BUGS IN ODOUR SPACE

How predatory mites respond to variation in herbivore-induced plant volatiles

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CONTENTS

ABSTRACT

In foraging for food, animals need information. Many natural enemies of herbivorous arthropods use herbivore-induced plant volatiles to locate their prey. The composition of a blend of herbivore-induced plant volatiles can vary with plant and herbivore species, and with other biotic and abiotic conditions. In this thesis I address the effects of variation in the composition of blends of herbivore-induced plant volatiles on the behavioural responses of a predatory mite and the role of phenotypic plasticity in predator responses.

To date, most studies on information use in tritrophic systems had been carried out in the laboratory in an odour-free background. However, in the field, the volatiles emitted by plants infested with suitable prey herbivores can mix with the volatiles from other plant-herbivore combinations. Moreover, a single plant may be infested with more than one herbivore species at the same time. Therefore, in the first part of this thesis I investigated the responses of the predatory mite *Phytoseiulus persimilis* to plant volatiles induced by its prey *Tetranychus urticae*, in an environment with volatiles induced by non-prey herbivores. The attraction of *P. persimilis* to volatiles from *T. urticae*-infested lima bean plants was not hampered by mixing with volatiles from *Pieris brassicae*-infested brussels sprouts plants, *Spodoptera exigua*-infested cucumber or lima bean plants. This was demonstrated in a Y-tube olfactometer set-up in the laboratory as well as in a 'semi-field' set-up in a greenhouse. Predatory mites were attracted significantly more to lima bean or cucumber plants that were infested with the combination of spider mites *T. urticae*, and caterpillars *S. exigua*, than to plants that were infested with one of the herbivore species alone.

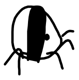
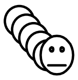
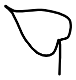
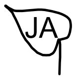
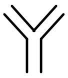
So far, it remained unknown which components of herbivore-induced volatile blends are of major importance in the foraging behaviour of the natural enemies of herbivorous arthropods. In part II of this thesis I demonstrate an important role for methyl salicylate in the foraging behaviour of *P. persimilis*. This compound attracts the predatory mites in a dose-dependent way. A qualitative, but not a quantitative, difference in methyl salicylate can explain the response of *P. persimilis* when it is offered a choice between two similar odour blends that differ in the presence of this compound. Both methyl salicylate and the terpenoid (3*E*,7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene can enable *P. persimilis* to discriminate between volatiles from lima bean plants infested with their prey *T. urticae* or the non-prey *S. exigua*. The spider mites induce significantly larger amount of these two compounds than the caterpillars. These are the first studies to demonstrate a significant role in the foraging behaviour of carnivorous arthropods for specific volatile compounds *within* a natural herbivore-induced volatile blend. In addition, 2-butanone was identified as an attractive compound but it did not play a role in enabling predatory mites to discriminate between volatiles from lima bean plants infested with *T. urticae* or *S. exigua*.

The third part of this thesis concentrated on the effect of previous experiences of predatory mites with herbivore-induced plant volatiles on their subsequent foraging behaviour. Phenotypic plasticity is expected because *P. persimilis* is confronted with highly variable

information about the presence of its prey. I showed that the plant species (either lima bean or cucumber) on which predatory mites developed and spent the first days of the adult phase influenced their attraction to: (1) the single compound methyl salicylate, (2) their choice between two volatile blends that differed in the presence of methyl salicylate, (3) their attraction to volatiles from lima bean or cucumber plants infested with the non-prey caterpillar *S. exigua*, and (4) their choice between the volatiles from lima bean plants infested with *T. urticae* or *S. exigua*. During the adult phase a non-rewarding experience (i.e. absence of prey) with volatiles induced by *S. exigua* had a small effect on the behaviour of *P. persimilis*, while a rewarding experience (i.e. presence of prey) with volatiles induced by *T. urticae* had a larger effect on its behaviour. Multiple experiences, i.e. a non-rewarding experience followed by a rewarding experience, had the strongest impact on the choices of *P. persimilis* between the volatiles from lima bean plants infested with *T. urticae* or *S. exigua*.

LIST OF SYMBOLS

Explanation of symbols that are used frequently in this thesis

	Predatory mite *Phytoseiulus persimilis*
	Two spotted spider mite *Tetranychus urticae*
	Beet armyworm *Spodoptera exigua*
	Lima bean *Phaseolus lunatus*
	Cucumber *Cucumus sativus*
	Lima bean infested with *T. urticae*
	Brussels sprouts infested with the cabbage white *Pieris brassicae*
	Cucumber infested with *S. exigua*
	Lima bean infested with *S. exigua*
	Lima bean treated with jasmonic acid
	Y-tube olfactometer
	Eppendorf vial

CHAPTER 1

General Introduction

Information

Information mediates all interactions between organisms, such as finding mates and food, or avoiding competitors and predators. The information can be visual, chemical, auditory or tactile: many arthropods use chemical cues (for example sex pheromones) to find a mate while birds often use visual (for example brightly coloured plumage) and auditory cues; vertebrate predators often use visual cues to locate their prey whereas many arthropod predators use chemical information. An important aspect of information use is discrimination of relevant signals from background noise. This idea is easy to grasp for auditory cues: it is harder to hear someone in a loud discotheque than in a quiet restaurant. Similarly, natural background noise can influence the ability of female frogs to discriminate between the advertisement calls of different conspecific males (Wollerman & Wiley 2002). The same applies to chemical cues. In their use of chemical information, animals need to filter out the relevant signals from the irrelevant background noise; for example predators need to discriminate between cues related to prey and cues that are not related to suitable prey. The topic of my thesis is the use of chemical information by carnivorous arthropods in simplified food webs consisting of plants, herbivorous and carnivorous arthropods, also called tritrophic systems.

Using indirect information to locate prey

In a tritrophic system, carnivores can use information from different sources to locate their herbivorous victims: information from the prey itself or information from the prey's food plant. Visual or chemical cues from the prey itself, e.g. from faeces, can give highly reliable information about prey presence and identity. However, these cues are often difficult to detect because arthropod herbivores are small components in the environment and they are under strong selection not to reveal themselves (e.g. Turlings *et al.* 1991; Vet & Dicke 1992; Du *et al.* 1996). Plants, on the other hand, are much larger than herbivorous arthropods and are therefore easier to detect both visually and chemically. Although information from plants is well detectable, in general it is a less reliable indicator of prey presence because plants also emit visual and chemical cues in the absence of suitable herbivores. However, the reliability of information from plants can be greatly enhanced when plants respond to herbivory with the emission of more specific information, for example visually via holes in plant tissue or yellow colouring, or chemically via the emission of volatiles that can be released by plants upon herbivore damage. The ability of plants to emit these so-called herbivore-induced plant volatiles has been shown for many plant species in a range of plant families, including Brassicaceae, Cucurbitaceae, Fabaceae, and Solanaceae (e.g. Dicke 1999a; Dicke & Vet 1999). Undamaged parts of a herbivore-damaged plant can also emit induced volatiles, and this systemic emission of volatiles increases the total amount of information emitted by the plant and thereby the detectability for carnivores (Turlings & Tumlinson 1992; Dicke *et al.* 1993; Potting *et al.* 1995; Röse *et al.* 1996). Herbivore-induced plant volatiles are used as foraging cues by a diverse range of carnivore species, including parasitoid wasps, and predatory bugs and mites (e.g. Sabelis & Van de Baan 1983; Dicke *et al.* 1990a; Turlings *et al.* 1990; Steinberg *et al.* 1992; Scutareanu *et al.* 1997; Van Loon *et al.* 2000a). These carnivores can reduce the herbivore pressure on plants and thereby have a positive effect on plant fitness (Dicke & Sabelis 1989; Van Loon *et al.* 2000b; Fritzsche Hoballah & Turlings 2001). Both plants and carnivores can thus benefit from herbivore-induced plant volatiles and therefore it can be expected that plants are under selection to emit a signal that attracts effective carnivores, while the carnivores, in turn, are under selection to optimise their responses to these plant signals (Vet 1999a).

How do carnivores use herbivore-induced plant volatiles to locate their prey?

Blends of herbivore-induced plant volatiles consist of mixtures of compounds, some of which are passively released and some of which are synthesised *de novo* upon herbivory (Paré & Tumlinson 1997a). Several factors influence the composition of the blends of volatiles released by herbivore-damaged plants: plant species or genotype, plant tissue and age, time of the day, herbivore species and developmental stage, attack by a second herbivore species or pathogen, and abiotic factors (e.g. Takabayashi et al. 1991;Turlings *et al.* 1993a; Takabayashi *et al.* 1994a; Loughrin *et al.* 1994, 1995; Takabayashi *et al.* 1995; Turlings *et al.* 1995; Geervliet *et al.* 1997; DeMoraes *et al.* 1998; Turlings *et al.* 1998; Dicke 1999a; Halitschke *et al.* 2000; Maeda *et al.* 2000; Shiojiri *et al.* 2001; Cardoza *et al.* 2002; Fritzsche Hoballah *et al.* 2002; Gouinguené & Turlings 2002; Schmelz *et al.* 2003a; Van den Boom *et al.* 2004). For carnivores, which require prey or hosts for reproduction, it is especially important to respond to the signal that identifies the presence of suitable herbivores feeding on a plant. It seems unlikely that the composition of the complete blend of volatiles emitted by a herbivore-infested plant constitutes the prey-location signal for foraging carnivores because these blends can consist of more than a hundred different compounds. Moreover, the composition of the volatile blend of a specific plant-herbivore combination can vary, e.g. when a second herbivore species infests the same individual plant, or with different abiotic conditions to which the plant is exposed. Hence, we conclude that the information – the herbivore-induced plant volatile blend – that is available to a foraging carnivore includes both a signal part (related to the presence of suitable prey) and a noise part (not related to the presence of suitable prey). In foraging for prey, carnivores have to discriminate between volatile blends that are induced by herbivore species that are suitable as prey and blends that are induced by other herbivore species or other biotic or abiotic factors. In other words, they have to discriminate between information that contains both signal and noise versus information that contains noise alone. Despite the extensive research on the responses of carnivores to herbivore-induced plant volatiles, it is not yet known which parts of the volatile blends constitute the signal that reveals the presence of prey to carnivores, and which parts are noise (e.g. Chadwick & Goode 1999; Vet 1999b; Dicke & Van Loon 2000; Degenhardt *et al.* 2003).

The following paragraphs describe three important gaps that exist in our knowledge on information use by the natural enemies of herbivorous arthropods, each related to the signal/noise issue described above. Each section introduces one of the research questions that will be addressed in this thesis.

Foraging for prey in a noisy environment: effect of volatiles induced by non-prey herbivores

In the field, plants are part of a community, in which most plants emit a blend of volatiles, which can be induced by herbivory, or by other biotic or abiotic factors. Wind turbulence can mix the volatiles emitted by two or more neighbouring plants and thereby create variation in the information that is available to a foraging carnivore (Figure 1A). In addition, more than one herbivore species can simultaneously infest the same individual plant, and this presumably influences the composition of the volatile blend (Figure 1B) (Shiojiri *et al.* 2001). To date, most studies on the responses of carnivores to herbivore-induced plant volatiles have been carried out in the laboratory in an odour-free background, with plants from a single species infested with a single herbivore species (Sabelis *et al.* 1999a; Hunter 2002). Little is known about the responses of carnivores to the prey-related signal when variation in information is caused by the presence of non-prey herbivores on heterospecific or conspecific plants (odour mixing), or on the same individual plant (multiple infestation) (but see Monteith 1960; Shiojiri *et al.* 2000, 2001, 2002; Gohole *et al.* 2003a,b; Perfecto & Vet 2003; Rodriguez-Saona *et al.* 2003). Odour mixing or multiple infestation could change the signal-to-noise ratio in the available information and thereby hamper the detection of the prey-related signal by carnivores, or influence the ability of carnivores to discriminate between this signal and noise. Addressing this issue could bridge the gap between the laboratory studies on the responses of carnivores to herbivore-induced plant volatiles and the actual – more noisy – situation in which they use this information to locate their prey in the field.

Question I: How are the foraging responses of carnivores to plants infested with suitable prey herbivores influenced by the presence of non-prey herbivores on neighbouring heterospecific or conspecific plants (odour mixing), or on the same individual plant (multiple infestation)?

Identifying the signal that enables carnivores to discriminate between two volatile blends

Plant species vary in the specificity of the herbivore-induced volatiles that they emit (e.g. Dicke 1999b; Vet 1999a; Dicke & Vet 1999). Some plant species emit the same compounds upon herbivory by different herbivore species, albeit in different ratios (quantitative differences), whereas other plant species emit novel compounds (qualitative differences). Carnivores can discriminate between plants infested with their prey or infested with non-prey herbivores based on herbivore-induced plant volatiles from plant species in both groups. For example, the parasitoid wasp *Cardiochiles nigriceps* prefers the volatiles from tobacco plants infested with the host caterpillar *Heliothis virescens* to the volatiles from tobacco infested with the non-host caterpillar *Helicoverpa zea*, although the volatile blends consist of the same compounds (DeMoraes *et al.* 1998). Similarly, the parasitoid *Aphidius ervi* prefers the volatiles from broad bean infested with the host aphid *Acyrthosiphon pisum* to the volatiles from broad bean plants infested with the non-host aphid *Aphis fabae* (Du *et al.* 1996, 1998). The volatile blend induced by *Ac. pisum* includes 6-methyl-5-hepten-2-one, whereas this compound has not been detected in the blend induced by *A. fabae*, suggesting that *A. ervi* could use this compound to discriminate between the two blends. Although these results indicate that carnivores may use the ratio between compounds and/or the presence of specific compounds to identify the presence of suitable herbivores on plants, it has not yet been demonstrated which compounds play a significant role in the foraging behaviour of carnivores *within* a natural herbivore-induced volatile blend (Figure 1C) (e.g. Dicke 1999b; Vet 1999a,b; Turlings & Fritzsche Hoballah 1999; Dicke & Van Loon 2000).

Question II: Which parts of herbivore-induced volatile blends from plants enable carnivores to discriminate between two volatile blends?

Phenotypic plasticity in foraging responses

The fact that information from plants infested with suitable prey herbivores displays temporal and spatial variation raises another important question: how can carnivores adapt their responses to these cues? Genetic adaptations to the information could include increased perception of the signal, more efficient

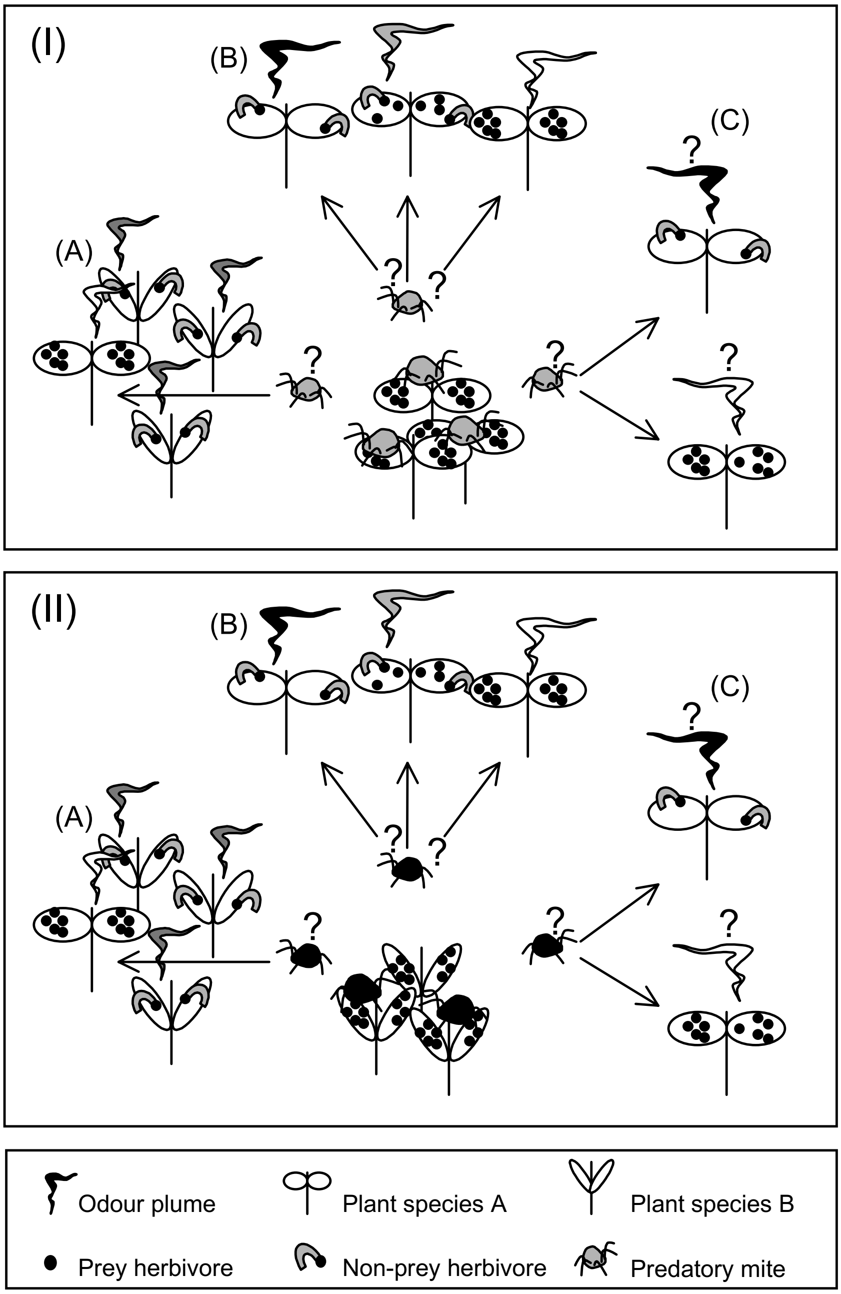


Figure 1: (I) Situations that carnivores can be confronted with in searching for a new prey patch in a natural environment: (A) The volatile blend from prey-infested plants can be mixed with the volatiles from heterospecific plants infested with non-prey herbivores (similarly with volatiles from conspecific plants infested with non-prey); (B) Prey and non-prey herbivores can simultaneously infest the same individual plant; (C) Choice between plants infested with prey or non-prey herbivores. The previous experiences of predatory mites with volatiles may influence their foraging responses in situations A-C. For example, as depicted in (II), the plant species on which predatory mites have developed may affect their foraging decisions.

processing of information or decision making, and enhanced discrimination between signal and noise (Vet 1999a). Studies on genetic adaptation in the responses of carnivores to herbivore-induced plant volatiles have been initiated recently, and genetic variation has been demonstrated for the predatory mites *Amblyseius womersleyi* and *Phytoseiulus persimilis*, and the parasitoid wasp *Cotesia glomerata* (Margolies *et al.* 1997; Maeda *et al.* 1999, 2001; Jia *et al.* 2002; Wang *et al.* 2003). Besides genetic adaptation, phenotypic plasticity can enable carnivores to cope with variation in prey-location cues between and within generations of carnivores (e.g. Via 1987; Dukas 1998). Phenotypic plasticity in responses to herbivore-induced plant volatiles has been well-studied for parasitoid wasps (reviewed by Turlings *et al.* 1993a; Vet *et al.* 1995). These wasps can learn to associate a specific volatile blend to the presence of a specific host herbivore, for example when they are exposed to this information during an oviposition in the host. In contrast, there is much less information on the role of learning of herbivore-induced plant volatiles in the foraging behaviour of predatory natural enemies of herbivorous arthropods (Figure 1) (but see Dicke *et al.* 1998; Drukker *et al.* 2000 a,b).

Question III: What is the role of learning in foraging behaviour of carnivores in relation to variation in herbivore-induced plant volatiles?

Aim and rationale

By addressing the three research questions introduced above, this thesis aims to increase the understanding of the behavioural responses of carnivores to herbivore-induced plant volatiles. Knowledge on how carnivores respond to herbivore-induced plant volatiles can give new insight in the selection pressures that act on the foraging behaviour of carnivores, and consequently also on the selection pressures that act on volatile production by plants in response to herbivory. Eventually, this knowledge can also lead to applications in agriculture (e.g. Dicke *et al.* 1990c; Lewis & Martin 1990; Kahn *et al.* 1997; Sabelis *et al.* 1999a; Degenhardt *et al.* 2003; Powell & Pickett 2003). Carnivore behaviour could be manipulated to increase the effectiveness of biological control of pest herbivores, for example by luring carnivores into the crop through volatiles that signal the presence of suitable herbivores, either by intercropping with plant species that emit such volatiles, or by engineering plant genotypes that show an enhanced emission of these compounds. Alternatively, prior to their release in the crop, carnivores could learn to respond to specific volatiles induced by the pest herbivore, or lines of carnivores could be selected that have a strong response to these volatiles.

Study system

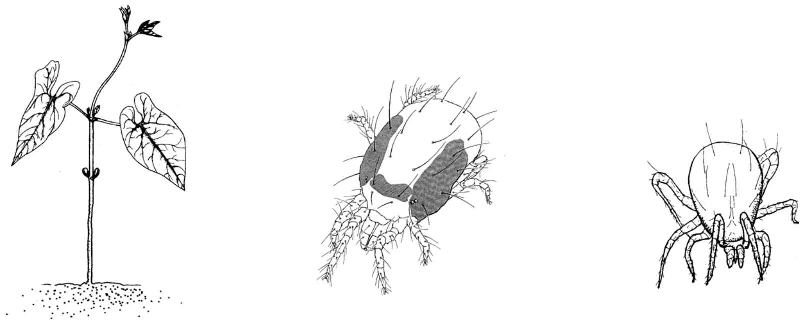
To address the research questions, I have studied a tritrophic system consisting of lima bean plants, two-spotted spider mites, and predatory mites (Figure 3). This tritrophic system has been a model system for the study of indirect plant defence through herbivore-induced volatiles for about two decades, and thus forms an ideal system to study my research questions (e.g. Sabelis & Van de Baan 1983; Dicke *et al.* 1990a).

The mechanisms of induced indirect defence through the emission of volatiles have been studied extensively in lima bean plants (Figure 2A). Upon infestation with herbivores, lima bean plants emit a blend of volatiles that differs in composition from the blend emitted upon mechanical damage (e.g. Dicke *et al.* 1990a; Ozawa et al. 2000a; Horiuchi *et al.* 2003). This blend of volatiles can attract carnivorous arthropods (e.g. Dicke *et al.* 1990a; Shimoda *et al.* 2002). Moreover, lima bean has been used as a model system to study the role of plant hormones in the induction of volatile emission (e.g. Hopke *et al.* 1994; Boland *et al.* 1999; Dicke *et al.* 1999; Koch *et al.* 1999; Ozawa *et al.* 2000a; Horiuchi *et al.* 2001), and to study the biosynthesis of induced volatiles (e.g. Donath & Boland 1994; Bouwmeester *et al.* 1999).

The two-spotted spider mite *Tetranychus urticae* (Figure 2B) is a polyphagous herbivore that can cause economical damage world wide to field and greenhouse crops, ornamentals or fruit trees (Van de Vrie *et al.* 1972; Helle & Sabelis 1985). Spider mites pierce their food plant with their stylets and feed on the contents of parenchyma cells. Feeding by *T. urticae* leads to the induction of volatiles in a range of plant species, including lima bean (e.g. Sabelis & Dicke 1985; Dicke & Sabelis 1988; Dicke *et al.* 1998; Van den Boom *et al.* 2004). Spider mites have a patchy distribution on plants; a spider mite patch consists of a group of colonies on different leaves of the same plant or of a group of plants. Because spider mite populations grow rapidly, they often overexploit their host plant, which can result in the death of annual plants. When that happens, spider mites have to colonise new plants by dispersal through the air, often on strands of silk that they produced (Helle & Sabelis 1985).

The predatory mite *Phytoseiulus persimilis* (Figure 2C) is a specialised natural enemy of spider mites in the genus *Tetranychus*. They preferably feed on the eggs of their prey; adult females can consume up to 30 prey eggs per day (Sabelis 1981). Development from egg to adult takes less than one week under favourable conditions and the adult females can lay up to 5 eggs per day (the weight of 5 eggs is equal to the weight of a female!), resulting in a high rate of population increase. *Phytoseiulus persimilis* is well known for its ability to exterminate local prey populations, and is now widely used for biological control of spider mites (Sabelis & Van der Meer 1986; Pels & Sabelis 1999). To locate new prey patches *P. persimilis* can disperse through the air. Upon landing, herbivore-induced plant volatiles are the main prey-location cues (Sabelis & Van de Baan 1983; Dicke *et al.* 1990a). Responses of *P. persimilis* to herbivore-induced volatiles from various plant species have been well documented, both in laboratory set-ups and in more realistic field-like set-ups in the greenhouse (e.g. Sabelis & Van de Baan 1983;

Janssen 1999; Van den Boom *et al.* 2002). *Phytoseiulus persimilis* uses its first pair of legs like antennae: these legs are moved more intensively upon stimulation with volatiles (Dicke *et al.* 1991). Transmission electron microscopy and electrophysiology have demonstrated the presence of olfactory chemoreceptors on the first pair of legs (Jagers op Akkerhuis *et al.* 1985; De Bruyne *et al.* 1991). Besides using herbivore-induced volatiles for long-distance foraging, *P. persimilis* can also use these volatiles within a prey patch by responding to the steep odour gradient at its border (Sabelis *et al.* 1984). In addition, they use contact cues from the spider mite webbing and from the spider mites themselves in prey location and prey acceptance within the patch. Several studies have shown that the responses of *P. persimilis* to herbivore-induced plant volatiles are plastic. Evidence for phenotypic plasticity comes from the differences in responses of predators that had been reared on different plant species (Takabayashi & Dicke 1992; Krips *et al.* 1999; Shimoda & Dicke 2000). Recently, Drukker *et al.* (2000a) were the first to show that *P. persimilis* can associate a specific blend of volatiles with the presence or absence of prey, thus demonstrating associative learning. Genetic variation in the responses of *P. persimilis* to herbivore-induced plant volatiles has also been demonstrated (Margolies *et al.* 1997; Jia *et al.* 2002).



(A) *Phaseolus lunatus* (B) *Tetranychus urticae* (C) *Phytoseiulus persimilis*

Figure 2: Schematic drawings of the species of the tritrophic system used in this thesis: (A) Lima bean plants (*Phaseoulus lunatus* L. Fabaceae); (B) The two-spotted spider mite (*Tetranychus urticae* Koch; Acari: Tetranychidae), adult female; (C) The predatory mite (*Phytoseiulus persimilis* Athias-Henriot; Acari: Phytoseiidae), adult female.

Thesis outline

The effects of background noise caused by non-prey caterpillars feeding on neighbouring plants (odour mixing) or on the same individual plant (multiple infestation) on the responses of *P. persimilis* to the volatiles from *T. urticae*-infested plants have been studied in **chapters 2** and **3** (***part I***).

In **chapter 2** we studied the behaviour of *P. persimilis* when the volatiles from *T. urticae*-infested lima bean plants were mixed with the volatiles from *Pieris* *brassicae*-infested brussels sprouts plants. The caterpillar *P. brassicae* is not a prey for the predatory mite, and brussels sprouts plants are not suitable as food plants for the prey spider mites. The volatile blends emitted by *T. urticae*-infested lima bean and *P. brassicae*-infested brussels sprouts are very different (Mattiacci *et al.* 1994; Dicke *et al.* 1999). Y-tube olfactometer experiments have been carried out to investigate the responses of predators to *T. urticae*-induced volatiles mixed with *P. brassicae*-induced volatiles. We also studied the behaviour of predatory mites in a greenhouse set-up that resembled the field situation more closely (Pallini *et al.* 1997; Janssen 1999). In **chapter 3** we studied the behaviour of *P. persimilis* when the volatiles from *T. urticae*-infested lima bean plants were mixed with the volatiles from *Spodoptera exigua*-infested cucumber or lima bean plants, or when *S. exigua* infested the same individual plant as *T. urticae*. The caterpillar *S. exigua* is not a prey for *P. persimilis*, but cucumber and lima bean are highly suitable food plants for *T. urticae*. We tested the hypothesis that the three situations were increasingly challenging for foraging predators. We expected that the volatile blends from *S. exigua*-infested cucumber or lima bean plants would be more similar to the blend from *T. urticae*-infested lima bean than was the blend of *P. brassicae*-infested brussels sprouts plants (Takabayashi *et al.* 1994a; Ozawa *et al.* 2000a). Besides behavioural experiments in a Y-tube olfactometer and a greenhouse set-up, we have also analysed the chemical composition of the volatile blends from lima bean and cucumber plants upon infestation with *T. urticae*, with *S. exigua*, or with the combination of *T. urticae* and *S. exigua*, to determine the similarity between these different blends.

The role of the composition of blends of herbivore-induced volatiles in enabling *P. persimilis* to discriminate between two volatile blends has been investigated in **chapters 4** and **5** (***part II***).

Chapter 4 describes the role of one volatile compound emitted by *T. urticae*-infested lima bean plants, i.e. methyl salicylate (MeSA), in the foraging behaviour of predatory mites. First, a dose-response experiment addressing the attraction of the predators to the single compound MeSA was carried out. Then, we investigated the effect of *quantitative* differences in the amount of MeSA on the foraging responses of *P. persimilis* by adding additional synthetic MeSA to the volatile blend from *T. urticae*-infested lima bean, which includes MeSA naturally (Dicke *et al.* 1999). Finally, we investigated whether a *qualitative* difference in the presence of MeSA affected the behaviour of predators by studying their preferences for the volatiles from *T. urticae*-infested lima bean versus jasmonic acid-treated lima bean. Both volatile blends are similar but, in contrast to *T. urticae*-infested lima bean, jasmonic acid-treated lima bean plants do not emit MeSA (Dicke *et al.* 1999). In **chapter 5** we studied which factors of volatile blend composition enable predatory mites to discriminate between plants infested with their prey *T. urticae*, and conspecific plants infested with the non-prey caterpillar *S. exigua*. We investigated the effect of the densities of herbivores that were used to infest the plants on the ability of *P. persimilis* to discriminate, and we have analysed the chemical composition of the volatile blends from lima bean plants infested with different densities of *T. urticae*, or with *S. exigua*. Chemical analysis revealed a change in the amounts of some compounds in the volatile blend induced by *T. urticae* between spider mite densities at which predators did or did not discriminate between *T. urticae*-induced and *S. exigua*-induced volatiles. We subsequently investigated the role of three of these compounds on the ability of the predators to discriminate between *T. urticae*-induced and *S. exigua*-induced volatiles from lima bean plants.

The role of phenotypic plasticity in the responses of *P. persimilis* to herbivore-induced plant volatiles has been studied in **chapters 6**, **7** and **8** (***Part III***).
In **chapter 6** we investigated the behavioural plasticity of responses of predatory mites to MeSA. We compared the responses of predators that had been reared on spider mites on lima bean (that does emit MeSA) or spider mites on cucumber (that does not emit MeSA) with the responses of predators that had been reared on spider mites on cucumber in the presence of synthetic MeSA. Responses to the single compound MeSA, and to volatile blends that differ in the presence of MeSA (induced by *T. urticae*-infestation or by JA-treatment in lima bean) were studied. **Chapter 7** addresses the effect of experience on the choices of predatory mites between the volatiles from *T. urticae*-infested or *S. exigua*-infested lima bean plants. First we studied the effect of experiences acquired during rearing on discrimination between prey and non-prey herbivores by comparing the responses of predators that had been reared on cucumber or lima bean. Then, we investigated which experiences during the adult stage influenced discrimination between prey and non-prey herbivores by subjecting predators to a non-rewarding experience (exposure to *S. exigua*-induced volatiles in the absence of prey), a rewarding experience (exposure to *T. urticae*-induced volatiles in the presence of prey), or both (multiple experience). In **chapter 8** we investigated how experience acquired during rearing affects the responses of *P. persimilis* to volatiles induced by a prey herbivore, volatiles induced by a non-prey herbivore, and volatiles induced by more than one herbivore species. To achieve that goal, we compared the responses of predators that had been reared on spider mites on lima bean or cucumber to the volatiles from *T. urticae*-infested, *S. exigua*-infested, or *T. urticae* + *S. exigua*-infested lima bean and cucumber plants. In addition, we studied the responses of cucumber-reared predatory mites to mixtures of volatiles from *T. urticae*-infested lima bean and *S. exigua*-infested lima bean or cucumber. To investigate whether the experience acquired during rearing influences the foraging responses of predatory mites to such mixtures of volatiles, we compared the

responses of the cucumber-reared predators (chapter 8) to those of lima bean-reared predators (chapter 3).

The last chapter of this thesis synthesises the main results and conclusions from **chapters 2** to **8**. I first summarise how predatory mites respond to volatiles from plants infested with their prey in an environment with non-prey herbivores, where odour mixing or simultaneous infestation of the same plant with prey and non-prey herbivores can occur. The possible consequences of these responses for plants, herbivores and carnivores are discussed. Second, I summarise the findings of our experiments on discrimination by predatory mites between two volatile blends. Finally, I discuss which previous experiences of predatory mites with herbivore-induced plant volatiles influence their responses when they are confronted with volatiles from plants infested with prey and/or non-prey herbivores, or mixtures of odours.

Acknowledgements
I thank Marcel Dicke, Maurice Sabelis and Remco Van Poecke for helpful comments on a previous version of this introduction.

Part I

Foraging in an environment with non-prey herbivores

CHAPTER 2

Mixed blends of herbivore-induced plant volatiles and foraging success of carnivorous arthropods

Abstract
Infochemicals can mediate direct and indirect interactions in a food web. It has been documented extensively that carnivorous arthropods exploit herbivore-induced plant volatiles during foraging for herbivorous prey. Most studies on the role of infochemicals in multitrophic interactions have been conducted against an odour-free background, although field studies show that carnivores also use herbivore-induced plant volatiles under more complex conditions. Here we investigated the effect of mixing the blends of volatiles emitted by two plant species on the foraging behaviour of the predatory mite *Phytoseiulus persimilis*. This was done in an olfactometer under laboratory conditions and in a semi-field set-up under greenhouse conditions. In 4 out of 5 olfactometer experiments the behaviour towards volatiles from spider mite (*Tetranychus urticae*) infested lima bean plants was not affected by mixing with volatiles from caterpillar (*Pieris brassicae*) infested brussels sprouts plants. In the fifth olfactometer experiment the response shifted significantly towards the volatiles from infested lima bean leaves without volatiles from infested brussels sprouts leaves. In the greenhouse set-up no effect of infested brussels sprouts plants or their volatiles on the location of spider mite-infested lima bean plants by predatory mites was recorded. The two odour blends used in this study, i.e. those from spider mite-infested lima bean plants and from caterpillar-infested brussels sprouts plants, are very different and there is no overlap in compounds that are known to attract the predators. The results are discussed in the context of other types of odour-blend mixing and the effects on food web interactions.

