FELIPE DE LEMOS

INDIRECT INTERACTIONS IN TOMATO ATTACKED BY *Tetranychus evansi*

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Entomologia, para obtenção do título de *Doctor Scientiae*.

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APROVADA: 30 de abril de 2015.

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Biografia

Felipe de Lemos, filho de Fátima Maria de Lemos e Adilson Pereira Nunes é natural de Itaguaí, Rio de Janeiro e nasceu em 4 de fevereiro de 1985. Em março de 2003 ingressou no curso de Agronomia na Universidade Federal de Viçosa (UFV). Em abril de 2004 iniciou suas atividades no Laboratório de Acarologia como estagiário voluntário sob orientação do Prof. Angelo Pallini e supervisão de Hamilton Gomes Oliveira e Marco Antônio Matiello Fadini. No período de julho de 2004 e maio de 2005 foi bolsista de iniciação tecnológica industrial sobre co-orientação do Dra. Madelaine Venzon na Empresa de Pesquisa Agropecuária de Minas Gerais (EPAMIG/CTZM). No período de junho 2005 a junho de 2006 foi estagiário de projeto de pesquisa da Universidade de Amsterdam sob orientação dos Prof. Arne Janssen e Angelo Pallini. Entre agosto de 2008 e julho de 2009 foi bolsista de Iniciação Científica do Laboratório de Acarologia sob orientação do Prof. Angelo Pallini. Em janeiro de 2009 graduou-se em Agronomia pela UFV e em março do mesmo ano ingressou no mestrado em entomologia pela UFV sob orientação do Prof. Angelo Pallini. Em fevereiro de 2011 terminou o mestrado defendendo a dissertação intitulada "Spider mite web protects prey and predator alike" e em março do mesmo ano ingressou no doutorado em entomologia sob orientação do Prof. Angelo Pallini.

Conteúdo

RESUMO

LEMOS, Felipe de, D.Sc., Universidade Federal de Viçosa, abril de 2015. **Interações indiretas em tomateiros atacados por *Tetranychus evansi***. Orientador: Angelo Pallini Filho. Coorientadores: Madelaine Venzon, Arnoldus Rudolf Maria Janssen e Eraldo Rodrigues de Lima.

Plantas apresentam inúmeras estratégias de defesa direta e indireta contra herbívoros. As defesas diretas atuam sobre os herbívoros enquanto as defesas indiretas beneficiam os inimigos naturais dos herbívoros. Para maximizar o seu fitness, plantas sob ataque de herbívoros fazem uso de ambas estratégias de defesa simultaneamente. No entanto, alguns herbívoros têm se adaptado para lidar com as defesas de plantas e o ácaro vermelho *Tetranychus evansi* é um exemplo. Esse herbívoro é capaz de manipular a defesa direta de plantas de tomate em seu próprio benefício. Nesta tese, foram investigados aspectos das interações indiretas entre plantas de tomate atacadas por *T. evansi* e os inimigos naturais de *T. evansi*, embora algumas interações diretas entre plantas e herbívoros e herbívoros e predadores também foram estudadas. No primeiro capítulo, foi investigado a influência da planta hospedeira na inadequação de *T. evansi* como alimento para o ácaros predador *Phytoseiulus persimilis*. Observou-se que a inadequação de *T. evansi* como alimento para esse predador não está relacionada com a planta hospedeira do herbívoro. No entanto, o efeito negativo da dieta de *T. evansi* no desempenho do ácaro predador foi reversível, indicando a ausência de um efeito tóxico ao longo prazo. No segundo capítulo, estudou-se como *T. evansi* poderia interferir com a defesa indirera de tomateiros pela indução de voláteis e atração de ácaros predadores. Foi observado que *T. evansi* induz a produção de compostos voláteis que são diferentes dos presentes na mistura produzida por plantas atacadas por *Tetranychus urticae*. Entretanto, a atratividade dos ácaros predadores (*P. persimilis*, *Phytoseiulus longipes* and *Phytoseiulus macropilis*) por odores de tomateiros atacados por *T. evansi* foi variável com a densidade de infestação de herbívoros. No terceiro capítulo desta tese, explorou-se a capacidade do ácaro predador *P. macropilis* em aprender a associar odores de plantas atacadas por *T. evansi* com a qualidade da presa. Juvenis de *P. macropilis* não se desenvolveram até a fase adulta, quando alimentados com ovos de *T. evansi*. No entanto, adultos de *P. macropilis* não evitaram voláteis de plantas atacadas por *T. evansi* mesmo após quatro dias consecutivos de experiência com essa presa de baixa qualidade. Em conclusão, estes resultados confirmam a notável capacidade de *T. evansi* em manipular a defesa de sua planta hospedeira e contornar a ameaça de inimigos naturais. A interação indireta entre ácaros predadores e plantas de

tomateiro infestados com *T. evansi* é prejudicada pela indução diferencial de voláteis que enganam os ácaros predadores.

ABSTRACT

LEMOS, Felipe de, D.Sc., Universidade Federal de Viçosa, April, 2015. **Indirect interactions in tomato attacked by *Tetranychus evansi***. Adviser: Angelo Pallini Filho. Co-advisers: Madelaine Venzon, Arnoldus Rudolf Maria Janssen and Eraldo Rodrigues de Lima.

Plants employs an array of direct and indirect strategies of defence against herbivores. Direct defence acts upon the herbivores, while indirect defence benefits the natural enemies of the herbivores. To maximize their fitness, plants under attack of herbivores are predicted to simultaneously make use of both direct and indirect defence. However, some herbivores have adapted to cope with plant defences. The red spider mite *Tetranychus evansi* was found to manipulate the direct defence of tomato plants to their own benefit. In this thesis, I focus on investigating indirect interactions between tomato plants attacked by *T. evansi* and their natural enemies, although some direct interactions between plants and herbivores and herbivores and predators were also studied. First, I studied the influence of host plant on the unsuitability of *T. evansi* as food for the predatory mite *Phytoseiulus persimilis*. I observed that this unsuitability was not related with the herbivore's host plant. The negative effect of *T. evansi* on the performance of predatory mites was reversible, indicating the absence of long-term toxic effects of prey on the predator. In the second chapter, I studied how *T. evansi* interferes with the indirect defence of tomato plants through induction of volatiles and attraction of predatory mites. I observed that damage by *T. evansi* induces the production of volatile organic compounds that are different from those present in the attractive blend of volatiles induced by *Tetranychus urticae*. The attractiveness of odours from tomato plants infested with *T. evansi* to predatory mites (*P. persimilis*, *Phytoseiulus longipes* and *Phytoseiulus macropilis*) varied with the density of mites on the plant. In the third chapter of this thesis, I explored the capacity of the predatory mite *P. macropilis* to learn to associate odours from plants infested with *T. evansi* with prey quality. Juveniles of *P. macropilis* were show to perform poorly when fed with eggs of *T. evansi*. However, adults of *P. macropilis* did not avoid odours from plants infested with *T. evansi*, even after four consecutive days of experience with the poor quality prey. In conclusion, these results confirm the remarkable ability of *T. evansi* to manipulate the plant defence and circumvent the threat of natural enemies. The indirect interaction between predatory mites and tomato plants infested with *T. evansi* is impaired by the differential induction of volatiles that mislead the predatory mites.

GENERAL INTRODUCTION

When studying populations of organism, ecologists are interested in understanding interactions that occurs in a specific ecosystem and how these interactions are important in structuring communities of populations. Species may interact through predation, resource competition, parasitism, mutualism etc. (Begon et al. 2006). These interactions may be direct, between two species (for instance, the herbivory of a caterpillar upon a plant or the parasitism of a caterpillar by a parasitoid). However, in the same habitat some other indirect interactions may appear (for instance, the attacked plant releases volatile compounds which act as cues for parasitoids to find their prey or the caterpillars sequestering a toxic compound from their host plants to use against parasitoids). The examples above illustrate direct interactions (parasitism and herbivory) and indirect interactions in a simple tritrophic food web. In nature (and in an artificial agroecosystem too), the food web are often more complex, with a myriad of interactions.

Direct interactions such as predation, parasitism and competition have historically received large attention from ecologists, especially those studying artificial agroecosystems aiming to develop strategies for biological control of pest (Hajek 2004). However, the understanding of indirect interactions among species is essential for the study of complex multi-trophic food webs (Janssen et al. 1998). The discussion about indirect interactions between plants and natural enemies of herbivores raised more attention after the publication of Hairston, Smith and Slobodkin (1960). The authors argued that plants are rarely depleted by herbivores, which indicates that plants are more than passive organisms, rooted to the soil and submissive to be attacked by herbivores (Hairston et al. 1960). With the findings of the induction of biochemical compounds of secondary metabolism in plants after herbivory, more attention was paid to the active role of plant defence (Green and Ryan 1972).

Plants have developed sophisticated mechanisms of defence against herbivores. Some mechanisms employed by plants act directly against the herbivores and are called direct defences (Rhoades and Cates 1976; Price et al. 1980). Direct defences may act on herbivores by reducing their access to plant tissue, as is observed on plants with many thorns or trichomes (Levin 1973). Another strategy of direct defence employed by plants interferes with the digestive performance of herbivores. Some plant secondary metabolites such as proteinase inhibitors and alcaloids reduce herbivore feeding on plants (Ryan 1973).

Plants also posses other mechanisms of defence, in which the employed strategy benefits the natural enemies of the herbivores. The natural enemies in turn protect the plants reducing the population of herbivore (Sabelis et al. 2001). This so called indirect defence occurs when plants provide shelter (Walter 1996; Agrawal and Karban 1997), food (Bentley 1977; Tilman 1978) and cues (Dicke and Sabelis 1988; Dicke et al. 1990; Turlings et al. 1990) to natural enemies of the herbivores. For instance, the domatia present on leaves help predators to survive longer on plants without prey (Walter and O'Dowd 1992; Walter 1996; Matos et al. 2006). Some plants also provide alternative food (or facilitate access to it) to natural enemies, keeping them around even in the absence of prey (Duarte et al. 2015). Additionally, plants attacked by herbivores release attractive blends of volatiles that are used by natural enemies to locate plants with prey (Dicke and Sabelis 1988; Turlings et al. 1990; De Moraes et al. 1998).

Many of these defensive traits are constitutively present in plants (Gatehouse 2002). However, some of them are induced after herbivory which lead to an increase in their concentration in plant tissue (Agrawal and Karban 1997; Walling 2000; Traw and Dawson 2002). The induction of defences is mainly regulated by two plant hormones: jasmonic acid and salicylic acid, but other hormones as ethylene and systemin are important too (Walling 2000). In general, a signalling cascade is activated by the increase in concentration of these

hormones due to herbivory (Kant et al. 2004). Plants can notice the attack of herbivores using cues from insect saliva (Alborn et al. 1997), mechanical damage (Mithöfer et al. 2005), volatiles released from attacked parts of plants (Arimura et al. 2000) and even cues from insect oviposition (Hilker and Meiners 2006). These cues are responsible for triggering the physiological cascade that leads to the final products of induced defence (Hilker and Meiners 2006). This general model of induction of defence is observed in almost all plants and the metabolic pathways are shared between direct and indirect defence (Alba et al. 2012).

Besides its importance in reducing the performance of herbivores, plant direct defence may also mediate direct and indirect interactions between plants and natural enemies of the herbivores (Kennedy 2003). These indirect interactions are expected to result in benefits to natural enemies, however some herbivores may be so well adapted to plant defence and use it against their predators and parasitoids (Chaplin-Kramer et al. 2011). The majority of chemical defences employed by plants do not really kill the herbivores. Most of the products of direct defence against herbivores are chemical compounds known to affect the performance of their attackers. For instance, the proteinase inhibitors induced after herbivory affect digestibility of plant material by herbivores (Broadway and Duffey 1986). This results in a lower performance of the herbivores (for instance, a longer development time of immature stages) on induced plants than as uninduced plants (Orozco-Cardenas et al. 1993). This slower developmental time, especially observed with immature stages, may turn these herbivores more susceptible to be attacked by natural enemies: the direct defence of plants against herbivores through the production of deterrent compounds may also positively affect the natural enemies of the herbivores, making their prey more susceptible to attack.

However, herbivores may also adapt to cope with these compounds, which mostly have antifeedant proprieties. In more derivate adaptation, herbivores may sequester plant

toxins and use them against their natural enemies (Chaplin-Kramer et al. 2011). The first example is the work of Campbell & Duffey (1979) with larvae of *Heliothis zea* (Lepidoptera: Noctuidae). They observed that the parasitoid wasp *Hyposoter exiguae* was hampered by the alpha-tomatine acquired from tomato plants by the larvae, which are less sensitive to this compound (Campbell and Duffey 1979). Many other examples were observed with aphids, caterpillar and mites that suffer lower predation due to utilization of chemical from host plants to their own benefit (Poelman et al. 2008; Hopkins et al. 2009; Chaplin-Kramer et al. 2011; Kos et al. 2012). These negative indirect interactions between plants and natural enemies of these herbivore are observed to affect the composition of herbivores communities in nature (Poelman et al. 2009; Newton et al. 2009).

Changes in blend of volatile organic compounds from plants may affects direct and indirect interactions between plants and herbivores and natural enemies respectively. For instance, Shiojiri et al. (2002) observed that cabbage plants attacked by multiple herbivores at the same time release a different blend of volatiles than plants attacked by either of the two herbivore species alone. This new blend is less attractive to the parasitoid of one of the herbivores. This explains the fact that this herbivore prefers to oviposit on cabbage plants under attack by their competitor, thus reducing the risk of parasitism of their offspring (Shiojiri et al. 2002).

The above examples of negative indirect interactions between plants and natural enemies mediated by plant defence involve herbivores that have adapted to overcome plant defences. In this thesis, I have investigated the effect of *Tetranychus evansi*, a herbivore that manipulate plants defence, on indirect interactions between tomato plants and predatory mites (Sarmento et al. 2011a; Sarmento et al. 2011b). The red spider mite *T. evansi* is a herbivore considered to be native from South America, but during the last two decades its geographical distribution has expanded overseas. First *T. evansi* has become an agricultural

problem in Africa from where it has spread to Spain and Portugal. Nowadays, it is present in 41 countries in six geographical areas (Boubou et al. 2012; Migeon and Dorkeld 2015). As *T. evansi* has high invasion capacity, it is considered a threat to agriculture. Much research was done to select good of biological control agents for this pest, however, the natural enemies tested often failed to control *T. evansi* (Navajas et al. 2013). As *T. evansi* constructs a dense and profuse web over their host plants, the predators were considered to have problems in dealing with the web of *T. evansi* (Lemos et al. 2010). Recently, a strain of the predatory mite *Phytoseiulus longipes* was found to be a good candidate to control this pest. Some first results from laboratory and screenhouse experiments indicate its high potential as an agent of control of *T. evansi* (Furtado et al. 2007; Silva et al. 2010).

I especially focused on indirect interactions mediated by volatile compounds and chemical characteristics of host plants. In the first chapter, I studied the role of host plant quality on the suitability of spider mites (*T. evansi* and *T. urticae*) for the predatory mite *P. persimilis*. I also explored the effect of diet composed of *T. evansi* eggs on behaviour and performance of *P. persimilis*. I observed that host plant species does not completely explain the poor performance of *P. persimilis* on *T. evansi*, as was hypothesized by other authors. I also showed that *P. persimilis* does not get intoxicated after feeding on *T. evansi*. However, this predatory mite was not able to completely avoid preying on the poor quality prey. These results suggest that plants co-infested by both spider mite species (*T. evansi* and *T. urticae*) could experience a reduced indirect defence by the predators of this herbivores, compared with a plant attacked only by *T. urticae*.

In the second chapter, I explored the influence of the herbivores *T. evansi* and *T. urticae* on induction of the production of volatile organic compounds in plants and their attractiveness for the predatory mites *P. macropilis* and *P. longipes*. I tested the attraction of predatory mites to volatiles from tomato plants infested with low and high densities of

herbivorous mites. Furthermore, I collected the volatiles from those plants and identified the organic compounds from the blend of volatiles.

In the last chapter, I investigated the capacity of the predatory mite *P. macropilis* to learn the association of plant odours with prey of different quality. I submitted predatory mites to consecutive feeding experiences on the poor quality prey *T. evansi* associated with odours from plants infested with this herbivore. Subsequently, I tested the preference of *P. macropilis* for odours from plants infested with *T. evansi* versus odours from plants infested with *T. urticae*. I observed that the predatory mites did not increase their avoidance of volatiles from plants infested with the poor quality prey *T. evansi* after four consecutive days of training.

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CHAPTER I - Unsuitability of *Tetranychus evansi* as prey for the predatory mite *Phytoseiulus persimilis*

Title: Unsuitability of *Tetranychus evansi* as prey for the predatory mite *Phytoseiulus persimilis*

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Abstract
Chemical compounds from the secondary metabolism of plants can act as defence against herbivores. However, these toxic compounds may also mediate indirect interactions between plants and the natural enemies of their herbivores. Here, we investigated the role of the host plant on the quality of the red spider mite *Tetranychus evansi* Baker and Pritchard as prey for the predatory mite *Phytoseiulus persimilis*. First, we tested the developmental rate of *P. persimilis* juveniles fed on eggs of *T. evansi* and *T. urticae* reared on three different host plants (tomato, common bean and cucumber). We found that the host plant affected the quality of *T. urticae* as prey for *P. persimilis,* but not of *T. evansi*. Adults of *P. persimilis* fed on a single diet of *T. evansi* eggs stopped laying eggs after two days. When fed on a mixed diet of *T. evansi* and *T. urticae* eggs*, P. persimilis* showed a preference for eggs of *T. urticae* but had a reduced oviposition rate compared to predators on a diet with eggs of *T. urticae* only. We subsequently studied the reversibility of these negative effects of feeding on *T. evansi* eggs in juveniles and adults of *P. persimilis*. Juveniles of *P. persimilis* restored their development when changing from a diet of eggs of *T. evansi* to a diet of eggs of *T. urticae*. Such a diet change also restored the performance of adult predatory mites. These results suggest that the unsuitability of *T. evansi* as prey for predatory mites is not exclusively determined by the host plant of *T. evansi* and is also not a long-term toxic effect. Possibly, the eggs of *T. evansi* lack some essential nutrient required for development and reproduction of *P. persimilis*.

