; Chesson 2000). Indeed, intraspecific competition is usually considered as a force driving niche expansion, whereas interspecific competition generally results in a narrower niche width (Connell 1983). Competition can be assessed by measuring the growth rate of one species in scenarios differing in the number of individuals (either of the same species or of different species) sharing a given amount of resources, or in scenarios where the number of competitors is maintained but the quantity of resources is manipulated (Inouye 2001; Rodrigues *et al.* 2016). For the above-mentioned reasons is clear that competition has a strong role in determining the distribution of species and community composition. Herbivorous arthropods are not exception, if the competing species have similar fundamental niches, they will prefer the same host plants and plant tissues. Thus, herbivores may be faced with a decision of choosing between lower quality plants or plants with competitors.

2.2.1. Host choice driven by the presence of competitors (and other antagonistic species)

Besides factors that are intrinsic to the plant, other organisms may also affect the performance of herbivores and, as a consequence, determine host choice. Indeed, the enemy-free space theory suggests that herbivores may avoid plants or plant tissues with higher nutritional quality or less defences if those are occupied by antagonistic species such as competitors, predators or pathogens (Ballabeni *et al.* 2001; Mulatu *et al.* 2004; Murphy 2004). Avoidance of plants with competitors has been shown for many species of herbivorous arthropods, including butterflies, thrips and mites (Pallini *et al.* 1997; Yoshimoto 2009; Zhang *et al.* 2019). However, herbivores may also use the presence of conspecifics, or heterospecific competitors, as a proxy for good

quality of the food resource. Indeed, some herbivores were shown to prefer plants with conspecifics against un-infested plants (Bolter *et al.* 1997; Landolt *et al.* 1999). Thus, avoidance or attraction to plants infested with other herbivores may depend on characteristics of the environment (i.e. the quality of neighbouring host plants) or on the strength of competitive interactions. Moreover, besides affecting choice between hosts, the presence of competitors may also affect choice for different strata within a plant (Dechert & Ulber 2004; Cédola *et al.* 2013; Gómez *et al.* 2014). Furthermore, other antagonistic species may affect the distribution of herbivores. Indeed, several species of herbivorous arthropods are known to avoid plant tissues with predators or to select host plants that provide better refuge and allow higher offspring survival (Ballabeni *et al.* 2001; Mulatu *et al.* 2004; Heisswolf *et al.* 2005; Ninkovic *et al.* 2013). Additionally, herbivorous arthropods are also known to avoid plant tissues with bacteria or parasitoids (Singer *et al.* 2004; Zélé *et al.* 2019).

Together, factors intrinsic to the plant and the presence of antagonistic species have a strong role in shaping the distribution of herbivorous arthropods between and with plants. However, the literature regarding the interactions between these different sources of plant quality on the choice of herbivorous arthropods is not extensive.

2.2.2. Alternatives to deal with the presence of competitors

Depending on the composition of the host plants available on a given environment, competition for the best quality hosts may be intense. Additionally, the quality of plants does not always decrease with herbivory (Karban & Baldwin 1997; Awmack & Leather 2002). Indeed, some herbivores suppress plant defences making the host more profitable (Musser *et al.* 2002; Sarmento *et al.* 2011a; Elzinga *et al.* 2014). Also, by responding to the presence of one herbivore species, plant defences may become impaired, and that host may be unable to respond to a second herbivore attack if the specificity of the secondary compounds is different (Thaler *et al.* 1999, 2002b; Stout *et al.* 2006; Erb *et al.* 2011). For these reasons, avoiding competition may not always be the most profitable strategy. Therefore, other mechanisms to deal with competitors have evolved in herbivorous arthropods.

Herbivorous arthropods may be considered as plant ectoparasites, as their populations grow as a result of the exploitation of the resources of their host plant and then they disperse to other host plants. Since the infection of one host plant is ephemeral, the fitness of one population infecting a plant is linked with the number of secondary infections (R_0), i.e. the number of new hosts it will infect (Anderson & May 1982). Therefore, the fitness of parasites depends both on

within-host dynamics (i.e. population growth within a host) and between host dynamics (i.e. dispersal to other hosts) and this relation is commonly described by the virulence transmission trade-off hypothesis. This hypothesis postulates that in order to transmit themselves to other hosts, a parasite increases in numbers by exploiting the host, which increases its virulence, i.e. the damage inflicted to the host (Anderson & May 1982). However, damage inflicted may lead to host death which may diminish the length of infection and, consequently, lead to lower transmission. Therefore, transmission is predicted to be maximized at intermediate levels of virulence (Anderson & May 1982), and this has been empirically demonstrated in parasites of arthropods and plants (de Roode *et al.* 2008; Doumayrou *et al.* 2013). By reducing the per capita amount of host resources, the presence of competitors affects the within-host growth rate of parasites, which hampers transmission to other hosts, decreasing the fitness of that parasite. One strategy some herbivorous arthropods may display to deal with competition is to increase competitive ability in the presence of heterospecifics, increasing consumption and oviposition rates (Joshi 2004; Sanders *et al.* 2005).However, this results in higher exploitation rates and increases damage on the host (Levin & Bull 1994; Choisy & de Roode 2010). Consequently, it may lead to a faster over-exploitation of the host that may potentiate its death, decreasing the infection duration (van Baalen & Sabelis 1995). Ultimately this may hamper the production of offspring and transmission to other hosts. Additionally, intraspecific competition may negatively affect within-host population growth of herbivorous arthropods, since the efficiency in converting host resources into offspring is density dependent (Ebert *et al.* 2000; Rotem & Agrawal 2003). Thus, for these parasites, independently of being in a single or in a co-infection, this strategy to increase exploitation rate may be more efficient in the beginning of the infestation period, whereas at later stages of infestation, avoiding competition by dispersing to other hosts may become a better option.

Choosing competition-free hosts and increasing the exploitation rate may not be sufficient to keep competitors at bay. Instead, some strategies may allow herbivorous arthropods to monopolize the resources and hamper exploitation by competitors (Weir & Grant 2004). This can be done by rapidly occupying several high quality host patches and taking advantage of inhibitory priority effects (Fukami 2015; de Meester *et al.* 2016). Indeed, the order of arrival on a host plant has strong effects on the outcome of competition between different species (Viswanathan *et al.* 2005; Stam *et al.* 2018; Peñaflor *et al.* 2019). In contrast, some herbivores are known to profit from aggregation by constructing structures that impede competitors to reach host resources (Inbar 1998; Morimoto *et al.* 2006). A well know example of this strategy

is gall formation by aphids (Inbar 1998; Wool *et al.* 1999). Galls are structures that aphids make on leaves and stems, that function as a sink of resources. Each colony of aphids grows inside the gall, profiting from the diversion of host resources and protects it against invasion by intraspecific and interspecific competitors (Inbar 1998; Wool *et al.* 1999). Another example is web making by spider mites. These herbivores are known to produce web on top of plant tissues, feeding and laying eggs below the web, thus, preventing competitors from using their feeding sites (Morimoto *et al.* 2006; Sarmento *et al.* 2011b). Some of these strategies, namely gall formation and web spinning, may also be used against other antagonistic species such as predators or parasitoids (Price *et al.* 1987; Lemos *et al.* 2010).

3. Study system

In my thesis I worked mainly with two species of herbivorous spider mites, *Tetranychus urticae* and *T. evansi*. In this section I will briefly provide a general description of their biology (3.1), as well as what is known regarding their interactions with host plants (3.2) and with competitor species infesting those hosts (3.3).

3.1. Biology of spider mites

Spider mites (Tetranychidae) are herbivorous arthropods which feed on the mesophyll of leaf cells by piercing their wall and sucking the content (Helle & Sabelis 1985). The life cycle of spider mites is usually short, going from egg to adult in less than 14 days, passing through one larvae and two nymph stages, intercalated with sessile quiescent periods (Helle & Sabelis 1985). Spider mites have sexual dimorphism and are haplodiploids with an arrhenotokous genetic system, where males are haploid, stemming from unfertilized eggs, whereas females are diploid, resulting from fertilized eggs (Helle & Sabelis 1985). The short life cycle and the fact that each female may lay up to 200 eggs lead to a fast population growth rate, possibly causing severe damage upon plants. During this process, gravid females disperse to other parts of the plant or to other host plants (Smitley & Kennedy 1985). Due to the fast-growing rate of their populations and because spider mites have a wide distribution and infest many economically important plants such as bean (*Phaseolus vulgaris*) and tomato (*Solanum lycopersicum*), they are considered major agricultural pests (Helle & Sabelis 1985). Furthermore, spider mites produce a silken web on top of the leaf surface, feeding and laying

eggs below this web, where they are protected from predators (Sabelis & Bakker 1992; Lemos *et al.* 2010). Spider mites are also known to rapidly adapt to pesticides (Van Leeuwen *et al.* 2012). The outbreaks of these species are, thus, hard to control, making the study of their interactions with and in their host plants very relevant because such knowledge may help in the management of the crops they infest.

Tetranychus urticae is a generalist species occurring in more than 1000 host species with a wide distribution around the world (Migeon *et al.* 2010). Populations of this spider mite rapidly adapt to new host species with little or no costs (Magalhães *et al.* 2007, 2009; Sousa *et al.* 2019).

Tetranychus evansi is a specialist of plants of the Solanaceae family, with some occurrences being registered on plants from other families (Migeon *et al.* 2010). Being endemic to South America, it has, on the past decades, invaded several countries in Africa and in the Mediterranean Basin, where it has spread rapidly and became a major economical concern (Boubou *et al.* 2012).

3.2. Interactions with host plants

The effect of herbivory by *T. urticae* is well described for several plant species, such as tomato, cucumber, maize, barley, grapevines, or *Arabidopsis*, both at physiological and molecular levels (Kant *et al.* 2004; Díaz-Riquelme *et al.* 2016; Rioja *et al.* 2017; Bui *et al.* 2018; Santamaría *et al.* 2018). In most reported cases, *T. urticae* induces secondary metabolites on the diverse plant species. Still, some genotypes that suppress the defences of tomato plants were identified from one population (Kant *et al.* 2008). Other strategies of *T. urticae* to deal with plant defences are well described. Sequencing of its genome revealed the presence of genes coding for several xenobiotic detoxification enzymes that can modify a wide range of compounds (Grbić *et al.* 2011; Wybouw *et al.* 2014). Moreover, the expression of different detoxifying enzymes was shown to depend on host adaptation, since the transcriptomic profile of *T. urticae* differs according to the species it is adapted to (Wybouw *et al.* 2015). Another mechanism found in *T. urticae* is the presence of genes coding for cleavage dioxygenases that may be used to metabolize aromatic compounds present in plant secondary metabolites (Grbić *et al.* 2011).

Although *T. urticae* is a generalist species, able to use many different plant species as a host, differences in plant quality still determine host choice in this species. Populations of this species adapted to or collected on some host plants showed low preference and acceptance of new host species (Yano *et al.* 1998; Agrawal *et al.* 2002; Magalhães *et al.* 2009). Moreover, when given the choice of plant tissues with different amounts of nitrogen, *T. urticae* was shown to prefer plant tissues with higher amounts of nitrogen (Hoffland *et al.* 2000; Chen *et al.* 2008).

Tetranychus evansi has the remarkable ability to suppress plant defences, leading to higher herbivore performances on those plants (Sarmento *et al.* 2011a). Most studies on the interactions of *T. evansi* with its host plants were performed using tomato plants. However, the suppression mechanism was recently described for other host species (Paulo *et al.* 2018). Little is known regarding this mechanism, even though it may have important consequences on the performance of suppressed plants and of herbivores infesting those plants. Additionally, knowledge regarding the effect of plant quality on host choice by *T. evansi* is also lacking.

Even though the interactions of spider mites with host plant defences are well described, especially those regarding tomato plants, little is known on how metal accumulation affects these herbivorous arthropods. So far, only two studies focus and this topic, showing that *T. urticae* is negatively affected by nickel accumulation by *Streptanthus polygaloides* and selenium accumulation by *Stanleya pinnata* and *Astragalus bisulcatus* (Jhee *et al.* 2006; Quinn *et al.* 2010). No information regarding the effect of metal accumulation on *T. evansi* is, to my knowledge, available. Some varieties of tomato plants are able to accumulate cadmium on their leaves (Gratão *et al.* 2008). Thus, it is a good candidate plant not only to unravel the effects of cadmium accumulation on these two species of spider mites but also to tackle the study of the possible interactions that metal accumulation and plant defences may have on their effects on herbivory.

3.3. Interactions with competitors

Since the invasion of the Mediterranean basin by *T. evansi*, the abundance and host range of several resident species, including *T. urticae*, has been reduced (Ferragut *et al.* 2013). In laboratory studies, *T. urticae* was shown to be outcompeted by *T. evansi* when sharing the same tomato plant (Sarmento *et al.* 2011b). However, later studies showed that the effects of *T. evansi* on the performance of *T. urticae* and vice versa may vary among populations and may also depend on the order or arrival on the host plant (Orsucci *et al.* 2017; de Oliveira *et al.*

2019). Furthermore, knowledge on how the presence of these species may affect host choice by the competitor is lacking. *T. urticae* has been shown to avoid plants with other herbivorous species (Pallini *et al.* 1997), thus, it might as well avoid plants with *T. evansi*. For *T. evansi*, no information regarding the effect of heterospecifics on host choice is, to my knowledge, available.

4. Outline of the thesis

The main goal of this thesis was to characterize the effects of different sources of plant quality on the performance and preference of two species of herbivorous spider mites, *T. uritcae* and *T. evansi*.

I begin by describing the creation of outbred and inbred populations of both species of spider mites, from diverse field populations collected in different locations in Portugal (Chapter II). These are important biological tools to characterize responses on laboratory conditions while maintaining a good representation of the responses one might find in natural conditions. Further on, I focus on how within plant differences in quality, intrinsic to leaf age, affect the performance of these two species of spider mites and choice between old leaves and young leaves, in the presence and absence of heterospecifics (Chapter III). Subsequently, I address how host damage and dispersal of *T. urticae* is affected by intraspecific competition (i.e. density on the host) and interspecific competition (i.e. the presence of *T. evansi*; Chapter IV). In the following chapters I describe the effect of cadmium accumulation by tomato plants on the performance of these herbivores, as well as the interactions between metal accumulation and induction / suppression of plant defences (Chapter V) and how simultaneous exposure to cadmium and spider-mite herbivory affects tomato plants and the growth rate of spider mites on those plants (Chapter VI). Finally, I focus on intraspecific variation of these two species for the response to the direct effects of cadmium and to the indirect effects of cadmium accumulation derived from physiological changes in tomato plants (Chapter VII). I conclude by summarizing and contextualizing the main results of this thesis, providing some possible future directions that may follow up on those results (Chapter VIII).

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Chapter II

Creating outbred and inbred populations in haplodiploids to measure adaptive responses in the lab

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Accepted for publication in Ecology and Evolution (doi: 10.1002/ece3.6454)

Abstract

Laboratory studies are often criticized for not being representative of processes occurring in natural populations. One reason for this is the fact that laboratory populations generally do not capture enough of the genetic variation of natural populations. This can be mitigated by mixing the genetic background of several field populations when creating laboratory populations. From these outbred populations it is possible to generate inbred lines, thereby freezing and partitioning part of their variability, allowing each genotype to be characterized independently. Many studies addressing adaptation of organisms to their environment, such as those involving quantitative genetics or experimental evolution, rely on inbred or outbred populations, but the methodology underlying the generation of such biological resources are usually not explicitly documented. Here, we developed different procedures to circumvent common pitfalls of laboratory studies, and illustrate their application using two haplodiploid species, the spider mites *Tetranychus urticae* and *T. evansi*. First, we present a method that increases the chance of capturing high amounts of variability when creating outbred populations, by performing controlled crosses between individuals from different field-collected populations. Second, we depict the creation of inbred lines derived from such outbred populations, by performing several generations of sib-mating. Third, we outline an experimental evolution protocol that allows the maintenance of a constant population size at the beginning of each generation, thereby preventing bottlenecks and diminishing extinction risks. Finally, we discuss the advantages of these procedures and emphasize that sharing such biological resources and combining them with available genetic tools will allow consistent and comparable studies that greatly contribute to our understanding of ecological and evolutionary processes.

Keywords: experimental evolution, quantitative genetics, laboratory studies; spider mites; biological resources

Introduction

Understanding the processes that shape individual traits and ecological processes in natural populations is arguably the ultimate aim of evolutionary ecology. This can be achieved by studying populations in their natural environment (Arnold 1983). However, this approach suffers from the difficulty in controlling several environmental variables simultaneously (Lauder et al. 1993). Laboratory studies, in contrast, while allowing for controlled variables, are often criticized for not being representative of the processes occurring in natural populations (Aguilar et al. 2005, Calisi and Bentley 2009, Melvin and Houlahan 2012). This is partly because it is not possible to recreate the complexity of the natural environment in the laboratory (Carpenter 1996, Calisi and Bentley 2009). Another reason is that laboratory populations often do not harbour sufficient variability to produce representative responses. Indeed, some studies have shown that laboratory populations have lower genetic variability than natural populations (Norris et al. 2001, Stohler et al. 2004, Bian et al 2015), possibly due to bottlenecks during the establishment and maintenance of the population, or to the long-term adaptation to the same environment, i.e. the laboratory conditions (Matos et al. 2000, Stohler et al. 2004, Aguilar et al. 2005, Santos et al. 2012). However, the lack of representativity of laboratory populations may also be related with the origin and the procedures involved in the creation of such populations (Berthier et al. 2010). Natural populations of the same species may be genetically differentiated and/or harbour different genetic compositions, shaped by different geographic and environmental factors (Aguilar et al. 2005, Nunes et al. 2008, Langley et al. 2012, Bian et al. 2015). Thus, laboratory populations founded by individuals collected from a single field population may not produce representative responses, even if the sample size is enough for the sample to be representative of that population.

To ensure that data obtained in the laboratory reflects the range of possible responses found in natural populations of the species under study, the ancestral population should reflect the variability found in the field (MacDonald and Long 2004, Nunes et al. 2008, Faria and Sucena 2017). Several studies have used laboratory populations founded by a large number of individuals collected in the field and maintained at high numbers in the laboratory (e.g., Mery and Kawecki 2002, Magalhães et al. 2007, Teotónio et al. 2009, Martins et al. 2013). However, this method falls short on accounting for potential geographical variation in trait values across populations. To ensure this, some authors have produced outbred populations by merging clones, inbred lines (Zbinden et al. 2008, Kover et al. 2009, King et al. 2012), or field

populations (Tucic et al. 1995, Fricke and Arnqvist 2007) collected at different locations. This option increases the chance of obtaining a population containing genotypes from different environments, thus potentially representing different subsets of the genetic variability of a species. Yet, this procedure does not preclude the possibility that one (or a set of) genotype(s) from a particular environment is over-represented in the final population. To circumvent this caveat, in sexual organisms, it is desirable to create an outbred population with an equitable representation of the genotypes present in several field populations, which can be achieved by performing controlled crosses between individuals of different populations.

Using outbred populations not only increases the representativity of the observed responses but also the robustness of comparisons between studies performed in different laboratories, if these populations are shared (Churchill et al. 2012). Thus, with the exception of studies on local adaptation, such as common garden and reciprocal transfers experiments (Kawecki and Ebert 2004, Blanquart et al. 2013), and of other studies aiming at comparing populations, most types of laboratory studies may benefit from using outbred populations. In particular, understanding the process of adaptation to specific selective pressures, in controlled laboratory conditions, with experimental evolution and quantitative genetic methodologies, requires the usage of populations with large amounts of variability (Kawecki et al. 2012, Svenson et al. 2012).

Experimental evolution follows adaptation of populations exposed to specific selection pressures in real time (Gibbs 1999, Kawecki et al. 2012). Hence, it allows measuring the process of adaptation itself instead of inferring it based on observed patterns, and to infer causality. This method consists in deriving populations from a common ancestral and exposing them to specific controlled environments during several generations, which enables (a) knowledge of the ancestral state of populations (i.e., the ancestral population from which all others were derived), (b) the possibility to define and control the environments that populations are exposed to, and (c) replication at the population level (Magalhães and Matos 2012). The explanatory power of experimental evolution can be used to unravel how populations adapt to environmental changes, to the presence of antagonists or to different population structures (Macke et al. 2011, Kawecki et al. 2012, Rodrigues et al. 2016, Zélé et al. 2018a). Additionally, this method can be used to measure convergent evolution of different populations to a common environment (e.g. the laboratory; Simões et al. 2008, Fragata et al. 2014). In any case, adaptation of non-microbial organisms to rapid environmental changes relies mostly on the standing genetic variation present in a population, rather than on the arrival of new mutations

(Hermisson and Pennings 2005, Barret and Schluter 2008, Sousa et al. 2019). Thus, for the establishment of experimental evolution populations, it is crucial to generate and maintain populations with large genetic variability in the laboratory, being the above-mentioned outbred populations an excellent tool for that purpose. Moreover, some populations may crash during the evolution process. Therefore, it is useful to design methods that maximize the prevention of such events.

Quantitative genetics uses several designs to evaluate the genetic vs. environmental contribution to a particular phenotype (Falconer and Mackay 1996). In such studies, it is important that the population used to infer these contributions is sufficiently variable. Some designs rely on a panel of inbred lines, which allows to identify any quantitative trait loci involved in one phenotype (Mackay 2004). To ensure that this panel is composed of different genotypes, it is important to derive it from a highly outbred population (e.g. Mackay et al. 2012, King et al. 2012). Such panel can then be used to measure the broad-sense heritability of a given trait, as well as genetic correlations between traits (e.g. Travers et al. 2015, Howick and Lazzaro 2017, Wang et al. 2017, Lafuente et al. 2018, Everman et al. 2019. Although the most famous and complete panels are found in *Drosophila* (DGRP – Mackay et al. 2012, DSPR - King et al. 2012; GDL – Grenier et al. 2015), this resource has also been used in plants (Kover et al 2009, Wills et al. 2013) and other animals (Table 1). Outbred populations themselves may also be useful in quantitative genetic designs (Svenson et al. 2012, Woods 2014). In contrast with inbred lines, where each line represents a fixed allelic combination, individuals from outbred populations are maintained in randomized recombinant crossings. Therefore, from an outbred population one can retrieve a much higher amount of allelic combinations, allowing a fine mapping of complex phenotypes (Svenson et al. 2012, Woods 2014).

Here, we describe the creation of the above-mentioned biological tools, outbred populations and inbred lines, using protocols focused on haplodiploid systems. The creation of hybrid populations using controlled crosses in haplodiploids has an extra layer of complexity (as compared to diploid species). This is because in these systems whereas females stem from fertilized eggs, haploid males stem from unfertilized eggs and thus crosses between different genotypes / populations only generate hybrid diploid females; hybrid males, instead can only stem from unfertilized eggs of these hybrid females. As a case study, we describe the creation of outbred populations for two species of haplodiploid spider mites. This was done by performing controlled single crosses between individuals of different populations, within each species, in round-robin and matched crosses designs. From one of these outbred populations

we also created inbred lines through 15 generations of sib-mating, for which we also present a method to calculate the coefficient of inbreeding through time, adapted to haplodiploid species, as well as a method to calculate the probability of having a fully inbred line. Finally, we provide a general description of an experimental evolution protocol, which includes a back-up for each experimental population that can be used to replenish the population when needed, maintaining a constant population size at each transfer and minimizing the risk of extinction of such populations. With this work, we aim to provide the community with protocols that can be easily applied, not only to this, but to other systems.

Collection of field populations

In order to maximize the representativity of responses observed in laboratory studies, field populations used to create outbred populations should be sampled at different locations. Here, we use spider mites (Acari: Tetranichidae) which are haplodiploid pests widespread in many agricultural crops (Migeon et al. 2010). Given their small size and life-cycle characteristics, these species are easily reared and maintained in high numbers in the laboratory. We surveyed several tomato (*Solanum lycopersicum*) fields and greenhouses in Portugal for the presence of Tetranychid mites between May and October 2017 (Fig. 1). Each location was sampled during ca. 1 hour. Tomato leaves infested with spider mites were collected and kept in a closed plastic box. If the tomato plants were free of spider mites, neighbouring plants from other species were also screened. All collected spider mite populations were established in the laboratory by transferring adult females (N= 32 to 463, depending on the density of spider mites found on a given location; Table 2) to a rearing cage containing tomato leaves (variety Moneymaker). Since arrival to the laboratory the populations were maintained under controlled conditions (23.5 ± 2 ºC, 60% RH, 16/8 h L/D) for a few generations (3 to 6), populations were left untouched to promote laboratory adaptation and thus foster an increase in population size (> 300 adult females). Subsequently, each population was identified at the species level by performing a multiplex PCR on a pool of 50-100 spider mites (Zélé et al. 2018b, detailed in Supp.1). A total of 27 populations were collected in 24 different locations (Table 2). Sixteen of those were identified as *Tetranychus cinnabarinus* (also referred to as the red form of *T. urticae*; Auger et al. 2013), 4 as *T. urticae* (green form) and 7 as *T. evansi*. In 8 locations, there were no spider mites infesting tomato plants but on 4 of those, spider mite populations were found on neighbouring plants (Table 2).

Table 1. Inbred line panels in different animal species

Organism	**Characteristics**	**# of lines**	**Reference**
Drosophila melanogaster	"DGRP" founded from 1 outbred population (1500 mated females).	192/205	Mackay et al. 2012; Mackay et al. 2018
Drosophila melanogaster	"DSPR" founded from 2 outbred populations, created from 15 isolines and recombined for 50 generations, followed by 25 generations of inbreeding	1600	King et al. 2012
Drosophila melanogaster	"GDL" founded from 5 populations coming from different geographic regions; inbred for 12 generations	84	Greenberg et al. 2010; Grenier et al. 2015
Drosophila simulans	Founded from mated females collected from a single population; 15 generations of sib-mating	170	Signor et al. 2017
Drosophila serrata	Founded from mated females collected from a single population; 17 to 20 generations of sib-mating	110	Reddiex et al. 2018
Mus musculus	"RIX" founded from 8 laboratory strains, combined during 3 generations and then inbred during 20 generations.	69	Churchill et al. 2004; Srivastava et al. 2017
Mus musculus	"BXD ARI" founded from 2 laboratory strains (after 9 to 14 generations of inter-crossing, followed by at least 14 generations of inbreeding	46	Peirce et al. 2004
Caenorhabditis elegans	Founded from 2 wild-type strains; 15 generations of inbreeding	73	van Swinderen et al. 1997
Caenorhabditis elegans	From 8 parental lines each initially crossed with males of one different line; 3 to 5 generations of backcrossing followed by 10 generations of selfing	90	Doroszuk et al. 2009
Caenorhabditis elegans	12 lines from 2 hybrid populations (6 from each); 6 from 11 generations of selfing and 6 from 22 generations of sib-mating	12	Teotónio et al. 2012
Caenorhabditis elegans	359 genotypes from reciprocal crosses between 2 parental lines; 3 generations of backcrossing followed by 10 generations of controlled matings between hybrids	359	Andersen et al. 2015
Caenorhabditis elegans	From 2 lines; 10 generations of selfing	120	Frézal et al. 2018
Callosobruchus maculatus	Founded from 215 mated females; 10 generations of inbreeding	~ 86 (40% of 215)	Bilde et al. 2009

# of lines - Number of lines available

Table 2. Geographic locations visited to sample spider mites. *For each location, the table includes the coordinates, the date and the host plants examined, as well as the number of females (# females) and their species when a population was found. n.a. – non-applicable; * tomato. Letters (A to H) correspond to the populations used in the creation of the outbred populations.*

Location	Date	Host plant	# females	Population name	Species	Coordinates
Biomimos, Almada	30.05.2017	*Solanum lycopersicum**	0	n.a.	n.a.	38.657101, -9.183984
		unknown	38	OB	*T. cinnabarinus*	
Quinta dos medronheiros, Sesimbra	30.05.2017	*Solanum lycopersicum**	0	n.a.	n.a.	38.543333, -9.101944
		Phaseoulus lunatos	54	MFV	*T. cinnabarinus*	
		Solanum muricatum	118	MPE	*T. cinnabarinus*	
Herdade do freixo do meio, Montemor-o-Novo	28.06.2017	*Solanum lycopersicum**	72	HFM (H)	*T. urticae*	38.703667, -8.325385
Quinta Vidigal, Montemor-o-Novo	28.06.2017	*Solanum lycopersicum**	146	MON (G)	*T. urticae*	38.706887, -8.327368
Chamusca	03.07.2017	*Solanum lycopersicum**	158	CHA1	*T. cinnabarinus*	39.317635, -8.506450
		*Solanum lycopersicum**	0	n.a.	n.a.	39.312217, -8.516502
		*Solanum lycopersicum**	0	n.a.	n.a.	39.330084, -8.492160
		*Solanum lycopersicum**	38	CHA2	*T. cinnabarinus*	39.333608, -8.498716
Agrial, Alpiarça	03.07.2017	*Solanum lycopersicum**	87	ALP (E)	*T. urticae*	39.221486, -8.572960
Quinta do Montalto, Ourém	04.07.2017	*Solanum lycopersicum**	0	n.a.	n.a.	39.698069, -8.598858
Gracieira, Óbidos	04.07.2017	*Solanum lycopersicum**	89	OTO	*T. cinnabarinus*	39.331097, -9.121093
		Phaseoulus vulgaris	300	OBE	*T. cinnabarinus*	
Campo Grande, Lisboa	11.07.2017	*Solanum nigrum*	320	CG	*T. evansi*	38.756088, -9.154691
Parque hortícola Aquilino Ribeiro Machado, Lisboa	11.07.2017	*Solanum lycopersicum**	207	LNEC	*T. cinnabarinus*	38.760753, -9.144024
Quinta da Granja, Lisboa	11.07.2017	*Solanum lycopersicum**	181	QG (C)	*T. evansi*	38.752069, -9.194166
Parque da boa Saúde, Lisboa	11.07.2017	*Solanum lycopersicum**	153	PBS (B)	*T. evansi*	38.753958, -9.177114
Póvoa de Santa Iria	11.07.2017	*Solanum lycopersicum**	65	PSI	*T. cinnabarinus*	38.866650, -9.062553
Alenquer	29.07.2017	*Cucumis sativus*	32	ALE	*T. cinnabarinus*	39.063222, -9.018111
Hortas comunitárias do Vale de Chelas, Lisboa	10.08.2017	*Solanum melongena*	76	VC	*T. evansi*	38.754530, -9.122054
		*Solanum lycopersicum**	0	n.a.	n.a.	
Ericeira	16.08.2017	unknown	94	ER	*T. evansi*	38.966135, -9.417877
Quinta das 6 Marias, Lagos	10.08.2017	*Solanum lycopersicum**	400	6M1 (A)	*T. evansi*	37.136506, -8.690885
		*Solanum lycopersicum**	93	6M2	*T. cinnabarinus*	
Quinta da pedra branca (Gradil)	14.08.2017	*Solanum lycopersicum**	74	GT	*T. cinnabarinus*	38.990052, -9.292190
		Carica papaya	100	GP	*T. cinnabarinus*	
Biobrotar, Mafra	19.09.2017	*Solanum lycopersicum**	0	n.a.	n.a.	38.975686, -9.350661
		unknown	72	BB	*T. cinnabarinus*	
Biofrade, Lourinhã	22.09.2017	*Solanum lycopersicum**	52	BF	*T. cinnabarinus*	39.244005, -9.312744
Casa da Caldeira, Rio Maior	22.09.2017	*Solanum lycopersicum**	0	n.a.	n.a.	39.343618, -8.797132
Horticilha, Alcochete	13.10.2017	*Solanum lycopersicum**	317	VIT (D)	*T. evansi*	38.672328, -8.876891
Agrolimoa, Lagoa	13.10.2017	*Solanum lycopersicum**	463	LIM	*T. cinnabarinus*	37.147162, -8.432982
Alvalade, Lisboa	13.10.2017	*Solanum lycopersicum**	300	DEF (F)	*T. urticae*	38.755673, -9.147124

Figure 1. Map of the field sampling of spider mites. *A) Total of 24 sites visited in Portugal, B) detailed Lisbon geographic region. This map was adapted from Google Maps.*

Characterization of field populations

In several organisms, different features of the field-collected populations can lead to reproductive incompatibilities between different populations/genotypes and may hamper the maintenance of variability along the creation of outbred populations. Identifying the source of such incompatibilities, and excluding or avoiding them, is thus a pre-requisite to the successful creation of outbred populations.

In many arthropod species, including spider mites, the presence of maternally-inherited bacterial endosymbionts may hamper the viability of offspring from inter-population crosses (Telschow et al. 2002, Duron et al. 2008, Engelstadter and Hurst 2009). Therefore, we assessed infection by the most common reproductive manipulators found in arthropods (Weinert et al. 2015; including spider mites, e.g. Zélé et al. 2018b), namely *Wolbachia*, *Cardinium* and *Rickettsia*, in most of the field-collected populations (cf. Table 3). Using a multiplex PCR method developed by Zélé et al. (2018c, detailed in Supp.1), we found *Wolbachia* in 6 out of the 14 populations screened, whereas the remaining populations were free of symbionts (Table 3). Subsequently, to avoid incompatibilities among populations due to the presence of endosymbionts, a subset (N > 300 females) of each population selected to create the outbred populations (see below) was cured from endosymbiont infection by heat shock (continuous exposure to 33 ºC) for 6 generations, a method previously used in *T. urticae* for the same purpose (van Opijnen and Breeuwer 1999). Due to potential side effects of the heat shock treatment, this procedure was used for all selected populations, independently of whether they

were initially infected by symbionts. All populations were re-tested after the heat shock treatment to confirm the absence of symbionts.

Table 3. Infection by endosymbionts and ITS type. *Spider mites of each population were tested in a pool (N= 50 to 100) for the presence of endosymbionts (Wolbachia, Cardinium and Rickettsia). In T. evansi, each population was characterized for its ITS type (T1 or T2).*

Species	**Population**	**Symbionts**	**ITS type**
T. urticae	HFM	uninfected	n.a.
	MON	*Wolbachia*	
	ALP	uninfected	
	DEF	uninfected	
T. evansi	CG	uninfected	T2
	ER	uninfected	T2
	PBS	uninfected	T1
	QG	*Wolbachia*	T1
	VC	*Wolbachia*	T2
	VIT	uninfected	T1
	6M1	uninfected	T1
T. cinnabarinus	6M2	uninfected	n.a.
	LIM	*Wolbachia*	
	LNEC	*Wolbachia*	

n.a. – non-applicable

Reproductive incompatibilities due to genetic differentiation among populations of the same species is a common feature in many organisms (Scopece et al. 2010, Jennings et al. 2011, Corbett-Detig et al. 2013, Harrison and Larson 2014), including spider mites (e.g. de Boer 1982, Gotoh and Tokioka 1996, Sugasawa et al. 2002, Knegt et al. 2017). In our case

study, to avoid well-known reproductive incompatibilities between the green and red forms of *T. urticae* (e.g. de Boer 1982, Gotoh and Tokioka 1996, Sugasawa et al. 2002), we used populations of the green form only, and discarded most populations of the red form (or *T. cinnabarinus*) after genetic identification. Additionally, in *T. evansi*, two highly incompatible major clades, I and II, have been identified based on the cytochrome oxidase complex I (COI) haplotypes and the internal transcribed spacer region (ITS; Boubou et al. 2011; Knegt et al. 2017). To avoid such incompatibility, we sequenced the ITS of *T. evansi* populations (Table 3; detailed in Supp.1) and used only the populations with ITS type T1, corresponding to clade I, to create the outbred population.

Creation of outbred populations in haplodiploids

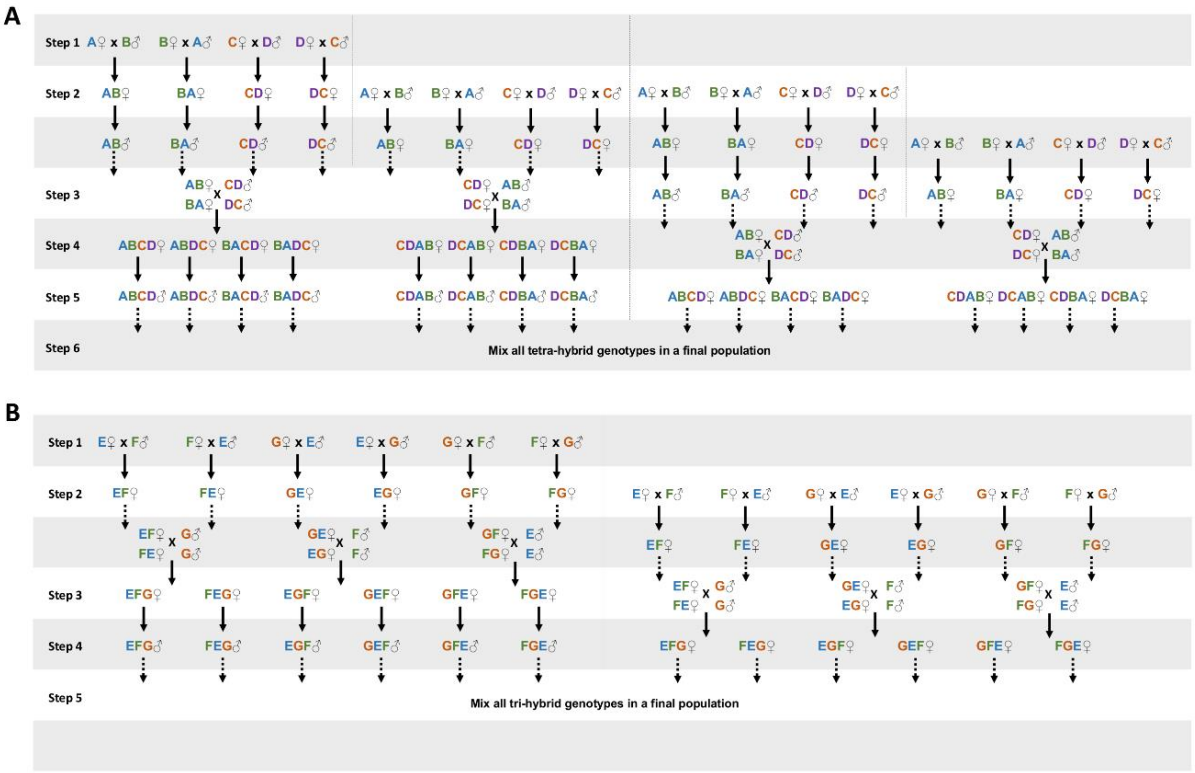
Using different field collected populations to create outbred laboratory populations allows including genetic variation from different geographic locations, which increases the chances of capturing high variability. However, the number of populations used is limited by the logistical feasibility of protocols involving controlled crosses between those populations. Moreover, the complexity of such designs increases in haplodiploid systems (c.f. below) as compared to diploid systems. Here, to create outbred populations with high levels of standing genetic variation, we used 4 symbiont-free à priori compatible populations of each species (the green form for *T. urticae* and Clade I for *T. evansi*). These populations were 6M1, PBS, QG and VIT for *T. evansi* and ALP, DEF, MON and HFM for *T. urticae* (Table 2; hereafter labelled A to D and E to H, respectively). The populations were merged performing inter-population crosses in a controlled match design, to avoid over-representation of genotypes from a given population (Fig. 2). To this aim, 200 females from population A were crossed with 200 males from population B and vice versa; while 200 females from population C were individually crossed with 200 males from population D and vice versa. In this way, we obtained a hybrid F1 (AB, BA; CD and DC). However, because spider mites are haplodiploid with an arrhenotokous genetic system (Helle and Sabelis 1985), only the F_1 female offspring resulting from these crosses are hybrids, as sons stem from unfertilized eggs. This characteristic of haplodiploid species adds one layer of complexity to controlled crossing design, as compared to diploid species. To form hybrid males, virgin hybrid F_1 females were collected during their last moult, allowed to emerge as adult female and to lay unfertilized eggs for 48h. Subsequently, their offspring (haploid hybrid males) developed until adulthood. To synchronize the generations at

which hybrid females and males were produced, a new generation of F_1 hybrid females (again AB, BA, CD and DC) was obtained by repeating the previous set of matings one generation later. These hybrid females were then crossed with hybrid males to produce a fully-hybrid F_2 (AB and BA hybrid females were crossed with CD or DC hybrid males and vice versa). Again, because males stem from unfertilized eggs, only the female offspring resulting from these crosses was a fully-hybrid combination of the 4 populations (e.g. ABCD). These fully-hybrid females were also isolated as virgin and their sons allowed to develop until adulthood. To synchronize the production of fully-hybrid adult males and females, another cross of AB and BA females and CD or DC males (and vice versa) was performed simultaneously (Fig. 2a). Finally, individuals of both genders of each of the 8 fully-hybrid combinations performed (ABCD, ABDC, BACD, BADC, CDAB, CDBA, DCAB and DCBA) were mixed to form the outbred population (Fig. 2a).

This procedure was done to ensure an equal genetic representation (nuclear and mitochondrial) of each field population in the resulting outbred population. The number of crosses of each type (200) was chosen to ensure that the outbred population was founded by a sufficient number of genotypes for it to be representative of a natural population (> 70 mated females; Sousa *et al.*, 2019). Because some crosses did not produce viable offspring of at least one sex, we opted to found the outbred population with an equal number of individuals from each different combination (ex. ABCD), corresponding to the minimum number of genotypes obtained in those combinations. This means that the *T. evansi* outbred was founded with 72 females and 72 males from each of the 8 combinations performed (a total of 576 genotypes of each sex from 4 different locations).