INTRODUCTION

Indirect interactions can have large effects on food web dynamics (Abrams *et al.* 1996; Polis & Strong 1996; Berlow 1999; Morin 1999) and adaptive foraging behaviour can play a crucial role in this (Sabelis & Dicke 1985; Abrams 1993). It is well-known that many arthropod predators exterminate local prey populations (e.g. Pels & Sabelis 1999 and references therein). Yet, indirect interactions mediated by the food plant of the prey may be an important factor in this prey extermination (Price *et al.* 1980). Plants respond to herbivory with the emission of a herbivore-induced blend of volatiles that attracts carnivorous enemies of the herbivores, which exterminate the local herbivore population (Sabelis & Van der Meer 1986; Dicke & Vet 1999; Sabelis *et al.* 1999).

There is abundant evidence that attraction of carnivorous arthropods to prey-infested plants is not the result of a response to volatile cues from their prey but of an attraction to plant volatiles induced by prey herbivores (for reviews see e.g. Vet & Dicke 1992; Turlings *et al.* 1995). In other words, indirect cues are more important for distant prey location by carnivores than direct prey-derived cues. Obviously, prey are under selection not to convey information to their enemies (Vet & Dicke 1992). In contrast, plants benefit in terms of Darwinian fitness from the attraction of carnivores through herbivore-induced volatiles (Dicke & Sabelis 1989; Van Loon *et al.* 2000a; Fritzsche-Hoballah & Turlings 2001). Herbivore-induced plant volatiles can provide carnivores with specific information. The volatiles are specific for the plant species and can also be specific for the herbivore species that damages the plant (Takabayashi & Dicke 1996). Different plant species that are infested with the same herbivore species emit qualitatively different blends that also share compounds with the blends emitted by other plant species. On the other hand, conspecific plants that are infested with different herbivore species usually emit qualitatively similar blends that differ in the ratios of the blend components (Dicke 1999a). Carnivores can discriminate between such different blends (DeMoraes *et al.* 1998; Dicke 1999c). However, the more similar two odour blends are, the more difficult it is for carnivores to discriminate between them (Geervliet *et al.* 1998a; Vet *et al.* 1998).

In the majority of studies to date the responses of carnivores have been tested against a minimal odour background in laboratory or simplified greenhouse set-ups (Hunter 2002, but see e.g. Drukker *et al.* 1995; Shimoda *et al.* 1997; DeMoraes *et al.* 1998; Thaler 1999; Geervliet *et al.* 2000; Kessler & Baldwin 2001 for field studies). In nature, plants are part of a community where infested plants are surrounded by other plants, either conspecifics or heterospecifics. These neighbouring plants, especially when damaged by herbivores, emit complex odour blends as well. As a consequence, the herbivore-induced blend of volatiles is mixed with blends emitted by other plants. This background odour varies with the local community surrounding the prey-infested plant. Thus, the plant community may influence the composition of an odour blend that drifts downwind. How this affects the responses of carnivorous arthropods remains to be investigated. This should be done through manipulative experiments. When the manipulations are based on information on the composition of the odour blends, the behaviourally active compounds, and carnivore responses, this allows an explanation of the observed responses. In the present study we address the effect of plant odour mixing on carnivore behaviour for a well-studied experimental system. Precise manipulations have been carried out with plants whose induced odour blends had previously been well characterised.

There is ample knowledge on the responses of predatory mites (Acari: Phytoseiidae) to prey-induced volatiles from many plant species during prey location (Dicke *et al.* 1998). The specialist predatory mite *Phytoseiulus persimilis* Athias-Henriot exterminates local prey populations and prey-induced plant volatiles play an important role in this (Sabelis *et al.* 1983; Sabelis & Dicke 1985; Sabelis & Van der Meer 1986). We have comparatively investigated the attraction of this predator towards volatiles from a prey-infested plant, either when offered against a background of clean air or against a background of volatiles from a different plant species infested with a non-prey caterpillar. The mixing of odour blends was done in two ways: (1) by thoroughly mixing of the odour blends in a closed system with a laminar air flow, or (2) by odour diffusion where prey-infested and non-prey infested plants were placed next to each other in an open system. Thorough odour mixing represents an extreme case where a novel odour blend is created by leading one odour blend through a container with a second odour source. In the diffusion experiment, the degree of mixing is lower because here the plants are spatially separated odour sources. Studies on insect pheromones have shown that odour blends emitted from an odour source travel as distinct odour packages and therefore the interference between odour sources will be most intense when the distance between odour sources is small (Murlis *et al.* 1992). We used two well-characterised odour blends: volatiles from lima bean plants infested with the spider mite *Tetranychus urticae* Koch, and volatiles from brussels sprouts plants infested with caterpillars of the large cabbage white, *Pieris brassicae* L. The blend of spider mite-infested lima bean plants is dominated by induced terpenoids, and females of *P. persimilis* are strongly attracted to this volatile blend (Dicke *et al.* 1999). The blend emitted by brussels sprouts plants infested with the non-prey *P. brassicae* is dominated by green leaf volatiles. The two odour blends mainly overlap in green leaf volatiles that do not attract the predatory mite (Dicke *et al.* 1990a). The compounds from infested lima bean plants that attract the predatory mite

(Dicke *et al.* 1990a) are not emitted by brussels sprouts plants (Mattiacci *et al.* 1994).

We hypothesised that the blend emitted by non-prey-infested brussels sprouts plants is not attractive to the predatory mites. Because of the large qualitative differences between the blends we expected that mixing the blend from brussels sprouts to the attractive blend from lima bean leaves would not severely affect predator attraction. However, if the ratio of attractive compounds to non-attractive compounds influences the predator's behaviour, odour mixing is expected to influence predator attraction.

MATERIALS AND METHODS

Plants and herbivores

Lima bean plants (*Phaseolus lunatus* L., cv Sieva) were grown in a greenhouse at 20-30 °C, 50-70% r.h., and 16 h of light. The plants were used when the two primary leaves were fully unfolded and the first trifoliate leaf started to unfold, about 10 to 15 days after sowing. Brussels sprouts plants (*Brassica oleracea* L. var *gemmifera* cv. Icarus) were grown in a greenhouse under similar conditions. They were used when they had ca. 6-10 expanded leaves, which was 6 to 8 weeks after sowing. Two-spotted spider mites (*Tetranychus urticae*) were reared on lima bean plants in a greenhouse under the same conditions as uninfested plants. *Pieris brassicae* caterpillars were reared on brussels sprouts plants in a climatised room at 20±2 °C, 50-70 % r.h., under long day conditions.

Predators

Predatory mites, *Phytoseiulus persimilis*, were originally obtained from Entocare CV, Wageningen, The Netherlands. In our laboratory they were reared on spider mite-infested lima bean leaves in Petri dishes in a climatised room at 22±1 °C, 50-70 % r.h. and L:D 16:8. Prior to an experiment, females were starved individually in an Eppendorf vial for 24 h.

Olfactometer

A closed-system Y-tube olfactometer was used to investigate the responses of predatory mites to two odour sources. The olfactometer has been described in detail by Takabayashi & Dicke (1992). In short, air was filtered through activated charcoal and split into two air streams that were led through two glass jars that contained the odour sources. The resulting odour flows (4 l/min) were introduced into the two arms of a Y-shaped glass tube. Individual adult female predators were introduced onto an iron wire that runs through the glass tube, parallel to the wall of the tube. When the predator reached the end of one of the olfactometer arms, a choice for the respective odour source was recorded. Each predator was observed for a maximum of five min. Predators that did not make a choice within five minutes were recorded as 'no-choice'. All predators were used only once. After every five predatory mites, the odour sources were interchanged to compensate for any unforeseen asymmetry in the set-up. All experiments were carried out in the laboratory at 21±2 °C, using 4 high-frequency fluorescent lights above the olfactometer (Philips 840, 36 W). Each experiment was repeated on several different days with new odour sources and new predatory mites on each day.

The following experiments were done:

(a) Nine brussels sprouts leaves each infested with 50 first-second instar *P. brassicae* caterpillars versus nine uninfested brussels sprouts leaves. The infested leaves had been infested for three days. This experiment was replicated on four days, with 11-18 predators per day.

(b) In this experiment we offered the predators two identical sets of *T. urticae*-infested lima bean leaves as odour sources. The two odour flows were either transferred to the olfactometer directly (Figure 1a – no odour mixing, control) or after one of the odour flows had been mixed with the odour of caterpillar-infested brussels sprouts plants (Figure 1b – with odour mixing, treatment). Mixing of odours was done by leading the odour from infested lima bean leaves through a glass jar with caterpillar-infested brussels sprouts leaves and subsequently to the olfactometer (Figure 1b). The response of the predators to the two sets of infested lima bean leaves (Figure 1a) was compared to the response to the same two sets of lima bean leaves of which one has been mixed with the odour of brussels sprouts leaves (Figure 1b). Details of the procedure are as follows:

  (i) Nine lima bean leaves infested with *T. urticae* spider mites versus nine spider mite infested lima bean leaves (Figure 1a). All spider mite-infested leaves (detached, placed individually in a vial with water) had been infested for three days by 50±5 adult female mites. The choice of 10 predators was recorded.

  (ii) Subsequently, nine brussels sprouts leaves, each infested with 50 first-second-instar *P. brassicae* caterpillars for three days, were placed in a glass container *downwind* from the set of lima bean leaves that had attracted the majority of the 10 predators (Figure 1b). Downwind from the other set of lima bean leaves an empty container was inserted (Figure 1b). The response of 20 predators was recorded, and then the container with the brussels sprouts leaves and the empty container downwind from the lima bean leaves were removed.

  (iii) Subsequently, the response of 10 predators was recorded towards the two sets of spider mite-infested lima bean leaves (Figure 1a) to establish

> whether the response to the two sets of infested lima bean leaves was still similar to the situation at the beginning of the experiment (see above under (i)). This was true for all replicate trials and therefore the responses of the two groups of 10 predators to the two sets of spider mite-infested lima bean leaves were combined.

The complete experiment was replicated on three different days. Variations on this experiment with different numbers of lima bean leaves or brussels sprouts leaves were carried out as follows: (1) nine lima bean leaves and three brussels sprouts leaves, (2) three lima bean leaves and nine brussels sprouts leaves, (3) six lima bean leaves and nine brussels sprouts leaves, (4) eight lima bean leaves and nine brussels sprouts leaves.

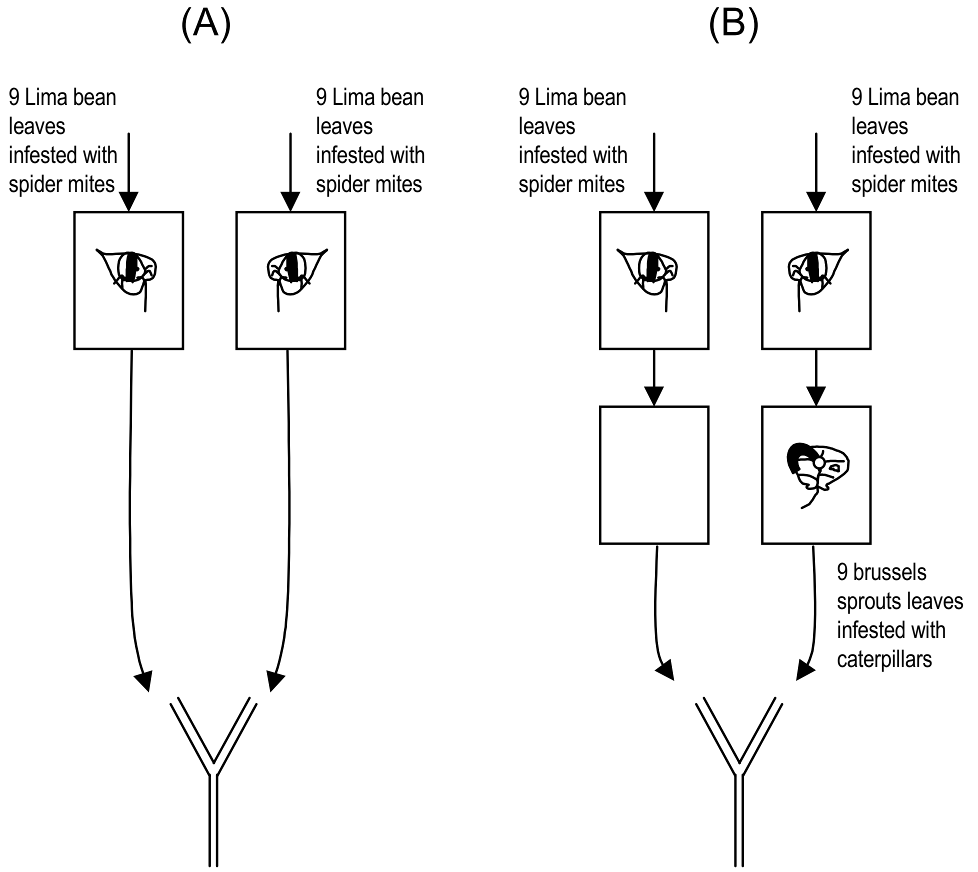


Figure 1: Experimental set-up of olfactometer experiments in which the odour of lima bean plants infested with spider mites (*T. urticae*, 50 females per leaf for 3 days) is mixed with the odour of brussels sprouts leaves infested with caterpillars (*P. brassicae*, 50 first-second instar caterpillars per leaf for 3 days). (A) Similar odour sources, consisting of 9 spider mite-infested bean leaves, are offered in each olfactometer arm; (B) Downwind to the set of lima bean leaves that attracted the most predators, a set of 9 caterpillar-infested brussels sprouts leaves was added, an empty container was added downwind of the other set of infested lima bean leaves.

Greenhouse set-up for olfactory responses

In this set-up we tested the effects of odour mixing on the ability of the predators to locate prey-infested lima bean plants in an open system with whole plants. Odour mixing in this set-up is not as controlled as in the closed-system olfactometer. In the open set-up in the greenhouse odour mixing is the result of diffusion rather than from directing the odour of infested lima bean leaves through a closed container with caterpillar-infested brussels sprouts leaves.

The set-up was based on the one described by Pallini *et al.* (1997) (Figure 2). In a greenhouse compartment (20-30 °C, r.h. 50-70% and L:D 16:8 – photoperiod from 6:00 AM until 22:00 AM) plants were placed in a container with soil. The surface dimensions of the arena represent ca 860 x 860 predator lengths. In the centre of the hexagon 100 adult female predatory mites (starved for 3 h) were released at 10:00 AM. Subsequently, the plants were monitored for the presence of predatory mites at 30 min intervals until 1:00 PM, and at 5:00 PM as well as 9:30 AM on the next day. At each observation, the predators that were found on the plants were removed and discarded. Each experiment had three replications on different days with different predatory mites and different plants. The positions of the plants were different for each replicate experiment to avoid any positional effects.

Four different experiments were carried out:

(a) *Three spider mite-infested lima bean plants versus no plants.* Three infested bean plants were introduced into the hexagon and the three other positions were left unoccupied. The spider mite infested plants were obtained by taking uninfested plants of which the first trifoliate leaf just started to unfold. Each plant was infested with spider mites by placing two heavily infested trifoliate leaves of plants from the spider-mite rearing on each plant, three days before the experiment. This served as the control without odour mixing.

(b) *Three spider mite-infested plants versus three caterpillar-infested brussels sprouts plants.* This experiment was similar to that described under (a) but here the open positions of the hexagon were filled with *P. brassicae*-infested brussels sprouts plants. Each brussels sprouts plant had been infested with 50 first-second instar *P. brassicae* caterpillars during three days. The spider mite-infested plants were prepared as described under (a). This experiment represents odour mixing through diffusion.

(c) *Three spider mite-infested lima bean plants versus three artificial plants.* Artificial plants were made from wooden sticks to which green paper leaves were attached with iron wire. The number and size of the paper leaves were similar to those of the brussels sprouts plants that were used in the experiments described under (b) and (d). This experiment was carried out to investigate the effect of the physical presence of plants in the absence of plant odours. The physical presence may influence air movements and thus exposure of the predators to the volatiles from the infested bean plants. Furthermore, the predators may walk on the paper plants, which may affect their time allocation. The spider mite-infested plants were prepared as described under (a).

(d) *Three spider mite-infested plants versus three upwind caterpillar-infested brussels sprouts plants.* This experiment was carried out to investigate the effect of the odour of infested brussels sprouts plants in the absence of the physical presence of plants in between the lima bean plants. The positions of the hexagon were occupied by spider mite-infested lima bean plants as in experiment (a). In this experiment three caterpillar-infested brussels sprouts plants were placed together 50 cm from the hexagon with lima bean plants. A fan generated a gentle airstream that blew the volatiles from the brussels sprouts plants towards the three lima bean plants. In this way the volatiles emitted by the brussels sprouts plants were present at the hexagon, but the brussels sprouts plants could not be reached by the predatory mites and thus the predators could not waste time on the brussels sprouts plants.

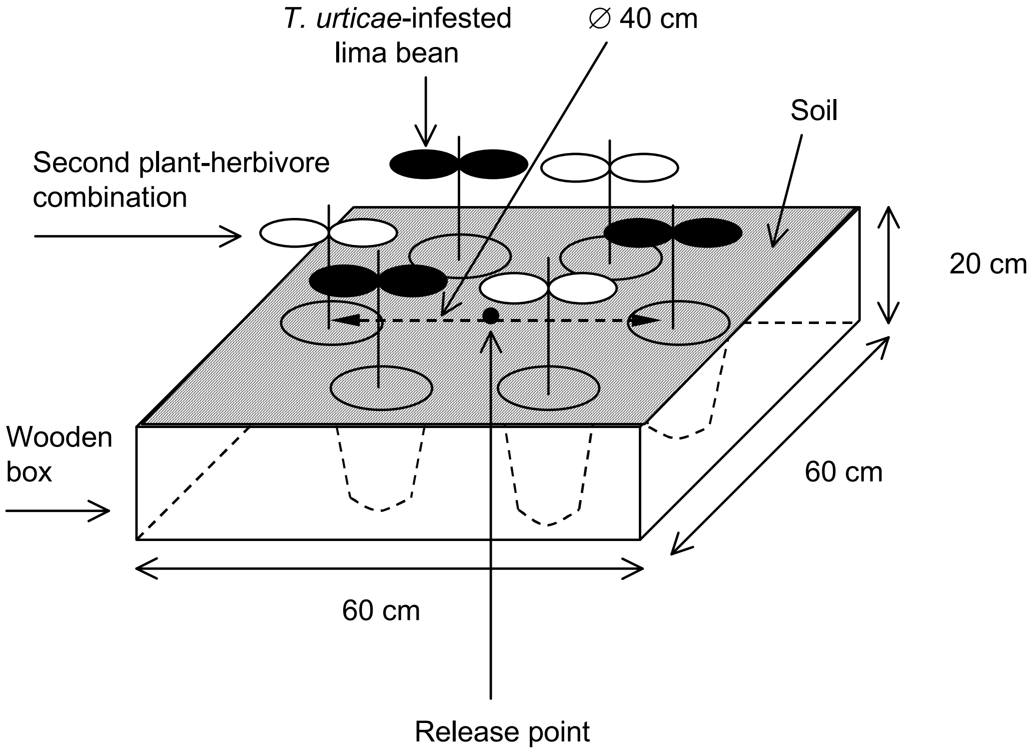


Figure 2: Experimental set-up for the greenhouse experiment. Six pots with or without plants could be placed in holes in the lid of the wooden box. The lid was then covered with a thin layer of soil to create an even-surfaced arena.

Statistical analysis

In the olfactometer experiments predatory mites that did not make a choice were excluded from the statistical analysis, this was only 2 % of 681 predatory mites. The choice of the predatory mites between two odour sources was statistically analysed with a two-sided binomial test to investigate whether the distribution of the predators differed from 50:50. The effect of odour mixing was comparatively analysed with a contingency table based on the Chi-square distribution.

In the greenhouse experiment, we statistically compared the cumulative numbers of predators recovered after one day on prey-infested lima bean plants among experiments with the Kruskal-Wallis test. Each experimental day was used as one replicate.

RESULTS

Y-tube olfactometer

The volatiles emitted by *P. brassicae*-infested brussels sprouts leaves were not attractive to 24 h-starved *P. persimilis*: 26 out of 57 predators chose for the volatiles from infested leaves and 31 predators chose for volatiles from uninfested leaves (P=0.60, binomial test). Therefore, *P. brassicae*-infested brussels sprouts leaves represent a neutral odour source to these predators. In subsequent experiments this neutral odour source was added to the odour plume emitted from spider mite-infested lima bean leaves.

Different numbers of lima bean leaves and brussels sprouts leaves were used in the odour mixing experiment. In four out of the five experiments (Figure 3A,C,D,E), adding one set of infested brussels sprouts leaves (Figure 1B) did not significantly change the choice of *P. persimilis* for the two sets of infested lima bean leaves. Only in the experiment where nine caterpillar-infested brussels sprouts leaves were added downwind to one of the two sets of nine spider mite-infested bean leaves, the response of the predators shifted significantly to the odour from lima bean leaves without brussels sprouts-leaf odour (Figure 3B, $P<0.001$, 2x2 contingency table). In two other experiments (Figure 3A,D) the response shifted towards the volatiles from the set of lima bean leaves with the clean downwind container, but this was not statistically significant ($P>0.05$, 2x2 contingency table).

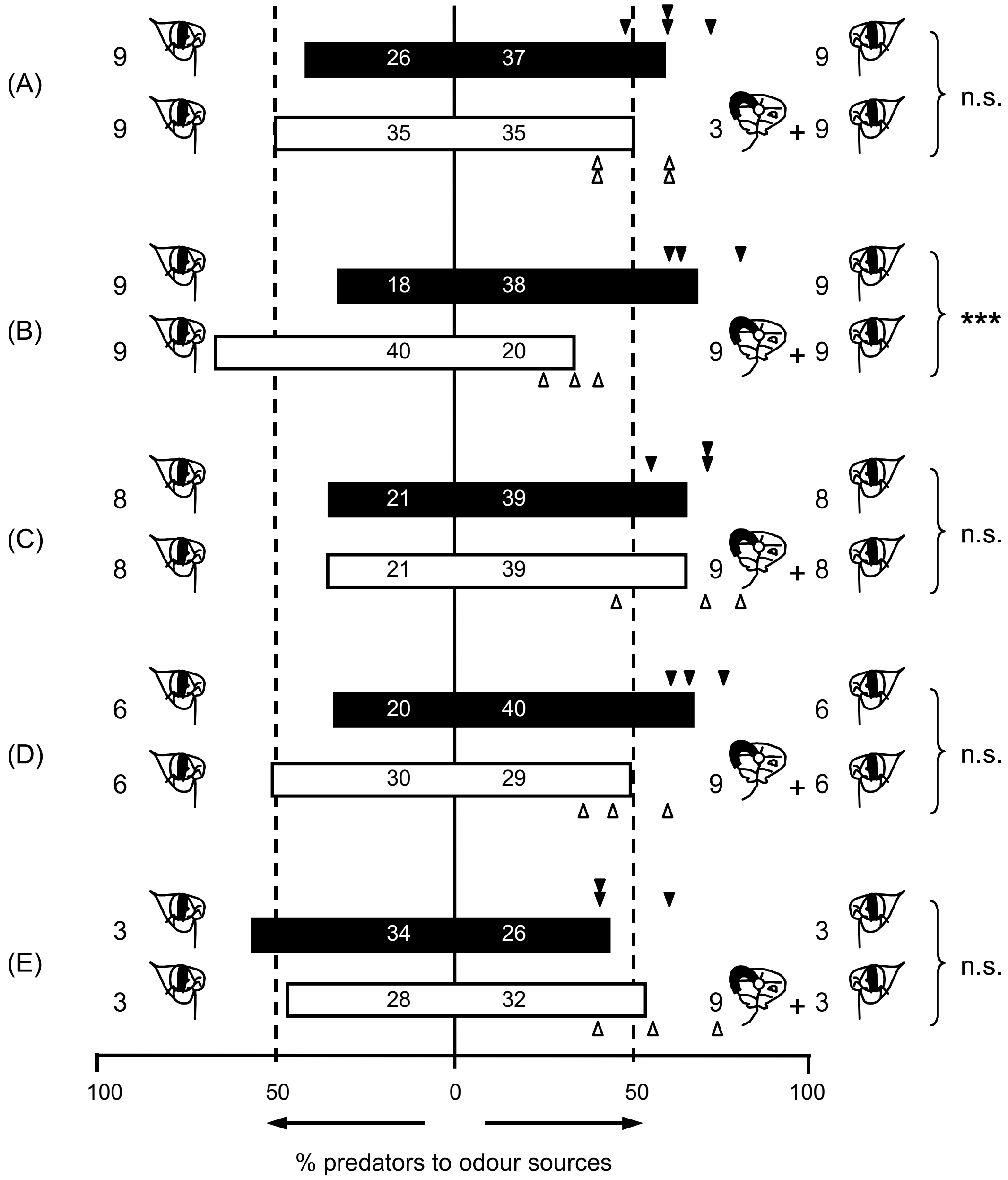


Figure 3: Response of adult female predatory mites in a Y-tube olfactometer to two standardised sets of spider mite-infested lima bean leaves with and without a downwind odour source consisting of caterpillar-infested brussels sprouts leaves (see Figure 1 for set-ups). Black bars present the percentages of the total number of predators choosing between the volatiles from two equal sets of *Tetranychus urticae*-infested lima bean leaves. White bars present the percentages of the total number of predators choosing between the volatiles from one set of *T. urticae*-infested lima bean leaves and *T. urticae*-infested lima bean leaves plus *Pieris brassicae*-infested brussels sprouts leaves. The number of leaves per experiment is indicated in front of the symbols used for the odour sources. Numbers in the bar segments represent the total numbers of predators responding to each odour source. The effect of odour mixing was analysed with a 2x2 contingency table test (n.s. $P>0.05$; *** $P<0.001$).

Greenhouse set-up

In the greenhouse set-up, ca 50% of the released predators was recovered on the plants after one day (Figure 4). The number of predators recovered was similar for all four treatments (Kruskal Wallis test, P=0.87). When three spider mite-infested lima bean plants were offered, 47 ± 7.8 predators (mean ± SD) were recovered. When three caterpillar-infested brussels sprouts plants were mixed with the lima bean plants, 44 ± 6.5 predators were recovered on the lima bean plants and 2 ± 1.7 on the brussels sprouts plants. When three artificial plants were mixed with 3 spider mite-infested lima bean plants, this yielded a recovery of 48 ± 7.5 predators on the lima bean plants and none on the paper plants. When the odour of three caterpillar-infested brussels sprouts plants was blown over the three lima bean plants the recovery was 52 ± 22.5 predators. The temporal patterns of predator recovery were also similar for the four treatments (data not shown). This experiment does not show an effect of mixing caterpillar-infested brussels sprouts plants, or their volatiles on the foraging success of the predatory mites.

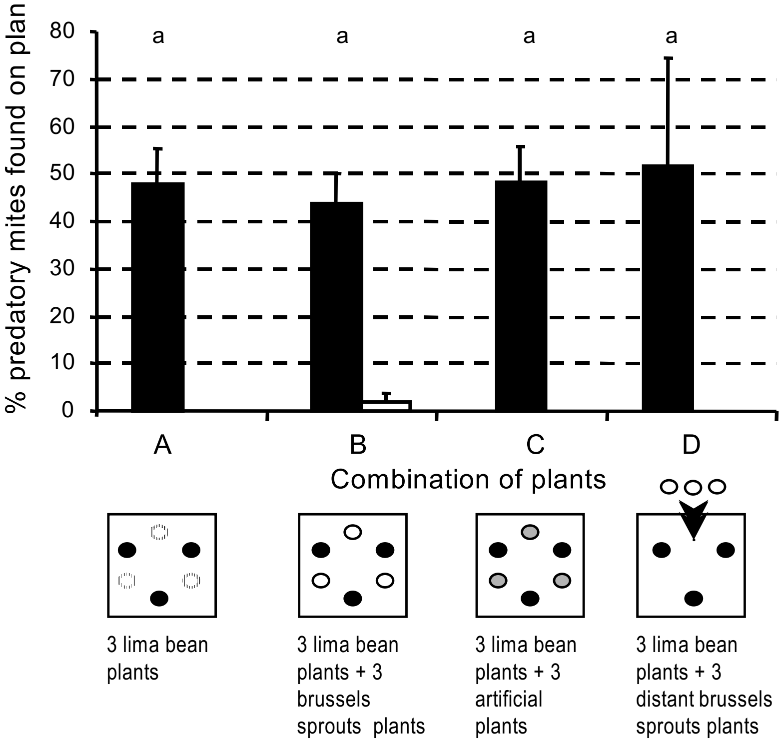


Figure 4: Recovery of predatory mites, 1 day since release, on *Tetranychus urticae*-infested lima bean plants (black bars) or brussels sprouts plants (white bars) in the greenhouse set-up (see Figure 2). Each experiment had three replicates. Four different experiments were carried out: (A) 3 spider mite-infested lima bean plants, (B) 3 spider mite-infested lima bean plants plus 3 *Pieris brassicae*-infested brussels sprouts plants, (C) 3 spider mite-infested lima bean plants plus 3 artificial plants, and (D) 3 spider mite-infested lima bean plants plus 3 upwind caterpillar-infested brussels sprouts plants from which the odour is blown over the bean plants. The recovery rates are not significantly different (Kruskal-Wallis test).

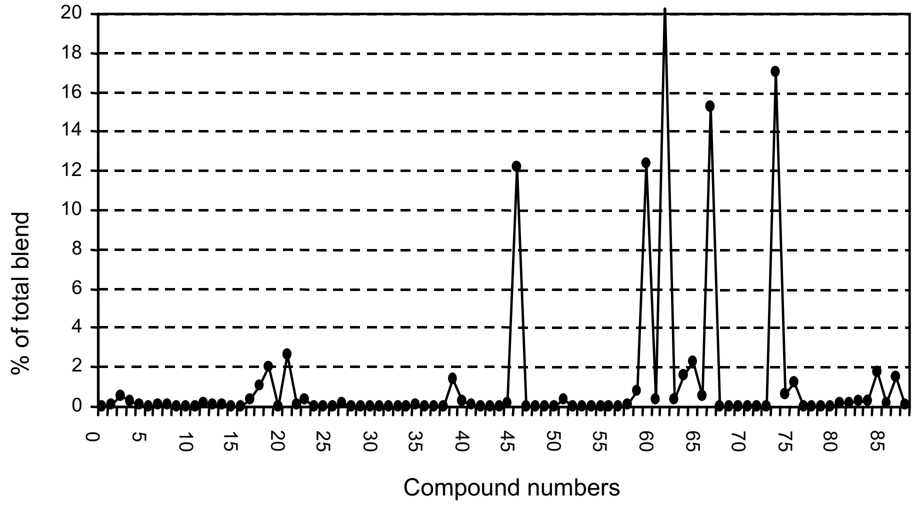
DISCUSSION

To investigate the potential effect of volatiles emitted by neighbouring plants on the attraction of a predatory mite to the volatiles from a prey-infested plant species we have used two experimental set-ups that have been extensively used in previous studies (e.g. Dicke *et al.* 1999; Janssen 1999). Neither in the olfactometer set-up, nor in the greenhouse set-up did volatiles from *P. brassicae*-infested brussels sprouts leaves interfere with the attraction of the predatory mites to volatiles from *T. urticae*-infested lima bean leaves. Although the results from the greenhouse set-up could be explained by incomplete odour mixing that results from the distance between the different odour sources (Murlis *et al.* 1992), this explanation does not hold for the results of the olfactometer set-up. Therefore, the data from the two set-ups show that volatiles from *P. brassicae*-infested brussels sprouts leaves do not interfere with the attraction of the predatory mites to volatiles from *T. urticae*-infested lima bean leaves. Information on the blend compositions can explain this. The volatile blends emitted by spider mite-infested lima bean plants and caterpillar-infested brussels sprouts plants are very different (Figure 5). The blend emitted by lima bean plants is dominated by terpenoids such as (*E*)-4,8-dimethyl-1,3,7-nonatriene, (*E*)-β-ocimene, (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene, methyl salicylate and the green leaf volatiles (*Z*)-3-hexenyl acetate and (*Z*)-3-hexenol (Dicke *et al.* 1999), while brussels sprouts plants mainly emit green leaf volatiles such as (*Z*)-3-hexenol, (*Z*)-3-hexenyl acetate and (*E*)-2-hexenal (Mattiacci *et al.* 1994). The predatory mites are attracted by several of the terpenoids and by methyl salicylate, but not by green leaf volatiles (Dicke *et al.* 1990a). Moreover, the predators' chemoreceptors respond to some of the terpenoids and to methyl salicylate, while no chemoreceptor activity has been recorded in response to two green leaf volatiles (De Bruyne *et al.* 1991). Thus, the volatiles from infested brussels sprouts plants may not activate the chemoreceptors and consequently do not affect the response to the attractive compounds from spider mite-infested lima bean plants.

The experiments under less carefully controlled conditions in the greenhouse set-up support the conclusion of other studies that carnivorous arthropods are attracted to prey-infested plants under conditions without a standardised laminar airflow (e.g. Steinberg *et al.* 1992; Drukker *et al.* 1995; Shimoda *et al.* 1997; DeMoraes *et al.* 1998; Janssen 1999; Thaler 1999; Kessler & Baldwin 2001). Other studies also provide evidence from open-system experiments that supports olfactometer studies under carefully controlled conditions (e.g. Janssen 1999; Zemek & Nachman 1999).

One might criticise our experiments by arguing that we have not studied the effect of odour mixing under natural conditions. However, we have deliberately chosen to carry out carefully manipulated experiments with odour blends that have been well-characterised for the following reasons: (1) Because we have mixed the odours of two species of plants in a controlled experiment in a closed system we can exclude that an absence of interference with predator behaviour can be explained by insufficient odour mixing in the set-up. A better mixing of the odours of different plants than obtained in the olfactometer experiments will be hard to achieve and therefore these experiments represent an extreme case. (2) We have applied well-proven set-ups (Dicke *et al.* 1999; Janssen 1999) to investigate the effect of odour mixing on the behaviour of carnivorous arthropods and so an absence of interference cannot be caused by an inappropriate set-up. The available knowledge on the experimental system is consistent with the data obtained in the two set-ups. This study cannot be the last step, however. Ultimately, the effects of plant community on foraging by carnivores should be investigated under field conditions (Hunter 2002). Before this can be done reliably, one needs an experimental system for which enough basic knowledge is available to allow the design of relevant manipulative experiments. For such manipulations basic knowledge of the induction process as well as the perception of plant volatiles by carnivores provides important tools (Baldwin 2001; Dicke *et al.* 2002; Roda & Baldwin 2003). This may be achieved by taking a lead from experiments carried out under carefully controlled laboratory or semi-field conditions and increasing the complexity of the experiments with finally doing field experiments. Alternatively, one may start with field experiments and subsequently reduce the complexity so as to test the explanations for the field results. The investigations of the role of herbivore-induced plant volatiles in *Phytoseiulus-Tetranychus* predator-prey interactions are a combination of these two approaches. Large scale population-dynamic studies of *Phytoseiulus-Tetranychus* interactions resulted in the hypothesis that chemical cues were essential to understand the spatial distributions leading to extermination of local prey populations (Sabelis *et al.* 1983; Sabelis & Van der Meer 1986). Subsequent laboratory experiments demonstrated that *Tetranychus*-induced plant volatiles and not prey-derived cues result in the attraction of *P. persimilis* to prey patches (Sabelis & Van de Baan 1983; Dicke *et al.* 1990a). The present study provides a new step in linking laboratory studies to investigations under field conditions.