Key words: *Phytoseiulus persimilis*, mixed diet, prey quality, host plant, toxicity.

Introduction

Populations of herbivores are regulated simultaneously by both top-down and bottom-up forces (Price et al. 1980; Hunter and Price 1992). Top-down forces are imposed by organisms from the third trophic level, such as predators or parasites of the herbivores (Murdoch et al. 1985; Polis and Strong 1996). These natural enemies use volatile chemical compounds released in the atmosphere by the attacked plants as cues to find their prey (Dicke and Sabelis 1988; De Moraes et al. 1998; Karban 2011). Bottom-up forces result from the action of plant chemical metabolites and/or physical structures, which negatively affect the performance of the herbivores (Karban and Baldwin 1997; Agrawal 1998). Either bottom-up or top-down forces reduces herbivory. However, there are several observations indicating a synergistic effect of these forces; for instance the induction of direct defence after herbivory (production of proteinase inhibitors) followed by the release of volatile compounds which are attractive to the natural enemies of the herbivore (De Moraes et al. 1998; Kant et al. 2004; Howe and Jander 2008).

Some herbivores may overcome defensive chemical metabolites from their host plant and use it against their own natural enemies (Ode 2006), which may reduce the beneficial synergism of bottom-up and top-down forces on the regulation populations of herbivores (Kos et al. 2012). For instance, populations of herbivores feeding from more toxic plants species suffer reduced predation risk due to a lower suitability to predators (Agrawal et al. 2002; Chaplin-Kramer et al. 2011). This was first observed by Eisner et al. (1974) with larvae of the sawfly *Neodiprion sertifer* that sequester and store a terpenoid resin from their host plant. When disturbed, the larvae discharge droplets of resin, which deters their predators (Eisner et al. 1974). A similar phenomenon was observed with the parasitoid wasp *Hyposoter exiguae*, which is

hampered by the alpha-tomatine acquired from tomato plants by its host *Heliothis zea*, which is less sensitive to this compound (Campbell and Duffey 1979).

The red spider mite *Tetranychus evansi* is known by its ability to down-regulate the plant defences (Sarmento et al. 2011a; Alba et al. 2015), which leads to higher performance on attacked plants. Additionally, this herbivore, which is most often observed on tomato plants (Bonato 1999; Navajas et al. 2013; Migeon and Dorkeld 2015), lacks efficient natural enemies, especially when compared with other Tetranychid mite species (Navajas et al. 2013). Many studies tested the ability of natural enemies to control *T. evansi*, but this spider mite proved to be unsuitable as prey for the natural enemies tested (de Moraes and McMurtry 1985; Sarmento et al. 2004; Oliveira et al. 2005; Escudero and Ferragut 2005; Koller et al. 2007). Among the tested predators was the predatory mite *Phytoseiulus persimilis* which is a common natural enemy of Tetranychidae spider mites (Helle and Sabelis 1985) and is widely used as biological control agent of many Tetranychid mites (Gerson et al. 2003). However, many tests showed that populations of *P. persimilis* failed to establish when feeding on *T. evansi* (de Moraes and McMurtry 1985; Escudero and Ferragut 2005).

In most of the studies cited above *T. evansi* was offered to predators on tomato leaves or in the few studies where *T. evansi* were offered to predators on a different host plant, they were fed with tomato plants during at least one stage of their life cycle (de Moraes and McMurtry 1985; Sarmento et al. 2004; Oliveira et al. 2005; Escudero and Ferragut 2005; Koller et al. 2007). Considering the correlation between the tomato host plant and unsuitability of *T. evansi* to predators, we argued if the lower performance of the predators tested for control of *T. evansi* is related with the species of the herbivore host plant. We investigated the influence of host plant species (tomato, bean and cucumber) on the suitability of *T. evansi* eggs as food for predators.

Developmental and survival rates of juveniles and prey preference, predation and oviposition rates of females of *P. persimilis* preying upon *T. evansi* and *Tetranychus urticae* were assessed.

Material and Methods

Organisms

Tomato seeds (*Solanum lycopersicum* var. Santa Clara I-5300) were sowed in a PVC tray (6 x 12 cells) once per week and plants were transplanted to plastic pots (2L) with soil substrate 14 days after germination. Common bean (*Phaseolus vulgaris* L. cv Speedy) were sowed and grown in plastic trays (50 X 40 cm) filled with soil substrate. Bean and tomato plants were kept in a glasshouse compartment (16:8 h, 25:18 °C, day:night, 50–60% relative humidity). Cucumber was sowed directly in plastic pots filled with soil substrate and were grown in a climate room (25 ºC, 70 – 80 % relative humidity) with controlled photoperiod (16:8 L:D). Tomato and cucumber plants were tied to wooden stakes inserted into the soil for support. We used detached leaves from plants to feed the colonies of spider mites and to prepare the leaf discs used in the experiments.

The populations of spider mites (*T. evansi* and *T. urticae*) were started from specimens supplied by the laboratory of acarology from the Federal University of Viçosa, Minas Gerais, Brazil. These strains of spider mites were collected in 2002 from infested tomato plants in Brazil (Sarmento et al. 2011a; Sarmento et al. 2011b). Spider mites were reared on detached tomato leaves kept in plastic trays (30 cm x 22 cm x 8 cm, LxWxH) containing water to maintain leaf turgor. These trays were placed inside larger trays (54 cm x 38 cm x 8.5 cm, LxWxH) filled with detergent and water (approximately 1:50, v/v) that prevent mite contamination among the populations. The colonies were maintained in a climate room (25 ºC, 70 – 80 % relative humidity) with controlled photoperiod (16:8 L:D). The spider mites were used to perform experiments and feed the colonies of predatory mites.

The strain of *P. persimilis* used here was obtained from Koppert and was kept on detached cucumber leaves infested with *T. urticae*. For our experiments, a colony of

P. persimilis was started from this stock colony on tomato leaves infested with the Brazilian strain of *T. urticae* one month before being tested. The colony of *P. persimilis* was kept in a plastic tray (30 cm x 22 cm x 8 cm, LxWxH) accommodated in larger PVC trays (54 cm x 38 cm x 8.5 cm LxWxH) filled with detergent and water. Two to three tomato leaves infested with spider mites were added to trays every 3-4 days. The colonies of predatory mites were maintained in a climate room (25 ºC, 70 – 80 % relative humidity, photoperiod of 16:8 hours L:D).

The predators (both eggs and adults) used in our experiments came from cohorts, obtained by taking approximately 30 adult females of *P. persimilis* from the stock colony and transferring them to tomato leaflets infested with *T. urticae* arranged on wet cotton wool on plastic trays. The females were allowed to feed and oviposit for 24 hours. Subsequently, the females were removed from the leaflets, which were incubated in a climate room (25 ºC, 70 – 80 % relative humidity, photoperiod of 16:8 hours L:D). After 7 to 9 days the cohort of predatory mites was adult and the females were mated.

Effect of the herbivor's host on predator development

To test if the quality of *T. evansi* and *T. urticae* as food for *P. persimilis* was related to the herbivore host plant, we studied the developmental time, survival and predation rate of the juvenile predatory mites feeding on these two spider mites species offered on three different host plants: tomato, bean and cucumber. The populations of *T. evansi* and *T. urticae* on bean and cucumber plants were established using infested leaflets from the stock colonies maintained on tomato leaves. The infested tomato leaflets were transferred to rearing trays (as described above) with leaves of either bean or cucumber. The colonies of spider mites on bean and cucumber leaves were kept as described above for the colonies on tomato. As new colonies were established one

month prior to the first experiment, the mites were reared on bean and cucumber for at least two generations.

Leaf discs (diameter of 24 mm) were cut from leaves of clean plants and arranged on wet paper tissue positioned on wet foam inside a tray (12.5 x 7.5 x 2.5 cm) filled with tap water. The leaf discs were infested with 20 adult females of *T. evansi* or *T. urticae* taken from stock colonies. The trays with the leaf discs were incubated in a climate room (same conditions as mentioned above) for 24 hours. Then, we used a painting brush (made of camel hair) to remove the females and the web from discs while the eggs of spider mites were kept. Subsequently, one egg from a cohort of *P. persimilis* was transferred to each leaf disc. The treatments with cucumber plants (*T. evansi*, *T. urticae* and no food) and *T. urticae* on bean plants were replicated 12 times. The treatments of *T. evansi* on bean and tomato plants were replicated 24 times while *T. urticae* on tomato and no food on bean and tomato 18 times.

As eggs of the spider mites take around four days to hatch at 25 ºC (Bonato 1999) and our objective was to evaluate the performance of juvenile *P. persimilis* when feeding on eggs of spider mites, the leaf discs were replaced every three or four days. The new leaf discs were prepared as described above and the predatory mites were transferred from the old discs to new ones of the assigned treatment.

We observed the development, survival and predation rate of the juveniles of *P. persimilis* from egg until the adult stage once a day. Evaluation of the development was based on observing the presence of exuviae on the discs. For both developmental time and survival, we used a time-to-event analysis (Cox proportional hazards regression model) using the function "coxph" of the library "survival" in R© (Therneau 2012; R Development Core Team 2015). The number of spider mite eggs preyed by juveniles of *P. persimilis* were compared among treatments with a linear mixed effects

model with treatments as fixed factor and disc identity as random factor to correct for repeated measures. Contrast were assessed by stepwise mode simplification (Crawley 2013). All statistical analyses were done using the computer software R© version 3.1.3 (R Development Core Team 2015).

Predation and oviposition on mixed and single diet

We tested the performance and preference of adult females of *P. persimilis* fed with a mixture of eggs of *T. evansi* and *T. urticae*. The experiment was performed only with tomato leaf discs cut from detached leaves (24 mm diameter). The discs were cut from the leaves in such a way that the main leaf vein divided the discs in two halves with approximately similar areas. The leaf discs were regularly arranged on wet filter paper, positioned on wet foam inside a tray (12.5 x 7.5 x 2.5 cm). Each disc half was infested with 20 adult females of either *T. evansi* or *T. urticae* from the stock colonies. To prevent that spider mites crossed from one half to the other half, we put a thin thread of wet cotton wool along de midrib in contact with the surrounding water to serve as a physical barrier. The trays with the leaf discs were incubated in a climate room (25 ºC, 70 – 80 % relative humidity, photoperiod of 16:8 hours L:D). After 24 hours of feeding, ovipositing and producing web, we used a brush to remove spider mites and web from discs while the eggs were kept and counted. Thereafter, we removed the cotton thread from the leaf midrib and inserted an entomological pin at the centre of the disc, drilling the midrib. We then took an adult female of *P. persimilis* from a cohort and carefully put it on the tip of the entomological pin. We observed that immediately after being transferred the predators moved down and started to forage on the leaf disc.

As controls, two other treatments were prepared in such a way that each prey species was offered alone. These treatments were prepared as described above, except

that we transferred 20 adult females of the same prey species (40 adult females per disc in total) to both of the two halves of the leaf discs. The predation and oviposition rates of *P. persimilis* were observed during four consecutive days. We used linear mixed-effects model (function "lme" of the library "nlme") to investigate the differences in the number of eggs preyed and produced on the two disc halves where *T. evansi* and *T. urticae* were offered simultaneously (prey preference by *P. persimilis*) and the leaf discs with the mixture of both prey or each prey. Data of predation and oviposition were log-transformed prior to analyses. The statistical computing was performed in R© (R Development Core Team 2015).

Long-term effects of previous diet

The previous experiments showed a negative effect of consuming *T. evansi* eggs on the performance of *P. persimilis*. We therefore investigated if the negative effects of *T. evansi* could be the product of a long-term toxic effect. All predatory mites were offered leaf discs with eggs of *T. urticae* for the first two days. This served to check whether the predatory mites could oviposit. Subsequently, we transferred the predatory mites to new leaf discs with one of three treatments: (1) leaf discs with eggs of *T. evansi*, (2) leaf discs with eggs of *T. urticae* and (3) leaf discs without food. The females were left on these leaf discs for two consecutive days. After this period, all female predators were transferred to new leaf discs with eggs of *T. urticae*. The predation and oviposition rate of *P. persimilis* were observed and counted daily during six days of the experiment. The numbers of eggs laid and preyed by *P. persimilis* was compared among treatments with linear mixed-effects models in R© (R Development Core Team 2015).

The leaf discs used here were cut from tomato leaves and arranged on plastic trays as described above. To prepare the discs with prey, 20 adult female spider mites

(*T. urticae* and *T. evansi*) from the stock colonies were used to infest leaf discs separately. The plastic trays with the infested leaf discs were incubated in a climate room for 24 hours, allowing the spider mites to feed, lay eggs and produce web. Subsequently, the spider mites and web were removed using a brush and spider mite eggs were counted. Leaf discs contained either eggs of *T. urticae*, eggs of *T. evansi* or no food, and each treatment was replicated 24 times.

In an experiment with juveniles, we prepared leaf discs and infested them with adult females of either *T. evansi* or *T. urticae* or the leaf discs were kept clean (treatment without food). Subsequently,, we incubated the discs in the climate room for 24 hours. After this period, we removed all the spider mites and silk from the leaf discs. Subsequently, we transferred a larva (one day old) of *P. persimilis* to each leaf disc. The discs were incubated in a climate room as above. We evaluated the survival and development of the juveniles of *P. persimilis* during two consecutive days. In the treatments with prey, we also counted the number of prey eggs killed. After these two days, we transferred the juveniles to new leaf discs with eggs of *T. urticae*. These leaf discs were prepared as above, but infested with *T. urticae*. Subsequently, the development and survival of the juveniles of *P. persimilis* were evaluated daily until they reached adulthood or died. The data on both development time and survival were analysed with a time-to-event analysis (Cox proportional hazards) using the function "coxph" of the library "survival" in R© (R Development Core Team 2015).

Results

Effect of herbivores host on predator development

All the juveniles of *P. persimilis* fed with eggs of *T. evansi* as well the juveniles kept without food failed to become adult (Figure 1). In contrast, most juveniles developed into adults when feeding on eggs of *T. urticae* (Figure 1). There was a

significant difference in developmental rate of juveniles depending on the host plant of *T. urticae* (Cox Proportional Hazards, logrank test = 15.1, d.f. = 2, P < 0.001). Juveniles of *P. persimilis* feeding on eggs of *T. urticae* on cucumber plants developed faster than predators feeding on prey eggs from bean and tomato plants.

We also observed a significant difference in juvenile survival among diets tested (Cox Proportional Hazards, logrank test = 36.2, d.f. = 8, P < 0.001). As expected, juveniles of *P. persimilis* kept without food showed the lowest survival rate. However, the survival rate of the juvenile predators fed with eggs of *T. evansi* and *T. urticae* did not differ significantly. In conclusion, juveniles of *P. persimilis* fed with eggs of *T. evansi* do not develop to adults but they live longer than the immature predators kept without food.

We observed a significantly higher predation rate on eggs of *T. evansi* than on eggs of *T. urticae* (linear mixed-effects model, F = 3.19, d.f. = 5, P = 0.01, Figure 2). Especially on the fourth day (when most of the predators feeding on *T. evansi* were protonymphs) the predation rate of eggs of *T. evansi* was four times higher than eggs of *T. urticae* (Figure 2).

Predation and oviposition on mixed and single diet

The oviposition and predation rates of adult female *P. persimilis* were highest on a diet of only *T. urticae*, intermediate on a mixed diet and lowest on a diet of *T. evansi* (Figure 3). On the mixed diet, predatory mites fed and oviposited preferentially on *T. urticae* compared with *T. evansi* (Linear mixed-effects model, L-ratio = 95.3, P < 0.0001 and L-ratio = 54.2, P < 0.0001 respectively, Figure 3). In average, *P. persimilis* preyed 6.7 times more eggs of *T. urticae* than *T. evansi* and laid 3.3 times more eggs on disc halves with eggs of *T. urticae*.

Long term effects of previous diet

The oviposition of *P. persimilis* did not differ significantly among the three treatments during the first and second day of the experiment (Linear mixed-effects model, L-ratio = 1.1, P = 0.58), besides the slight difference of predation rate among the treatments (GLM, F = 4.03, d.f. = 2, P = 0.02). Hence, predatory mites from the three treatments (assigned as *T. evansi*, *T. urticae* and No Food) had the same performance when fed exclusively with *T. urticae*. After being fed with eggs of *T. evansi* or kept without food, *P. persimilis* showed a significantly lower oviposition rate than predators feeding on eggs of *T. urticae* (GLM, Deviance = 13.81, d.f. = 2, P < 0.01 and Deviance = 21.82, d.f. = 2, P < 0.01 for 3rd and 4th days respectively, Figure 4). However, after receiving eggs of *T. urticae* as food (days 5th and 6th) *P. persimilis* showed an increase on both oviposition and predation rates, reaching values similar to those predators fed only with *T. urticae* along all the experiment (Figure 4).

Regarding the development of juvenile predators, we observed a significant difference in the developmental rate among diets (X^2 = 29.3, d.f. = 2, P < 0.001). Juvenile *P. persimilis* fed exclusively with eggs of *T. urticae* developed faster than juveniles that were starved or fed with eggs of *T. evansi* before receive eggs of *T. urticae* as prey. This suggests that feeding on *T. evansi* does not completely block the development to adulthood of *P. persimilis*.

Discussion

We found no evidence that the unsuitability of *T. evansi* as prey for the predatory mite *P. persimilis* depends on the host plant species. In addition, the restoration of predator performance indicates the absence of a long-term toxic effect of *T. evansi* to *P. persimilis*.