During the creation of the *T. urticae* outbred population, hybrid breakdown was detected between the population HFM and the three others (i.e. ca. 75% of F_2 offspring were inviable). The protocol was thus adapted to merge 3 populations instead of 4 (Fig. 2b), by using a round-robin design in which females / males of population E mated with males / females from population F or population G. These crosses produced hybrid females that would mate with males from the population not included in the parental crossing. The outbred population was founded with 51 females from each of 6 different combinations, corresponding to a total of 306 females. Because the total number of males was low (N=197), we opted to use them all, even though the number across genotypes was not even.

Figure 2. Creation of outbred populations on haplodiploids. *Outbred populations of haplodiploid spider mites were created by performing controlled crosses between four (**A**) or three (**B**) populations collected in different locations. Letters represent the population from which the individuals stemmed (see Table 1). Each step represents the production of offspring to use in the crosses for the following generation: females were obtained from crosses between different genotypes and males from virgin females of a given genotype. Bold arrows represent the development of the offspring forming the next generation, dashed arrows represent the use of hybrids for the subsequent crosses within a generation.*



Creation of inbred lines in haplodiploids via full sib mating

A panel of inbred lines is an important biological tool as it allows determining the broad-sense heritability of any trait and assessing the genetic correlations among traits. Inbred lines can also be used to test how different degrees of variation among traits affect ecological processes. For the knowledge provided by such tool to be more robust, it is important to maximize the coefficient of inbreeding of each line when designing their creation and to derive lines from the same population, as a way of partitioning its genetic variation across the lines. Below we describe a method to create inbred lines in haplodiploids through 15 generations of sib-mating,

as well as an estimation of their coefficient of inbreeding and the probability of obtaining a fully inbred line.

Inbred lines were initiated by isolating a mated female randomly sampled from the outbred population. Given full first-male sperm precedence in spider mites, all descendants of a female stem from the same father (Rodrigues et al. 2020), which reduces genetic variance in the offspring as compared to species with mixed paternity. This protocol can easily be adapted to species with mixed paternity by initially isolating virgin males and females. In haplodiploids, a maximum of three different alleles (e.g. x, y and z) can be initially sampled at each locus, independently of the number of alleles available for that locus in a population. Hence, 4 different types of mated females can be sampled, which correspond to 4 possible types of crosses: (A) a heterozygous female mated with a male that does not share any allele with her (e.g. [xy]*[z]); (B) a heterozygous female mated with a male that shares one of her alleles (e.g. [xy]*[x]); (C) a homozygous female mated with a male that does not share an allele with her (e.g. [xx]*[y]) or (D) a homozygous female mated with a male that has the same allele as her for that locus (e.g. [xx]*[x]).

Because we do not have access to the genotype of the mated female that initiated each line, we assume the most heterozygotic situation, i.e. that we collected a [xy] female mated with a [z] male. By doing so, we conservatively underestimate the coefficient of inbreeding. This cross (type A) will produce ½ [xz] + ½ [yz] females and ½ [x] + ½ [y] males in the F_1. If these daughters and sons mate randomly among themselves, crosses among sibs will occur with the following probability: ¼ [xz]*[x] (type B) + ¼ [xz]*[y] (type A) + ¼ [yz]*[x] (type A) + ¼ [yz]*[y] (type B). Thus, at each time step, type A crosses will result in sons and daughters that, if mated randomly among themselves, will produce ½ type A crosses and ½ type B crosses. Following the same reasoning, type B crosses, e.g. [xy]*[x], will produce ½ [xx] + ½ [xy] females and ½ [x] + ½ [y] males. If these daughters and sons mate randomly, crosses among siblings will occur with the following probability: ¼ [xx]*[x] (type D) + ¼ [xx]*[y] (type C) + ¼ [xy]*[x] (type B) + ¼ [xy]*[y] (type B). Thus, type B crosses will result in sons and daughters that, if mated randomly among themselves, will produce ½ type B crosses, ¼ type C crosses and ¼ type D crosses. From a type C cross, e.g. [xx]*[y], only [xy] females and [x] males will be produced and, thus, only [xy]*[x] crosses (type B) will occur. Finally, type D crosses, e.g. [xx]*[x], will only produce [xx] females and [x] males, and, thus only type D crosses will occur.

Therefore, the frequencies of each type of cross, at each generation (t+1), are given by the following equations:

$$A_{t+1} = \frac{A_t}{2}$$

$$B_{t+1} = \frac{A_t}{2} + \frac{B_t}{2} + C_t$$

$$C_{t+1} = \frac{B_t}{4}$$

$$D_{t+1} = \frac{B_t}{4} + D_t$$

The coefficient of inbreeding (f_t), which corresponds to the probability that two alleles at one locus are identical by descent (Wright 1921), is subsequently given by the following equation:

$$f_t = C_t + D_t$$

Alternatively, for full sib-mating in haplodiploids, this coefficient can also be obtained directly as:

$$f_t = \frac{1}{4} + \frac{1}{4} f_{t-2} + \frac{1}{2} f_{t-1}$$

where the first two terms correspond to the probability of both alleles coming from the grandmother, being the alleles equal (first term), so that f_t = 1, or different (second term), so that f_t is equal to that of the grandmother f_{t-2}, and the third term corresponds to the probability of one allele coming from the grandmother and the other from the grandfather, so that f_t is the same as that of the mother f_{t-1}.

Both methods yield the same result, and assuming that generation 0 starts with a [xy] female mated with a [z] male (i.e. $A_0 = 1$ with the first method and $f_1 = f_2 = 0$ with the second method), we obtain a coefficient of inbreeding of 95.1% after 15 generations (Fig. 3). However, the first method also allows estimating the probability of having a fully inbred line, which is given by the frequency of individuals stemming from fully homozygous crosses (D_t). Again, assuming the most heterozygotic scenario, we obtain a probability of having a fully inbred line of 93.6% after 15 generations (Fig. 3).

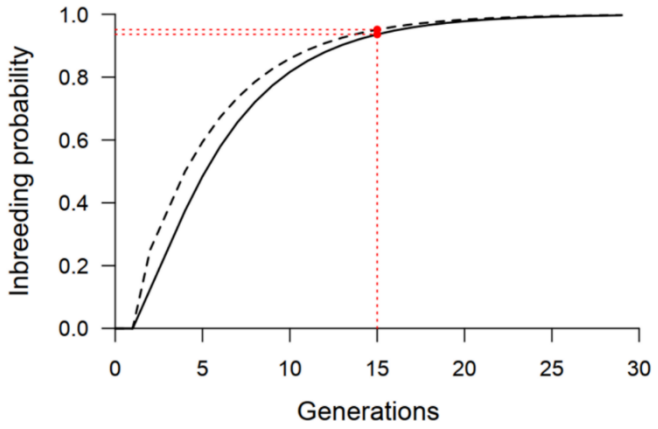


Figure 3. Inbreeding estimates from full sib-mating crosses in haplodiploids. *Coefficient of inbreeding (f_t; dashed line) and probability of having a fully inbred line (D_t; full line) for each discrete generation of full sib-mating, starting from the most heterozygotic combination at one locus (e.g. a [xy] female mated with a [z] male). After 15 generations of sib-mating, inbred lines of haplodiploids have a coefficient of inbreeding of 95.1% and a probability of being fully inbred of 93.6% (red lines and dots).*

To create inbred female lines from the *T. evansi* outbred population, we randomly sampled 450 mated females, 2 generations after the creation of the population. These females were installed individually on leaf patches, where they laid eggs for 48h. The offspring of each female was then allowed to develop until adulthood (10 to 12 days) and to mate on that patch (i.e. sib-matings). After 14 days, 3 mated females from each patch were isolated on 3 new patches and the same procedure was repeated. On the following generation, 3 sib-mated females from one of the three patches only, were isolated on 3 new patches and allowed to oviposit for 48h. The entire procedure was then repeated for 15 discrete generations. Having 3 replicates per line decreases the chances that lines are lost at each generation. However, despite this, many lines were lost due to the death of the female, null fecundity, no egg hatching, or no female or male offspring produced by a given female (Fig. 4). After 15 generations of sib-mating, each of the remaining inbred lines (N = 59) was transferred to individual patches of tomato plants kept on water-soaked cotton in petri dishes and maintained in small numbers thereafter.

Experimental evolution protocol

Experimental evolution is not only a powerful method to detect adaptation to specific controlled factors but can also be combined with next-generation sequencing techniques in order to identify and quantify individual loci contributing to adaptation (Magalhães and Matos 2012, Schlötterer et al. 2015). For this purpose, several parameters of the experimental design,

such as the number of founders, the number of generations and the number of replicates per selection regime, must be carefully considered according to the system involved (Kofler and Schlötterer 2014). However, during the course of the experiment these parameters may be affected due to predictable and unpredictable events (for example, the loss of replicates due to the extinction of an experimental population). Here we present a protocol that helps maximizing the prevention of such events. This experimental evolution protocol consists in transferring 220 randomly collected females from the outbred population to a box corresponding to a given selection regime. This number ensures > 200 living (due to mortality in the transfer < 5%) females founding each experimental population, which is the number needed to maximize the probability of detecting and quantifying responses to selection in spider mites (Sousa *et al.* 2019). This procedure is repeated 5 times per selection regime, as the replicate unit in experimental evolution studies is the population (Kawecki et al. 2012). All populations are maintained under the same environmental conditions except for variables that corresponded to each selection regime. Each generation, 220 randomly selected mated females are transferred to a new box with the same characteristics. The remaining individuals are maintained in the original box until the next transfer, creating a back-up population (t-1) for each replicate of each selective regime. Thus, if the 220 females cannot be found in a given box at the time of transfer, the remaining number is transferred from the back-up population. If the sum of females found in the experimental population and its respective t-1 back-up does not add up to 220, the remaining number of females should be transferred from the base outbred population. This procedure allows maintaining the same population size for each replicate, preventing bottlenecks and diminishing the chances of losing those populations. However, because every generation each experimental population may receive a different number of migrants from the t-1 back-up population and/or the base population (i.e. a different number of individuals are exposed to a different number of generations of selection), the average number of generations of selection of each replicate population might differ. The effective number of generations of selection can be estimated for each replicate population using the following equation:

$$Gen_{t+1} = 1 + \frac{N_t * Gen_t + N_{t-1} * Gen_{t-1} + N_0 * Gen_0}{N_{total}}$$

Where Gen_{t+1} corresponds to the effective number of generations of selection underwent by the individuals at the next generation. Gen_t, Gen_{t-1} and Gen_0 correspond, respectively, to the effective number of generations of selection underwent by the current generation, the previous

generation and the base populations (i.e. not adapted to the new environment, so $Gen_0 = 0$). N_t, N_{t-1} and N_0 correspond to the number of individuals transferred from the current generation of selection, the back-up t-1 box and the base population, respectively, and N_{total} to the total number of adult females transferred. We provide an example of this formula by applying it to our system in Fig. 5.

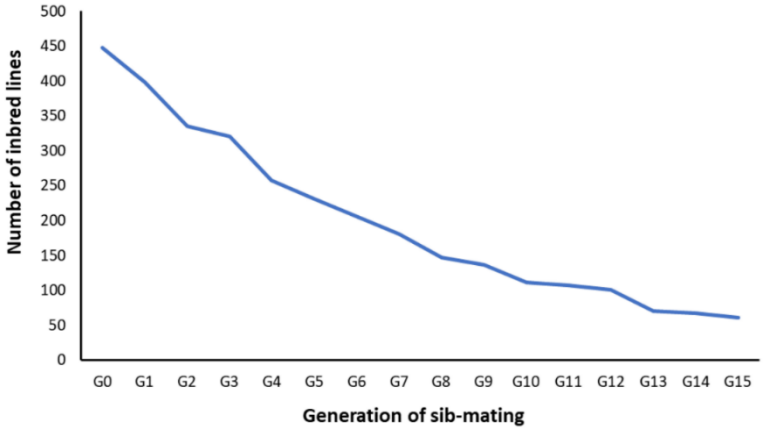
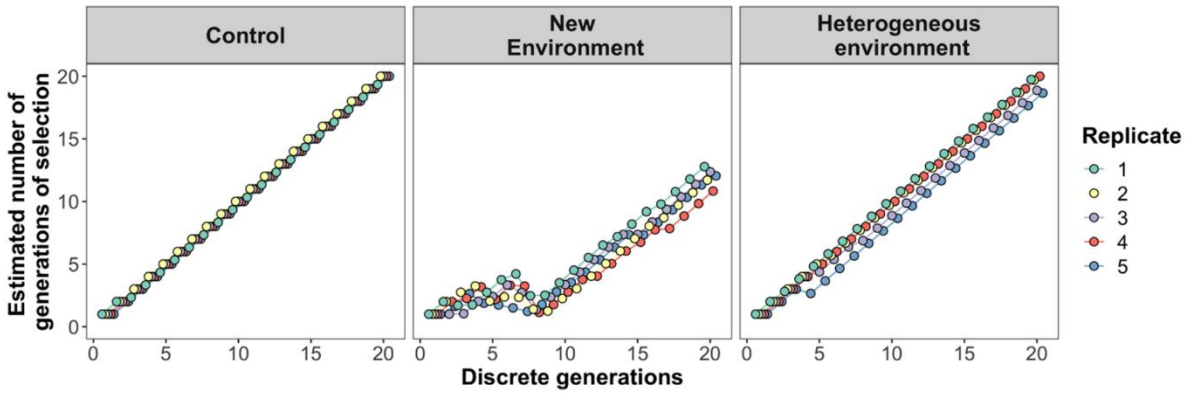


Figure 4. Loss of inbred lines during their creation. *Number of inbred lines of T. evansi across 15 generations of sib-mating.*

Figure 5. Estimated number of effective generations of selection during experimental evolution of spider mite populations exposed to different environments*. Populations were exposed to an environment similar to that of the ancestral population (control), to a new environment, or to a mixture of both (heterogeneous environment). Because individuals from the t-1 and base populations were often added in the selection regime corresponding to a new environment, the estimated number of generations decreases considerably relative to the other selection regimes. However, this procedure allowed populations to overcome the initial reduction in population size and to subsequently adapt to the selection regime imposed.*



Discussion

We describe the creation of biological resources (outbred and inbred populations) that maximize the maintenance of standing genetic variation in laboratory populations and, thus, increase the representativity of the responses described in laboratory conditions. As a case study, we present the creation of outbred populations for two spider mite species, *T. urticae* and *T. evansi*, by performing controlled crosses between recently collected field populations. In addition, we report a procedure to calculate the inbreeding coefficient, but also the probability of having a full inbreed line when performing full sib-mating in haplodiploids and apply it to the creation of inbred lines of *T. evansi*. Finally, we provide an outline of an experimental evolution protocol allowing the maintenance of constant densities across generations of selection, thereby reducing the risk of bottlenecks. Even though the methods we described are applied to haplodiploid species, they can easily be adapted to diploid systems as well. Thus, these protocols may be used in any species that can easily be sampled, have a relatively short generation time and can be maintained in the laboratory conditions in high numbers.

Undoubtedly the method we present here is time and work consuming. However, we believe that the advantages of creating such powerful tools, as we describe here, compensate the effort in the long run. The creation of outbred populations through controlled crosses allows to a) incorporate genetic variability from different geographic collections, b) detect reproductive incompatibilities between different genotypes and populations and c) incorporate an equal representativity of each population merged. All of these characteristics maximize the chances of maintaining a high amount of standing genetic variation in the outbred populations. By deriving the inbred lines from an outbred population, one also increases the change of keeping that diversity among the lines. Additionally, the time spent with the creation of outbred populations gives the populations time to adapt to the laboratory conditions, a requirement before testing adaptive responses to other environments (Matos et al. 2002, Simões et al. 2008, Fragata et al. 2014). Moreover, while developing these tools, the populations collected may be thoroughly characterized, providing a preview of the variability expected in the derived outbred population and inbred lines.

Here, to illustrate the application of these methods, populations were collected from nearby locations. Therefore, the resulting populations do not encompass large areas of potential geographical variation, unlike for example the populations used to create the DSPR and the GDL panels, where the founding genotypes have distant geographic origins (King et al. 2012, Grenier et al. 2015). This may limit the standing genetic variation available, because of similar environmental conditions, and/or migration among populations. However, this is not very likely in the case of spider mites, as (a) experimental evolution studies performed with populations from a single location have repeatedly shown responses to selection (reviewed in Sousa et al 2019), and (b) populations of spider mites show high genetic differentiation even within small geographical scales (e.g. Bailly et al. 2004, Carbonelle et al. 2007). Additionally, by founding the outbred populations with more than 500 individuals, the chances of obtaining large amounts of standing genetic variation are high.

Performing controlled crosses among field populations maximizes the chances of obtaining a highly outbred laboratory population. Indeed, this method allows controlling for assortative mating and for differences in fitness and/or mating competitive ability between genotypes. Therefore, it ensures an equal genetic representation of the nuclear and mitochondrial genomes of each population by equalizing the number of genotypes from each field population that will be incorporated in the outbred population. Finally, it allows the detection of reproductive incompatibilities between populations/genotypes, and the consequent exclusion of inviable crosses. Indeed, throughout the process of creation of these outbred populations, several crosses resulted in infertile females, or inviable offspring, indicating possible incompatibilities. Specifically, we detected hybrid breakdown (i.e. postzygotic reproductive isolation where F_2 hybrids are inviable or sterile) between one population of *T. urticae* (HFM) and the others. None of these potential causes of reduction of the genetic variability in the final population would have been detected and prevented if the outbred population had been founded by mixing several individuals of different populations without controlling for the outcome of such crosses at the individual level, as previously done in other studies (Tucic et al. 1995, Fricke and Arnqvist 2007, Rodrigues et al. 2018, biorxiv).

Using outbred populations increases the chance that the responses observed are representative of the study species, which is a common shortcoming of laboratory studies. For example, Vala et al. (2004) found that *T. urticae* females that are not infected with *Wolbachia* prefer uninfected over infected males, thereby potentially reducing the costs of incompatible mattings. However, this result was based on a single line, whereas a later study using an outbred

population stemming from several field populations does not recapitulate this result (Rodrigues et al. 2018, biorxiv). Another example concerns the interaction between *T. urticae* and tomato plant defences. Although this herbivore generally induces plant defences, some field-collected lines were shown to supress them instead (Kant et al. 2008). Therefore, capturing and maintaining natural variation in laboratory studies is highly relevant for understanding the ecology and evolution of the interaction between study organisms, such as spider mites, and many environmental factors, such as symbionts or plants. A particular example of studies which may profit from using outbred populations are those using experimental evolution. As genetic variance is the raw material for selection to act upon, having a highly outbred population to initiate experimental evolution will increase the chances of observing fast responses to selection. However, many experimental evolution studies have been performed with populations or strains collected from a single location, and in some cases from a small number of individuals (but see Fricke and Arnqvist 2007, Zbiden et al. 2008). Spider mites are no exception to such contingencies (reviewed in Sousa et al. 2019). Therefore, the responses obtained may be idiosyncratic of the genetic background used. Providing the community with highly outbred laboratory populations may be very useful to test the generality of the responses reported, and to perform future studies on other topics with a larger representation of the genetic variation of the species.

Moreover, in experimental evolution studies the initial diversity harboured by the ancestral populations may be quickly lost due to selection and/or stochastic events, leading to the extinction of experimental populations. Indeed, in environments that impose a strong selection pressure there is a high probability that the populations adapting to those conditions crash in a few generations. Additionally, unpredictable logistical problems that lead to the loss of experimental populations may occur. Here we outline an experimental evolution protocol that allows using populations from the previous generation of selection (back-up t-1 populations) to ensure that the total population size remains constant across generations, thereby allowing populations to overcome the initial reduction in population size. In this way, it is possible to avoid losing replicates, as commonly occurs in experimental evolution studies (Simões et al. 2007, Cooper and Lenski 2010, Schlötterer et al. 2015). Such populations can thus be rescued and subsequently adapt to the selection regime imposed (Fig. 5). In this case, it is important to, calculate the effective number of generations of selection that populations have undergone, such as to correctly compare responses among selection regimes. Note that

these t-1 back-up populations can be kept under relaxed conditions, thereby minimizing the workload necessary to maintain them.

Clearly, performing laboratory studies with outbred populations adds to their robustness. However, to characterize the different responses found in such outbred populations due to genetic variance, it is necessary to fix this variance along a panel of inbred lines, such that each genotype can be studied independently. For this purpose, one can derive a panel of inbred lines from an outbred population source, as done in *Drosophila melagonaster* (King et al. 2012, Mackay et al. 2012). The main advantage of this method is that the high genetic variability of the outbred population can be maintained among the isogenic lines, while keeping the same genetic background. In particular, studying these lines allows a clear understanding of the phenotypic and genotypic variability for traits that may be relevant in many different contexts. Importantly, inbred lines can also be used to assess genetic correlations and trade-offs between different traits (e.g. Travers et al. 2015, Howick and Lazzaro 2017, Wang et al. 2017, Lafuente et al. 2018, Everman et al. 2019), including those measured in different environments (Howick and Lazzaro 2014, Unckless et al. 2015, Ørsted et al. 2018). Indeed, because all individuals of a given inbred line represent roughly the same genotype, responses of each genotype can be measured in different contexts. Additionally, such inbred lines can be used as a fixed genetic background against which the response of another population is studied. This may be particularly useful in the context of the evolution of biological interactions. For example, unravelling the evolution of sexual conflicts can be done by exposing individuals of the evolving sex to inbred lines of the non-evolving sex (e.g. Macke et al. 2014). Also, the magnitude of GxG (genotype by genotype) in host parasite interactions has been addressed by exposing different lines of hosts and/or parasites to each other (Lambrechts, Fellous and Koella 2006, de Roode and Altizer 2010, Carpenter et al. 2012, Hudson et al. 2016). Finally, there is a recently increasing interest on how inter-individual variation affects several ecological characteristics, such as species persistence and coexistence (Lichstein et al. 2007, Agashe 2009, Lankau 2009, Bolnick et al. 2011, Forsman and Wennersten 2016, Hart et al. 2016). Within this context, the creation of inbred lines may also be a useful tool. Finally, having the same background in the outbred population and the inbred lines allows comparing results stemming from both types of populations when tackling a common question.

The power of the biological resources described here, that can easily be adapted to other organisms, can be further potentiated if they are shared with collaborative laboratories and combined with increasingly fast advances on the genetic and genomic resources available. This will allow consistent and comparable studies that unquestionably will provide great advances in many different frameworks.

Acknowledgements

We would like to thank Margarida Matos and Élio Sucena for important inputs on the design of the crosses for the creation of the outbred populations, Lucie Sousa and Inês Santos for growing the plants and keeping the field-collected populations and all the members of the MITE2 group for stimulating discussions.

This study was funded by an ERC Consolidator Grant (COMPCON, GA 725419) to SM and by an FCT Ph.D. scholarship PD/BD/114010/2015 to DPG.

Conflict of interests

The authors declare no conflict of interest.

Data availability statement

The dataset has been deposited in Figshare repository (dx.doi.org/10.6084/m9.figshare.12263777).

Author's contribution

DPG and SM designed the study with help from LRR and FZ. DPG and LRR collected the spider mite populations. The creation of the outbred and inbred populations was performed by DPG, MAC, MCM, JTP and CE. FZ and IF developed the formula to calculate the coefficient of inbreeding and the effective number of generations of selection. The manuscript was written by DPG and SM with considerable contributions from all authors.

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Supplementary information 1. Identification of *Tetranychus* species and detection of endosymbiont infection.

1. DNA extraction

A pool of 50-100 female spider mites from each population was used to extract the DNA using the Sigma-Aldrich protocol (GenElute™ Mammalian Genomic DNA Miniprep Kit, Sigma-Aldrich, St. Louis, MO, USA). This DNA was then used for identification of spider mite species and *T. evansi* ITS type, and for the diagnostic of endosymbiont infection. Note that previous sensitivity tests showed that the multiplex PCRs used for species identification and symbiont infections allowed successful detection of target genes at very low density (up to 1%; i.e. 1 mite in a pool of 100; Zélé et al. 2018c).

2. Species identification

To identify each population of spider mites at the species level, we used the multiplex PCR developed by Zélé et al. (2018c). Briefly, we used a *Tetranychus*-generalist forward primer and different reverse primers specific to each target species (Table S1). The amplification conditions were the following: 15 min at 95 °C, followed by 35 cycles of 94 °C for 30 s, 58 °C for 1 min 30 s (annealing), 72 °C for 1 min and a final step at 72 °C for 10 min.

Table S1 List of primers used in multiplex to identify *Tetranychus urticae, T. ludeni* and *T. evansi*

Species	**Target gene**	**Primer name**	**Sequence (5´- 3´)**
Tetranychus sp.	5.8S	TspG_F	TAATCGGTGCGAATTGCAGG
T. urticae	28S	TuS_R	ATGTTTATTTGTGTTGTTTGCAAGC
T. ludeni	ITS2	TlS_R	GAATGAAATAGATACTATTTGTGATTC
T. evansi	ITS2	TeS_R	GATTCATGTATACAYATATAAATATATGC

Identification of the ITS2 types of each population of *T. evansi* was subsequently performed by PCR amplification and sequencing of a fragment of the nuclear ribosomal DNA (rDNA) ITS2 region using the primers ITS2a (5'-TACCAATCGATGAAGAACGTAGC-3') and ITS2b (5'ATATGCTTAAATTCAGGGGG-3') developed by Hurtado et al. (2008). The

conditions for amplification were the same than for the multiplex PCR, except for the annealing temperature that was 50ºC. The PCR products were sent to Stabvida (Caparica, Portugal) for purification and sequencing.

3. Endosymbiont detection

To detect infection by *Wolbachia*, *Cardinium* and/or *Rickettsia* in our populations, we used multiplex PCR developed by Zélé et al. (2018c). Briefly, we used forward and reverse primers for one specific gene of each endosymbiont, as well as spider mite generalist primers to distinguish uninfected individuals from PCR failure (Table S2). The amplification conditions were the same as for the species identification multiplex PCR, except for the annealing temperature that was 65 ºC.

Table S2 List of primers used in multiplex to detect simultaneous infection by *Wolbachia*, *Cardinium* and / or *Rickettsia*

Target gene	**Primer name**	**Sequence (5´- 3´)**
Tetranychus ITS1 (partial)	ITS1G_F	AGGTGAACCTGCGGAAGGATCATTAACG
	ITS1G_R	CCTTCTTTAAACCTTGCCGTCAGCATAAGC
Wolbachia wsp	WSPTG_F	GTTGGTGTTGGTGCAGCGTATGTAAGC
	WSPTG_R	AGTGCTGTAAAGAACTTTGATTCCGCCATC
Cardinium 16S rRNA	CARDTG_F	GGCGGCTTATTAAGTCAGTTGTGAAATCCT
	CARDTG_R	GCTGCCTACGCTATTGGTATTCCTTATGAT
Rickettsia gtlA	RICTG_F	AGGCTAATGGGCTTTGGTCATCGTGTAT
	RICTG_R	TGTGCCATCCAGCCTACTGTTCTTGC

Chapter III

The distribution of herbivores between leaves matches their performance only in the absence of competitors

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Submitted in Ecology and Evolution

Abstract

Few studies have tested how plant quality and the presence of competitors interact in determining how herbivores choose between different leaves within a plant. We investigated this in two herbivorous spider mites sharing tomato plants: *Tetranychus urticae*, which generally induces plant defences, and *T. evansi*, which suppresses them, creating asymmetrical effects on co-infesting competitors. On un-infested plants, both herbivore species preferred young leaves, coinciding with increased mite performance. On plants with heterospecifics, the mites did not prefer leaves on which they had a better performance. In particular, *T. urticae* avoided leaves infested with *T. evansi*, which is in agreement with *T. urticae* being out-competed by *T. evansi*. In contrast, *T. evansi* did not avoid leaves with the other species, but distributed itself evenly over plants infested with heterospecifics. We hypothesize that this behaviour of *T. evansi* may prevent further spread of *T. urticae* over the shared plant. Our results indicate that leaf age determines within-plant distribution of herbivores only in absence of competitors. Moreover, they show that this distribution depends on the order of arrival of competitors and on their effects on each other, with herbivores showing differences in behaviour within the plant as a possible response to the outcome of those interactions.

K**ey-words:** host-plant quality; interspecific competition; plant defences; spider mites; within-plant distribution

Introduction

The quality of plants as food for herbivores is highly variable, even among plants of the same species (Underwood & Rausher 2000; Wetzel *et al.* 2016; Koricheva & Hayes 2018) and among parts of the same plant (Coley 1980; Raupp & Denno 1983; Coley *et al.* 2006). Such variation depends on traits that are intrinsic to the plant, such as the nutritional value of different tissues, or differential defences in various tissues (Wetzel *et al.* 2016). Additionally, plant quality hinges on the damage caused by intraspecific and interspecific herbivores (Karban & Baldwin 1997; Awmack & Leather 2002).

The distribution of herbivores over different plants is expected to match differences in their performance on these plants (Jaenike 1978; Singer *et al.* 1988; Thompson 1988). Herbivore performance may be affected by different characteristics of the plant, be it physical, such as the presence of trichomes, or chemical, such as nutritional quality and inducible defences (Walling 2000; Awmack & Leather 2002). For instance, the performance of herbivorous arthropods is commonly positively correlated with the amount of nitrogen in the plants (Mattson 1980; Hoffland *et al.* 2000; Chen *et al.* 2008) and negatively affected by secondary metabolites produced by plants (Bennett & Wallsgrove 1994; Walling 2000; Mithöfer & Boland 2012).

Other factors can also affect the host plant choice of herbivores. For example, they may avoid plants with competitors (Pallini *et al.* 1997; Yoshimoto 2009; Zhang *et al.* 2019). This may be because herbivorous arthropods alter the quality of their host plant (Karban & Myers 1989; Awmack & Leather 2002). Indeed, many herbivore-induced changes in the plant are detrimental both to conspecific and heterospecific consumers, be it via the consumption of plant tissues or via the induction of plant defences (Karban & Myers 1989; Awmack & Leather 2002; Ohgushi 2005; Kant *et al.* 2015). To avoid the negative effects of competition, some herbivores may choose to oviposit on less crowded host plants, even if those have lower nutritional quality (Valladares & Lawton 1991; Ellis 2008). Alternatively, they may choose hosts where the performance of offspring is not density-dependent such as to avoid possible costs of future over-crowding (Wetzel & Strong 2015). Moreover, some herbivores can also have positive effects on the performance of competitors (Awmack & Leather 2002), for example by supressing plant defences (Sarmento *et al.* 2011a; Matsukura, Matsumura and Tokuda, 2012; Takemoto *et al.*, 2013; Godinho *et al.*, 2016). These dissimilar herbivore-induced effects on plant quality may not only result in changes of the niche of the herbivore species that modifies plant defences, but also of that of its competitors (Hutchinson 1959;

Shimada & Fujii 1985), and thus, their distribution among plants. Furthermore, these asymmetrical interspecific interactions via plant quality may affect the outcome of competition between herbivores and their distribution.

Another factor that may affect the distribution of herbivores is the order of arrival on a plant (Viswanathan *et al.* 2005; Stam *et al.* 2018; Peñaflor *et al.* 2019). The first species to colonize a plant may have a numerical advantage over its competitors, negatively affecting species that arrive later on, due to priority effects (Fukami 2015; de Meester *et al.* 2016). These negative effects can be exacerbated by the induction of plant defences. Alternatively, the presence of one species may have positive effects on the species subsequently arriving on the same host plant, facilitating its establishment (Callaway 1995; Bruno *et al.* 2003), which is the case of species that suppress plant defences. In any case, the presence of a competitor may not only affect the establishment of a herbivore on a given host plant but also lead to changes in distribution at larger scales, affecting community composition (Stam *et al.* 2018).

Factors affecting herbivore distribution may also vary within plants (Coley 1980; Stout *et al.* 1996; Coley *et al.* 2006; Travers-Martin & Müller 2008; Meng *et al.* 2018). For example, young, growing leaves often have higher nutritional value than old leaves, (e.g. a higher amount of N; Coley, 1980; Stout et al., 1996; Coley et al., 2006). Such differences among leaves may cause uneven performance and preference of herbivorous arthropods within a plant (Cannon & Connell 1965; Wiktelius 1987; Opit *et al.* 2003; Chen *et al.* 2007; Cornelissen & Stiling 2008). Leaves may also contain different (concentrations of) secondary metabolites, affecting the preference of herbivores, depending on whether they are negatively affected by such metabolites or can tolerate or sequester them (van der Meijden 1996). Furthermore, some herbivores move within plants to avoid antagonists such as predators (Magalhães *et al.* 2002; Walzer *et al.* 2009) or competitors (Dechert & Ulber 2004; Anderson & Agrell 2005; Cédola *et al.* 2013; Gómez *et al.* 2014). This may benefit to herbivores, as they avoid competitors and other antagonists at low costs relative to moving to another plant. However, it may be less efficient than moving to another plant because antagonists may easily follow them within plants. Moreover, the presence of competitors in one stratum may affect plant quality in other strata (i.e. systemic plant defences Stout, Workman and Duffey, 1996; Sarmento *et al.*, 2011a). Even though differences in the quality of plant strata and the presence of competitors are known to affect performance of herbivores within plants, the effect of the presence of heterospecific competitors on the within-plant distribution of herbivores has not been studied extensively (Dechert & Ulber 2004; Gómez *et al.* 2014). We aimed to fill this gap by studying the

performance and preference of two herbivorous spider mites co-infesting old or young leaves of the same host plant.

Fig. 1 *The spider mites T. urticae (right) and T. evansi (left) co-infesting a plant. Picture by Anna Beber.*

Spider mites (Fig. 1) are herbivorous arthropod pests of many crops (Migeon *et al.* 2010). *Tetranychus evansi* and *T. urticae* coexist in the Mediterranean Basin, where the former is invasive (Boubou *et al.* 2012). *Tetranychus evansi* is a specialist of solanaceous plants and suppresses the defences of tomato plants to levels lower than those of un-infested plants (Sarmento *et al.* 2011a). This results in higher performance of *T. evansi*, but also of *T. urticae*, on those plants (Sarmento *et al.* 2011a; Godinho *et al.*, 2016; Schimmel *et al.*, 2017). In contrast, most strains of *T. urticae* induce the defences of tomato plants, leading to lower herbivore performance on infested plants (Li *et al.* 2002; Ament *et al.* 2004; Kant *et al.* 2004). *Tetranychus urticae* and *T. evansi* thus have contrasting effects on host plant defences. This results in oviposition rates on plants infested by the two species that are intermediate to those on plants colonized by either the inducer or the suppressor species (de Oliveira *et al.* 2016, 2019; Schimmel *et al.* 2017b). Additionally, one laboratory study shows that *T. urticae* is out-competed by *T. evansi* on tomato plants (Sarmento *et al.*, 2011b). However, these previous studies did not consider how choices of herbivores for certain leaves within a plant may affect the interaction between these mite species. The distribution of mites within a plant may be affected by the presence of the interspecific competitors, by the induction or suppression of plant defences by these competitors, but also by the quality of different plant parts for the herbivores. Addressing how interspecific competition interacts with leaf age to define the within-plant distribution of spider mites will not only shed light on this study system, but also contribute to our general understanding of how changes in within-plant distribution may shape herbivore communities.

Materials and methods

Mite and plant cultures

Tomato plants (*Solanum lycopersicum*, var. Moneymaker) and kidney bean plants (*Phaseolus vulgaris*, var. prelude) were sown in a 5:1 mixture of garden soil and vermiculite in a greenhouse, where they grew for 4 weeks (25 ºC, 16:8 LD). The plants used in the experiments had five leaves; the 2nd and 4th leaf were used for experiments and are referred to as old leaf and young leaf, respectively.

The *T. evansi* population used in these experiments was collected from tomato plants in a greenhouse in Brazil (Sarmento *et al.*, 2011a). Because *T. urticae* has a wider host range than *T. evansi* (Migeon *et al.* 2010), it is more likely for the former to originate from another host plant species when invading tomato plants. To account for this heterogeneity, we used a population of *T. urticae* collected on *Ricinus communis* in the Netherlands and reared on bean plants. Mites were maintained on detached leaves of tomato (*T. evansi*) or bean plants (*T. urticae*) and placed on wet cotton wool in isolated boxes under controlled conditions (25 Cº, 16:8 LD) in a climate room

Cohorts of adult female spider mites were obtained by allowing groups of adult females to lay eggs on leaves of their rearing host plant for 48h. The adult females that emerged from those eggs were used in the experiments. All experiments were carried out in a climate-controlled chamber under the same conditions as the rearings.

Effect of leaf age and infestation by heterospecific competitors on the performance of spider mites

We measured the effect of heterospecifics, of leaf age, and their interaction on the performance of spider mites. Tomato plants were infested with 40 female mites of one species (*T. urticae* or *T. evansi*) either on an old leaf (2nd) or a young leaf (4th; Supp.1 Fig. S1), while the other leaves were not infested. To prevent mites from moving to different parts of the plant, the leaves were previously isolated with lanolin paste applied to the petiole. Mites were allowed to feed and oviposit on the plants for 48 hours. Un-infested plants similarly treated with lanolin paste, were used as control (N = 10 per treatment). Subsequently, females, eggs and web were removed from the leaves. Ten leaf discs of circa 4 cm^2 were made from each leaf (old and young) of each plant (N = 10 per treatment; Supp.1 Fig. S1) and placed on top of wet cotton wool in Petri dishes with the abaxial surface facing up (Supp.1 Fig. S1). One female of the species that had not previously infested the plant was placed on each disc and allowed to oviposit for 4 days. The total number of eggs and daily survival of females were recorded. For each leaf, the

average daily oviposition rate was determined by dividing the total number of eggs laid per female by the number of days on which the female was alive.

Effect of leaf age and infestation by heterospecific competitors on the distribution of spider mites across leaves

To assess the preference of spider mites of both species for old or young leaves, either previously infested by heterospecifics or not, plants were infested with one of the two spider mite species as in the previous experiment or left uninfested (N = 16 per treatment; Supp.1 Fig. S2). After 48h, old and young leaves, either with spider mites and their cues or uninfested, were connected with a nylon string of equal length (35 cm) to a small Petri dish (Ø 35 mm), placed on the soil (Supp.1 Fig. S2). Subsequently, 100 female mites of the species that was not present on the plant were released in the Petri dish and allowed to climb up the strings and choose between the leaves. Twenty-four hours after release, the number of mites of the focal species on each leaf was recorded (Supp.1 Fig. S2).

Statistics

All statistical analyses were performed with the software package R 3.0.2. Models were simplified by removing non-significant interactions. This was determined by comparing the full model including the non-significant interactions and factors, to a model excluding a given non-significant interaction, using the anova function in R. When models were not significantly different, the model with the lowest AIC (Akaike Information Criterion) was kept (Crawley 2007).

To assess the effect of leaf age and the presence of heterospecifics on the performance of spider mites, oviposition rates of each species, averaged per leaf, were compared using a general linear mixed-effects model (lme). Leaf age (old or young), infestation treatment (un-infested plant, plant with old leaf infested with heterospecifics, plant with young leaf infested with heterospecifics), and their interactions were coded as fixed factors and plant was coded as a random factor. Because there was a significant interaction between leaf age and infestation treatment for *T. urticae*, differences in oviposition rate between leaves of different ages were compared for each infestation treatment using the testInteractions function (phia package, de Rosario-Martinez, 2015).

Within un-infested plants, the preference of each species for old and young leaves was tested by comparing differences in the number of mites on each leaf, using a generalized linear

model (glm) with a quasi-Poisson error distribution to correct for overdispersion. Tested species (*T. urticae* and *T. evansi*), leaf age and their interaction were used as fixed factors. With this model, we also aimed to test whether the two species showed similar preference. In order to test if the preference between old and young leaves was affected by the presence of heterospecifics on one of those leaves, the distributions within plants with different infestation status were compared using a glm with a quasibinomial error distribution (to correct for the overdispersion of the residuals). The tested species (*T. urticae* or *T. evansi*), infestation treatment (as described above) and their interactions were used fixed factors. Because there was a significant interaction between the tested species and the infestation treatment, differences in the within-plant distribution among different infestation treatments were assessed separately for each species using the testInteractions function (phia package, de Rosario-Martinez, 2015). Additionally, the effect of the presence of heterospecifics on the proportion of mites arriving on a plant was tested using a glm with a quasibinomial error distribution (due to the overdispersion of the residuals), where the total number of mites found on the plant (old + young leaves) and the number of mites missing (*i.e.*, the number released – the number found on the plant) were used as the response variable. The species tested (*T. urticae* or *T. evansi*), infestation treatment (coded as above) and their interactions were used as fixed factors.

Results

Effect of leaf age and infestation by heterospecific competitors on the performance of spider mites

The oviposition rate of *T. urticae* was significantly affected by the interaction among infestation treatment and leaf age ($F_{2,10} = 5.85$, $P = 0.007$). The oviposition rate was 0.72-fold higher on young leaves than on old leaves of un-infested plants (Fig. 2, $\chi^2_1 = 8.05$, $P = 0.009$). On plants of which the young leaf had been infested with *T. evansi*, the same pattern was found, with the oviposition rate 0.64 fold higher on young leaves (Fig. 2, $\chi^2_1 = 23.82$, $P < 0.001$), but no such difference was found for plants of which the old leaf had been infested (Fig. 2, $\chi^2_1 = 0.002$, $P = 0.96$).