(A) *T. urticae*-infested lima bean



(B) *P. brassicae*-infested brussels sprouts

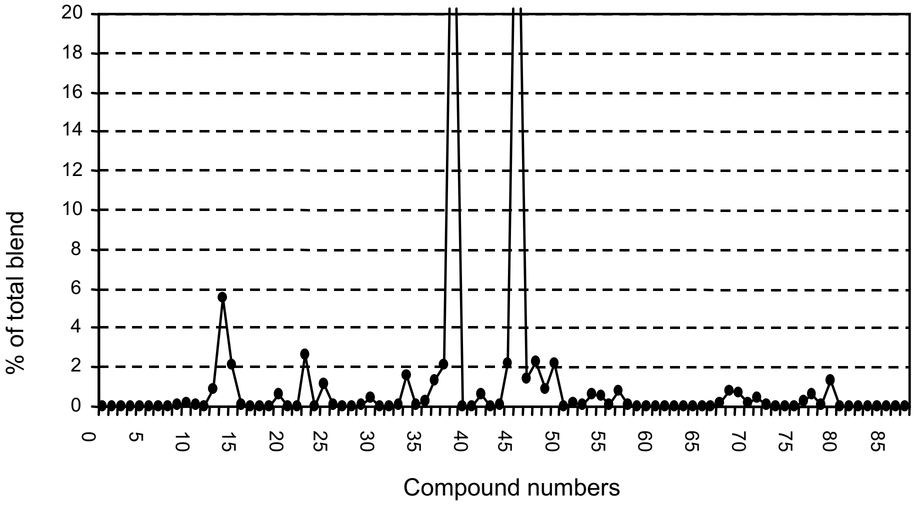


Figure 5: Odour blend composition of (a) spider mite (*T. urticae*) infested lima bean plants and (b) caterpillar (*P. brassicae*) infested brussels sprouts plants. Compound numbers: 1= 2 methyl-propanal-O-methyloxime; 2= 2 methyl butanal-O-methyloxime; 3= 3 methyl butanal-O-methyloxime; 4= 2 methylpropanenitrile, 5= 2-methylbutanenitrile, 6= 3-methylbutanenitrile; 7= rose furan; 8= 2-butenal; 9= (*E*)-2-pentenal; 10= (Z)-2-pentenal; 11= 2 methyl-2-propenal; 12= hexanal; 13= (*E*)-2-hexenal; 14= (Z)-2-hexenal; 15= 2,4-hexadienal; 16= octanal; 17= nonanal; 18= decanal; 19= 1-cyclopropyl-2-propen-1-one; 20= 2-butanone; 21= 3-butene-2-one; 22= 3-pentanone; 23= 3 methyl 3-buten-2-one; 24= 1-penten-3-one; 25= 3-penten-2-one; 26= 3-heptanone; 27= 3-octanone; 28= ethanol; 29= 2-methyl-1-propanol; 30= 2-butanol; 31= 2 methyl 3-buten-2-ol; 32= 3-methyl 1-butanol; 33= 1-penten-3-ol; 34= 1-pentanol; 35= 3-pentanol; 36= 1-hexanol; 37= (*E*)-2-hexen-1-ol; 38= (*Z*)-3-hexen-1-ol; 39= 1-octen-3-ol; 40= 1-nonanol; 41= 3-methyl-1-buten-1-yl acetate; 42= pentyl acetate; 43= isopentyl acetate; 44= hexyl acetate; 45= (*Z*)-3-hexen-1-yl acetate; 46= (*E*)-2-hexen-1-ol acetate; 47= (*Z*)-3-hexen-1-yl butanoate; 48= (*Z*)-3-hexen-1-yl

So far, no other study addressed the effect of odour mixing on attraction of carnivores to herbivore-induced plant volatiles. Yet, odour masking has been reported in a study on odours of undamaged plants. The parasitic fly *Drino bohemica* is attracted to volatiles from its host's (undamaged) food plant. However, this is not the case when the host's food odours are mixed with volatiles from another plant species (Monteith 1960). The mechanism underlying this effect has not been investigated. If the blends share important compounds that the fly uses, this may explain the negative effect of mixing the blends. This mechanism has been reported to underlie interference in host plant selection by the herbivorous beetle *Leptinotarsa decemlineata*: mixing odours from plants that largely overlap in blend components also interferes with food location by the herbivorous beetle (Visser & Avé 1978). Moreover, altering the ratios of blend components by adding individual components of the volatile blend from a suitable food plant to the total blend resulted in interference with the attraction of the beetles (Visser & Avé 1978). This shows that altering the ratio of attractive volatiles affects the behaviour of these herbivorous insects. Whether this is also true for the responses of carnivorous arthropods in general, and of the predatory mite *P. persimilis* in particular, still needs to be investigated. Our present experiments represent an extreme. The odour blends that were mixed have very different compositions. The next step in the research should be to use two plant species whose odour blends are more similar.

(Figure 5 continued)
isobutanoate; 49= (*Z*)-3-hexen-1-ol isovalerate; 50= (*Z*)-3-hexen-1-ol 2-methylbutanoate; 51= 3-hexen-1-yl propanoate; 52= 3-hexen-1-yl caproate; 53= (*Z*)-3-hexen-1-yl hexanoate; 54= isobutyric acid; 55= caproic acid; 56= isovaleric acid; 57= myrcene; 58= (*Z*)-β-ocimene; 59= (*E*)-β-ocimene; 60= (*Z*)-4,8-dimethyl-1,3,7-nonatriene; 61= (*E*)-4,8-dimethyl-1,3,7-nonatriene; 62= α-copaene; 63= linalool; 64= β-caryophyllene; 65= 4,8,12-trimethyl-1,3,7,11-tridecatetraene isomer; 66= (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene; 67= α-thujene; 68= sabinene; 69= limonene; 70= 1,8-cineole; 71= β-elemene; 72= α-farnesene; 73= methyl salicylate; 74= benzyl cyanide; 75= indole; 76= 2-ethyl furan; 77= dimethyldisulfide; 78= dimethyltrisulfide; 79= methyl-isothiocyanate; 80= unknown 55,83,84B; 81= unknown 91B,107,135; 82= unknown 91,93,95B,150, 83= unknown 41,55,69,70B,83,134; 84= unknown 41,69B,79,95,109,149,164; 85= unknown 41,69B,107,147,218; 86= unknown 43B,79,93,94,148,151,166; 87= unknown 67,71,82B. Based on Dicke *et al.* (1999) and Mattiacci *et al.* (1994).

Interestingly, there is evidence that mixtures of two odour blends can interfere with the attraction of carnivores when the blends are more similar. This evidence comes from experiments with different herbivore species on the same plant species, rather than from mixing odours from different plant species. Host location by the parasitoid *Cotesia glomerata* is hampered in a habitat in which non-host herbivores (*Plutella xylostella* caterpillars) occur together with hosts (*Pieris rapae* caterpillars), compared to a habitat without the non-host (Shiojiri et al. 2000; Vos et al. 2001). The volatile blends emitted by cabbage plants infested with different herbivore species are very similar (Geervliet et al. 1997; Shiojiri et al. 2001) and this may explain the disturbing effects. The interference with parasitoid foraging behaviour implies that *P. rapae* would benefit from preferentially selecting a habitat that has been infested with *Pl. xylostella*. Although this oviposition preference has not been recorded, females of *Pl. xylostella* do prefer to oviposit on plants infested with *P. rapae* (Shiojiri *et al.* 2002). On *P. rapae*-infested plants, *Pl. xylostella*-larvae may run a lower risk of parasitisation because their natural enemy *C. plutellae* prefers plants infested with its host alone over plants infested with both caterpillar species. Moreover, *C. plutellae* parasitises fewer hosts on plants infested with *P. rapae*. Consequently, interference with indirect plant defence may be exploited by herbivores. Many herbivorous arthropods are attracted to herbivore-induced plant volatiles (Dicke & Vet 1999), and this includes responses to plants infested with conspecifics and plants infested with heterospecifics. This may be unexpected in the context of direct interactions, as it leads to exploitative competition. The functional explanation for such attraction to competitor-infested plants may be an associated protection within the infochemical web such as reported by Shiojiri *et al.* (2002).

In the considerations discussed above, the carnivores were implicitly assumed to have a fixed searching behaviour in response to mixed odour blends. However, arthropods can learn to respond to complex blends of chemical information (reviewed by Papaj & Lewis 1993; Vet *et al.* 1995). So far, there are no reports on the effects of experience with hosts in mixed versus single plant species communities on subsequent foraging behaviour. Such phenotypically flexible behaviour may differentially affect interactions in a food web. For instance, experience with hosts as well as experience with non-hosts may enable carnivorous arthropods to discriminate between blends of volatiles that have only small differences (Vet *et al.* 1998).

It has long been known that the composition of plant communities can affect herbivore population dynamics in various ways, either through effects on herbivores directly or through effects on carnivores (e.g. Tahvanainen & Root 1972; Atsatt & O'Dowd 1976; Sheehan 1986; Abrams 1993; Abrams *et al.* 1996; McCann 2000). Food webs are overlaid with infochemical webs that mediate direct and indirect interactions (Dicke & Vet 1999; Sabelis *et al.* 1999). The infochemical web may modify interaction strengths and therefore, infochemical webs may have important consequences for food webs (McCann *et al.* 1998; Vos *et al.* 2001). Such potential effects of the infochemical web on the dynamics and persistence of a food web have mostly been neglected in food web studies (e.g. McCann 2000). This is unfortunate, because taking the infochemical web into account is likely to improve our understanding of direct and indirect interactions within a community and thus our understanding of ecosystem functioning.

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CHAPTER 3

Carnivore attraction to herbivore-induced plant volatiles: effects of mixing volatile blends and multiple infestation of plants

Abstract

Plants can be simultaneously attacked by a range of herbivorous arthropods and pathogens. Whereas it is well established that plants infested with a single herbivore species can attract specific carnivores through the emission of herbivore-induced volatiles, it remains to be investigated whether this indirect defence mechanism functions under more complex and realistic conditions. We investigated the responses of the predatory mite *Phytoseiulus persimilis*, a specialised natural enemy of spider mites, to (1) spider mite-induced plant volatiles mixed with volatiles induced by non-prey herbivores on heterospecific or conspecific plants, and (2) volatiles induced upon simultaneous infestation with spider mites and non-prey herbivores on the same individual plant. The responses of predators were not influenced by mixing the volatiles from spider mite-infested lima bean with the volatiles from *Spodoptera exigua*-infested cucumber or lima bean plants. This was shown both in a greenhouse experiment and in a Y-tube olfactometer in the laboratory. Simultaneous herbivory by spider mites and non-prey herbivores did not hamper the responses of predatory mites either; predators preferred the volatiles from lima bean or cucumber plants induced upon multiple species herbivory to the volatiles induced upon single species herbivory. Chemical analysis showed that upon single or multiple species herbivory lima bean and cucumber plants emitted volatile blends that differed mainly quantitatively. We conclude that lima bean and cucumber plants effectively attract the natural enemy of spider mites upon multiple species herbivory, or when neighbouring plants are infested with *S. exigua*.

INTRODUCTION

Apart from defence mechanisms that have a direct effect on herbivores, plants can also employ the natural enemies of their enemies. Such indirect defence mechanisms include provision of alternative food, shelter, or prey-location cues that can promote the effectiveness of carnivorous arthropods (Price *et al.* 1980). The emission of prey-location cues is induced upon herbivore attack and has been demonstrated for plants in several families (e.g. Dicke *et al.* 1990a; Turlings *et al.* 1990; Dicke 1999a). Many parasitoid wasps, predatory mites and bugs are known to use herbivore-induced plant volatiles to locate their prey or hosts (reviewed by Dicke 1999a; Dicke & Vet 1999; Sabelis *et al.* 1999a). Predatory arthropods can have a large impact on local herbivore populations and thereby also on plant fitness (Sabelis & Van der Meer 1986; Dicke & Sabelis 1989; Pels & Sabelis 1999; Van Loon *et al.* 2000a; Fritzsche Hoballah & Turlings 2001). Hence, herbivore-induced plant volatiles can have an important influence on the interactions between plants, herbivores and carnivores in food webs (Dicke & Vet 1999). Despite the wealth of knowledge on the production of volatiles and the responses of carnivores to volatiles as recorded for single plant species upon attack by a single herbivore species, little is known about the effectiveness of herbivore-induced volatiles under the more complex and realistic conditions that occur in a natural environment (e.g. Janssen 1999; Shiojiri *et al.* 2001; Hunter 2002).

In their natural environment, plants are part of a community in which some plants can be infested with one herbivore species while other plants (from the same or from different species) can be infested with another herbivore species. Consequently, mixing of volatile blends can occur and this may affect the responses of predators to the volatiles induced by their prey (chapter 2). When certain compounds are present in two volatile blends, mixing of these volatile blends may change the ratio between compounds. The ratios between compounds can be important in indicating the presence of prey to a foraging carnivore (e.g. Takabayashi *et al.* 1991; DeMoraes *et al.* 1998), and thus mixing of two volatile blends may result in the carnivore no longer recognising or detecting this information. Such effects of mixing volatile blends have been shown to disturb host plant location by herbivorous arthropods (Visser & Avé 1978).

In addition to mixing of volatile blends, a single plant can be attacked by more than one herbivore species at the same time (multiple herbivory). Multiple herbivory can affect both the production of herbivore-induced volatiles, the response of predators to these volatiles, and the predation rate on herbivores, as has been shown for two parasitoid wasp species (Shiojiri *et al.* 2000, 2001, 2002; Vos *et al.* 2001). Altered parasitoid responses as a consequence of multiple herbivory may result in an enemy-free space for herbivores (Shiojiri *et al.* 2002), or affect the persistence of parasitoid populations (Vos *et al.* 2001). These studies show the potential impact of multiple herbivory on the interactions in the studied food webs and thereby underline the importance of studying plant and carnivore responses to multiple herbivory.

In this study, we assess whether herbivore-induced plant volatiles effectively attract the natural enemy of one herbivore species when a second, non-prey, herbivore species feeds on another plant in the same environment or on the same individual plant. In particular, we ask how predators respond to volatiles induced by prey herbivores when these are mixed with volatiles induced by a non-prey herbivore feeding on:

(i) A *heterospecific* plant?
(ii) A *conspecific* plant?
(iii) The same individual plant?

The degree of overlap between the volatile blends induced by prey and non-prey herbivores is likely to affect how much the searching efficiency of carnivores is hampered by volatiles induced by non-prey herbivores (chapter 2). Such overlap will be larger between the volatile blends from conspecific plants upon attack by a single, but different, herbivore species (single herbivory) than between the volatile blends from heterospecific plants (Dicke 1999a). Similarly, it is likely that the overlap between the volatile blends from conspecific plants upon single and multiple herbivory is larger than between the volatile blends from conspecific plants upon single herbivory by different herbivore species. Thus, we hypothesise that situations (i) to (iii) represent increasingly challenging situations for a foraging predator. We test this hypothesis using lima bean (*Phaseolus lunatus*) and cucumber (*Cucumis sativus*), the two-spotted spider mite (*Tetranychus urticae*) and the beet armyworm (*Spodoptera exigua*), and the predatory mite *Phytoseiulus persimilis*, a specialised natural enemy of spider mites. We study the behaviour of predators in a Y-tube olfactometer in the laboratory, as well as in a greenhouse, using a set-up that mimics the field situation more closely. In addition, we make chemical analyses of the volatile blends of lima bean and cucumber plants upon single herbivory by the different herbivore species and upon multiple herbivory to test whether the degree of overlap of volatile blend composition indeed increases from situations (i) to (iii).

MATERIALS AND METHODS

Plants and herbivores

Lima bean plants (*Phaseolus lunatus* L. cv Sieva) and cucumber plants (*Cucumis sativus* L. cv Lange Groene Giganten) were grown in a greenhouse at 20-30 °C, 50-70 % r.h. and 16 h of light. Lima bean plants were used when their primary leaves had unfolded (and were about 8-12 cm wide), 10 to 15 days after sowing. Cucumber plants were used when their first two leaves were about 8 to 12 cm wide, 15 to 35 days after sowing. Two-spotted spider mites, *Tetranychus urticae* Koch (Acari: Tetranychidae), were reared on lima bean plants in a greenhouse-compartment under the same conditions as uninfested plants. Eggs of the beet armyworm *Spodoptera exigua* Hübner (Lepidoptera: Noctuidae) were obtained from a stock colony reared on artificial diet at the laboratory of Virology, Wageningen University, The Netherlands (Smits *et al.* 1986). Two to three egg batches were placed in a Petri dish together with a lima bean or cucumber leaf in a climate cabinet at 23±1 °C. Larvae were used in experiments within 24 h of hatching.

Predators

A stock colony of predatory mites, *Phytoseiulus persimilis* Athias-Henriot (Acari: Phytoseiidae), was kept on spider mite-infested lima bean leaves in a climate cabinet at 23±1 °C, 50-70 % r.h. and continuous light. To obtain females of the same age, five to six gravid females from this stock colony were introduced into each of a series of Petri dishes with a spider mite-infested lima bean leaf. These females were allowed to oviposit for two days and were subsequently removed. Fresh spider mite-infested leaves were added every two days to rear the offspring. The offspring females were used in experiments 9 days after initiation of the colonies, i.e. 1-2 days after the final moult. Prior to experiments, females were kept individually in Eppendorf vials without food but with a small droplet of water, for 24±2 h at 23±1 °C, unless stated otherwise.

Plant treatments

Three days before each experiment, the two oldest leaves of lima bean or cucumber plants were infested with herbivores, or left uninfested. After infestation, plants were incubated in a climate room at 24±1 °C, 50-70% r.h. and 16L:8D. Plastic cages were used to keep plants of the different treatments separated. We used the following four treatments:

(1) Infestation with *T. urticae*: Adult female spider mites from the greenhouse culture on lima bean were transferred to the experimental plants. 20 *T. urticae* females per leaf were used on lima bean (unless stated otherwise); 100 *T. urticae* females per leaf were used on cucumber. We used a higher number of spider mites on cucumber than on lima bean because the same number of spider mites result in a lower attraction of predators to cucumber than to lima bean (Dicke *et al.* 1990b).

(2) Infestation with *S. exigua*: Newly hatched *S. exigua* larvae were placed in a clip cage (constructed from two perspex cylinders, 2.5 cm ∅, and a hairpin; the

upperside was covered with gauze and the underside with Parafilm) to keep the caterpillars on the leaves. Lima bean plants were infested with two larvae in one clip cage per leaf, cucumber plants were infested with two or nine larvae per leaf (two larvae in one clip cage or nine larvae in two clip cages). Clip cages were moved daily to a new position on the same leaf and any missing larvae were replaced. The clip cages were supported by sticks to prevent the leaves from bending and incurring damage due to the weight of the cages.

(3) Infestation with *T. urticae* and *S. exigua*: Two newly hatched *S. exigua* larvae in a clip cage and 20 (lima bean) or 100 (cucumber) *T. urticae* females were placed on each leaf.

(4) Uninfested plants.

When clip cages were used to keep caterpillars in place, *T. urticae*-infested or uninfested plants were treated in the same way with empty clip cages to avoid any differences related to the use of the cages.

Greenhouse experiment

The behaviour of predatory mites was studied in a semi-field set-up in the greenhouse (23±2 °C, 30-50 % r.h., 16L:8D) (Janssen 1999; chapter 2). Six plants were placed in a wooden box in a hexagon of 40 cm ∅. A group of 100 well-fed predatory mite females was collected in a pipet-tip from the stock colony on lima bean about 1-2 h before the start of the experiment. The pipet tip was placed in the centre of the hexagon of plants in upright position, the wide side pointing upwards. The upper side of the pipet tip was open so that the predators could leave and forage in the experimental arena. Predatory mites were released into the arena at 10:30 AM. Each plant was subsequently examined for the presence of predators once per hour until 16:30 PM and once at 10:30 AM on the following day. Every predator that was found was removed from the set-up. We used six different combinations of plants in the hexagon set-up, different combinations were tested on different days. Three places of the hexagon were occupied by *T. urticae*-infested lima bean plants (20 spider mites per leaf) in every experiment, the other three places were occupied by:

a) Empty pots (6 independent replicates, on different days)
b) Uninfested cucumber plants (5 replicates)
c) Caterpillar-infested cucumber plants (2 *S. exigua* per leaf) (3 replicates)
d) Caterpillar-infested cucumber plants (9 *S. exigua* per leaf) (3 replicates)
e) Uninfested lima bean plants (3 replicates)
f) Caterpillar-infested lima bean plants (2 *S. exigua* per leaf) (3 replicates)

Positions of the plants were changed on different days of the experiments to avoid positional effects on the number of predators recaptured.

Y-tube olfactometer experiments

A closed system Y-tube olfactometer set-up was used to test the choices of predatory mites between two odour sources (Takabayashi & Dicke 1992; chapter 2). Odour sources consisted of four leaves of the same treatment that were cut from the plant just before an experiment, their petioles wrapped in wet cotton wool and aluminium foil, and placed in a 2 l glass container. We observed the behaviour of an individual predator for a maximum of 5 min and recorded a choice for one of the two odour sources when it reached the finish line halfway of one of the olfactometer arms. When the female did not make a choice within 5 min, a "no-choice" was recorded. Per experimental day, new groups of predatory mites and new sets of leaves were used. Sixteen to 23 predators were tested per experimental day and each experiment was repeated on four different days.

We tested the responses of predatory mites to *T. urticae*-induced volatiles in the following three experiments: (1) prey-induced volatiles mixed with non-prey-induced volatiles from a heterospecific plant; (2) prey-induced volatiles mixed with non-prey-induced volatiles from a conspecific plant; (3) volatiles from multiple-herbivore-infested plants versus volatiles from plants infested with a single herbivore species. In addition, we tested the attraction of the predators to the volatiles from lima bean and cucumber plants induced by prey or non-prey herbivores. The different combinations of odour sources, number of leaves and herbivores that we used for these experiments are specified for each experiment in the figure legends.

In experiments 1 and 2, the procedure of odour mixing was as follows: First, the choice of 16 to 18 predators between the volatiles from four *T. urticae*-infested lima bean leaves and the volatiles from four *S. exigua*-infested lima bean or cucumber leaves was determined. A set of eight *S. exigua*-infested cucumber or lima bean leaves was then placed in an odour container upwind of the original sets of *T. urticae-* and *S. exigua*-infested leaves. The air stream that was led through this odour container was split into two equal air streams, each representing the volatiles from four *S. exigua*-infested leaves. One air stream was led through the container with the original *T. urticae*-infested lima bean leaves, and the other one through the container with the original *S. exigua*-infested leaves. This resulted in a choice situation for the predators between the combination of the volatiles from four *T. urticae*-infested lima bean leaves plus four *S. exigua*-infested cucumber (or lima bean) leaves versus the volatiles from eight *S. exigua*-infested cucumber (or lima bean) leaves. In this second choice-situation a new set of 16 to 18 predatory mites was tested.

Volatile collection and analysis

Volatiles from the plants of the different herbivore-treatments (except for uninfested lima bean) were collected on the same day, but lima bean and cucumber were sampled on different days. Just prior to volatile trapping, plants were cut above the soil and the stem of each individual plant was wrapped in wet cotton wool and aluminium foil. All clip cages, and caterpillars and their products were removed before the collection of volatiles. Five plants of one treatment were transferred to a 5 l glass vessel. A viton O-ring and a metal clamp were used to attach the glass lid airtight to the vessel. Purified air (filtered through silica, a molecular sieve, activated charcoal, and 90 mg Tenax) was split into two air streams of about 100 ml/min and led into the vessels through teflon tubing. In this way, volatiles from two treatments could be collected simultaneously in parallel. The system was purged for 30 min before attaching a tube filled with Tenax (90 mg for lima bean samples, 200 mg for cucumber samples) to the air outlet in the lid to trap the volatiles. Lima bean volatiles were collected for 25 min, cucumber volatiles were collected for 3 h. For lima bean, the volatile collection was repeated 4 times for each treatment. For cucumber, volatile collections were made 7 times for the uninfested plants, 6 times for the *T. urticae*-infested plants, and 5 times for the *S. exigua*-infested plants and the multiple-herbivore-infested plants. During the analysis we lost one sample of the "*T. urticae*" and one of the "*S. exigua*" treatment of cucumber.

The volatiles from lima bean and cucumber were analysed in two different GC-MS systems:

Lima bean – Volatiles were released from the tenax traps with a thermodesorption coldtrap set-up (Chrompack, Middelburg, The Netherlands) by heating at 250 °C for 10 min, with a He-flow of 12 ml/min. The desorbed compounds were collected in the cold trap at –90 °C. Volatiles were injected in splitless mode into the DB5 column (60 m x 0.25 mm ID, 0.25 µm film thickness) by heating of the cold trap to 220 °C. After an initial column temperature of 40 °C for 4 min, the temperature was raised to 280 °C at 4 °C/min. The column was directly coupled to the ion source of a Finnigan MAT 95 mass spectrometer, which was operating in the 70 eV EI ionisation mode and scanning from mass 24 to 300 at 0.5 scans/sec. Compounds were identified by comparison of mass spectra with those in the NIST 98 library and in the Wageningen Mass Spectral Database of Natural Products, and by checking the retention indices.

Cucumber – Volatiles were released from the tenax traps with a thermodesorption coldtrap set-up (Markes, UK) by heating at 200 °C for 10 min, with a He-flow of 30 ml/min. The desorbed volatiles were collected in the cold trap at –10 °C. Volatiles were injected in splitless mode into the RTX-5Silms column (Restec, 30 m x 0.32 mm ID, 0.33 µm film thickness) by heating of the coldtrap to 270 °C. After an initial column temperature of 40 °C for 2 min, the temperature was raised to 95 °C at 3 °C/min, then to 165 °C at 2 °C/min, and subsequently to 250 °C at 15 °C/min. The column was directly coupled to the ion source of a Finnigan quadrupole mass spectrometer, which was operating in the 70 eV EI ionisation mode and scanning from mass 33 to 300 at 3 scans/sec. Compounds were identified by comparison of mass spectra with those in the NIST 98 and Wiley 7th edition spectral libraries, and by checking the retention indices.

For lima bean and cucumber separately, we quantified the amounts of the compounds that were present in at least N-1 replicates of at least one of the treatments.

Statistical analysis

To compare the numbers of predatory mites recaptured on the *T. urticae*-infested lima bean plants in the greenhouse experiments for the different combinations of plants, we used a one-way ANOVA. For this analysis, we used the fractions of the released predators that were recaptured on prey-infested lima bean plants, assuming a normal distribution for the range of data that we obtained, and checking the validity of this assumption by means of the residual plot.

The choices of predatory mites between two odour sources in the Y-tube olfactometer were analysed with a two-sided binomial test to investigate whether the distribution of the predators differed from 50:50. Predators that did not make a choice in the Y-tube olfactometer were excluded from this statistical analysis; this related to only 3 % of 1268 predators. To analyse the effect of mixing *T. urticae*-induced volatiles with *S. exigua*-induced volatiles in the Y-tube olfactometer (experiments 1 and 2) we used a generalised linear model, under the null-hypothesis that odour mixing had no effect (GLM, GenStat 6.1; McCullagh & Nelder 1989). The number of predators choosing for the volatiles from *T. urticae*-infested lima bean per day of the experiment was taken as the response variate. The total number of choosing predators per day was used as the binomial total. To check the validity of the model, we subjected the deviance of the residual to a χ^2-test. We used replicate and odour source as predicting factors for each separate experiment.

Differences in the emission of each volatile compound between the three herbivore treatments (single herbivory by *T. urticae*, or *S. exigua*, or multiple herbivory by *T. urticae* and *S. exigua*) were analysed with the non-parametric Kruskal-Wallis test, for lima bean and cucumber separately.

RESULTS

Greenhouse experiment

In the greenhouse-experiment, we recaptured predatory mites at different times of the day over a period of 24 h. However, time did not affect the difference in cumulative number of predators recaptured on the prey-infested lima bean plants and thus we present the cumulative results over 24 h. Prey-infested plants mixed with no other plants yielded the highest fraction of predators recaptured: 68 % (Figure 1). Significantly fewer predators were recaptured on *T. urticae*-infested lima bean plants when they were mixed with uninfested or *S. exigua*-infested lima bean plants, or with cucumber plants infested with 2 *S. exigua* larvae per leaf ($P<0.05$, ANOVA). In these treatments less than 40 % of the predators was found back on prey-infested plants. When *T. urticae*-infested lima bean plants were mixed with uninfested cucumber plants or cucumber plants infested with 9 *S. exigua* per leaf, around 50 % of the predators was recaptured on prey-infested plants. These two treatments did not differ significantly from any of the other treatments ($P>0.05$, ANOVA).

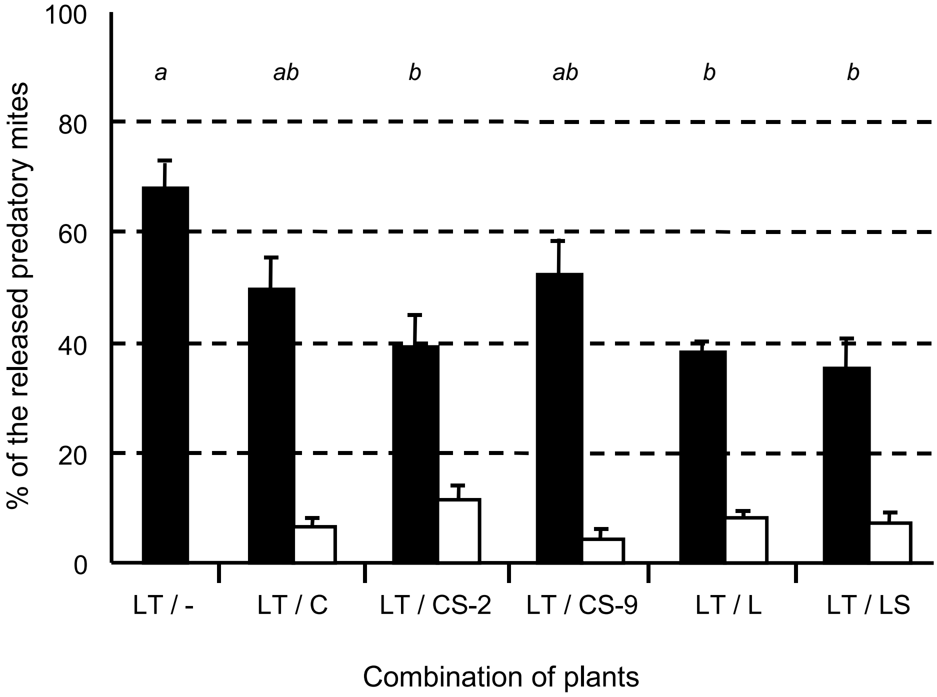

Figure 1: Greenhouse experiment on the searching efficiency of the predatory mite *Phytoseiulus persimilis* towards *Tetranychus urticae*-infested lima bean plants mixed with conspecific or heterospecific plants that were uninfested or infested with the non-prey herbivore *Spodoptera exigua*. Three positions of a hexagon set-up were occupied by spider mite-infested lima bean plants (LT) in all tests (20 spider mites were used per leaf). The other three positions were left unoccupied (–), or were occupied by uninfested cucumber plants (C), cucumber plants infested with 2 or 9 *S. exigua* per leaf (CS-2 and CS-9 respectively), uninfested lima bean plants (L), or lima bean plants infested with 2 *S. exigua* per leaf (LS). Black bars present the average cumulative percentage of predatory mites that was recaptured on prey-infested lima bean plants after 24 h. White bars present the average percentage of predators that was recaptured on the second plant in the set-up. Error bars represent the standard error. Treatments marked with a different letter differed significantly in the percentage predators recaptured on prey-infested lima bean plants ($P<0.05$, one-way-ANOVA).

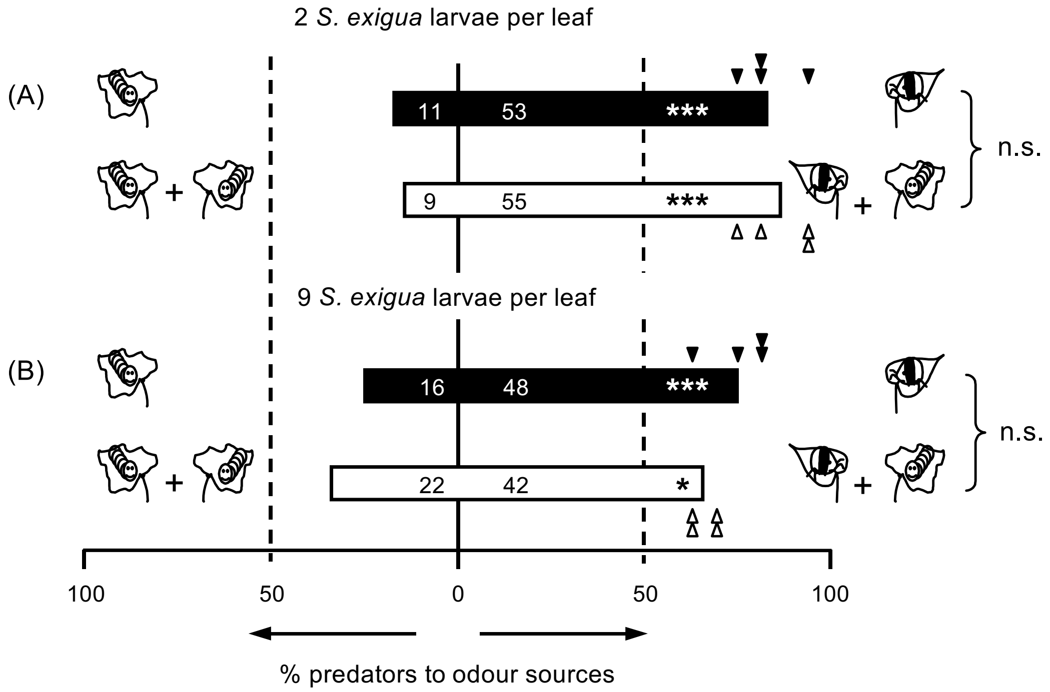

Figure 2: Effect of mixing the volatiles from *Tetranychus urticae*-induced lima bean with the volatiles from *Spodoptera exigua*-induced cucumber on the foraging responses of the predatory mite *Phytoseiulus persimilis* in the Y-tube olfactometer. Bars present the overall percentages of predators choosing for each odour source; numbers in bars are the total numbers of predators responding to each odour source. Triangles indicate the percentage predators choosing for one of the odour sources per day of the experiment. Odour sources: (A) Black bar: four *T. urticae*-infested lima bean leaves (20 per leaf) vs. four *S. exigua*-infested cucumber leaves (2 per leaf), white bar: four *T. urticae*-infested lima bean leaves (20 per leaf) plus four *S. exigua*-infested cucumber leaves (2 per leaf) vs. eight *S. exigua*-infested cucumber leaves (2 per leaf); B) Black bar: four *T. urticae*-infested lima bean leaves (20 per leaf) vs. four *S. exigua*-infested lima bean leaves (9 per leaf), white bar: four *T. urticae*-infested lima bean leaves (20 per leaf) plus four *S. exigua*-infested cucumber leaves (9 per leaf) vs. eight *S. exigua*-infested lima bean leaves (9 per leaf). Choices between odour sources were analysed with a two-sided binomial test; a GLM was used to analyse the effect of mixing prey-induced with non-prey-induced volatiles, the significance of which is indicated behind the brackets (predicting factors: replicate + odour source). (n.s. $P>0.05$; * $P<0.05$; *** $P<0.001$).