Juveniles of *P. persimilis* were unable to develop until adulthood when fed with *T. evansi*. Similar results were found with juveniles of the ladybird *Cycloneda sanguinea*, which did not reach adulthood and died within 11 days when fed exclusively with *T. evansi* (Oliveira et al. 2005). It has been suggested that the unsuitability of *T. evansi* for predators is related with the host plant of the herbivore (usually solanaceae) or be to the profuse weebing produced by *T. evansi* (de Moraes and McMurtry 1985; de Moraes and McMurtry 1987a; de Moraes and McMurtry 1987b; Oliveira et al. 2005). We tested for the effect of host plants by offering prey reared on three different host plants to the predators. These host plants are expect to differ in chemical composition, which could interfere with the natural enemy via herbivore (Agrawal et al. 2002; Suzuki et al. 2011). For instance, the proportion of juveniles of *Neoseiulus womersleyi* reaching adulthood was lower when they were fed with larvae of *Tetranychus kanzawai* reared on oleander plants, than when fed with prey from bean plants (Suzuki et al. 2011). Indeed, we observed an effect of host plant species of *T. urticae* on the performance of *P. persimilis*. However, our results showed no indication that host plant species of *T. evansi* affect its suitability as prey for *P. persimilis*.

The absence of juvenile development of *P. persimilis* fed with *T. evansi* could be physiologically explained as a matter of lack of energy or nutrients required for juveniles predators to develop. This low nutrition effect can be the result of a reduced access of predators to prey items (for instance some physical obstruction or inhibition). Our experiment was performed with a surplus of food and the juveniles did not face

obstructions to find and reach the prey items: the experiment was performed on small leaf discs without prey web. This easy access to prey items was confirmed by the high predation rate of the juveniles of *P. persimilis* on eggs of *T. evansi*. This higher predation rate on eggs of *T. evansi* also indicates that juveniles do not suffer from a physical impediment from the eggs. For instance, a harder and thicker eggshell would act as a physical obstruction, increasing the handling time and consequently negatively affecting feeding rate of juveniles. The presence of any sticky material in eggs could also reduce the feeding rate, gluing the mouthparts of predators, making harder to attack new eggs. However, our results indicate that eggs of *T. evansi* are not better protected from predation than eggs of *T. urticae* in the absence of web.

The predation rate of *P. persimilis* juveniles showed a peak at the fourth day, when most of the predators were protonymphs, which could be explained as a compensatory feeding. It has been observed that herbivores increase the consumption rate of low quality food, trying to compensate for the low quality by increasing their intake rate (Cruz-Rivera and Hay 2003; Simpson et al. 2004). Predatory mites also showed a higher predation rate upon the less suitable prey (*T. urticae* reared on a resistant cultivar of eggplant) (Khanamani et al. 2014). The longer survival of juveniles fed with eggs of *T. evansi* compared with starved individuals and the higher predation rate of juveniles of eggs of *T. evansi* than of eggs of *T. urticae* provide clues of a beneficial effect of compensatory feeding. However, this high intake rate did not help juvenile predators to complete their development. It is possible that this compensatory feeding was constrained by toxic effects of prey.

Prey toxicity is another hypothesis to explain why no juvenile predator fed with eggs of *T. evansi* becomes adult. A toxic factor from prey could lead to a reduction of digestive processes and absorption of nutrient. When increasing their consumption

rate to acquire nutrients, predators may consequently increase their rate of ingestion of toxic compounds from their diet (Cruz-Rivera and Hay 2003), which could have been the case in our system. If eggs of *T. evansi* contain some small amount of a digestibility blocker, may not matter as much eggs the predators eat, their compensatory feeding will be constrained by prey toxicity.

When adult females of *P. persimilis* received eggs of *T. evansi* plus eggs of *T. urticae*, the predatory mites showed a clear preference for eggs of *T. urticae*. However, this preference was not absolute: predators preyed upon some eggs of *T. evansi*. This diet mixing reduced the performance of *P. persimilis* compared with the single diet with the high quality prey. Diet mixing is mostly observed as a behaviour that results in better performance (Lefcheck et al. 2013; Marques et al. 2015) by balancing the nutrients that are limited in each diet item or reducing the negative effect of a toxic item by complex the harm elements. However, there are some examples of diet mixing by predators causing a decrease in performance(Malcolm 1989; Bilde and Toft 2001; Oelbermann and Scheu 2002; Fisker and Toft 2004). The negative effect of mixing a high quality diet with low quality prey items may be expressed in lower juvenile development and survival (Toft and Wise 1999; Oelbermann and Scheu 2002), a decrease of total predation rate and a change of the foraging behaviour of predators (Malcolm 1989).

The preference of *P. persimilis* to prey on eggs of *T. urticae* indicates that this predatory mite can discriminate between the two prey species. The predators may use chemical cues left from adult females of spider mites on the leaf surface or cues from the eggs to find and decide which prey to eat. However, it is intriguing why *P. persimilis* did not avoid preying on eggs of *T. evansi*. We would expect from predators to optimize their performance, learning to avoid low quality prey items (Traugott and Stamp 1996).

However, the number of eggs of *T. evansi* preyed remained constant during the four days of the experiment, indicating an absence of learned avoidance of low quality food. Maybe *P. persimilis* cannot learn to associate the two prey species in a short period of four days.

The fast recovery of oviposition and juvenile development after changing their diet from *T. evansi* to *T. urticae* indicates that predators previously fed with *T. evansi* recovered their normal predation and oviposition rates when fed eggs of *T. urticae* as fast as individuals kept without food. Juveniles of *P. persimilis* also restored their development and reached adulthood after stopping to feed on *T. evansi* and starting feeding exclusively on eggs of *T. urticae*. Such a change of prey quality affecting the performance of the predators was observed by Suzuki et al. (2011) with spider mites reared on two host plants with different degree of suitability. Prey transferred from oleander to bean plants for one day became better food for predators, which resulted in a better performance of the predators (Suzuki et al. 2011).

In conclusion, our findings do not support the hypothesis that unsuitability of *T. evansi* to predators is caused by sequestration of toxins from its host plant. The strong association of *T. evansi* with solaneceous plants (Navajas et al. 2013) is not the main factor determining its unsuitability as prey for a common predator of Tetranychidae. Similar observations of unsuitability of eggs of *T. evansi* as diet for juveniles was observed with a related species of predatory mite *Phytoseiulus macropilis* (Chapter III). It seems that a combination of lack of nutrients and a short-term toxic effect would explain this unsuitability of *T. evansi* to many natural enemies. Nevertheless, other defensive traits of *T. evansi*, such as the profuse web covering their colonies can contribute to reducing their predation (Venzon et al. 2009). Additionally, our results suggest that *T. evansi* can benefit from an enemy-free space on different

host plants. However, it is intriguing why *T. evansi* is not so often observed on host plants from other botanical families than Solanaceae, considering that they may not suffer from strong top-down regulation on these other hosts. On tomato, *T. evansi* can down-regulate the direct defence (Sarmento et al. 2011a) and outcompete *T. urticae* (Sarmento et al. 2011b). Maybe this manipulation of bottom-up forces and competitive advantage do not occur on plants other than Solanaceae. Still to be explored the amplitude of host plant manipulation by *T. evansi* on different host plant species.

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Figure legends

Figure 1. The cumulative proportion of live individuals of *P. persimilis* that reached adulthood when fed on eggs of *T. evansi* (continuous lines), *T. urticae* (dashed lines) or kept without food (dotted lines). The spider mites were reared on three different host plants: bean (lines with 'X' mark), tomato (lines with closed circles) and cucumber (lines with closed triangles). The letters close to the itens of the legend denote significant differences among the treatments in accordance with the time-to-event analysis.

Figure 2. Predation rate of *P. persimlis* juveniles on eggs of *T. evansi* (continuous lines) and *T. urticae* (dashed lines). The spider mites were reared on three different host plants: bean (lines with 'X' mark), tomato (lines with closed circles) and cucumber (lines with closed triangles). Different letters in the legend indicate significant differences ($P < 0.05$) among treatments.

Figure 3. Predation (A) and oviposition (B) of adult females of *P. persimilis* on single diets of eggs of *T. evansi* (grey bars named 'Only *T. evansi*') or eggs of *T. urticae* (dark bars named 'Only *T. urticae*') and on a mixed diet of both *T. evansi* and *T. urticae* eggs 'Mixed'. The letters above the bars denote significant differences ($P < 0.05$) among treatments through multiple comparisons of means with Tukey test.

Figure 4. Predation and oviposition of adult females of *P. persimilis* which fed exclusively on eggs of *T. urticae* for six days (line with closed triangles) or received eggs of *T. urticae* on the first two days, then eggs of *T. evansi* (line with closed squares) or no food (line with closed circles) for two days and then eggs of *T. urticae* again for two

more days. The vertical dotted lines indicate when the predators were transferred to new leaf discs. The small letters close to the markers denote significant difference ($P < 0.05$) among the treatments through multiple comparisons of means with Tukey test on each day of the experiment.

Figures

Figure 1

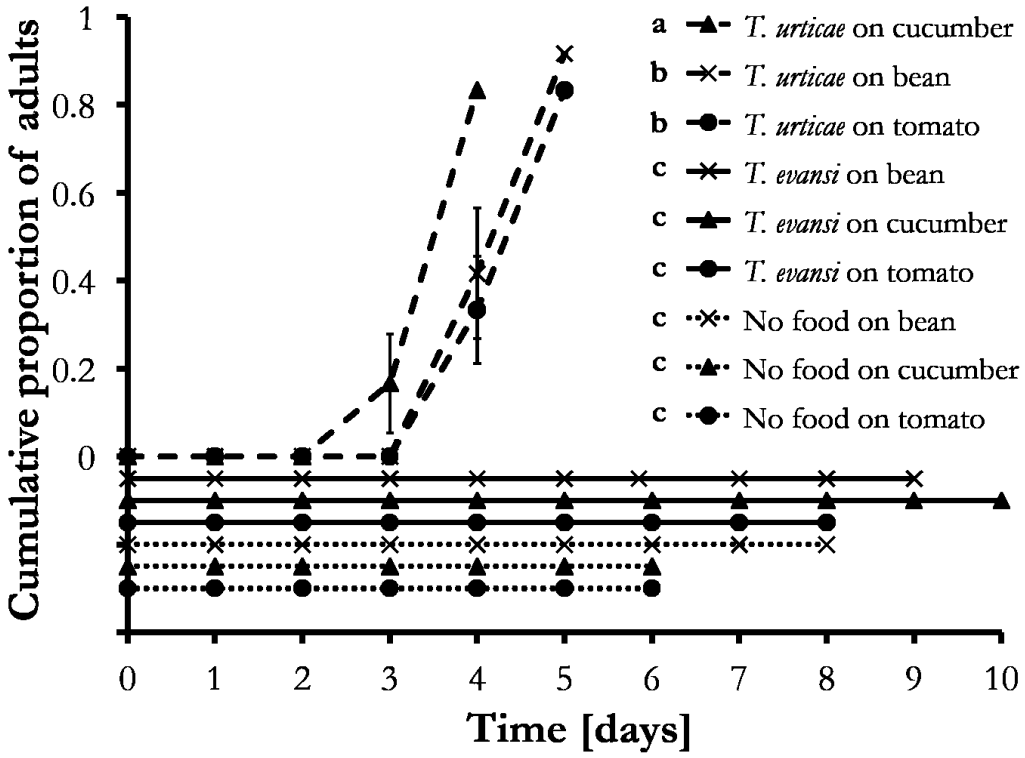


Figure 2

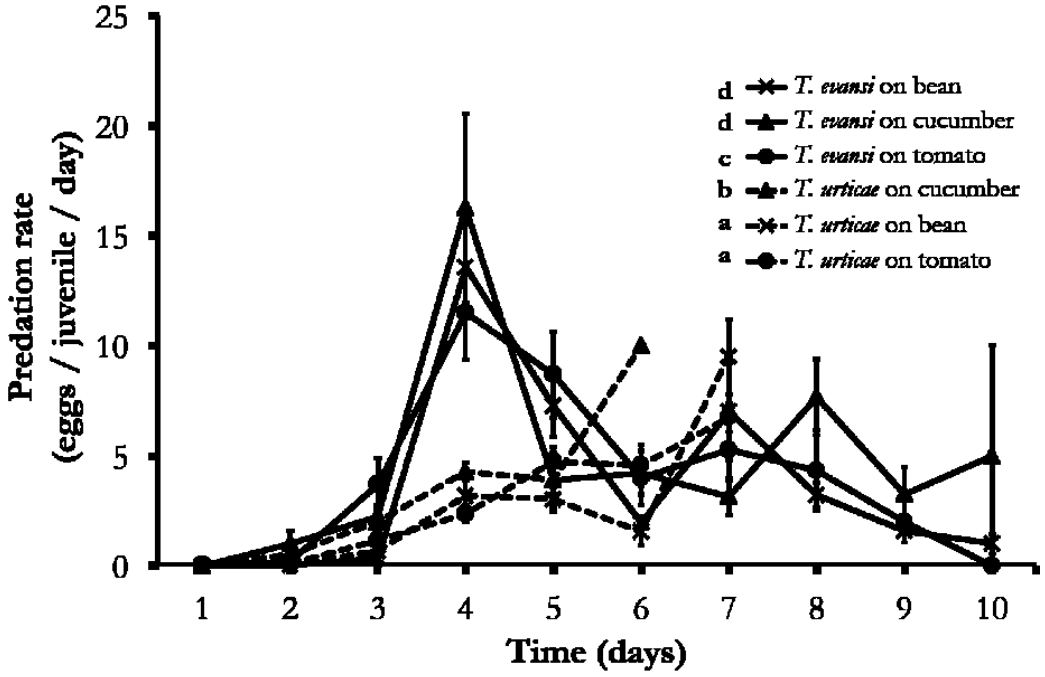

25
20
15
10
5
0
Predation rate
(eggs / juvenile / day)
1
2
3
4
5
6
7
8
9
10
Time (days)
d T. evansi on bean
d T. evansi on cucumber
c T. evansi on tomato
b T. urticae on cucumber
a T. urticae on bean
a T. urticae on tomato

Figure 3

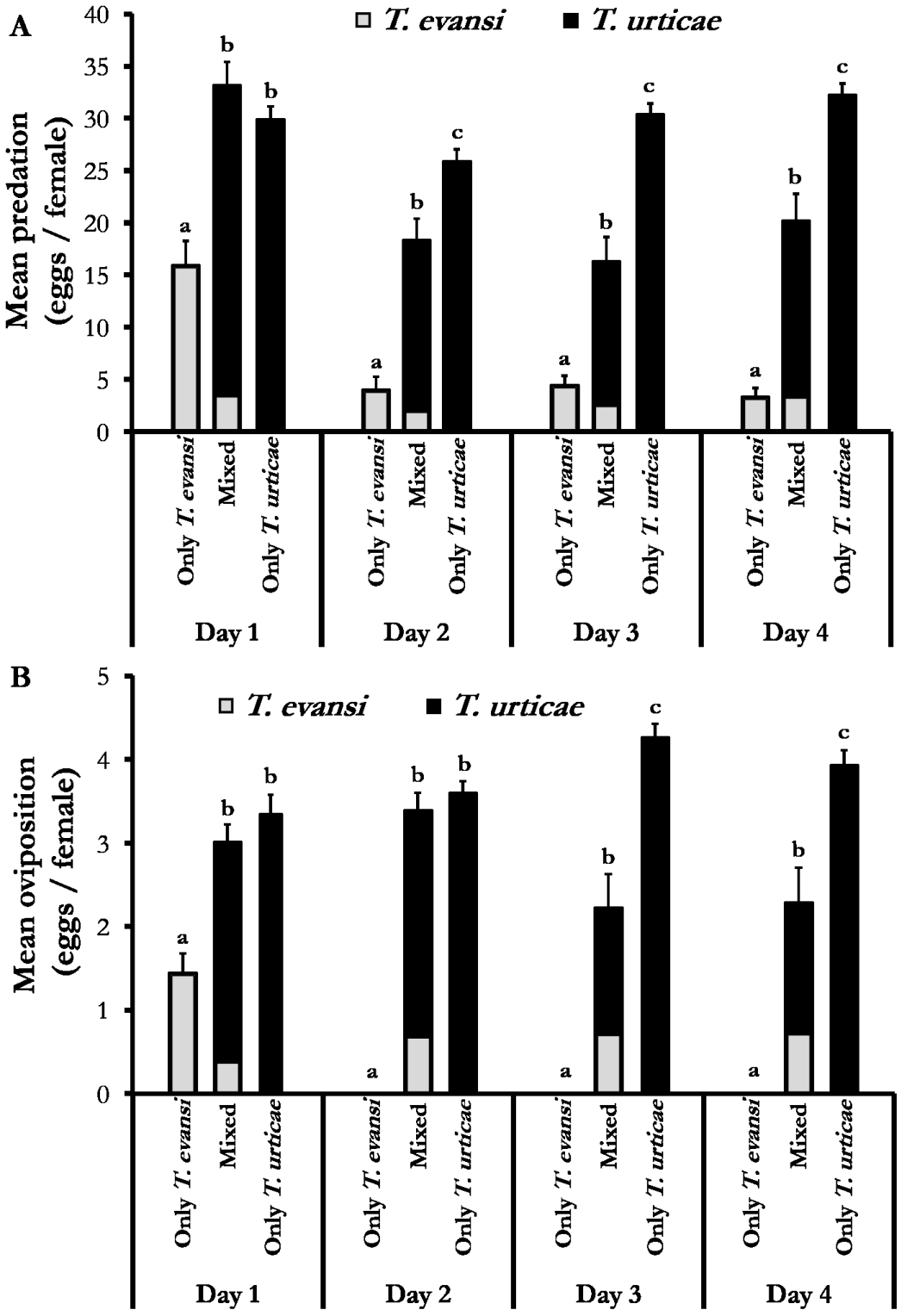
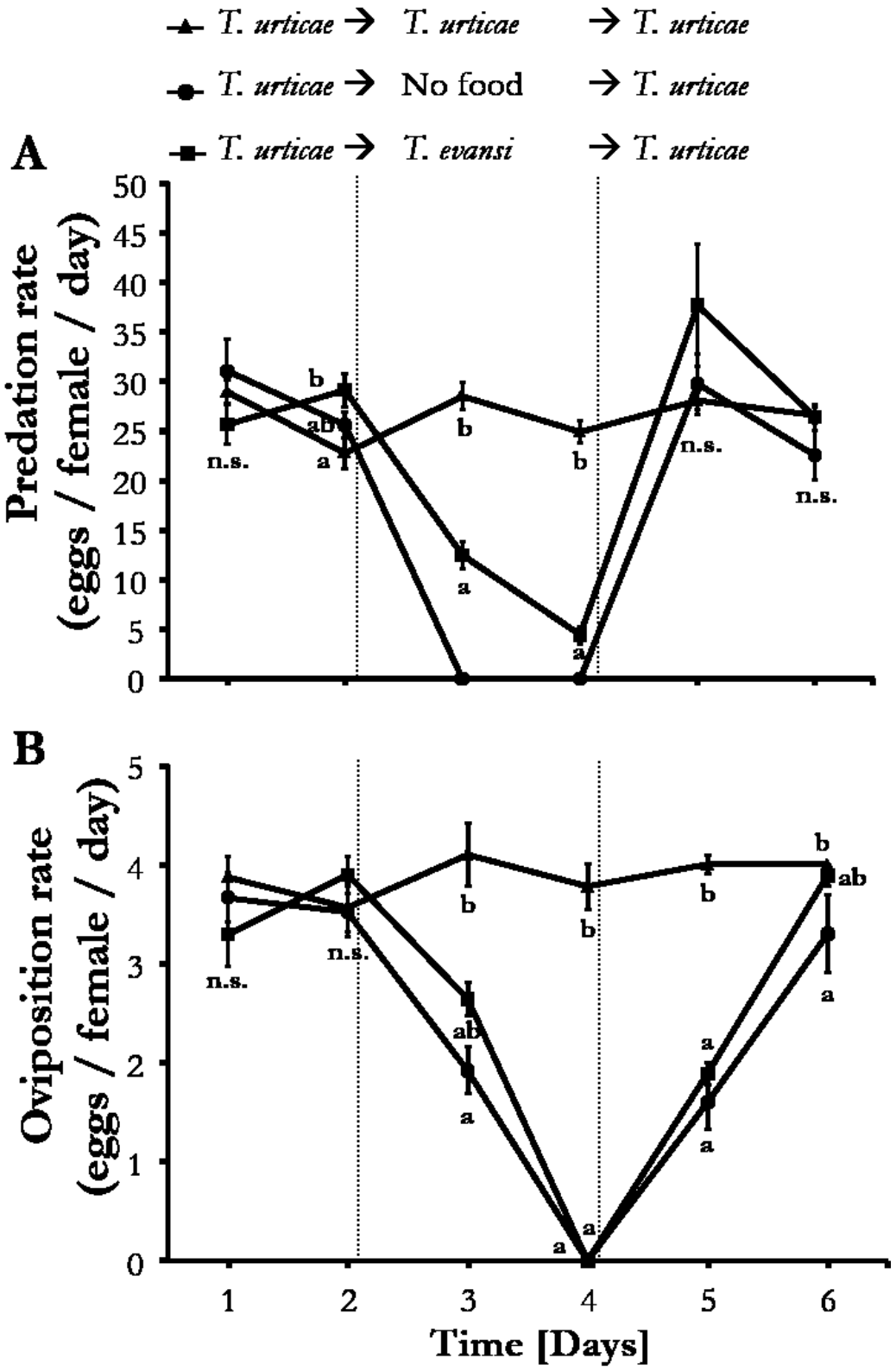