In contrast, the oviposition rate of *T. evansi* was not significantly affected by the interaction between infestation treatment and leaf age ($F_{2,10} = 0.29$, $P = 0.74$) or by previous infestation by *T. urticae* (Fig. 3, $F_{2,10} = 0.17$, $P = 0.84$). Overall, oviposition was significantly (1.26-fold) higher on young than on old leaves (Fig. 3, $F_{1,10} = 33.11$, $P < 0.001$).

Fig. 2 Oviposition rates of T. urticae on tomato plants. *Average oviposition rates (eggs per female per day) of T. urticae (± s.e. 10 females per leaf, 10 plants) on old (dark gray) and young (light gray) leaves of tomato plants. Each set of two bars corresponds to one infestation treatment, i.e. plants that were either uninfested or had previously been infested with 40 heterospecifics on the old leaf (old leaf infested) or on the young leaf (young leaf infested). Significance between leaves of different ages among treatments: * - P < 0.05; n.s. - not significant.*

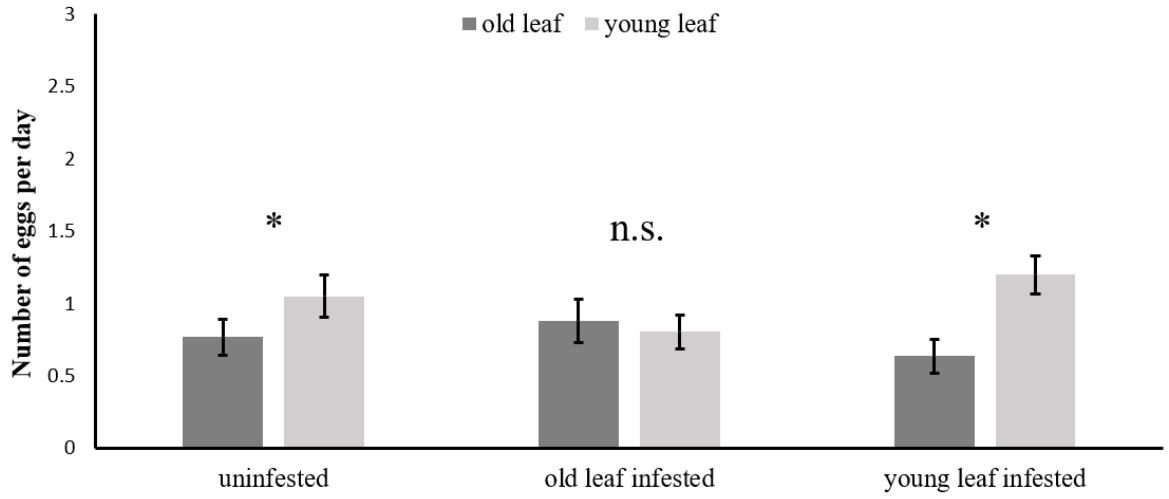
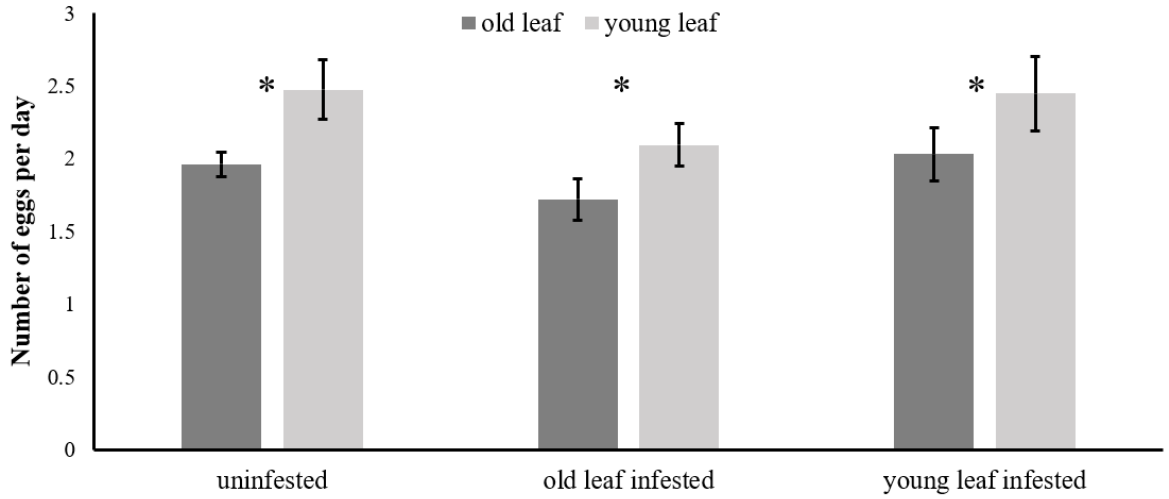


Fig. 3 Oviposition rates of T. evansi on tomato plants. *Average oviposition rates (eggs per female per day) of T. evansi (± s.e. 10 females per leaf, 10 plants) on old (dark gray) and young (light gray) leaves of tomato plants. Each set of two bars corresponds to one infestation treatment, i.e. plants that were either uninfested or had previously been infested with 40 heterospecifics on the old leaf (old leaf infested) or on the young leaf (young leaf infested). Significance between leaves of different ages among treatments: * - P < 0.05; n.s. - not significant.*



Effect of leaf age and infestation by heterospecific competitors on the distribution of spider mites across leaves

On un-infested plants, both species showed a similar distribution across leaves (Fig. 4, $F_{1,16}$ = 0.01, P = 0.92); the number of *T. urticae* and *T. evansi* females was 2.51 and 2.69 fold higher on younger leaves, respectively (Fig. 4, un-infested plants; $F_{1,16}$ = 155.66, P < 0.001). The recovery rate, hence host acceptance, did not significantly differ between species ($F_{1,16}$ = 0.33, P = 0.56) and was not affected by the presence of heterospecific competitors ($F_{2,16}$ = 3.21, P = 0.28). The distribution of the mites between leaves was affected by the presence of competitors (Fig. 4; $F_{2,16}$ = 33.96, P < 0.001). *Tetranychus urticae* and *T. evansi* were differently affected, however, by the presence and position of the competitor on the plant (Fig. 4, interaction between tested species and infestation treatment $F_{2,16}$ = 16.06, P < 0.001). The distribution of the females of *T. urticae* did not differ between un-infested plants and plants of which the older leaf was infested (Fig. 4A; $F_{1,16}$ = 3.01, P = 0.08). In contrast, the proportion of *T. urticae* on younger leaves was 0.61-fold lower when plants were infested with *T. evansi* than when plants were un-infested (Fig. 4A; $F_{1,16}$ = 44.15, P < 0.001). When younger leaves of plants were infested with *T. urticae*, the proportion of *T. evansi* females on those leaves was 0.66-fold lower than on un-infested plants (Fig. 4B; $F_{1,16}$ = 59.52, P < 0.001). Additionally, when old leaves were infested with *T. urticae*, the proportion of *T. evansi* on young leaves (47.1 ± 5.2 %) was 0.80-fold lower than on un-infested plants (71.4 ± 4.8 %) (Fig. 4B; $F_{1,16}$ = 32.87, P < 0.001).

Discussion

This study shows that both *T. urticae and T. evansi* prefer to colonize younger rather than older leaves of un-infested tomato plants, and this was matched by their high oviposition rates on these leaves. Their preference changed when plants were infested with heterospecifics: *T. urticae* avoided leaves infested with *T. evansi*, even when the latter occupied young leaves and *T. evansi* did not avoid leaves with *T. urticae* but showed a more even within-plant distribution than on un-infested plants. Moreover, the within-plant distribution of spider mites did not reflect the differences in oviposition rate between leaves of different ages on plants infested with heterospecifics.

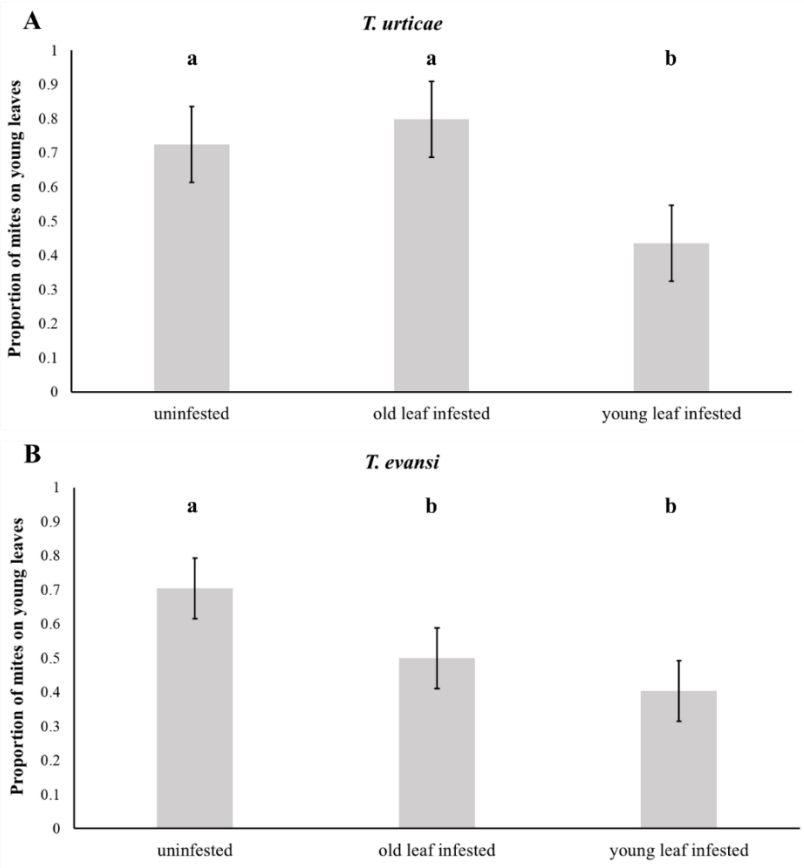


Fig. 4 Distribution of T. urticae and T. evansi within tomato plants either uninfested or infested with heterospecifics on the old leaf or the young leaf. *Shown is the proportion (± s.e.) of mites on young leaves of tomato plants (16 replicate plants per treatment; 100 mites per replicate). Plants were either uninfested or infested with 40 heterospecifics on the old leaf (old leaf infested) or on the young leaf (young leaf infested). A: Proportion of T. urticae on young leaves of uninfested plants or plants infested by T. evansi. B: Proportion of T. evansi on young leaves of uninfested plants or plants infested with T. urticae. Significant differences in preference between infestation treatments are indicated with different letters above each bar (P < 0.05).*

On un-infested plants, *T. urticae* had a lower oviposition rate than *T. evansi*. This was expected given that (a) this is generally the case on tomato plants (Sarmento *et al.*, 2011b; Alba *et al.*, 2015; Schimmel *et al.*, 2017), and (b) our *T. urticae* population was cultured on bean, whereas *T. evansi* was reared on tomato. The higher performance and the preference of both species for young leaves, which have lower C/N ratios (Supp.2), are in agreement with previous studies that show a preference of *T. urticae* for leaves with lower C/N ratios (Hoffland *et al.* 2000; Chen *et al.* 2007). However, other differences between leaves of different ages could explain the preference of spider mites for younger leaves. For example, differences in specific

nutrients or secondary metabolites, may affect the observed differences in performance and preference. In any case, our results suggest that both spider mite species preferred host plant leaves on which their oviposition rate was highest, at least in the absence of competitors. Independently of the proximate cues that trigger this behaviour, the similarity in preference for younger leaves in the two mite species on un-infested plants suggests that both species will preferentially colonize the same leaves on a plant, at least in the absence of competitors. Moreover, the percentage of mites recovered on the plant was similar between species (and treatments), indicating that the ability to find a host plant does not differ substantially between these two spider-mite species, at least not in the experimental set-up used. Hence, the competitive interactions of the invasive species *T. evansi* with resident species such as *T. urticae* are potentially intense, as they prefer to infest the same leaves, thus having similar fundamental niches, even within plants.

On infested plants, differences in quality are not only due to differences in leaf age but are also affected by herbivory (Karban & Myers 1989; Walling 2000; Awmack & Leather 2002). Here, infestation by spider mites did not affect variation in C/N ratio among different leaves (Supp.2), confirming previous results in this system (Ximénez-Embún *et al.* 2016, 2017). Still, infestation by spider mites may lead to physiological changes in the plant that are not detected by this ratio (Hamilton *et al.* 2001), such as the concentration of free sugars, proteins or amino acids (Ximénez-Embún *et al.* 2016, 2017) and these, in turn, could entail differences in performance. In any case, the two spider mite species were expected to distribute themselves according to differences in their performance. However, in contrast to what was observed on un-infested plants, the distribution of mites on plants with competitors did not reflect the differences in performance on different leaves.

The population of *T. evansi* used here was shown to suppress plant defences and this resulted in higher performance of con- and heterospecifics on infested leaves than on un-infested leaves (Sarmento *et al.* 2011a; Godinho *et al.* 2016). Here, in contrast, the performance of *T. urticae* was not always higher on leaves infested with *T. evansi* than on un-infested leaves. Possibly, this was due to intermediate expression of plant defences caused by the co-infestation of suppressors and inducers (de Oliveira *et al.* 2016, 2019; Schimmel *et al.* 2017b). Additionally, the effect of infestation by *T. evansi* on the performance of *T. urticae* varied with the age of the infested leaf. Indeed, infestation of old leaves by *T. evansi* led to a reduction in the differences in *T. urticae* oviposition rates on young and old leaves. By suppressing plant defences, *T. evansi* possibly increases the quality of old leaves. As old leaves are of lower quality than young leaves, this leads to a reduction in the differences between the two types of

leaves. Consequently, *T. urticae* should have less pronounced preferences for plant strata on plants where old leaves are infested by *T. evansi*. However, when *T. evansi* was present on young leaves, the differences in oviposition rates of *T. urticae* between leaves were not attenuated, because oviposition was already higher on young leaves. Based on these results, we expected *T. urticae* to still prefer young leaves when those were infested by *T. evansi*. Instead, we found that *T. urticae* avoided leaves infested with *T. evansi*. Likewise, the distribution of *T. evansi* on plants infested with heterospecifics did not match the differences in plant quality intrinsic to leaf age or differential oviposition of mites. In contrast to *T. urticae*, however, *T. evansi* distributed itself evenly between old and young leaves, hence, it did not avoid leaves with the competitor, independently of the position of the latter on the plant. Essentially, it seems that these herbivores chose leaves according to differences in their oviposition rate on un-infested plants, but not on plants infested with heterospecific competitors. Which cues trigger such differences in behaviour remains to be investigated.

Possibly, spider mites did not respond to the current plant quality, but instead, to cues associated with the presence of the competitor. There is evidence that *T. urticae* is outcompeted by *T. evansi* on tomato plants (Sarmento *et al.*, 2011b) and that *T. evansi* interferes with the reproduction of *T. urticae* (Clemente *et al.* 2018). Therefore, cues associated with *T. evansi* may be indicative of the presence of a competitor that will probably impose strong fitness costs upon *T. urticae* individuals. Avoiding heterospecifics within a plant may allow *T. urticae* to increase in numbers locally, to then disperse to other host plants before being out-competed by *T. evansi*. This may contribute to the coexistence of *T. urticae* with its invading competitor in the Mediterranean Basin (Boubou *et al.*, 2012; Ferragut, Garzón-Luque and Pekas, 2013; Zélé *et al.*, 2018a;). Because *T. evansi* possibly outcompetes *T. urticae* (Sarmento *et al.* 2011b), selection of *T. evansi* to avoid leaves with heterospecifics may be low, which is consistent with their behaviour in our experiments By distributing themselves evenly over different leaves in the presence of a competitor, *T. evansi* occupies more leaves, and by subsequently producing dense webbing (Sarmento *et al.*, 2011b), *T. evansi* can monopolize more feeding sites on a plant. In this way, it may prevent *T. urticae* from benefiting from defence suppression and from inducing defences in these leaves (Schimmel *et al.* 2017b). These results imply that the order of arrival is important for the outcome of the interspecific interactions between these species, as was found for other herbivorous arthropods (Erb *et al.* 2011; Miller-Pierce & Preisser 2012; Huang *et al.* 2017; Stam *et al.* 2017; Schaeffer *et al.* 2018), re-enforcing the idea that priority effects may play an important role in determining the composition of herbivore communities (Stam *et al.* 2018).

In conclusion, we show that the distribution of herbivores within a plant are affected by the presence of heterospecifics on this plant and does not reflect their performance. Possibly, this within-plant preference has been shaped by the asymmetries of their interspecific interactions. We speculate that these behaviours affect the outcome of those interactions and the potential for invasion. Indeed, the behaviour described here for *T. evansi*, allowing monopolization of local resources, may enhance the ability to colonize novel sites (Holway *et al.* 1998; Drescher *et al.* 2011). In addition, the within-plant avoidance behaviour of *T. urticae* may contribute to the resilience of the resident herbivore community. In any case, such changes in the settling behaviour may affect the distribution of species at larger spatial scales.

Acknowledgments

We thank Felipe Lemos for sending mite strains, Flore Zélé for improving the greenhouse facilities, Ana Janeiro for the C/N content analyses, Inês Santos for taking care of plants and mite populations and Leonor Rodrigues for aesthetical inputs on the supplementary schemes.

Funding This work was funded by an FCT-ANR project (FCT-ANR//BIA-EVF/0013/2012) to SM and Isabelle Olivieri, by a consolidator grant from the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation programme (COMPCON, grant agreement N. 725419) to SM and by an FCT PhD scholarship PD/BD/114010/2015 to DG.

Author contributions

DPG, SM and AJ conceived the project; DPG collected the data, with help from DL; CC provided logistical and technical support; DPG, SM and AJ analysed the data; DPG, AJ and SM wrote the manuscript. All authors gave final approval for publication.

Declarations

The authors declare no conflict of interest.

All data used in this work will be archived in FigShare data depository upon acceptance of the manuscript for publication.

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Supplementary information 1. Experimental design

Figure S1. Experimental design for the measurement of the C/N ratio of each leaf and the performance of spider mites infesting those leaves. On the left panel, each square represents a different treatment. Plants were infested with either *T. urticae* or *T. evansi*, on the old leaf (2nd) or the young leaf (4th) and un-infested plants were used as control. On the right panel, the material used per plant (replicate) to collect data is represented. The performance of the species that had not previously infested each plant was determined using 10 individual discs made from each leaf, old and young. The remaining leaf material was used to determine the C/N content.

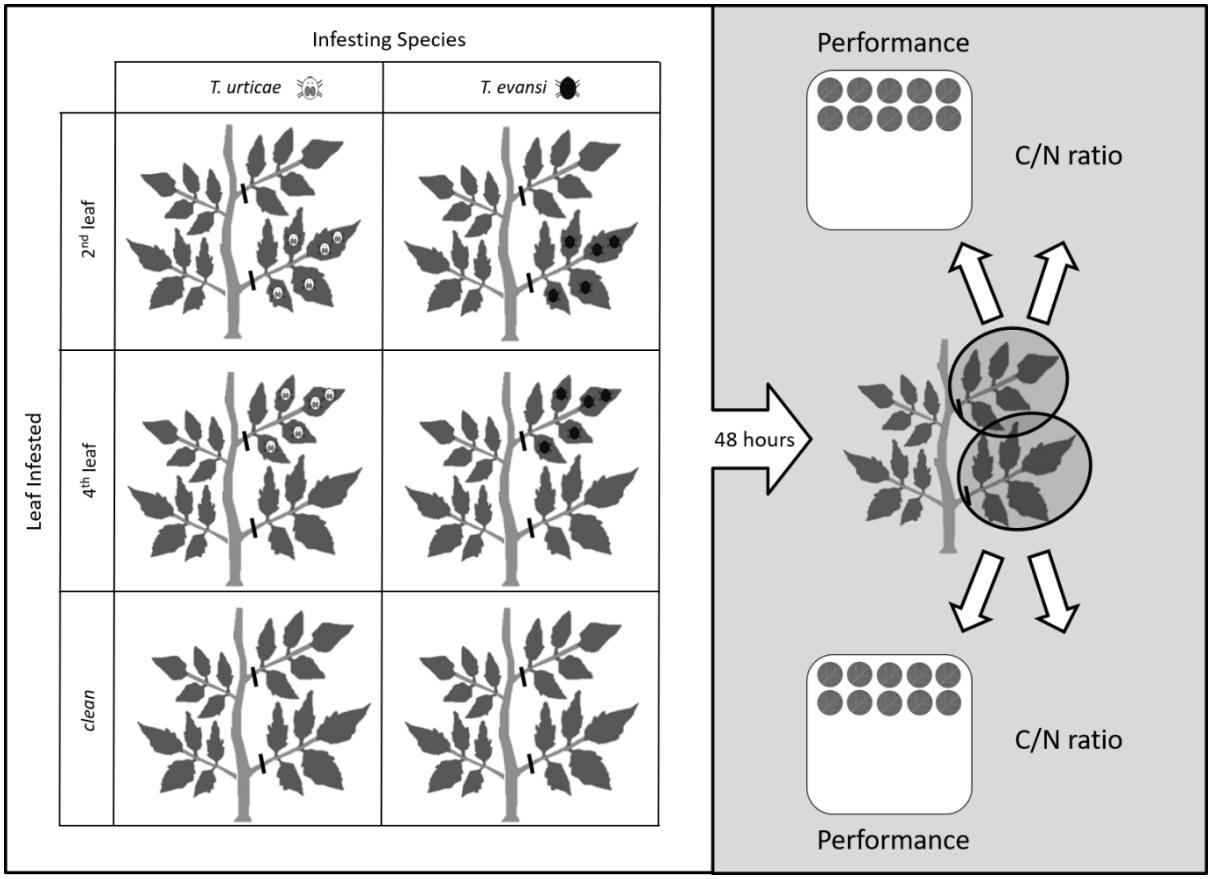
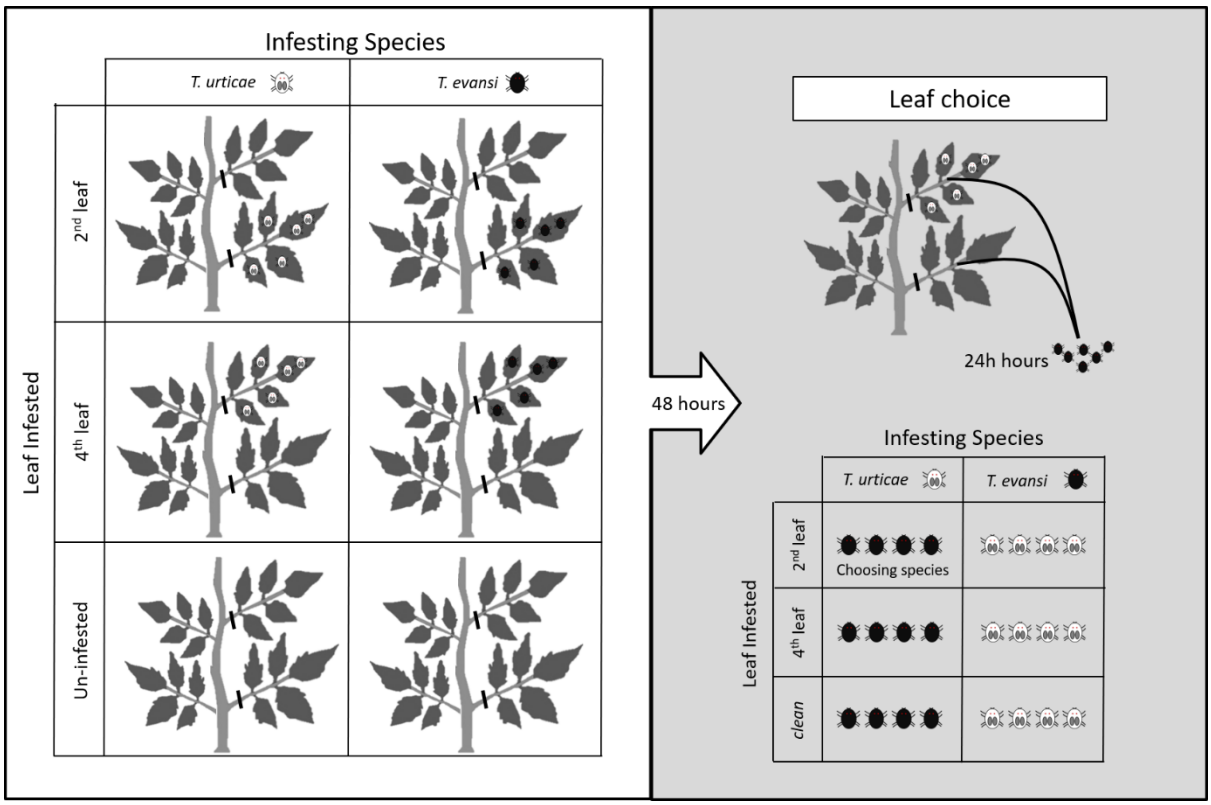


Figure S2. Experimental design for the choice between leaves. On the left panel, each square represents a different treatment. Plants were infested with either *T. urticae* or *T. evansi*, on the old leaf (2^{nd}) or the young leaf (4^{th}) and un-infested plants were used as control. On the right panel, the collection of data for the choice assay is represented. Both leaves of each plant were connected to an arena where 100 females of the species that was not infesting the plant (focal species) were released. 24h after later the number of females of the focal species on each leaf was registered.



Supplementary information 2. Effect of leaf age and spider-mite infestation on carbon to nitrogen ratio

To assess if the C/N ratio varied among leaves of difference ages and if spider mite infestation affected this ratio, the remaining material of each leaf used in the experiment to measure the performance of spider mite was dried at 60°C until constant mass, and ground. The ratio between the total carbon and nitrogen contents of old and young leaves of each plant was determined for 0.5g of each sample, by dry combustion using an elemental analyzer (EuroVector, Italy, Rodrigues *et al.*, 2009).

To assess the effect of leaf age and spider mite infestation on the C/N content of tomato leaves we used a general linear mixed-effects model (lme) with a Gaussian error distribution. C/N ratio was log transformed and used as the response variable. Leaf age (old or young), infestation treatment (un-infested plant, old leaf infested, young leaf infested), and their interaction were used as fixed factors and replicate (plant) as a random factor. Because infestations with different species were performed separately in this experiment, using different un-infested plants as controls, this analysis was performed separately for each species.

Leaf C/N ratio was not affected by infestation with *T. urticae* (Table 1; $F_{2,10} = 0.49$, $P = 0.61$) independently of the age of the infested leaf (interaction between leaf age and infestation treatment: $F_{2,10} = 0.94$, $P = 0.40$). The same pattern was observed for *T. evansi* (Table 1; $F_{2,10} = 2.32$, $P = 0.12$), also independently of the age of the infested leaf (interaction between leaf age and infestation treatment: $F_{2,10} = 0.69$, $P = 0.51$). The C/N ratio was 1.27-fold higher in old leaves than in young leaves, irrespective of their infestation status (Table 1; $F_{1,10} = 87.82$, $P < 0.001$ for infestations with *T. urticae* and $F_{1,10} = 81.82$, $P < 0.001$ for infestations with *T. evansi*).

Table S1. C/N content in old and young eaves of tomato plants.

Species infesting	*T. urticae*			*T. evansi*		
Infestation treatment	Un-infested plant	Old leaf infested	Young leaf infested	Un-infested plant	Old leaf infested	Young leaf infested
Young leaf	5.87 ± 0.14 **a**	5.97 ± 0.25 **a**	6.33 ± 0.31 **a**	6.10 ± 0.22 **A**	6.31 ± 0.23 **A**	6.04 ± 0.11 **A**
Old leaf	7.59 ± 0.25 **b**	7.29 ± 0.22 **b**	8.45 ± 0.56 **b**	7.44 ± 0.20 **B**	7.76 ± 0.38 **B**	7.94 ± 0.24 **B**

Mean C/N ratios (± SEM; 10 plants) in old and young leaves of tomato plants that were either un-infested or had one leaf (the old leaf or the young leaf) infested with *T. evansi* or *T. urticae*. Different letters indicate significant differences (lower case letters for plants infested with *T. urticae* and capital letters for plants infested with *T. evansi.*

Chapter IV

Effects of within-host competition on genetic variation for virulence, transmission and their correlation in herbivorous spider mites

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Abstract

Although the correlation between virulence and transmission is key to host-parasite interactions, whether it has a genetic basis, and how this may be affected by within-host competition remains elusive. Here we disentangle between genetic and environmental effects of within-host intra- and interspecific competition on virulence, parasite load, transmission and their potential genetic correlations, using 14 inbred lines of the ectoparasite *Tetranychus urticae*. Five, 10 or 20 females from each line were placed on a bean leaf patch, either alone or with 10 females of *T. evansi*, a heterospecific competitor. Virulence was assessed as the leaf area damaged 4 days later. The number of adult females on those patches was counted two weeks later, as a measure of parasite load. Transmission was measured in a separate assay, by placing females on a leaf patch connected to two other patches and measuring the number of females on each patch after 1, 2, 3, 6 and 9 days. We found genetic variance for virulence, parasite load and transmission. However, these traits were not genetically correlated. Additionally, the mean adult offspring per *T. urticae* female was lower in patches with higher conspecific densities, but not on patches with the heterospecific competitor, *T. evansi*. Transmission of *T. urticae* increased with the density of conspecifics and with the presence of *T. evansi*. Overall, different lines responded similarly to intra- and interspecific competition. Our results show that virulence, parasite load and transmission are genetically and environmentally labile. This may allow parasites to cope with heterogeneous environments.

Introduction

The virulence-transmission trade-off is a central tenet of host-parasite interactions (Alizon *et al.* 2009). According to this hypothesis, transmission should be maximized at intermediate virulence levels, as low virulence is correlated with low parasite load, hence few parasites to be transmitted, whereas high virulence should entail lower transmission due to excessive harm to the host via high parasite load (Anderson and May 1982; Antia *et al.* 1994).

Although not explicit in the original models, the relationship between virulence and transmission is assumed to have a genetic basis, given that it is expected to affect the evolution of the interaction between parasites and their hosts (May & Anderson 1983). This supposes that virulence, parasite load and transmission have a genetic basis, which has been indeed documented in a few systems (Mackinnon & Read 1999; de Roode *et al.* 2007; Lind *et al.* 2007; de Roode & Altizer 2010; Louhi *et al.* 2013; Stefansson *et al.* 2014; Clerc *et al.* 2015; Stewart *et al.* 2017). However, few empirical studies directly test the relationship between virulence and transmission (meta-analysed in Acevedo et al. 2019) and even fewer find evidence that supports the trade-off hypothesis (Mackinnon & Read 1999; de Roode *et al.* 2008; Doumayrou *et al.* 2013; Williams *et al.* 2014). Moreover, these studies measured transmission from infected hosts, making transmission dependent of the effects of virulence on that host, rather than being only dependent on the genetic variance for transmission of the parasite. Therefore, it is hard to pinpoint whether the relationship between virulence and transmission is due to a genetic correlation between these traits or due to differential impacts of virulence on within-host dynamics.

Indeed, within-host competition is also expected to affect virulence, parasite load and transmission, as well as their genetic variance and correlations (Frank 1996; Alizon *et al.* 2013; Sofonea *et al.* 2015; Ben-Ami 2017). Regarding intraspecific competition, theoretical models predict that, since host resources are finite, within-host competition will negatively affect the number of offspring produced by individual parasites, thus parasite load and, consequently, virulence and/or transmission (Anderson & May 1978) Such density-dependent parasite growth rate has been empirically shown in some systems (Ebert *et al.* 2000; Hughes *et al.* 2004). However, competition will generally select for an overall higher virulence. Indeed, theory predicts that within-host competition for resources may lead to higher replication rates and higher virulence (Levin & Bull 1994; Choisy & de Roode 2010). However, higher virulence is expected to lead to increased host mortality, reducing the period of multiple

infections and thus, the prevalence of co-infections in a population, lowering the selective pressure of interspecific competition on virulence (van Baalen & Sabelis 1995; Kamiya *et al.* 2018; Clay & Rudolf 2019). Some empirical studies indicate that competitors with higher replication rates and/or virulence are often less affected by competition and they are, thus, transmitted at higher rates (de Roode *et al.* 2005, 2008; Duncan *et al.* 2015), supporting the idea that multiple infections select for higher virulence. Some empirical studies showed that, virulence of co-infections was higher than that of single infections (Taylor *et al.* 1998; De Roode *et al.* 2005; Bell *et al.* 2006), or at least equivalent of that of the most virulent competitor (Ben-Ami *et al.* 2008, 2011). However, other empirical studies showed lower overall virulence in co-infections as compared to that of single infections, either because the most competitive strains were less virulent (Gower & Webster 2005; Ojosnegros *et al.* 2010), or due to spiteful interactions between competitors (Garbutt *et al.* 2011). Clearly, many theoretical and empirical studies show that virulence and transmission may be affected by within-host dynamics. However, knowledge on how these processes affect the genetic correlation between virulence and transmission is lacking.

To fill this gap, we measured virulence, parasite load and transmission in 14 inbred lines of the ectoparasitic spider mite *T. urticae* under different competition scenarios. This spider mite species is a generalist, infesting multiple host plants and its damage negatively impacts many crops (Helle & Sabelis 1985). The population growth of *T. urticae* is negatively affected by the density of conspecifics (Rotem & Agrawal 2003). Moreover, *T. urticae* often co-infests plants with the congeneric *T. evansi* (Zélé *et al.* 2018b). Such coinfections may lead to increased or decreased parasite load of one or the other species, or have no effect, depending on the populations tested (Sarmento *et al.* 2011; Orsucci *et al.* 2017). Additionally, *T. urticae* dispersal propensity varies with relatedness and density (Li and Margolies 1993a; Bitume et al. 2011, 2013; Bonte et al. 2014; Van Petegem et al. 2018), and population structure and host quality (Fronhofer et al. 2014, Li and Margolies 1993a; Dahirel et al. 2019). Additionally, within-plant movement of these two species is affected by the presence of one another (c.f. Chapter III). Nevertheless, to our knowledge, only one study assesses both virulence and transmission in this system. There, disperser mites were shown to inflict less damage on the host plant than non-dispersing mites, which suggests a possible negative correlation between these traits (Dahirel *et al.* 2019). However, it is unclear whether this negative correlation has a genetic basis.

Here we propose to characterize the effect of intraspecific and interspecific competition on virulence (i.e. host damage), parasite load (i.e. number of daughters) and transmission (i.e. dispersal) of 14 inbred lines of *T. urticae*, at different densities and in presence or absence of the congeneric *T. evansi*. Additionally, we used these inbred lines to test genetic correlations between virulence, parasite load and transmission and the effect of intra- and interspecific competition on those correlations.

Materials and Methods

Biological material

Tetranychus urticae was sampled on different host plants, in Portugal in 2013 (Zélé *et al.* 2018a), and reared since on bean plants (*Phaseolus vulgaris*, variety Prelude), at the University of Lisbon. From 5 of these field populations, 200 individuals were collected and mixed to form an outbred population (Rodrigues et al. 2018 BioRxiv) that was kept at high densities (>1000) also on bean. From this population, 20 inbred lines were created by sib mating for 15 generations. This was performed by isolating mated females on small leaf patches (18 mm diameter) where they laid eggs for 2 days. The offspring of each female remained isolated for 14 days. After this period, from each patch, 1 sib-mated female was transferred to a new patch. This procedure was repeated for 15 generations, ensuring an inbreeding coefficient above 95% (Godinho *et al.* 2020). A subset of 14 inbred lines was then transferred to the University of Montpellier, where they were reared on bean plants (variety Pongo) prior to, and during this study.

The *Tetranychus evansi* population was collected in October 2010 in the Alpes Maritimes (43.75313 N, 7.41977 E) on *Solanum nigrum*. A subset was transferred to the University of Montpellier on January 2018, where they were maintained on bean.

Prior to each experiment, cohorts of spider mites from each inbred line were created by isolating 40 mated females of each line on a bean patch (2-3 leaves placed together). These females were allowed to lay eggs for 48h. 14 days after, the mated daughters of these females, of approximately the same age, were used in the experiments. The same procedure was used to create cohorts of *T. evansi*. All spider-mite populations, inbred lines and cohorts used in these experiments were maintained on bean leaves (variety Pongo) placed on water saturated cotton

wool, in small plastic boxes (255 mm length x 183 mm width x 77 mm height), at 25˚C with a 16:8 L: D cycle, at 60% relative humidity.

In two separate assays, we measured the virulence and dispersal of each of the inbred lines at 3 different densities (5, 10 or 20 females), in the presence or absence of interspecific competition with *T. evansi*.

1. Virulence & parasite load

Females of each inbred line were randomly assigned to one of 3 different 'intraspecific density' treatments (5, 10 and 20 females), with or without 'interspecific competition' (10 *T. evansi* females), and virulence and the number of female offspring reaching adulthood was measured. In all treatments, females were placed on 2x2 cm bean leaf patches placed on wet cotton wool in plastic boxes (N=7 per block, 3 blocks) in a 6 x 8 design (48 patches per box, inbred lines randomly assigned to locations within boxes). There were 3 to 13 replicates for each inbred line per treatment combination (intraspecific density + interspecific competition) distributed across 3 blocks. Additionally, there were 16 patches, randomly distributed among the experimental boxes of all blocks, with 10 *T. evansi* females alone in order to assess average traits for this species. All females were allowed to feed and lay eggs on their leaf patches for 4 days. After this period, females were killed, and a photograph of each patch taken using a Canon EOS 70D camera. The damage inflicted by the spider mites, used as a measure of virulence, was determined using imageJ (Schneider *et al.* 2012) and Ilastik 1.3 (Sommer *et al.* 2011). Briefly, the background from each photo was removed in imageJ, subsequently using Ilastik we distinguished damaged area from healthy leaf and then in imageJ the damaged area was calculated taking advantage of the colour contrast between damaged and undamaged leaf tissue. Because some leaf veins were assigned as damage by Ilastik, clean bean leaf patches (uninfested by spider-mites) were left in the experimental boxes for the same period of time and photographed. These control patches were used to establish an average baseline "damage", which was subtracted from each measurement. After a period of 14 days, the female offspring surviving on each patch was counted. Only females were counted because the males of both species are not easily distinguishable and in these species mated females are the main dispersers (Smitley & Kennedy 1985).

2. Transmission

Adult females of each *T. urticae* inbred line were randomly assigned to the same 'intraspecific density' and 'interspecific competition' treatments as in the 'virulence' experiment, being placed on a 2x2 cm bean leaf patch. However, in this assay each patch was connected to two similar 4 cm^2 leaf patches in a line, joined to one another with 3 x 1 cm Parafilm bridges placed on wet cotton wool. Note that on day 1 all females were placed on the first patch in the line. This experimental setup was replicated across several boxes (N=23 per block, 2 blocks) in a design of 2 x 6 per box, with the position of patches within boxes randomized for inbred line and treatment. Additionally, 16 patches with only 10 *T. evansi* females were randomly distributed among the experimental boxes to assess average traits for this species. Females were allowed to feed and disperse across the patches, and the number of mites on each patch counted on days 1, 2, 3, 6, and 9 after the beginning of the experiment. Dispersal of mites to new patches was used as a measure of transmission (see details below). There were 3 to 13 replicates for each inbred line per treatment combination (density + heterospecifics) distributed across 2 blocks.

Due to logistical constrains, the dispersal data was only collected for 12 of the 14 inbred lines used in this study. Thus, even though the virulence traits were assessed for 14 inbred lines, the correlations between virulence and dispersal could only be done with 12 lines.

Statistical analysis

All analyses were preformed using the software R, version 3.6.0 and, all models were simplified removing non-significant interactions and factors.

Effects of intraspecific density and interspecific competition on total virulence

Because it is impossible to distinguish between damage inflicted by *T. urticae* and *T. evansi*, we compared differences in total host damage among lines, for 3 different treatments: 10 *T. urticae* females, 10 *T. urticae* females + 10 *T. evansi* females and 20 *T. urticae* females. These densities were chosen to compare virulence on the different patches when the density of mites was the same (20 mites, 20 Tu vs 10 Tu + 10 Te) and when the density of *T. urticae* was the same (10 *T. urticae* alone or in competition). Differences in the total percentage of damage inflicted were tested using a general linear mixed model (lmer function, lme4 package in R) with a Gaussian error distribution and an identity link function. The identity of the line and

treatment (10 *T. urticae* females, 10 *T. urticae* females + 10 *T. evansi* females, 20 *T. urticae* females) were used as fixed factors and block as a random factor. Because the distribution of the residuals was not normal, we transformed the data using the boxcox function (MASS package in R, Brian et al. 2019). Post-hoc contrasts between treatments with a Bonferroni adjustment were performed using the glht function (multcomp package in R, Hothorn et al. 2007). Additionally, broad-sense heritability, $H^2 = \frac{Var\ (G)}{Var\ (G)+Var\ (E)}$ (Falconer & Mackay 1996), was determined using a generalized linear mixed model fitted with a Markov Monte Carlo Chain approach (MCMCglmm package in R, Hadfield 2010). The percentage of damage inflicted was used as the response variable, 'intraspecific density' and 'interspecific competition' treatments as fixed factors and the identity of the inbred line as a random factor, so that the genetic variance for this trait could be calculated. The model included 300.000 iterations, with a burning of 10000 iterations and thinning of 10.