The non-prey herbivore feeding on a heterospecific plant

In the Y-tube olfactometer, the volatiles from lima bean infested with prey and cucumber plants infested with non-prey herbivores were mixed to form one new odour source. Mixing the volatiles from *T. urticae*-infested lima bean with the volatiles from *S. exigua*-infested cucumber did not influence the behavioural responses of predatory mites (Figure 2, $P \geq 0.24$, GLM). Predators preferred the volatiles from lima bean infested with 20 *T. urticae* per leaf to the volatiles from cucumber infested with 2 or 9 *S. exigua* per leaf ($P<0.001$, binomial test). This preference remained unaffected when both odour sources were mixed with volatiles from *S. exigua*-infested cucumber.

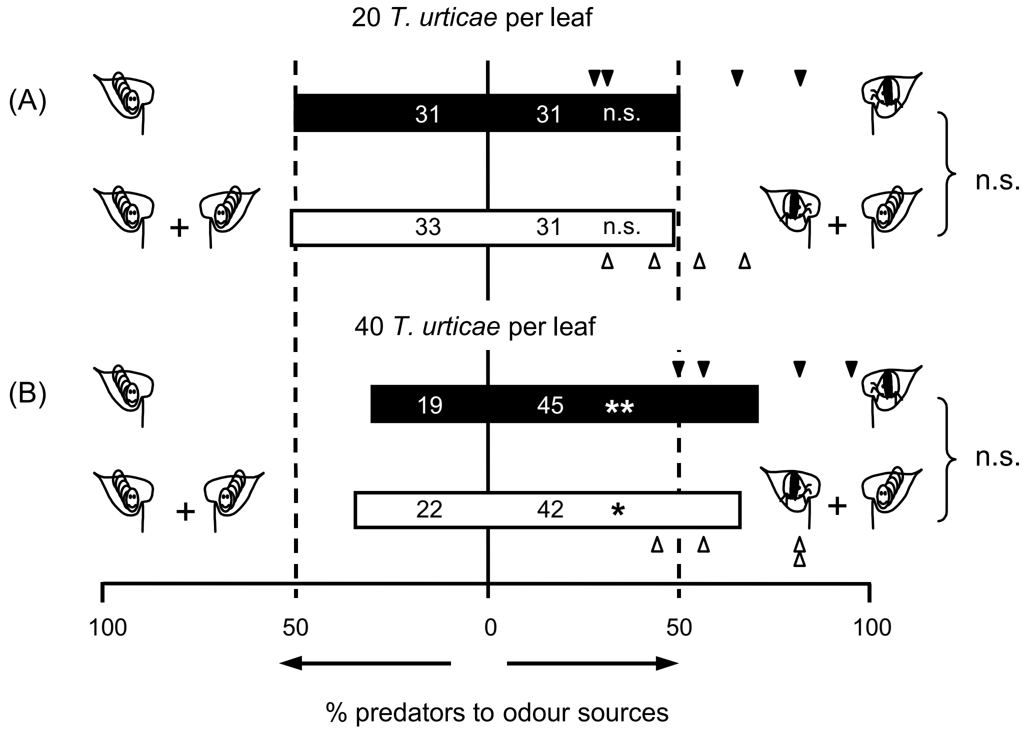

Figure 3: Effect of mixing the volatiles from *Tetranychus urticae*-induced lima bean with the volatiles from *Spodoptera exigua*-induced lima bean on the foraging responses of the predatory mite *Phytoseiulus persimilis* in the Y-tube olfactometer. Bars present the overall percentages of predators choosing for each odour source; numbers in bars are the total numbers of predators responding to each odour source. Triangles indicate the percentage predators choosing for one of the odour sources per day of the experiment. Odour sources: (A) Black bar: four *T. urticae*-infested lima bean leaves (20 per leaf) vs. four *S. exigua*-infested lima bean leaves (2 per leaf), white bar: four *T. urticae*-infested lima bean leaves (20 per leaf) plus four *S. exigua*-infested lima bean leaves (2 per leaf) vs. eight *S. exigua*-infested lima bean leaves (2 per leaf); (B) Black bar: four *T. urticae*-infested lima bean leaves (40 per leaf) vs. four *S. exigua*-infested lima bean leaves (2 per leaf), white bar: four *T. urticae*-infested lima bean leaves (40 per leaf) plus four *S. exigua*-infested lima bean leaves (2 per leaf) vs. eight *S. exigua*-infested lima bean leaves (2 per leaf). Choices between odour sources were analysed with a two-sided binomial test; a GLM was used to analyse the effect of mixing prey-induced with non-prey-induced volatiles, the significance of which is indicated behind the brackets (predicting factors: replicate + odour source). (n.s. $P>0.05$; * $P<0.05$; ** $P<0.01$).

The non-prey herbivore feeding on a conspecific plant

Mixing the volatiles from *T. urticae*-infested lima bean with the volatiles from *S. exigua*-infested lima bean did also not influence the behavioural responses of predatory mites (Figure 3, $P \geq 0.55$, GLM), but the preference of the predators for spider mite-induced volatiles depended on prey density. The predators did not discriminate between the volatiles from lima bean plants infested with 20 *T. urticae* per leaf to the volatiles from lima bean plants infested with 2 *S. exigua* per leaf in the Y-tube olfactometer ($P=1$, binomial test). Mixing both odour sources with volatiles from *S. exigua*-infested lima bean did not result in discrimination

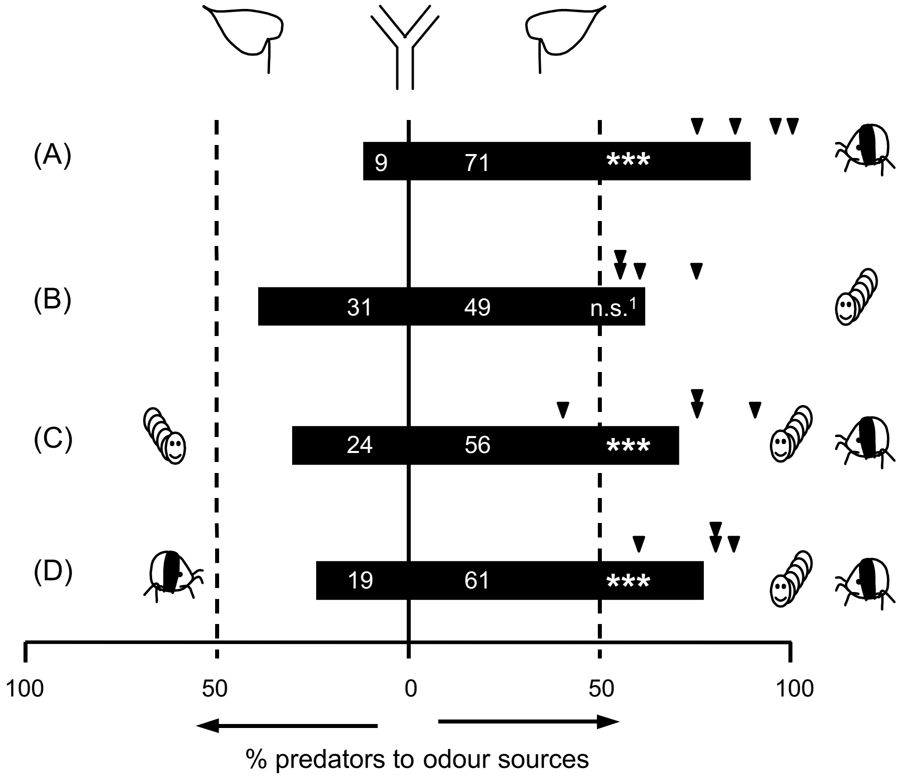

Figure 4: Responses of the predatory mite *Phytoseiulus persimilis* to volatile blends from lima bean plants infested *Tetranychus urticae* and/or *Spodoptera exigua* in the Y-tube olfactometer. Four (A and B) or nine (C and D) leaves were used per odour source, infestation level per leaf: (A) 20 *T. urticae* vs. uninfested; (B) 2 *S. exigua* vs. uninfested; (C) 20 *T. urticae* and 2 *S. exigua* vs. 2 *S. exigua*; (D) 20 *T. urticae* and 2 *S. exigua* vs. 20 *T. urticae*. Bars present the overall percentages of predators choosing for each odour source. Numbers in bars are the total numbers of predators responding to each odour source. Triangles indicate the percentage of predatory mites choosing for one of the odour sources per day of the experiment. Choices between odour sources were analysed with a two-sided binomial test. (n.s.[1] P=0.056; *** P<0.001).

either (P=0.90). However, predators did prefer the volatiles from lima bean plants infested with 40 *T. urticae* per leaf to the volatiles from lima bean plants infested with 2 *S. exigua* per leaf (P=0.002). This preference remained unaffected when both odour sources were mixed with volatiles from *S. exigua*-infested lima bean.

The non-prey herbivore feeding on the same individual plant: multiple herbivory

On lima bean, predatory mites preferred the volatiles induced upon multiple herbivory to volatiles induced by either *T. urticae* or *S. exigua* alone (Figure 4, P<0.001, binomial test). The volatiles induced by infestation of *T. urticae* alone on lima bean attracted the predators (P<0.001), whereas the attraction to the volatiles induced by infestation of *S. exigua* alone was almost significant (P=0.06).

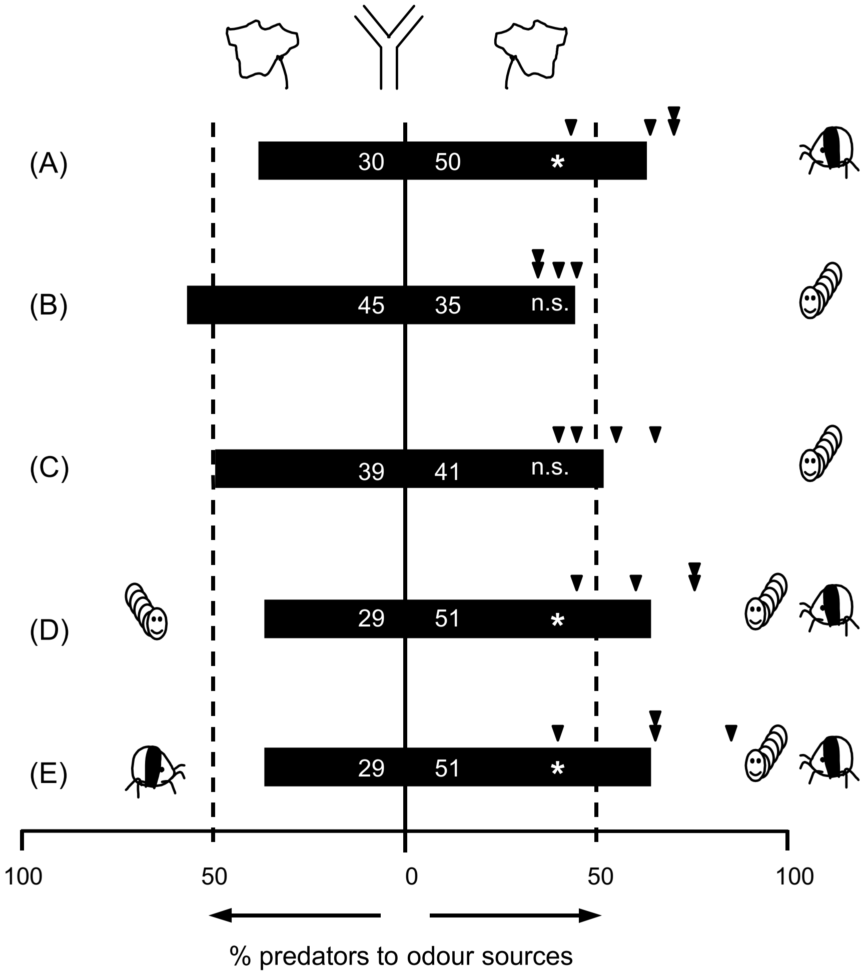

Figure 5: Responses of the predatory mite *Phytoseiulus persimilis* to volatile blends from cucumber plants infested with *Tetranychus urticae* and/or *Spodoptera exigua* in the Y-tube olfactometer. Four leaves were used per odour source, infestation level per leaf: (A) 100 *T. urticae* vs. uninfested; (B) 2 *S. exigua* vs. uninfested; (C) 9 *S. exigua* vs. uninfested; (D) 100 *T. urticae* and 2 *S. exigua* vs. 2 *S. exigua*; (E) 100 *T. urticae* and 2 *S. exigua* vs. 100 *T. urticae*. Bars present the overall percentages of predators choosing for each odour source. Numbers in bars are the total numbers of predators responding to each odour source. Triangles indicate the percentage of predatory mites choosing for one of the odour sources per day of the experiment. Choices between odour sources were analysed with a two-sided binomial test. (n.s. $P>0.05$; * $P<0.05$).

On cucumber, predatory mites also preferred the volatiles induced upon multiple herbivory to volatiles induced by either *T. urticae* or *S. exigua* alone (Figure 5, $P\leq0.02$, binomial test). The volatiles induced upon single herbivory by *T. urticae* on cucumber attracted the predators ($P=0.03$). Volatiles induced upon single herbivory by *S. exigua* on cucumber plants, on the other hand, did not attract the predators ($P\geq0.31$).

Volatile emission upon single or multiple herbivory

The main compounds emitted by *T. urticae*-lima bean plants were methyl salicylate (MeSA), (3*E*)-4,8-dimethyl-1,3,7-nonatriene [(*E*)-DMNT] and (3*E*,7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene [(*E*,*E*)-TMTT] (Figure 6A). Herbivory by *S. exigua* alone resulted in the emission of similar amounts of (*E*)-DMNT, but amounts of MeSA and (*E*,*E*)-TMTT were small (Figure 6B). In addition, *S. exigua*-infested lima bean leaves emitted large amounts of (*Z*)-3-hexen-1-ol acetate and (*E*)-β-ocimene. Multiple herbivory on lima bean plants resulted in a volatile blend that was qualitatively similar to the volatile profile upon herbivory by *S. exigua* alone, but different from herbivory by *T. urticae* alone. Multiple-herbivore-infested plants emitted larger amounts of all volatile compounds than plants infested with a single herbivore species (Figure 6C), except for (*Z*)-3-hexen-1-ol which was emitted in larger amounts by *S. exigua*-infested plants and MeSA which was emitted in larger amounts by *T. urticae*-infested plants.

In lima bean, herbivore treatment had a statistically significant effect on the emission of (*E*)- and (*Z*)-β-ocimene, (*Z*)-3-hexen-1-ol acetate, (*Z*)-3-hexen-1-ol, hexyl acetate, (*E*)-2-hexen-1-ol acetate, 1-octen-3-ol, 2-methylbutanal-O-methyl oxime, linalool, β-caryophyllene, indole, 2 unknown compounds, and the total of unidentified compounds ($P<0.05$, Kruskal-Wallis test). These compounds were all emitted in larger amounts upon herbivory by *S. exigua* alone and/or upon multiple herbivory than upon herbivory by *T. urticae* alone.

The main compounds emitted by *T. urticae*-infested cucumber plants were (*E*)-DMNT, (*E*)-β-ocimene, (*E*,*E*)-α-farnesene, (*Z*)-3-hexen-1-ol acetate, and 3-methylbutanal-O-methyl oxime (Figure 7B). The major volatile compounds emitted by *S. exigua*-infested cucumber plants were (*E*)-DMNT, (*E*)-β-ocimene, (*E*,*E*)-α-farnesene, 3-methylbutanal-O-methyl oxime, and decanal (Figure 7C). Multiple herbivory resulted in a volatile blend that was qualitatively similar to the blends induced upon herbivory by a single herbivore species (Figure 7D). Several compounds were emitted in the largest amount by multiple-herbivore-infested plants (e.g. (*E*)-β-ocimene and 3-methylbutanal-O-methyloxime), some by *T. urticae*-infested plants ((*Z*)-3-hexen-1-ol acetate and (*Z*)-3-hexen-1-ol), and some by *S. exigua*-infested plants (nonanal and decanal). (*E*,*E*)-TMTT was present upon herbivory by *T. urticae* alone and upon multiple herbivory, whereas the amount of (*E*,*E*)-TMTT upon single herbivory by *S. exigua* was small. No MeSA was detected in any of the cucumber samples.

In cucumber, herbivore treatment had a significant effect on the emmision of (*E*)-DMNT and 3-methylbutanal-O-methyloxime ($P<0.05$, Kruskal-Wallis test). Both compounds were emitted in larger amounts upon herbivory by *T. urticae* alone or in combination with *S. exigua* than upon herbivory by *S. exigua* alone.

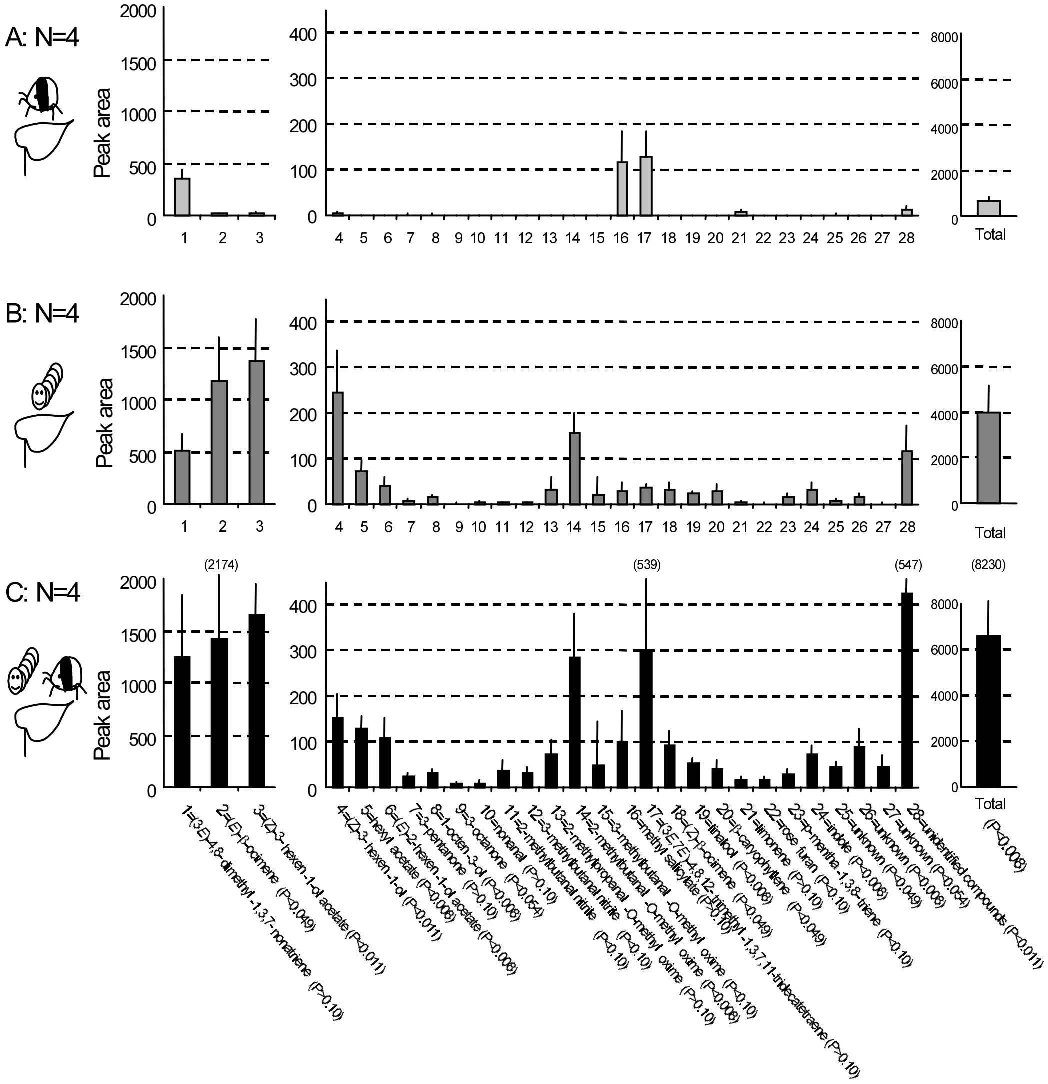

Figure 6: Volatile profiles of lima bean plants upon single herbivory by *Tetranychus urticae* (A: light grey bars, 20 mites per leaf), or *Spodoptera exigua* (B: dark grey, 2 larvae per leaf), or upon multiple herbivory by *T. urticae* and *S. exigua* (C: black). Bars present the mean amount of volatiles, error bars represent the standard error. P-values of the Kruskal-Wallis test are given for comparison of the different herbivore treatments.

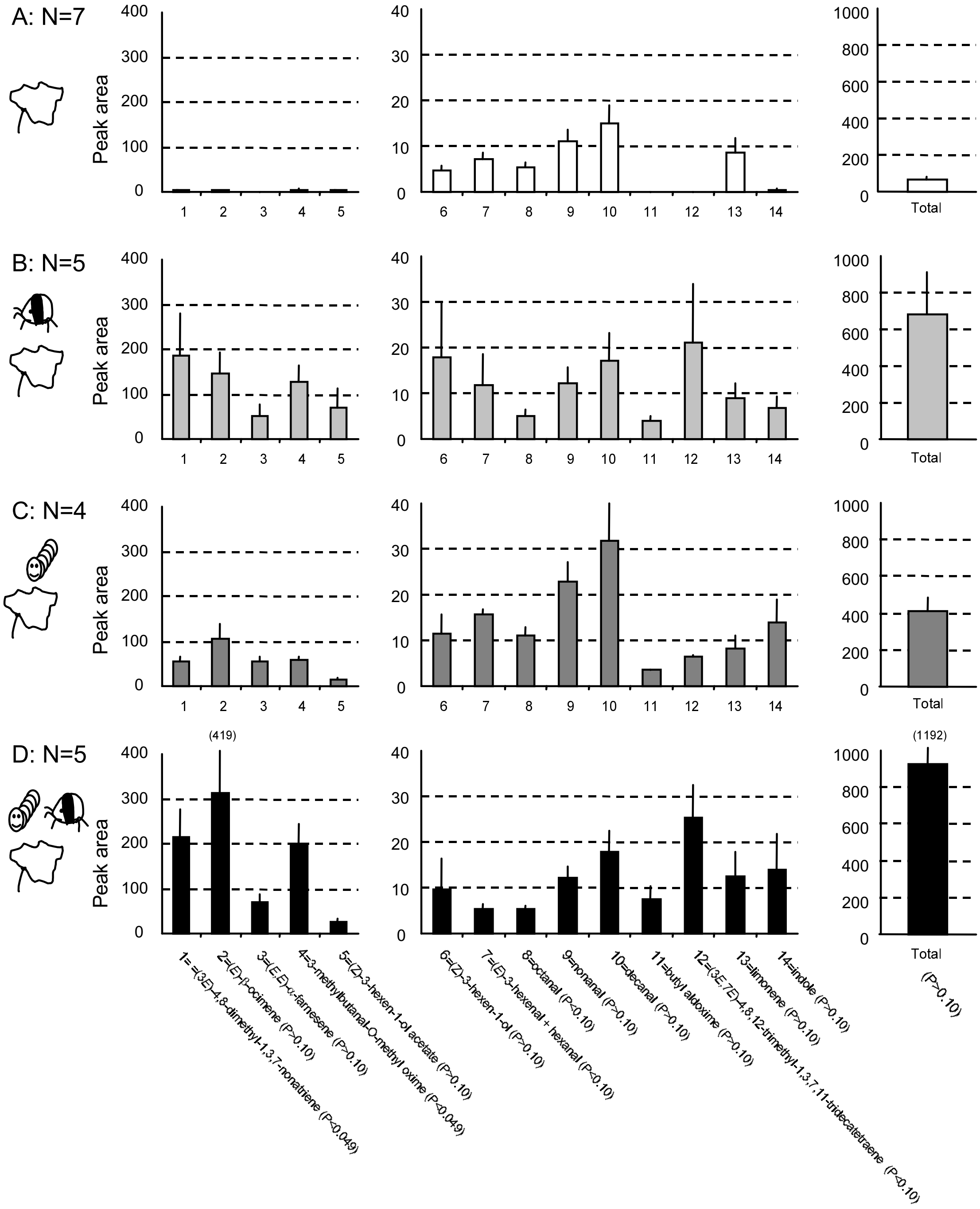

Figure 7: Volatile profiles of uninfested cucumber plants (A: white bars) and cucumber upon single herbivory by *Tetranychus urticae* (B: light grey, 100 mites per leaf), or by *Spodoptera exigua* (C: dark grey, 2 larvae per leaf), or upon multiple herbivory by *T. urticae* and *S. exigua* (D: black). Bars present the mean amount of volatiles, error bars represent the standard error. P-values of the Kruskal-Wallis test are given for comparison of the different herbivore treatments (B, C and D).

DISCUSSION

Effectiveness of herbivore-induced plant volatiles

In the greenhouse set-up the searching efficiency of predatory mites was reduced by the presence of a second plant, but this effect was not significant for all combinations of plants that we tested (Figure 1). Moreover this effect did not depend on the presence of *S. exigua* on the second plant. It is not likely that the physical structure of the second plant in the set-up interfered with the responses of *P. persmilis* because a previous greenhouse experiment showed that mixing prey-infested plants with artificial plants did not influence the number of predators recaptured (chapter 2). Possibly, the foraging responses of predatory mites were hampered by volatiles emitted by uninfested as well as by *S. exigua*-infested cucumber and lima bean plants. The Y-tube olfactometer experiments confirmed that *S. exigua*-induced volatiles from cucumber or lima bean did not influence the attraction of predatory mites to *T. urticae*-infested lima bean (Figures 2 and 3). Therefore, we conclude that mixing *T. urticae*-induced volatiles from lima bean with *S. exigua*-induced volatiles from heterospecific or conspecific plants does not interfere with the predator's foraging responses. The same conclusion was drawn from previous work on the responses of *P. persimilis* to the volatiles from *T. urticae*-infested lima bean plants mixed with the volatiles from *Pieris brassicae*-infested brussels sprouts plants (chapter 2).

Simultaneous herbivory by the prey and the non-prey herbivore resulted in the emission of volatiles that were more attractive to *P. persimilis* than volatiles induced upon infestation by one of the herbivore species alone (Figures 4 and 5). To the predator, these responses mean that they can still locate their prey in an environment where multiple herbivory by their prey *T. urticae* and the non-prey *S. exigua* occurs. However, it should be noted that the total level of damage inflicted on multiple-herbivore-infested plants was higher than on plants infested with a single herbivore species. It remains to be investigated how the predators respond to the volatiles from multiple-infested versus single herbivore-infested plants when the amount of damage is equal. Although a multiple-herbivore-infested plant appears easier to detect for predators than plants infested with the prey herbivore alone, there may also be disadvantages. On plants infested with both herbivore species, the caterpillars may pose a direct danger to the predators, for example by accidentally feeding on predator eggs. Moreover, the quality or quantity of prey may be lower on caterpillar-infested plants because the spider mites compete for food with the caterpillars, and the caterpillars may accidentally eat some spider mite eggs. However, these latter effects are also disadvantageous for the spider mites themselves and in addition multiple-herbivore-infested plants attract a larger number of predators. Spider mites are thus expected to select plants without caterpillars. In a preliminary experiment, we found that *T. urticae* females indeed preferred to oviposit on uninfested lima bean leaf discs compared to leaf discs from plants that had been infested with *S. exigua*, whereas in a no-choice situation they laid the same number of eggs on *S. exigua*-infested or control leaf discs (JG De Boer, G Van de Wiel, D De Wit, unpublished). A comparable finding has been reported for *Plutella xylostella*. Although the development of caterpillars was not influenced by previous infestation with heterospecific caterpillars, the females preferentially oviposited on *P. rapae*-infested versus uninfested plants (Shiojiri *et al.* 2002). The authors concluded that this is an adaptive response because multiple herbivory by *P. rapae* and *Pl. xylostella* resulted in a lower attraction and a lower parasitisation rate by the parasitoid wasp *Cotesia plutellae* thus creating an enemy-free space. To conclude about the effects of multiple herbivory on the interactions in a tritrophic system, one should thus investigate the responses of all species involved.

Production of herbivore-induced plant volatiles

We analysed the composition of the volatile blends of lima bean and cucumber plants upon single and multiple herbivory to test whether the degree of overlap increased in the three experiments for which we studied the responses of predatory mites. The overlap between the volatile blends from *T. urticae*-infested and *S. exigua*-infested *conspecific* plants was indeed larger than between *T. urticae*-infested and *S. exigua*-infested *heterospecific* plants (Table 1, Figures 6 and 7). However, within one plant species, the differences between the volatile blends induced by *T. urticae*, *S. exigua*, or the combination of both herbivore species were mainly quantitative. Therefore, the degree of overlap between the volatile blends from multiple herbivore-infested plants and conspecific plants infested with one of the herbivore species alone was similar to the degree of overlap between the blends emitted upon infestation with either of the herbivore species alone. Only one other study had previously compared the emission of volatiles upon multiple and single herbivory: multiple herbivory on cabbage by the caterpillars *P. rapae* and *Pl. xylostella* resulted in the emission of volatiles that were qualitatively similar to single herbivory (Shiojiri *et al.* 2001). In addition, Cardoza *et al.* (2002) have studied the emission of volatiles by peanut plants that were simultaneously infested with a fungus and caterpillars, or by one of the attackers alone. Single and multiple attack resulted in the emission of volatile blends that were both qualitatively and quantitatively different.

In conclusion, variation between volatile blends induced upon single and multiple species herbivory appear to be mainly quantitative. This suggests that the defence pathways activated by the different attackers at least partly overlap. Moreover, these results suggest that during prey location, carnivores are confronted with quantitative variation in herbivore-induced volatiles.

Table 1: Volatile compounds emitted by lima bean and cucumber plants upon infestation with *T. urticae* or *S. exigua* alone, or simultaneously with *T. urticae* and *S. exigua*.

	Lima bean			Cucumber		
Compound[1]	*T*[2]	*S*	*T & S*	*T*	*S*	*T & S*
Methyl salicylate	++[3]	+	++	–	–	–
(*E*,*E*)-α-farnesene	–	–	–	++	++	++
(*E*)-β-ocimene	+	+++	+++	++	++	+++
(*E*)-DMNT	++	++	+++	+++	+	+++
(*E*,*E*)-TMTT	++	+	+++	++	+	++
(*Z*)-3-hexen-1-ol acetate	+	+++	+++	++	+	+
(*Z*)-3-hexen-1-ol	+	+++	+++	++	+	+
Oxime	–	+	+	++	+	+++

[1] (*E*)-DMNT=(3*E*)-4,8-dimethyl-1,3,7-nonatriene; (*E*,*E*)-TMTT=(3*E*,7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene; Oxime=3-methylbutanal-*O*-methyl oxime

[2] *T*=*T. urticae*-infested (20 per leaf on lima bean, 100 per leaf on cucumber), *S*=*S. exigua*-infested (2 per leaf on both plant species), *T&S*=*T. urticae & S. exigua*-infested (20 or 100 spider mites per leaf on lima bean and cucumber respectively plus 2 caterpillars per leaf).

[3] Compounds have not been recorded (–), or emitted in minor (+), moderate (++), or major (+++) amounts.

How do predatory mites use herbivore-induced plant volatiles?

Despite the extensive literature on the responses of carnivores to herbivore-induced plant volatiles, it is not yet known *how* they perceive and interpret volatile blend composition (e.g. Turlings & Fritzsche Hoballah 1999; Dicke & Van Loon 2000a). Previous research has shown that *P. persimilis* can use the ratio between compounds as well as the presence of specific compounds in the volatiles blend to identify the presence of its prey on plants (Takabayashi *et al.* 1991; see also chapters 4 and 5). Although the chemical analyses showed that *T. urticae*-induced and *S. exigua*-induced volatiles from conspecific plants were more similar than from heterospecific plants, we could not conclude from behavioural responses that these situations were increasingly challenging for predatory mites. This can be explained by the fact that the *S. exigua*-induced volatiles from lima bean or cucumber did not or only weakly attract *P. persimilis*, suggesting that the compounds that identify the presence of prey to the predators were not induced by *S. exigua*. The strong preference of *P. persimilis* for the volatiles from multiple-herbivore-infested plants may be explained by the relatively large amount of several compounds in the multiple-herbivore-induced volatile blend. In lima bean, candidate compounds could for example be (*E*)-DMNT, MeSA, and (*E*,*E*)-TMTT, and in cucumber (*E*)-β-ocimene (Figures 6 and 7, Table 1). MeSA is a known attractant for *P. persimilis* (Dicke *et al.* 1990a; chapter 4) and it can play an important role in enabling predatory mites to discriminate between volatile blends that differ in the presence of this compound (chapter 4). (*E*)-DMNT and (*E*)-β-ocimene are also known to attract *P. persimilis* but their role within a volatile blend remains to be investigated (Dicke *et al.* 1990a). We will further examine the role of specific compounds in the foraging behaviour of predatory mites in chapter 5. Experience with volatiles is known to affect foraging responses of carnivores (e.g. Vet *et al.* 1995; Dicke *et al.* 1998; Drukker *et al.* 2000a). In the present article, we used predators that had been reared on lima bean and thus had experienced the volatile blend from spider mite-infested lima bean. How experience with herbivore-induced volatiles affects the responses of predatory mites to volatiles induced by prey or non-prey herbivores, or induced by more than one herbivore species simultaneously remains to be investigated. This issue will be addressed in chapters 7 and 8.