A
T. evansi
T. urticae
Mean predation (eggs / female)
0
5
10
15
20
25
30
35
40
a
b
b
a
b
c
a
b
c
a
b
c
Only T. evansi
Mixed
Only T. urticae
Day 1
Day 2
Day 3
Day 4
B
T. evansi
T. urticae
Mean oviposition (eggs / female)
0
1
2
3
4
5
a
b
b
a
b
b
a
b
c
a
b
c
Only T. evansi
Mixed
Only T. urticae
Day 1
Day 2
Day 3
Day 4

Figure 4


T. urticae → T. urticae → T. urticae
T. urticae → No food → T. urticae
T. urticae → T. evansi → T. urticae
A
Predation rate (eggs / female / day)
B
Oviposition rate (eggs / female / day)
Time [Days]
n.s.
a
ab
b

CHAPTER II - Suppression or differential induction? Indirect defence of tomato attacked by *Tetranychus evansi*

Title: Suppression or differential induction? Indirect defence of tomato attacked by *Tetranychus evansi*

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Abstract

Plants have developed sophisticated mechanisms of defence against herbivores. These defensive traits may acts directly against herbivores, thus reducing their performance, or indirectly by attracting the herbivore's natural enemies. However, herbivores can adapt to plant defence in many different ways. The red spider mite *Tetranychus evansi* is known to suppress direct defences of tomato plants to their own benefit. Here, we investigated the interference of *T. evansi* with the indirect defence of tomato plants, consisting of the attraction of predatory mites to odours from plants attacked by spider mites. In olfactometer experiments we observed that volatiles from tomato plants infested with approximately 350 *T. evansi* were not attractive to the predatory mites *Phytoseiulus macropilis*, *P. longipes* and *P. persimilis*, whereas volatiles from plants infested with 3.5 times more spider mite were significantly attractive. We also observed that odours from tomato plants infested with the inducer *Tetranychus urticae* (both with low and high densities) were attractive to and preferred by these predatory mites. Additionally, we identified the volatile chemicals released by clean plants and plants attacked by herbivores. The compounds of the blends were identified and quantified. We observed that *T. evansi* did not induce those volatile compounds that are often observed in attractive blends from plants attacked by the inducers. Despite the absence of attraction of predatory mites to odours from plants infested with low densities of *T. evansi*, these blends contained some compounds induced by *T. evansi* (but not by *T. urticae*). These results indicate that *T. evansi* may manipulate the indirect defence of tomato plants by inducing and suppressing the production of different volatile organic compounds.

Keywords: Herbivore-induced plant volatiles, predatory mites, plant manipulation, olfactometer, GC-MS

Introduction

As sessile organisms, plants are susceptible to attack by mobile herbivores. However, plants have developed sophisticated mechanisms of defence against these attackers. Some mechanisms deployed by plants act directly against herbivores (i.e. thorns, trichomes and toxic compounds) (Levin 1973; Ryan 1973; Agrawal 1998; Agrawal 1999; Howe and Jander 2008). Other mechanisms of plant defence know as indirect defence benefits the natural enemies of the herbivores (i.e. attractive volatiles, shelter and alternative food to natural enemies of herbivores) (Dicke and Sabelis 1988; Dicke et al. 1990; Turlings et al. 1990; Agrawal and Karban 1997; Heil 2008; Alba et al. 2012). Many of these defensive traits are constitutively present in plants, even under basal levels (Gatehouse 2002). However, some of them are induced after herbivory (Karban and Baldwin 1997; Traw and Dawson 2002). The physiological processes of induction of defensive traits in plants are regulated by the phytohormones jasmonic acid, salicylic acid, ethylene and systemin (Walling 2000). Cues associated with herbivory, such as herbivore saliva (Alborn et al. 1997), mechanical damage (Mithöfer et al. 2005) and volatiles released from attacked parts of plants (Arimura et al. 2000) are responsible to induce these hormones, thus triggering the physiological cascade that leads to the final products of induced defence (Walling 2000; Kant et al. 2004; Ament et al. 2004; Hilker and Meiners 2010).

Among the mechanisms of indirect defence, the release of volatile organic compounds attractive to natural enemies of herbivores is observed in most, if not all, plants (Dicke et al. 1998; Allison and Hare 2009; Kant et al. 2009; Baldwin 2010; Hare 2011). The volatiles act as cues, helping predators and parasitoids of herbivores to discriminate between plants harbouring prey from plants without herbivore or plant with a non-prey herbivore (Dicke et al. 1990; Turlings et al. 1990; De Moraes et al. 1998). To be considered as a defensive strategy, the fitness of plants that release volatiles organic compounds after the attack of herbivores is expected to be higher than plants that do not induce volatiles after

herbivory (Sabelis et al. 2001). This better performance from induced plants is assumed to be product of attraction of more natural enemies to attacked plants, leading to a reduction in the damage caused by herbivores (Agrawal 1999; Sabelis et al. 2001; Allison and Hare 2009). Besides the large body of evidence, this model of evolution of induced volatiles as indirect defence was never empirically proven (Allison and Hare 2009).

Volatile organic compounds are continuously released into the atmosphere and rhizosphere by different plant organs, such as leaves, fruits, flowers and roots (Maffei 2010). However, after herbivory, very often the blend released by plants is different in composition of the organic compounds in the mixture and the concentration of each specific chemical compound (Turlings et al. 1990; Paré and Tumlinson 1997; De Moraes et al. 1998; Kant et al. 2009). This induced response begins with the activation of plant secondary metabolic pathways which lead to the production of new compounds or increases the production of constitutive compounds (Kant et al. 2004; Ament et al. 2004). As is the case for direct defence, the induction of volatile organic compounds is regulated upstream by jasmonic acid, but salicylic acid is also required (Kant et al. 2004; Ament et al. 2004).

The herbivore *Tetranychus evansi* was found to supress direct defence of tomato plants to their own benefit (Sarmento et al. 2011; Alba et al. 2015). *Tetranychus evansi* supresses the signalling cascades triggered by both jasmonic acid and salicylic acid in attacked tomato plants, reducing the production of secondary metabolites responsible for negative effects performance of herbivores (Sarmento et al. 2011; Alba et al. 2015). As direct and indirect defence are both regulated upstream by jasmonic acid and salicylic acid, interferences on signalling of these two hormones will affect both direct and indirect defences simultaneously (Kant et al. 2004; Ament et al. 2004; Ament et al. 2006). Therefore, the suppression of jasmonic acid and salicylic acid defences by *T. evansi* is expected to suppress the indirect defence, besides direct defence. Indeed, we previously observed that *T. evansi* supressed the

production of an important volatile organic compound (the homoterpene 4,8,12-trimethyltrideca-1,3,7,11-tetraene, TMTT) and downregulated the expression of a gene related with the synthesis of terpenes on plants (GPPS-1). Despite this suppression, tomato plants attacked by *T. evansi* were attractive to herbivores and predatory mites (Sarmento et al. 2007; Sarmento et al. 2011).

Here, we investigated the interference of the spider mite *T. evansi* with the indirect defence of tomato plants. We compared the indirect defence of plants attacked with *T. evansi* to plants attacked with the putative inducer *Tetranychus urticae*. As the mechanism used by *T. evansi* to suppress the defence from its host plants is not clear so far, the infestation of plants by spider mites were controlled for two levels (20 or 60 spider mites per leaflet). We tested the response of three species of predatory mites (*Phytoseiulus longipes*, *Phytoseiulus macropilis* and *Phytoseiulus persimilis*) to the volatiles from attacked plants in olfactometer experiments. Furthermore, we analysed the chemical composition of the blend of volatiles released by these attacked plants.

Material and Methods

General explanations and organisms

One part of the olfactometer experiments was performed in 2013 in Amsterdam (Science Park, University of Amsterdam, Amsterdam, The Netherlands) and the other part in 2014 in Viçosa (Federal University of Viçosa, Viçosa, Minas Gerais, Brazil). For both sets of experiments, we used the same variety of tomato plants (*Solanum lycopersicum* var. Santa Clara I-5300) to rear the mites and to perform the experiments. The populations of *T. evansi* and *T. urticae* were collected in 2002 from naturally infested tomato plants in Viçosa (Sarmento et al. 2011). In 2010, a sample of *T. evansi* was sent to Amsterdam where it was reared on tomato leaves (*Solanum lycopersicum* L. cv Castlemart). In 2012, a sample of *T. urticae* was sent to Amsterdam where they were reared on leaves of common bean. In January, 2013, a rearing of *T. evansi* and *T. urticae* was established on leaves of tomato Santa Clara I-5300. The populations of *T. evansi* and *T. urticae* in Viçosa were continuously reared on the same tomato variety (*S. lycopersicum* var. Santa Clara I-5300) since 2002.

The olfactometer experiments performed in Amsterdam used two species of predatory mite: *P. persimilis* and *P. longipes* (hereafter refereed as *P. longipes*-ams). For olfactometer experiments carried out in Viçosa, we used *P. macropilis* and *P. longipes* (hereafter referred as *P. longipes*-vic).

Rearing conditions

In Amsterdam, plants used for the mite rearing were grown in a greenhouse with controlled photoperiod (16:8 h, L:D), temperature (25:18 °C, L:D) and relative humidity (50–60%) while plants used in olfactometer tests were grown in a sealed climate room with controlled climate and artificial light (Temp. 25 ºC, relative humidity 70–80 %, photoperiod (16:8 L:D). In Viçosa, plants used for mite rearing and experiments were grown in a greenhouse with natural daylight and a temperature ranging from 15ºC (during the coldest

nights in winter) to 34 ºC (during the hottest days in summer) and relative humidity ranging from 60-90%.

Every week, we sowed tomato seeds in a commercial plant substrate (Viçosa: Tropstrato HT Hortaliças, Vida Verde Indústria e Comércio de Insumos Orgânicos Ltda, Mogi Mirim, SP; Amsterdam: Jongkind Grond BV, Aalsmeer, The Netherlands). We transplanted the seedlings to plastic pots (2 L) with soil substrate after the first pair of leaves had expanded, approximately twenty days after sowing. The plants grown in Viçosa were water-fertilized once a week with a solution of 20g of N-P-K (20-05-20) and 40g of superphosphate simple in 20 L of water. Plants grown in Amsterdam were not fertilized. Approximately ten days after transplanting, we used cotton yarns to tie plants to wooden stakes inserted into the soil for support.

Colonies of spider mites were kept in PVC trays (54 cm x 38 cm x 8.5 cm LxWxH) filled with detergent and water (1:50, v/v), which served to prevent mite escapes and invasion of mites and other non-flying arthropods. We used detached leaves from tomato plants of at least 40-days old to feed the colonies of spider mites. To prevent desiccation, fresh tomato leaves used to feed the colonies had their petiole inserted into water, provided either in small plastic trays (30 cm x 22 cm x 8 cm, Amsterdam) or in PVC tubes (Viçosa, 3 cm x 9 cm DxH). In Amsterdam, the colonies of spider mites were maintained in a climate room (25 ºC, 70 – 80 % relative humidity) with controlled photoperiod (16:8 L:D). In Viçosa, the spider mite colonies were maintained in a rearing room (temperature ranging from 18 to 28 ºC and relative humidity of 70 – 90 %) with controlled photoperiod (12:12 L:D).

Colonies of predatory mites were established in a PVC tray (44 cm x 30 cm x 8 cm) inside a larger tray (54 cm x 38 cm x 8.5 cm). The larger tray was filled with a mixture of water plus detergent to deter escaping of the predatory mites and contamination with other species. We fed the predatory mites *P. persimilis* and *P. macropilis* with *T. urticae* and *P. longipes*

with *T. evansi*. We put, at least three times per week, tomato leaves from the stock colony of spider mites containing a mixture of all stages and web. In Amsterdam, the colonies of predatory mites were maintained in a climate room (25 ºC, 70 – 80 % relative humidity, photoperiod of 16:8 hours L:D) while in Viçosa, mites were kept in a rearing room (temperature ranging from 18 to 28 ºC and relative humidity of 70 – 90 %) with controlled photoperiod (12:12 L:D).

Response of predatory mites to volatiles of spider mite-infested tomato plants

We used a Y-tube olfactometer to assess the response of the predatory mites to volatiles from the plants with spider mites (Sabelis and van de Baan 1983). The olfactometer consists of a glass tube (3.5 cm diameter) in the form of a "Y". Each of the two arms of this Y-tube was connected with plastic tubes to a glass container (43 x 36 x 50 cm) in which the plants were arranged. The base of the Y-tube was connected thought a plastic tube to a vacuum pump, which was used to generate a constant air flow. The air entered in the glass containers through an air inlet, passed over the plants and then carried the odours through the plastic tubes to the Y-tube. The airflow in each arm of the olfactometer was calibrated to 0.50 m/s (VelociCalc® Air Velocity Meter 9545-A). Each odour source consisted of three tomato plants that were infested for seven days either with *T. evansi* or *T. urticae* or were uninfested. The plants were carefully placed in the glass containers to avoid mechanical damage and/or cross infestations. Because leaves and stems of tomato are covered with glandular trichomes with possible repellent compounds, we transferred the plants to glass containers at least 12 hours prior to the tests with the vacuum pump connected to remove any odours produced during handling of the plants.

We used tomato plants of 30 to 60-days-old. Plants were transfered to the laboratory and a fine brush was used to transfer spider mites from infested leaves from the stock colonies to the plant. Plants were infested with two different densities of the spider mites *T.*

evansi or *T. urticae*: 20 adult females per leaflet or 60 adult females per leaflet. On average, plants infested with *T. evansi* had 354±29 and 1269±71 respectively and plants with *T. urticae* received 335±63 and 1176±64 spider mites per plant. After being infested, plants were incubated in a climate room for 6 days until they were used for olfactometer experiments (Amsterdam: 25±1 ºC, 70 – 80 % RH, 16:8 L:D; Viçosa: 26±2 ºC).

The predatory mites tested were taken from the rearing and were starved for at least two hours prior to the test. After being tested, the predatory mites were discarded. The predatory mites were tested individually in the olfactometer and each trial consisted of twenty adult female predatory mites that reaches the end of the arm of the Y-tube within five minutes. Females that did not respond within five minutes were recorded, but not included in the calculation of the preferences. Using a fine brush, each female was put on the metal wire inside the Y-tube after disconnecting the vacuum pump. The metal wire served as a bridge positioned in the centre of the Y-tube, connecting its base to the end of each arm. After reconnecting the vacuum pump to the base of the Y-tube, the predatory mites were observed for five minutes.