Effects of intraspecific density and interspecific competition on parasite load

As the number of *T. urticae* differed between treatments, we determined the number of daughters per capita, a relative measure of the production of transmissible stages that contribute to the parasite load. We compared differences between treatments with different intraspecific densities (5, 10 and 20) and with / without interspecific competition, using a general linear mixed model (lmer function, lme4 package in R) with a Gaussian error distribution and an identity link function. The identity of the line, 'intraspecific density' and 'interspecific competition' treatments were used as fixed factors and block as a random factor. Because the distribution of the residuals was not normal, we transformed the data using the boxcox function (MASS package in R, Brian et al. 2019). Broad-sense heritability was determined using the per capita number of daughters as the response variable fitted with the MCMCglmm function with 'intraspecific density' and 'interspecific competition' treatments as fixed factors, the identity of the inbred line as a random factor and the parameters defined as before.

Effects of intraspecific density and interspecific competition on transmission

Differences in transmission between inbred lines were determined using two measurements: the dispersal propensity and the time to half dispersal.

For dispersal propensity, each female on every replicate (group of 3 patches) was coded for each time point with a 0 if she remained on the initial patch and a 1 if she had left it. Differences in the number of females that left the initial patch through time were assessed using

a mixed effects Cox proportional-hazards model (coxme package in R, Therneau 2018). The identity of the line, 'intraspecific density' and 'interspecific competition' treatments were used as fixed factors and block as a random factor.

Time to half dispersal was measured as the average number of days it took for half the females to disperse from the initial patch. How this measure varied between lines, when exposed to each condition (density and competition) was assessed using a generalized linear mixed model (glmer function, lme4 package in R) with a Poisson error distribution and a log link function. The identity of the line, 'intraspecific density' and 'interspecific competition' treatments were used as fixed factors and block as a random factor.

Broad-sense heritability was calculated for both transmission traits as before, using dispersal propensity and time to half dispersal as the response variables fitted with MCMCglmm function with 'intraspecific density' and 'interspecific competition' treatments as fixed factors, the identity of the inbred line as a random factor and the parameters defined as before.

Genetic correlations between virulence, parasite load and transmission

Genetic correlations between virulence, parasite load and transmission were performed using a multi-response generalized linear mixed model fitted with a Markov Monte Carlo Chain approach (MCMCglmm package in R, Hadfield 2010). For parasite load, the number of daughters per capita was used as the response variable. For virulence, the average total damage inflicted was used as the response variable. Because there was no correlation between dispersal propensity and time to half dispersal (Supp. 1), we correlated both measurements against virulence. However, because the results were consistent (Fig. 4 and Supp. 2), we opted to show only the correlations between virulence and dispersal propensity. In these models 'intraspecific density' and 'interspecific competition' treatments were included as fixed factors, and the identity of the inbred line was included as a random factor, in order to estimate genetic variance attributable to the correlation between these traits. The model included 300.000 iterations, with a burning of 10000 iterations and thinning of 10.

Transmission of T. evansi

The dispersal propensity of *T. evansi* was assessed as a measure of transmission and determined in the same ways as for *T. urticae*, with each female in each replicate being coded at every time point with a 0 if she remained on the initial patch and a 1 if she had left it. The effect of

intraspecific variation (i.e. line identity) and density of *T. urticae* on the dispersal ratio of *T. evansi* was determined using a mixed effects Cox proportion-hazards model (coxme package in R, Therneau 2018). The identity of the *T. urticae* inbred line and density were used as fixed factors and block as a random factor.

Results

Effects of intraspecific density and interspecific competition on total virulence

We found significant genetic variance for total virulence (Fig. 1B), measured as the overall damage inflicted on the host patch, as this trait varied significantly among inbred lines ($F_{13, 319.03} = 3.63$, $P < 0.001$). Comparing an increase in density due to conspecifics or heterospecifics we found that, for all lines, total damage inflicted increased with intraspecific density (contrast between 10 and 20 *T. urticae*: $z = 7.12$, $P < 0.001$; Fig. 1A), maintaining roughly the same per capita damage, but not when the increase in density was due to heterospecifics (contrast between 10 *T. urticae* and 10 *T. uticae* + 10 *T. evansi*: $z = -1.2$, $P = 0.79$; Fig. 1A). This is further supported by the fact that for the same total parasite density, damage inflicted was higher in patches with 20 *T. urticae* than in patches with 10 *T. urticae* + 10 *T. evansi* (contrast between 20 *T. urticae* was and 10 *T. urticae* + 10 *T. evansi* : $z = 4.49$, $P < 0.001$; Fig. 1A). The broad sense heritability of damage inflicted was 0.07 (95% HDPI: 0.0007, 0.17).

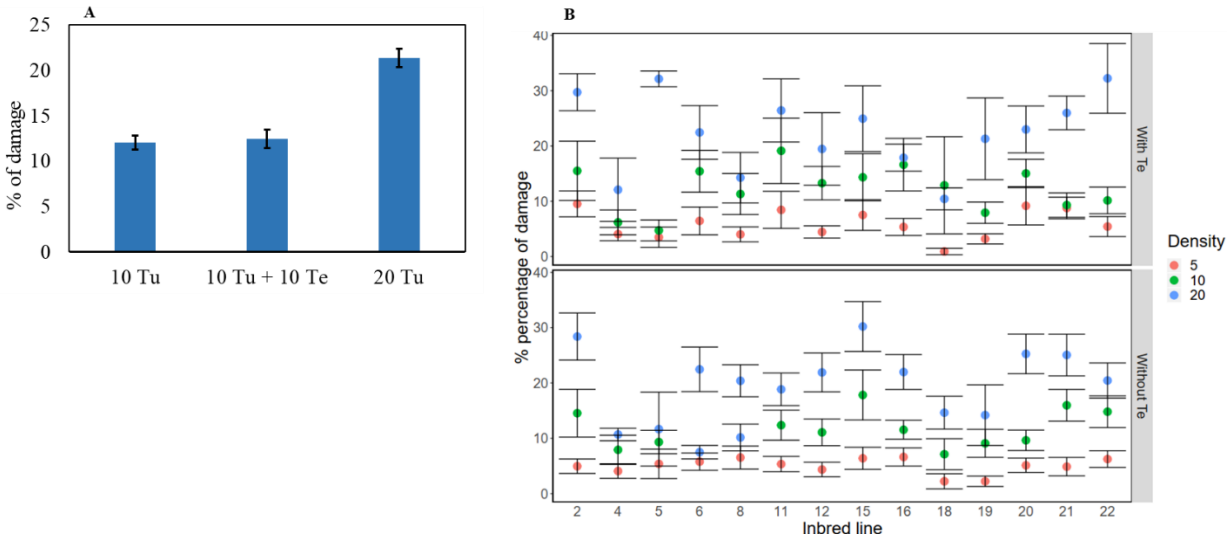


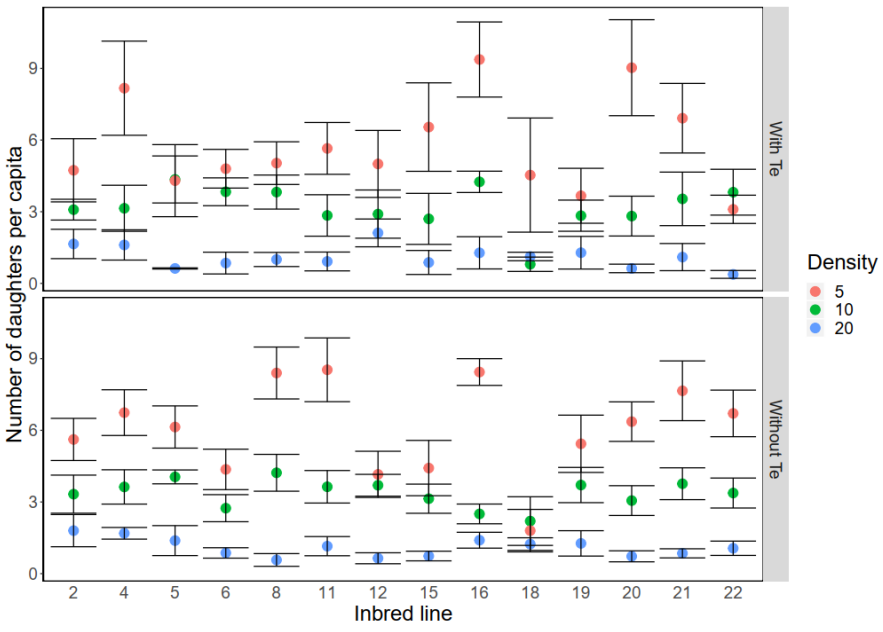
Figure 1. *Average percentage of leaf area damaged on 4 cm^2 bean leaf patches infested for 4 days. A) Comparison of intraspecific competition with interspecific competition. Host patches were either*

infested with 10 T. urticae females (10 Tu), 10 T. urticae females plus 10 T. evansi females (10 Tu + 10 Te) or 20 T. urticae females (± standard errors - vertical bars, N= 3 to 13 per line per treatment, 14 lines per treatment). B) Genetic variance in T. urticae (± standard error - vertical bars, N= 3 to 13 per inbred line per treatment) in the presence (With Te) or absence (Without Te) of 10 T. evansi females. Colours represent different densities (red = 5, green = 10 and blue = 20 females) of T. urticae on the patch.

Effects of intraspecific density and interspecific competition on parasite load

The per capita average number of adult daughters produced per patch after 14 days, used as a measure of the contribution to parasite load, differed significantly among inbred lines ($F_{13, 605.04} = 4.36$, $P < 0.001$; Fig. 2), hence, there was genetic variability for this trait as well. The broad sense heritability for this trait was 0.06 (95% HDPI: 0.0002, 0.13). Each line responded differently to female density on the patch (line * 'intraspecific density': $F_{26, 605.05} = 1.96$, $P = 0.003$; Fig. 2), but this was, in general, a difference in magnitude with rank order differences mostly being the same for each line (higher per capita offspring production at 5 females per patch, ≥ 10 ≥ 20; Fig. 2). Interspecific competition did not affect the number of daughters that reached adulthood ('interspecific competition': $F_{1, 605.65} = 0.15$, $P = 0.69$; line * 'interspecific competition': $F_{13, 592.02} = 1.4$, $P = 0.15$ Fig. 2).

Figure 2. *Average per capita number of daughters of T. urticae inbred lines (± standard error - vertical bars, N= 3 to 13 per line per treatment) on 4 cm² bean leaf patches in the presence (With Te) or absence (Without Te) of 10 T. evansi females. Colours represent different densities (red = 5, green = 10 and blue = 20 females) of T. urticae on the patch.*



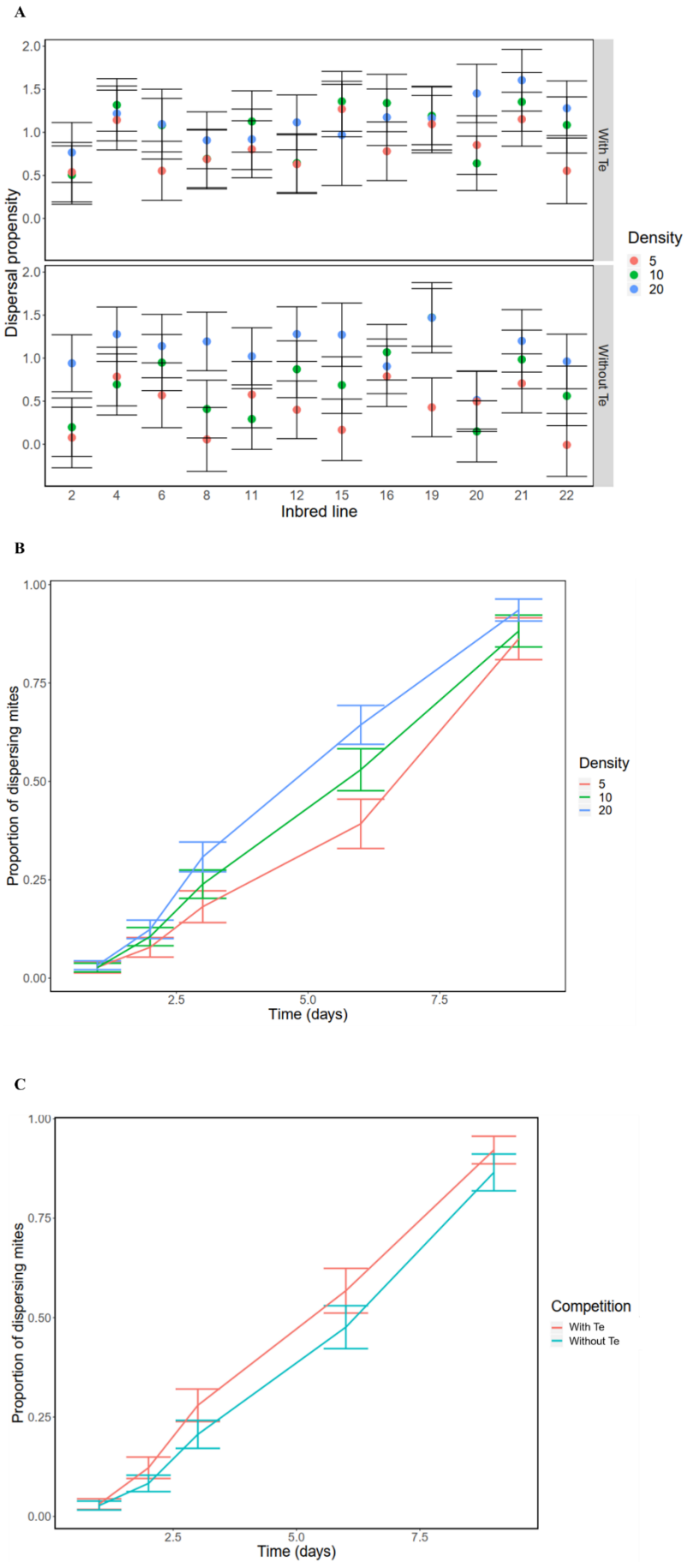


Figure 3. *A) Average dispersal propensity (± standard error - vertical bars, N= 3 to 13 per line per treatment) of inbred lines at different densities (red = 5, green = 10 and blue = 20), in the presence (With Te) or absence (Without Te) of 10 T. evansi females; B) and C) Proportion of dispersing mites through time (±standard errors - vertical bars, N= 3 to 13 per line per treatment, 12 lines per treatment), averaged per density (B; regardless of inbred line identity and of competition regime) and per competition regime (C; regardless of inbred line identity and of density).*

Effects of intraspecific density and interspecific competition on transmission

The dispersal propensity was significantly different across inbred lines ($X^2_{11} = 51.68$, $P < 0.001$; Fig. 3A, Supp. 3), indicating genetic variability for this trait. Overall, dispersal propensity increased with intraspecific density on the patch (line * 'intraspecific density': $X^2_{22} = 22.16$, $P = 0.45$; 'intraspecific density': $X^2_2 = 55.87$, $P < 0.001$; Fig. 3B) and the presence of heterospecific competitors (line * 'interspecific competition': $X^2 = 15.86$, $P = 0.15$; 'interspecific competition' $X^2_1 = 24.73$, $P < 0.001$; Fig. 3C). The heritability for dispersal propensity was 0.25 (95% HDPI: 0.005, 0.49).

We found a tendency for differences in time to half dispersal, across inbred lines ($X^2_{1\,1} = 18.03$, $P = 0.08$). The average time to half dispersal increased with the intraspecific density on the patch and this effect was similar across lines ('intraspecific density': $X^2_2 = 26.41$, $P < 0.001$; line * 'intraspecific density': $X^2_{2\,2} = 24.30$, $P = 0.33$). However, it was not affected the presence of heterospecific competitors ('interspecific competition': $X^2_1 = 1.22$, $P = 0.27$; line* 'interspecific competition': $X^2_{11} = 4.44$, $P = 0.95$). The heritability of time to half dispersal was 0.016 (95% HDPI: 0.0009, 0.058).

Genetic correlation between virulence, parasite load and transmission

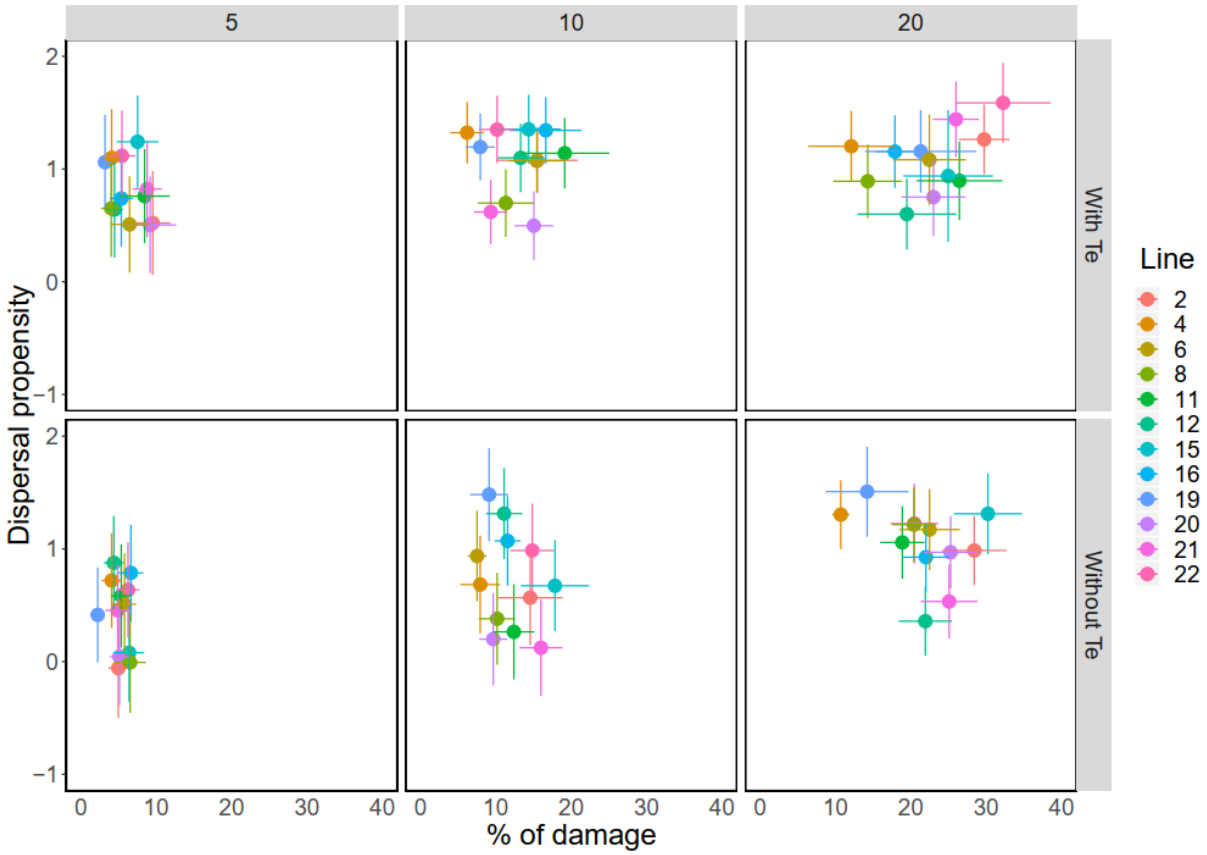
There were no genetic correlations between virulence, measured as percentage of leaf damage, and parasite load, measured as the number of daughters per capita (95% HDPI: -0.68, 0.62) and between parasite load and transmission, measured as dispersal propensity (95% HDPI: -0.65, 0.57).

There was also no genetic correlation between virulence, measured as percentage of leaf damage, and transmission, measured as dispersal propensity (95% HDPI: -0.57, 0.63; Fig. 4). Furthermore, neither intraspecific density on the patch nor the presence of *T. evansi* had an effect on the relationship between virulence and transmission (Simplest model had a lower DIC = 733.0, as compared to the DIC of the model with 'intraspecific density' as a fixed factor = 774.2 and the DIC of the model with 'interspecific competition' as a fixed factor = 774.3).

Transmission of T. evansi

The dispersal propensity of *T. evansi* was not affected by the identity of the inbred lines ($X^2_{1\,1} = 3.07$, $P = 0.98$), nor their density ($X^2_2 = 0.59$, $P = 0.74$; Supp. 4, Fig. S3).

Figure 4. *Co-variation between dispersal propensity and percentage of leaf damage inflicted on the host plant, for inbred lines of T. urticae, at different densities (5, 10 and 20), in the presence (With Te) or absence (Without Te) of 10 T. evansi females (± standard error - vertical bars, N= 3 to 13 per line per treatment).*



Discussion

In this study, using inbred lines from a population of the herbivorous arthropod *T. urticae* on bean plants, we showed that virulence (damage inflicted on the plant), the number of daughters produced per capita (as a surrogate for parasite load) and transmission (dispersal propensity) harbour genetic variance. However, we did not find a genetic correlation between these traits. Moreover, virulence (damage inflicted) and the number of adult females produced per capita on a patch of *T. urticae* were affected by intraspecific density, but not by the presence of the heterospecific competitor *T. evansi*. In contrast, the transmission of *T. urticae* (i.e. dispersal propensity) increased both with intraspecific density and with interspecific competition. For virulence and dispersal, all lines responded in the same way to intraspecific density and interspecific competition, despite significant genetic variability. The per capita number of

daughters was the only trait for which inbred lines responded differently to the density of intraspecific individuals, but not to the presence of heterospecifics. Finally, all inbred lines of *T. urticae* affected similarly transmission of *T. evansi*, independently of their density on the host.

The damage inflicted by *T. urticae* on the host patch (i.e. virulence) and the per capita number of daughters varied among inbred lines, revealing genetic variation for these traits, albeit low. Even though it was previously shown that different lines created from a field population differed in the virulence they inflicted on tomato plants (Kant et al. 2008), the extent of genetic variance for this trait was, to our knowledge, unknown. Dispersal propensity (i.e. transmission) of *T. urticae* also varied among inbred lines, with a heritability similar to that previously observed for this species (Bitume *et al.* 2011). The maintenance of genetic variance for these different traits may be explained by three, non-exclusive, hypotheses. First, spider-mites often live in heterogeneous environments and dispersal propensity may be more or less favourable depending on the spatial/temporal structure of the environment (Wolinska & King 2009; Tack *et al.* 2014) as they may encounter more or less suitable hosts. Second, parasitizing many hosts may help maintaining high variance for virulence, since traits that make parasites virulent on one host may hamper their virulence on another host (Gandon 2004). However, this is unlikely since in this species there is consistent evidence for the absence of trade-offs in fitness on different hosts (reviewed in Sousa et al. 2019). Additionally, by parasitizing different host species, genetic variance for virulence may be maintained by the trade-off between virulence and transmission, if the optimal virulence for transmission in one host differs from that on a different host. Finally, genetic variance for transmission may be preserved in populations maintained in laboratory conditions for a long time, such as those used in this study, where the costs of transmission may be reduced and, therefore, selection pressure for transmission is expected to be relaxed.

Using inbred lines increases the robustness of studies designed to detect genetic correlations between traits, as compared to studies using parasite isolates from different field / laboratory populations, since it ensures the same genetic background and evolutionary history for all genotypes assessed. Although there was genetic variance for virulence and transmission, we found no genetic correlation between these traits. It may be that virulence and transmission are uncoupled in this system. The relationship between virulence and transmission is hypothesized to be mediated via parasite load and, interestingly, we also did not find genetic correlations between the per capita number of daughters produced and virulence or

transmission. This decoupling between virulence and transmission might occur if the infection period is long enough to allow for genotypes with different virulence levels to disperse at equal rates, before the host is killed. Whereas this may be the case for long-living hosts such as citrus trees infected with *T. urticae*, it is not very likely to apply to bean (the host plant used in this study). As referred before, being multi-host parasites may help maintain genetic variance for virulence if the optimal virulence that maximizes transmission differs among host species. For the same reason, using many hosts may disrupt the overall relation between virulence and transmission in those parasites. Alternatively, the relation between virulence and transmission may depend on within-host dynamics, which are a consequence of virulence. Indeed, previous studies in other systems, despite using different parasite genotypes / strains, measure transmission after within-host parasite growth (Mackinnon & Read 1999; Mackinnon *et al.* 2002; de Roode *et al.* 2005, 2008; Doumayrou *et al.* 2013; Williams *et al.* 2014; Ben-Ami 2017). This measures the ability to transmit after within-host exploitation and damage. In other words, transmitting parasites are exposed to and experience the consequences of the damage they inflict. Additionally, most of these studies do not actually measure transmission per se but, instead, use a proxy for transmission, such as the number of transmission spores produced, or the number of vectors infected (Mackinnon & Read 1999; Mackinnon *et al.* 2002; de Roode *et al.* 2005, 2008). These transmission stages may fail to infect other hosts, compromising the reliability of the proxy (Alizon & Michalakis 2015). Other studies determine the proportion of infected hosts, which may be dependent on the density of available hosts and / or other epidemiological factors (Doumayrou *et al.* 2013; Williams *et al.* 2014). Here, we have used a different approach, in which we measure transmission traits, such as, the propensity for dispersal and the time it takes for half of the parasites to disperse to a new host patch. These measures of transmission were taken from hosts that had no prior damage, i.e. the transmission stage (adult females) was placed on uninfested leaf patches. Thus, we could disentangle whether the differences in dispersal detected were due to genetic differences or to different within-host dynamics, which we mimicked by using different initial densities. Note, however, that both traits used are a proxy for the parameters we wish to measure. Indeed, it should be proved that more damage results in lower plant fitness and that higher dispersal results in more hosts being colonized. The surrogates we have used are similar to those used in most studies on this topic, still, they may blur the detection of a genetic correlation (Alizon & Michalakis 2015). Additionally, it is possible that, even in the absence of a genetic correlation, the relationship between virulence and transmission is mediated by within-host dynamics.

At the same time, our results underscore the importance of the effects of intraspecific and interspecific competition on virulence and transmission. Indeed, we show that intraspecific density decreases the per capita production of female offspring of *T. urticae*, whereas within-host interspecific competition does not. Possibly, *T. evansi* is not a strong competitor on bean, the host plant used in this study. Indeed, they lay few eggs on this host plant (personal observations). Accordingly, the overall virulence was not affected by the presence of *T. evansi*, being equivalent to that of the most virulent species, as found in other studies (Massey et al. 2004; Ben-Ami et al. 2008, 2011, Duncan et al. 2015). This has important consequences both for *T. urticae*, which is not hampered by interspecific competition, and for the host, since interspecific competition does not result in higher overall virulence. However, this might not be the case if the strength of competition is more balanced between the involved species, for example on plants to which both *T. urticae* and *T. evansi* are equally adapted.

We found that both intra- and interspecific competition increased transmission. This may seem at odds with the fact that *T. evansi* does not affect the production of female offspring of *T. urticae*, nor the total damage inflicted on the host. However, in our design, hosts on which mites were placed to measure their transmission were not damaged. Therefore, it seems that transmission of *T. urticae* was affected by the density of competitors, independently of any effect that competitors may have on the host plant. This in accordance with previous results on a different host, tomato, which show that *T. urticae* avoids competitors, responding to their presence rather than to the effects of the competitor on the host (c.f. Chapter III). Interestingly, we found no differential effect of *T. urticae* inbred lines, or their density, on transmission of *T. evansi*. As bean is probably not a very suitable host for *T. evansi*, it may be that they are unable to retrieve sufficient resources from this host, in order to increase dispersal. Alternatively, these two species may have asymmetric effects on the dispersal, or movement decisions, of the other species. This has been shown on tomato plants, in which *T. urticae* avoids leaves previously infested with *T. evansi*, whereas the reverse was not observed (c.f. Chapter III). Here, however, as we mainly focus on *T. urticae*, we do not have an orthogonal design exposing both species to the same treatments. Therefore, further research on this topic is needed to clarify this issue.

Given the observed effects of intraspecific and interspecific competition on virulence and transmission, it may still be possible to find, in this system, a phenotypic correlation between virulence and transmission if the latter is measured from hosts where virulence was inflicted. We found little evidence for genotype by density interactions, with the only exception being for the number of female offspring produced per capita. Even in this case, the interaction

appears to be due to the magnitude of the effect (and not crossing reaction norms), as the rank order of the effect of density on this trait is the same for most inbred lines. Thus, inbred lines should be similarly affected by the damage inflicted on the host and, as such, more virulent lines may have higher dispersal propensities.

In the absence of a genetic correlation between virulence and transmission in this system, we expect those traits to be more genetically and environmentally labile. Here we show that virulence and transmission are affected by intraspecific and interspecific competition. Likewise, these traits may be affected by other environmental factors such as host-parasite interactions (e.g. the production of plant defences) and/or the spatial and temporal structure of host community. The lack of a genetic correlation between virulence and transmission suggests that parasites with the same virulence may transmit more or less (and vice-versa) depending on the environmental conditions, this way maintaining higher levels of variance in those traits. This may have relevant epidemiological consequences, leading to heterogeneous levels of virulence within and among host populations and allowing parasites to cope better with variability in host populations.

Acknowledgements

We would like to Inês Santos for sending the isogenic lines from Lisbon to Montpellier, Alain Migeon who provided the *T. evansi* population, Inês Fragata for insights on the statistical analyses and all the members of the MITE2 group for stimulating discussions.

This study was funded by an ERC Consolidator Grant (COMPCON, GA 725419) to SM, by a PHC-PESSOA grant (38014YC) to SM and AD and by an FCT Ph.D. scholarship PD/BD/114010/2015 to DPG.

Author's Contributions: DPG, AD and SM designed the experiments. LRR created the inbred lines. DPG, SL and AD performed the experiments with some help from LRR. DPG, SL and AD processed the data. DPG and SM analysed the data and wrote the manuscript with considerable help from AD.

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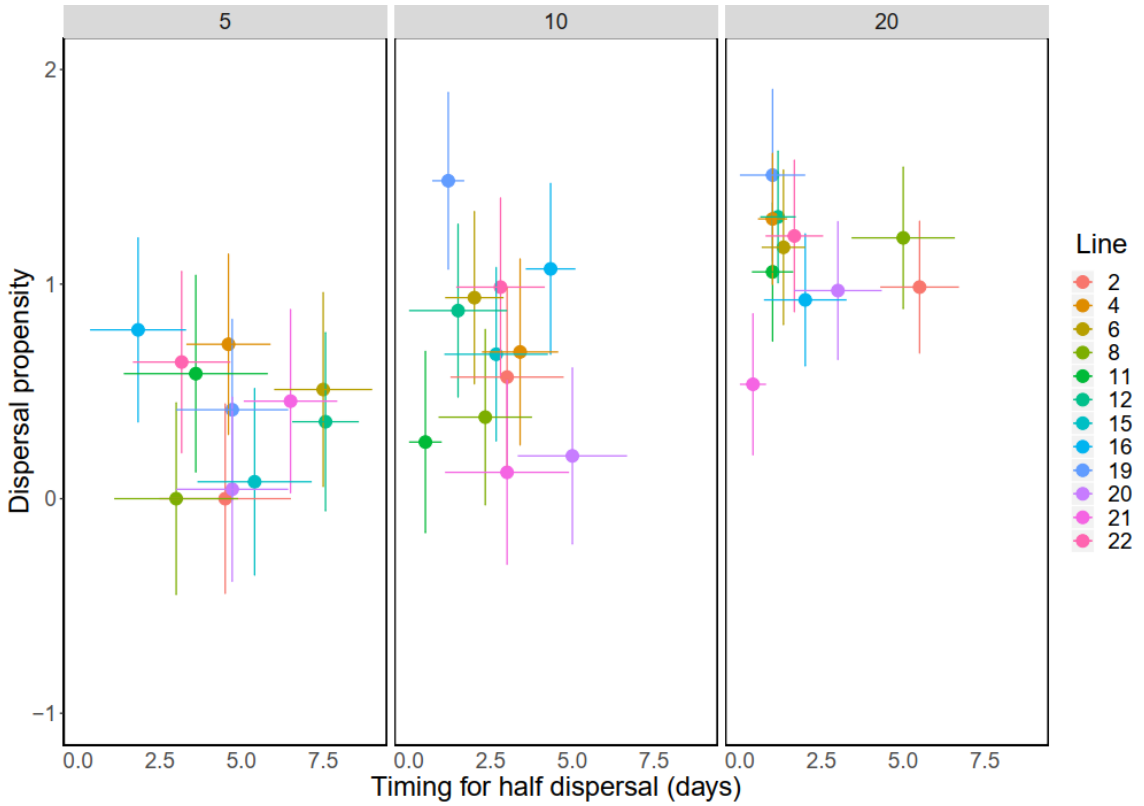
Supplementary information 1. Correlation between dispersal propensity and time to half dispersal

There was no significant genetic correlation between dispersal propensity and the time to half dispersal (95 % HDPI: -0.80 to 0.38).

Figure S1. Co-variation between dispersal propensity and time to half dispersal of inbred lines of *T. urticae*, at different densities (5, 10 and 20 females).



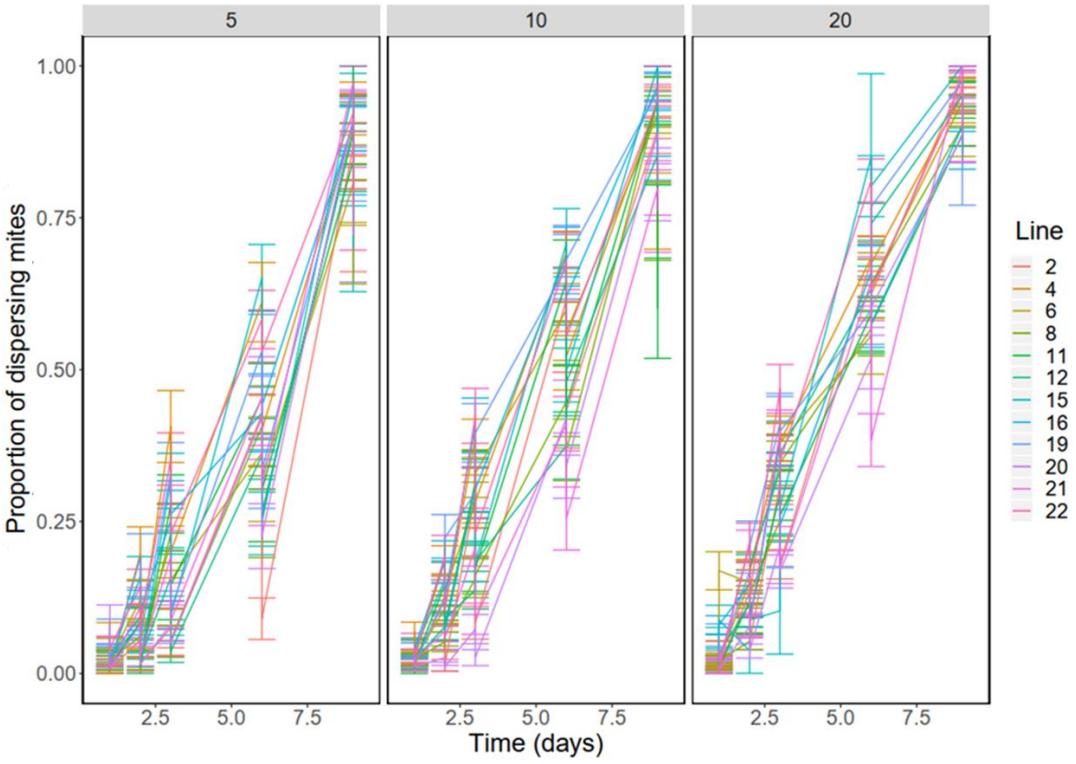
Supplementary information 2. Correlation between time to half dispersal and virulence

There was no genetic correlation between time to half dispersal and the damage inflicted on the host patch (95 % HDPI: -0.68 to 0.62).

Supplementary information 3. Dispersal propensity in individuals of *T. urticae* inbred lines

The dispersal propensity was significantly different across inbred lines (X^2_{11} = 51.68; P < 0.001), indicating genetic variability for this trait. Overall, dispersal propensity increased with intraspecific density on the patch but in a similar way across lines (line * 'intraspecific density': X^2_{22} = 22.16; P = 0.45; 'intraspecific density': X^2_2 = 55.87; P < 0.001)

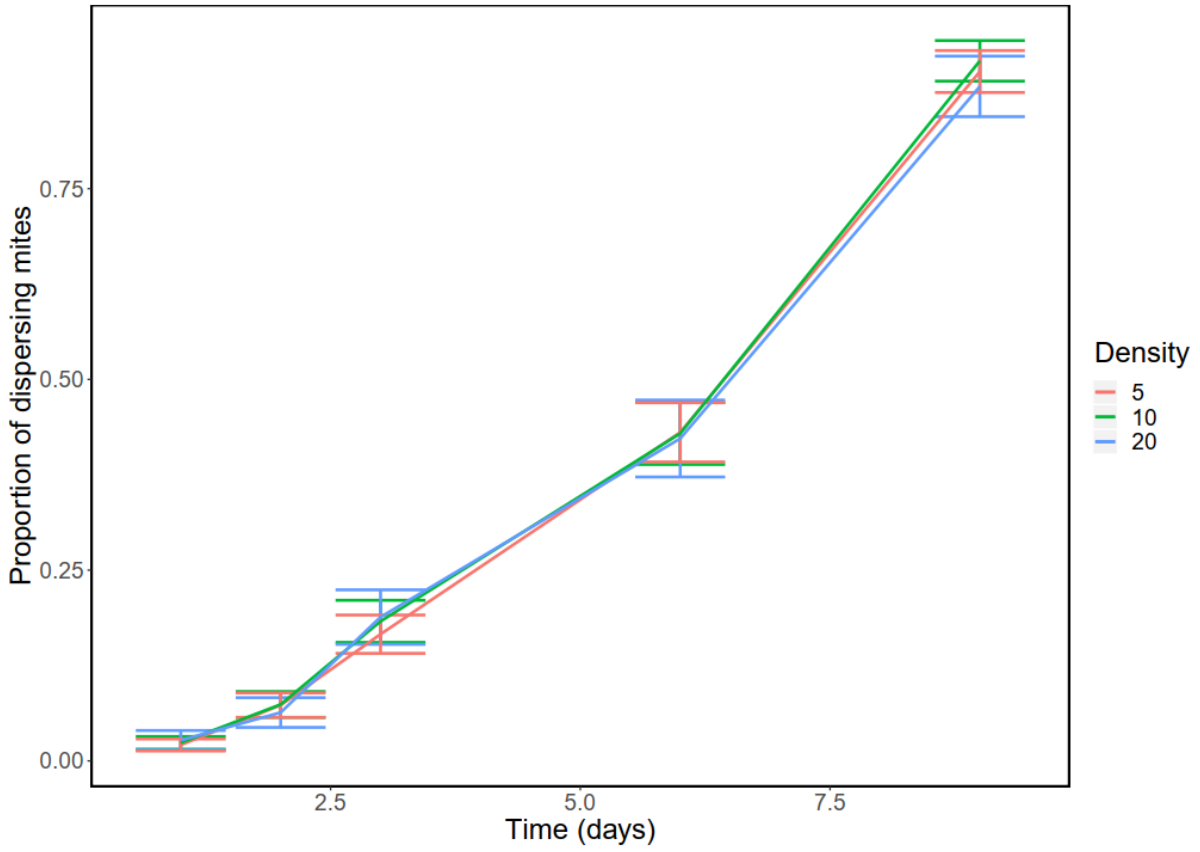
Figure S2 Average (± standard error - vertical bars, N= 3 to 13 per line per treatment) proportion of disperal from patches with different densities (5, 10 and 20 females) through time, for *T. urticae* inbred lines.



Supplementary information 4. Effect of the density of *T. urticae* on the dispersal of *T. evansi*

The dispersal propensity of *T. evansi* was not affected by the identity of the inbred lines (X^2_{11}= 3.07, P = 0.98), nor their density (X^2_2 = 0.59, P = 0.74).

Figure S3. Average (± standard error - vertical bars, N= 3 to 13 per line per treatment) proportion of *T. evansi* females dispersing through time from patches with either 5, 10 or 20 individuals from different *T.urticae* inbred lines, at different densities.



Chapter V

Effect of cadmium accumulation on the performance of plants and of herbivores that cope differently with organic defences

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Published in Frontiers in Plant Science

frontiers in Plant Science

ORIGINAL RESEARCH
published: 28 November 2018
doi: 10.3389/fpls.2018.01723

Effect of Cadmium Accumulation on the Performance of Plants and of Herbivores That Cope Differently With Organic Defenses

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Some plants are able to accumulate in their shoots metals at levels that are toxic to most other organisms. This ability may serve as a defence against herbivores. Therefore, both metal-based and organic defences may affect herbivores. However, how metal accumulation affects the interaction between herbivores and organic plant defences remains overlooked. To fill this gap, we studied the interactions between tomato (*Solanum lycopersicum*), a model plant that accumulates cadmium, and two spider-mite species, *Tetranychus urticae* and *Tetranychus evansi* that, respectively, induce and suppress organic plant defences, measurable via the activity of trypsin inhibitors. We exposed plants to different concentrations of cadmium and measured its effects on mites and plants. In the plant, despite clear evidence for cadmium accumulation, we did not detect any cadmium effects on traits that reflect the general response of the plant, such as biomass, water content, and carbon/nitrogen ratio. Still, we found effects of cadmium upon the quantity of soluble sugars and on leaf reflectance, where it may indicate structural modifications in the cells. These changes in plant traits affected the performance of spider mites feeding on those plants. Indeed, the oviposition of both spider mite species was higher on plants exposed to low concentrations of cadmium than on control plants, but decreased at concentrations above 0.5 mM. Therefore, herbivores with contrasting responses to organic defences showed a similar hormetic response to metal accumulation by the plants. Additionally, we show that the induction and suppression of plant defences by these spider-mite species was not affected by the amount of cadmium supplied to the plants. Furthermore, the effect of cadmium on the performance of spider mites was not altered by infestation with *T. urticae* or *T. evansi*. Together, our results suggest no interaction between cadmium-based and organic plant defences, in our system. This may be useful for plants living in heterogeneous environments, as they may use one or the other defence mechanism, depending on their relative performance in each environment.