Concluding remark

We expected that foraging for prey would be increasingly difficult for predatory mites in an environment where spider mite-induced volatiles were mixed with the volatiles from non-prey-infested heterospecific or conspecific plants, or where non-prey herbivores infested the same individual plant as spider mites. However, in all tested situations spider mite-infested plants effectively attracted predators. These results imply that indirect plant defence against herbivorous arthropods through the attraction of predatory arthropods is effective in more complex and realistic situations than have been tested so far. This is an important new step to unravel the interactions between plants and the natural enemies of herbivores (Shiojiri *et al.* 2001; Vos *et al.* 2001; chapter 2; this study). Incorporating these situations in studies on plant defence narrows the gap between laboratory research on the mechanisms of defence and the field situation in which plants are confronted with their multiple attackers. Similarly, by studying these complex situations we will gain better insight in the complex foraging behaviour of carnivorous arthropods. Ultimately, the hypotheses based on the laboratory and greenhouse experiments will have to be tested in the field (e.g. Sabelis *et al.* 1999a; Hunter 2002).

Acknowledgements

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Part II

Discriminating between volatile blends: the role of volatile blend composition

CHAPTER 4

The role of methyl salicylate in prey searching behaviour of the predatory mite *Phytoseiulus persimilis*

Abstract

Many carnivorous arthropods use herbivore-induced plant volatiles to locate their herbivorous prey. These plant volatiles are blends of up to hundreds of compounds. It is often unknown which compounds in such a complex volatile blend represent the signal that reveals the presence of prey to the foraging carnivore. In this paper, we studied the role of methyl salicylate (MeSA) as part of the volatile blend in the foraging behaviour of the predatory mite *Phytoseiulus persimilis*, using a Y-tube olfactometer. MeSA is one of the compounds released by lima bean plants infested with *Tetranychus urticae* – a prey species of the predatory mite. MeSA attracted satiated predatory mites in a dose-dependent way with the optimum attraction at a dose of 0.2 μg. Predatory mites did not discriminate between a volatile blend from *T. urticae*-infested lima bean plants and the combination of this natural blend plus synthetic MeSA. However, they preferred a MeSA-containing volatile blend (induced by *T. urticae*) to an otherwise similar but MeSA-free blend (induced by jasmonic acid). Adding synthetic MeSA to the MeSA-free blend significantly increased the choice of predatory mites for this odour source such that they no longer preferred the volatiles from plants infested with their prey, strongly suggesting an important role for MeSA. This study is a new step towards unravelling the role of herbivore-induced plant volatiles in the foraging behaviour of predatory arthropods.

INTRODUCTION

Animals base their foraging decisions on information from their environment. One would expect animals in search of food to focus on the parts of the available information that are associated with their food, to maximise the efficiency of information use (Bernays & Wcislo 1994; Bernays 2001). However, it is often unknown which parts of the information contain the relevant signals for a foraging animal (see for example Dicke 1999b). We studied this issue for a predatory mite that uses herbivore-induced plant volatiles to locate its herbivorous prey.

Indirect information

Most prey species are inconspicuous: they are under selection to minimise the information that they emit to reduce the risk of predation. Consequently, various carnivorous animals use information that is not emitted by their prey but instead by their prey's direct environment. A well-explored example of this phenomenon is the use of information from the prey's food plant by natural enemies of arthropod herbivores (Vet & Dicke 1992; Paré & Tumlinson 1999). Although plants can produce well-detectable and reliable information about the presence of herbivorous arthropods, herbivore-induced plant volatiles can be highly variable. Many factors influence the composition of herbivore-induced volatile blends: plant species, plant cultivar, leaf age and plant tissue, but also herbivore species or even developmental stage (e.g. Takabayashi *et al.* 1994a; Takabayashi *et al.* 1995; De Moraes *et al.* 1998; Turlings *et al.* 1998; Gouinguené *et al.* 2001). A pathogen infection in addition to herbivore damage may also affect volatile blend composition (Cardoza *et al.* 2002). Furthermore, environmental conditions that affect the plant's physiology, such as light and water, contribute to variation in volatile production (Takabayashi *et al.* 1994b; Gouinguené & Turlings 2002).

Given the large variation in the composition of herbivore-induced volatile blends, the question arises *which* parts of the volatile blends are used by the carnivores to detect the presence of their prey. Little is yet known about *how* carnivores perceive and interpret volatile blend composition and its variation (Dicke 1999b; Dicke & Vet 1999; Turlings & Fritzsche 1999; Vet 1999b). Because of the large overlap in herbivore-induced volatiles between different plant-herbivore complexes (for example Turlings *et al.* 1993a; Paré & Tumlinson 1999; Van den Boom *et al.* 2004), it is generally assumed that most carnivores do not use a single key-compound to detect their prey. Indeed, carnivores perceive and respond to more than one compound of herbivore-induced plant volatile blends (Dicke *et al.* 1990a; De Bruyne *et al.* 1991; Smid *et al.* 2002). On the other hand, it is also not expected that all parts of a volatile blend contain relevant information for a searching carnivore because not all variation in blend composition is related to the presence of the prey herbivore.

Several studies have addressed the question which compounds in a complex odour blend cause the attractiveness of a blend to carnivorous arthropods (Dicke *et al.* 1990a; Turlings *et al.* 1991; Scutareanu *et al.* 1997). A good method to narrow down the range of potentially active compounds in a volatile blend is gas chromatography coupled with electroantennography (GC-EAG) (e.g. Du *et al.* 1998; Weissbecker *et al.* 2000). Using this technique, volatiles that are perceived by the sensory system of the arthropod can be identified. However, activity of compounds at the sensory level is not always directly correlated to behavioural activity of compounds (Li *et al.* 1992). The ecological significance of potentially interesting compounds should therefore always be evaluated in behavioural set-ups.

In the field, a foraging carnivore will have to make choices between volatile blends induced by their prey, and volatile blends that have been induced by other factors, such as non-prey herbivores. The question what variation between volatile blends enables the carnivores to make such a choice has not yet been answered (Vet 1999a,b; Dicke & Van Loon 2000). We address this question by using two plant volatile blends of known similar composition, and between which a carnivore can discriminate. By restoring one volatile blend with the compounds that it misses compared to the other volatile blend, the compounds that play a role in discrimination by the carnivore can be identified.

The role of methyl salicylate in the foraging behaviour of predatory mites

We studied the attraction of the predatory mite *Phytoseiulus persimilis* to lima bean plants infested with the herbivorous spider mite *Tetranychus urticae*. The interactions in this tritrophic system have been studied extensively (e.g. Sabelis & Van de Baan 1983; Dicke *et al.* 1990a; Drukker *et al.* 2000a). *Phytoseiulus persimilis* is a specialised natural enemy of spider mites in the genus *Tetranychus*. Plant volatiles are important foraging cues for *P. persimilis* (Sabelis & Van de Baan 1983; Sabelis & Dicke 1985; Dicke *et al.* 1990a). The spider mite *T. urticae* is a highly polyphagous herbivore that feeds on many different plant species (Helle & Sabelis 1985) and thus *P. persimilis* is potentially confronted with a large variation in herbivore-induced volatile blends.

We specifically investigated the influence of both *quantitative* and *qualitative* differences in the amount of one compound of the herbivore-induced volatile blend of lima bean plants – methyl salicylate (MeSA) – on the foraging behaviour of *P. persimilis*. Several studies have presented data that indicate a role for MeSA in foraging by *P. persimilis*. First, MeSA is attractive to *P. persimilis* when offered as a single compound (Dicke *et al.* 1990a). Second, *P. persimilis* prefers the volatiles from *T. urticae*-induced lima bean to the volatiles from jasmonic acid (JA)-induced lima bean and the absence of MeSA in the latter blend is one of the most important differences between the two volatile blends (Dicke *et al.* 1999).

In this article we study the responses of *P. persimilis* to:

(i) Different doses of MeSA

(ii) Two natural *T. urticae*-induced lima bean volatile blends, one of which was combined with synthetic MeSA

(iii) A natural volatile blend (*T. urticae*-infested lima bean), an incomplete blend (JA-induced lima bean) and an incomplete but restored blend (JA-induced lima bean plus MeSA) versus uninfested lima bean volatiles

(iv) The natural *T. urticae*-induced lima bean volatile blend versus the incomplete JA-induced blend, that was or was not restored with synthetic MeSA

MATERIALS AND METHODS

Plants and mites

Lima bean plants (*Phaseolus lunatus*, cv Sieva) were reared in a greenhouse (25±5 °C, 50-70 % r.h., photoperiod 16L:8D). They were used for experiments when the primary leaves had unfolded (10 to 15 days after sowing). A culture of the two-spotted spider mite, *Tetranychus urticae* Koch, was kept on lima bean plants under greenhouse conditions. The predatory mites, *Phytoseiulus persimilis* Athias-Henriot, were reared on lima bean leaves infested with *T. urticae* in Petri dishes (9 cm Ø) in a climate room (23±1 °C, 50-70 % r.h., 16L:8D). In all experiments, we used mated adult females, one to two days after the final moult. Females were kept individually in Eppendorf vials for 2-5 h (satiated) or 22-26 h (starved) before an experiment, at 23±1 °C. We used both satiated and starved predatory mites in this study because the starvation level may affect their searching behaviour (Dicke *et al.* 1998; Shimoda & Dicke 2000).

Y-tube olfactometer

In all experiments, the responses of the predatory mites were tested in a Y-tube olfactometer (Takabayashi & Dicke 1992; chapter 2). Individual predators were observed in the olfactometer, females that did not make a choice for one of the two odour sources within 5 min were recorded as "no choice". Each experiment was repeated on 4 to 6 different days with new groups of about 20 predatory mites and new odour sources per day. In experiments where we compared the responses of satiated and starved predatory mites, the mites of the two groups were tested alternately with the same odour sources. All olfactometer experiments were conducted at 21±2 °C.

Responses to methyl salicylate

Methyl salicylate (MeSA, Merck, 99 % pure) was diluted in n-hexane (Merck, pro-analysi). In all experiments 0.1 ml of the diluted MeSA was applied on a piece of filter paper (15 cm^2) and placed in the last section of one of the arms of the Y-tube olfactometer. A filter paper with 0.1 ml hexane was placed at the same position in the other arm as a control. A predatory mite was introduced into the olfactometer after 30 to 60 seconds when most of the solvent had evaporated. New filter papers with MeSA or hexane were used for each predatory mite. Different doses of MeSA (0.002 µg, 0.02 µg, 0.2 µg, 2 µg, 20 µg and 200 µg MeSA dissolved in 0.1 ml hexane) were tested on different days. In a separate experiment we compared the responses of satiated and starved predatory mites to 0.2 µg MeSA.

Quantitative differences in amount of methyl salicylate

In this experiment, we studied the effect of varying the relative amount of MeSA in a volatile blend on the behaviour of predatory mites. This was done by adding MeSA to a natural, MeSA-containing, volatile blend emitted by spider mite-infested lima bean leaves. Primary lima bean leaves were detached, individually placed with their petiole in glass vials filled with water (15 ml) and infested with 50 adult spider mite females per leaf. The leaves were incubated in a plastic cage in a climate room for three days at 23±1 °C, 50-70 % r.h. and 16L:8D. One odour source consisted of four *T. urticae*-infested leaves from four lima bean plants, the alternative odour source consisted of the other infested primary leaves from the same four plants. In this way, we standardised the two sets of infested leaves to minimise differences between the two odour sources. Each odour source was placed in a glass jar (2 l) and connected to the Y-tube olfactometer with plastic tubing. Although we expected the two sets of infested lima bean leaves to be equally attractive, we first determined the response of 10-20 *P. persimilis* females to both odour sources in the Y-tube olfactometer. After the relative attraction of both odour sources was determined, a filter paper with 0.1 ml MeSA solution was placed in the olfactometer arm downwind of the set of infested lima bean leaves that had attracted 50 % or more of the predators in the previous relative attraction test. It should be noted that the lima bean leaves were not exposed to the MeSA so that MeSA cannot have affected the volatile emission by these leaves. A filter paper with 0.1 ml hexane was placed at the same position in the other arm of the Y-tube. New filter papers with MeSA and hexane were used for every predatory mite. The influence of different amounts of synthetic MeSA on the choice between two natural volatile blends was studied: 0.2 µg, 2 µg, 20 µg and 200 µg. In a separate experiment, the responses of starved predators were tested at a MeSA dose of 0.2 µg only.

Qualitative differences in amount of methyl salicylate

To obtain odour sources that differed qualitatively in the presence of MeSA, we used the plant hormone jasmonic-acid (JA). Lima bean plants incubated with JA emit a volatile blend that is similar to the volatile blend released by *T. urticae*-induced lima bean, except for the presence of MeSA (Dicke *et al.*, 1999).

The following four comparisons were made to investigate the importance of a qualitative difference in the amount of MeSA:

(1) JA-induced lima bean versus clean lima bean: Young lima bean plants were cut at soil level and placed with the stem in a glass vial containing a 15 ml solution of jasmonic acid (1 mM, JA, Sigma-Aldrich) in water. Plants were incubated for two days in a plastic cage in a climate room (23±1 °C, 50-70 % r.h., 16L:8D). The air was sucked from the cage by house vacuum. Clean lima bean plants, incubated in the same way in vials with water only, served as the control odour source. Nine leaves were used per odour source. We used the same odour sources to test the responses of satiated and starved predators alternately on the same days.

(2) JA-induced lima bean plus synthetic MeSA versus clean lima bean: One odour source consisted of nine JA-induced lima bean leaves (see above) in a glass jar and a filter paper with 0.2 μg MeSA in the last section of the Y-tube (i.e. downwind of the lima bean leaves, to avoid that the leaves were exposed to MeSA). The control odour source consisted of nine uninfested lima bean leaves in a glass jar and a filter paper with 0.1 ml hexane downwind in the olfactometer-arm. We used the same odour sources to test the responses of satiated and starved predators alternately on the same days.

(3) *T. urticae*-induced lima bean versus clean lima bean: Odour sources consisted of four lima bean leaves, each infested with 50 *T. urticae* females, and four uninfested lima bean leaves. Leaves were incubated as described above in the section 'Quantitative differences in amount of methyl salicylate'. We used the same odour sources to test the responses of satiated and starved predators alternately on the same days.

(4) *T. urticae*-induced lima bean versus JA-induced lima bean plus synthetic MeSA: Young lima bean plants were cut at soil level and placed with their stem in a glass vial with JA dissolved in water (1 mM, 15 ml). Plants were incubated for two days in a plastic cage in a climate room (23±1 °C, 50-70 % r.h., 16L:8D). Plants that were infested with 50 *T. urticae* females per leaf were incubated in the same way in water only. Nine leaves were used per odour source. After testing the choice of 10 predators in the Y-tube olfactometer, a filter paper with MeSA (0.2 μg in 0.1 ml hexane) was placed in the olfactometer arm, downwind of the JA-induced lima bean leaves. A filter paper with hexane was placed in the other arm as a control. Then, the response of a new set of 20 predators was tested to the volatiles from *T. urticae*-infested lima bean leaves plus hexane versus the volatiles

from JA-induced lima bean leaves plus MeSA. Subsequently the last sections of both olfactometer arms were replaced by clean parts to exclude effects of any MeSA or hexane left on these parts. Then the response to *T. urticae*-induced volatiles versus JA-induced volatiles was tested for another 10 predators. The responses of the two groups of 10 predators that were used to determine their choices between JA-induced and *T. urticae*-induced volatiles, were not different (2x2 contingency table, $\alpha=0.05$) and thus the summed results of these two groups are presented. The responses of satiated and starved predators were tested in experiments carried out on different days.

Statistical analysis

Contingency table analysis (nx2 contingency tables, where n=number of experimental days) showed that results from replicate tests, done on different days with different batches of odour sources and different sets of predators, do not differ significantly. Therefore, the data from different experimental days were pooled for each experiment.

A two-sided binomial test was used to analyse the choices of predators in the olfactometer (i.e. a difference from a 50:50 distribution over the two odour sources) for all experiments. Predators that did not make a choice were excluded from the statistical analysis. Overall the percentage of satiated predators that did not make a choice was 11 % (of 1787 predators); less than 4 % of the 645 starved predators did not make a choice.

The response of satiated predatory mites to different doses of MeSA was analysed by fitting a generalised linear model (GLM), using the binomial distribution and logit-link-function with log-dose and square of log-dose as predicting factors (Crawley 1993). To test the effect of starvation level on the responses of predatory mites to MeSA, JA-induced lima bean volatiles (plus MeSA), and *T. urticae*-induced lima bean volatiles, we fitted a GLM with replicate and starvation level as predicting factors. The same procedure was used to test the effect of adding synthetic MeSA on the choice of predators between two *T. urticae*-induced volatile blends or between JA-induced and *T. urticae*-induced volatiles (predicting factors: replicate + odour source).

RESULTS

Response to methyl salicylate

Methyl salicylate offered as a single compound was attractive to satiated *P. persimilis* females (Figure 1). Although a low dose of 0.002 μg did not attract *P. persimilis* ($P=0.21$, binomial test), doses from 0.02 μg to 20 μg attracted the predators ($P\leq0.018$). The predatory mites were repelled by the highest dose of

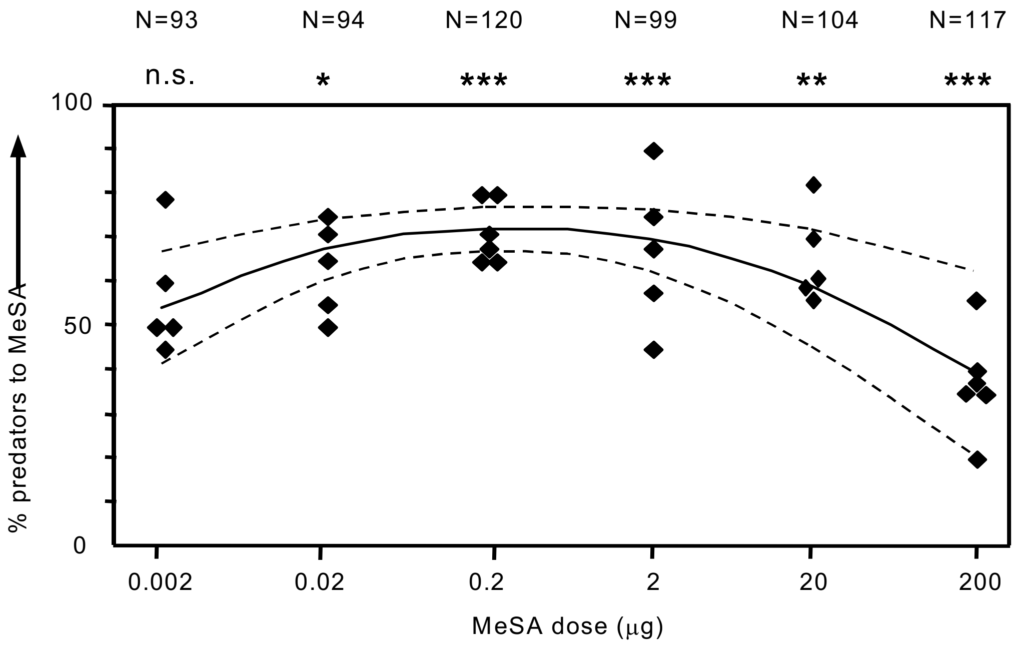

Figure 1: Responses of satiated female *Phytoseiulus persimilis* to different amounts of MeSA in the Y-tube olfactometer versus control (hexane). Each square represents the percentage of about 20 female predators choosing for MeSA in one replicate experiment. The solid curve shows the predicted values of the generalised linear model (see Materials and Methods) for the attraction of predators to different doses of MeSA; the dotted curves indicate the 95-confidence limits of the predicted values of the model. The total number of predators that made a choice is given for each dose of MeSA. Results were statistically analysed with the binomial test of which the two-sided P-value is given for each dose of MeSA (n.s. $P>0.05$; * $P<0.05$; ** $P<0.01$; *** $P<0.001$).

MeSA (200 µg; $P<0.001$). The maximum attraction to MeSA was found at 0.2 µg, where on average 72 % of the mites were attracted. The percentage of predators attracted to MeSA was thus dose-dependent. Both log-dose and square-log-dose were highly significant as factors predicting attraction ($P<0.001$). The dose-response curve as described by the model is shown in Figure 1.

Starved predatory mites were also attracted to 0.2 µg MeSA (Figure 2, $P<0.001$, binomial test). Their response was not significantly different from the response of satiated predators to 0.2 µg MeSA ($P=0.37$, GLM).

Quantitative differences in amount of methyl salicylate

To evaluate the effect of the relative amount of MeSA in the volatile blend, satiated predatory mites were offered a choice between the volatiles from two sets of *T. urticae*-infested lima bean leaves – one having an additional amount of synthetic MeSA, and the other not. The extra amount of MeSA did not affect the attraction of *P. persimilis* to the natural volatile blend except when the highest, repellent, dose of MeSA (200 µg) was added (Figure 3). In the latter case, the

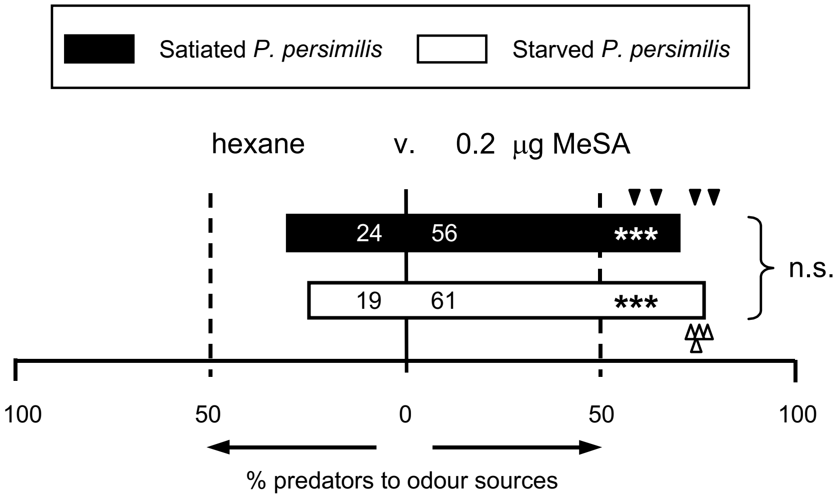

Figure 2: Responses of satiated (black bar) and starved (white bar) *Phytoseiulus persimilis* to 0.2 µg MeSA. The numbers in the bars are the absolute numbers of predators that made a choice for MeSA or the control. The triangles represent the percentage of predators in each replicate experiment that was attracted to MeSA. Choices between odour sources were analysed with a two-sided binomial test, and the effect of starvation level was analysed with a GLM, the significance of which is indicated behind the bracket (n.s. $P>0.05$; *** $P<0.001$).

average attraction towards the volatiles from the most attractive set of *T. urticae*-infested lima bean leaves was 61 %, while after adding 200 µg MeSA to this set of leaves the attraction dropped to 31 % ($P=0.003$, GLM).

Adding 0.2 µg MeSA to one of two sets of *T. urticae*-infested lima bean leaves did not influence the response of starved *P. persimilis* to the volatiles from these two sets of infested leaves either (Figure 3, $P=0.32$, GLM).

Qualitative differences in amount of methyl salicylate

The volatiles from JA-induced lima bean leaves attracted satiated *P. persimilis* females (Figure 4, 68 %, $P=0.002$, binomial test) but starved predators were not attracted (55 %, $P=0.44$). A direct statistical comparison of the responses of both groups of mites showed that the effect of starvation level was almost significant ($P=0.060$, GLM). However, in three out of four replicate experiments, the satiated predator mites were attracted more to the volatiles from JA-induced lima bean than starved mites.

The satiated predatory mites were strongly attracted to the volatiles from JA-induced lima bean leaves plus 0.2 µg MeSA (Figure 4, 87 %, $P<0.001$, binomial test), whereas starved predators were not attracted (60 %, $P=0.11$). The behavioural responses of satiated and starved predators to JA-induced volatiles plus MeSA were significantly different ($P<0.001$, GLM). In all four replicate experiments more satiated than starved predators preferred the volatiles from JA-induced lima bean plus MeSA to uninfested lima bean.

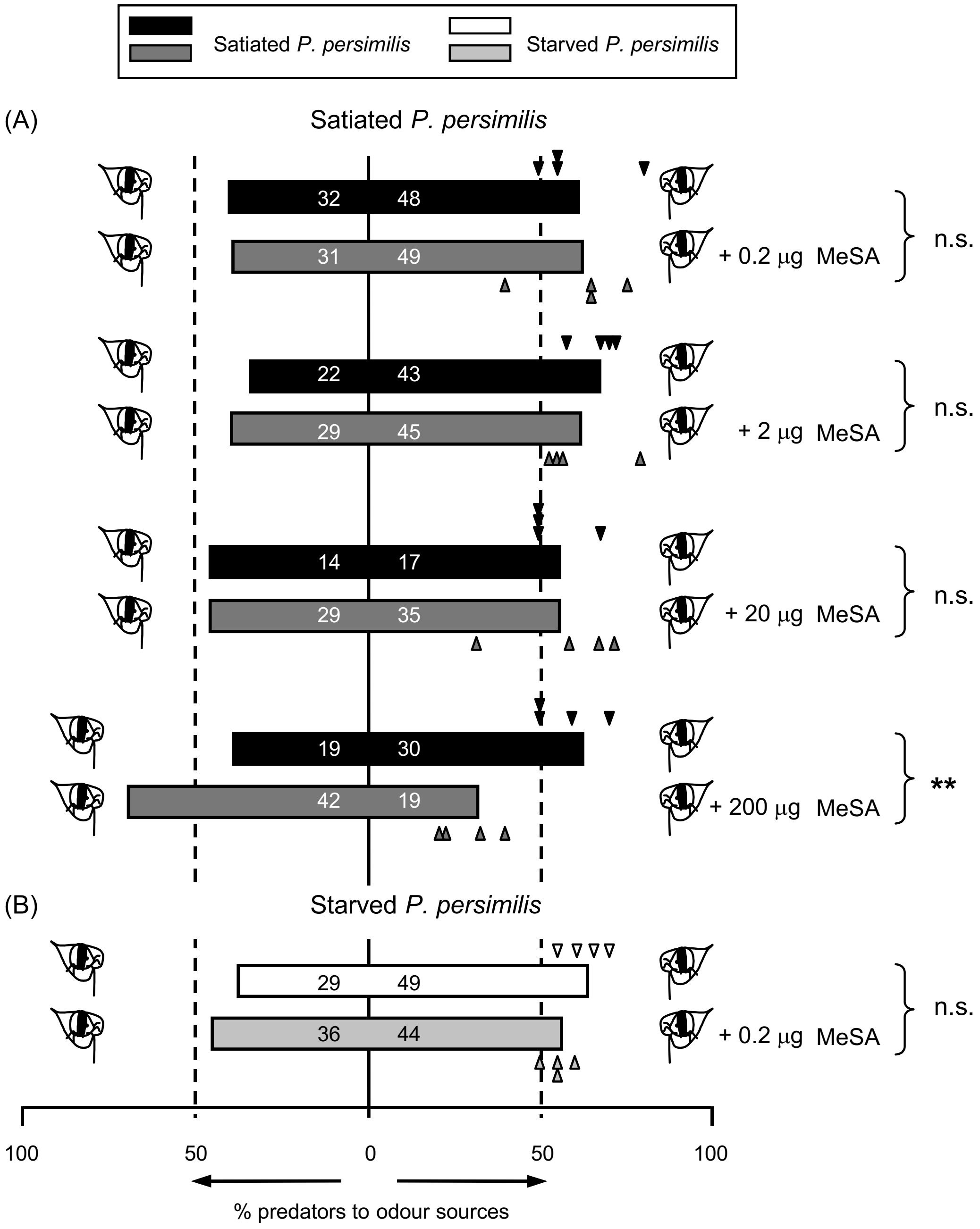

Figure 3: Influence of the relative amount of MeSA on the choices of satiated (A) and starved (B) *Phytoseiulus persimilis* between the volatiles from two sets of *Tetranychus urticae*-infested lima bean leaves. Black (A) or white (B) bars represent the overall percentages of predators choosing between the volatiles from two equal sets of *T. urticae*-infested lima bean leaves, grey bars present the overall percentages of predators choosing between the *T. urticae*-induced lima bean volatiles and the combination of *T. urticae*-induced volatiles plus synthetic MeSA (0.2 µg, 2 µg, 20 µg, 200 µg) downwind of one of them. The triangles indicate the percentage of predators in each replicate experiment that was attracted to the volatiles from the set of *T. urticae*-infested lima bean leaves to which MeSA was added. Numbers in bars are the absolute numbers of predators responding to each odour source. The effect of adding MeSA on the choices of the predators was compared with a GLM, the significance of which is indicated behind the bracket (n.s. P>0.05; ** P<0.01).

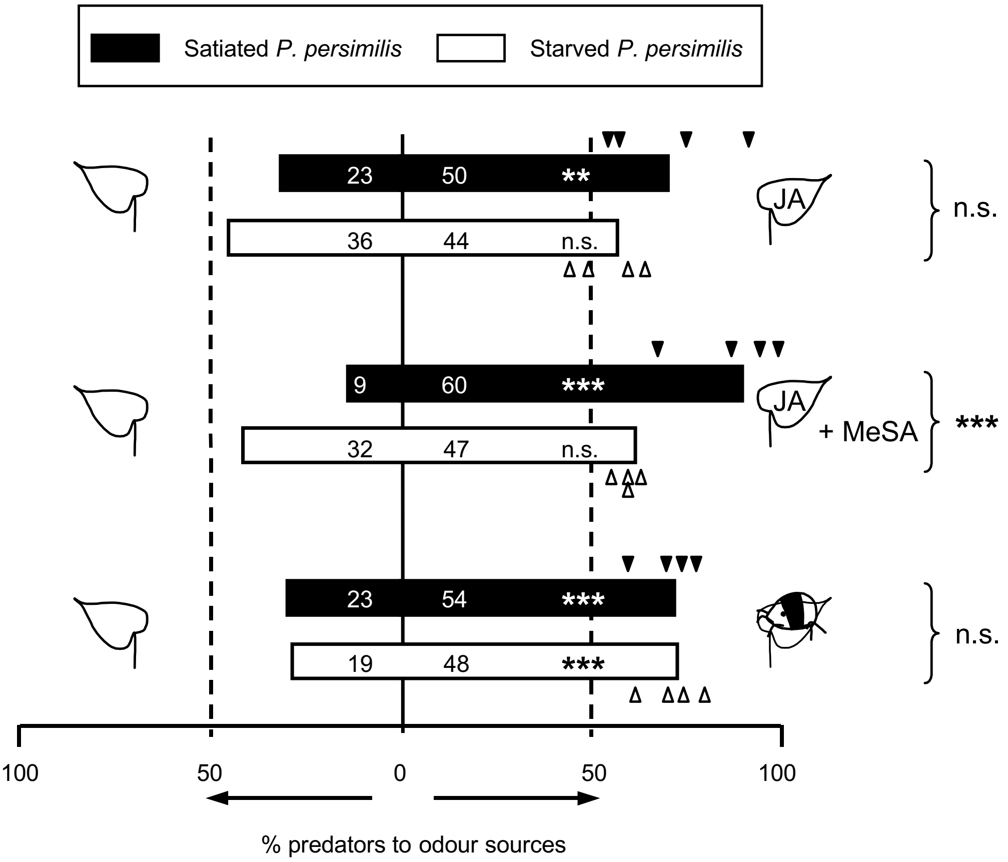

Figure 4: Effect of a qualitative difference in the amount of MeSA on the response of satiated (black bars) and starved (white bars) *Phytoseiulus persimilis*. Attraction of *P. persimilis* to the volatiles from nine jasmonic-acid (JA)-induced lima bean leaves, the combination of the volatiles from nine JA-induced lima bean leaves and MeSA (0.2 μg), and the volatiles from four *Tetranychus urticae*-infested lima bean leaves, all tested versus clean lima bean leaves. The triangles represent the percentage of predatory mites in each replicate experiment that was attracted to the volatiles from treated lima bean leaves. Numbers in bars are the absolute numbers of predatory mites responding to each odour source. Choices between odour sources were statistically analysed with a two-sided binomial test, the effect of starvation level was analysed with a GLM, the significance of which is indicated behind the bracket (n.s. $P>0.05$; ** $P<0.01$; *** $P<0.001$).

Both satiated and starved *P. persimilis* females were attracted to the volatiles from *T. urticae*-infested lima bean leaves (Figure 4, $P<0.001$, binomial test).

Satiated predators preferred *T. urticae*-induced lima bean volatiles to JA-induced volatiles (Figure 5, $P<0.001$, binomial test). However, when 0.2 μg MeSA was added to the JA-induced volatiles the preference of *P. persimilis* shifted towards this odour source (from 30 to 70 %, $P<0.001$, GLM). In all four replicates of this experiment, more predatory mites were attracted to the volatiles from JA-induced lima bean leaves when MeSA was added.