We used replicated goodness-of-fit G-test to access statistical deviance on the responses of predatory mites on olfactometer experiments from an expected fraction of 0.5:0.5. Values of Gt <0.05 indicates a deviation from the expected distribution of 0.5:0.5 in the fractions of the predatory mites moving to each of the volatiles sources offered to them (Sokal and Rohlf 1995). This G-statistic also tests for deviation from expected fractions of the overall polled number of predatory mires (Gp < 0.05 means significant preference for polled results from all replicates) and for the degree of heterogeneity among the replicates (Gh < 0.05 means different distribution of the predatory mites among the replicates).

Volatile analysis

We collected the headspace volatiles from tomato plants infested as described above. Plants were carefully put inside glass chambers and care was taken to reduce mechanical damage. As the manipulation of plants could damage some glandular trichomes, the plants were aerated for at least 2 hours before attaching the volatile trap. We used a 'push–pull' headspace collection glass chamber for collecting the organic volatile compounds from entire plants. An aircompressor was used to push air through a charcoal filter, subsequently passing through a flowmeter (set to 2 L/min) and entering the glass chamber from the top. At the bottom of the glass chamber, a volatile trap with 50 mg of Super Q (80/100 mesh; Alltech, Deerfield, IL, USA) was connected to a vacuum pump (air flow set to 0.5 L/min). The time of aeration among replications varied from 24 to 63 hours (30.4 ± 3.1 hours on average).

The aerations were simultaneously performed with groups of plants with different treatments. Whenever possible, we collected the volatiles from all treatments simultaneously (plants infested with *T. evansi*, with *T. urticae* and clean plants). However, for logistical reason, we sometimes collected the volatiles only from two treatments at the same time. In these cases, we used an infested plant (either by *T. evansi* or *T. urticae*) and a clean plant to correct for unforeseen differences in the volatile blend. In summary, we had 5 samples with 20 *T. urticae* per leaflet and 5 with 60 *T. urticae*, 5 samples with 20 *T. evansi* per leaflet and 6 samples with 60 *T. evansi* and 11 samples from clean tomato plants.

Before use, the filters were rinsed with 2 mL of HPLC grade n-hexane (Sigma-Aldrich, St Louis, MO, USA). After plant aeration, volatiles were washed from the filters with 0.2 mL of HPLC grade n-hexane (Sigma-Aldrich), collected in sealed 2 mL glass vials and stored at -20 ºC. Subsamples were then prepared from each sample by collecting an aliquot of 8 μL of the stock sample and adding 2 μL of an internal standard (η-heptyl acetate at 40 ng/μL) and stored in 100 μL glass inserts. Subsequently, an aliquot of 1 μL of the sample with internal standard was injected into the GC-MS (QP2010, Shimadzu). The

temperature program started from 35°C (hold for 5 min) raising at 8°C/min to 280°C (hold for 5 min).

To identify/analyse the compounds from the volatile blends, we first visually inspected the peaks of the chromatograms generated after sample readings. The mass spectra of each peak were compared with the mass spectra from commercial standard compounds. Compounds that did not match with the standards available in our lab, were tentatively identified by comparing the mass spectra from identified peaks in samples with spectra available in NIST08, WILEY and FFNSC1.3 databases. To this end, we performed quantitative analyses of the compounds present in the blend of volatiles. The concentration of each compound was determined by equivalence of its area with the area of the internal standard of each sample (η-heptyl acetate at 8 ng/μL). To compare the difference in concentrations of each compound among the treatments, we performed ANCOVAs with treatment as the main factor (clean, *T. evansi*, and *T. urticae*) and time of aeration and plant weight as covariates (Crawley 2013). Contrasts among the treatments were determined with a post hoc Tukey test implemented by the package 'multcomp' (Hothorn et al. 2008). We used the software R© to perform the statistical analysis (R Development Core Team 2015).

Results

Response of predatory mites to volatiles of spider mite-infested tomato plants

The volatiles of tomato plants infested with 20 adult females of *T. evansi* per leaflet were not significantly attractive for any of the predatory mite species tested (Figure 1). However, volatiles from plants infested with 60 adult females of *T. evansi* per leaflet were attractive to *P. macropilis* ($Gt_{df=3}$ = 23.88 P < 0.01, $Gp_{df=1}$ = 15.70 P < 0.01, $Gh_{df=2}$ = 8.18 P = 0.02), *P. persimilis* ($Gt_{df=4}$ = 19.34 P < 0.01, $Gp_{df=1}$ = 13.17 P < 0.01, $Gh_{df=3}$ = 6.18 P = 0.10) and to *P. longipes*-ams ($Gt_{df=3}$ = 33.89 P < 0.01, $Gp_{df=1}$ = 29.11 P < 0.01, $Gh_{df=2}$ = 4.78 P = 0.09), but not to *P. longipes*-vic ($Gt_{df=4}$ = 3.64 P = 0.46, $Gp_{df=1}$ = 3.22 P = 0.07, $Gh_{df=3}$ = 0.42 P = 0.94).

All four populations of predatory mites tested here (*P. longipes*-vic, *P. longipes*-ams, *P. macropilis* and *P. persimilis*) showed a preference for volatiles from plants attacked by *T. urticae* relative to plants with *T. evansi* (Figure 2). A significant preference for odours from plants infested with either 20 and with 60 *T. urticae* per leaflet was observed with *P. persimilis* ($Gt_{df=5}$ = 43.19 P < 0.01, $Gp_{df=1}$ = 38.55 P < 0.01, $Gh_{df=4}$ = 4.64 P = 0.33 and $Gt_{df=2}$ = 23.08 P < 0.01, $Gp_{df=1}$ = 18.35 P < 0.01, $Gh_{df=1}$ = 4.72 P = 0.03 respectively) and with *P. longipes*-ams ($Gt_{df=2}$ = 6.58 P < 0.04, $Gp_{df=1}$ = 6.58 P < 0.01, $Gh_{df=1}$ = 0.00 P = 1.00 and $Gt_{df=2}$ = 25.54 P < 0.01, $Gp_{df=1}$ = 25.31 P < 0.01, $Gh_{df=1}$ = 0.23 P = 0.63, respectively). The predatory mites *P. macropilis* and *P. longipes*-vic showed a significant preference to volatiles from plants attacked by *T. urticae* (20 mites per leaflet, $Gt_{df=3}$ = 13.75 P < 0.01, $Gp_{df=1}$ = 11.65 P < 0.01, $Gh_{df=2}$ = 2.10 P = 0.35 to *P. macropilis* and $Gt_{df=4}$ = 13.34 P < 0.01, $Gp_{df=1}$ = 6.13 P = 0.01, $Gh_{df=3}$ = 7.21 P = 0.07 to *P. longipes*-vic). The preference of *P. longipes*-vic between odours from plants infested with 60 *T. evansi* per leaflet versus plants infested with 60 *T. urticae* was tested only once and showed slight trend of preference for plants with *T. urticae* while *P. macropilis* was not tested for this combination of odours.

We observed a significant preference for odours from plants attacked by T. urticae when offered together with clean plants (Figure 3). The predatory mite *P. macropilis* showed a significant preference for volatiles from plants infested with two densities of infestation, 20 mites per leaflet ($Gt_{df=4}$ = 16.12 P < 0.01, $Gp_{df=1}$ = 14.92 P < 0.01, $Gh_{df=3}$ = 1.20 P = 0.75) and 60 mites per leaflet ($Gt_{df=2}$ = 11.00 P < 0.01, $Gp_{df=1}$ = 10.46 P < 0.01, $Gh_{df=1}$ = 0.54 P = 0.46). Volatiles from plants infested with 20 females of *T. urticae* were also attractive to *P. persimilis* ($Gt_{df=5}$ = 39.79 P < 0.01, $Gp_{df=1}$ = 21.98 P < 0.01, $Gh_{df=4}$ = 17.81 P = 0.01). However, *P. longipes*-vic did not show any significant preference for volatiles from clean plants when offered together with volatiles from plants infested with 20 or 60 adult females of *T. urticae* per leaflet ($Gt_{df=4}$ = 6.24 P = 0.18, $Gp_{df=1}$ = 1.81 P < 0.18, $Gh_{df=3}$ = 4.43 P = 0.22 and $Gt_{df=2}$ = 5.43 P = 0.07, $Gp_{df=1}$ = 3.66 P = 0.06, $Gh_{df=1}$ = 1.78 P = 0.18, respectively). Although repeated only once, the experiment testing the preference of *P. persimilis* and *P. longipes*-ams to odours from plants infested with 60 females of *T. urticae* per leaflet showed a clear attraction by odours from infested plants.

Volatile analysis

35 organic volatile compounds were present in the volatiles of which 29 were identified (Table 1). The concentration of 9 out of the 35 compounds found were higher in blends from infested plants than in samples from clean plants. The green leaf volatiles (Z)-3-Hexen-1-ol and Hex-(3Z)-enyl butyrate were significantly induced only in plants infested with 60 females of *T. urticae* per leaflet ($F_{4,25}$ = 3.62, P = 0.01 and $F_{4,25}$ = 4.64, P < 0.01 respectively). The compounds Dodecane and Caryophyllene were significantly induced in plants infested either with *T. evansi* or with *T. urticae* with the two densities of infestation ($F_{4,25}$ = 5.48, P < 0.01 and $F_{4,25}$ = 6.11, P < 0.01 respectively). The compound TMTT was significantly induced in plants infested with 60 adult females of spider mite per leaflet (both,

T. evansi and *T. urticae*) while the concentration in plants infested with 20 mites per leaflet was not significantly different from clean plants ($F_{4,25} = 4.96$, $P < 0.01$).

We also observed that the concentration of Tridecane was significantly higher only in the blend from tomato plants attacked with *T. evansi* ($F_{4,25} = 3.76$, $P = 0.02$). We also observed a slight induction (but not significant) of methyl salicylate in plants infested with 60 females of *T. urticae* per leaflet in relation to clean plants and plants infested with *T. evansi* ($F_{4,25} = 2.36$, $P = 0.08$).

Discussion

We observed that odours from tomato plants attacked by T. evansi were attractive to predatory mites only when plants were highly infested with spider mites. However, odours from plants infested with both low (20 females per leaflet) and high densities of *T. urticae* (60 females/leaflet) were significantly attractive to predatory mites. Moreover, the predatory mites clearly preferred for odours from plants infested with *T. urticae* to plants with *T. evansi*. Our volatile analysis confirmed a differential induction of volatiles in plants infested with *T. urticae* and *T evansi* compared with volatiles from clean plants.

Previously, we observed a suppression of the gene GGPS-1 in tomato plants attacked by *T. evansi* (Sarmento et al. 2011). This gene encodes for precursors of some (but not all) volatile terpenoids present in the attractive blend of herbivore-infested tomato plants (Kant et al. 2004; Ament et al. 2004), acting as a marker for indirect defence (Alba et al. 2012). In tomato, GGPS-1 is upregulated by jasmonic acid, although a basal level of salicylic acid is also required (Ament et al. 2006). Indeed, the suppression of GGPS-1 correlates with the suppression of the volatile organic compound TMTT in the volatile blend from plants attacked by *T. evansi* (Sarmento et al. 2011). However, odours from plants infested with *T. evansi* were attractive to predators (Sarmento et al. 2007; Sarmento et al. 2011), indicating that other volatiles, not downstream of GGPS-1, may be induced in plants infested with *T. evansi*. Here we observed that among the compounds induced by the spider mites on tomato, TMTT, Caryophyllene, Hex-(3Z)-enyl butyrate, 3,7-Dimethyldecane and (Z)-3-Hexen-1-ol were not significantly different between plants infested with *T. evansi* (d20) and clean plants. From those compounds, 3,7-Dimethyldecane, Caryophyllene and TMTT were significantly induced in tomato plants infested with 60 adult females of *T. evansi* per leaflet. Furthermore, plants infested with 20 *T. evansi* per leaflet were not attractive to predatory mites while plants infested with 60 spider mite per leaflet were attractive. This indicates that *T. evansi* induces

the production of attractive organic volatile compounds in tomato. However, this significant induction only occurs with higher levels of herbivory.

The compounds Decanal, Dodecane and Tridecane were induced in plants infested with *T. evansi* but not in plants infested with *T. urticae*. Their concentrations were significantly higher in blends of volatiles from plants infested with 20 females of *T. evansi* compared with clean plants and plants infested with *T. urticae*. As these plants (*T. evansi* d20) were not attractive to predatory mites, these compounds may be not important for attraction of predatory mites. Dodecane and Tridecane are hydrocarbons acting as pheromone or allomone in insect communication (El-Sayed 2014). Here, we observed the presence of Dodecane and Tridecane also in samples from clean tomato plants, indicating that spider mites were not the source of this compound and consequently it is not a pheromone (Dicke and Sabelis 1988). Moreover, the concentration of Dodecane and Tridecane in odours from plants infested with *T. urticae* was lower than in clean plants. It is possible that these compounds act as a repellent to predatory mites. Hence, the suppression of these compounds in plants attacked by *T. urticae* is an adaptive plant strategy to attract predatory mites. Additionally, the induction of Decanal, Dodecane and Tridecane in plants infested with *T. evansi* may be part of a mechanism employed by *T. evansi* to manipulate indirect defence in tomato. Moreover, the induction of Decanal, Dodecane and Tridecane may act as attractant of conspecifics to plants supressed by *T. evansi* (Sarmento et al. 2011).

It is known that some predatory mite species perceive odours as a synthetic whole (van Wijk et al. 2011). This means that predatory mites do not recognize a specific volatile organic compound in a blend, but the mixture of many compounds (the blend) as a whole. Sometimes, a mixture of a subset of some compounds induced by herbivory may elicit positive chemotaxis, but is not as attractive as the complete blend (van Wijk et al. 2011). If this holds for all predators tested here, it is not important to look for the presence of specific

compounds as markers of the induction of indirect defence by herbivores on plants. This synthetic perception is especially important to understand the chemotaxis of natural enemies that forage for generalist prey that can feed on many different plant species. This is the case of Phytoseiid mites from the genus *Phytoseiulus*. These predatory mites are known to be important natural enemies of *Tetranychus* mites. Some species from this genus, as it is the case of *T. urticae*, are known to be hosted by more than 1100 plant species (Migeon and Dorkeld 2015). It is also known that the same species of herbivore induces the production of different blends of volatiles in different plant species (van den Boom et al. 2004). Nevertheless, the natural enemies are able to use these myriad of odours to locate plant harbouring their prey, showing a high plasticity in the use of plant odours as cues (van den Boom et al. 2002; Alba et al. 2012). Here, we confirmed this plasticity with two species of predatory mites, *P. persimilis* and *P. macropilis*. These species are known important natural enemies of *T. urticae* (Helle and Sabelis 1985; Oliveira et al. 2007). However, they show a very poor performance when fed with *T. evansi* (Chapter I and Chapter III). Nevertheless, *P. persimilis* and *P. macropilis* showed a positive response to volatiles from plants highly infested with *T. evansi* when tested against volatiles from clean plants. However, when *P. persimilis* was offered the choice between volatile from plants infested with *T. evansi* or *T. urticae*, it clearly showed a preference for their favourable prey. This indicates that predatory mites may fine-tune their response to induced volatiles from less favourable prey to odour from a more favourable prey.

The density of infestation with herbivores is known to positively correlate with the attraction of predators and parasitoids to odours from attacked plants (Gols et al. 2003; Girling et al. 2011). The increase in attraction is explained by qualitative and quantitative differences in blends from plants with increased herbivory (Girling et al. 2011). A common density used to infest plants for induction of volatiles production is about 100 (or more) mites per plant (Sabelis and van de Baan 1983; Janssen et al. 1997; Dicke 1999; Arimura et al. 2000; Gols et al. 2003). Here we observed that volatiles from plants infested with

approximately 1269±71 individuals of *T. evansi* (d60) were attractive to predatory mites while volatile from plants infested with 354±29 mites per plant were not. Our findings contrast with previous results that volatiles from tomato plants infested with 200 mites were attractive to predatory mites (Sarmento et al. 2011). However, in this previous work, plants were infested by putting four infested tomato leaves from stock colonies over the leaves from the clean plant (Sarmento et al. 2011). If laboratory colonies of spider mites reach a stable age distribution after some time, it is expected that populations of spider mites on leaves consisted approximately of 66% eggs, 26% immature and 8% adults (Carey 1982). As the leaves were left on the plants for 3 to 4 days (F. Lemos, personal information), it is possible that many immatures emerged from the eggs and moved from infested leaves to the plants, and consequently the level of infestation in these experiments were underestimated.

In conclusion, we found that a herbivore which manipulates the direct defence of its host plants also interferes with the indirect plant defence to its own benefit. This interference occurs through the suppression of the production of volatile organic compounds usually present in attractive blends from plants attacked by a putative inducer. However, the suppression of indirect defence seems to be limited to the density of herbivores on the plant. Probably, plants with high levels of herbivory suffer so many physiological changes that suppression may not be achieved.

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Table 1. Volatile organic compounds from the headspace of clean tomato plants (n=11) and tomato plants infested with 20 adult females of *T. evansi* (n=5) and *T. urticae* (n=5) in each leaflet or with 60 females of *T. evansi* (n=6) and *T. urticae* (n=5) per plant leaflet. The volatile organic compounds are ordered according to their calculated Kovats index. The lines with values in bold show compounds with significantly different amounts among treatments.