Keywords: metal accumulating plants, plant defence, tomato, spider mites, elemental defence hypothesis

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Plant Microbe Interactions, a section of the journal Frontiers in Plant Science

Received: *08 August 2018*
Accepted: *06 November 2018*
Published: *28 November 2018*

Citation:
Godinho DP, Serrano HC, Da Silva AB, Branquinho C and Magalhães S (2018) Effect of Cadmium Accumulation on the Performance of Plants and of Herbivores That Cope Differently With Organic Defenses. Front. Plant Sci. 9:1723. doi: 10.3389/fpls.2018.01723

INTRODUCTION

Plants are exposed to an array of abiotic and biotic stresses. The mechanisms that allow them to survive these adversities imply physiological and structural transformations that can be costly to the plants, affecting negatively their growth and fitness (Boyer, 1982; Wang et al., 2003). One such stress is high bioavailable metal concentrations in soil, either naturally (geochemical anomalies) or due to anthropogenic activities. Although these high concentrations are toxic to most organisms, some plant species or populations, termed metallophytes, thrive in such environments. They achieve this either by limiting the metal uptake or the translocation to the shoot (excluders), or by storing metals in their shots (accumulators; Baker, 1987). However, these strategies entail costs that may be reflected in the plant performance, namely in plant biomass, in the water content of the shoots, and/or in the root to shoot ratio (Kastori et al., 1992; Das et al., 1997; Larbi et al., 2002; Chaffei et al., 2004; Devi et al., 2007). In addition, the stress caused by metal toxicity may lead to disturbances in the carbon and nitrogen metabolism, affecting the nutritional status of various plant parts (Larbi et al., 2002; Chaffei et al., 2004; Wahid et al., 2007), and potentially changing the accumulation of soluble sugars, either leading to increased (Devi et al., 2007; Rosa et al., 2009; Mishra et al., 2014) or decreased (Scheirs et al., 2006; Shackira and Puthur, 2017) sugar concentrations in the shoots. These physiological changes in the plant may also affect the performance of the herbivores feeding on those plants (White, 1984; Scheirs et al., 2006).

Besides being costly for the plant, accumulation of some metals is highly toxic to herbivores as well. Indeed, due to their elemental nature, they cannot be degraded by chemical counter-defenses of the herbivores (Boyd, 2004). Therefore, metal accumulation by the plants may be detrimental to herbivores (Martens and Boyd, 1994; Boyd and Moar, 1999; Behmer et al., 2005; Kazemi-Dinan et al., 2014) and this accumulation has thus been suggested to serve as a defense against herbivory (Boyd, 2004; Poschenrieder et al., 2006; Hörger et al., 2013). If metal accumulation does not compromise the production of organic defenses, the combination of both defense strategies may give accumulating plants an advantage over non-accumulating competitors (Boyd, 2007). It has been shown that metal exposure may directly increase the activity of some organic plant defenses, such as proteases (Pena et al., 2006; Lin et al., 2010), having possible indirect effects on herbivores. However, because both types of defenses may be costly to the plant, the production of effective metal-based defenses may lead to fewer organic plant defenses being produced. Indeed, some studies show that metal-accumulating plants produce fewer organic defenses upon pathogen attack when they are supplied with metals (Farinati et al., 2011; Fones et al., 2013). This suggests a trade-off between metal-based and organic defenses, although more evidence is needed to establish causality and determine its prevalence.

Most herbivores induce the production of organic plant defenses (Karban and Myers, 1989; Walling, 2000; Awmack and Leather, 2002). However some are able to suppress them (Musser et al., 2002; Abramovitch et al., 2006; Sarmento et al., 2011). Likewise, metal defenses vary in their effects upon herbivores. For example, the effectiveness of metal accumulation as an anti-herbivore defense varies with herbivore feeding guilds (Jhee et al., 2005; Vesk and Reichman, 2009; Konopka et al., 2013), as well as between specialist and generalist herbivores (Kazemi-Dinan et al., 2014). However, it is yet unknown whether metal-based defenses affect differently herbivores that induce or suppress organic defenses, and this may shed new light into the study of potential interactions between metal-based and organic defenses.

The model system composed of tomato plants (*Solanum lycopersicum,* L.) and herbivorous mites is ideal to test the abovementioned issues. When growing on soils with cadmium (Cd), tomato plants show higher tolerance than other species (Bingham et al., 1974; Khan and Khan, 1983; Kuboi et al., 1986) and inclusively are able to accumulate this metal in their shoots, sometimes over the Cd-hyperaccumulation threshold (100 $mg.kg^{-1}$; Gratão et al., 2008; López-Millán et al., 2009). Among spider mites, *Tetranychus urticae* is negatively affected by the accumulation of different metals by some host plants (Jhee et al., 2005; Quinn et al., 2010), but information concerning the effects of metals on other spider-mite species is as yet lacking. Additionally, different species within the Tetranychidae show contrasting effects on the induction of organic defenses of tomato plants. Indeed, *T. urticae* induces the production of jasmonate defenses, such as proteinase inhibitors, leading to lower performance of herbivores infesting those plants (Li et al., 2002; Ament et al., 2004; Kant et al., 2004). In contrast, *Tetranychus evansi* suppresses the production of such defenses (Sarmento et al., 2011; Alba et al., 2015), leading to higher performances of herbivores on subsequent infestations (Sarmento et al., 2011; Godinho et al., 2016). These differences allow testing the possible effect of metal accumulation on the inducibility of organic plant defenses. To this aim, we assessed the effects of Cd accumulation on the performance of tomato plants and on the spider mites that infest those plants. Additionally, we evaluated the effect of herbivory on jasmonate defenses and subsequent infestations by spider mites, on plants exposed to different Cd concentrations.

MATERIALS AND METHODS

Biological Materials and Rearing Conditions

Plants

Tomato plants (*Solanum lycopersicum*, var. Moneymaker) were sowed in a climate chamber (25°C, photoperiod 16/8 h light/darkness), in a soil (pH 5.0–6.0; Siro, professional substrates, Portugal)/vermiculite mixture (4:1) and watered 3 times per week for the first 2 weeks. In the third and fourth weeks, plants were watered once a week with tap water and twice a week with 60 mL of a Cd chloride solution with two ranges of concentrations: a wide range: 0, 0.01, 0.1, 0.5, 1, 2, or 10 mM; and a narrow range: 0, 0.1, 0.25, 0.5, 0.75, 1, or 1.5 mM. Using the wide range, we tested the effects of high Cd concentrations in the plant and on spider mites. Using the narrow range allowed us to

measure plant and spider-mite traits with higher resolution. At the end of the fourth week, plants were used in the experiments.

Spider Mites

Tetranychus urticae was collected from tomato plants in Portugal in 2010, and reared on bean plants (*Phaseolus vulgaris*, L.) since then (Clemente et al., 2016). In January 2016, a sub-set of the population (>300 mated females) was transferred to tomato plants and maintained on this host for six generations, before being used in the subsequent experiments. *T. evansi* was collected from *Datura stramonium*, L. in 2013, in Portugal, and reared on tomato plants ever since (Zélé et al., 2018). The two species were maintained, separately, in plastic boxes containing two entire tomato plants, in a climate chamber with conditions identical to those of the plant growing compartment (25°C, photoperiod 16/8 h light/darkness). Once a week, one plant was removed, and its leaves were cut and placed on top of the leaves of a new plant, allowing spider mites to migrate to new intact plants. To ensure that females used in the experiments were approximately of the same age, adult females where isolated on separate leaves and allowed to lay eggs for 48 h. Twelve days later, the adult females resulting from these cohorts were used in the experiments.

General Methodology

The performance of plants and spider mites was assessed using plants exposed to both ranges of Cd supply. For every assay the plants were between 4 and 5 weeks old, but to control for the effect of leaf age, we also always used the third leaf from below (third older) for the several measurements.

Plant Performance

Because the plant material collected was not enough to use in every assay, measurements were performed with different plants: Plants exposed to the wide range of concentrations (0–10 mM, $N = 6$ per Cd concentration) were used to determine Cd accumulation on the leaf, as well as the amount of calcium (Ca) and magnesium (Mg). As Cd^{2+} uses the same transporters as these ions, their assimilation by the plant may be hampered by Cd, which is not the case in hyperaccumulating plants (Gomes et al., 2013). From the narrow range (0–1.5 mM), half the plants ($N = 6$ per Cd concentration) were used to obtain the biomass parameters (root/shoot; specific leaf area and water content), however, due to technical problems, the plants supplied with 1.0 mM of Cd could not be used in this assay. The remaining plants ($N = 6$ per Cd concentration) were used to measure the amount of soluble sugars and to determine the carbon (C) to nitrogen (N) ratio. Nevertheless, for each plant, and before any destructive assay, we determined the spectral reflectance of the leaf, a non-invasive method that provides a general assessment of plant stress (Carter, 1993; Carter and Knapp, 2001).

Spectral analysis

The spectral reflectance was measured on one leaf from each plant, five measurements per leaf, using a UniSpec spectroradiometer (PP Systems, Haverhill, MA, United States). The spectral data generated by these measurements was analyzed by calculating spectral reflectance factors (R) for each wavelength (between 300.4 and 1148.1 nm with intervals of 3.4 nm). These factors were obtained by normalizing the reflected radiation from the leaves by a reflectance white standard. Several vegetative indices can be determined using reflectance data and used as a proxy of plant stress, being the most commonly used the Normalized Difference Vegetation Index (NDVI) as it reflects the efficiency of the photosynthetic system (Sridhar et al., 2007). Therefore, we here measured NDVI ((R810–R680)/(R810+R680)). In addition, we measured the SC index, which is representative of structural changes (SC) in leaf cells caused by accumulation of Cd (R1110/R810; Sridhar et al., 2007). Moreover, as it has been proposed that plants respond similarly to UV-B light exposure and herbivory, such as producing phenolic compounds (Roberts and Paul, 2006; Izaguirre et al., 2007), we also analyzed the spectral data under those wavelengths. For that we averaged, for each plant, the spectral reflectance factors of all UV-B wavelengths (R300.4–R313.9), referred afterward simply as UV-B reflectance.

Cadmium, calcium, and magnesium quantification

One leaf from each plant was dried for 72 h at 60°C until constant mass and uniformly ground. The elements were then quantified using Inductively Coupled Plasma – Atomic Emission Spectrometry (ICP – AES, Agilent 7500ce – Eurofins, Spain), after nitric acid digestion, with a detection limit of 0.1 μg/L.

Root to shoot ratio, specific leaf area and plant water content

All leaves and roots of each plant were collected, then the area of each leaf was measured with a laboratory leaf meter (LI-COR Biosciences). Next, the fresh weight of leaves and roots was obtained. Each leaf and the roots were then separately dried for 72 h at 60°C until constant mass and again weighed. The ratio between the dry weight of the roots and the dry weight of the leaves (root/shoot) was determined as well as the specific leaf area (SLA, total leaf area/total leaf dry weight) and plant water content (fresh weight–dry weight/fresh weight).

Carbon to nitrogen ratio

One leaf from each plant was dried at 60°C until constant mass and again weighed. The total carbon (C) and nitrogen (N) contents (grams of C or N per 100g of leaf dry weight) of each leaf was determined by dry combustion using an elemental analyser (EuroVector, Italy; Rodrigues et al., 2009).

Soluble sugar contents

One leaf disk (Ø 12 mm) was stored at −80°C and subsequently used to quantify the amount of soluble sugars. These were extracted from the leaf disk using 2 mL of 80% ethanol at 80°C and then quantified through changes in absorbance, at 405 nm for sucrose, using the resorcinol (1,3-dihidroxybenzene) method (de Carvalho et al., 2015), and 490 nm for glucose and fructose, using DNS (de-nitrosalicilic acid) as an oxidizing agent (Santos et al., 2017).

Spider-Mite Performance

Six leaf disk arenas (Ø 12 mm) were cut from one leaf (third from below) of each plant ($N = 6$ per Cd concentration for the wide range, $N = 12$ per Cd concentration for the narrow range) and placed on a petri dish on top of wet cotton wool.

One female spider mite of one of the two species was placed on each arena (three arenas per species per plant) and allowed to feed and oviposit for 4 days. Daily survival and fecundity of each female were recorded. The daily fecundity of spider mites was obtained by dividing the number of eggs laid by the number of days the female lived. In a previous study, it has been shown that this measurement is highly correlated with total lifetime fecundity (Clemente et al., 2018). Therefore, this measure can also be considered as an indication of the overall performance of spider mites.

Interaction Between Cd Accumulation and Inducibility of Jasmonate Organic Defenses

To test whether the effect of Cd and jasmonate organic defenses on herbivores are independent, tomato plants were exposed to three different Cd concentrations (0, 0.5, and 1.5 mM) as described before. Next, plants from the three treatments were infested for 48 h with either 100 *T. evansi* or *T. urticae* females on the third leaf (from below), or they were left un-infested (N = 12 plants per treatment; 9 treatments: 3 Cd concentrations vs. 3 infestation status – un-infested plants, plants infested with *T. urticae* and plants infested with *T. evansi*). Afterward, the plants were cleaned by removing all the mites, web, and eggs with a brush.

The performance of spider mites was determined as above.

Activity of trypsin inhibitors (TIs)

Plant material from the leaf used to determine the performance of spider mites was stored at −80°C and used, later, to quantify the activity of TIs, as a proxy for inducibility of the jasmonic acid pathway by spider mites (Sarmento et al., 2011; Godinho et al., 2016; Paulo et al., 2018). Approximately 300 mg of the leaf material stored at −80°C was weighed, ground, and homogenized with 600 μL of extraction buffer (0.1 M Tris-HCl, pH 8.2; 20 mM $CaCl_2$; 1:3). Each sample was centrifuged at 4°C, 16.0 × g for 25 min, and the supernatant was separated from the pellet and used in the spectrophotometer assay. This assay, adapted from Paulo et al. (2018) consisted in measuring the changes in absorbance at 405 nm caused by the activity of trypsin upon its substrate N-α-Benzoyl-DL-arginine 4-nitroanilide hydrochloride (BApNA).

Statistical Analyses

All statistical analyses were performed with the software package R 3.0.2. The normality of the residuals of each model was tested using a Shapiro–Wilk normality test and, when needed, a Box-Cox transformation to the data was performed. Models were simplified by sequentially removing non-significant interactions and factors. Due to logistic constraints, each experiment was repeated in blocks of three plants per treatment. Block was thus included in the models as a random factor.

The effects of Cd exposure on NDVI, SC index (R1110/R810) and reflectance under the UV-B spectrum were determined using general linear mixed models (lmm) with, respectively NDVI, SC index or UV-B reflectance as response variables, Cd supplied as a fixed factor and block as a random factor.

The relation between the Cd contained in the solution administrated to the soil and the Cd contained in the leaves was determined with a Spearman correlation, due to the non-normality of the data. Furthermore, the relation between Cd contents and the amount of calcium (Ca) and magnesium (Mg) present on the leaves was assessed with a Pearson correlation.

The effects of Cd on specific leaf area, water and soluble sugar contents were tested using general linear mixed models (lmm) with Cd supplied as a fixed factor and block as a random factor, whereas differences in root/shoot and in C/N were determined using a generalized linear mixed model (glmm) with a binomial distribution, and the same factors as above.

The effects of Cd on daily fecundity of spider mites were determined for each range, using general linear mixed models (lmm) with species tested and Cd supplied as fixed factors and block as a random factor. Additionally, because the soluble sugar contents and the spectral SC index (R1110/R810) were affected by Cd, we tested whether changes in those traits influenced the daily fecundity of spider mites using a multivariate analysis of variance with distance matrices (adonis function, vegan package; Oksanen et al., 2013). The fecundity of *T. evansi* and *T. urticae* were used as response variables, the amount of sucrose and glucose plus fructose or the spectral SC index (R1110/R810), were used as fixed factors.

The statistical analysis of the interactions between Cd accumulation and jasmonate organic defenses were performed using general linear mixed models (lmm) with daily fecundity of *T. evansi* or the amount of trypsin inhibited as response variables, Cd supplied (0 mM; 0.5 mM; 1.5 mM) and infestation status (un-infested plants; plants previously infested with *T. urticae*; plants previously infested with *T. evansi*) used as fixed factors and block as a random factor.

RESULTS

Effect of Cd on the Performance of Tomato Plants

Cadmium exposure had no effect on NDVI (**Table 1**). However, significant differences were detected for the SC index (R1100/R810), (**Table 1**) on plants exposed to 2 mM or 10 mM of Cd (**Table 2**), suggesting structural changes in the leaf cells. The same pattern was detected when analyzing the narrow range of Cd concentrations (**Table 1**) but only for plants exposed to 1 mM and not for plants exposed to 1.5 mM (**Table 2**). Additionally, the UV-B reflectance of plants was significantly affected by Cd exposure (**Table 1**), for concentrations higher than 1 mM in the wide range (**Table 2**) and higher than 0.75 mM for the narrow range (**Table 2**).

The concentration of Cd accumulated in tomato leaves correlated positively with the Cd concentrations that plants were exposed to, in a linear way ($y = 52.299x + 21.165$, rho = 0.945, $P < 0.001$; **Figure 1**). The amount of Ca and Mg in the leaves did not change significantly with Cd accumulation ($R^2 = 0.25$, $P = 0.11$ for Ca and $R^2 = 0.23$, $P = 0.14$ for Mg).

Cadmium supplied to plants did not significantly affect water content of the leaves, SLA and root/shoot ratio (**Tables 3**, **4** and

TABLE 1 | Statistical analyses of the effect of cadmium on leaf reflectance.

Variable of interest	Data subset	Exploratory variable	*Df*	*F*	*P*-value
NDVI	Wide range	Cd supplied	6	0.21	0.97
	Narrow range		5	0.83	0.53
SC index	Wide range	Cd supplied	6	3.19	**0.015**
	Narrow range		5	4.49	**0.001**
UV-B reflectance	Wide range	Cd supplied	6	11.44	**<0.001**
	Narrow range		5	10.15	**<0.001**

p-values < 0.05 are indicated in bold.

TABLE 2 | *A posteriori* contrasts on the effect of cadmium on leaf reflectance.

Variable of interest	Data subset	Contrast	*Z*-value	*P*-value
SC index	Wide range	0 mM vs. 0.01 mM	1.23	0.88
		0 mM vs. 0.1 mM	1.80	0.54
		0 mM vs. 0.5 mM	2.87	0.06
		0 mM vs. 1 mM	−1.89	0.49
		0 mM vs. 2 mM	−0.18	**0.001**
		0 mM vs. 10 mM	−0.13	**0.008**
	Narrow range	0 mM vs. 0.1 mM	−0.79	0.97
		0 mM vs. 0.25 mM	−0.20	0.99
		0 mM vs. 0.5 mM	0.84	0.96
		0 mM vs. 0.75 mM	−3.38	**0.009**
		0 mM vs. 1.5 mM	−1.43	0.71
UV-B reflectance	Wide range	0 mM vs. 0.01 mM	1.67	0.64
		0 mM vs. 0.1 mM	2.12	0.34
		0 mM vs. 0.5 mM	3.91	**0.002**
		0 mM vs. 1 mM	−0.11	**<0.001**
		0 mM vs. 2 mM	−0.13	**<0.001**
		0 mM vs. 10 mM	−0.17	**<0.001**
	Narrow range	0 mM vs. 0.1 mM	1.50	0.66
		0 mM vs. 0.25 mM	2.35	0.17
		0 mM vs. 0.5 mM	4.07	**<0.001**
		0 mM vs. 0.75 mM	−5.28	**<0.001**
		0 mM vs. 1.5 mM	−5.74	**<0.001**

p-values < 0.05 are indicated in bold.

Figures 2A,B). No effect of Cd exposure was observed in the C/N content of the tomato leaves up to 1.5 mM (**Table 3** and **Figure 2C**). However, the amount of soluble sugars in the leaves was affected by the concentration of Cd to which plants were exposed (**Table 3** and **Figure 2D**). The amount of both sucrose and glucose plus fructose decreased in plants exposed to low concentrations of Cd, having the lower values at 0.5 mM (**Table 5** and **Figure 2D**). In plants exposed to 0.75 mM of Cd, the levels of sugars peaked to values higher than in control un-exposed plants but then decreased again for higher concentrations to values lower than on control plants (**Table 5** and **Figure 2D**).

Effect of Cd Accumulation on the Performance of Spider Mites

The oviposition of spider mites on leaf disks was significantly affected by the Cd supplied to the plants used to make those disks (**Table 6** and **Figure 3**). Additionally, both spider-mite species were similarly affected by the Cd concentration that plants were exposed to (**Table 6**). Both species increased their oviposition with low amounts of Cd until a threshold concentration, 0.5 mM (**Table 6** and **Figure 3**). From this concentration onward, Cd had a negative effect on the oviposition rate of spider mites, reaching, values lower than in control plants at 2 mM for the wide range and at 1.5 mM for the narrow range, respectively (**Table 6** and **Figure 3**).

The amount of sucrose in the leaves did not affect the fecundity of spider mites ($F_1 = 0.008$, $P = 0.71$). In contrast, the amount of glucose plus fructose affected this trait ($F_1 = 0.19$, $P = 0.003$). Additionally, the reflectance SC index (R1110/R810) affected the fecundity of spider mites ($F_1 = 0.07$, $P = 0.005$).

Interaction Between Cd Accumulation and Inducibility of Jasmonate Organic Defenses

The oviposition rate of *T. evansi* was affected by both Cd concentration and previous infestation with conspecifics or

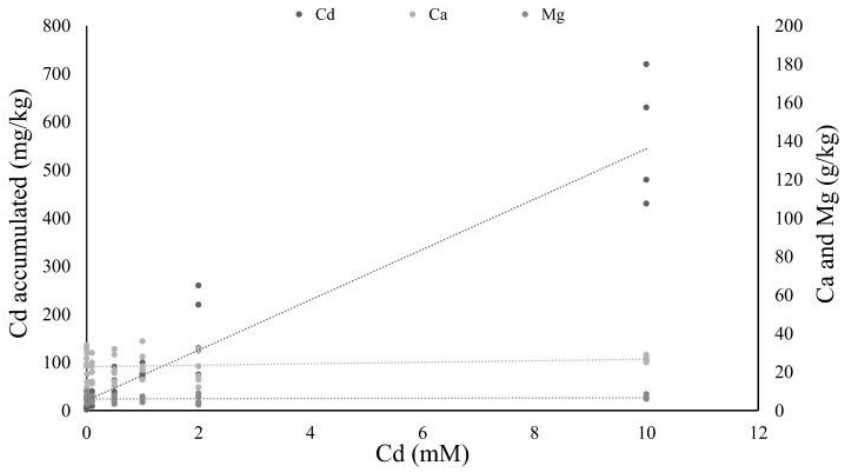

FIGURE 1 | Relation between cadmium supplied in soil solution in relation to cadmium, calcium, and magnesium concentration on tomato leaves. Lines represent linear regressions between the concentration of cadmium, calcium or magnesium solutions supplied to the plant (0, 0.01, 0.1, 0.5, 1, 2, and 10 mM; six plants per concentration) and the cadmium accumulated on the leaves, or the calcium and magnesium present on those leaves.

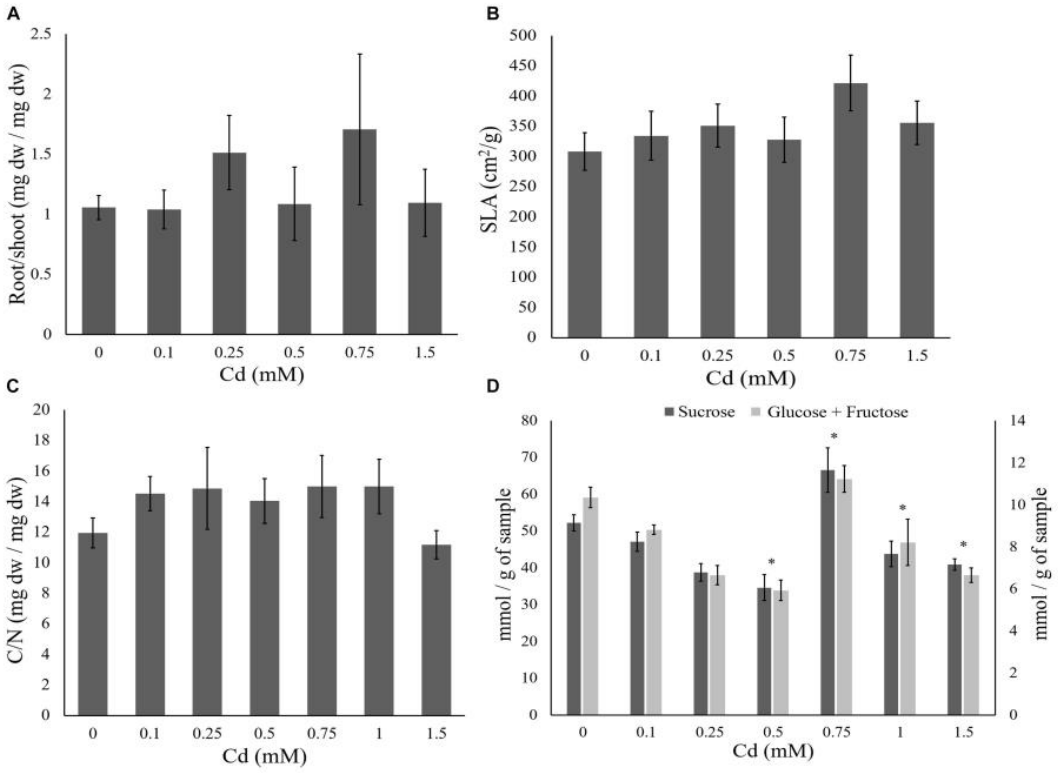

FIGURE 2 | Effect of cadmium on the performance of tomato plants. Tomato plants were supplied with different cadmium concentrations (0, 0.1, 0.25, 0.5, 0.75, 1, or 1.5 mM). The plant traits (±standard error – vertical bars; six plants per concentration) were: **(A)** average root to shoot ratio; **(B)** average specific leaf area (SLA, cm^2/g); **(C)** average carbon to nitrogen ratio of the leaves; **(D)** average glucose and fructose (light gray bars) and sucrose (dark gray bars) concentration (mmol per gram of leaf fresh weight). *Represents significant differences from the control plants.

TABLE 3 | Statistical analyses of the effects of cadmium on plant performance traits.

Variable of interest	Explanatory variable	*df*	χ^2/F	*P*-value
Water content	Cd supplied	5	34.03	0.31
SLA (specific leaf area)	Cd supplied	5	1.70	0.16
Root/shoot	Cd supplied	5	34.01	0.81
C/N	Cd supplied	6	4.42	0.65
Sucrose	Cd supplied	6	9.77	**<0.001**
Glucose + fructose	Cd supplied	6	13.21	**<0.001**

p-values < 0.05 are indicated in bold.

TABLE 4 | Effect of cadmium on plant biomass.

Biomass	Contrast	Fresh weight (mg ± SE)	Dry weight (mg ± SE)
Shoots	0 mM	48.44 ± 0.35	5.34 ± 0.06
	0.1 mM	52.23 ± 0.61	5.23 ± 0.09
	0.25 mM	41.52 ± 0.68	4.48 ± 0.09
	0.5 mM	47.00 ± 0.48	5.23 ± 0.08
	0.75 mM	37.04 ± 0.76	3.65 ± 0.09
	1.5 mM	44.83 ± 0.54	4.41 ± 0.08
Roots	0 mM	91.34 ± 0.94	5.56 ± 0.07
	0.1 mM	79.23 ± 0.90	5.00 ± 0.08
	0.25 mM	88.87 ± 0.89	5.79 ± 0.08
	0.5 mM	100.41 ± 1.04	5.00 ± 0.09
	0.75 mM	78.48 ± 0.62	4.73 ± 0.13
	1.5 mM	72.98 ± 0.80	4.36 ± 0.08

Average biomass of plants exposed to the narrow range of cadmium (N = 6).

TABLE 5 | *A posteriori* contrasts for the effects of cadmium on soluble sugar contents.

Variable of interest	Contrast	*t*-Value	*P*-value
Sucrose	0 mM vs. 0.1 mM	−1.07	0.29
	0 mM vs. 0.25 mM	−2.81	**0.008**
	0 mM vs. 0.5 mM	−3.67	**<0.001**
	0 mM vs. 0.75 mM	2.98	**0.005**
	0 mM vs. 1.5 mM	−2.36	**0.024**
Glucose + fructose	0 mM vs. 0.1 mM	1.61	0.12
	0 mM vs. 0.25 mM	4.72	**<0.001**
	0 mM vs. 0.5 mM	−6.06	**<0.001**
	0 mM vs. 0.75 mM	−0.79	**0.043**
	0 mM vs. 1.5 mM	−4.59	**<0.001**

p-values < 0.05 are indicated in bold.

heterospecifics (**Table 7** and **Figure 4**). However, the interaction between these factors was not significant (**Table 7**). The oviposition rate of *T. evansi* increased with previous infestation by conspecifics and decreased with previous infestation by *T. urticae* (**Table 7** and **Figure 4**), independently of the concentration of Cd to which plants were exposed before. Moreover, the oviposition rate of *T. evansi* increased on plants exposed to 0.5 mM of Cd and decreased on plants exposed to 1.5 mM of Cd (**Table 7** and **Figure 4**), compared to control plants, as observed in the previous results (**Figure 3**).

Additionally, the activity of trypsin inhibitors was modified by infestation by spider mites, independently of the concentration of Cd supplied to the plants (**Table 7** and **Figure 5**). Cadmium accumulation did not significantly affect the activity of trypsin inhibitors (**Table 7** and **Figure 5**).

DISCUSSION

Our results show that within the tested ranges, Cd exposure did not affect tomato growth (specific leaf area, root/shoot, water content, NDVI). However, variables related to leaf structure (SC index) or sugar content were affected, suggesting structural and biochemical changes in leaf cells. Spider mites infesting those plants were affected by Cd concentrations, albeit in a non-linear way. Indeed, both spider mite species had increased performance on plants mildly exposed to Cd, as compared to un-exposed plants, but lower performance after a given threshold, revealing a hormetic effect, which is a dose response phenomenon with stimulatory effects of mild concentrations and inhibitory effects at higher concentrations (Calabrese and Blain, 2009). Finally, the interaction of both spider mites with jasmonate defenses was not affected by the level of Cd that the plants were exposed to. Together, these results suggest that metal accumulation and the production of the studied plant organic defenses against herbivores do not interact with each other.

Studies regarding Cd accumulation by tomato plants reveal high variability in this trait (Hartke et al., 2013), with some plants accumulating amounts below the hyperaccumulation threshold (<100 mg/kg, Pollard, 2000), even at high concentrations of Cd supply (Ammar et al., 2007, 2008) and others accumulating above this threshold (Dong et al., 2006; Gratão et al., 2008; López-Millán et al., 2009). Here we observe that Cd accumulated linearly in the leaves of tomato plants, up to values above the hyperaccumulation threshold, suggesting that *Moneymaker*, the variety of tomato used in our study, is as a facultative hyperaccumulator. This is further confirmed by the values of Ca and Mg in the leaves which remain stable with increasing Cd in the leaves, as seen for other hyperaccumulator plants (Gomes et al., 2013; Pereira et al., 2017).

Here we report the absence of an immediate negative impact on plant growth, although there was an effective uptake of Cd into the leaves. Moreover, we also observe no differences in the carbon to nitrogen ratio in leaves of plants exposed to different Cd concentrations, indicating no shifts in the growth/defense balance (Herms and Mattson, 1992). This contrasts with previous studies showing a negative impact of Cd on tomato plant growth, for Cd accumulation values within the ranges used here (Dong et al., 2006; Ammar et al., 2007; Gratão et al., 2008; López-Millán et al., 2009). Possibly, the variety of tomato we used in this experiment is more tolerant to Cd than most other varieties. Indeed, the few studies using this variety observe no signs of toxicity (Petit and Van de Geijn, 1978; Petit et al., 1978). Another possibility is that the growing substrate affected these results. Indeed, most studies on this topic used continuously aerated hydroponics, creating an artificial situation for the plants such as

TABLE 6 | Statistical analyses on the effect of cadmium on the performance of spider mites.

Variable of interest	Data subset	Explanatory variable	*df*	*F*	*P*-value
Daily fecundity	Wide range	Cd supplied × tested species	6	47.04	0.14
		Cd supplied	6	64.13	**<0.001**
Daily fecundity	Narrow range	Cd supplied × tested species	6	0.12	0.99
		Cd supplied	6	10.62	**<0.001**
Variable of interest	**Data subset**	**Contrast**		***Z*-value**	***P*-value**
Daily fecundity	Wide range	0 mM vs. 0.01 mM	–	0.32	0.99
		0 mM vs. 0.1 mM		−0.94	0.96
		0 mM vs. 0.5 mM	–	−3.14	**0.028**
		0 mM vs. 1 mM		−1.34	0.83
		0 mM vs. 2 mM	–	−3.99	**0.012**
		0 mM vs. 10 mM	–	−4.82	**<0.001**
Daily fecundity	Narrow range	0 mM vs. 0.1 mM	–	−0.58	0.99
		0 mM vs. 0.25 mM	–	−1.45	0.77
		0 mM vs. 0.5 mM	–	−3.26	**0.018**
		0 mM vs. 0.75 mM	–	0.50	0.99
		0 mM vs. 1 mM	–	−1.43	0.78
		0 mM vs. 1.5 mM	–	3.93	**0.001**

p-values < 0.05 are indicated in bold.

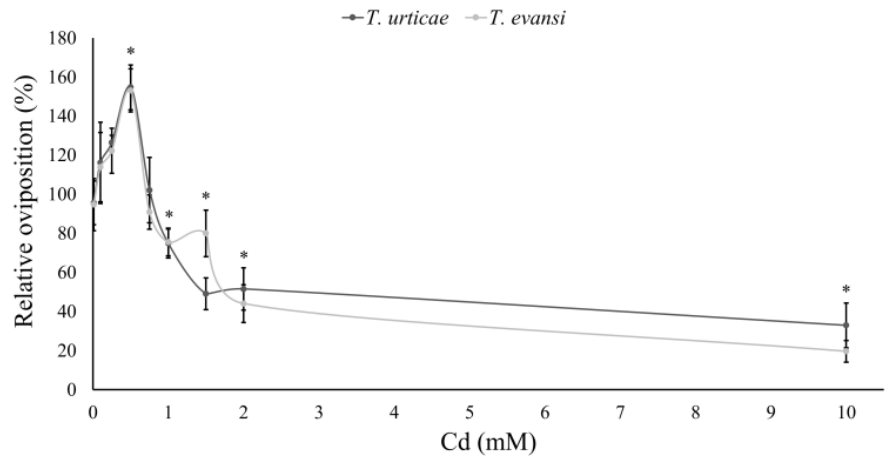

FIGURE 3 | Performance of spider mites on leaves of tomato plants exposed to cadmium. Average relative oviposition rate of *T. evansi* (light gray) and *T. urticae* (dark gray) females on tomato plants (±standard error – vertical bars; 6/12 plants, 3 disks per species per plant). For each range of cadmium solutions, a (0, 0.01, 0.1, 0.5, 1, 2, or 10 mM, *N* = 6) and b (0, 0.1, 0.25, 0.5, 0.75, 1, or 1.5 mM, *N* = 12) the oviposition of spider mites was normalized to the control (no cadmium) and merged in the same panel. *Represent significant differences to the control.

the absence of microbiota around the root, and here we used soil as a substrate as in natural conditions. Growing in soils may be advantageous to plants, given that soil microbiota may regulate the process of metal accumulation in the shoots (de Souza et al., 1999; Farinati et al., 2009), reducing the costs involved in this process for the plant (Farinati et al., 2009).

In contrast to most plant traits that did not respond to Cd, we found changes in soluble sugar contents and leaf reflectance. Changes in the amount of soluble sugars in the leaves with Cd were non-linear. Soluble sugars are generally associated with an initial response to plant stress, with changes in their accumulation, either increasing or decreasing, affecting the REDOX reactions originated by environmental stress (Couée et al., 2006). Cadmium supply may lead to either an increase (Mishra et al., 2014) or a decrease (Scheirs et al., 2006; Shackira and Puthur, 2017) in the amount of soluble sugars in the shoots of exposed plants. The fluctuations we observe in the soluble sugars content may indicate that these are being affected by different processes in the plant, and this may help to reconcile the contrasting observations in the literature. Additionally, plants

TABLE 7 | Statistical analyses of the effect of cadmium and spider mite infestation on daily fecundity and concentration of trypsin inhibitors.

Variable of interest	Explanatory variable	*df*	*F*	*P*-value
Daily fecundity	Cd supplied x infestation status	4	0.47	0.76
	Infestation status	2	27.56	**<0.001**
	Cd supplied	2	32.18	**<0.001**
Trypsin inhibitors	Cd supplied × infestation status	4	0.03	0.97
	Infestation status	2	4.49	**0.014**
	Cd supplied	2	0.77	0.38
Variable of interest	**Contrast**		***Z*-value**	***P*-value**
Daily fecundity	Un-infested vs. *T. evansi*	–	–3.22	**0.021**
	Un-infested vs. *T. urticae*	–	3.49	**0.009**
	0 mM vs. 0.5 mM	–	4.19	**<0.001**
	0 mM vs. 1.5 mM	–	–3.88	**<0.001**
Trypsin inhibitors	Un-infested vs. *T. evansi*	–	–3.26	**0.018**
	Un-infested vs. *T. urticae*	–	3.93	**0.021**

p-values < 0.05 are indicated in bold.

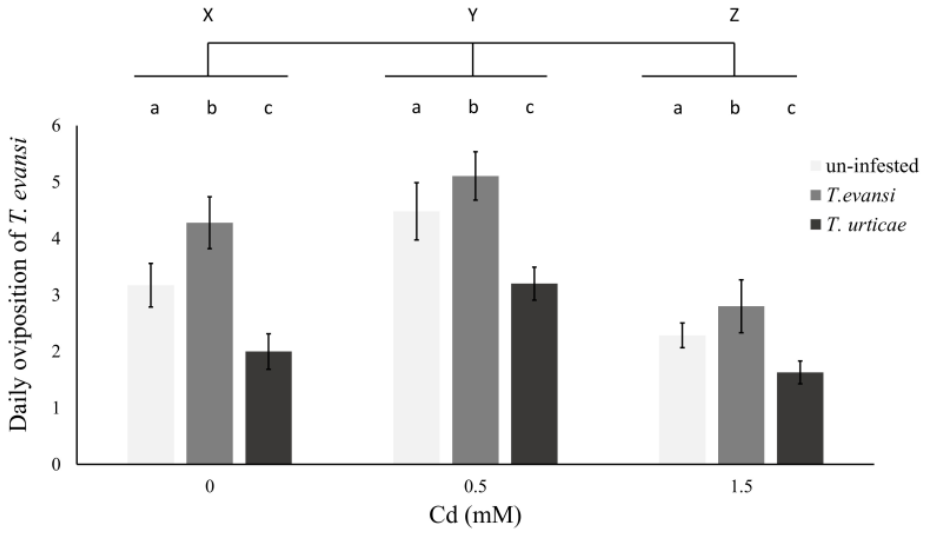

FIGURE 4 | Effect of cadmium exposure and herbivory on the performance of subsequent infestations. Average number of eggs laid per day by *T. evansi* females on un-infested plants (light gray), plants infested with 100 *T. evansi* females (gray) or with 100 *T. urticae* females (dark gray) for 48 h. Plants (±standard error – vertical bars; 12 plants, 3 disks per species per plant) were exposed to a range of cadmium concentrations (0, 0.5, or 1.5 mM). Small case letters (a,b,c) represent significant differences between infestation treatments and upper case letters (X,Y,Z) between cadmium treatments, there were no significant interactions between the two factors.

may also have a hormetic response to abiotic stressors, increasing their performance with small amounts of Cd until a threshold where the negative effects caused by this metal exceed the positive ones (Siddhu et al., 2008; Poschenrieder et al., 2013). If this is the case, the response of the plant to this stress may be different below and above this threshold, and this in turn could be reflected in the soluble sugar content. Corroborating this hypothesis requires more controlled experiments and a systematic measurement of Cd concentrations in the leaves. Moreover, higher concentrations of Cd exposure caused changes in leaf reflectance (SC index), which have been linked to structural changes on their leaf cells (Sridhar et al., 2007). Additionally, plants exposed to the higher concentrations of Cd showed a significant increase in the reflectance of UV-B light, which is possibly related to the production of phenolic compounds known to protect plants against abiotic stresses (Roberts and Paul, 2006; Izaguirre et al., 2007). Our results thus highlight the need to collect different measures of plant performance in response to a single abiotic stress.