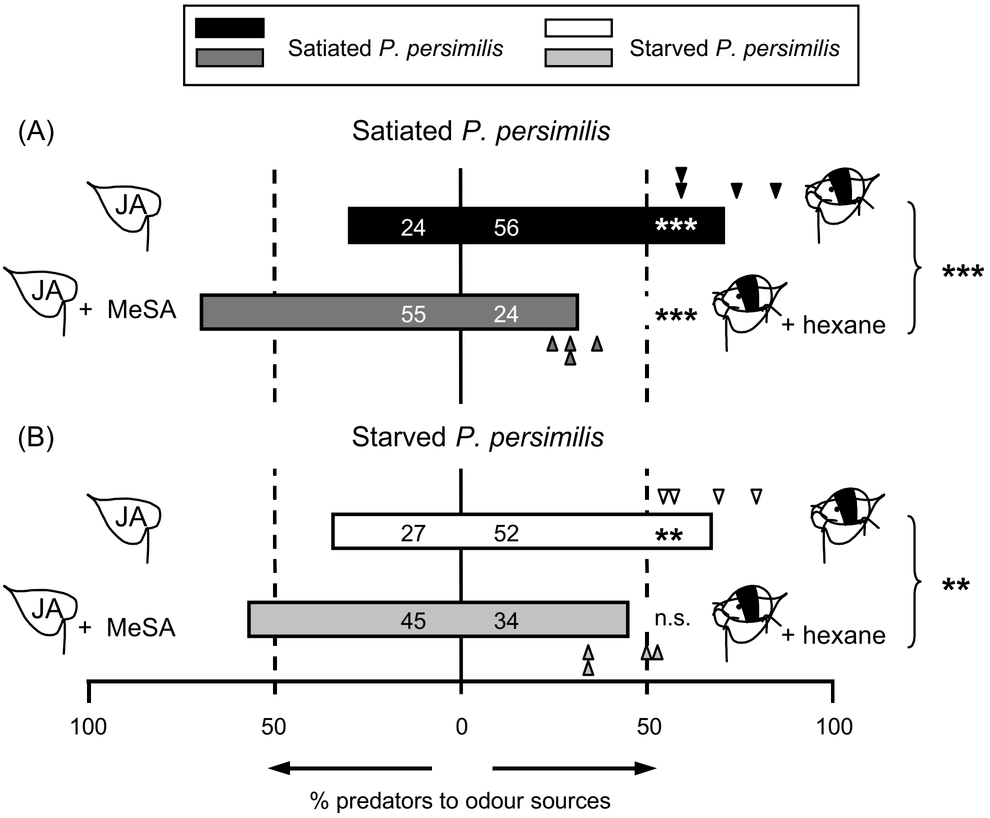

Figure 5: Choices of satiated (A) and starved (B) *Phytoseiulus persimilis* between the volatiles from *Tetranychus urticae*-induced lima bean leaves and JA-induced lima bean leaves (black bars in A, white bars in B), and between the volatiles from *T. urticae*-induced lima bean leaves and the combination of JA-induced lima bean leaves plus 0.2 μg MeSA (grey bars). The triangles represent the percentage of predators in each replicate experiment that was attracted to *T. urticae*-infested lima bean leaves. Numbers in bars are the absolute numbers of predatory mites responding to each odour source. Choices between odour sources were statistically analysed with a two-sided binomial test, the effect of adding MeSA on the responses of *P. persimilis* was analysed with a GLM, the significance of which is indicated behind the bracket (n.s. P>0.05; ** P<0.01; *** P<0.001).

Starved *P. persimilis* females also preferred the volatiles from *T. urticae*-induced lima bean leaves to the volatiles from JA-induced leaves (Figure 5, P=0.007). Adding MeSA to the JA-induced volatile blend significantly increased the percentage of predatory mites attracted to this odour source (from 34 % to 57 %, P=0.004, GLM). The starved females did not discriminate between the combination of JA-induced volatiles plus MeSA and the *T. urticae*-induced lima bean volatiles (P=0.26, binomial test).

DISCUSSION

In this paper we studied the role of methyl salicylate as part of the volatile blend induced by prey herbivores, in the foraging behaviour of the predatory mite *P. persimilis*. We showed that the presence of MeSA rather than its relative contribution to a blend of volatiles appeared to be important in enabling the predators to discriminate between two volatile blends.

Dose-dependent attraction to methyl salicylate

The single compound MeSA was attractive to the predatory mite over a range of doses. The most attractive dose matches roughly with the amount of MeSA emitted by *T. urticae*-infested lima bean plants (estimated from Dicke *et al.* 1999: 10 *T. urticae*-induced lima bean leaves produced 0.2 to 0.4 μg MeSA in 30 minutes). The predators were repelled when they were offered a large, biologically unrealistic, amount of MeSA (200 μg). A low dose of 0.002 μg was not attractive to the predators and may have been under the detection level. De Bruyne *et al.* (1991) demonstrated a dose-dependent sensitivity of chemoreceptor cells on the anterior tarsi of *P. persimilis* to MeSA and this could be the basis for a dose-dependent behavioural response to MeSA by the predatory mites.

Quantitative and qualitative differences in volatile blends

Most plant species that have been investigated so far showed quantitative differences in volatile blends when they are infested with different herbivore species (e.g. Geervliet *et al.* 1997; DeMoraes *et al.* 1998; Turlings *et al.* 1998). Because plants infested with prey or non-prey herbivores are very different in profitability for a carnivore, one might hypothesise that quantitative variation is thus important to a foraging carnivore. However, our experiments showed that *P. persimilis* females discriminate between volatile blends that differed *qualitatively* with respect to the amount of MeSA, but not between blends with a *quantitative* difference in the amount of MeSA.

Both satiated and starved predators preferred *T. urticae*-induced lima bean volatiles to JA-induced volatiles. The absence of MeSA in the JA-induced blend is one of the major differences when compared to the *T. urticae*-induced volatile blend (Dicke *et al.* 1999). After adding MeSA to the JA-induced blend, the preference of the predators changed significantly. This change in preference, after restoring the JA-induced blend with MeSA, indicates an important role for MeSA as a signal to the foraging predatory mites. The amount of MeSA that we added to the set of JA-induced lima bean leaves was of the same order of magnitude as the amount that the set of *T. urticae*-induced leaves produced (based on Dicke et al. 1999, see above). The preference of satiated predators shifted towards the combination of JA-induced volatiles and MeSA, whereas the response of the starved predators shifted to no discrimination between the two odour sources. This result is in agreement with the significantly stronger preference of satiated compared to starved predatory mites for the combination of JA-induced lima bean leaves plus MeSA when tested against uninfested lima bean leaves.

In contrast, neither satiated nor starved *P. persimilis* females discriminated between *T. urticae*-induced odour blends that differed quantitatively in the amount of MeSA. The response of satiated predatory mites to *T. urticae*-infested lima bean leaves was only affected when a very large amount of MeSA (200 μg) was added. This large, repellent, amount of MeSA may have masked the presence of the natural volatile blend. The results do not imply that the predatory mites are not able to discriminate between blends with a quantitative difference in MeSA or other compounds. They may not always need to discriminate, because not all odour variation represents a different value in terms of the availability of prey or non-prey herbivores. Ozawa *et al.* (2000a) showed that *T. urticae* but not the caterpillar *S. exigua* induces the production of MeSA in lima bean, i.e. an example of a qualitative difference induced by different herbivore species on the same plant. Alternatively, *P. persimilis* females may need to learn to discriminate between odour sources that differ quantitatively in the amount of MeSA or another compound. The parasitoid *Leptopilina heterotoma* did not discriminate between two odour sources that differed in the quantity of a single component of the blend, unless it had learned to do so (Vet *et al.* 1998).

Response to incomplete information

We included both satiated and starved predatory mites in our study because the level of starvation may influence the foraging behaviour of *P. persimilis* (Dicke *et al.* 1998; Shimoda & Dicke 2000). We observed a difference in response of satiated and starved *P. persimilis* females in experiments with JA-induced lima bean volatiles: (1) The volatile blend of JA-induced lima bean attracted satiated but not starved predators. (2) Satiated predators were significantly more attracted to a combination of JA-induced lima bean volatiles plus MeSA than starved predators. (3) Satiated predators preferred a combination of JA-induced lima bean volatiles plus MeSA to *T. urticae*-induced lima bean volatiles whereas starved predators did not discriminate between these odour sources. These data lend some support to the hypothesis that starved predators are more selective than satiated predators in their responses to volatiles (Shimoda & Dicke 2000). These authors suggested that it would be adaptive for satiated predatory mites to respond to incomplete volatile information, i.e. only a part of the volatile blend that is emitted by prey-infested plants. For example, they could use such a subset of volatiles when they return into a prey patch that they left but which had not been completely wiped out and is still nearby (Sabelis *et al.* 1984; Shimoda & Dicke 2000). Surprisingly, both groups of predators were attracted to the single compound MeSA – also an incomplete volatile blend – supporting an important role for this compound in the foraging behaviour of *P. persimilis*. Several studies demonstrated that the rearing history of *P. persimilis* influences its subsequent choices (Takabayashi & Dicke 1992; Krips *et al.* 1999; Shimoda & Dicke 2000). In our study, the predatory mites had been reared on spider mites on lima bean. The predators had thus been exposed to MeSA during the rearing (Dicke *et al.* 1990a). This probably explains their attraction to the single compound MeSA. The effects of rearing history and learning on the responses of predatory mites to MeSA need to be investigated (see chapter 6).

Conclusions

This study is a new step in unravelling which parts of the complex herbivore-induced volatile blends contain information that carnivores use to locate their prey. Using the combined knowledge of the composition of two volatile blends and the differential responses of a carnivore, we studied the role of MeSA in the foraging behaviour of the predatory mite *P. persimilis*. We conclude that MeSA can be a signal that determines the preference of predators in a choice situation between two volatile blends that differ in the presence of MeSA. However, MeSA can clearly not be the only compound used by *P. persimilis* to identify the presence of spider mites. The common presence of MeSA in herbivore-induced volatile blends, and the absence of MeSA in the spider mite-induced volatile blends of some plant species, rule out a role for MeSA as the single compound to reveal the presence of spider mites on a plant (e.g. Scutareanu *et al.* 1997; Turlings *et al.* 1998; Gols *et al.* 1999; Van den Boom *et al.* 2004). Other herbivore-induced volatiles are known to attract satiated *P. persimilis* females as a single compound: linalool, β-ocimene and 4,8-dimethyl-1,3(*E*),7-nonatriene (Dicke *et al.* 1990a). The role of these compounds as part of a volatile blend has not yet been investigated. These compounds may be important when MeSA is not present or in combination with MeSA.

Using chemical analytical methods, electroantennograph studies, and behavioural assays, it will be possible to make a directed search for compounds that may contain relevant information for foraging carnivores. The role of different parts of an odour blend may then be analysed using a similar approach as employed here.

Acknowledgements

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CHAPTER 5

Identification of volatiles that are used in discrimination between plants infested with prey or non-prey herbivores by a predatory mite

Abstract

Carnivorous arthropods can use herbivore-induced plant volatiles to locate their herbivorous prey. In the field, carnivores are confronted with information from plants infested herbivores that may differ in their suitability as prey. In this article, we first show that discrimination by the predatory mite *Phytoseiulus persimilis* between volatiles from lima bean plants infested with its prey *Tetranychus urticae* or plants infested with the non-prey caterpillar *Spodoptera exigua* depends on the herbivore densities used to infest the plants. Second, we have analysed the chemical composition of the volatile blends from *T. urticae*-infested lima bean plants at different densities of spider mites, and from *S. exigua*-infested lima bean plants. Based on the behavioural preferences of *P. persimilis* and the volatile profiles of lima bean plants, we selected compounds that potentially enable the predatory mite to discriminate between *T. urticae*-induced and *S. exigua*-induced volatiles. We demonstrate that the large amount of methyl salicylate and (3*E*,7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene emitted by *T. urticae*-infested lima bean plants compared to *S. exigua*-infested lima bean plants enable the predators to make this choice. This is the first study that presents conclusive behavioural evidence for a role of specific compounds within a complex herbivore-induced volatile blend in the foraging behaviour of predatory arthropods.

INTRODUCTION

Carnivores that search actively for food need information on the whereabouts of their prey. The information emitted by the prey is often minimal because of selection to reduce the risk of predation. Carnivores can solve this detectability problem by using information from the prey's direct environment instead of information from the prey itself. An intriguing and well-established example of this phenomenon is the use of information from plants by carnivorous arthropods. Upon attack by herbivorous insects or mites, plants emit a blend of volatiles that can attract a range of different carnivore species, including parasitoid wasps, and predatory mites and bugs (e.g. Dicke *et al.* 1990a; Turlings *et al.* 1990; Vet & Dicke 1992; Paré & Tumlinson 1997b; Scutareanu *et al.* 1997; Drukker *et al.* 2000a).

Information emitted by the food plants of prey herbivores is relatively easy to detect because compared to herbivores, plants have a large biomass and they can benefit from the emission of volatiles by attracting carnivorous arthropods (Dicke & Sabelis 1989; Vet & Dicke 1992; Van Loon *et al.* 2000). However, this indirect information is often more variable than information from the prey itself. Variation in the composition of herbivore-induced plant volatile blends can be caused by plant species and cultivar, herbivore species, multiple infestation by another herbivore species or pathogen, and abiotic factors (e.g. Takabayashi *et al.* 1994b; DeMoraes *et al.* 1998; Gouinguené & Turlings 2002; Cardoza *et al.* 2002; Schmelz *et al.* 2003a; chapter 3). To foraging carnivores, it is especially important to attend to the differences or variation in volatile blends that are associated with herbivore species because the herbivores may differ in their suitability as prey. Field and laboratory studies have shown that carnivores can use herbivore-induced plant volatiles to discriminate between plants infested with prey and plants infested with non-prey herbivore species (e.g. DeMoraes *et al.* 1998; Du *et al.* 1996). However, no studies have yet conclusively shown which differences in volatile blend composition enable carnivores to make this choice between volatile blends that are induced by prey and non-prey herbivores (e.g. Chadwick & Goode 1999; Dicke & Van Loon 2000).

In this chapter, we identify which differences between the volatile blends of lima bean plants infested with the two-spotted spider mite (*Tetranychus urticae*) or the beet armyworm (*Spodoptera exigua*) may enable the predatory mite *Phytoseiulus persimilis* – a specialised natural enemy of spider mites – to discriminate between these blends. We do this by testing the preferences of the predators between the volatile blends from *T. urticae*-infested and *S. exigua*-infested lima bean plants, and by analysing the chemical composition of these blends. Subsequently, we demonstrate that two specific compounds play a role in this discrimination by the predatory mite.

MATERIAL AND METHODS

Plants and herbivores

Lima bean plants (*Phaseolus lunatus* L. cv. Sieva) were grown in the greenhouse at 20-30 °C, 50-70% r.h. and 16 h of light. They were used in experiments when their primary leaves had expanded, 10 to 15 days after sowing. A colony of the two-spotted spider mite *Tetranychus urticae* Koch (Acari: Tetranychidae) was maintained on lima bean in the greenhouse under the same conditions. Eggs of *Spodoptera exigua* Hübner (Lepidoptera: Noctuidae) were obtained from the laboratory of Virology, Wageningen University, The Netherlands (see Smits *et al.* 1986). Two to three egg batches were placed in a Petri dish with a lima bean leaf and kept in a climate cabinet at 23±1 °C and 50-70% r.h. Larvae were used to infest plants within 24 h of hatching.

Predators

A stock colony of the predatory mite *Phytoseiulus persimilis* Athias-Henriot (Acari: Phytoseiidae) was kept on spider mite-infested lima bean leaves in a climate cabinet at 23±1 °C, 50-70% r.h. and continuous light. To obtain females of the same age, we transferred about 5 gravid females from the stock colony to a fresh *T. urticae*-infested lima bean leaf in each of a series of Petri dishes. The females were allowed to oviposit for one or two days and were then removed. Fresh spider mite-infested leaves were provided every two days to rear the offspring. Offspring females were used in experiments nine days after initiation of the colonies, i.e. 1 to 2 days after their final moult. Prior to an experiment females were kept individually in Eppendorf vials for 24±2 h, without food but with a small droplet of water. Petri dishes and Eppendorf vials were kept under the same conditions as the stock colony of predatory mites.

Y-tube olfactometer

Responses of predatory mites to herbivore-induced volatiles were tested in a closed system Y-tube olfactometer (Takabayashi & Dicke 1992; chapter 2). Each individual predatory mite was observed for a maximum of 5 min; when the predator did not make a choice for one of the two odour sources within this time we recorded a “no-choice”. Each experiment was repeated on 3 to 8 different days with new odour sources and new sets of predatory mites on each day.

Experiments

The following describes the preparation of the odour sources for all experiments: The two primary leaves of a lima bean plant were infested with caterpillars or spider mites 3 days before an experiment. Newly hatched caterpillars were placed in a clip cage that was attached to a leaf. Adult spider mite females from the greenhouse colony on lima bean were transferred to the test plants using a fine paintbrush. A clip cage was also attached to each spider mite-infested leaf. Clip cages were supported with sticks to prevent the leaves from bending down and perhaps incurring damage by the weight of the cages. We moved clip cages to a new position on the same leaf daily, checked the number of caterpillars and if necessary added a new caterpillar of the same age. Plants were incubated in a climate room at 24±1 °C, 50-70% r.h. and 16L:8D. Plants of different treatments were kept separate in plastic cages. Just before the experiment, leaves were cut from the plant and wrapped with their petioles in wet cotton wool and aluminium foil to prevent wilting. Odour sources consisted of four leaves of the same treatment in all experiments; the numbers of herbivores that we used are specified below for each experiment.

(1) Herbivore density

This experiment was conducted to study the effect of varying the densities of prey and non-prey herbivores on discrimination between prey and non-prey by predatory mites. First, we compared the attractiveness of the volatiles from one set of lima bean leaves infested with 20 *T. urticae* per leaf to the volatiles from each of three sets of lima bean leaves infested with 1, 2, or 4 *S. exigua* larvae per leaf within one experiment. The sequence of caterpillar densities was changed on the different days of this experiment. Second, we compared the attractiveness of the volatiles from one set of lima bean leaves infested with 2 *S. exigua* per leaf to the volatiles from each of three sets of lima bean leaves infested with 10, 20 or 40 *T. urticae* per leaf within one experiment, changing the sequence of spider mite densities on the different days of this experiment. Both experiments were repeated on six different days, such that each density sequence was repeated on two days. We used 10-12 predatory mites per spider mite or caterpillar density per day.

(2) The role of specific volatiles in the responses of *P. persimilis* to volatile blends induced by prey and non-prey herbivores

Based on the preferences of predatory mites between the volatile blends from *T. urticae*-infested and *S. exigua*-infested lima bean plants at different herbivore densities (see above) and the chemical analyses of these volatile blends (see below), we selected three volatile compounds to test their role in the foraging behaviour of *P. persimilis*. These compounds were selected because (1) they were emitted in a significantly larger amount by *T. urticae*-infested lima bean plants than by *S. exigua*-infested plants at the spider mite density that resulted in discrimination between the two odour sources by *P. persimilis* (methyl salicylate [MeSA] and (3*E*,7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene [(*E*,*E*)-TMTT]), or (2) they were emitted in a larger amount by *T. urticae*-infested plants at a spider mite density that resulted in discrimination by the predators than at a spider mite density that did not result in discrimination (2-butanone) (see results). MeSA (Merck, 99% pure) and 2-butanone (Sigma, ≥99% pure) were obtained commercially; (*E*,*E*)-TMTT was provided by Dr. W. Boland of the Max Planck Institute for Chemical Ecology, Jena, Germany. The purity of (*E*,*E*)-TMTT was measured with gas-chromatography and was at least 95%. We released the synthetic compounds into the olfactometer using a microcapillary. To this end a 0.5 µl glass microcapillary (Drummond Scientific Co., USA) was filled with a pure compound and was placed in the last glass compartment of the olfactometer arm at an angle of ~45° relative to the direction of the air stream. The release rates of the synthetic compounds from the microcapillaries were estimated to be 10 µg/h for MeSA, 0.5 µg/h for (*E*,*E*)-TMTT, and 4 mg/h for 2-butanone. Because 2-butanone was highly volatile the microcapillary had to be refilled after testing two to three predatory mites; microcapillaries with the other compounds were not replaced for the duration of one experiment.

First, we tested the attractiveness of the three single compounds. A microcapillary with a synthetic volatile was presented in one arm of the olfactometer, while no odour was presented in the other arm of the olfactometer. Different compounds were tested on different days. The experiments were repeated on 5 to 8 different days with 8 to 14 predatory mites per day.

Second, we evaluated the contribution of the three compounds to the attractiveness of the volatile blend induced by their prey by offering predatory mites the choice between natural *T. urticae*-induced lima bean volatiles versus one to three synthetic volatiles. Lima bean leaves were infested with 40 spider mites per leaf. The alternative odour source consisted of (A) no odour, (B) MeSA, (C) (*E*,*E*)-TMTT, (D) 2-butanone, (E) MeSA + (*E*,*E*)-TMTT, (F) MeSA + (*E*,*E*)-TMTT + 2-butanone. The control (A) was repeated on 18 different days with 8 to 10 predators per day. The other combinations were each repeated on 3 to 6 different days with 8 to 20 predators per day. Two to four combinations of synthetic compounds were tested on one day against the volatiles from the same set of *T. urticae*-infested leaves.

Finally, we investigated the role of the three compounds in enabling predatory mites to discriminate between the volatiles from plants infested with *T. urticae* or *S. exigua*. Lima bean leaves were infested with 40 *T. urticae* or 2 *S. exigua* larvae per leaf. First we determined the choice of about 10 predators between these two odour sources. Then we placed a microcapillary with one of the synthetic volatiles in the olfactometer, downwind of the caterpillar-infested leaves. In this way, we tested the response of about 20 predators to the *T. urticae*-induced volatiles versus the combination of *S. exigua*-induced volatiles plus the synthetic volatile. Subsequently, we removed the microcapillary with the synthetic volatile and replaced the olfactometer for a clean one. After this, another set of about 10 predators was tested for their choice between *T. urticae*-induced and *S. exigua*-induced volatiles. Different synthetic volatiles were tested on different days. We repeated this experiment on 4 to 8 different days for each of the three synthetic compounds.

Volatile collection and analysis

Plants for volatile collection were infested and incubated in the same way as described for the olfactometer experiments. We collected the volatiles of uninfested lima bean plants, *T. urticae*-infested plants (with 10 or 40 spider mites per leaf), and *S. exigua*-infested plants (2 caterpillars per leaf). After three days of incubation, plants were cut and their stems were wrapped in wet cotton wool and aluminium foil and used in headspace sampling immediately. The sampling procedure is described in detail in chapter 3. Volatiles from the four different treatments were sampled on the same day; the volatiles from two different treatments were collected simultaneously, in parallel. We repeated the volatile sampling on four different days but, during the analysis, we lost one replicate of the “uninfested” and “10 spider mites” treatments each. The procedure of GC-MS analysis is described in chapter 3. The peak areas were determined of compounds that were present in at least N-1 replicates of at least one of the treatments.

Statistical analysis

Predatory mites that did not make a choice (in total 4 % of 1570 predators) were excluded from the statistical analysis. To investigate whether the choices of predatory mites between the odour sources in the olfactometer differed from a 50:50 distribution we used a two-sided binomial test on the pooled data per experiment.

To analyse the effect of herbivore density on discrimination between prey and non-prey by predatory mites, we used a generalised linear model (GLM) with a binomial distribution and a logit-link function, testing the null-hypothesis that herbivore density does not affect the behaviour of the predators (GenStat 6.1, McCullagh & Nelder 1989). The number of predatory mites choosing for the volatiles from *T. urticae*-infested lima bean per day was set as the response variate. The total number of predators that made a choice per day was taken as the binomial total. We checked the validity of the models by comparing the deviance of the residual with the critical χ^2-value at $\alpha=0.05$. We also used a GLM to comparatively analyse the effect of combining *S. exigua*-induced volatiles with a synthetic compound, testing the null-hypothesis that adding the extra compound does not change the behaviour of predatory mites. The predicting factors and/or variates that we used to analyse each experiment are indicated in the figure legends.

Differences in the emission of volatile compounds from lima bean plants infested with 40 *T. urticae* or with 2 *S. exigua* were analysed with a Mann-Whitney U test.

RESULTS

Herbivore density

We varied the densities of herbivores to identify in which situations predatory mites did or did not discriminate between volatiles from lima bean induced by prey or non-prey herbivores.

Predatory mites did not discriminate between *T. urticae*-induced and *S. exigua*-induced volatiles at densities of 1, 2 or 4 caterpillars per leaf and a density of 20 spider mites per leaf (Figure 1, $P \geq 0.12$, binomial test). Varying caterpillar density did not significantly change the preference of predatory mites for *T. urticae*-induced volatiles ($P=0.12$, GLM).

Predatory mites did also not discriminate between *T. urticae*-induced and *S. exigua*-induced volatiles at densities of 10 or 20 spider mites per leaf versus a density of 2 caterpillars per leaf (Figure 2, $P \geq 0.09$, binomial test). However, when 40 spider mites per leaf were used, predators significantly preferred *T. urticae*-induced to *S. exigua*-induced volatiles ($P=0.006$, binomial test). The attraction of predatory mites to volatiles induced by their prey significantly increased with increasing spider mite density ($P<0.001$, GLM).

Volatiles emitted by T. urticae*-infested and* S. exigua*-infested lima bean plants*

Because *T. urticae* density had a significant influence on discrimination between prey and non-prey herbivores by predatory mites, we analysed the volatile blends emitted by lima bean plants upon infestation with the different densities of *T. urticae*, and one density of *S. exigua*.

Plants that had been left uninfested or had been infested with 10 *T. urticae* per leaf emitted small amounts of (*Z*)-3-hexen-1-ol acetate, methyl salicylate (MeSA), (3*E*)-4,8-dimethyl-1,3,7-nonatriene [(*E*)-DMNT] and (3*E*,7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene [(*E,E*)-TMTT] (Figure 3A and B). Volatile emission of lima bean plants infested with 20 *T. urticae* per leaf was similar to that of uninfested plants and plants infested with 10 *T. urticae* per leaf (Figure 3C; data from chapter 3). When 40 spider mites per leaf were used, a much larger range of compounds was detected in addition to the compounds mentioned before: 2-butanone, (*E*)-β-ocimene, (*Z*)-DMNT, a TMTT-isomer, ethyl salicylate and two

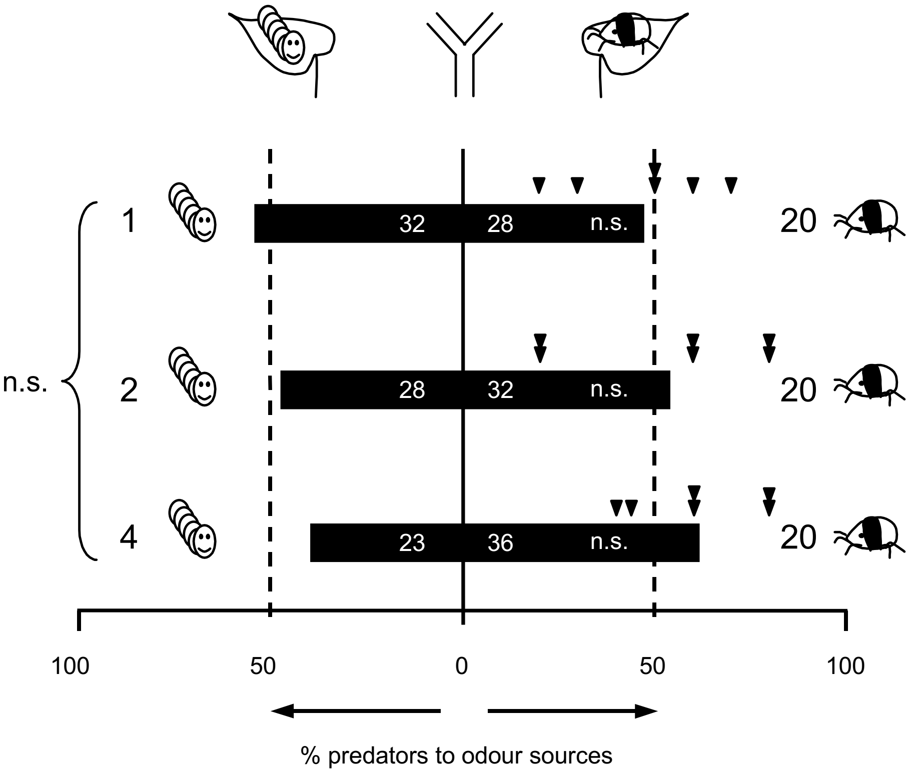

Figure 1: Choices of the predatory mite *Phytoseiulus persimilis* between the volatiles from *Tetranychus urticae*-infested and *Spodoptera exigua*-infested lima bean in the Y-tube olfactometer at different densities of caterpillars per leaf and a density of 20 spider mites per leaf. Numbers in front of the herbivore symbols indicate the number of herbivores per leaf. Bars present the overall percentages of predatory mites choosing for each odour source; triangles present the percentages predators choosing for the volatiles from *T. urticae*-infested leaves per day of the experiment. Numbers in bars are the total numbers of predators choosing for each odour source. Choices between odour sources were analysed with a two-sided binomial test; the effect of *S. exigua* density was analysed with a GLM, the significance of which is indicated in front of the bracket (predictors: replicate [factor] + caterpillar density [variate]). (n.s. $P>0.05$).

unknown compounds. Moreover, the emission of MeSA, (*E*)-DMNT and (*E*,*E*)-TMTT increased about six-fold (Figure 3D).

The major compound emitted by plants that had been infested with 2 *S. exigua* larvae per leaf was (*E*)-β-ocimene (Figure 3E). Large amounts of (*Z*)-3-hexen-1-ol acetate, and (*E*)-DMNT were emitted as well. A range of compounds that were not detected or only in trace amounts upon spider mite-infestation, was emitted upon caterpillar-infestation: 2- and 3-methylbutanal-O-methyl oxime, (*Z*)-β-ocimene, β-caryophyllene, p-mentha-1,3,8-triene and indole.

Compared to plants infested with 40 spider mites per leaf, significantly smaller amounts of MeSA, (*E*,*E*)-TMTT, and decanal were emitted by caterpillar-infested plants, whereas significantly larger amounts of (*E*)- and (*Z*)-β-ocimene were emitted by caterpillar-infested plants (Mann-Whitney U test, $P<0.05$).

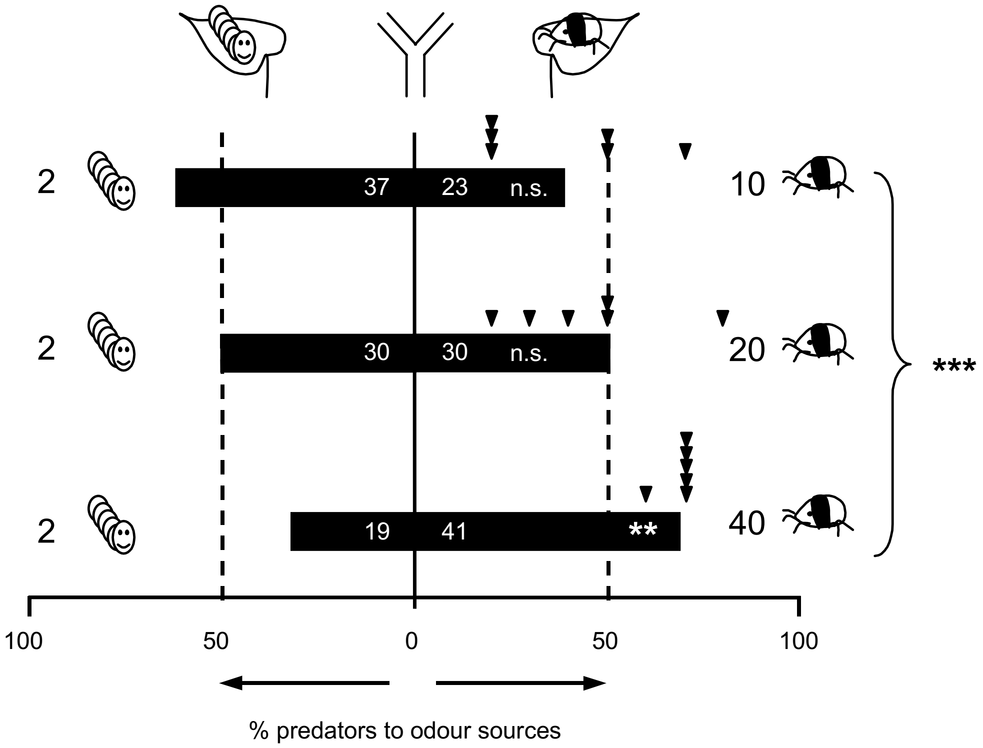

Figure 2: Choices of the predatory mite *Phytoseiulus persimilis* between the volatiles from *Tetranychus urticae*-infested and *Spodoptera exigua*-infested lima bean in the Y-tube olfactometer at different densities of spider mites per leaf and a density of 2 caterpillars per leaf. Numbers in front of the herbivore symbols indicate the number of herbivores per leaf. Bars present the overall percentages of predatory mites choosing for each odour source; triangles present the percentages predators choosing for the volatiles from *T. urticae*-infested leaves per day of the experiment. Numbers in bars are the total numbers of predators choosing for each odour source. Choices between odour sources were analysed with a two-sided binomial test; the effect of *S. exigua* density was analysed with a GLM, the significance of which is indicated behind the bracket (predictors: replicate [factor] + spider mite density [variate]). (n.s. $P>0.05$; ** $P<0.01$; *** $P<0.001$).

The role of specific volatiles in the responses of P. persimilis *to volatile blends induced by prey and non-prey herbivores*

The major differences in the volatile blends emitted by *S. exigua*-infested and *T. urticae*-infested lima bean plants at densities that resulted in discrimination by *P. persimilis* (2 caterpillars and 40 spider mites per leaf respectively, Figure 2) were the significantly larger amount of MeSA and (*E*,*E*)-TMTT induced by *T. urticae* (Figure 3). In addition, at the high *T. urticae* density (40 per leaf) 2-butanone was detected as the fourth largest peak whereas it was not detected at all at the low *T. urticae* densities (10 or 20 per leaf). Therefore, these three compounds were selected to investigate their role in the foraging behaviour of predatory mites.