Compounds	Calculated KI	Clean tomato plants	*T. evansi* infested		*T. urticae* infested	
			d20	d60	d20	d60
		ng / µL / plant (mean ± s.e.m.)				
(Z)-3-Hexen-1-ol[a]	**862.6**	**< 0.01a**	**0.01±0.01a**	**< 0.01a**	**0.35±0.35a**	**1.70±0.97b**
4-Methyloctane[b]	868.0	0.08±0.04	0.1±0.04	0.08±0.02	0.18±0.13	0.05±0.02
Nonane[b]	899.0	< 0.01	< 0.01	0.10±0.07	< 0.01	< 0.01
α-Pinene[b]	936.8	0.25±0.18	0.15±0.03	0.31±0.05	0.17±0.08	0.23±0.05
Sabinene[a]	976.9	0.01±0	0.01±0	0.01±0	0.01±0.01	< 0.01
(-)-β-pinene[a]	979.8	< 0.01	0.01±0	0.01±0	< 0.01	< 0.01
β-Myrcene[a]	991.9	0.11±0.08	0.07±0	0.05±0	0.16±0.07	0.10±0.02
1,2,3-Trimethylbenzene[b]	994.2	0.01±0.01	0.06±0.02	0.05±0.04	< 0.01	< 0.01
Decane[b]	998.0	0.05±0.03	0.13±0.05	0.06±0.05	0.03±0.02	< 0.01
2-Carene[b]	1000.8	1.87±1.26	1.13±0.26	1.47±0.21	1.36±0.43	2.17±0.21
α-Phellandrene[b]	1004.8	0.37±0.26	0.22±0.04	0.17±0.03	0.30±0.08	0.32±0.07
α-Terpinene[b]	1018.7	0.19±0.15	0.08±0.02	0.07±0	0.13±0.04	0.14±0.02
p-Cymene[a]	1027.6	0.03±0.02	0.03±0.01	0.04±0.01	0.01±0.01	0.02±0
β-phellandrene[b]	1032.8	7.59±4.7	5.81±0.72	6.5±0.67	7.3±1.96	8.71±1.03
NotIdent1[a]	1040.4	< 0.01	< 0.01	< 0.01	0.02±0.02	0.02±0.02
trans-β-Ocimene[a]	1051.5	0.05±0.04	0.02±0.01	0.04±0.01	0.06±0.04	0.07±0
3-Ethyl-3-methylheptane[b]	1059.1	0.19±0.12	0.26±0.13	0.10±0.07	0.46±0.3	0.11±0.04
3,7-Dimethyldecane[b]	**1059.7**	**0.02±0.01a**	**0±0a**	**0.05±0.01b**	**0±0a**	**0.01±0.01a**

γ-Terpinene[a]	1064.7	0.06±0.03	0.05±0.02	0.04±0.01	0.10±0.07	0.03±0.01
Terpinolene[a]	1091.6	0.05±0.03	0.01±0	0.01±0	0.01±0.01	0.02±0
3,7-Dimethylundecane[b]	1100.0	0.09±0.04	0.13±0.1	0.09±0.04	0.14±0.13	0.06±0.05
Nonanal[a]	1105.1	0.16±0.03	0.26±0.07	0.27±0.02	0.21±0.11	0.12±0.01
NotIdent2	1171.9	0.02±0	0.08±0.05	0.05±0.01	0.02±0.01	0.02±0.01
Menthol[b]	**1178.1**	**0.06±0.01a**	**0.63±0.38b**	**0.17±0.01ab**	**0.04±0.01a**	**0.22±0.1ab**
Hex-(3Z)-enyl butyrate[b]	**1187.3**	**< 0.01±0a**	**0.04±0.03a**	**0.04±0.02a**	**0.02±0.02a**	**0.33±0.16b**
Dodecane[b]	**1198.0**	**0.35±0.06a**	**0.79±0.22c**	**0.48±0.06ac**	**0.24±0.06ab**	**0.10±0.01ab**
Methyl Salicylate[a]	1202.2	0.09±0.06	0.02±0.01	0.05±0	0.07±0.04	0.29±0.11
Decanal[a]	**1206.6**	**0.07±0.01a**	**0.11±0.03ab**	**0.18±0.01b**	**0.06±0.03a**	**0.06±0.01a**
Tridecane[b]	**1297.8**	**1.29±0.19ab**	**2.51±0.9b**	**1.49±0.13ab**	**0.84±0.16a**	**0.55±0.05a**
NotIdent3	1388.2	0.09±0.02	0.09±0.04	0.07±0.01	0.05±0.03	0.04±0
Caryophyllene[a]	**1435.1**	**0.1±0.04a**	**0.41±0.14ab**	**0.3±0.06b**	**0.71±0.32b**	**0.29±0.05b**
NotIdent4	1456.7	< 0.01	< 0.01	0.01±0	< 0.01	0.01±0
NotIdent5	1539.8	0.15±0.04	0.24±0.04	0.13±0.02	0.12±0.05	0.04±0
TMTT[b]	**1581.7**	**0.06±0.01a**	**0.57±0.44a**	**1.69±0.55b**	**0.40±0.26a**	**3.15±1.3b**
NotIdent6	1601.9	0.03±0.01	0.02±0.01	0.01±0	0.05±0.03	< 0.01

5

[a] Identification according to retention times and mass spectra in comparison with authentic standards

[b] Identification according to mass spectra and Kovats retention indices (KIs) in comparison with reference libraries and comparison of KIs on similar columns

Figure legends

Figure 1. Attraction of predatory mites by odours of tomato plants attacked by *Tetranychus evansi*. Bars represent the fraction of predatory mites choosing for the odours of plants infested with 20 (white bars) or 60 (grey bars) adult females of *T. evansi* per leaflet when volatiles of un-infested tomato plants were given as alternative odour source. A fraction of 0.5 would indicate no preference. Fractions were averaged (± SEM) over replicates (numbers given inside the bars), carried out using different sets of three plants and different groups of 20 mites. The volatiles from plants infested with 20 spider mites per leaflet were not significantly attractive to any of the species of predatory mites tested. The odours from plants infested with 60 females of spider mite per leaflet were attractive to *P. macropilis*, *P. persimilis* and to the population of *P. longipes* from Amsterdam.

Figure 2. Attraction of predatory mites by odours of tomato plants attacked by *Tetranychus evansi*. Bars represent the fraction of predatory mites choosing for the odours of plants infested with 20 (white bars) or 60 (grey bars) adult females of *T. evansi* per leaflet. Volatiles of tomato plants infested with *T. urticae* were given as alternative odours. A fraction of 0.5 would indicate no preference. Fractions were averaged (± SEM) over replicates (numbers given inside the bars), carried out using different sets of three plants and different groups of 20 mites. No significant preference to volatiles of plants infested with *T. evansi* were observed; the three species of predatory mites showed a significant preference to odours from plants infested with *T. urticae* instead. Data were analysed with a replicated goodness-of-fit test and the symbols following de bar denotes: *** $P < 0.001$;** $P < 0.01$; * $P < 0.05$; n.s.: not significant.

Figure 3. Attraction of predatory mites by odours of tomato plants attacked by *Tetranychus urticae*. Bars represent the fraction of predatory mites choosing for the odours of plants infested with 20 (white bars) or 60 (grey bars) adult females of *T. urticae* per leaflet. Volatiles of un-infested tomato plants were given as alternative odours. A fraction of 0.5 would indicate no preference. Fractions were averaged (± SEM) over replicates (numbers given inside the bars), carried out using different sets of three plants and different groups of 20 mites. The predatory mite *P. longipes* showed a trend of preference the for volatiles from plants attacked with *T. urticae*. The other two species (*P. macropilis* and *P. persimilis*) were significantly attracted to odours of plants infested with *T. urticae*. Data were analysed with replicated goodness-of-fit test and the symbols following de bar denotes: *** $P < 0.001$;** $P < 0.01$; * $P < 0.05$; • marginal significance, $P < 0.10$; n.s.: not significant.

Figures

Figure 1

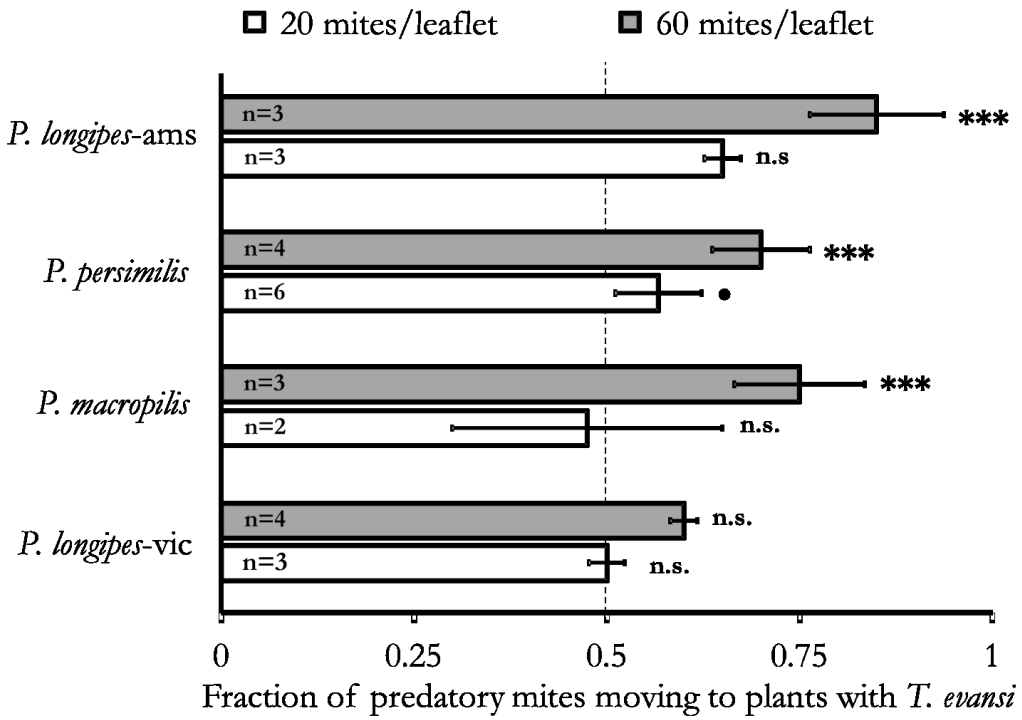


Figure 2

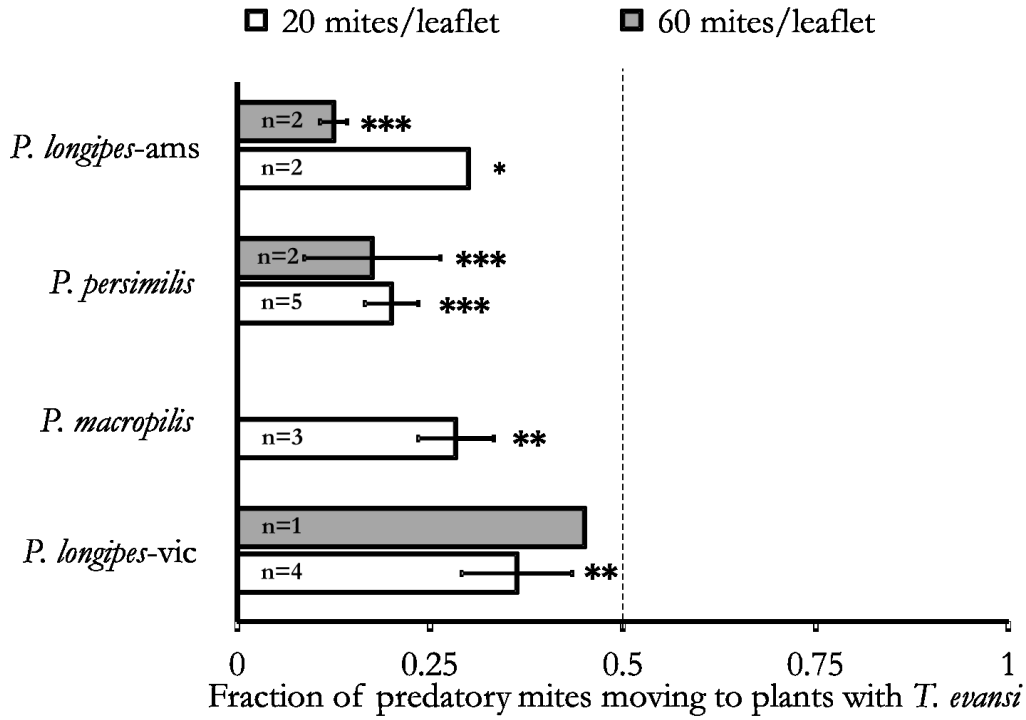
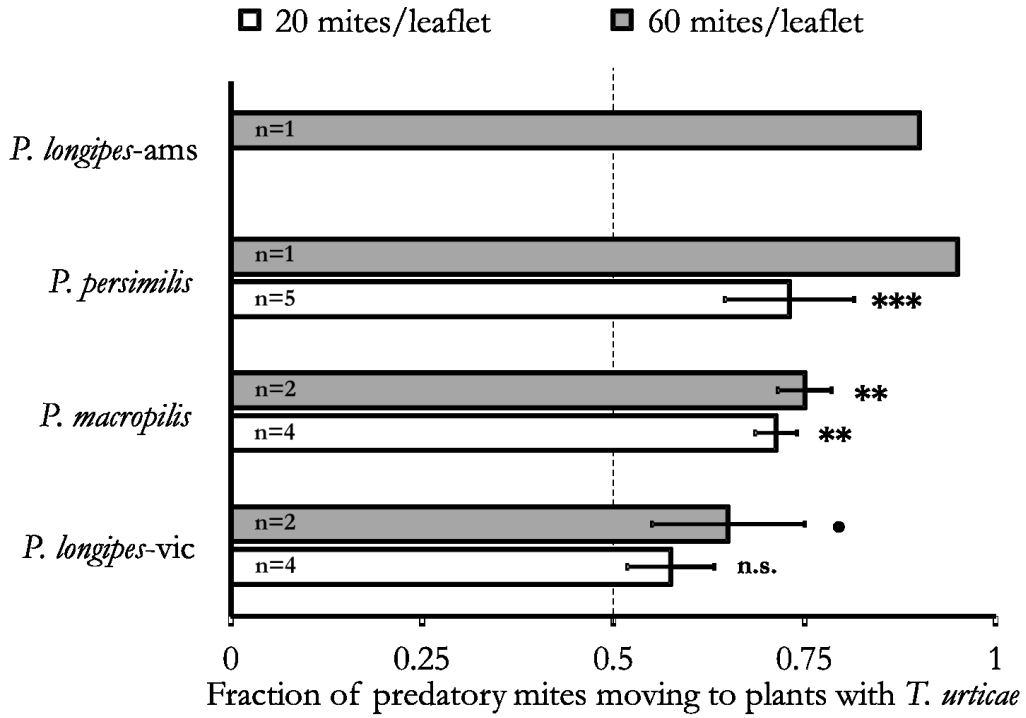

20 mites/leaflet
60 mites/leaflet
P. longipes-ams
P. persimilis
P. macropilis
P. longipes-vic
n=2
n=2
n=2
n=5
n=3
n=1
n=4
***
*
***
***
**
**
0
0.25
0.5
0.75
1
Fraction of predatory mites moving to plants with T. evansi

Figure 3


20 mites/leaflet
60 mites/leaflet
P. longipes-ams
P. persimilis
P. macropilis
P. longipes-vic
n=1
n=1
n=5
n=2
n=4
n=2
n=4
***
**
**
•
n.s.
0
0.25
0.5
0.75
1
Fraction of predatory mites moving to plants with T. urticae

CHAPTER III - The predatory mite *Phytoseiulus macropilis* is misled by spider mite-induced tomato volatiles

Title*: The predatory mite *Phytoseiulus macropilis* is misled by spider mite-induced tomato volatiles

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Abstract

Plants are not passive to attacks of herbivores and can defend themselves by producing chemical compounds or physical structures, which can directly or indirectly interfere with herbivores. A strategy of indirect defence employed by plants is the release of blends of volatile organic compounds after herbivory, which act as cues to carnivore forages find plants infested with prey. Different herbivores induce the production of different blends in the same host plant. To cope with the different volatile blends and the quality of prey, predators are expected to learn the association of each volatile signal with profitable prey to fine-tune their foraging behaviour. We studied the associative learning of the predatory mite *Phytoseiulus macropilis*. We used two species of prey, the two spotted spider mite *Tetranychus urticae* and the red spider mite *Tetranychus evansi*. First, we observed that *T. evansi* is a diet of inferior quality for *P. macropilis*, whereas *T. urticae* is a good diet. More than 88% of the juveniles of *P. macropilis* developed into adults after 6 days when feeding on eggs of *T. urticae* but none of the predatory mites fed with eggs of *T. evansi* reached adulthood. *Phytoseiulus macropilis* did not show significant innate preference when offered a choice between odours from plants infested with *T. evansi* or with *T. urticae*. We subsequently tested associative learning of *P. macropilis* between prey quality and plant volatiles. Adult females of *P. macropilis* were tested in olfactometer for volatiles from plants infested with *T. urticae* or with *T. evansi*. Predators that had chosen odours from plants with *T. evansi* were given *T. evansi* as prey. The same association was used for predators choosing odours from plants with *T. urticae*. Twenty-four hours later, the predators were tested again in the olfactometer with odours from new plants infested with either *T. urticae* or *T. evansi*. This procedure was repeated on four consecutive days. *Phytoseiulus macropilis* failed to show the association of prey quality with plant volatiles, even after having fed on *T. evansi* for four consecutive days. In conclusion, these results show

that *T. evansi* does not completely suppress the indirect defence of tomato plants. However, volatiles induced by *T. evansi* in tomato may result in a negative indirect interaction between plants and natural enemies, misleading *P. macropilis*.

Keywords: Indirect defence, insect learning, olfactometer, *Tetranychus evansi*,

Introduction

Upon attack by herbivores, plants can defend themselves with two different mechanism of induced defence (Karban and Baldwin 1997; Walling 2000). These mechanisms are the so called direct and indirect defences (Rhoades and Cates 1976; Price et al. 1980). The former is expressed by the plant as a direct action against herbivores through the production of trichomes (Levin 1973) or chemical compounds with antifeedant or even toxic effects on the herbivores (Ryan 1973). These alterations lead to a reduction of the performance of the herbivores feeding on the plants (Karban and Baldwin 1997). Indirect defence, instead, is based on an interaction between plants and carnivores. The plants can both attract and arrest carnivores by providing alternative food, shelter or cues (Dicke and Sabelis 1988; Dicke et al. 1990; Turlings et al. 1990). The attraction of carnivores is accomplice through the production of chemical cues known as volatile organic compounds, which can be used by members of the third trophic level to locate plants infested by herbivores (Dicke and Sabelis 1988; Turlings et al. 1990). As a consequence, the population of herbivores is reduced, thus protecting the plants (De Moraes et al. 1998; Kessler and Baldwin 2001).