The performance of spider mites was also affected by Cd accumulation in tomato leaves. Both species had a non-linear, hormetic response to this metal. Most herbivores are negatively

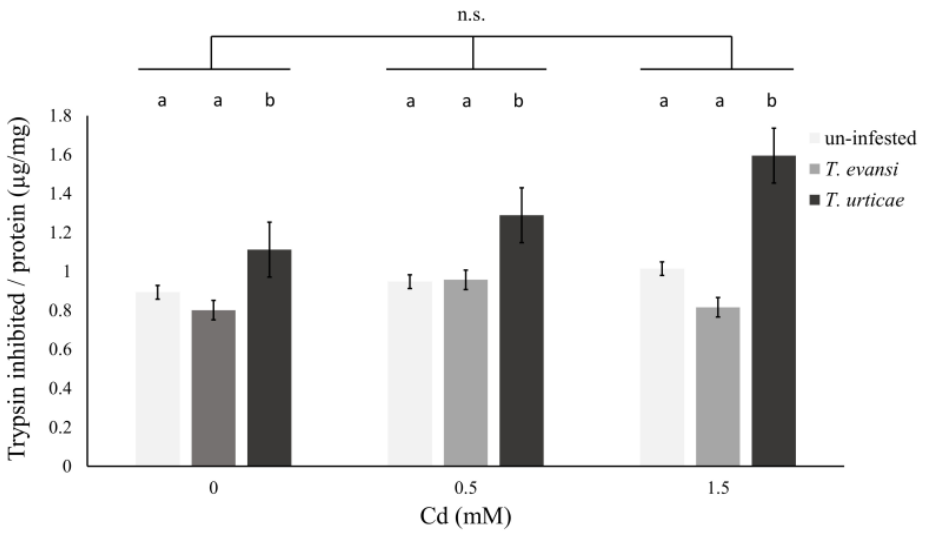

FIGURE 5 | Effect of cadmium exposure and herbivory on organic plant defenses. Amount (µg) of trypsin inhibited per mg of protein in leaf samples of un-infested plants (light gray), plants infested by 100 *T. evansi* females (gray) for 48 h or 100 *T. urticae* females (dark gray) for 48 h. Plants (±standard error – vertical bars; 12 plants, 3 disks per species per plant) were exposed to a range of Cd concentrations (0, 0.5, or 1.5 mM). Lower case letters (a,b) represent significant differences between infestation treatments, Cd supplied had no significant effect, neither was the interaction between the two factors significant.

affected by metal accumulation in the leaves of their host plants (Hanson et al., 2003; Freeman et al., 2007; Quinn et al., 2010; Stolpe et al., 2017). Still, there are some examples of higher abundances of herbivores on sites with intermediate concentrations of toxic metals (Zvereva et al., 1995; Kozlov, 2003), under natural conditions. Nevertheless, this is, to our knowledge, the first report of a hormetic effect of metals on herbivore performance. This phenomenon may significantly affect the evolution of metal accumulation, as selection will favor plants that accumulate amounts of metal above the threshold for herbivore inhibition. Such pattern may complement the "defense enhancement hypothesis" (Boyd, 2007). Indeed, given the hormetic effect of metals upon herbivores, plants are expected to reach the protective threshold of metal accumulation only when the costs of accumulation (i.e., a positive effect of metals upon herbivores) are surpassed by the benefits of metals reducing herbivory (Boyd, 2012; **Figure 6**). This hormetic pattern may be due to direct effects of the metal on the spider mites, or indirectly, through changes in plant quality. We observed no effect of Cd on plant biomass and C/N ratio, however, the amount of soluble sugars in the leaf significantly affected the performance of spider mites. The performance of spider mites has been reported as positively (Ximénez-Embún et al., 2016, 2017) or negatively (Wermelinger et al., 1985; Joutei et al., 2000; Scheirs et al., 2006) correlated to sugar content, indicating that this may depend on the host plant species or on other physiological responses that were not assessed. Although this correlation does not imply causation, it does suggest that Cd may indirectly affect mite performance via an effect on sugars or other physiological changes associated with them, a hypothesis that requires further tests. Additionally, we observed that the daily fecundity of both species correlated with the leaf spectral SC index, indicating a possible effect of structural changes in leaf cells caused by Cd. Still, our experiments do not exclude a possible direct effect of the metal on the performance of spider mites. Whether these correlations imply causality is a relevant question that calls for future studies.

The similarity in the hormetic pattern of the two spider mite species suggests they both may prefer to establish on plants with intermediate Cd concentrations rather than on un-contaminated plants. Moreover, their higher oviposition probably indicates that their growth rate is higher on those plants (Clemente et al., 2018). This may entail a faster saturation of that environment, relative to others. If this hormetic effect is extended to other herbivore and pathogenic species, then plants are expected to pay a high cost of mild Cd accumulation. Thus, given enough time, plants may be selected to "avoid" the level of Cd accumulation that results in better performance for the herbivores, being selected to accumulate higher amounts of metal, becoming hyperaccumulators, or to not accumulate metal at all, becoming excluders.

Because the two spider-mite species have dissimilar interactions with jasmonate organic defenses, the fact that they perform best on plants with the same Cd supply also suggests no interaction between metal accumulation and the inducibility of jasmonate defenses. Furthermore, the contrasting effects of these two spider-mite species on the activity of trypsin inhibitors and its effect on subsequent infestations were consistent across Cd environments, revealing no interference of Cd on protease activity, in contrast to what was seen in other

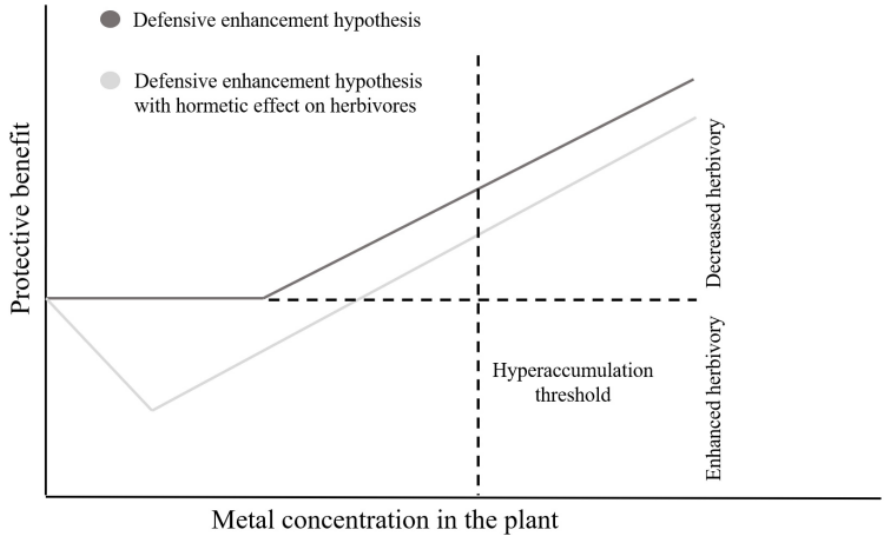

FIGURE 6 | The impact of hormesis on the protective effect of metals. Schematic comparison of the protective benefit of metal accumulation to the plant between the defensive enhancement scenario and a scenario with a hormetic effect of the metal on herbivores.

plant species (Pena et al., 2006; Lin et al., 2010). The plants used in these experiments showed little evidence of Cd toxicity. Possibly, the effect of Cd was not strong enough to induce the protective protease activity reported for other plants (Pena et al., 2006; Lin et al., 2010). Additionally, the effect of metal supply on spider mite performance was not affected by previous infestation. Together, these results suggest that metal based and organic plant defenses do not interfere with each other, serving the same purpose. Although some studies reveal that the expression of organic defenses is lower with high metal supply (Davis et al., 2001; Tolra et al., 2001; Farinati et al., 2009, 2011; Sun et al., 2009; Fones et al., 2013; Stolpe et al., 2017; Tewes et al., 2018), how herbivores are affected by the interaction between metal accumulation and organic defenses remains poorly studied (but see Stolpe et al., 2017). Still, if metal accumulation provides the same function as organic defenses, and if the production of organic defenses is costly, this may select for a reduction in organic defenses in plants under high metal supply. The opportunity for such selection to be effective is much higher on obligate metal accumulators (Poschenrieder et al., 2006), which is not the case of tomato. Alternatively, plants may be suffering from metal accumulation, hence they may lack the necessary resources to trigger organic defenses (Farinati et al., 2009, 2011; Fones et al., 2013). As we did not observe negative effects of Cd on plant growth, it may be that cost on tomato plants due to metals was not sufficient to lead to a trade-off between these two types of defenses. Possibly, long-term exposure to this contaminant, or exposure to higher concentrations, would cause significant costs to the plant affecting its growth rate or posing constrains in fruit production. Still, a recent field work found little evidence for trade-offs between organic and inorganic defenses (Kazemi-Dinan et al., 2015).

Another possible explanation for the absence of a trade-off in our study is that the effect of metal accumulation on herbivores was non-linear. Thus, if plants would produce fewer organic plant defenses, as metal accumulation increased, herbivores would have an extra-advantage at intermediate metal concentrations, benefiting from both a high performance in response to metals, and a low exposure to organic defenses. This, in turn, would pose a strong selective pressure upon plants not to shut down organic defenses. In the absence of an interaction between metal-based and organic defenses, plants occurring in heterogeneous environments may fine tune these strategies depending on their relative performance in each environment. Possibly, plants accumulate more metal when exposed to herbivores that suppress their organic defenses, overcoming the positive effects that low concentrations may have on these herbivores. This hypothesis awaits to be tested.

In sum, our results show that spider mites with different effects on the organic defenses of tomato plants have a similar hormetic response to Cd accumulation. This suggests that the community of spider mites on tomato plants will be similar in contaminated and un-contaminated soils. Our results highlight the importance of studying the interactive effects of metal based and organic plant defenses on herbivores, using metal concentrations below the hyperaccumulating threshold, which allows using more facultative accumulator species, including some of agricultural importance, such as tomato plants.

DATA AVAILABILITY

All data used in this work is archived in Dryad at doi: 10.5061/dryad.f274gs3.

AUTHOR CONTRIBUTIONS

DG, SM, and CB conceived the study. DG, SM, and CB designed the experiments with help from HS and ADS. DG collected the data with assistance from HS and ADS. DG analyzed the data with help from all authors. DG and SM led the writing of the manuscript, with significant help from CB and contributions by all authors. All authors gave final approval for publication.

FUNDING

Funds were provided by an ERC Consolidator Grant (COMPCON, GA 725419) to SM, by UID/BIA/00329/2013 (2015-2017) and UID/MULTI/04046/2013 center grants from FCT, Portugal to cE3c and BioISI, respectively, and by an FCT Ph.D. scholarship PD/BD/114010/2015 to DG.

ACKNOWLEDGMENTS

We would like to thank Plant Biology Department of the Sciences Faculty of the University of Lisbon for the space we used in the plant climatic chamber, Inês Santos for helping with plant growing and maintenance of spider mite populations, Alice Nunes for support in the statistical analyses, and all members of "Adaptation to heterogeneous environments" and "eChanges" groups for stimulating discussions and scientific inputs.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Chapter VI

Decrease in spider-mite growth rate despite no increase in cadmium accumulation by tomato plants

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Abstract

Although the interaction between plants, metals and herbivores are well documented, the consequences of the simultaneous exposure of plants to both types of stress are yet poorly studied. Here, we shed light on this topic using cadmium-accumulating tomato plants (*Solanum lycopersicum*) infested with two herbivorous spider mites, *Tetranychus urticae* or *T. evansi*. Plants, either continuously exposed to cadmium or not at all,were infested with 100 spider mites for 14 days and the development of their offspring until adulthood was followed. The number of spider mite adult offspring was lower on plants with vs without cadmium. Interestingly, even though the performance of *T. evansi* was higher than that of *T. urticae* on un-exposed plants, this was not the case on plants exposed with cadmium. The positive effect of low cadmium concentrations on oviposition rate, seen in previous work, was possibly countered by long term exposure to this metal, and/or cadmium affecting negatively the development of juveniles. Plants were affected by both cadmium toxicity and herbivory, as shown by the reflectance of the leaves. However, these stresses affected different plant traits as the differences in reflectance were not in the same wavelengths. In contrast, the amount of ROS on the plant, which is an outcome of the initial response of plants to both abiotic and biotic stresses, was unaffected by cadmium exposure or herbivory after 14 days. Finally, plants infested with spider mites did not accumulate more cadmium, suggesting that in this system metal accumulation is not inducible by herbivory, unlike organic defences.

Introduction

Plants use a wide variety of defence mechanisms against herbivores (Bostock 2005; Fujita *et al.* 2006). Some defences are constitutive, such as physical structures, which hinder herbivores the access to tissues; others are biochemical signals that deter herbivores, or toxins that hamper their metabolism (Kempel *et al.* 2011; Mithöfer & Boland 2012). Since defence production and maintenance is costly, it may entail trade-offs with growth or reproduction (Strauss *et al.* 2002). In contrast, inducible defences are expressed only upon exposure to a given stress. (Karban & Baldwin 1997; Walling 2000; Mithöfer & Boland 2012). This may reduce the cost of defence maintenance. However, inducible defences are specific to a particular stress, thus, by responding to that stress plants may be more susceptible to other types of stress, if there is a trade-off in the responses (Thaler *et al.* 2002; Denno & Kaplan 2007).

Plants in natural conditions are simultaneously exposed to several stresses (abiotic and biotic), and their response to some of these combinations is well described, both at a physiological and at a molecular level (e.g. Ben Rejeb *et al.*, 2014; Pandey *et al.*, 2015; Guo *et al.*, 2016; Nguyen *et al.*, 2016a,b; Ximénez-Embún *et al.*, 2016, 2017). However, most experiments (in controlled conditions) have exposed plants to abiotic and biotic stresses sequentially, instead of simultaneously (Ben Rejeb *et al.* 2014; Pandey *et al.* 2015; Nguyen *et al.* 2016a, b; Ximénez-Embún *et al.* 2016, 2017). With these desings it is hard to disentangle, wheter the observed effects on herbivory are due to a previous response of the plant to the abiotic stress, followed by a response to herbivory, or due to a concomitant response of the plant to both stressors.

One particular abiotic stress that plants experience is metal toxicity, occurring in geochemically-specific areas or in areas of anthropogenic disturbance. Some plants respond to this stress by uptaking the metal from the soil and accumulating it into their shoots (Baker 1987). This process is suggested to be a defence against herbivores (Martens & Boyd 1994; Boyd 2004). Many studies have shown the effectiveness of metal accumulation against herbivory in controlled experiments (Martens & Boyd 1994; Boyd & Moar 1999; Hanson *et al.* 2003; Jhee *et al.* 2005; Freeman *et al.* 2006, 2007; Rathinasabapathi *et al.* 2007; Quinn *et al.* 2010; Konopka *et al.* 2013), and under natural conditions (Martens & Boyd 2002; Freeman *et al.* 2007; Galeas *et al.* 2008; Kazemi-Dinan *et al.* 2014). Indeed, metal accumulating plants have been shown to harbour fewer herbivores than neighbouring plants, suggesting that metal accumulation not only hampers the performance of herbivores but it can also serve as a

deterrent (Freeman *et al.* 2007; Gonçalves *et al.* 2007; Galeas *et al.* 2008; Kazemi-Dinan *et al.* 2014). Moreover, because metal accumulation has costs for the plant, reducing growth and health (Foy *et al.* 1978; Maestri *et al.* 2010), some plants may be selected to accumulate higher amounts of metals only when facing herbivory. Indeed, even though metal accumulating plants are more tolerant to metal toxicity than their non-accumulating counterparts (Maestri *et al.* 2010), increasing the amounts of metals accumulated may take plants to a threshold upon which the negative effects of metal accumulation are higher than the positive ones (Boyd 2012). Only two studies address whether plants accumulate more metal when facing herbivory (Plaza *et al.* 2015; Stolpe *et al.* 2017). In these studies, *Arabidopsis halleri* was shown to accumulate more cadmium in their leaves when exposed to herbivory by *Pieris rapae* (Plaza *et al.* 2015) and in the phloem when exposed to aphid herbivory (Stolpe *et al.* 2017). Still, it is yet unclear if metal accumulation, as an active defence mechanism, can be generalized to other metal accumulating plant species and other herbivores.

There are many methods to analyse the response of plants to stress, however, most techniques are destructive, limiting the amount of analyses perfomed to a single plant and limiting the desings aiming to obtain the repeated measures over time. For this reason, we chose to analyse the multispectral reflectance of tomato leaves. This technique is non-invasive and allows for the same plant to be measured at several points in time (Carter 1993; Carter & Knapp 2001). It has been used successfully to assess the effect of metal toxicity, or herbivory, on plant performance (Horler *et al.* 1980; Peñuelas *et al.* 1995; Grant *et al.* 2003; Dunagan *et al.* 2007; Luedeling *et al.* 2009; Martin *et al.* 2015).

Changes in the Reactive Oxygen Species (ROS) are involved in the initial steps of the response of plants to both abiotic and biotic stresses. However, in the long-term, the involvement of ROS in response to abiotic and biotic stresses is conflicting (Mittler 2002). Whereas abiotic stress induces the production of ROS-scavenging enzymes, which reduce the amount of ROS over time, biotic stress may suppress the activity of these enzymes, leading to an over-accumulation of ROS over time (Mittler 2002). Despite this conflict, little is known about how plants manage ROS production and scavenging when exposed to both stresses simultaneously.

Here we address the above-mentioned issues by infesting tomato plants (*Solanum lycopersicum*) exposed, or not, to cadmium, with two species of herbivorous spider mites,

Tetranychus urticae and *T. evansi*. Previously, we found that exposure to up to 1.5 mM of cadmium chloride entailed no differences in growth and biomass of tomato plants (Godinho *et al.* 2018). Also, the two spider-mite species interact differently with tomato plants: while *T. urticae* infestation triggers the induction of plant organic anti-herbivore defences, the infestation by *T. evansi* suppresses those defences(Kant *et al.* 2004; Sarmento *et al.* 2011a; Godinho *et al.* 2016). This pattern of induction or suppression was unaffected by plant cadmium accumulation (Godinho *et al.* 2018). Additionally, cadmium accumulation by tomato plants had a hormetic effect on the oviposition rate of both species of spider mites, which increased with mild concentrations of cadmium but decreasing at higher concentrations (Godinho *et al.* 2018). However, in that study, spider mite infestation occurred after plants have been exposed to cadmium. Thus, it is yet unknown what changes in the response of tomato plants when exposed to cadmium and spider mites simultaneously and if tomato plants can increase the cadmium uptake from the soil while simultaneously infested by spider mites. Here, by using a full orthogonal design, with plants being exposed to cadmium, to spider mites or both stressors simultaneously, we expected to shed some light on the joint effects of metal toxicity and herbivory on plants.

Material and methods

Biological material

Tomato plants (*Solanum lycopersicum*, var. Moneymaker) were sown in a climate chamber (25 °C, photoperiod 16/8 h light/darkness), in a soil/vermiculite mixture (4:1). After two weeks, plants were watered twice a week with 60 ml of either distilled water or a cadmium chloride solution (0.5 mM). All plants were watered an additional time per week with tap water to compensate for micro-nutrients deficiencies.

The populations of spider mites used in this study were collected nearby Lisbon, Portugal: *Tetranychus urticae* in 2010 (Clemente *et al.* 2016) and *T. evansi* in 2013 (Zélé *et al.* 2018). Both spider-mite species were maintained in plastic cages containing entire tomato plants as described in Godinho *et al.* (2018). From these stock populations, cohorts of spider mites were prepared by isolating groups of 75 females on tomato leaves, placed on wet cotton wool in Petri dishes, laying eggs for 48 h and being removed afterwards. The daughters of these females were used in the subsequent experiments 14 days later.

General methodology

The experimental assays were done using 4.5 week-old plants. The spectral reflectance of the fourth oldest leaf of each plant was measured using a UniSpec spectroradiometer (PP Systems, Haverhill, MA, United States). The spectral data generated by these measurements was determined by calculating spectral reflectance factors (R) for each wavelength (between 300.4 and 1148.1 nm with intervals of 3.4 nm). These factors were obtained by normalizing the reflected radiation from the leaves with a reflectance white standard.

Afterwards, the same leaf (of plants with or without cadmium) was infested with 100 mated females of either *T. urticae* or *T. evansi*, or it was left un-infested, resulting in 6 treatments (N = 12 per treatment). The infestation with spider mites lasted 14 days, (approximately the generation time of these spider-mites), then the spectral reflectance was measured again. By measuring each plant in two different time points, before and after exposure to cadmium, exposure to spider mites or exposure to both, we obtained the response of each individual plant to these stressors. During the exposure period, plants were subjected to the same watering conditions as before. Subsequently, the number of adult females (the offspring of the infesting females) was registered for each plant.

To quantify the amount of cadmium on those plants, spider mites, web and eggs were removed (when present) and the leaf was dried at 60 ºC, until constant mass, and grinded. Cadmium was then quantified using Inductively Coupled Plasma – Atomic Emission Spectrometry (ICP – AES; LAIST, Portugal), after nitric acid digestion, with a detection limit of 1 mg/kg.

Additionally, fresh leaf material was frozen at -80 ºC, in liquid nitrogen to quantify ROS (in the form of H_2O_2). For the extraction, frozen leaf material was weighted (~100 mg tissue/0.5 ml assay buffer) and ground in liquid nitrogen (Antioxidant Assay Kit CS0790, Sigma-Aldrich). The macerate was placed in microtubes (2 ml), centrifuged and the supernatant used to determine the concentration of ROS using a modification of the assay protocol of the manufacturer (Antioxidant Assay Kit CS0790, Sigma-Aldrich). The concentration of H_2O_2 in the supernatant was determined through changes in absorbance, using a mixture of 50 µL extract in Assay buffer, 20 µL myoglobin working solution, and 150 µL ABTS substrate solution [2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) with Phosphate-Citrate buffer]. Values were then standardized with an H_2O_2 calibration curve prepared using 50 µL Assay buffer, 20 µL myoglobin working solution, and 0, 25, 50, 75 or

150 µL ABTS with 3.25 mM H_2O_2 (total volume 150 µL, diluted with ABTS without H_20_2). Absorbance was measured for all reactions at 405 nm in an Epoch 2 Microplate Photometer (BioTek, BioSPX®).

The assays were done in four blocks, separated in time, each consisting of three replicate plants per experimental treatment.

Statistical analyses

The number of female mites alive on the plant after 14 days was compared using a generalized linear mixed model (glmer) with a poisson distribution. Exposure to cadmium, infesting species (*T. urticae* or *T. evansi*) and their interaction were used as fixed factors and block as a random effect. Because there was a significant interaction between the infesting species and exposure to cadmium, comparisons among treatments within each factor were performed using the phia package in R (de Rosario-Martinez 2015).

The amount of cadmium (mg per Kg of sample) and the amount of H_2O_2 (µmol per gram of sample) present in the fourthe leaf of each plant were compared between treatments using general linear mixed models (lmer) with a gaussian distribution. Exposure to cadmium, infestation (infested with *T. urticae*, infested with *T. evansi* or uninfested) and their interaction were used as fixed factors and block as a random effect.

The effect of spider mite infestation and exposure to cadmium, on the spectral reflectance of leaves of tomato plants, was analysed using a multivariate analysis of variance with distance matrices (adonis function, vegan package; Oksanen, 2015). For each plant, the difference in reflectance, for each wavelength (between 300.4 and 1148.1 nm with intervals of 3.4 nm), was determined between the beginning and the end of the experiment, and was used as the response variable. Spider mite infestation, the cadmium concentration supplied, and their interaction, were used as fixed factors. Since there was a signigicant effect of spider mite infestation on leaf reflectance and because the Adonis function does not support post hoc contrasts, we compared the effect of the two spider mites species using the same model but with the data a subset of the data excluding uninfested plants. Because cadmium accumulation was shown to affect the reflectance of tomato plants at the UV wavelengths (Godinho *et al.* 2018), we repeated the analysis using only the reflectance factors of wavelengths between 300.4 and 395 nm. Changes in the visible spectra (400 to 700 nm) have been used to detect the damage of spider mites on several plant species (Peñuelas *et al.* 1995; Luedeling *et al.* 2009;

Martin *et al.* 2015) Additionally, leaf chemistry can be determined through the reflectance in the near infrared region of the spectrum (780 to 1400 nm), this method being used to assess the intensity of herbivory (Meuret *et al.* 1993; Gillon *et al.* 1999; Foley 2009). Therefore, we also analysed changes in reflectance within those wavelengths. Due to technical issues, the reflectance of plants of the last block could not be measured at both time points, thus we excluded them from the analysis, including only nine plants per treatment (from the three other blocks).

Results

The number of mites of each species was differently affected by cadmium accumulation (Fig. 1; interaction between cadmium exposure and infesting species: $\chi^2_1 = 97.01$; $P < 0.001$), being *T. evansi* more affected by cadmium than *T. urticae*. Still, the number of mites on the plant after 14 days was significantly lower on plants exposed to cadmium than on un-exposed plants, both for *T. urticae* (Fig. 1; $\chi^2_1 = 842.28$, $P < 0.001$) and for *T. evansi* (Fig. 1; $\chi^2_1 = 192.2$, $P < 0.001$). On un-exposed plants the number of *T. evansi* after 14 days was higher than that of *T. urticae* (Fig 3; $\chi^2_1 = 235.68$, $P < 0.001$) but this was not the case on plants exposed to cadmium (Fig. 1; $\chi^2_1 = 0.55$, $P = 0.46$).

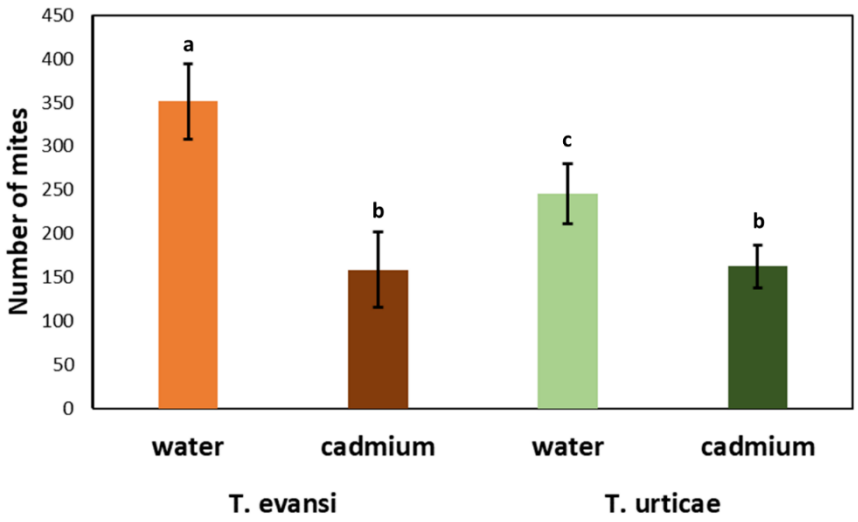
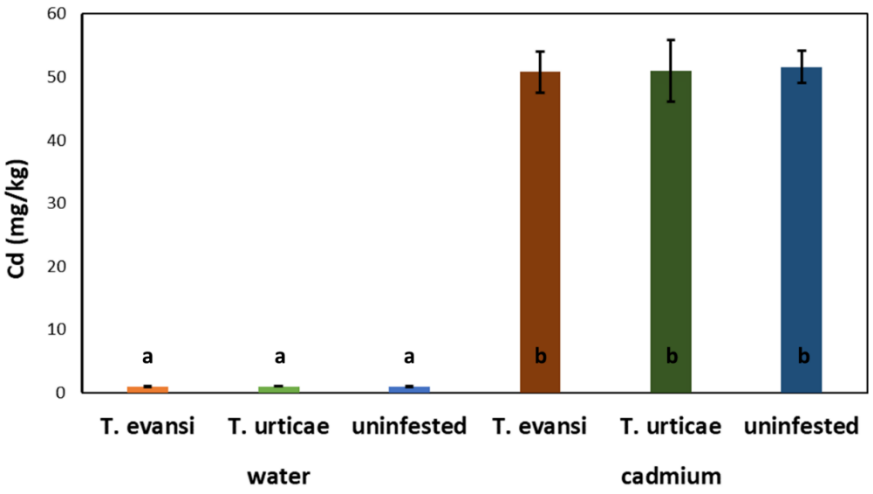


Figure 1. *Average number of mites (T. evansi - orange; T. urticae - green) on tomato plants after 14 days of infestation (± se, N = 12 plants per treatment). Plants were watered with or without 0.5 mM of cadmium chloride twice a week. Letters represent significant differences in* a posteriori *contrasts (P < 0.05).*

Plants exposed to cadmium accumulated higher amounts of this metal on their leaves (≈ 50 mg Cd/kg), as compared to un-exposed plants (< 1 mg Cd/kg; Fig. 2; $F_{1, 65} = 648.16$; $P <$

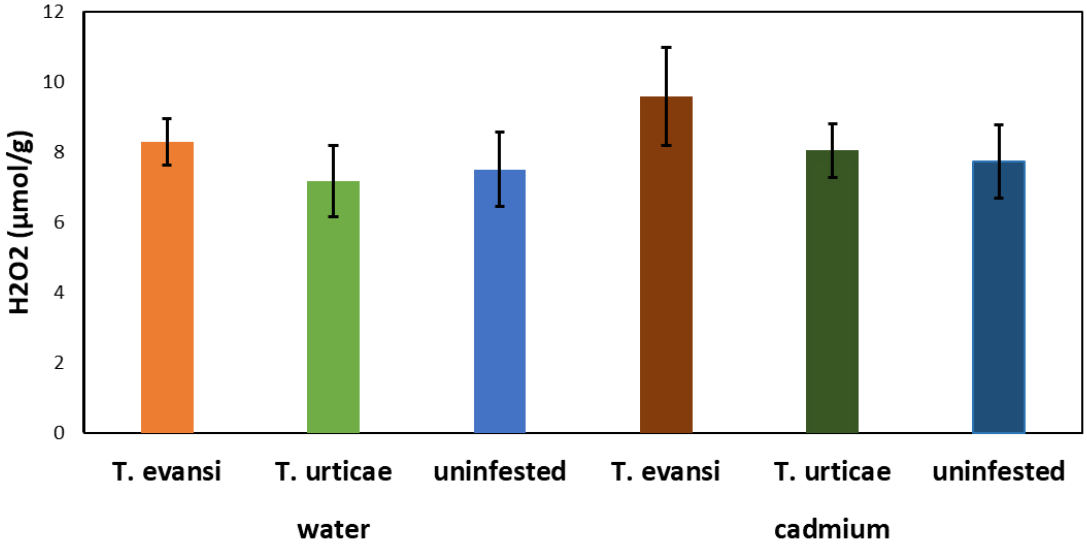
0.001). However, this accumulation was unaffected by plant infestation (interaction between cadmium exposure and spider mite infestation: Fig. 2, $F_{2, 65} = 0.06$, P = 0.94).

Figure 2. *Average accumulation of cadmium (± se, N = 12 plants per treatment) on leaves of plants exposed or not to 0.5 mM of cadmium chloride. Those leaves were either un-infested (blue) or infested with T. evansi (orange) or T. uritcae (green) for 2 weeks. Letters represent significant differences (P < 0.05).*



The amount of H_2O_2 (nmol per gram of sample) present in the leaves of tomato plants was not affected by cadmium exposure (Fig. 3; $F_{1,41} = 0.97$, $P = 0.933$), spider-mite infestation (Fig X; $F_{2,41} = 1.12$, P = 0.31) nor by the interaction between these two stressors (Fig. 3; $F_{2,39} = 0.14$; P = 0.87).

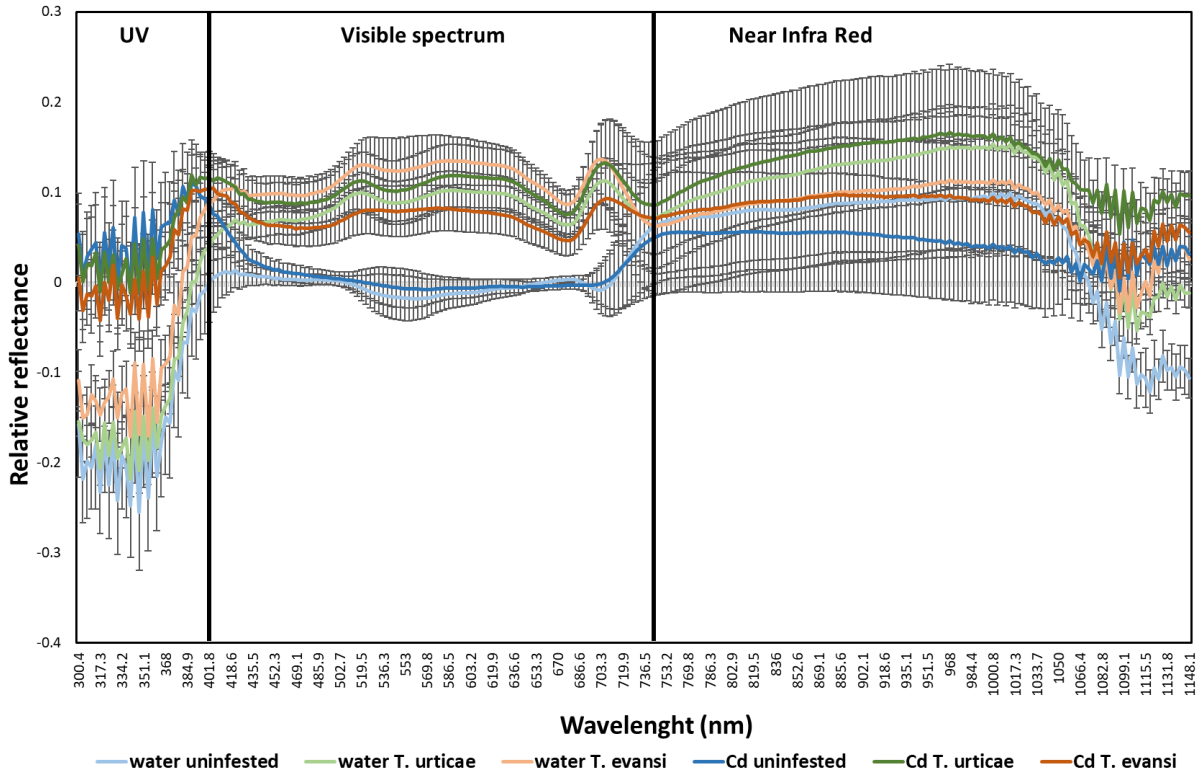
Figure 3. *Accumulation of H_2O_2 (± se, N = 12 plants per treatment) on leaves of on plants exposed or not to 0.5 mM of cadmium chloride. Those leaves were either un-infested (blue) or infested with T. evansi (orange) or T. uritcae (green), for 2 weeks. There was no significant effect of either cadmium exposure or infestation by spider mites on the amout of H_2O_2 accumulated after 14 days in the plant.*



The overall spectral reflectance of plants was significantly affected by spider-mite infestation ($F_{2,50}$ = 2.81; P = 0.045; Fig. 4), independently of cadmium exposure (interaction between infestation and cadmium: $F_{2,48}$ = 0.42; P = 0.76), and of the infesting species (diferences between plants infested with *T. urticae* and plants infested with *T. evansi*: $F_{1,34}$ = 0.36; P = 0.66; Fig. 4). Exposure to cadmium had no effect on the overall spectral reflectance of tomato plants ($F_{1,50}$ = 2.41; P = 0.10; Fig. 4).

In the UV area (300 nm-395 nm), exposure to cadmium had a significant effect on plant reflectance ($F_{1,50}$ = 24.51; P < 0.001; Fig.4), independently of the infestation by spider mites (interaction between infestation and cadmium: $F_{2,48}$ = 1.41; P = 0.24). Infestation by spider mites had no effect on the reflectance on the UV spectral reflectance zone ($F_{2,50}$ = 0.52; P = 0.61; Fig. 4).

Figure 4. *Average difference in leaf spectral reflectance (wavelengths between 300.4 and 1148.1 nm; ± se, N = 9 plants per treatment) between the beginning and the end of the experiment. During the trial, plants were exposed or not to 0.5 mM of cadmium chloride and were either un-infested (blue) or infested with 100 T. evansi mated females (orange) or 100 T. urticae mated females (green).*



In the visible spectral zone (400nm -700nm), exposure to cadmium had no effect on plant reflectance ($F_{1,50}$ = 0.36; P = 0.57; Fig. 4) but infestation by spider mites affected

significantly the reflectance of these wavelengths ($F_{2,50}$ = 14.68; P < 0.001; Fig. 4), as compared to uninfested plants, independentely of cadmium exposure (interaction between infestation and cadmium: $F_{2,48}$ = 1.65; P = 0.20). Still, the reflectance on the visible spectral zone was similarly affected by *T. urticae* and by *T. evansi* ($F_{1,34}$ = 0.06; P = 0.88; Fig. 4).

Finally, for the near infrared spectral reflectance zone, there was no effect of either infestation with spider mites ($F_{1,50}$ = 1.06; P = 0.36; Fig. 4) or exposure to cadmium ($F_{1,50}$ = 0.69; P = 0.42; Fig. 4).

Discussion

In this study, we show that the fitness advantage of *T. evansi* over its congeneric *T. urticae* is lost when infested tomato plants are exposed to cadmium.The levels of ROS on plants after 14 days were not affected by herbivory nor by cadmium exposure. However, the spectral reflectance of tomato leaves reveals that plants were affected by herbivory and cadmium exposure, but these effects were independent of each other. We also show that tomato plants do not accumulate more cadmium when infested with spider mites.

Tetranychus evansi is a specialist of Solanaceae plants, being its reproductive performance on tomato plants regularly higher than that of other spider mites, including *T. urticae* (Godinho *et al.* 2016, 2018; Schimmel *et al.* 2017; Paulo *et al.* 2018), leading to the exclusion of *T. urticae* on tomato plants with *T. evansi* (Sarmento *et al.* 2011b). Here, we recapitulate this result, but we also show that this reproductive advantage is lost when tomato plants are exposed to cadmium. Previously, we have shown that induction and suppression of tomato defences, by *T. urticae* and *T. evansi*, respectively, are not affected by cadmium accumulation (Godinho et al., 2018). Thus, the similar fitness of *T. evansi* and *T. urticae* when feeding on tomato plants exposed to cadmium suggests that the effect of this metal overrules that of plant defences. This is in agreement with previous results showing that the direct effect of cadmium on spider mites is stronger than those stemming from physiological changes in the plant (c.f. Chapter VII). This suggests that *T. urticae* is less susceptible than *T. evansi* to the long-term effects of cadmium. Possibly, suppression of plant defences is costly to *T. evansi*, reducing the energy available to cope with cadmium toxicity, as compared to *T. urticae*, being therefore more affected, and/or *T. urticae* has a higher metal detoxifying ability, being less affected.

Previously we showed, using this system, that under controlled conditions, spider mites had a hormetic response to tomato plants with cadmium, with higher oviposition observed at intermediate cadmium values (Godinho et al., 2018). However, here, we do not find a positive effect of cadmium accumulation on either spider mite species, even though the cadmium supplied to the plants was within the same range of that of this previous work (Godinho *et al.* 2018). The differences observed in the response of spider mites to cadmium concentration in plants could be a result of, i) plants having been exposed to both cadmium and spider mites simultaneously, and/or ii) the longer exposure period of spider mites to cadmium from this experiment (14 days) compared to the previous (4 days) (Godinho et al., 2018). Indeed, the longer duration of the current experiment might have resulted in plants accumulating more cadmium than before, over time, surpassing the level of beneficial concentrations to the spider mites. However, the the amount of cadmium accumulated in the leaves in this study is among the levels observed in the leaves for previous experiments, which varied between 25 and 150 mg/kg for the same supplied concentration, 0.5 mM (Godinho *et al.* 2018). Thus, we suggest that spider mites have decreased performance in the current study due to continuous exposure to plants with cadmium, eating over a longer period in plants with cadmium might increase the concentration of this metal on spider mites beyond the hormetic effect, reaching toxic concentrations. This may have important ecological and evolutionary consequences. Indeed, if the environment is heterogeneous, spider mites may alternate between eating plants with metals for a short period of time and plants without metals, increasing in this way their overall fitness. In contrast, in environments, only with plants accumulating metals, the fitness of spider mites is overall reduced, even for mild metal concentrations. Alternatively, cadmium concentration might affect differentially different stages of development of spider mites. We know that tomato plants with cadmium induce a hormetic response on oviposition rate in spider mites, as shown in Godinho et al. (2018). However, the juvenile stages of these species may be negatively affected by plants with cadmium. These negative effects of metal accumulation on juveniles were also observed in other systems (Martens & Boyd 1994; Boyd & Moar 1999; Freeman *et al.* 2006; Konopka *et al.* 2013). If this is the case, exposure of spider mites to plants with cadmium for 4 or 14-days is expected to have different outcomes. In any case, these two hypotheses, regarding the effects of short vs long term feeding and regarding differential effects on different life stages, are non-exclusive and disentangling between them could shed light on the mechanisms of the effect of metals on herbivores.