First, we tested the attraction of the predators to the three volatiles as single compounds. Predators were strongly attracted to the pure compounds MeSA

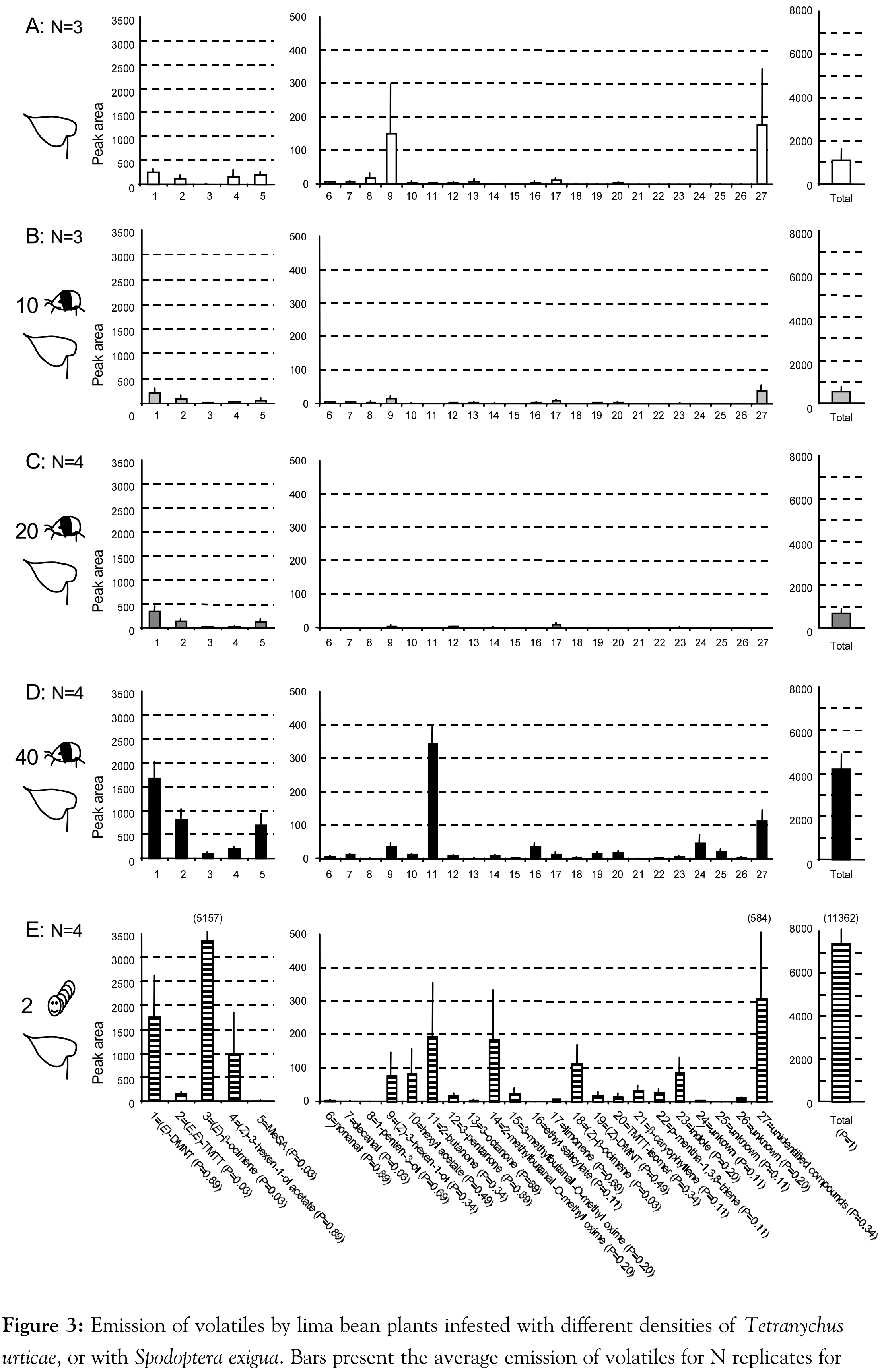

Figure 3: Emission of volatiles by lima bean plants infested with different densities of *Tetranychus urticae*, or with *Spodoptera exigua*. Bars present the average emission of volatiles for N replicates for uninfested lima bean plants (A: white bars), lima bean plants infested with 10 (B: light grey), 20 (C: dark grey)[1] or 40 *T. urticae* per leaf (D: black), or 2 *S. exigua* per leaf (E: striped). Error bars represent the standard error. The quantity of compounds emitted by plants infested with 40 *T.*

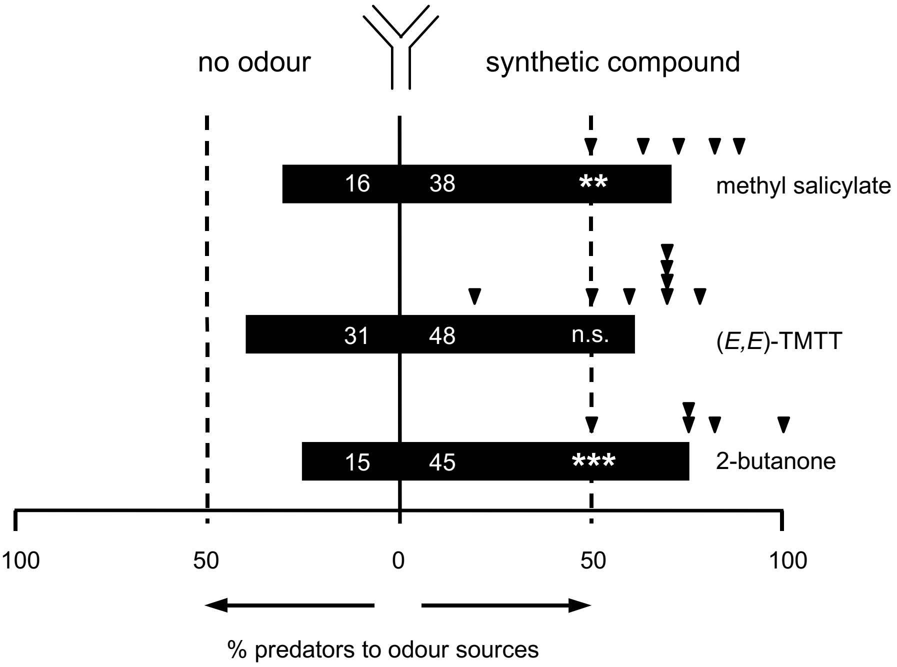

Figure 4: Attractiveness of the single synthetic compounds MeSA, (*E,E*)-TMTT, (*E*)-DMNT, and 2-butanone to the predatory mite *Phytoseiulus persimilis*. Release rates were 10 μg/h for MeSA, 0.5 μg/h for (*E,E*)-TMTT, and 4 mg/h for 2-butanone. Bars present the overall percentages of predatory mites choosing for each odour source; triangles present the percentages predators choosing for the synthetic volatiles per day of the experiment. Numbers in bars are the total numbers of predators choosing for each odour source. Choices between odour sources were analysed with a two-sided binomial test. (n.s. $P>0.05$; ** $P<0.01$; *** $P<0.001$).

(Figure 4, $P=0.004$, binomial test) and 2-butanone ($P<0.001$), while the attraction of *P. persimilis* to (*E,E*)-TMTT bordered significance ($P=0.07$).

Second, we tested the attractiveness of the three synthetic compounds versus the natural blend of spider mite-induced volatiles. The choice of predatory mites between synthetic MeSA and the natural volatile blend from *T. urticae*-infested lima bean leaves was not significantly different from a 50:50 distribution (Figure 5, $P=0.20$, binomial test). (*E,E*)-TMTT or 2-butanone did not have such an effect: the predators significantly preferred the volatiles from *T. urticae*-infested leaves to either of these pure synthetic compounds ($P<0.001$, binomial test). MeSA was part of both mixtures of synthetic compounds that we tested and the predators did not discriminate between these blends of synthetic volatiles and the volatiles from *T. urticae*-infested leaves ($P\geq0.19$, binomial test).

(Figure 3 continued)

urticae (D) or 2 *S. exigua* (E) per leaf was analysed with a Mann-Whitney U test, of which the P-values are given in brackets following the compound names. [1] Data for 20 *T. urticae* per leaf from chapter 3.

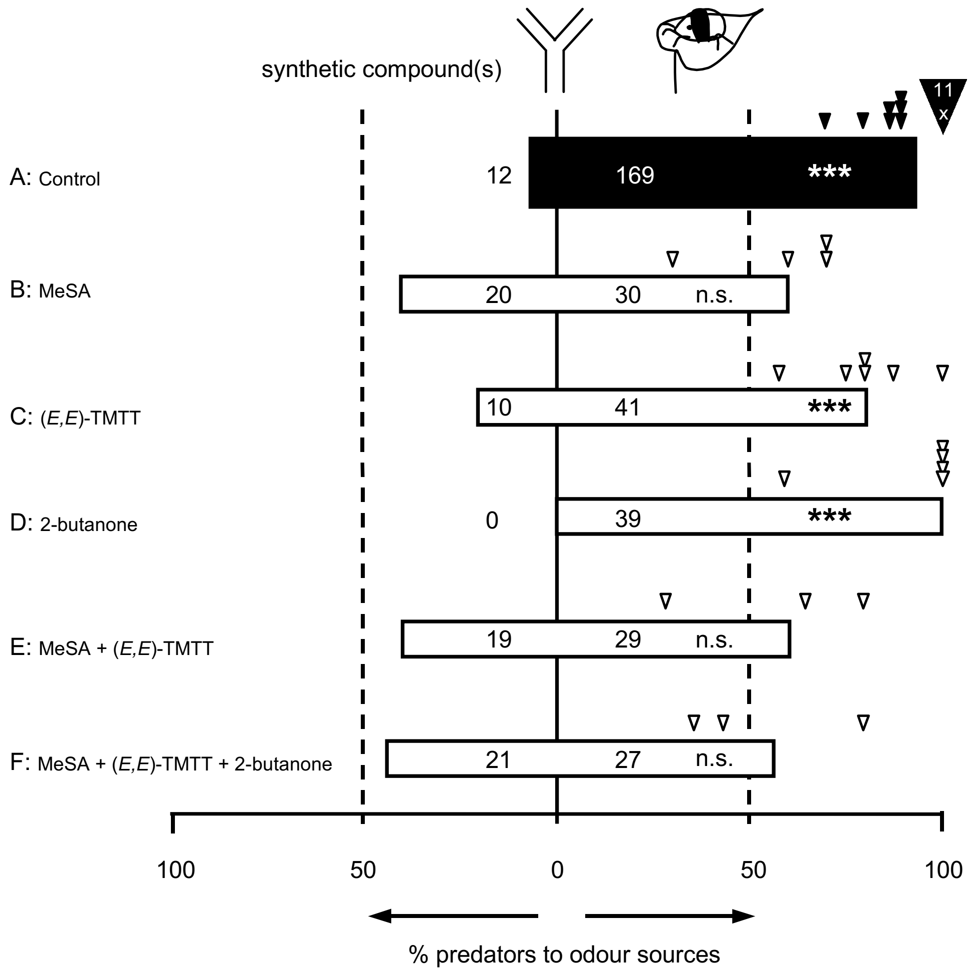

Figure 5: The choices of predatory mites between the natural volatile blend induced by their prey in lima bean, and one to three synthetic. The black bar presents the overall result of 18 replicates to test the attractiveness of the volatiles from *Tetranychus urticae*-infested lima bean leaves (40 spider mites per leaf) versus no odour (A). The white bars present the choice of predatory mites between the volatiles from *T. urticae*-induced lima bean and: (B) MeSA, (C) (*E*,*E*)-TMTT, (D) 2-butanone, (E) MeSA plus (*E*,*E*)-TMTT, (F) MeSA plus (*E*,*E*)-TMTT plus 2-butanone. Release rates were 10 µg/h for MeSA, 0.5 µg/h for (*E*,*E*)-TMTT, and 4 mg/h for 2-butanone. Triangles present the percentage of predators choosing for *T. urticae*-induced volatiles per day of the experiment. Numbers in bars are the absolute numbers of predators choosing for each odour source. Choices between odour sources were analysed with a two-sided binomial test. (n.s. $P>0.05$; *** $P<0.001$).

Finally, we investigated the role of MeSA, (*E*,*E*)-TMTT and 2-butanone in enabling *P. persimilis* to discriminate between the volatiles blends induced by its prey *T. urticae* and the non-prey caterpillar *S. exigua*. Because the choices of predatory mites between *T. urticae*-induced and *S. exigua*-induced volatiles did not differ before and after adding a synthetic volatile ($P>0.05$, GLM, predicting factors replicate + odour source) we pooled the results of these two sub-experiments. The pooled results show that on average 68 % of the predators

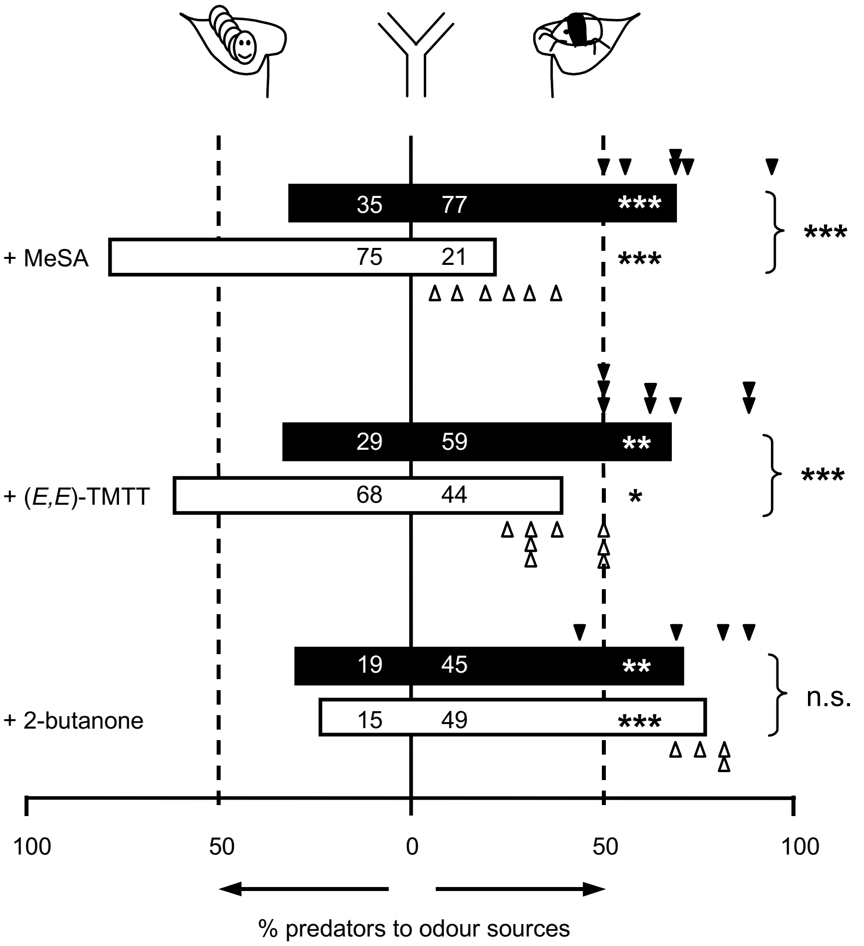

Figure 6: The role of MeSA, (*E*,*E*)-TMTT, and 2-butanone in the choices of predatory mites between the volatiles from *Tetranychus urticae*-infested and *Spodoptera exigua*-infested lima bean plants. Herbivore densities were 40 *T. urticae* and 2 *S. exigua* per leaf. Release rates of synthetic volatiles were 10 μg/h for MeSA, 0.5 μg/h for (*E*,*E*)-TMTT, and 4 mg/h for 2-butanone. Black bars present the choices between the volatiles from *T. urticae*-infested versus *S. exigua*-infested lima bean leaves; white bars present the choices between the volatiles from *T. urticae*-infested leaves versus the combination of *S. exigua*-infested leaves and the synthetic compound indicated on the left side of the white bar. Numbers in bars are the total numbers of predators responding to each odour source. Triangles present the percentage of predators choosing for *T. urticae*-induced volatiles per day of the experiment. Choices between odour sources were analysed with a two-sided binomial test. The effect of adding the synthetic volatile to *S. exigua*-induced volatiles was analysed with a GLM, the significance of which is indicated behind the bracket (predicting factors: replicate + odour source). (n.s. P>0.05; * P<0.05; ** P<0.01; *** P<0.001).

preferred the volatiles induced by *T. urticae* to those induced by *S. exigua* (Figure 4, P≤0.002, binomial test). After adding MeSA to the *S. exigua*-induced volatiles, as much as 78 % of the predators preferred this combination to *T. urticae*-induced volatiles (P<0.001, binomial test). (*E*,*E*)-TMTT had a similar effect: after adding (*E*,*E*)-TMTT to the *S. exigua*-induced volatiles, 61 % of the predators preferred this combination of volatiles to *T. urticae*-induced volatiles (P=0.03, binomial test). Both MeSA and (*E,E*)-TMTT had a significant effect on the choice of *P. persimilis* between the volatiles from *T. urticae*-infested and *S. exigua*-infested lima bean leaves ($P<0.001$, GLM). 2-Butanone, a compound emitted by both *T. urticae*-infested and *S. exigua*-infested lima bean plants, did not have such an effect ($P=0.41$, GLM). When 2-butanone was added to the *S. exigua*-induced volatiles from lima bean, the predators still preferred the volatiles induced by their prey *T. urticae* (77%, $P<0.001$, binomial test).

DISCUSSION

We have investigated which compounds emitted by lima bean plants enable the predatory mite *P. persimilis* to distinguish between plants infested with its prey *T. urticae* and plants infested with the non-prey caterpillar *S. exigua*. It was well established that carnivores can use herbivore-induced plant volatiles to discriminate between plants infested with herbivores that are suitable prey and plants that are infested with non-prey herbivores (e.g. Agelopoulos & Keller 1994; Du *et al.* 1996, 1998; DeMoraes *et al.* 1998; Shimoda & Dicke 2000). Several differences in volatile blends that potentially played a role in discrimination had been identified, but it had not yet been clearly demonstrated which differences or variation in volatile blends enables carnivores to differentiate. For example, Du *et al.* (1998) identified 6-methyl-5-hepten-2-one as a potentially important compound in discrimination by the aphid parasitoid *Aphidius ervi* between volatile blends induced by a host and a non-host aphid. The parasitoid perceived 6-methyl-5-hepten-2-one and was attracted to the single synthetic compound, but a role of 6-methyl-5-hepten-2-one within the natural volatile blend was not shown. To our knowledge, our experiments provide the first clear evidence for a role of specific volatile compounds *within* a complex herbivore-induced volatile blend in the foraging behaviour of carnivorous arthropods.

By integrating behavioural preferences (Figure 2) with chemical analyses of the volatile blends (Figure 3) we selected three potentially important compounds for discrimination between prey and non-prey by *P. persimilis*: MeSA, (*E,E*)-TMTT, and 2-butanone. These three compounds are commonly induced upon spider mite infestation in a range of plant species (e.g. Krips *et al.* 1999; Horiuchi *et al.* 2003; Van den Boom *et al.* 2004), and in the same and other plant species upon infestation with different herbivore species (e.g. Scutareanu *et al.* 1997; Van Poecke *et al.* 2001; Fritzsche Hoballah *et al.* 2002). The commonness of these compounds suggests that they may not be reliable indicators of the identity of the herbivore inducing the volatile emission. However, our data show that MeSA and (*E,E*)-TMTT can cause predatory mites to discriminate between volatiles induced by herbivores that differ in their suitability as prey. Adding one of these compounds to the *S. exigua*-induced volatile blend increased the attractiveness of this blend so much that predatory mites lost their preference for *T. urticae*-induced volatiles (Figure 6). The strong effect of MeSA on the choices of *P. persimilis* is also supported by the loss of preference of the predatory mites for the volatiles from lima bean infested with *T. urticae* when MeSA was offered as an alternative odour source (Figure 5). MeSA had been identified as an attractant for predatory arthropods in previous studies – for example predatory mites, Anthocorid bugs, and a green lacewing (Dicke *et al.* 1990a; Scutareanu *et al.* 1997; James 2003). Moreover, MeSA was identified as the major determinant of the preference of *P. persimilis* when given a choice between the volatiles from *T. urticae*-infested and jasmonic acid-treated lima bean plants (chapter 4). The effect of (*E*,*E*)-TMTT on the preference of *P. persimilis* for *T. urticae*-induced volatiles was less pronounced than that of MeSA (Figures 5 and 6). This cannot be explained by the difference in attractiveness of the two compounds, because the attractiveness of a compound does not necessarily explain the role of the compound within the total blend. Although 2-butanone was highly attractive as a single compound, it did not have a significant effect on the attraction of *P. persimilis* to *T. urticae*-induced volatiles (Figures 4, 5 and 6). The lack of an effect of 2-butanone on discrimination between prey and non-prey by predatory mites can be explained by the fact that both *S. exigua*-infested and *T. urticae*-infested lima bean plants emitted 2-butanone (Figure 3).

Our data suggest that the combination of MeSA and (*E*,*E*)-TMTT in the *T. urticae*-induced lima bean blend revealed the presence of prey to *P. persimilis*. When only one of these compounds (for example MeSA) was offered in combination with *S. exigua*-induced volatiles, predatory mites lost their preference for the *T. urticae*-induced blend, despite the larger amount of the other compound ([*E*,*E*]-TMTT) in the *T. urticae*-induced versus the *S. exigua*-induced volatile blend. In fact, *P. persimilis* even preferred the combination of *S. exigua*-induced volatiles and MeSA or (*E*,*E*)-TMTT to *T. urticae*-induced volatiles. We estimated that the concentrations of synthetic MeSA and (*E*,*E*)-TMTT that we used (released from the microcapillary) were respectively 40 and 2 times higher than the concentrations of MeSA and (*E*,*E*)-TMTT emitted by *T. urticae*-infested lima bean plants. We previously showed that a quantitative difference in the amount of MeSA does not affect discrimination by *P. persimilis* between two odour sources (chapter 4), and thus it seems unlikely that the higher concentration of MeSA in the combined blend of *S. exigua*-induced volatiles and synthetic MeSA explains the preference of *P. persimilis* for this odour source versus the blend of *T. urticae*-induced volatiles. The effect of quantity of (*E*,*E*)-TMTT on the choices of *P. persimilis* has not yet been investigated but it seems unlikely that a two-fold difference in concentration can explain the preference of predators for the combination of *S. exigua*-induced volatiles plus (*E,E*)-TMTT versus *T. urticae*-induced volatiles. Alternatively, a combined effect of MeSA or (*E*,E)-TMTT with one or more of the *S. exigua*-induced volatiles could explain this preference of predatory mites. For example, *S. exigua*-infested lima bean plants emit a large amount of (*E*)-β-ocimene (Figure 3), and this compound is a known attractant for *P. persimilis* (Dicke *et al.* 1990a).

An important question that remains to be answered is which compounds can be used by predatory mites when MeSA or (*E,E*)-TMTT are not differentially induced by prey or non-prey herbivores, for example on other plant species or between spider mites and other herbivore species feeding on lima bean. Apart from the presence of specific compounds, the ratios between compounds in the volatile blends could play a role here. Quantitative differences in volatile blends have been found in a range of plant-herbivore combinations, including infestation of maize, tobacco or cotton with the caterpillars *Helicoverpa zea* and *Heliothis virescens* (DeMoraes *et al.* 1998), infestation of apple with the spider mites *T. urticae* or *Panonychus ulmi* (Takabayashi *et al.* 1991), and infestation of cabbage with the caterpillars *Pieris rapae* or *Plutella xylostella* (Shiojiri *et al.* 2001). Carnivorous arthropods can indeed discriminate between plants infested with these different herbivore species, which suggests a role for the ratios between compounds in carnivore foraging behaviour. Alternatively, in such situations carnivores may use the presence of specific compounds that are not detected by the GC-MS equipment; arthropods are known to be more sensitive than the detector of the GC-MS (Pickett *et al.* 1998).

The predatory mites used in our study had been reared on lima bean and had thus experienced the volatile blend of spider mite-infested lima bean, including MeSA, (*E,E*)-TMTT and 2-butanone. This most likely explains the attraction of the predators as well as the role of these compounds in discrimination between *T. urticae*-induced and *S. exigua*-induced volatiles because previous experiences with volatiles can influence the foraging behaviour of *P. persimilis* (Dicke *et al.* 1990b; Krips *et al.* 1999; Drukker *et al.* 2000a). An important question that needs to be addressed is what the role of experience or learning is in discrimination between prey and non-prey by predatory arthropods, and whether and how experience influences the compounds that are used in discrimination. In three forthcoming chapters, we show that experience indeed influences the foraging responses of *P. persimilis*, including whether or not they discriminate between the volatile blends from plants infested with prey or non-prey herbivores (chapters 6, 7 and 8).

In conclusion, our data provide first evidence for a role of specific volatile compounds within a natural volatile blend in discrimination between prey and non-prey by a predatory arthropod. It is important to understand how carnivores use herbivore-induced plant volatiles to discriminate between plants infested with prey or non-prey herbivores because this choice is highly relevant in an ecological context. In making these choices predators select the plants on which they will found new colonies and on which they may eventually exterminate the herbivore populations (e.g. Pels & Sabelis 1999), or parasitoids select the plants on which they will parasitise their victims. Plants can benefit from the attraction of predators or parasitoids in terms of reproductive success (Dicke & Sabelis 1989; Van Loon *et al.* 2000; Fritzsche Hoballah & Turlings 2001) and thus it will be at their advantage to emit herbivore-specific information which helps carnivores to locate their prey in an environment where non-prey herbivores are also present. Unravelling the volatiles that are used in discrimination between prey and non-prey by carnivores can therefore not only shed light on the selection pressures that shape the foraging behaviour of carnivorous arthropods but also on the way the emission of volatiles by plants has evolved as an indirect defence mechanism.

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Part III

Phenotypic plasticity in the foraging behaviour of predatory mites

CHAPTER 6

Experience with methyl salicylate affects behavioural responses of a predatory mite to blends of herbivore-induced plant volatiles

Abstract

Many natural enemies of herbivorous arthropods use herbivore-induced plant volatiles to locate their prey. These foraging cues consist of mixtures of compounds that show considerable variation within and among plant-herbivore combinations, a situation that favours flexibility in foraging behaviour of the natural enemies. In this paper, we address the flexibility in behavioural responses of the predatory mite *Phytoseiulus persimilis* Athias-Henriot (Acari: Phytoseiidae) to herbivore-induced plant volatiles. We specifically investigated the effect of experience with one component of a herbivore-induced volatile blend: methyl salicylate (MeSA).

We compared responses of three groups of predatory mites: (1) reared from egg to adult on *Tetranychus urticae* Koch (Acari: Tetranychidae) on lima bean (*Phaseolus lunatus* L., which emits MeSA), (2) reared on *T. urticae* on cucumber (*Cucumus sativus* L., which does not emit MeSA), and (3) reared on *T. urticae* on cucumber in the presence of synthetic MeSA. Experience with MeSA during the rearing period (groups 1 and 3) resulted in attraction to the single compound MeSA in a Y-tube olfactometer. Moreover, experience with MeSA affected the choice of predatory mites between two volatile blends that were similar except for the presence of MeSA. Predators reared on lima bean preferred the volatile blend from *T. urticae*-infested lima bean (including MeSA) to the volatile blend from jasmonic-acid treated lima bean (lacking MeSA), but predators reared on cucumber preferred the volatile blend from the latter. Predators reared on cucumber in the presence of synthetic MeSA did not discriminate between these two blends. Experience with MeSA during three days in the adult phase, after rearing on cucumber, also resulted in attraction to the single compound MeSA.

We conclude that a minor difference in the composition of the volatile blend to which a predatory mite is exposed can explain its preferences between two complex odour sources.

INTRODUCTION

Many carnivorous arthropods use herbivore-induced plant volatiles to locate their herbivorous prey (e.g. Vet & Dicke 1992). These prey herbivores have been selected to release as little information as possible about their presence. The host plants, on the other hand, may profit from releasing information about their attackers by attracting natural enemies of the herbivores (Van Loon *et al.* 2000a; Fritzsche-Hoballah & Turlings 2001). Herbivore-induced plant volatiles can provide specific information to a foraging carnivore with respect to the herbivore species that is feeding on the plant (e.g. Turlings *et al.* 1993b; DeMoraes *et al.* 1998). Plants may even emit volatile blends that are specific for different stages of the same herbivore species, thereby guiding a parasitoid to the most suitable host stage (Takabayashi *et al.* 1995; but see Mattiacci & Dicke 1995, and Gouinguené *et al.* 2003). However, the composition of volatile blends is not only determined by the attacking herbivore. Herbivores may feed on several host plant species that may all emit a different volatile blend upon herbivore-attack (e.g. Van den Boom *et al.* 2004). Similarly, different cultivars of the same plant species may emit different volatile blends when attacked by the same herbivore species (Takabayashi *et al.* 1991; Gouinguené *et al.* 2001). In addition, plant age and type of tissue, and also abiotic factors such as light and water can influence the composition of the volatile blend (Takabayashi *et al.* 1994a,b; Scutareanu *et al.* 1997; Gouinguené & Turlings 2002; Gnanvossou *et al.* 2003), as can a simultaneous attack by another herbivore species or a pathogen (Shiojiri *et al.* 2001; Cardoza *et al.* 2002). In short, natural enemies of herbivorous arthropods have to handle variable information from plants infested with prey during foraging. The information that indicates the presence of prey often varies between carnivore generations, for example when different herbivore generations use different host plant species throughout the year. Consequently, the selection for a certain foraging response of the carnivores may not be constant and genetic adaptation to the environment may not occur. In such a situation, phenotypic plasticity in foraging behaviour is favoured (Vet *et al.* 1995; Dukas 1998; Dukas & Bernays 2000).

Learning – as a form of phenotypic plasticity – of herbivore-induced volatiles by parasitoid wasps has been demonstrated in many studies (e.g. Lewis & Tumlinson 1988; Turlings *et al.* 1993a; Vet *et al.* 1995; Geervliet *et al.* 1998b; Powell *et al.* 1998). These wasps can learn to associate a blend of volatiles with the presence of a host. They do so after short moments of exposure to the plant volatiles, for example during an oviposition in a host. Subsequently, the experienced parasitoids are more responsive to herbivore-induced plant volatiles or adjust their preference in a choice-situation. Evidence for similar learning mechanisms in arthropod predators is still scarce (but see Drukker *et al.* 2000a, 2000b). Several studies, however, have demonstrated an influence of experience with volatiles during the entire rearing period on the foraging behaviour of adult predators (e.g. Dicke *et al.* 1990a; Takabayashi & Dicke 1992; Krips *et al.* 1999). Irrespective of the learning mechanism, it is often also unknown *what* cues are actually learned. Carnivores may learn the complete composition of a new volatile blend or they may learn only certain parts.

In this paper, we study the responses of predatory mites to herbivore-induced plant volatiles. We specifically ask how experiences with volatile blends that differ in the presence of a single compound affect the responses of the predators. The predatory mite *Phytoseiulus persimilis* Athias-Henriot (Acari: Phytoseiidae) is a specialist natural enemy of spider mites in the genus *Tetranychus*. *Phytoseiulus persimilis* uses herbivore-induced volatiles to locate its prey (e.g. Sabelis & Van de Baan 1983; Dicke *et al.* 1990a; Takabayashi *et al.* 1991; Janssen 1999), which are polyphagous herbivores that can feed on hundreds of plant species (Helle & Sabelis 1985). Consequently, *P. persimilis* may have to handle highly variable information when it searches for prey, and thus flexibility in foraging behaviour is expected (Vet & Dicke 1992; Sabelis *et al.* 1999a). Indeed, several studies demonstrated that the foraging behaviour of the predatory mite *P. persimilis* can be affected by their previous experience with volatiles (Takabayashi *et al.* 1994a; Krips *et al.* 1999; Drukker *et al.* 2000a).

We focus on the presence of one specific compound – methyl salicylate – in the volatile blend that the predators experience. Methyl salicylate (MeSA) is emitted as part of the volatile blend of spider mite-infested lima bean plants (Dicke *et al.* 1990a, 1999; chapters 3 and 5). However, not all plant species emit MeSA upon spider mite infestation. For example, gerbera and cucumber plants do not emit MeSA when they are attacked by *T. urticae* (Takabayashi *et al.* 1994a; Krips *et al.* 1999). Thus, the question is whether MeSA can be an important cue for *P. persimilis* from an environment with plants that do not produce MeSA. In other words, do all predators of this species respond to MeSA or do they have to learn to respond? We hypothesise that the predatory mites have to learn to respond to MeSA. To test this hypothesis, first we compare the responses of three groups of predatory mites to the single compound MeSA: (1) reared from egg to adult on spider mites on lima bean (that does produce MeSA), (2) reared on spider mites on cucumber (that does not produce MeSA), (3) reared on spider mites on cucumber in the presence of synthetic MeSA. We expect only the predators that have been reared in the presence of MeSA (groups 1 and 3) to respond to this compound. Second, we investigate whether exposure to MeSA during only the adult phase, after rearing on cucumber, affects the responses of predatory mites to MeSA. Finally, we study the influence of rearing-experience with MeSA on the response of the predator towards two volatile blends that are similar except for the presence of MeSA. We expect that predatory mites that have experienced MeSA can use this compound to discriminate between the volatile blend from *T. urticae*-infested lima bean (which includes MeSA) and the volatile blend from jasmonic acid-induced lima bean (which lacks MeSA), but that predators that have not experienced MeSA cannot do so (Dicke *et al.* 1999; chapter 4).

MATERIAL AND METHODS

Mites

Two-spotted spider mites (*Tetranychus urticae*) were reared on lima bean (*Phaseolus lunatus* L. cv Sieva) and cucumber (*Cucumis sativus* L. cv Lange Groene Giganten) plants in a greenhouse (20-30 °C, 50-70 % r.h., 16 h light).

A stock colony of the predatory mite, *Phytoseiulus persimilis*, was kept on spider mite-infested lima bean leaves in Petri dishes. Petri dishes with predators were kept in a climate room (23±1 °C, 50-70 % r.h., 16L:8D).

Bioassay

Olfactory preferences of predatory mites were tested in a glass Y-tube olfactometer (Takabayashi & Dicke 1992; chapter 2). The behaviour of an individual predatory mite females was observed for a maximum of 5 min. If the predator did not make a choice for one of the two odour sources within this time, it was recorded as a “no choice”. Each experiment was repeated on four to seven days. New odour sources were prepared and new sets of predatory mites were reared for each day of the experiment. Predators from different treatments were tested alternately on the same day with the same odour sources to exclude any possible influence of time of testing. All experiments were performed at 21±1 °C under laboratory conditions.

Experiments

(1) Rearing history and starvation level

In this experiment, we tested the effect of rearing history and starvation level on the responses of predatory mites to MeSA. Four groups of predators were prepared: reared on spider mites on lima bean (volatile blend including MeSA) or on cucumber (volatile blend lacking MeSA), and either satiated or starved. We compared predatory mites of different starvation levels because starvation may influence the choices that they make (Shimoda & Dicke 2000). To obtain predators of the same age for the experiment, about 5 females from the stock colony on lima bean were transferred to spider mite-infested cucumber or lima bean leaves in each of a series of Petri dishes. Females were allowed to oviposit for two days and were subsequently removed. Offspring adult females were collected eight days after initiation of the colonies, i.e. 1-2 days after their final moult.

Predators were collected 2-4 h before a bioassay (referred to as satiated) or 22-26 h in advance (referred to as starved). All predators were kept individually in Eppendorf vials with a small droplet of water, until the experiment. Eppendorf vials and Petri dishes were kept under the same conditions as the stock colony. Throughout this paper we will use the word "rearing-experience" to indicate the experience that predatory mites acquired with the herbivore-induced volatiles from the plant species on which they were reared; this includes the entire developmental period and 1 to 2 days of the adult phase.