Volatile organic compounds are continuously produced by plants (Kant et al. 2009), but when plants are attacked by herbivores, secondary metabolic pathways are elicited, which lead to production of mixtures of specific volatile compounds (Dicke and Sabelis 1988; Turlings et al. 1990; De Moraes et al. 1998). Each combination of plants of a certain species and particular herbivore species results in the production of a unique blend of plant volatiles, with qualitative and quantitative differences in the composition of the blends (Dicke et al. 1998; van den Boom et al. 2004). When these volatiles are released into the atmosphere, they can be perceived and used by both herbivores and natural enemies of the herbivores (Dicke et al. 2003). This release of odours, which can benefit the plants by attracting the natural enemies of the

herbivores, is called indirect induced defence of plants (Khan et al. 1997; De Moraes et al. 2001; Kessler and Baldwin 2001; Turlings and Ton 2006).

The idea behind indirect defence of plants is that this new blend of volatiles is a signal, which elicits a behavioural response in the natural enemies of the herbivores (*i.e.* attraction or arrestment) by establishing a channel of communication between the organisms of the first and third trophic levels (Scott-Phillips 2008; Allison and Hare 2009). However, to be an evolutionarily stable strategy, the indirect induced defence system of plants must be based on the release of honest signals (van Baalen and Jansen 2003). Such honest signals should reliably indicate that plants are infested with a profitable prey (van Baalen and Jansen 2003; Sabelis et al. 2007). However, there are many examples in the literature of predators and parasitoids being attracted to volatiles from plants infested with non-prey or non-host herbivores (Shimoda and Dicke 2000; van Poecke et al. 2003). This is observed mainly with natural enemies of generalist herbivores that feed on a large range of host plants species (Allison and Hare 2009). Since a given species of herbivore attacks different plants species, thus inducing the production of different blend of volatiles, it is suggested that natural enemies of generalist herbivores must cope with different signals that are all associated with the presence of their prey (van den Boom et al. 2002; van den Boom et al. 2004). It is therefore expected that these natural enemies have the ability to learn the association of each signal with the correct prey to fine-tune their foraging (De Boer et al. 2005; Allison and Hare 2009; Kant et al. 2009; Janssen et al. 2014).

Here, we investigated the ability of the predatory mite *Phytoseiulus macropilis* (Banks) (Acari: Phytoseiidae) to learn the association between two different odours produced by tomato plants attacked by two species of spider mites and the reward that each prey represents to them. This predatory mite has been described not to feed on

the red spider mite *Tetranychus evansi* Baker & Pritchard (Acari: Tetranychidae) (de Moraes and McMurtry 1985; Rosa et al. 2005), whereas they have a high performance when feeding on the two spotted spider mite *Tetranychus urticae* Koch (Acari: Tetranychidae) (Oliveira et al. 2007; Oliveira et al. 2009). Hence, they were expected to discriminate between volatiles of plants attacked by either of the two species.

Both spider mites cause damage to many crops and have a wide geographical distribution (Bolland et al. 1998; Navajas et al. 2013; Migeon and Dorkeld 2015). Besides this economic importance, *T. evansi* has captured the attention of ecologists because of its intriguing capacity of manipulating the defence system of tomato plants to its own benefit (Sarmento et al. 2011). In contrast to other herbivores such as *T. urticae*, *T. evansi* supresses defence in plants to such an extent that it results in increased performance on attacked plants compared to that on clean plants (Sarmento et al. 2011). Plants attacked by *T. evansi* show lower activity of defensive compounds such as proteinase inhibitor and no induction of defensive genes such as *WIPI-II*, *GGPS-1* and *PR-P6*, whereas these are all upregulated in tomato plants attacked by the closely related species *T. urticae* (Kant et al. 2004; Sarmento et al. 2011). The *GGPS-1* gene is involved in indirect plant defence, i.e. the production of the volatile compound (E,E)-4,8,12-trimethyltridecae-1,3,7,11-tetraene (TMTT), which is considered one of the main volatiles responsible for the attraction of predatory mites to plants attacked by spider mites (Kant et al. 2004; Sarmento et al. 2011). Indeed, the production of this volatile by plants attacked by *T. evansi* was not significantly different from that of clean plants, while plants attacked by *T. urticae* produced significantly more TMTT than un-infested plants (Sarmento et al. 2011).

Despite the suppression of volatiles and genes involved in the indirect defence system, odours from tomato plants attacked by *T. evansi* were found to be attractive

for predatory mites and ladybirds, as well for conspecifics (Sarmento et al. 2007; Sarmento et al. 2011). Hence, there is an apparent contradiction between the absence of induction of gene expression and volatile production by *T. evansi* and the attractiveness of plants attacked by this species (Sarmento et al. 2011). Moreover, the predatory mite *Phytoseiulus longipes* Evans (Acari: Phytoseiidae) did not show a preference when offered a choice between volatiles from plants infested with *T. evansi* or *T. urticae*.

In this study, we first assessed the performance of *P. macropilis* when feeding on *T. evansi*. Subsequently, we tested the preference of the predatory mites for *T. evansi* or *T. urticae* by recording their response to volatiles from plants with these herbivores and finally we measured their predation and oviposition rates on eggs of these spider mites. Lastly, we gave *P. macropilis* experience with *T. evansi* in the presence of the plant volatiles and studied whether such experience changed its response towards these volatiles. We expected that experiencing the association between the volatiles and a low-quality prey would result in reduced attraction of the predators to these volatiles.

Material and Methods

Organisms

Every week, we sowed tomato seeds (*Solanum lycopersicum* var. Santa Clara I-5300) in a commercial plant substrate (Bioplant®) in polystyrene trays (8 x 16 cells) in a greenhouse. We transplanted the seedlings to plastic pots (2 L) with soil substrate after the first pair of leaves had expanded, approximately twenty days after sowing. The plants were watered daily and were fertilized with 20g of N-P-K (20-05-20) and 40g of superphosphate simple in 20 L of water every seven days.

We obtained the populations of spider mites (*T. evansi* and *T. urticae*) in 2002 from a natural infestation of tomato plants in a greenhouse in Viçosa, Minas Gerais, Brazil. Herbivores were reared on tomato leaves with their petioles inserted in a PVC tube filled with water to prevent desiccation of the leaves. Tubes with infested leaves were kept in PVC trays filled with detergent and water (1:50, v/v), which served to prevent mite escapes and invasion of mites and other non-flying arthropods. The mass culture was maintained in a rearing room (25 ± 3 ºC, 70 – 90 % relative humidity) with controlled photoperiod (12:12 L:D).

We started the laboratory culture of the predatory mite *P. macropilis* with individuals collected from a palm tree infested with *Tetranychus* sp. on the campus of the Federal University of Viçosa in 2012. Colonies were maintained in the laboratory (25 ± 5 ºC, 70 ± 20 % relative humidity), fed at least three times per week with *T. urticae* on tomato leaves from the stock colony spider mite.

Performance experiments

To test the quality of *T. evansi* and *T. urticae* as food for *P. macropilis,* we measure the developmental time and survival of juvenile predatory mites feeding on these two spider mites species. Leaf discs (diameter 2.4 cm) were cut from tomato leaflets and

arranged on wet filter paper, positioned on wet foam inside a tray (12.5 x 7.5 x 2.5 cm) with water. We then infested the leaf discs with 20 adult females of *T. evansi* or *T. urticae*. The trays with the discs were incubated in a room with controlled temperature (23 ± 2 ºC) and photoperiod (14:10 L:D). The females were allowed to feed and oviposit for 24 hours. Subsequently, the females and the web were removed from the discs while the eggs remained, and one egg of *P. macropilis* was added to each disc. The eggs of the predatory mites were taken from a cohort of 1 day old. To prepare this cohort, we took around 30 adult females of *P. macropilis* from the mass rearing and transferred them to tomato leaflets infested with *T. urticae* arranged on wet cotton on Petri dishes. The predatory mites were allowed to feed and oviposit for 24 hours and then we collected these eggs using a fine brush and transferred them to the leaf discs with spider mite eggs.

Using a stereoscopic microscope, we observed the development and survival of the juveniles from eggs to adult once per day. Differences in development and survival were tested using a time-to-event analysis with Cox proportional hazards regression model using the function "coxph" of the library "survival" in R© (R Development Core Team 2015).

Prey preference of P. macropilis

To observe the preference of *P. macropilis* for *T. evansi* or *T. urticae*, we conducted two different experiments. First, the response of the predatory mites for volatiles from plants attacked by either *T. evansi* or *T. urticae* was observed in an olfactometer experiment. Secondly, predation and oviposition rates of *P. macropilis* were observed on tomato leaf discs containing eggs of each species of the two herbivores spatially separated on two halves of a leaf discs.

We used a Y-tube olfactometer to observe the response of the predatory mites to volatiles from plants infested with spider mites (Sabelis and van de Baan 1983). The olfactometer consists of a glass tube (3.5 cm diameter) in the shape of a "Y". Each of the two arms of the Y-tube was connected with plastic tubes to a glass container (43 x 36 x 50 cm) in which the volatile sources were arranged. The base of the Y-tube was connected through plastic tubes to a vacuum pump, which was used to generate a constant air flow. The air entered the glass containers through an air inlet, passed over the plants and then carried the odours through the plastic tubes to the Y-tube. The air flow in each arm of the olfactometer was calibrated to 0.50 m/s (VelociCalc® Air Velocity Meter 9545-A). Each odour source consisted of four tomato plants that were infested for seven days with either *T. evansi* or *T. urticae*. Previous experiments showed that seven days of infestation were enough to induce the production of volatiles (Sarmento et al. 2007; Sarmento et al. 2011). To infest plants, we put infested leaflets taken from stock colonies on the leaves of the experimental plants (approximately 1-2 leaflets per plant leaf). The infested leaflets were left on the plants for 3-4 days to allow mites to move to the plants. The plants were put in the glass containers at least 12 hours prior to the tests. In each trial, twenty adult females of *P. macropilis* were tested and the experiments were repeated four times.

The predatory mites were tested individually in the olfactometer. Using a fine brush, each female was put on a metal wire inside the Y-tube after disconnecting vacuum pump. The metal wire serves as a bridge positioned in the centre of the Y-tube, connecting its base to the end of each arm. After reconnecting the vacuum pump to the base of the Y-tube, the predatory mites were observed for 5 minutes. When the females reached the end of one of the arms of the Y-tube within this period, it was considered a response. Females that did not respond in five minutes were recorded but not included in the calculation of the preferences (2 out of 82 *P. macropilis* tested).

The predatory mites were taken from the rearing and were starved for at least two hours prior to being tested. After being tested, the predatory mites were killed to avoid accidental testing of the same individual in a next trial.

For the small-scale preference experiment, leaf discs (2.4 cm diameter) were cut from the basal part of tomato leaflets and arranged on wet filter paper, positioned on wet foam inside a tray (12.5 x 7.5 x 2.5 cm) filled with water. Leaf discs were cut so that the midrib divided them into two halves. A thin yarn of wet cotton wool was placed along the midrib with the ends touching the wet filter paper. This connection with the water kept the cotton yarn wet and prevented the spider mites from crossing to the other disc half. Subsequently, the leaf disc halves were infested with 20 *T. evansi* or 20 *T. urticae* females, which were allowed to oviposit and produce web for 24 hours. Subsequently, the females and web were removed with a thin brush. We then counted the number of spider mite eggs on each disc half and removed the cotton yarn from the midrib. Subsequently, we inserted an entomological pin at the centre of the disc, drilling the leaf midrib. With a fine brush, we took an adult female of *P. macropilis* from the laboratory colony and released her on the head of the pin. The age of the females tested was not standardised, but we took only mites with an expanded opisthosoma, indicating they were carrying a (nearly) full-grown egg. After 24 hours, the numbers of spider-mite eggs remaining and *P. macropilis* eggs laid on each disc half were recorded. Each replicate (from a total of 17) consisted of the response of one predatory mite on one disc.

The data of the olfactometer experiment were analysed with a log-linear model for contingency tables with Generalized Linear Models (GLM) using a Poisson error distribution (Crawley 2013). For the leaf disc experiment, we used a GLM with a binomial error distribution to test if there was a difference in the numbers of *T. evansi*

and *T. urticae* eggs preyed by *P. macropilis*. The numbers of eggs laid on each disc half were compared with a GLM with a Poisson error distribution. All analyses were performed with the statistical software R© (R Development Core Team 2015).

Learning experiments

To test whether *P. macropilis* could discriminate the volatiles of plants infested with *T. evansi* from the volatiles of plants infested with *T. urticae* after a period of experience with these prey, we conducted a series of associative learning experiments, using the olfactometer. The learning experiments consisted of giving *P. macropilis* consecutive feeding experiences with the two prey species that differ in quality as food to predator. On the first day of the experiment, females of *P. macropilis* were taken from the mass rearing and kept in Petri dishes without food for at least two hours prior to being tested in the olfactometer. We tested the preference of *P. macropilis* to odours from tomato plants infested by either *T. evansi* or *T. urticae* during four consecutive days. The plants were infested with spider mites as described before.

After being tested, each predator was collected at the end of the Y-tube and transfred to a tomato leaflets infested with all stages and web of the spider mite species it had chosen in the olfactometer. The infested leaflets were arranged on wet foam inside plastic pots (500 mL). To seal the pots while allowing the flow of air between the pot and the environment, we used plastic lids with a circular hole in the centre, covered with a fine mesh. The pots were arranged inside the glass containers together with the same plants that were previously used as odour sources. Thus, the predatory mites were allowed to feed on the chosen species while experiencing the odours from the plants and the leaflets for at least 12 hours prior being tested again. To avoid high humidity, clean air was pumped through the interior of the glass containers at a constant flow (5 litres per minute). The next day, the predatory mites were tested again,

using new infested plants as odour sources. This procedure was repeated on four consecutive days. In this way, we followed the consecutives choices of the predatory mites after experiencing a period of feeding on the prey species that were chosen based on plant volatiles. The expectation was that the predators would associate the feeding on an inferior prey species with one volatile blend, and feeding on a superior prey species with the other blend, and that the preference for this latter blend would increase throughout the experiment.

The learning experiment was carried out in two series. The first series was started with 180 individuals and the second with 120 individuals of *P. macropilis*. Since no significant difference was observed in the proportion of predatory mites choosing each volatile source between the two series of the experiment (GLM, $F_{1,55} = 2.24$, $P = 0.14$), we pooled the data of the two series for the statistical analysis. The data were analysed with a log-linear model for contingency table analysis with GLM with a Poisson error distribution. We tested whether there was a significant difference in the number of predatory mites choosing each odour source and whether the previous choices affected the next choice (Crawley 2013). The analysis was performed with the statistical software R© (R Development Core Team 2015).

Results

Development and survival of P. macropilis

88.5% of all *P. macropilis* were adult on the 6th day after egg hatching when fed with eggs of *T. urticae* (Figure 1). In contrast, all the mites died before the 9th day and none of them became adult when fed with eggs of *T. evansi* (Figure 1). Not surprisingly, the survival to adulthood of *P. macropilis* fed with *T. evansi* was significantly lower than on a diet of *T. urticae* (Likelihood ratio = 4.04, d.f. = 1, P = 0.04).

Prey preference of P. macropilis

The predators did not show a significant preference for volatiles from plants infested with *T. evansi* or *T. urticae* (GLM, Deviance = 0.45, d.f. = 1, P = 0.50). Out of 80 females tested, 53% chose volatiles from tomato plants infested with *T. evansi*. No significant difference between the replicates was observed, indicating homogeneity of the responses among the four replicates (GLM, Deviance = 5.99, d.f. = 3, P = 0.11).

Phytoseiulus macropilis showed a significant preference to lay eggs on the leaf disc halves with eggs of *T. evansi* instead of *T. urticae* (GLM, Deviance = 38.29, d.f. = 1, P < 0.01, Figure 2). However, the predation rate of eggs of *T. urticae* or *T. evansi* did not differ significantly (GLM, $F_{1,32} = 0.51$, P = 0.48, Figure 2).

Learning in P. macropilis

Phytoseiulus macropilis showed a slight, but significant preference for volatiles from plants infested with *T. evansi* on the first day of this experiment (GLM, Deviance = 12.91, d.f. = 1, P < 0.01, Figure 3). Of all the 300 predatory mites tested on the first day of the experiment, 60.33% showed a preference for volatiles from plants infested with *T. evansi*.

On the second day of the experiment, 52.34% of the predatory mites chose volatiles from plants infested by *T. urticae*, and there was no significant preference (GLM, Deviance = 0.56, d.f. = 1, P = 0.45). Furthermore, no significant interaction between the choice of the predatory mites on the first and second days was observed (GLM, Deviance = 0.98, d.f. = 1, P = 0.32). The lack of such an interaction indicates that the experience given to the predatory mites on the first day did not influence the choice on the second day.

On the third day, there was again no significant preference for any of the two odour sources: 48% of all the mites chose volatiles from plants infested with *T. urticae* (GLM, Deviance = 0.32, d.f. = 1, P = 0.57). Again no significant interaction between the previous choices made by the predatory mites and the choice on the third day was observed (GLM, Deviance = 0.23, d.f. = 1, P = 0.89).

On the fourth day, again no significant interaction between the previous choices and the present choice made by the predatory mites was observed (GLM, Deviance = 3.22, d.f. = 1, P = 0.67). Forty-six percent of the predatory mites chose volatiles from plants infested with *T. urticae*, showing no significant preference (GLM, Deviance = 0.82, d.f. = 1, P = 0.36).

Discussion

The predatory mite species tested here did not show a preference for volatiles from plants infested with either *T. urticae* or *T. evansi*. Although *T. evansi* is a prey of poor quality, the adult females of this predatory mite were attracted by volatiles from plants infested with this spider mite, even after consecutive feeding experiences associated with these volatiles.