Moreover, we showed that tomato plants did not accumulate more cadmium when exposed to herbivory. This suggests that metal accumulation in plants is not inducible by spider mites infestation. This result contrasts with what was found for *Arabidopsis halleri*, which increases the accumulation of cadmium and zinc in the leaves and in the phloem, as a response to herbivory (Plaza *et al.* 2015; Stolpe *et al.* 2017). However, herbivory on *A. thaliana*, did not induce cadmium uptake (Plaza *et al.*, 2015), suggesting that this could be a plant-species specific trait. One possibility is that tomato plants are more sensible to cadmium than *A. helleri* and in this case, the accumulation of cadmium could reach a threshold above which there is toxicity for tomato plants. However, the amount of cadmium accumulated by these plants, even though being above the hyper-accumulation threshold (Pollard 2000), was within the values found on plants which growth rate and biomass production were not affected (Godinho *et al.* 2018), suggesting little or no costs of cadmium for this concentration. Another hypothesis is that since the amount of cadmium accumulated by plants was enough to diminish the effects of herbivory, there was no need for the plant to induce more metal uptake. Indeed, even though the supplied concentration of cadmium (0.5 mM) was shown to have a positive effect on the oviposition rate of these spider mites (Godinho *et al.* 2018), this did not prevail in longer exposures of spider mites to this metal, affecting negatively the subsequent generation.

The latter hypothesis in agreement with the fact that we did not find differences in ROS accumulation among plants. Neither cadmium toxicity, herbivory, nor the combination of both led to an increase in ROS accumulation. This suggests that either the plants were not stressed by these factors or, that the important role of ROS mediating the early response of plants to spider mites (Santamaría *et al.* 2012, 2018) and cadmium (Mishra *et al.* 2014; Gratão *et al.* 2015) is not swayed through time, possibly because plants recovered from the initial response to those stressors and regained the homeostatic balance.

Although leaf reflectance in the visible region was not affected by the cadmium concentrations used (photosynthetic pigments region) suggesting no significant stress, there was a decrease of reflectance in the UV region compared to the normal ageing of plants without cadmium, thus indicating some effect either in leaf texture (e.g. rugosity, glandular trichomes) and/or decrease of some chemical molecule (e.g. waxes, secondary compounds) originating on the plant response to accumulate ca. 50 mg Cd/kg in the leaves (Carter 1993; Carter & Knapp 2001; Grant *et al.* 2003). In contrast, leaf reflectance was broadly affected by herbivory, in particular in the visible spectrum (400 to 700 nm), which is indicative of damage on the photosynthetic apparatus (Peñuelas *et al.* 1995; Luedeling *et al.* 2009; Martin *et al.* 2015).

These effects of spider-mite infestation were independent of cadmium exposure. Interestingly, the effects of cadmium accumulation on UV reflectance were also independent of spider mite infestation. The effects on spectral reflectance, both by cadmium exposure and by spider mite infestation, support the hypothesis that the plants were indeed stressed by these factors and possibly responded with a initial burst in ROS, but with time the basal levels of ROS were regained. The initial burst in ROS is also used in the signaling mediating the phytohormonal pathways that produce stress specific compounds and these may be used by the plants in longer responses (Baxter *et al.* 2014). However, the observed effects on spectral reflectance suggest that cadmium exposure and spider-mite infestation affect different plant traits. It is unclear if tomato plants are able to produce simultaneously specific compounds for both stressors. However, because metal accumulation hampers spider-mite growth, tomato plants may only need to produce compounds to deal with cadmium stress. More studies on this topic are required to enlighten this issue. In any case, our results show that spectral reflectance is a useful tool to disentangle between the effects of different stressors when these occur simultaneously. Finally, the spectral reflectance results show that, even though the growth rate of spider mites was hampered by cadmium accumulation, plants were similarly affected by herbivory, independently of being exposed to cadmium or not. Thus, female adults introduced on the plant were able to damage cadmium exposed plants as much as un-exposed plants during the 14-day period in which they were infesting the plant, suggesting minor negative effects of cadmium on the spider mites. Despite this, the growth rate of spider mites was hampered in cadmium exposed plants, therefore, these results advocate the hypothesis that juvenile stages are more susceptible to cadmium than adult spider mites.

In conclusion, we show that the duration of cadmium exposure may have different outcomes in the performance of spider mites. This may have important ecological consequences for plant-herbivore interactions, depending on the heterogeneity of the environment. Whereas in homogeneous metal polluted environments metal accumulation may hamper the growth rate of herbivores, in heterogeneous environments metal accumulation may enhance overall herbivory. Furthermore, we show that tomato plants do not accumulate more cadmium when facing spider-mite herbivory, suggesting that the hormetic response of herbivores cannot be countered by the plant. Understanding how common is metal accumulation an inducible or a passive mechanism, in other plant-herbivore systems, may disclose key aspects of the ecology and evolution of metal accumulation as a defence against herbivory. Finally, we show that metal accumulation may even out differences in fitness of

herbivores in the same plant. Since some herbivores are more affected by metal accumulation than others, this may affect differently their distributions among plants with and without the metal, which may have important eco-evolutionary consequences for the competition and coexistence of those herbivores.

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Chapter VII

Cadmium is the direct cause of intraspecific variation in the hormetic response of spider mites to metal-accumulating tomato plants

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Abstract

Herbivores differ in how they cope with metal-based defences. However, knowledge on the range and causes of intraspecific variation in this response is lacking, despite clear implications for the ecology and evolution of herbivore-plant interactions. Here, we test variation in response to cadmium-accumulating tomato plants in 17 spider mite populations and identify the factor responsible for such variation. We recapture a non-linear, hormetic response of mites to plants with cadmium and to artificial diets with this metal in some, but not all populations. Artificial diets with sugars do not recover this pattern. This suggests that herbivores on metal-accumulating plants respond to metals, and not to physiological changes in the plant. Moreover, intraspecific variation and non-linearities are key, yet overlooked aspects of herbivore responses to metal-based defences, with important consequences for the ecology and evolution of this interaction.

Keywords: elemental defence; hormesis; plant-herbivore interactions; variability; *Tetranychus urticae; Tetranychus evansi; Solanum lycopersicum*

Introduction

Plants can grow in heavy metal contaminated soils either by limiting metal translocation in the roots, or by storing metals in their aerial parts, a process termed metal accumulation (Baker 1987). Metal accumulation may affect negatively plant health and growth (Foy *et al.* 1978; Maestri *et al.* 2010), but it may also serve as a defence against herbivores and pathogens (Boyd 2004; Poschenrieder *et al.* 2006). Herbivores feeding on metal accumulating plants generally induce damage and have reduced fecundity and/or survival under laboratory conditions (Martens & Boyd 1994; Boyd & Moar 1999; Behmer *et al.* 2005; Freeman *et al.* 2007; Rathinasabapathi *et al.* 2007; Quinn *et al.* 2010; Kazemi-Dinan *et al.* 2014). In natural conditions, metal accumulating plants have been shown to suffer less herbivory than neighbouring plants (Martens & Boyd 2002; Freeman *et al.* 2007; Noret *et al.* 2007; Galeas *et al.* 2008; Kazemi-Dinan *et al.* 2015).

Some herbivores circumvent the toxicity of metals (Jhee *et al.* 2005; Freeman *et al.* 2006; Vesk & Reichman 2009; Konopka *et al.* 2013), via metallothionein enzymes (Cobbett & Goldsbrough 2002) or by accumulating non-toxic methylated forms of the metal (Freeman *et al.* 2006). Moreover, different feeding guilds respond differently to metal accumulating plants, with chewing herbivores being affected by nickel and cadmium accumulation, but not phloem-sucking aphids (Jhee *et al.* 2005; Vesk & Reichman 2009; Konopka *et al.* 2013). Finally, the response also differs with niche width, generalists being more affected by metal accumulation than specialists (Wall & Boyd, 2006; Kazemi-Dinan *et al.*, 2014; but see Godinho *et al.*, 2018).

In contrast with the ample evidence of interspecific variation in herbivore responses to metal-accumulating plants, data on intraspecific variation lags behind. Some studies suggest that populations of plant pathogens that are exposed to metal-rich environments cope better with metal-accumulating plants (Fones *et al.* 2016, 2019). Additionally, Freeman and colleagues (2006) showed that *Plutella xylostella* (diamond moth) collected in selenium-rich environments were more tolerant to accumulation of this metal by *Stanleya pinnata* than other populations. These studies provide evidence for intraspecific variation in the response to metal accumulation due to local adaptation of populations more heavily exposed to this stress. Still, intraspecific variability may derive from several other causes and result in patterns more complex than those associated with local adaptation only. Therefore, more studies are needed to shed further light on this topic.

Metal-accumulating plants may be better defended against herbivores due to the direct harmful effects of metals on herbivores (Boyd 2004). However, many physiological and metabolomic changes occur in the plant due to metal toxicity (Villiers *et al.* 2011; Syed *et al.* 2018) and these may have indirect negative effects on herbivores as well. Indeed, the photosynthetic apparatus is damaged by high levels of heavy metals (Sanità Di Toppi & Gabbrielli 1999), leading to slower plant growth, thus, less food for herbivores. Heavy metals also disturb homeostasis (Clemens 2001), affecting the water and nutrient content of plant tissues (Barcelò & Poschenrieder 1990; Sanità Di Toppi & Gabbrielli 1999), thereby reducing the nutritional quality of plants for herbivores. Finally, the response of plants to metal toxicity affects the redox balance leading to changes in enzymatic activities and the accumulation of soluble sugars on plant tissues (Scheirs *et al.* 2006; Bielen *et al.* 2013; Mishra *et al.* 2014), which in turn have been shown to affect herbivore performance (Scheirs *et al.* 2006; Bartoli *et al.* 2013). Despite all these studies, which of these factors are responsible for the observed variation in the response of herbivores to metal-accumulating plants is as yet unknown.

One under-exploited feature of the interaction between herbivores and metal-accumulating plants is the analysis of the response of herbivores to different concentrations of the metal supplied to the plant. Indeed, most studies used plants exposed to one or two concentrations of each contaminant(s) (Martens & Boyd 1994; Boyd & Moar 1999; Hanson *et al.* 2003; Jhee *et al.* 2005; Freeman *et al.* 2006, 2007; Rathinasabapathi *et al.* 2007; Quinn *et al.* 2010; Kazemi-Dinan *et al.* 2014). Therefore, it is often difficult to establish the shape of the response of herbivores to varying levels of metals in plants. In particular, metals, like other toxic compounds, may have a hormetic effect on the performance of herbivores, where mild doses enhance the performance whereas higher doses have negative effects (Calabrese *et al.* 1999; Zhang *et al.* 2009; Cordeiro *et al.* 2013; Guedes & Cutler 2014). Indeed, spider mites showed a hormetic response to cadmium accumulation by tomato plants (Godinho *et al.* 2018). The few other studies measuring the performance of herbivores on plants exposed to several metal concentrations show linear effects (Behmer *et al.* 2005; Fones *et al.* 2010) or fluctuating effects that might suggest hormesis, although this hypothesis was not tested (Konopka *et al.* 2013). In general, non-linear responses require more concentration levels to be detected, so hormesis might have been missed in some studies. Identifying the factors underlying such responses is key to our understanding of the interaction between herbivores and metal-accumulating plants.

One potential means to understand the factors responsible for the response of herbivores to metal-accumulating plants is to expose those herbivores to artificial diets with specific compounds. Indeed, some studies have shown that the performance of herbivores is reduced when these are fed with artificial diets containing metals (Boyd & Moar 1999; Jhee *et al.* 2006; Fones *et al.* 2010; Cheruiyot *et al.* 2013, 2015; Konopka *et al.* 2013; Stolpe & Müller 2016) suggesting a direct effect of metals on herbivores. Additionally, some authors tested artificial combinations of metals and organic compounds (Jhee *et al.* 2006; Cheruiyot *et al.* 2015), showing that, for some combinations, the effect was higher than that of the individual compounds, but for other combinations this was not the case. However, most studies do not compare the performance of herbivores on artificial diets to that on plants exposed to the same contaminants. Thus, addressing whether herbivore performance is due to direct effects of metals or to indirect effects via physiological and metabolically changes in the plant is compromised (but see Boyd & Moar, 1999; Fones *et al.*, 2010; Konopka *et al.*, 2013).

Here, we aimed to address the degree of intraspecific variability in herbivore responses to metal-accumulating plants as well as to unravel the possible causes of such variation. To this end, we studied the variation in the response of several field populations of two species of herbivorous spider mites, *Tetranychus urticae* and *Tetranychus evansi*, to tomato plants (*Solanum lycopersicum*) that accumulate cadmium. These two spider-mite species are herbivorous pests of many crops (Migeon et al. 2010), including tomato. Both species respond similarly to cadmium accumulation by tomato plants, showing an increase in their oviposition rate with mild cadmium concentrations and a decrease at higher concentrations, to levels below those on un-exposed plants (Godinho *et al.* 2018). This similarity suggests that the hormetic response to metal accumulation might be a common pattern in spider mites. Moreover, cadmium accumulation in tomato plants was associated with changes in the amount of soluble sugars in the plant, which was correlated with the oviposition rate of the spider mites on those plants (Godinho *et al.* 2018). This suggests that the hormetic pattern observed might be caused by metabolic changes in the plant. Still, the direct effect of cadmium cannot be excluded.

To disentangle between the direct effect of the metal on herbivores and the indirect effects inherent to changes in the plant, we exposed different populations of herbivorous spider mites to an artificial diet composed of different concentrations of cadmium or of soluble sugars. We then tested if the hormetic pattern found in some populations when feeding on tomato plants exposed to cadmium could be recaptured by the response to the metal, to the soluble sugars or both.

Material and methods

Study organisms

Tomato plants (*Solanum lycopersicum*, var. Moneymaker) were sowed in a climate chamber (25◦C, photoperiod 16/8 h light/darkness), in a soil/vermiculite mixture (4:1).

Two weeks after being sowed, tomato plants were watered for another two and a half weeks with 60 mL of distilled water, either without cadmium or with a given cadmium chlorine solution (0.25, 0.5, 0.75 or 1.5 mM) twice a week. All plants were watered an additional time per week with tap water to compensate for micro-nutrients deficiencies These 4 and a half week old plants were then used in the experiments.

The populations used in this study were: (a) a population of *Tetranychus evansi* long adapted to laboratory conditions, hereafter Te QL, which was collected from *Datura stramonium*, L. in 2013 in Portugal (Godinho *et al.* 2018; Zélé *et al.* 2018) and for which the response to cadmium accumulation on tomato has been previously described (Godinho *et al.*, 2018); (b) 7 natural populations of *T. evansi* and of *T. urticae* collected on tomato plants in 2017 and (c) an outbred population of each species created in the laboratory by merging 3 or 4 natural populations of *T. urticae* and *T. evansi*, respectively (Godinho et al., 2020; Table S1).

All spider-mite populations were maintained separately in plastic boxes containing leaves of tomato plants, in a climate chamber with conditions identical to those of the plant-growing compartment (25 ºC, photoperiod 16/8 h light/darkness). To ensure that females used in the experiments were approximately of the same age, groups of adult females where isolated on separate leaves and allowed to lay eggs for 48 h. 14 days later, mated adult females resulting from these cohorts were used in the experiments.

Performance of spider mites on plants exposed to cadmium

Discs (Ø 16 mm) were made from the 3rd oldest leaf of each plant (N = 7 to 10 per cadmium concentration). One female spider mite was placed on each disc and allowed to feed and oviposit for 3 days. Each plant served to form leaf discs for mites of several populations and each population was tested on leaf discs from different plants. Daily survival and fecundity of each female were recorded. The oviposition rate of spider mites was obtained by dividing the number of eggs laid by female survival.

Performance of spider mites on artificial diet

To distinguish whether the response of spider mites to cadmium accumulating tomato plants was due to their response to sugars or cadmium, we exposed a subset of the spider-mite populations used on entire plants to artificial diets.

The artificial diet was created using a protocol adapted from Zélé *et al.*, 2019 by mixing 0.446 mL of Schneider medium with 0.544 ml of a given pre-prepared solution and 0.01 ml of food colouring dye. Two sets of solutions were prepared, one with several cadmium chlorine concentrations (0, 50, 150, 300, 500 and 750 mg/kg), the other with an array of glucose solutions (0, 5, 8, 10, 15, 20 and 25 mmol), which is a range of concentrations wider than that found of tomato plants exposed or not to cadmium (Godinho et al. 2018).

Parafilm squares (circa 1 cm^2) were placed in a metallic well microplate connected to a vacuum pump, creating a parafilm bubble. 30 µL of a given solution were then inserted in the bubble with a micropipette, and the bubble was subsequently closed with tape (Zélé *et al.* 2019). Each bubble (N = 6 per population, per treatment) was placed in an arena consisting of a small Petri dish, together with 30 female spider mites of a given population (Table 1). The Petri dish was then closed with a fine fabric mesh and the females were allowed to feed for 24h. The females that had fed on the artificial diet could be distinguished via a blue coloration in their gut due to the dye. 10 of those females were then collected (per arena; N = 60 females per population per treatment) and isolated individually on a tomato leaf disc (Ø 16 mm). 24h later, the number of eggs was registered.

Statistical analysis

Dose-response curves were modelled using the "mselect" function of drc package in R (Ritz *et al.* 2016), which compares the fit of the data to different dose-response curves (Ritz *et al.* 2016). However, our data did not fit any of the models with 4 or 5 parameters (including or not the hormetic effect, Table S2 and S3). Therefore, we tested spider-mite responses using general linear models.

To test the effect of cadmium accumulation by tomato plants on spider-mite performance, we performed a general linear mixed model with a gaussian distribution, using population and concentration of cadmium as fixed factors and plant as a random factor. To test whether an artificial diet with varying concentrations of either cadmium or glucose affected spider-mite performance, we performed general linear mixed models with gaussian distributions, using population as a fixed factor, cadmium or glucose concentration as a fixed factor and the arena

as a random factor. With these analyses, we observed that glucose did not affect the performance of spider mites, hence there was no hormetic (or other) significant response (cf. results). In contrast, cadmium had a significant effect on the performance of spider mites, both via plant and via artificial diets, and this was population dependent. To test if the response of each population was hormetic we performed:

a) Independently for each population, a general linear mixed model with a gaussian distribution, with concentration of cadmium as fixed factors and plant or arena (for the artificial diets) as a random factor. Using the previous models, we performed à posteriori planned post-hoc contrasts with Bonferroni adjustments between the control and each of the cadmium concentrations, using the mutlcomp package in R (Hothorn *et al.* 2007). The populations that had, on any cadmium concentration, an average oviposition rate significantly higher than that on un-exposed control plants were considered to have a hormetic response.
b) We estimated the slope of the response of each population to the cadmium concentration applied to the plants, or to cadmium concentrations in the artificial diet, using a general linear mixed model in which the spider mite population was used as a fixed factor and concentration was nested within population to specify that each population could have a different response slope. Plants or arenas were included in the model as random factors. To account for hormesis, the range of cadmium was divided in two, from 0 to the inflection point (0.5 mM in whole plants and 150 mmol in the artificial diet) and from the inflection point to the highest concentration. These inflection points were chosen with the contrasts performed for the previous analysis. For each range, the slope of the response of each population was compared to 0 by adjusting the model accordingly (Bates *et al.* 2019). The populations that had a slope significantly higher than 0 on the first range of cadmium concentrations were considered to have an hormetic response. We then assessed whether the performance pattern observed in spider mites fed with cadmium-accumulating plants or with an artificial diet with cadmium matched, by comparing the slopes of both tests (plants vs artificial diet) for each population. This was performed with a general linear model with oviposition rate as the response variable, cadmium concentration as a co-variate and test (plant or artificial diet) as a fixed factor.

Results

Performance of spider mites on plants exposed to cadmium

The oviposition rate of spider mites was significantly affected by the cadmium concentration the plants were exposed to (F_4= 77.12, P< 0.001). This effect differed significantly across populations (interaction between cadmium and population: F_{60}= 1.86, P< 0.001, Fig. 1A, Fig. S2).

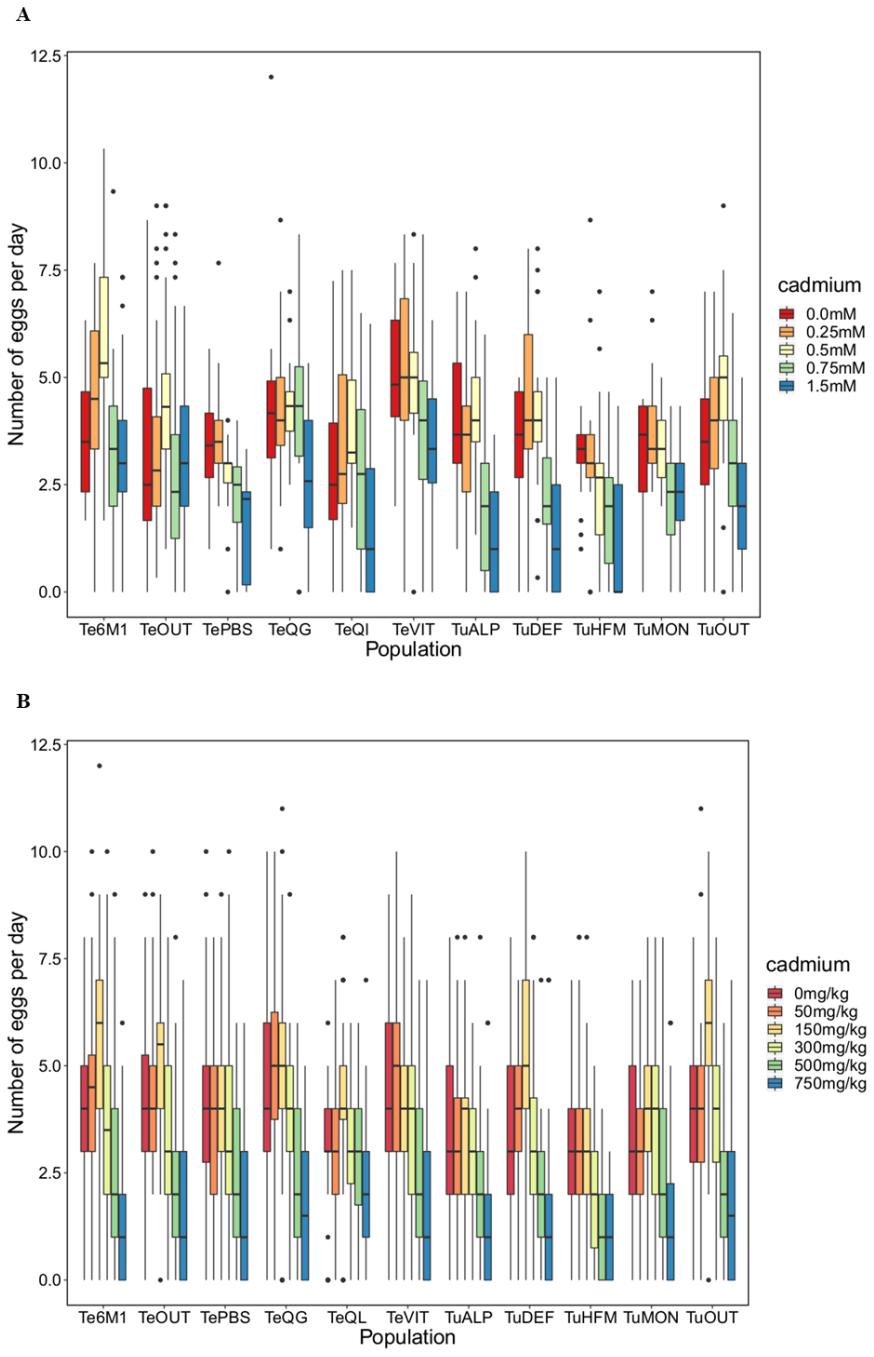
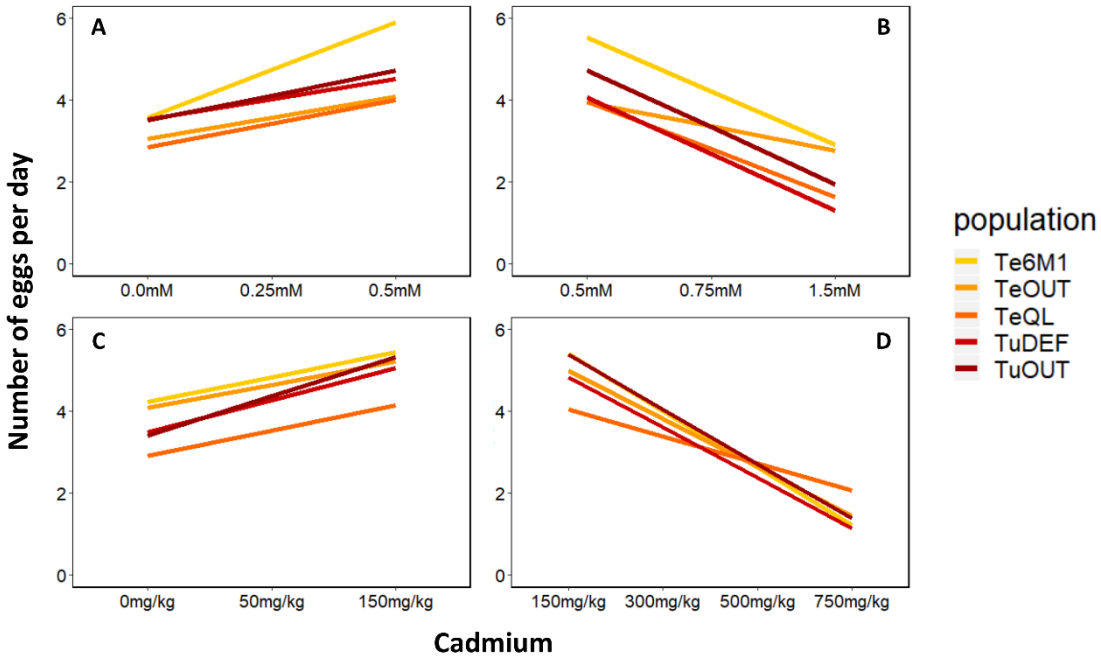


Figure 1 *Performance of spider-mite populations on A) plants exposed to different concentrations of cadmium and B) artificial diets containing different concentrations of cadmium. Bars represent boxplots of the oviposition rate of different spider mite populations. In tests on plants (N = 7 to 10 per concentration), 3 to 5 spider mites were used; tests on artificial diets were done with 10 spider mites per arena (N = 6 per concentration).*

A hormetic response to cadmium accumulation was observed for some populations of both species (Te 6M1, Te CG, Te QL, Te OUT, Tu 6M2 and Tu OUT). For those, the oviposition on plants exposed to 0.5 mM of cadmium was significantly higher than that on un-exposed plants (Tables S4 to S6). Two of these populations had a significantly lower oviposition rate on plants exposed to higher concentrations of cadmium, as compared to that on un-exposed plants (Te QL and Tu OUT), whereas the others did not (Te 6M1, Te CG, Te OUT, Tu 6M2 and Tu OUT). In agreement, within the first range of cadmium concentrations, from 0 to the inflection point (0.5 mM), the slope of the response to cadmium of these populations was significantly higher than 0 (Table 1, Fig. 2A), revealing an increase in oviposition rate for those populations with increasing cadmium concentrations. These populations were, however, negatively affected by higher concentrations of cadmium, since the response slope was significantly lower than 0 for the second range of concentrations, from 0.5 mM to 1.5 mM (Table 1, Fig. 2B).

Figure 2 *Slopes of the responses of spider-mite populations with a hormetic response to cadmium via A) and B) plants or C) and D) artificial diets.*

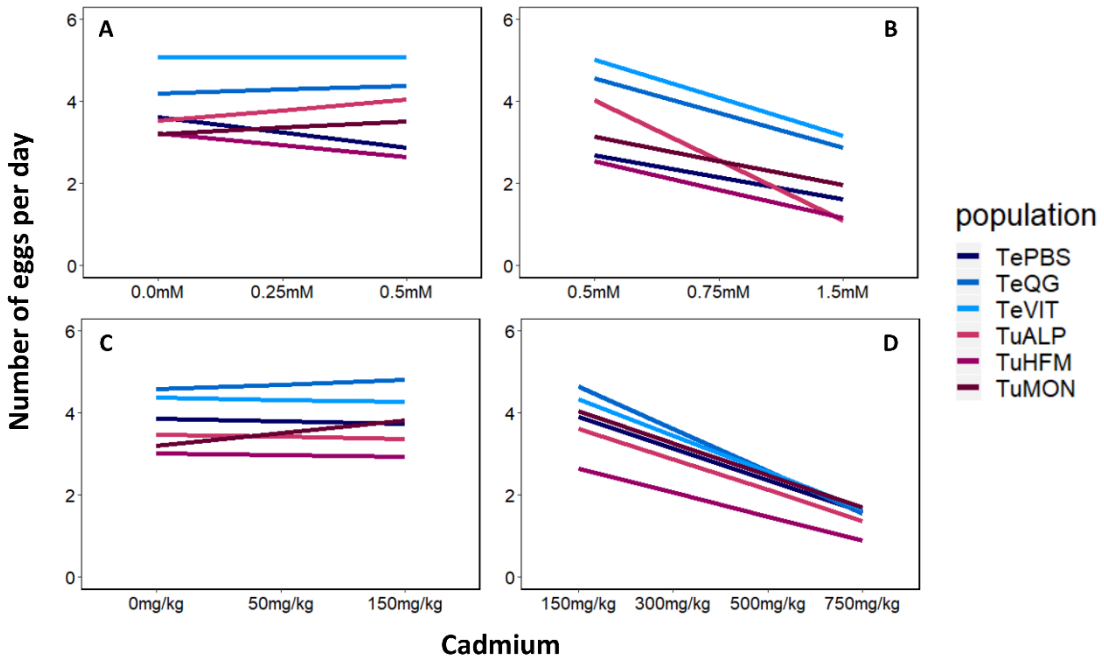


The remaining populations (TeER, TePBS, TeQG, TeVC, TeVIT, TuALP, TuDEF, TuHFM, Tu MON, TuLIM and TuLNEC) did not have, on plants exposed to any concentration of cadmium, oviposition rates significantly higher than those on un-exposed plants (Tables S4 to S6). Most of these populations (except Tu MON) were negatively affected by cadmium at higher concentrations (Tables S4 to S6). In agreement, the response slope of these populations

for the range of concentrations from 0 to the inflection point (0.5 mM), was not significantly different from 0 (Table 1, Fig. 3A). Additionally, the response slopes for the second range of cadmium, from 0.5 mM to 1.5 mM, were significantly lower than 0 (Table 1, Fig. 3B), except for TeVIT and TuMON (Table 1, Fig. 3B).

Note that the oviposition rate of TuLIM was un-affected by cadmium concentration in the plants. Thus, it was not considered in the analyses to detect hormesis.

Figure 3 *Slope of the response of spider-mite populations with no hormetic response to cadmium via A) and B) plants or C) and D) artificial diets.*



Performance of spider mites on artificial diets

a) Effect of cadmium

The oviposition rate of spider mites was significantly affected by the cadmium concentration on the artificial diet (F_5= 160.80, P< 0.001). This effect differed significantly across populations (interaction between cadmium and population: F_{50}= 3.02, P< 0.001, Fig. 1B).

The oviposition rate of some populations (Te6M1, TeQL, TeOUT, TuDEF and TuOUT) was higher when mites were feeding on an artificial diet with 150 mg/kg of cadmium than when they were feeding on an artificial diet without cadmium (Tables S5 and S6).

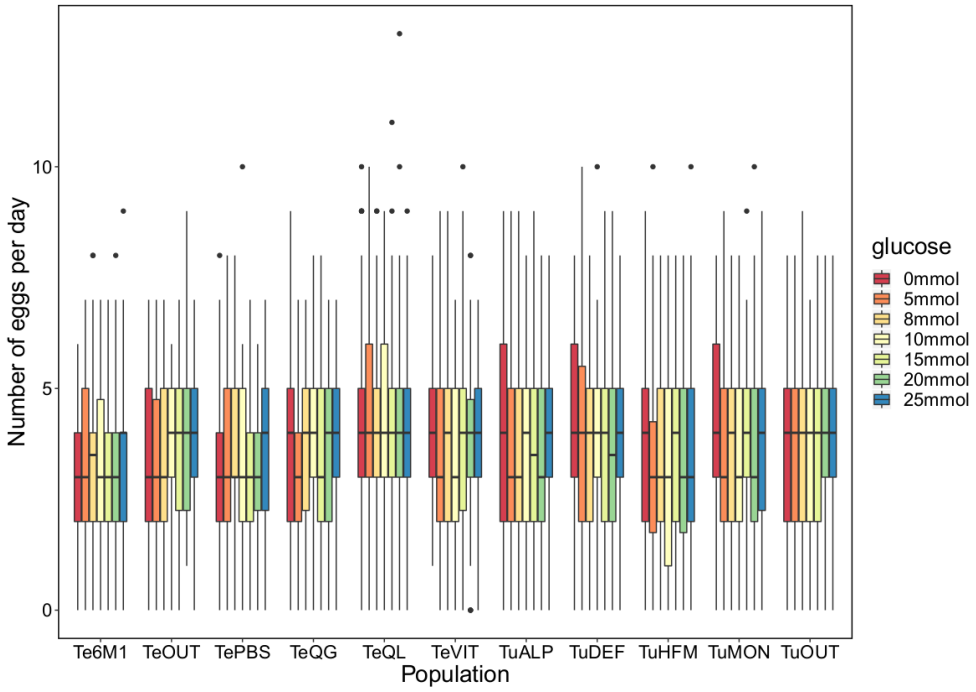
Consistently, their response slope within the first range of cadmium concentrations, from 0 to 150 mg/kg, was significantly higher than 0 (Table 2, Fig. 2C). These populations were negatively affected by higher concentrations of cadmium, since the response slope was significantly lower than 0 for the second range of concentrations, from 150 to 750 mg/kg (Table 2, Fig. 2D).

None of the remaining populations (TePBS, TeQG, TeVIT, TuALP; TuHFM and TuMON) had, on artificial diets with any concentration of cadmium, oviposition rates significantly higher than those on artificial diets without cadmium. Additionally, these populations were not affected by cadmium within the first range of concentrations, from 0 to 150 mg/kg, as the response slope was not significantly different from 0 (Table 2, Fig. 3C). All of these populations were negatively affected by higher concentrations of cadmium, having response slopes for the second range of cadmium, from 150 to 750 mg/kg, significantly lower than 0 (Table 2, Fig. 3D).

b) Effect of glucose

The oviposition rate of all spider mite populations was not affected by the concentration of glucose on the artificial diet (F_6= 0.61, P= 0.72, Fig. 4).

Figure 4 *Performance of spider mites on artificial diets with different glucose concentrations. Bars represent boxplots of the oviposition rate of different spider mite populations. 10 spider mites per arena were used (N = 6 per concentration).*



Comparison of the hormetic response between plants and artificial diets

Within the first range of the hormetic curve, where cadmium increases the oviposition rate of spider mites, the response of most populations was similar between plants and artificial diets. Indeed, the oviposition rate of those populations was not significantly affected by the interaction between test (plants or artificial diets) and cadmium concentration (TeQL: F = 2.46, P = 0.12; TeOUT: F = 0.63, P = 0.43; TuDEF: F = 0.03, P = 0.86 and TuOUT: F = 0.002, P = 0.96). A single population did not comply to this pattern: Te6M1, for which the response slope on artificial diets was steeper than that on plants (interactions between cadmium concentration and test: F = 6.79, P = 0.01). Note that even though the oviposition rate of TuDEF was not significantly higher on plants exposed to the different cadmium concentrations than that on un-exposed plants (Table S6), a marginally significant tendency was found (P = 0.08). As, we found, a hormetic response on the artificial diets for this population (Table S6), we included it in the current analysis.

Table 1. *Results of the GLMM testing the hormetic response of each population of spider mites when feeding on plants exposed to cadmium. Values in bold represent response slopes significantly different from 0.*

Species	**Population**	**Cd range: 0 to 0.5 mM**		**Cd range: 0.5 to 1.5 mM**		**Hormesis**
T. evansi	QL	t = 1.90	**P = 0.05**	t = -3.42	**P < 0.001**	yes
T. evansi	6M1	t = 4.12	**P < 0.001**	t = -3.62	**P < 0.001**	yes
T. evansi	PBS	t = -1.21	P = 0.23	t = -1.69	**P = 0.009**	no
T. evansi	QG	t = 0.30	P = 0.76	t = -2.97	**P = 0.003**	no
T. evansi	VIT	t = 0	P = 1	t = -2.72	**P = 0.006**	no
T. evansi	OUT	t = 3.06	**P = 0.002**	t = -2.38	**P = 0.017**	yes
T. urticae	ALP	t = 0.92	P = 0.355	t = -4.48	**P < 0.001**	no
T. urticae	DEF	t = 1.74	P = 0.08	t = -4.28	**P < 0.001**	possibly
T. urticae	HFM	t = -1.02	P = 0.31	t = -2.37	**P = 0.018**	no
T. urticae	MON	t = 0.56	P = 0.57	t = -1.56	P = 0.12	no
T. urticae	OUT	t = 3.59	**P < 0.001**	t = -7.27	**P < 0.001**	yes
T. evansi	CG	t = 3.92	**P < 0.001**	t = -4.16	**P < 0.001**	yes
T. evansi	ER	t = 1.09	P = 0.27	t = -2.95	**P = 0.003**	no
T. evansi	VC	t = -0.01	P = 0.99	t = -1.64	P = 0.10	no
T. urticae	6M2	t = 4.29	**P < 0.001**	t = -4.64	**P < 0.001**	yes
T. urticae	LIM	t = 0.53	P = 0.60	t = -2.09	**P = 0.037**	no
T. urticae	LNEC	t = -1.63	P = 0.10	t = -2.83	**P = 0.005**	no

Table 2. *Results of the GLMM testing for the hormetic response of each population of spider mites when feeding on artificial diets with cadmium. Values in bold represent response slopes significantly different from 0.*

Species	**Population**	**Cd range: 0 to 150 mg/kg**		**Cd range: 150 to 750 mg/kg**		**Hormesis**
T. evansi	QL	t = 3.36	**P < 0.001**	t = -5.27	**P < 0.001**	yes
T. evansi	6M1	t = 3.59	**P < 0.001**	t = -13.09	**P < 0.001**	yes
T. evansi	PBS	t = -0.12	P = 0.9	t = -7.30	**P < 0.001**	no
T. evansi	QG	t = 0.41	P = 0.68	t = -9.70	**P < 0.001**	no
T. evansi	VIT	t = -0.50	P = 0.62	t = -8.56	**P < 0.001**	no
T. evansi	OUT	t = 3.27	**P < 0.001**	t = -10.93	**P < 0.001**	yes
T. urticae	ALP	t = 0.01	P = 0.99	t = -7.13	**P < 0.001**	no
T. urticae	DEF	t = 4.55	**P < 0.001**	t = -11.37	**P < 0.001**	yes
T. urticae	HFM	t = -0.25	P = 0.81	t = -5.34	**P < 0.001**	no
T. urticae	MON	t = 1.91	P = 0.06	t = -7.50	**P < 0.001**	no
T. urticae	OUT	t= 5.81	**P < 0.001**	t = -12.43	**P < 0.001**	yes

Discussion

In this study, we show that populations of two species of herbivorous spider mites, *T. urticae* and *T. evansi*, differ in their response to cadmium accumulation by tomato plants. Whereas some populations showed a hormetic response to this metal, others did not. Additionally, for all populations studied (except for TuDEF), the presence or absence of a hormetic response to cadmium accumulation by tomato plants was recaptured in the response to cadmium on artificial diets, suggesting that hormesis is caused directly by the metal and not by metabolic changes in the plants exposed to this metal. In agreement with this, the oviposition rate of spider mites was not affected by artificial diets with different concentrations of glucose, revealing that the fluctuations on the amount of soluble sugars found on plants with different cadmium concentrations (Godinho *et al.* 2018) were not responsible by the pattern observed in the herbivores.

Some authors have previously used artificial diets to assess the direct effect of metal toxicity on herbivores (Boyd & Moar 1999; Jhee *et al.* 2006; Cheruiyot *et al.* 2013, 2015; Konopka *et al.* 2013; Stolpe & Müller 2016). However, comparing the direct response to metals with a response of herbivores on metal accumulating plants has seldom been done. An exception is the study by Boyd and Moar (1999), which used artificial diet to show that the

nickel concentration that is lethal to *Spodoptera exigua* is much higher than that found in *Streptanthus* plants. Therefore, the negative effect of plants with nickel on the performance of *S. exigua* should result from an interaction with other plant processes. In accordance, Konopka and colleagues (2013) found that the negative effects of cadmium were more pronounced when *Trichoplusia ni* was feeding on an artificial diet than on *Brassica juncea* plants, which also suggests that such plants affect herbivore performance via an interaction with other plant processes. Although these studies provide important insight on the interaction between herbivores and metal-accumulating plants, the range of metal concentrations used on entire plants was limited. Therefore, it was not possible to test whether the seemingly hormetic response found with the artificial diet in the latter study could be recovered on entire plants.

In the spider-mite populations we studied, the presence or absence of the hormetic response to cadmium accumulation by tomato plants was remarkably recaptured, for all populations (except for TuDEF), when using an artificial diet with different cadmium concentrations. However, when feeding on the artificial diet, the effect of the metal was clearer than when feeding on plants. Indeed, the population TuDEF showed an increase in oviposition rate both when feeding on the artificial diets and on plants, but the latter was not significant ($P = 0.08$). Still, the response slope of this population was not significantly different between tests, suggesting that the hormetic pattern is present in both responses, even though it is clearer when mites feed on artificial diets. Additionally, some populations that were unaffected by cadmium on the plants were significantly affected by higher concentrations of cadmium on artificial diets. Possibly, the range of cadmium used in the artificial diet was wider than the concentrations accumulated in the whole plants, reaching values that had a negative impact even on the most tolerant populations. Remarkably, the tipping point for hormesis, i.e. the concentration where the oviposition rate reaches a peak, was the same for all populations, 0.5 mM on the plants and 150 mg/kg on the artificial diet. However, 150 mg/kg is higher than the average amount of cadmium accumulated by plants exposed to 0.5 mM (Godinho *et al.* 2018). Possibly, some metabolic processes in the plant increase the overall effect of cadmium on herbivores, in agreement with what was observed for nickel (Boyd & Moar 1999).In any case, the slope of the response of all populations (except Te6M1) was similar on plants and on artificial diets, thus, our results reveal that the hormetic response is caused directly by cadmium and not by some metabolic change occurring in the plant as a response to this metal.

Studies using other organisms has also shown some positive effects of cadmium on animal physiology. Indeed, low concentrations of cadmium were shown to stimulate growth

on aquatic invertebrates, leading to higher reproductive biomass and, therefore, an increase in offspring production (Stebbing 1981; Bodar *et al.* 1988; Correia *et al.* 2001; Lefcort *et al.* 2008). Additionally, cadmium has been shown to affect the levels of ecdysteroids, which is a hormone that affects reproduction in *Daphnia magna* (Bodar *et al.* 1990). However, whether these effects are extensive to other invertebrates such as spider mites, is still unknown.

Moreover, using an artificial diet, we show that glucose does not affect the oviposition rate of any spider mite population of both species, even though we tested a range of glucose concentrations wider than that found in tomato leaves (Godinho *et al.*, 2018). This is at odds with earlier findings, showing that the performance of spider mites is correlated with the amount of sugars on the plant (Joutei *et al.* 2000; Ximénez-Embún *et al.* 2016, 2017; Godinho *et al.* 2018). However, such studies were done on entire plants. Our results suggest that sugars are not the direct cause of such changes in performance. Indeed, the performance of spider mites on plants stressed by abiotic factors was shown to be also correlated with other changes in the plant, such as nitrogen content (Joutei *et al.* 2000) and non-essential proteins such as proline, which is known as a reproductive stimulant in arthropods (Ximénez-Embún *et al.* 2016, 2017). Although herbivores do respond to these physiological changes in plants, it is not obvious that they will do so on metal accumulating plants, because these are known to suffer less from metal toxicity, having a much higher threshold for the effects of metals, as compared to the herbivores feeding on them (Boyd 2004; Maestri *et al.* 2010). Thus, the metabolic changes that might happen in the plant may not be sufficiently strong to have a pronounced effect on the performance of herbivores. Therefore, we expect that the pattern observed here may be extendable to other metal accumulating plant species and other metals.

We also found that hormesis was not universal, being present in only some populations of each herbivore species. Given that the species studied have the same feeding mode (i.e. cell piercing) and being *T. urticae* a generalist species and *T. evansi* a specialist of host plants of the Solanaceae family, neither the feeding type nor the host range seem to be responsible for the hormetic response. Hormesis was also not associated with a particular *T. evansi* haplotype, as it was present in populations of both Cytochrome Oxidade I haplotypes (Godinho *et al.* 2020), nor with one of the *T. urticae* morphotypes (green and red). Thus, the hormetic response is widespread among different populations of each species, even among populations with distinct phenotypic and/or genotypic profiles. Given this scenario, we speculate that hormesis may be a common response of many species of herbivores to metal accumulation in plants.

Hormesis is hypothesized to be a mechanism to buffer the negative effects of a given stressor on some life history traits by stimulating another fitness component, thus, maintaining the overall fitness (Forbes 2000). Therefore, exposure to a stressor may select for hormesis. Indeed, in different *Drosophila* species the hormetic response to ethanol seems to be positively correlated with exposure to this compound in rotting fruit (Holmes *et al.* 1980; Parsons 2001). Therefore, the evolutionary history may explain the intraspecific variability for the presence or absence of the hormetic response in spider mites. Moreover, hormesis is also proposed to be linked to phenotypic plasticity and acclimation, having an important role in responses to changing environments (Forbes 2000; Costantini *et al.* 2010). Thus, populations growing in more unstable or heterogeneous environments may be more prone to a hormetic response than populations having a more constant recent evolutionary history. Alternatively, it is possible that hormesis results from a negative effect on parasites or diseases if those have a lower threshold for metal toxicity that the hosts (Bengtsson 1985, Forbes 2000). Thus, differences in the microbiome may explain the presence or absence of hormesis.

The hormetic response of herbivores to metal accumulation is a pattern that may have a strong influence not only on the interactions between plants and herbivores in metal contaminated environments, but also on the ecology and evolution of both parties. Indeed, if mild amounts of metal accumulated on the shoots lead to higher herbivore reproductive performances, plants may be pressed to accumulate more metals to surpass these beneficial effects of mild concentrations or to not accumulate metals at all, becoming excluders. (Boyd 2012). On the herbivore side, if their reproductive performance is enhanced in plants with mild metal concentrations, this may lead to an adaptive advantage over competitors that feed on neighbouring plants that do not accumulate metals, explaining why, in some situations, more herbivores are found in environments with intermediate concentrations of pollutants (Zvereva *et al.* 1995; Kozlov 2003). Here, we show that several populations of the same species may exhibit a hormetic response to metal accumulation, suggesting that this pattern may be more common than previously thought.

In sum, metals have a direct effect on herbivores, independently of the metabolic changes occurring in the plants. Additionally, there is intraspecific variation in the response of herbivores to metals, with some populations displaying a hormetic response and others not. This interspecific variation may have important consequences for the ecology and evolution of both plants and herbivores. Our study clearly underscores the need for considering variation in

the response to metals and hormesis when studying the interactions between plants and herbivores.

Acknowledgements

We thank Maud Charlery for some help in the experiments, Flore Zélé and Gonçalo Matos for assistance with the protocol to create the artificial diets, Miodrag Grbic for the well microplate adapter used to produce the parafilm bubbles, José Feijó for the vacuum pump and all the members of the MITE2 group for stimulating discussions.

This study was funded by an ERC Consolidator Grant (COMPCON, GA 725419) to SM and by an FCT Ph.D. scholarship PD/BD/114010/2015 to DPG.

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Supplementary information 1

Figure S1 Oviposition rate of the spider mite populations not used on the artificial diet test on plants with different cadmium concentrations. 3 to 5 spider mites of each population were tested on each plant (N= 7 to 10 per concentration).

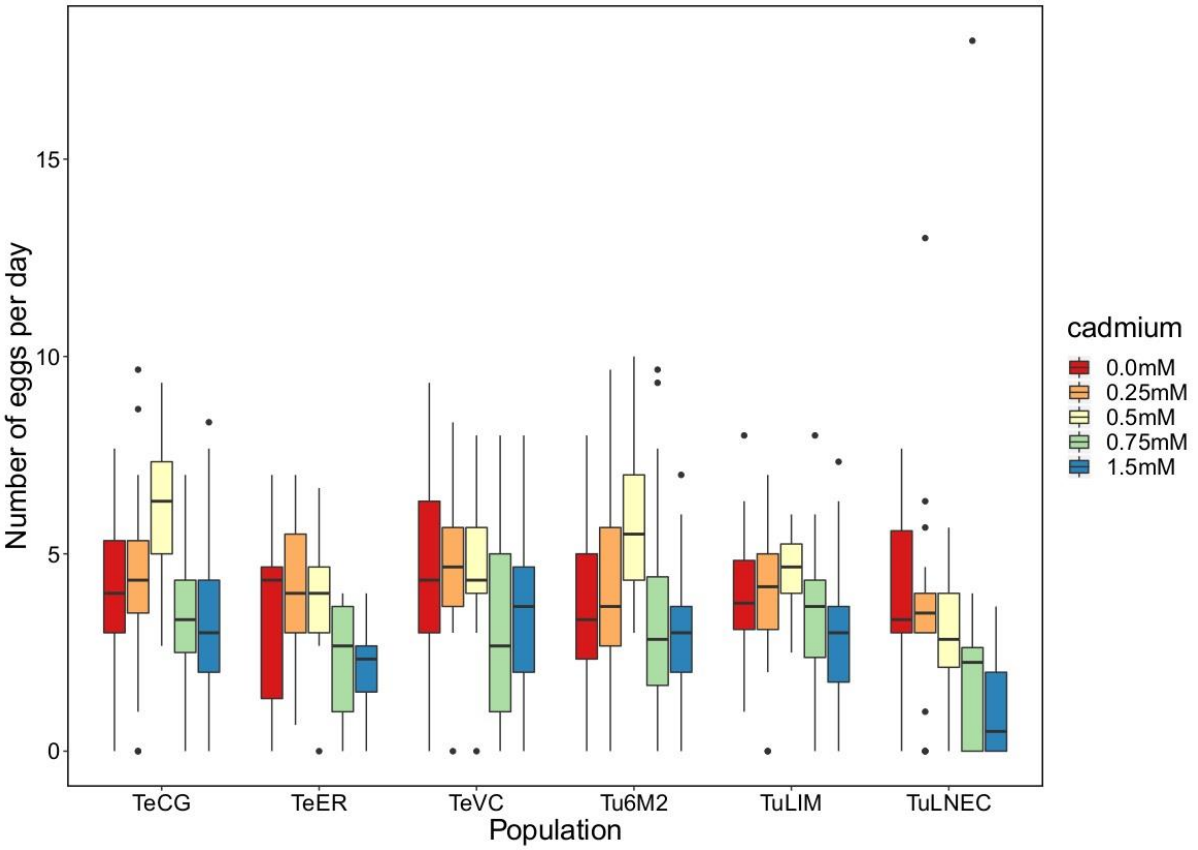

Table S1. Comparison of models fitting the oviposition of spider mites feed on whole plants exposed to different Cd concentrations. Model selection was performed using drc R package "mselect" function (Ritz et al. 2010). Original model: drm(oviposition rate ~ dose, population, fct=LL.4), where dose represents de concentration of Cd and population the population of spider mites.

Model	**LogLik**	**IC**	**Lack of fit (P-value)**	**Response variance**
CRS.4b	-4220.8	8579.595	1.70E-16	3.54543
BC.4	-4223.6	8585.175	1.62E-17	3.554982
CRS.4c	-4226	8590.002	2.11E-18	3.563266
LL.4	-4590.1	9318.184	1.76E-164	5.06208
W1.4	NA	NA	NA	NA
CRS.4a	NA	NA	NA	NA
BC.5	-4193.6	8559.219	NaN	3.483203
LL.5	NA	NA	NA	NA
CRS.5c	NA	NA	NA	NA
CRS.5a	NA	NA	NA	NA
CRS.5b	NA	NA	NA	NA

NA – Model did not converge

Table S2. Comparison of models fitting the oviposition of spider mites feed on an artificial diet with different Cd concentrations. Model selection was performed using drc R package "mselect" function (Ritz et al. 2010). Original model: drm(oviposition rate ~ dose, population, fct=LL.4), where dose represents de concentration of Cd and population the population of spider mites.

Model	**LogLik**	**IC**	**Lack of fit (P-value)**	**Response variance**
BC.4	-7874.2	15838.41	3.10E-04	3.447496
CRS.4a	-7905.2	15900.4	3.04E-14	3.503096
CRS.4b	-7915.8	15921.57	4.73E-18	3.522285
W1.4	-7944.0	15978.03	1.20E-28	3.573982
LL.4	-8639.8	17369.66	2.516222e-317	5.118239
CRS.4c	NA	NA	NA	NA
BC.5	-7870.6	15853.13	5.28E-06	3.450939
CRS.5a	-7893.1	15898.25	2.40E-14	3.491356
CRS.5b	-7909.6	15931.2	8.55E-21	3.521169
CRS.5c	-7921.5	15954.96	1.56E-25	3.542825
LL.5	NA	NA	NA	NA

NA – Model did not converge

Table S3. *A posteriori* contrasts for the oviposition rate of spider mites on tomato plants exposed to different cadmium concentrations. These populations were not used in the test with artificial diets. Significant differences are represented in bold.

Species	Population	Contrast	Z-value	P-value
T. evansi	CG	0 vs 0.25	z = 0.76	P = 1
		0 vs 0.5	z = 3.39	**P = 0.003**
		0 vs 0.75	z = -0.74	P = 1
		0 vs 1.5	z = -1.04	P = 1
	ER	0 vs 0.25	z = 1.46	P = 0.57
		0 vs 0.5	z = 1.25	P = 0.84
		0 vs 0.75	z = -2.34	P = 0.08
		0 vs 1.5	z = -2.84	**P = 0.02**
	VC	0 vs 0.25	z = -0.16	P = 1
		0 vs 0.5	z = -0.01	P = 1
		0 vs 0.75	z = -2.67	**P = 0.03**
		0 vs 1.5	z = -2.05	P = 0.16
T. urticae	6M2	0 vs 0.25	z = 0.92	P = 1
		0 vs 0.5	z = 3.64	**P < 0.001**
		0 vs 0.75	z = 0.27	P =1
		0 vs 1.5	z = 0.76	P = 1
	LNEC	0 vs 0.25	z = -0.25	P = 1
		0 vs 0.5	z = -1.16	P = 0.98
		0 vs 0.75	z = -1.44	P = 0.59
		0 vs 1.5	z = -3.07	**P = 0.01**

Table S4. *A posteriori* contrasts for the oviposition rate of ***T. evansi*** on tomato plants exposed to different cadmium concentrations and on artificial diets with different cadmium concentrations. Significant differences are represented in bold.

	Plants (mM)			Artificial diets (mg/kg)		
Population	**Contrast**	**Z-value**	**P-value**	**Contrast**	**Z-value**	**P-value**
QL	0 vs 0.25	z = 0.89	P = 1	0 vs 50	z = -0.07	P = 1
	0 vs 0.5	z = 2.35	**P = 0.05**	0 vs 150	z = 3.53	**P = 0.002**
	0 vs 0.75	z = 0.43	P = 1	0 vs 300	z = -0.43	P = 1
	0 vs 1.5	z = -1.74	**P = 0.03**	0 vs 500	z = -1.52	P = 0.64
				0 vs 750	z = -2.36	**P = 0.05**
6M1	0 vs 0.25	z = 1.49	P = 0.54	0 vs 50	z = 0.64	P = 1
	0 vs 0.5	z = 3.79	**P < 0.001**	0 vs 150	z = 3.32	**P = 0.005**
	0 vs 0.75	z = -0.55	P = 1	0 vs 300	z = -1.73	P = 0.42
	0 vs 1.5	z = -0.46	P = 1	0 vs 500	z = -4.46	**P < 0.001**
				0 vs 750	z = -8.42	**P < 0.001**
PBS	0 vs 0.25	z = 0.68	P = 1	0 vs 50	z = -1.08	P = 1
	0 vs 0.5	z = -1.86	P = 0.25	0 vs 150	z = -0.33	P = 1
	0 vs 0.75	z = -3.01	**P = 0.01**	0 vs 300	z = -2.06	P = 0. 20
	0 vs 1.5	z = -4.54	**P < 0.001**	0 vs 500	z = -4.63	**P < 0.001**
				0 vs 750	z = -6.64	**P < 0.001**
QG	0 vs 0.25	z = 0.35	P = 1	0 vs 50	z = 1.14	P = 1
	0 vs 0.5	z = 0.30	P = 1	0 vs 150	z = 0.59	P = 1
	0 vs 0.75	z = 0.01	P = 1	0 vs 300	z = -2.64	**P = 0.04**
	0 vs 1.5	z = -2.46	**P = 0.05**	0 vs 500	z = -5.14	**P < 0.001**
				0 vs 750	z = -7.92	**P < 0.001**
VIT	0 vs 0.25	z = -0.03	P = 1	0 vs 50	z = 0.84	P = 1
	0 vs 0.5	z = 0	P = 1	0 vs 150	z = -0.26	P = 1
	0 vs 0.75	z = -1.82	P = 0.28	0 vs 300	z = -1.15	P = 1
	0 vs 1.5	z = -2.87	**P = 0.02**	0 vs 500	z = -4.91	**P < 0.001**
				0 vs 750	z = -6.89	**P < 0.001**
OUT	0 vs 0.25	z = 0.03	P = 1	0 vs 50	z = 0.94	P = 1
	0 vs 0.5	z = 2.36	**P = 0.05**	0 vs 150	z = 3.20	P = 0.007
	0 vs 0.75	z = -1.18	P =0.95	0 vs 300	z = -1.83	P = 0.33
	0 vs 1.5	z = -0.31	P = 1	0 vs 500	z = -4.14	**P < 0.001**
				0 vs 750	z = -6.84	**P < 0.001**

Table S5. *A posteriori* contrasts for the oviposition rate of ***T. urticae*** on tomato plants exposed to different cadmium concentrations and on artificial diets with different cadmium concentrations. Significant differences are represented in bold.

	Plants (mM)			Artificial diets (mg/kg)		
Population	**Contrast**	**Z-value**	**P-value**	**Contrast**	**Z-value**	**P-value**
ALP	0 vs 0.25	z = -0.84	P = 1	0 vs 50	z = -1.64	P = 0.51
	0 vs 0.5	z = 1.11	P = 1	0 vs 150	z = -0.32	P = 1
	0 vs 0.75	z = -3.57	**P < 0.001**	0 vs 300	z = -2.11	P = 0.18
	0 vs 1.5	z = -5.1	**P < 0.001**	0 vs 500	z = -4.63	**P < 0.001**
				0 vs 750	z = -7.32	**P < 0.001**
DEF	0 vs 0.25	z = 2.07	P = 0.54	0 vs 50	z = 1.20	P = 1
	0 vs 0.5	z = 1.58	P = 0.45	0 vs 150	z = 4.50	**P < 0.001**
	0 vs 0.75	z = -2.02	P = 0.17	0 vs 300	z = -1.01	P = 1
	0 vs 1.5	z = -3.57	**P < 0.001**	0 vs 500	z = -4.54	**P < 0.001**
				0 vs 750	z = -6.12	**P < 0.001**
HFM	0 vs 0.25	z = 0.38	P = 1	0 vs 50	z = -0.06	P = 1
	0 vs 0.5	z = -1.26	P = 0.83	0 vs 150	z = -0.30	P = 1
	0 vs 0.75	z = -2.5	**P = 0.05**	0 vs 300	z = -4.63	**P < 0.001**
	0 vs 1.5	z = -4.26	**P < 0.001**	0 vs 500	z = -5.94	**P < 0.001**
				0 vs 750	z = -6.83	**P < 0.001**
MON	0 vs 0.25	z = 1.66	P = 0.39	0 vs 50	z = 0.10	P = 1
	0 vs 0.5	z = 0.81	P = 1	0 vs 150	z = 1.91	P = 0.28
	0 vs 0.75	z = -2.39	P =0.07	0 vs 300	z = 0.46	P = 1
	0 vs 1.5	z = -2.15	P = 0.07	0 vs 500	z = -2.73	**P = 0.03**
				0 vs 750	z = -5.09	**P < 0.001**
OUT	0 vs 0.25	z = 0.33	P = 1	0 vs 50	z = 0.36	P = 1
	0 vs 0.5	z = 4.32	**P < 0.001**	0 vs 150	z = 5.85	**P < 0.001**
	0 vs 0.75	z = -2.44	P = 0.06	0 vs 300	z = 0.76	P = 1
	0 vs 1.5	z = -5.55	**P < 0.001**	0 vs 500	z = -4.37	**P < 0.001**
				0 vs 750	z = -6.05	**P < 0.001**

Chapter VIII

Discussion

The work presented in this thesis contributes to the understanding of the interactions between the herbivorous spider mites *Tetranychus urticae* and *T. evansi* and between them and their host plant tomato (*Solanum lycopersicum*). So far, the study of these interactions has been mostly focused on how these spider mites affect and are affected by tomato organic defences. Our work was the first to unravel the effects of cadmium accumulation by tomato plants on the interactions of this plant with herbivorous spider mites. We have thus established a new model system to study the ecological consequences of metal accumulation by plants. By unravelling the similarities and disparities of the interactions between the two spider-mite species and this host plant, our work contributed to the underpinning of some of the causes of niche overlap between them as well as of some possible mechanisms that may minimize the effects of interspecific competition caused by such overlap. Below I summarize the main results from this work and point to potential research directions that stem from our findings.

1. Key results

1.1 Niche overlap

Tetranychus urticae and *T. evansi* have different effects on tomato plant defences. Whereas most strains and populations of *T. urticae* induce tomato plant defences, *T. evansi* suppresses such defences (Kant *et al.* 2004; Sarmento *et al.* 2011). Because the quality and quantity of plant defences differs within a plant and the performance and distribution of other herbivorous arthropods was shown to be affected by such differences, we expected that these two species might display different distributions within a plant. Instead, we found that, in the absence of competition, *T. urticae* and *T. evansi* have similar niches within tomato plants, performing better on young leaves as opposed to old leaves and, in accordance, choosing young over old leaves when colonizing an uninfested plant (Chapter III). Differences in quality between leaves may stem from the differential composition of plant defences, nutritional composition and the possible interactions between both. Whatever the plant characteristics behind the observed pattern, both herbivore species prefer the same leaves, which suggests that competition may be intense between the two species.

Likewise, laboratory populations of *T. urticae* and *T. evansi* were similarly affected by cadmium accumulation by tomato plants (Chapter V). Both species responded to the amount of cadmium accumulated with a hormetic pattern, where mild concentrations increased their

oviposition rate and higher concentrations hampered that trait. For both species, the point of inflexion of the curve, corresponding to the cadmium concentration that maximized the oviposition rate was the same. This result suggests that the distribution of *T. uritcae* and *T. evansi* among and within tomato plants differing on the amount of cadmium accumulated may be identical.

However, both species showed intraspecific variability in their response to cadmium accumulation. Indeed, the hormetic response was recaptured in several, but not all, tested field populations of each species (Chapter VI). This intraspecific variation may be explained by the evolutionary history of each population. Different populations may have been exposed to different host plants and / or different amounts of cadmium or other toxic compound that may affect the response to cadmium. This variability may stem from the composition of the community of host plants, from the presence of cadmium or other contaminants on the environment and / or from the presence of competitors or other antagonistic species that may affect the distribution of these spider mites among and within plants. Which factors (or combination of factors) has a stronger role in determining the observed intraspecific variability are yet to disentangle.

1.2 Metal accumulation as an effective defence against spider mites

Tomato plants are able to extract cadmium from the soil and accumulate it in their leaves (Gratão *et al.* 2008). However, whether this characteristic could serve as a defence against herbivores was not known for this plant. Our work showed that tomato plants can use cadmium accumulation as a defence against spider mites. First, even though low amounts of cadmium increased the oviposition rate of both *T. urticae* and *T. evansi*, higher concentrations hindered this rate (Chapter V). Second, the growth rate of spider mites on tomato plants for one generation, from egg to adult, was lower on plants exposed to cadmium as compared to that on control unexposed plants, at the concentrations that maximize oviposition rate (Chapter VI). Our results showed that unlike what was shown for *Arabidopsis halleri* (Plaza *et al.* 2015; Stolpe *et al.* 2017a), cadmium accumulation by tomato plants is a passive mechanism, depending only on the bio-available cadmium and not being induced by herbivory, in contrast with many plant secondary metabolites (Chapter VI). Furthermore, we showed that this defensive strategy, metal accumulation, may be employed without apparent substantial costs to the plant under greenhouse conditions, since growth rate and many physiological traits of

the plant were not affected by cadmium accumulation, within the concentrations used in our study and for the parameters evaluated (Chapter V). In addition, we showed that the negative effects of cadmium accumulation on spider mites are mostly mediated directly by cadmium toxicity, rather than by physiological changes in the plant. In fact, field populations that presented a hormetic response to cadmium accumulation in tomato plants showed a similar pattern when feeding on artificial diets with different cadmium concentrations and field populations that did not have an hormetic response to cadmium accumulation also did not reveal this pattern when fed artificial diets with this metal (Chapter VII).

1.3 Interactions between metal-based and organic defences

Antagonistic and synergistic effects of metal accumulation and organic based defences have been shown in different systems (Fones & Preston 2013; Cheruiyot *et al.* 2015; Stolpe *et al.* 2017b; Sahu *et al.* 2018). Our results show that cadmium accumulation in tomato plants does not interact with the production of proteinase inhibitors, a plant secondary metabolite induced upon herbivory (Chapter V). Indeed, induction of these plant defences by *T. urticae* and suppression by *T. evansi* did not vary among environments with different amounts of cadmium and, in accordance, the effect of cadmium accumulation on the oviposition rate of spider mites was not affected by induction or suppression of plant defences by *T. urticae* and *T. evansi* respectively (Chapter V). This may have important consequences for the ecology and evolution of plant herbivore interactions in metal polluted environments, since in the absence of trade-offs plants may use one defence mechanism without compromising the other, or even use both simultaneously, as hypothesized in the joint effect theory (Boyd 2007).

1.4 Dealing with interspecific competition

Herbivorous arthropods may display different strategies to deal with competition. Whereas some may avoid plants and / or tissues with competitors, others may invest in monopolizing and protecting host resources (Ballabeni *et al.* 2001; Weir & Grant 2004). In our work we showed that these two species of spider mites, *T. urticae* and *T. evansi*, display different strategies in the face of competition with heterospecifics. Indeed, *T. urticae* circumvented competition with *T. evansi* by avoiding tomato leaves infested with the latter (Chapter III) and by increasing dispersal from bean patches where this competitor is present (Chapter IV). In contrast, *T. evansi* did not avoid tomato leaves infested with *T. urticae*, nor did higher densities

of the latter on bean patches lead to increased dispersal by *T. evansi* (Chapters III and IV). Instead, *T. evansi* distributed evenly among leaves of tomato plants infested with *T. urticae*, diminishing the opportunity of *T. urticae* to occupy "free" portions of the shared host plant (Chapter III). One possible explanation for these differences in behaviour could be asymmetric competition. Indeed, *T. urticae* had lower oviposition rates than *T. evansi* on tomato plants and this disadvantage could trigger avoidance of a competitor that is fitter on tomato. However, *T. evansi* did not have a reproductive advantage on bean plants and *T. urticae* displayed behavioural avoidance anyway. Another possibility is that *T. urticae* has a better colonizing ability in detriment of its competitive ability, which would fall into the competition-colonization trade-off hypothesis (Levins & Culver 1971). Additionally, *T. urticae* being a generalist species, it has higher chances of dispersing to a host plant that is within its host range, as compared with *T. evansi* which is a specialist of plants of Solanaceae family. In this sense, the costs of dispersing to a new host plant may be lower for *T. urticae*. Nevertheless, even though these predictions were shown empirically in many systems, they may hinge on the environmental grain (Stevens *et al.* 2014).

Furthermore, the reproductive advantage of *T. evansi* over *T. urticae*, when feeding on tomato plants was lost when these plants were supplied with cadmium, since the growth rate of the two species on those plants was similar. This may have important implications for the outcome of competition between these two spider-mite species. Indeed, coexistence between them via minimization of fitness differences (Chesson 2018) is expected to be more likely on plants with cadmium than on plants without metals.

1.5 Absence of a genetic correlation between virulence and transmission

The virulence transmission trade-off is a cornerstone hypothesis in the host-parasite interactions literature (Alizon *et al.* 2009). In our work (Chapter IV) we show that even though there is genetic variance for virulence and transmission in *T. urticae*, there is no genetic correlation between those traits. Still, within-host dynamics derived from intraspecific and interspecific competition affected virulence and transmission, which suggests that, in this system, the relation between virulence and transmission may still follow the predictions of the trade-off but mediated by within-host dynamics rather than by a genetic correlation.

2. Main perspectives

The results from this thesis point out to new interesting directions in the study of plant herbivore interactions and of competitive interactions between herbivorous arthropods. Below I present some of those directions.

2.1 Causes for hormesis

Hormesis is an interesting mechanism with many relevant implications on how species adapt to toxins or other stresses (Mattson & Calabrese 2009), yet the mechanisms involved on this process are poorly understood (Małkowski *et al.* 2020). It may be triggered by a compensation of organisms to oxidative stress when under mildly stressful environments. This could be tested by measuring the level of oxidative stress in mites exposed to plants with different cadmium concentration. An alternative hypothesis for hormesis is that low concentrations of a given toxin do not have negative effects on the studied organism but may hinder the growth rate of microorganisms that hamper the performance of the study organism (Hörger *et al.* 2013). Therefore, by releasing the study organism from this pressure, its performance increases. This hypothesis can be tested using a protocol similar to that used in chapter VII, creating artificial diets of different concentrations of cadmium with and without the addition of large spectrum antibiotics and testing the performance of spider mites when feeding on those diets.

2.2 Intraspecific variability for hormesis

The intraspecific variability for the hormetic response in field populations of the two spider mite species also motives further investigation. The causes of such variability are intriguing (c.f. section 1.1), however, the data obtained in our work (Chapters II and VII) does not suffice to satisfy such curiosity. For that, extensive field work would be advisable. Such study would require a thorough characterization of plant communities in each location, as well as the amount of cadmium and other contaminants, such as other metals or pesticides, on that environment. Furthermore, the presence of spider mites and antagonistic species should be monitored over time (c.f. section 1.1). Since spider mites are seasonal, those factors should be followed at least during the course of one season (from late spring to autumn). These logistical requirements are very demanding and would involve the commitment of many people. Therefore, experiments in the laboratory or in semi-natural conditions, such as greenhouses, controlling specific

variables, may meanwhile enlighten some issues (c.f. section 2.3). Additionally, the presence / absence of a microbe with a negative effect on spider mites, may also be one of the variables behind the interspecific variability found for hormesis (c.f. next section 2.1). Even though the populations used in chapter VII were tested for the presence of *Wolbachia*, *Cardinium* and *Rickettsia*, the presence of other non-tested microbes could vary among populations, affecting the response to cadmium.

2.3 Metal accumulation as mediator of plant-herbivore and herbivore-herbivore interactions

Some of the main results from this thesis revealed key aspects of metal accumulation as a mechanism not only mediating plant-herbivore interactions but also, possibly, mediating the competition between different herbivore species. Metal accumulation affects herbivores mostly through the toxicity of the metal and not via physiological changes in the plant and, in the absence of a trade-off with organic defences, both types of defence may be used by plants simultaneously. Therefore, herbivore populations may be exposed to two distinct selective forces that should influence their evolutionary trajectories when adapting to such metal-accumulating plants. Therefore, studying phenotypic and genomic changes in populations adapting to metal accumulating plants via experimental evolution may unravel important aspects of plant-herbivore interactions in metal polluted environments. As far as we know, no studied addressed this topic so far.

Driven by the results of this thesis and making use of the outbred populations created during the thesis (Chapter II), we decided to follow this path and, as such, we are currently performing experimental evolution of spider mites adapting to cadmium-accumulating tomato plants. Moreover, motivated by the similarities in the fundamental niche of *T. urticae* and *T. evansi* and by the disparities in the response of these two species to interspecific competition, we decided to study as well, adaptation of these two species to spatially heterogeneous environments, i.e. composed of plants with cadmium plus plants without cadmium, in the presence and absence of heterospecifics. Preliminary results show that after 13 discrete generations of selection, *T. urticae* is adapting to cadmium. Indeed, populations evolving on plants supplied with 2 mM cadmium have higher oviposition rates on those plants than control populations, i.e. evolving on plants not exposed to cadmium (Fig 1A). Additionally, this adaptation to cadmium is not costly for *T. urticae*, since populations evolving on cadmium, did not have oviposition rates lower than control populations, when tested on plants without

cadmium (Fig 1A). In contrast, the populations of *T. evansi* evolving on plants with cadmium did not show a significant increase in oviposition rate on this environment, compared to control populations (Fig 1B). This suggests that *T. urticae* may adapt at a faster rate to plants with cadmium, gaining an advantage over its competitor on cadmium polluted environments. Yet, populations of *T. evansi* adapting to heterogeneous environments had higher oviposition rates than control populations on plants supplied with cadmium (Fig 1B). Note however that oviposition rates were measured over short periods of time (48 hours). Possibly *T. evansi* evolving in heterogeneous environments can compensate for the negative effects of cadmium by feeding alternatingly on both types of plants. Thus, when feeding on cadmium for longer periods of time, this increase in oviposition rate may not be maintained. Interestingly, this increase in oviposition rate of *T. evansi* populations evolving in heterogeneous environments, on plants supplied with cadmium, was lost in populations evolving in the presence of heterospecifics (Fig. 2). This shows that, in this case, the presence of heterospecific competitors hinders adaptation to new environments. Another interesting preliminary result was that hormesis was lost, for both species, in populations evolving in environments with cadmium and in heterogeneous environments. This suggests that the hormesis may occur only in populations naïve to cadmium. This may be either because hormesis is not beneficial to populations adapted to cadmium or because adaptation to cadmium entails a cost that hampers the hormetic response. Detailing the mechanisms behind the causes of hormesis (c.f. section 2.1) may help to shed some light on this topic. For instance, if the cause of hormesis relies on hampering negative effects of microbes (c.f. section 2.1), populations of spider mites that are not naïve to cadmium may be already "free" from those microbes or may be infected with cadmium adapted microbes. In any case, these spider-mite populations would not benefit from the negative effect of cadmium on their parasites and, thus, hormesis would not be observed.

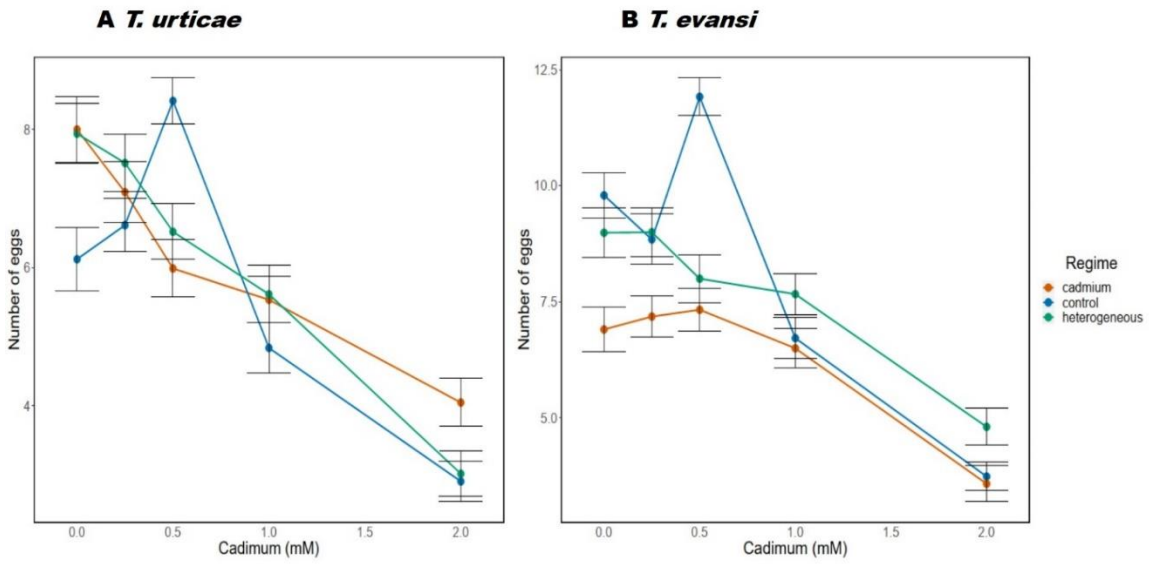

Figure 1. *Oviposition rate on leaf discs from plants with different cadmium concentrations of spider mites (A: T. urticae, B: T. evansi) evolving under different selection regimes: control (plants not exposed to cadmium), cadmium (plants exposed to 2 mM of cadmium) or heterogeneous (environment composed of plants with and without cadmium within each generation). Females (20 per replicate population, 5 replicates per selection regime) were placed individually on leaf discs and allowed to feed and oviposit for 48h.*

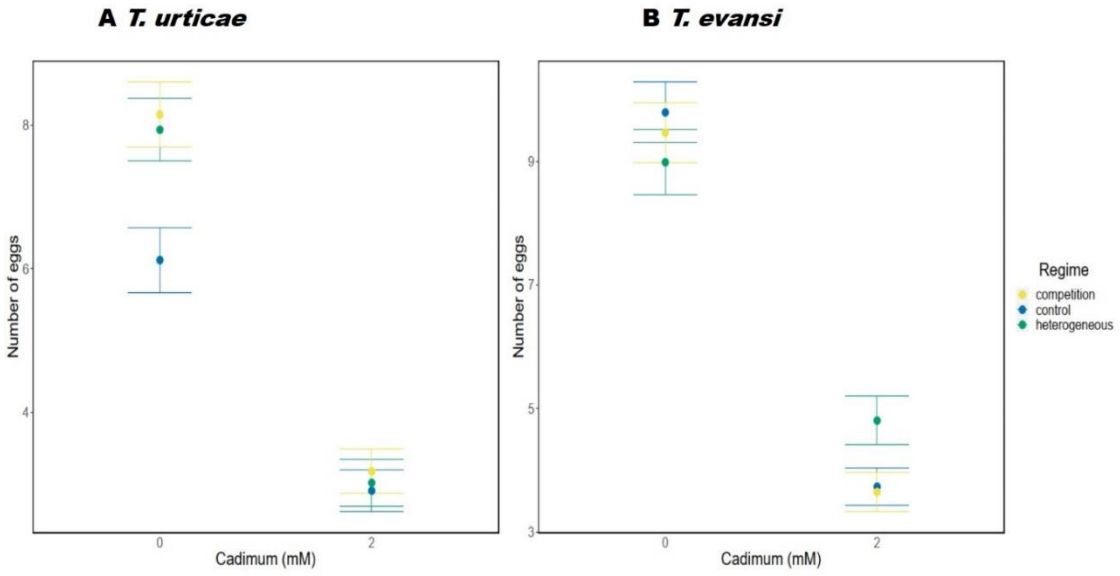

Figure 2. *Oviposition rate of spider mites from different selection regimes: control (plants not exposed to cadmium, <u>no</u> heterospecific competitors), heterogeneous (environment composed of plants with and without cadmium within each generation; <u>no</u> heterospecific competitors) and competition (environment composed of plants with and without cadmium within each generation ; <u>presence</u> of heterospecific competitors) on plants exposed to 2 mM of cadmium or not. Females (20 per replicate, 5 replicates per selection regime) were placed individually on leaf discs and allowed to feed and oviposit for 48h.*

2.4 Differential responses to interspecific competition

The differential responses of *T. urticae* and *T. evansi* to interspecific competition (Chapter III) raise some interesting questions. One open question that rises from our work is if metal accumulation affects the competition interactions between these two species. To shed some light on this issue, one could measure the strength of competition in environments with and without cadmium. This could be pursued by performing typical tests of competition, in which individuals of one species are exposed to increasing numbers of conspecifics or of heterospecifics (Hart *et al.* 2018), and this could be done in plants with or without cadmium. Another open issue is if the alternatives avoiding competitors vs. monopolizing resources are related with the strength of competition between the two species. For example, one could test if these behaviours are recaptured in populations of *T. urticae* that are differently affected by competition by *T. evansi*. Alternatively, one could test for the presence of such behaviours in other host plants where *T. urticae* has similar or higher fitness than *T. evansi*. Our results on bean (Chapter IV) suggest that *T. urticae* avoids *T. evansi* on this host plant, even though it has a higher fitness than *T. evansi*. However, because we focused on the traits of *T. urticae* only (our design was not orthogonal), and because of differential rearing conditions prior to the experiments, we cannot compare the behaviour of the two species. Thus, more studies on this topic are required to enlighten this issue. Furthermore, it would be interesting to test if these species follow the colonization-competition trade-off by comparing the competitive ability and dispersal traits of these two species. Additionally, one could test if this trade-off is due to a negative genetic correlation between traits that increase competitive ability and traits that increase dispersal That could be pursued by using isogenic lines *T. urticae* against an outbred population of *T. evansi* and vice-versa, and correlating the competitive ability of those lines with dispersal traits.

2.5 Virulence transmission trade-off

As referred before (c.f. section 1.5), we did not find a genetic correlation between virulence and transmission in *T. urticae*. Still, the relation between these traits may follow the predictions of the trade-off hypothesis via within-host dynamics. This can be tested with a similar protocol as that used in chapter IV but measuring transmission from the patches where virulence was inflicted. Alternatively, one could infest plants with different lines and follow the number of secondary infections, i.e. new hosts infected, until the death of the original host. Then, the

number of secondary infections could be compared among lines with different levels of virulence and the optimal virulence for transmission could be determined for this system.

3. Conclusion

The results obtained in this work contribute to the knowledge on how the heterogeneity in the environment, mediated by plants, affects responses by different herbivore species that may influence the competitive interactions among them. Our work has provided answers to some issues in the topics of plant-herbivore and herbivore-herbivore interactions but also raised new questions that pave the way to further research. Hopefully, this will contribute to refining the framework of plant-herbivore interactions and herbivore-herbivore interactions in heterogeneous environments. Spider mites are important crop pests living in a world intensively modified by human action, not only in terms of agricultural practices but also in terms of pollution and environmental contamination. Therefore, the work I present in this thesis may provide useful applicable knowledge in the context of agroecological systems.

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