Even with *T. evansi* not being a suitable prey, it was observed that *P. macropilis* has a significant preference for the volatiles from tomato plants infested with this spider mite when tested against volatiles from clean plants (Sarmento et al. 2011). One could argue that it is adaptive for predatory mites to have an innate response to odours from plants infested with any herbivore rather than move towards a non-infested plant. For instance, when the predatory mite *P. persimilis* was tested for preference between volatiles from plants damaged by caterpillar of *Spodoptera exigua* (Hubner) (Lepidoptera: Noctuidae) or from clean plants, predators showed a significant preference for odours induced by the non-prey herbivore (De Boer and Dicke 2006). However, this is not a rule. Fadini et al. (2010) observed that *P. macropilis* avoided volatiles from strawberry plants infested with the red mite *Oligonychus ilicis* (McGregor) (Acari: Tetranychidae), showing a significant preference to volatiles from non-infested plants. As with *T. evansi*, a diet of *O. ilicis* did not allow the development of *P. macropilis* juveniles (Fadini et al. 2010). These examples illustrate how variable can be the innate response of predators to odour from plants infested with non-prey herbivores.

Tetranychus evansi is known to downregulate the signalling cascade of both jasmonic acid and salicylic acid in tomato (Sarmento et al. 2011; Alba et al. 2015). Besides suppressing the direct defence in attacked plants, *T. evansi* also downregulates genes involved in production of volatile terpenes (Alba et al. 2015). For instance, *T. evansi* suppresses the gene *GGPS-1*, which transcribes to enzymes involved in the

synthesis of the attractive monoterpene TMTT (Ament et al. 2006; Sarmento et al. 2011). As a consequence, this volatile organic compound is present in basal levels in odours from plants infested with *T. evansi*, whereas its concentration is significantly higher in odours from plants infested with *T. urticae* (Sarmento et al. 2011). Additionally, a screening of the volatiles from plants infested with *T. evansi* and *T. urticae* showed a differential induction of compounds by these two herbivores (Chapter II). These blends differ in concentration of at least nine compounds (Chapter II).

Despite this difference in blends, *P. macropilis* did not show a preference when offered a choice between volatiles from plants infested with *T. urticae* or volatiles of plants with *T. evansi*. Similar results were observed with the predatory mite *P. longipes* and the ladybird *Cycloneda sanguinea* (L.) (Coleoptera: Coccinellidae) (Sarmento et al. 2011; Fonseca 2012). However, in Chapter II we observed that volatiles from tomato plants infested with a low density of *T. evansi* were not attractive to three species of predatory mites (*P. longipes*, *P. macropilis* and *P. persimilis*) while odours from plants highly infested with this spider mite were attractive. Moreover, the predatory mites *P. macropilis* and *P. persimilis* showed a preference for volatiles from plants infested with *T. urticae* when tested against odours from plants with *T. evansi* with the same level of infestation (Chapter II).

The difference among these results may be a consequence of the different levels of herbivory among the experiments. It is know that *T. evansi* can cause more damage on tomato leaves than *T. urticae* causes (Alba et al. 2015). Additionally, tomato plants respond differentially to different densities of infestation. For instance, plants infested with 60 spider mites (*T. evansi*) per leaflet were attractive to predatory mites and released volatiles that were not induced by plants infested with 20 spider mites per leaflets, which were not attractive to predatory mites (Chapter II). In our experiments,

plants were infested with an unknown number of mites per plant. After seven days of infestation, plants were visually damaged by spider mites and we never noticed any clear difference in population size and leaf damage caused by *T. evansi* and *T. urticae*. However, it is possible that the methodology of infestation employed by us here has led to important differences in levels of plants infestation. As higher levels of plant infestation with *T. evansi* induce attractive odours (Chapter II), it is possible that in our experiments plants harbouring *T. evansi* had significantly more individuals than plants harbouring *T. urticae*.

Another possible explanation for the absence of preference between these two volatile blends is related with the ecological characteristics of the predatory mite tested here. *Phytoseiulus macropilis* is considered a specialized predator of spider mites of the genus *Tetranychus* (McMurtry and Croft 1997; Gerson et al. 2003). In Brazil, *P. macropilis* has been described associated with *T. urticae*, which is a generalist herbivore, feeding from more the 1.000 plant species (Migeon and Dorkeld 2015). Predatory organisms that feed on different prey species or on prey that have a wide range of host plants must cope with the challenge of being able to detect, and discriminate between, different blends of volatiles (van den Boom et al. 2002; Sabelis et al. 2007; Allison and Hare 2009). They are therefore expected to show a general innate response to a large range of volatile blends (van den Boom et al. 2002), which can subsequently be fine-tuned through associative learning (Steidle and van Loon 2003). However, we did not observe associative learning by *P. macropilis*, even after having fed on *T. evansi* for four consecutive days. Associative learning of volatiles and prey quality in predators would be observed as an increase in the number of predatory mites avoiding/choosing volatiles from plants infested with *T. evansi*/*T. urticae* after having experienced feeding on *T. evansi*. However, the fractions of *P. macropilis* choosing *T. evansi* or *T. urticae* were

not significantly different from 0.5:0.5 along the four days of odour-prey associative experiences.

In contrast, such an ability to learn the association of food with plant volatiles has been demonstrated in the related species *Phytoseiulus persimilis* Athias-Henriot (Acari: Phytoseiidae (Drukker et al. 2000; De Boer and Dicke 2004). Both *P. persimilis* and *P. macropilis* are known to attack spider mites, in particular *T. urticae*, on many different host plants (Gerson et al. 2003). Hence, there is no obvious difference in the ecology of both species that would explain large differences in the ability to learn the association between herbivore-induced plant volatiles and the quality of the food. Moreover, to our knowledge, this is the first attempt to study learning in *P. macropilis* and we cannot argue that this species is not able to learn.

In the small-scale choice test, *P. macropilis* did not show preference for preying on eggs of *T. evansi* or *T. urticae*. However, the females showed a significant preference to oviposit on disc halves with eggs of *T. evansi*. The preference for *T. evansi* could be a strategy to avoid hyper-predation: *T. evansi* does not have as many natural enemies as *T. urticae* (de Moraes and McMurtry 1985; Rosa et al. 2005; Navajas et al. 2013) and produces a dense web that cannot be penetrated by many predators (Moraes and Lima 1983; Venzon et al. 2009). Therefore, ovipositing in patches of *T. evansi* may protects the offspring of *P. macropilis* from intraguild predation. Indeed the web of *T. evansi* was observed to give protection against intraguild predation by *P. macropilis* to immature *P. longipes* (Lemos et al. 2015).

In conclusion, the volatiles released by plants infested with *T. evansi* can mislead the predatory mite *P. macropilis*. Besides the difference in volatiles induced by *T. evansi* and *T. urticae*, *P. macropilis* did not show associative learning between these odours and prey quality. This shows that *T. evansi* does not completely suppress the indirect

defence of tomato plants. However, this induction of volatiles by *T. evansi* may not result in reliable communication between plants and natural enemies.

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Figure legends

Figure 1. The development from egg to adult of *P. macropilis* fed on eggs of *T. evansi* or *T. urticae*. On a diet of *T. evansi*, none of the predators survived beyond the 9th day and none reached adulthood. The results are expressed as mean proportions (±S.E.M.) of alive individuals that reached adulthood.

Figure 2. Prey preference of *P. macropilis* for eggs of *T. evansi* or *T. urticae*. Bars indicate the mean number (± S.E.M.) of eggs preyed ("Predation") and mean numbers (± S.E.M.) of eggs laid by *P. macropilis* on the disc halves with either of the two prey species (*T. evansi* in white and *T. urticae* in grey).

Figure 3. Result of the series of olfactometer experiments with repeated testing of *P. macropilis*. Pie charts indicate the proportions of predatory mites, which chose volatiles from plants infested with *T. evansi* (white) or *T. urticae* (grey). Numbers inside pie halves indicate the number of predatory mites that chose for each volatile cue. The day of the experiment is indicated at the bottom of the graph. Combinations of letters at the left indicate the history of choices made by this specific group of predatory mites (i.e. UEEE means that these predatory mites chose *T. urticae* on the first day and *T. evansi* on the following three days). There was a significant preference for the volatiles from plants infested by *T. evansi* on the first day of the experiment (indicated by the asterisk).

Figures

Figure1

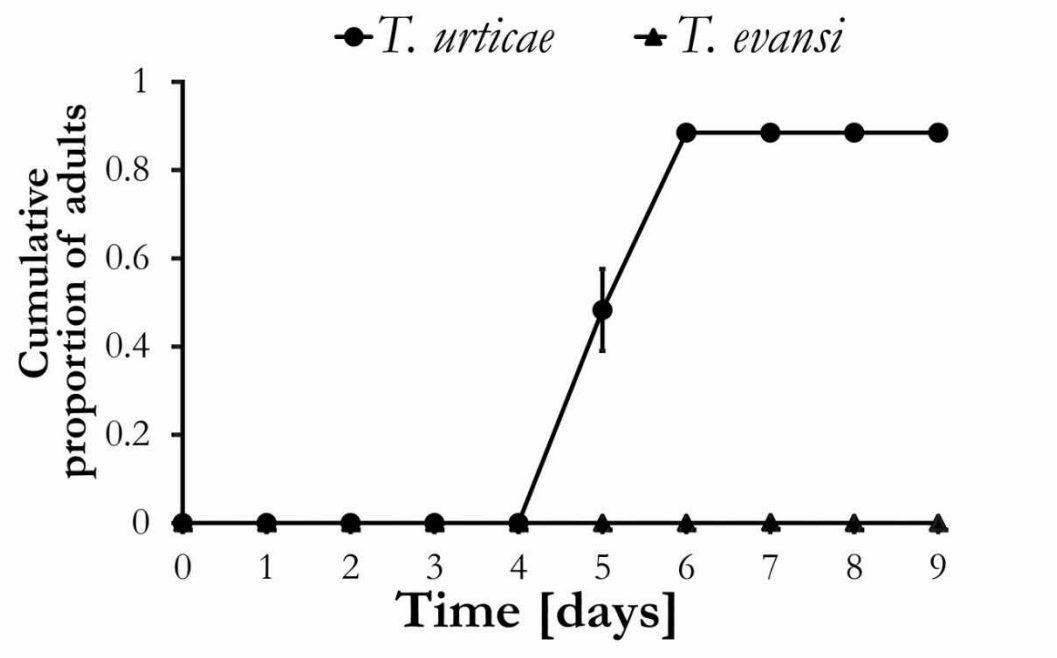


Figure 2

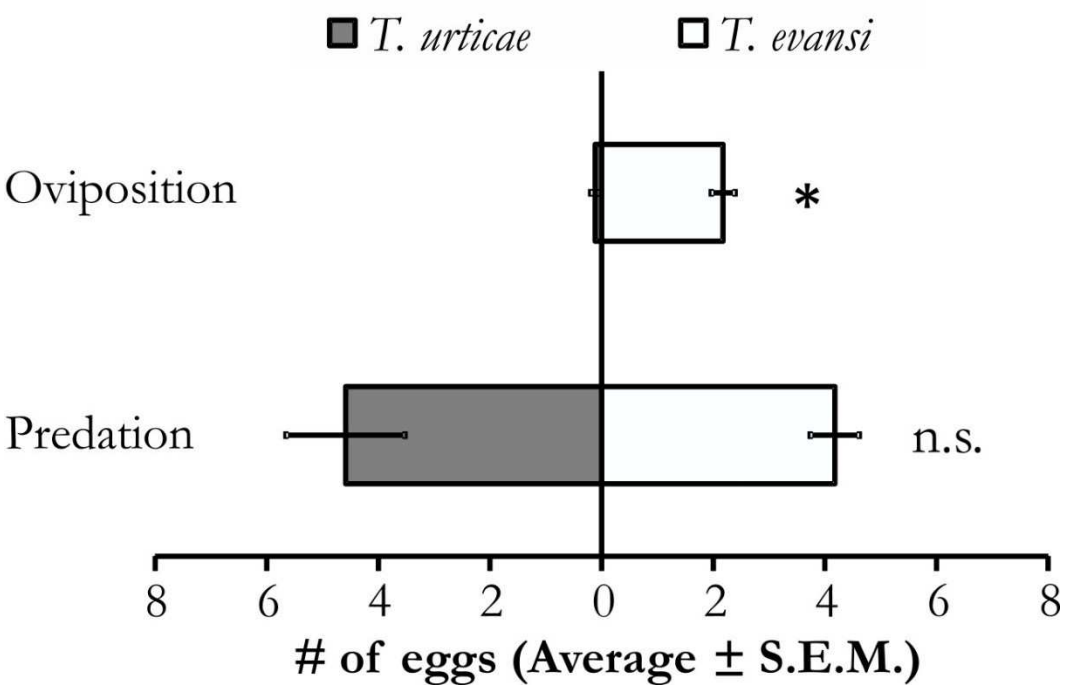
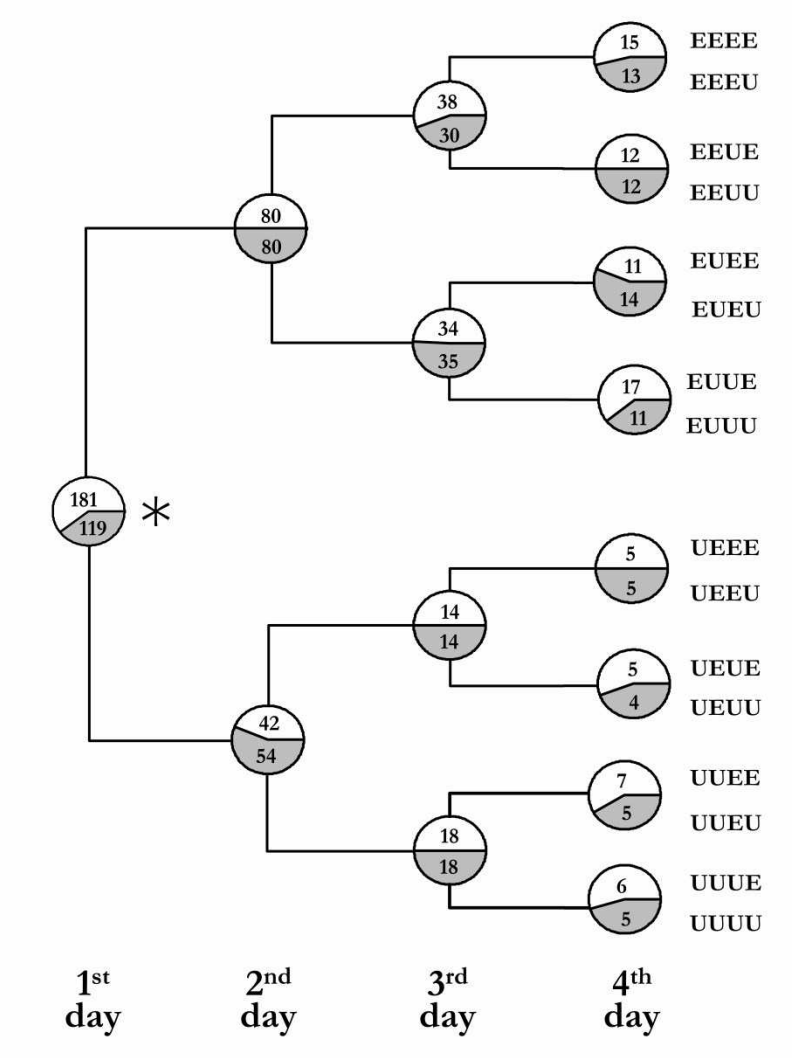

T. urticae
T. evansi
Oviposition
*
Predation
n.s.
8
6
4
2
0
2
4
6
8
# of eggs (Average ± S.E.M.)

Figure 3


15
13
EEEE
EEEU
38
30
12
12
EEUE
EEUU
80
80
11
14
EUEE
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34
35
17
11
EUUE
EUUU
181
119
*
5
5
UEEE
UEEU
14
14
5
4
UEUE
UEUU
42
54
7
5
UUEE
UUEU
18
18
6
5
UUUE
UUUU
1st day
2nd day
3rd day
4th day

General Conclusions

Chemical compounds from plant secondary metabolism can act as defence against herbivores. However, these toxic plant compounds may also mediate indirect interactions between plants and the natural enemies of their herbivores. Apparently, this was not the case for the interaction between *T. evansi* and tomato plants. Plant quality did not indirectly affect the predators of *T. evansi*. However, this indirect interaction between plant defence and natural enemies of herbivores may occur with other species of herbivores as *T. urticae*. The performance of the predatory mites feeding on *T. urticae* is different depending on the host plants used by its prey.

The chemical compounds involved in direct plant defence are not the only ones that mediate indirect interactions with natural enemies. The volatile organic compounds from plants are well known to be part of the plant mechanism of indirect defence, connecting plants and the natural enemies of herbivores. To ensure a higher fitness to plants attacked by herbivores, plant volatiles must be reliable enough and attractive to natural enemies of these herbivores. However, herbivores may manipulate the indirect defence of their host plant. *Tetranychus evansi* is known to manipulate the direct defence of tomato plants and it is also able to manipulate the induction of volatile organic compounds on attacked plants.

The manipulation of indirect defence by *T. evansi* is restricted to the level of herbivory. High levels of infestation by *T. evansi* make these plants attractive to predatory mites. However, these volatile are attractive to predatory mites that cannot develop when preying upon *T. evansi*. This exemplifies a disruption of indirect defence of plant triggered by the induction of the production of attractive volatiles that do not provide reliable information to natural enemies.

Together, these results confirm the amazing ability of *T. evansi* to manipulate the plant defence and circumvent the threat of natural enemies. The ecological implications from these manipulation remains to be explored and the mechanism used by *T. evansi* still needed to be understand in details. However, the threat that *T. evansi* represents to agriculture, especially regarding the implementation of an efficient biological control program, is even clearer.