We tested the attraction of predatory mites to methyl salicylate (MeSA, Merck, 99 % pure). Methyl salicylate was dissolved in hexane (pro analysi, Merck) to obtain a concentration of 2 μg/ml. A filter paper (15 cm^2) with 0.1 ml of dissolved MeSA (=0.2 μg) was used as one odour source, a filter paper with 0.1 ml hexane served as the alternative odour source. The MeSA dose is based on dose-response data obtained in chapter 4. The filter papers were placed in the arms of the olfactometer, about 10 cm upwind from the finish line. A predator was introduced into the olfactometer 30 to 60 seconds after introducing the filter papers, when most of the solvent had evaporated. New filter papers were used for every predator. We repeated this experiment on four different days with 20-25 females per treatment per day.

(2) Exposure to methyl salicylate during rearing

To study whether any difference in response between lima bean-reared and cucumber-reared predatory mites could be attributed to the presence of methyl salicylate in the volatile blend from spider mite-infested lima bean, the following experiment was designed. Predators were reared for eight days on cucumber with or without adding synthetic MeSA to the plant odour and the two groups were compared for their attraction to the single compound MeSA in the olfactometer. We introduced predatory mite females from the stock colony on lima bean onto fresh *T. urticae*-infested cucumber leaves in Petri dishes. A filter paper with 0.2 mg MeSA (in 0.1 ml water) was placed in half of the Petri dishes. As a control, a filter paper with water was placed in the other Petri dishes. After two days the predatory mite females were discarded and their offspring was left in the Petri dish. Every two days we added fresh food and we replaced filter papers with MeSA-solution or water. Offspring adult females were collected eight days after initiation of the colonies and were placed individually in Eppendorf vials 2-4 h before an experiment (i.e. they were satiated). Predators were tested in the olfactometer for their attraction to 0.2 μg MeSA (see above). We repeated this experiment on five different days with 19-25 females per group per day.

(3) Exposure to methyl salicylate during the adult phase

We conducted this experiment to study whether exposing adult predatory mites to one novel synthetic compound – MeSA– in addition to the prey-induced volatile blend could change the adults' behavioural responses to this compound. Predators were reared on cucumber. Seven to eight days after initiation of the colonies, females were divided into two groups. A filter paper with 0.2 mg MeSA was placed in the Petri dishes of one group; a filter paper with 0.1 ml water was placed in the Petri dishes of the other group (see above). A fresh *T. urticae*-infested cucumber leaf was also added to each Petri dish. Adult female predators were exposed to MeSA for one day before they were collected individually in Eppendorf vials. The satiated (i.e. collected 2-4 h before an experiment) predators of the control-cucumber group and of the MeSA-exposed group were tested in the olfactometer for their response to 0.2 µg MeSA (see above). We repeated this experiment on six days with 12-22 females per day of the MeSA-exposed group and on seven days with 8-20 females per day of the control-cucumber group.

In another experiment, adult predators that had been reared on cucumber were exposed to MeSA for three days. The procedure was the same as described above. We repeated this experiment on six days with 16-23 females of the MeSA-exposed group and 7-24 females of the control-cucumber group per day.

(4) Responses towards volatile blends with and without methyl salicylate

In this experiment, we assessed the responses of predatory mites with different rearing experiences towards volatiles from *T. urticae*-infested and jasmonic acid-treated lima bean leaves. Jasmonic acid (JA)-treated lima bean leaves do not produce MeSA but otherwise their volatile blend is similar to the blend of spider mite-infested lima bean leaves (Dicke *et al.* 1999).

Lima bean plants were grown in the greenhouse (20-30 °C, 50-70 % r.h., 16 h light) and were used when they were 10 to 15 days old. The primary leaves were detached and placed with their petiole in a vial. Half of the leaves was placed in a vial with water and infested with 50 female *T. urticae* per leaf. The other leaves were placed in a vial filled with a JA (Sigma) solution in water (0.5 mM). Plants of the two treatments were incubated in separate plastic cages in a climate room (24±1 °C, 50-70 % r.h., 16L:8D) for three days. Before an experiment, the petiole of each leaf was wrapped in wet cotton wool and aluminium foil and the leaves were placed in the 2 l vessels connected to the Y-tube olfactometer. Six leaves were used per odour source. Predators reared on lima bean or cucumber were collected individually in Eppendorf vials 2-4 h before the experiment. Fifteen to 19 females of each group were tested and the experiment was repeated on four different days.

In the second part of this experiment, we compared the responses of cucumber-reared predators to the responses of cucumber-reared predators that had been exposed to MeSA during their development (8 days). The predatory mites of these groups were reared in the same way as described above. First, we observed the choices between the volatiles from *T. urticae*-infested lima bean and JA-induced lima bean of 17-19 control-cucumber predators and 15-22 MeSA-exposed mites. Subsequently, we added synthetic MeSA (0.2 μg in 0.1 ml hexane on a filter paper, see above) downwind of the JA-induced lima bean leaves, a filter paper with 0.1 ml hexane was placed downwind of the *T. urticae*-infested leaves as a control. Then we observed the choices of another set of 10-14 predators of both groups. New filter papers were used for every predator. This experiment was repeated on six days.

Statistical analysis

We investigated whether the choices of predatory mites between the two odour sources in the olfactometer differed from a 50:50 distribution with a two-sided binomial test, using the pooled results of the different replicates per experiment. Within an experiment the responses of the different groups of predators were comparatively analysed using a generalised linear model (GLM, GenStat 6.1; McCullagh & Nelder 1989) with a binomial distribution and a logit-link function. The number of predators that made a choice for MeSA, or *T. urticae*-induced volatiles per day of the experiment, was taken as the response variate (i.e. 4 to 7 replicates per experiment). The total number of predators that made a choice per day was taken as the binomial total. We analysed the effect of treatment (experience) on the fraction of predatory mites choosing MeSA (when tested vs. control hexane) or *T. urticae*-infested lima bean leaves (when tested vs. JA-treated lima bean leaves), in an additive model using replicate and predator treatment as explanatory factors. The null hypothesis was that treatment had no effect on the responses of the predatory mites. The deviance of the residual was subjected to the χ^2-test to check the validity of the model ($\alpha=0.05$).

The statistical analyses were performed on the numbers of predatory mites that made a choice for one of the odour sources because the percentages of predators that did not make a choice were not influenced by treatment in any of the experiments ($P>0.05$, GLM, predicting factors: replicate + treatment). In total, 15 % of 1470 predators did not make a choice.

RESULTS

Rearing history and starvation level

Both satiated and starved predatory mites that had been reared on lima bean were attracted to 0.2 μg MeSA (Figure 1A, P=0.01 and P=0.03 respectively, binomial test). In contrast, predators that had been reared on cucumber from egg to adult were not attracted to MeSA (P≥0.58). A statistical comparison with a generalised linear model showed that the effect of rearing history was almost significant (P=0.053, GLM) and the starvation level did not affect the attraction of the predators to MeSA (P=0.73).

Exposure to methyl salicylate during rearing

When predatory mites had been reared on cucumber in the presence of synthetic MeSA for eight days, they were attracted to MeSA (Figure 1B, P=0.01, binomial test). In contrast, the predators of the control-cucumber group, that had not been exposed to MeSA, were not attracted to MeSA; the majority of predators even chose for the control arm but this was not significant (P=0.08, binomial test). The responses of the two groups of predators were significantly different (P=0.009, GLM).

Exposure to methyl salicylate during the adult phase

Exposing cucumber-reared predatory mites to synthetic MeSA for only one day during the adult phase did not result in attraction to MeSA (Figure 2, P=0.29, binomial test). The response of the MeSA-exposed group was not significantly different from the control-cucumber group (GLM, P=0.63).

When cucumber-reared predators had been exposed to MeSA for three days during the adult phase, they were attracted to MeSA (Figure 2, P<0.001, binomial test), whereas mites that had not been exposed to MeSA were not attracted (P=0.23). However, a direct statistical comparison showed that the responses of both groups of predators to MeSA were not significantly different (P=0.21, GLM).

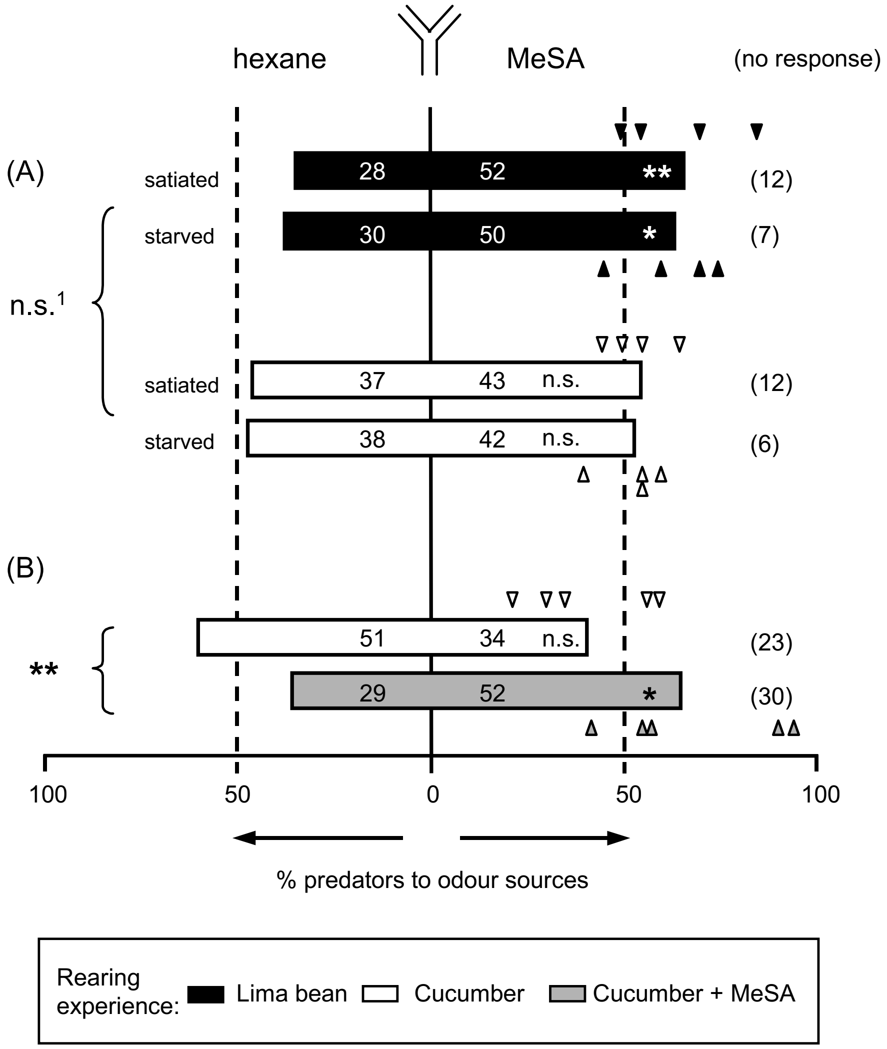

Figure 1: Effect of rearing history and starvation level on the responses of *Phytoseiulus persimilis* to 0.2 µg methyl salicylate (MeSA) in a Y-tube olfactometer. (A) Response of satiated and starved predators reared on spider mites on lima bean (black bars) or on cucumber (white bars). (B) Response of satiated predators reared on spider mites on cucumber with (grey bar) or without (white bar) additional synthetic MeSA. Numbers in bars are the total numbers of predators that chose for MeSA or the control hexane. Triangles indicate the percentage of predators attracted to MeSA in each replicate. The number in brackets behind each bar is the number of predators that did not make a choice. Choices between odour sources were analysed with a two-sided binomial test, and the effect of experience acquired during rearing on the responses of the predators was analysed with a GLM, the significance of which is indicated in front of the brackets (predicting factors: replicate + treatment) (n.s. $P>0.05$ [[1] $P=0.053$]; * $P<0.05$; ** $P<0.01$).

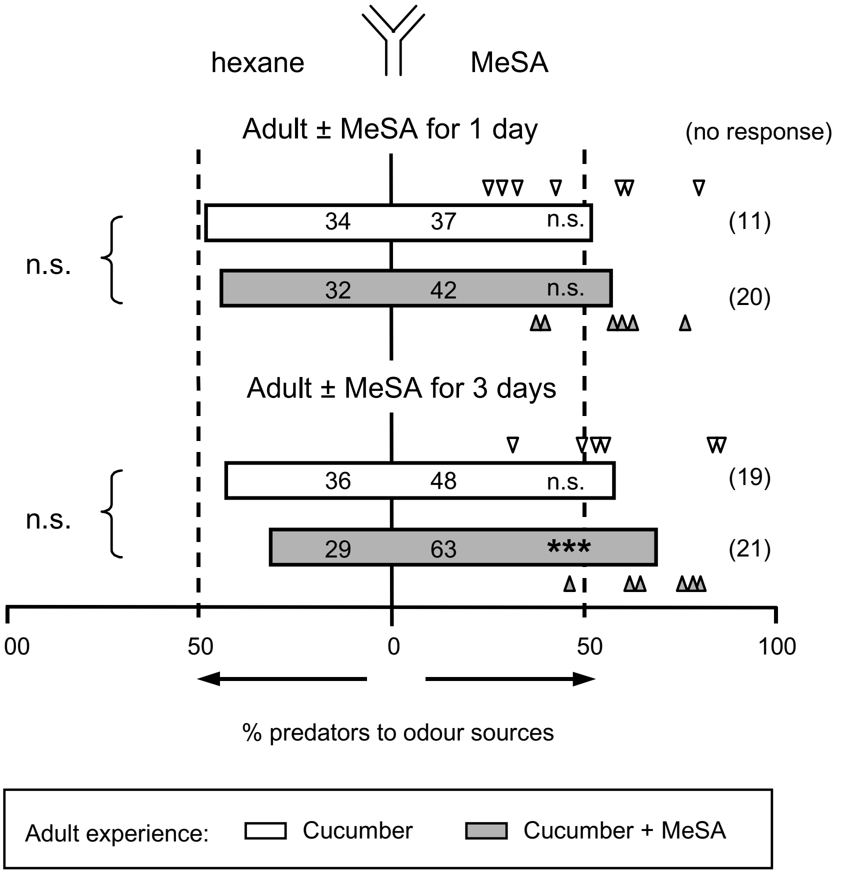

Figure 2: Influence of adult-experience with methyl salicylate on the responses of *Phytoseiulus persimilis* to 0.2 μg MeSA in the Y-tube olfactometer. The white bars represent the choice of the control-cucumber groups (predatory mites reared on spider mites on cucumber); the grey bars represent the choice of the cucumber-reared predators that had been exposed to MeSA during 1 or 3 days in the adult phase. Numbers in bars are the total numbers of predators that chose for MeSA or hexane. Triangles indicate the percentage of predators attracted to MeSA in each replicate. The number in brackets behind each bar is the number of predators that did not make a choice. Choices between odour sources were analysed with a two-sided binomial test, and the effect of exposure to MeSA on the responses of the predators was analysed with a GLM, the significance of which is indicated in front of the brackets (predicting factors: replicate + treatment) (n.s. $P>0.05$; *** $P<0.001$).

Responses towards volatile blends with and without methyl salicylate

Rearing history of the predatory mites had a large influence on their choices between the volatile blends from *T. urticae*-infested and JA-treated lima bean (Figure 3A, $P<0.001$, GLM). Lima bean-reared predators strongly preferred the volatiles from spider mite-infested lima bean leaves ($P<0.001$, binomial test), while cucumber-reared predators preferred the volatiles from JA-induced lima bean leaves ($P=0.03$, binomial test). The responses of the predators reared on cucumber in the presence of synthetic MeSA were intermediate: they did not discriminate between the volatiles from *T. urticae*-infested lima bean leaves and

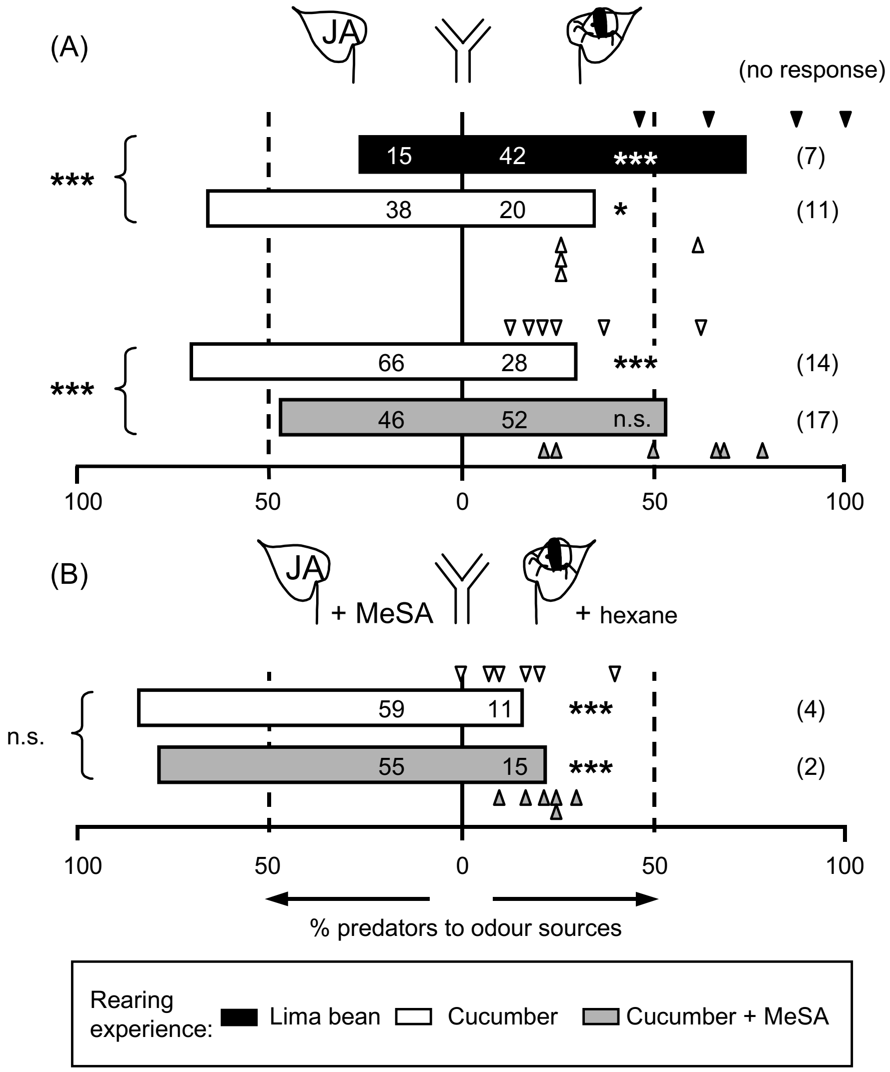

Figure 3: Effect of rearing experience with methyl salicylate of *Phytoseiulus persimilis* on its responses to two lima bean volatile blends with or without MeSA in the Y-tube olfactometer. (A) Response of lima bean-reared predators (black bars), cucumber-reared predators (white bars), and cucumber-reared predators that had been exposed to MeSA (grey bars) to the volatile blends from *T. urticae*-induced lima bean (MeSA present) and JA-induced lima bean (MeSA absent). (B) The choice of control-cucumber predators (white bar) and MeSA-exposed mites (grey bar) between the volatiles from *T. urticae*-infested lima bean and the combination of JA-induced lima bean volatiles plus 0.2 μg MeSA. Numbers in bars are the total numbers of predators making a choice. Triangles indicate the percentage of predators attracted to *T. urticae*-induced lima bean in each replicate. The number in brackets behind each bar is the number of predators that did not make a choice. Choices between odour sources were analysed with a two-sided binomial test, and the effect of experience acquired during rearing on the responses of the predators was analysed with a GLM, the significance of which is indicated in front of the brackets (predicting factors: replicate + treatment) (n.s. $P>0.05$; * $P<0.05$; *** $P<0.001$).

JA-treated leaves ($P=0.61$, binomial test). The effect of exposing the cucumber-reared predators to MeSA during their development was significant ($P=0.001$, GLM).

When the JA-induced lima bean volatile blend was complemented with synthetic MeSA (0.2 μg), both MeSA-exposed and control-cucumber predators significantly preferred this odour source to the volatiles from spider mite-infested lima bean leaves (Figure 3B, $P<0.001$, binomial test). The responses of the two predator groups were not significantly different in this choice-test ($P=0.38$, GLM).

DISCUSSION

Our results demonstrate that the responses of *P. persimilis* to the individual compound MeSA are affected by experience with this compound. Furthermore, the results show that the composition of the herbivore-induced volatile blend to which predatory mites are exposed during rearing has a large impact on the choices of adult predators between two volatile blends emitted by differently treated lima bean plants.

Attraction to methyl salicylate

Predatory mites that had been reared on cucumber were not attracted to the single compound MeSA, in contrast to predators that had been reared on lima bean. Predators reared on cucumber had not been exposed to MeSA (Takabayashi *et al.* 1994a; chapter 3) whereas predators that had been reared on lima bean had been exposed to MeSA (Dicke *et al.* 1990a, 1999; chapters 3 and 5). The role of experience with MeSA was demonstrated explicitly by the difference in response between predatory mites reared on spider mites on cucumber with or without additional synthetic MeSA: only the predators that had been exposed to MeSA were attracted to MeSA. Apart from the presence or absence of MeSA, all other treatments, such as origin and diet, were identical for the two groups of predator. Selection for a genetically different response during the period of experience cannot explain the results because the mothers from the females used in experiments were all taken from the same stock colony, the same numbers of offspring were produced on lima bean and on cucumber, and we did not observe differential mortality between the different treatments. Starvation level did not affect the responses of *P. persimilis* to MeSA; the same result was found in chapter 4. These data show that a preference for experienced compounds can be retained for at least 24 h.

A similar result has been found for the bug *Anthocoris nemoralis* (Drukker *et al.* 2000b). Predatory bugs that had been collected on psyllid-infested pear trees (which do emit MeSA, Scutareanu *et al.* 1997) from the field were attracted to the single compound MeSA. However, when the bugs had been reared in the laboratory on *Ephestia* eggs in the absence of volatiles they were not attracted to MeSA. Exposing the bugs to synthetic MeSA in the absence of other volatiles during the rearing resulted in attraction to MeSA. In the same study, adults could learn either to be attracted or to avoid MeSA by exposing them to MeSA in the presence or absence of food prior to the olfactometer test.

In our study we also found some support that predatory mites can learn to respond to MeSA during the adult stage. Cucumber-reared predators that had been exposed to MeSA for three days during the adult stage were attracted to the single compound MeSA, but their response was not significantly different from that of control-cucumber predators. Adult females that had been kept for one day on spider mite-infested cucumber with synthetic MeSA were not attracted. Several other studies also indicate that adult predatory mites need more than one day of experience to learn a new volatile blend. For example, it took seven days of feeding on lima bean to shift the preference of cucumber-reared predators from *T. urticae*-induced cucumber volatiles to *T. urticae*-induced lima bean volatiles (Dicke *et al.* 1990b). In contrast, Drukker *et al.* (2000a) showed that adult *P. persimilis* females could learn to respond to spider mite-infested lima bean volatiles after only one day of experience with this volatile blend. Differences in the expression of adult learning can be explained in two ways: (1) The expression of adult learning depends on pre-adult experiences with herbivore-induced volatiles. This hypothesis is based on the finding that learning in honey bees of a component of a volatile blend can be hindered or blocked by pre-training with another component (Smith & Cobey 1994). The predatory mites in our experiments had been reared on cucumber and thus had associated the *T. urticae*-induced cucumber volatiles with the presence of their prey. It may therefore take longer to associate the presence of the new volatile compound (MeSA) to the presence of the prey as well. Drukker *et al.* (2000a,b) used predators that had been reared in total absence of volatiles and thus these predators could not associate any volatile foraging cues to the presence of their prey. During the adult stage, these predators quickly associated the volatile(s) to which they were exposed in the presence of their prey. (2) Starvation level may influence the rate of adult learning (Drukker *et al.* 2000a). Predatory mites that had been starved for 24 h in the presence of a herbivore-induced volatile blend and subsequently had been allowed to feed for 24 h in the presence of another volatile blend shifted their preferences accordingly. The adult predators in our experiments were not starved before they were exposed to MeSA so this difference might explain the weak effect of adult learning in our experiments. However, Drukker *et al.* (2000a) also showed that predators that had been exposed to *T. urticae*-infested lima bean volatiles in the presence of prey for only 16 h without a previous starvation period learned to respond to these volatiles, which does not support their hypothesis of slow learning by well fed predators. As yet, no studies have conclusively demonstrated an effect of starvation level on the expression of adult learning in predatory mites.

Discriminating between volatile blends

Predatory mites reared on lima bean prefer the volatiles from lima bean infested with their prey *T. urticae* to volatiles from lima bean treated with the plant hormone jasmonic acid (JA) (Dicke *et al.* 1999; chapter 4). MeSA has not been detected in the volatile blend from JA-induced lima bean leaves (Dicke *et al.* 1999). After complementing the JA-induced volatile blend with synthetic MeSA, the predators no longer preferred the volatiles from prey-infested leaves, which showed the important role of MeSA in predator foraging behaviour (chapter 4). We hypothesised that, unlike lima bean-reared predators, cucumber-reared predators can not use MeSA to discriminate between the volatiles from *T. urticae*-infested and JA-treated lima bean leaves because they have not had any experience with this compound. Indeed, cucumber-reared predators did not prefer *T. urticae*-induced to JA-induced volatiles; in fact, their response was the opposite, they preferred JA-induced volatiles. Cucumber-reared predators that had been exposed to MeSA during the rearing period (eight days) did not discriminate between JA-induced lima bean volatiles and the volatiles from *T. urticae*-infested leaves. In this situation, the behavioural response of the MeSA-exposed cucumber-reared predators was intermediate between that of lima bean-reared and cucumber-reared predators. This suggests that the experience with MeSA can partly explain the difference in response of predators reared on lima bean or cucumber to volatiles from JA-induced and *T. urticae*-infested lima bean leaves, but that other compounds experienced by the predators played a role as well. Indeed, JA-treated and *T. urticae*-infested plants differ in the emission of several other compounds (Dicke *et al.* 1999). For example, two oximes and (*E*)-β-ocimene are present in significantly larger amounts in the volatile blend from JA-induced lima bean than in the volatile blend from *T. urticae*-infested lima bean (Dicke *et al.* 1999). Because these compounds are also emitted in a much larger amount by *T. urticae*-infested cucumber than by *T. urticae*-infested lima bean (Takabayashi *et al.* 1994a; chapter 3), predators reared on cucumber have experienced them more than predators reared on lima bean and this may explain the different responses of both groups. We confirmed that the MeSA-exposed cucumber-reared predators really responded to MeSA by complementing the JA-induced volatile blend with MeSA. The response of the predatory mites then shifted from no discrimination towards a preference for the combination of JA-induced volatiles plus MeSA over *T. urticae*-infested lima bean volatiles. Once MeSA is present in both odour sources, the choice of MeSA-experienced predators is no longer influenced by this compound and their preference for JA-induced volatiles can be explained in the same way as for the control-cucumber predators (see above).

In conclusion, our data show that predatory mites can learn to respond to one novel compound within a complex volatile blend. The large effect of such a small difference in volatile blend composition on the choices of the adult predatory mite between complex volatile blends is an important step in understanding how predatory mites handle variation in herbivore-induced plant volatiles. This flexibility in the foraging behaviour can enable the predator to adjust its responses to subtle differences in volatile information in a natural ecosystem where their prey feeds on a range of different plant species that all emit a different herbivore-induced volatile blend. Most likely similar effects of experience with prey-induced plant volatiles that play an important role in carnivore foraging behaviour can be found for other tritrophic systems. To address this issue, the components of the volatile blend that are actually important in the foraging behaviour of carnivores need to be revealed first because this is not yet known in most tritrophic systems (e.g. Dicke & Van Loon 2000).

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CHAPTER 7

Predatory mites learn to discriminate between plant volatiles induced by prey and non-prey herbivores

Abstract

Many carnivorous arthropods can use chemical information from plants to locate their herbivorous prey. The composition of blends of herbivore-induced plant volatiles can vary with plant and herbivore species and thus carnivores are confronted with variable information about the presence of their prey. Such environmental variation is expected to favour learning. We investigate the learning ability of the predatory mite *Phytoseiulus persimilis*, a specialised natural enemy of polyphagous spider mites. First we show that the choices of predatory mites between the volatiles from lima bean plants infested with the prey herbivore *Tetranychus urticae* or the non-prey caterpillar *Spodoptera exigua* are affected by the plant species on which the predators have been reared. Predators reared on lima bean prefer the volatiles induced by the prey herbivore whereas predators reared on cucumber do not. Second, we demonstrate that a non-rewarding experience during the adult phase (i.e. starvation in the presence of *S. exigua*-induced volatiles from lima bean) has a small effect on the foraging behaviour. However, a rewarding experience (i.e. feeding in the presence of *T. urticae*-induced volatiles) has a much larger impact on the foraging responses. Predatory mites with multiple experiences (i.e. a non-rewarding experience followed by a rewarding experience) have the strongest preference for *T. urticae*-induced versus *S. exigua*-induced volatiles. We conclude that the demonstrated learning abilities of *P. persimilis* enable the predatory mites to forage in an environment where their prey can feed on a different food plant species than the one on which the predator developed, and where non-prey caterpillars are also present.

INTRODUCTION

The ability to learn enables animals to adapt their responses to a variable environment (Stephens 1993; Dukas 1998). Arthropods have proven to be valuable subjects to study the mechanisms and ecology of learning (Papaj & Prokopy 1989; Papaj & Lewis 1993). Recently, the adaptive significance of learning was demonstrated for a grasshopper, a spider mite and a parasitoid wasp (Dukas & Bernays 2000; Dukas & Duan 2000; Egas & Sabelis 2001). In a study on experimental evolution in the fruitfly *Drosophila melanogaster*, Mery & Kawecki (2002) showed that an improved learning ability indeed evolves in a variable environment. Compared to the wealth of knowledge on learning in several groups of insects, such as social Hymenoptera (e.g. Menzel 1983; Real 1991), phytophagous insects (reviewed by Papaj & Prokopy 1989), parasitoid wasps (reviewed by Turlings *et al.* 1993a; Vet *et al.* 1995), fruit flies, cockroaches and crickets (e.g. Barron & Corbet 1999; Lomassese *et al.* 2000; Sakura *et al.* 2002), relatively little is known about learning in predatory insects and mites (Bernays 1993; Dicke *et al.* 1998). In this article, we explore the learning abilities of a predatory mite that uses chemical information to locate its prey.

Using indirect information to locate food

Prey are often under strong selection not to reveal themselves and are therefore difficult to detect. Predators can solve this problem by using information from the prey's environment instead of from the victim itself. Foraging behaviour involving such indirect information is well documented for carnivorous arthropods that use herbivore-induced plant volatiles to locate their herbivorous prey or hosts (Vet & Dicke 1992). Herbivore-induced plant volatiles are blends of volatile compounds released by plants that are attacked by herbivorous insects or mites. The composition of these blends is influenced by plant and herbivore species (e.g. Takabayashi *et al.* 1994b; DeMoraes *et al.* 1998; Van den Boom *et al.* 2004). Because many herbivorous arthropods can feed on several different plant species, carnivores have to be able to handle variable information about the presence of the same prey, a situation that is expected to favour learning. Learning to associate herbivore-induced plant volatiles with the presence of suitable host herbivores has been demonstrated extensively for hymenopteran parasitoids (reviewed by Turlings *et al.* 1993a; Vet *et al.* 1995). This learning mechanism allows parasitoids to specialise temporarily on the food plants used by the host at a certain place and time.

Although several studies have shown that the responses of predatory bugs and mites are influenced by previous experiences with herbivore-induced plant volatiles (e.g. Dicke *et al.* 1990b; Takabayashi & Dicke 1992; Krips *et al.* 1999; Drukker *et al.* 2000a,b), little is yet known about the mechanisms of learning and the role of learning in the foraging behaviour of arthropod predators (Bernays 1993; Dicke *et al.* 1998).

Can predatory mites learn to discriminate between volatile blends?

The specialised predatory mite *Phytoseiulus persimilis* uses herbivore-induced plant volatiles to locate its prey: spider mites in the genus *Tetranychus* (Sabelis & Van de Baan 1983; Dicke *et al.* 1990a). Because spider mites are polyphagous herbivores that induce volatile blends of variable composition in different host plant species (Van de Vrie *et al.* 1972; Van den Boom *et al.* 2004), *P. persimilis* is potentially confronted with variable information on the presence of its prey on a plant, and flexibility in its foraging responses is expected. Indeed, it has been shown that the attraction of *P. persimilis* to herbivore-induced volatiles exhibits genetic variation and is also influenced by previous experiences with volatiles (Margolies *et al.* 1997; Dicke *et al.* 1998; Drukker *et al.* 2000a; Jia *et al.* 2002). In the present paper, we study whether learning influences the ability of *P. persimilis* to discriminate between the volatiles from plants infested with its prey, the two-spotted spider mite *Tetranychus urticae*, or with the non-prey caterpillar, *Spodoptera exigua*. To this end, we test the following four hypotheses:

(i) The volatile blend to which predatory mites are exposed during rearing influences their choices between the volatiles from *T. urticae*-infested or *S. exigua*-infested plants.

(ii) During the adult phase a non-rewarding experience (i.e. exposure to *S. exigua*-induced volatiles in the absence of prey) or a rewarding experience (i.e. exposure to *T. urticae*-induced volatiles in the presence of prey) result in a stronger preference of *P. persimilis* for the volatiles from *T. urticae*-infested plants.

(iii) Multiple experiences (i.e. a non-rewarding experience followed by a rewarding experience) have a larger impact on foraging responses than a non-rewarding or a rewarding experience alone (Vet *et al.* 1998).

(iv) The starvation level influences the impact of a rewarding experience (Drukker *et al.* 2000a).

MATERIAL AND METHODS

Plants and herbivores

Lima bean plants (*Phaseolus lunatus* L. cv. Sieva) and cucumber plants (*Cucumis sativus* L. cv. Lange Groene Giganten) were grown in a greenhouse at 20-30 °C, 50-70% r.h. and 16 h of light. Lima bean plants were used in experiments when their primary leaves had expanded, which was 10 to 15 days after sowing. Cucumber plants were used when the first two leaves were about 10 cm wide, which was 15 to 30 days after sowing. Colonies of the two-spotted spider mite

Tetranychus urticae Koch (Acari: Tetranychidae) on lima bean and cucumber were maintained in a greenhouse under the conditions described above. Eggs of *Spodoptera exigua* Hübner (Lepidoptera: Noctuidae) were obtained from the laboratory of Virology, Wageningen University, The Netherlands (see Smits *et al.* 1986). Two to three egg batches were placed in a Petri dish with a lima bean leaf and kept in a climate cabinet at 23±1 °C and 50-70% r.h. Larvae were used in experiments within 24 h of hatching.

Predators

A stock colony of predatory mites, *Phytoseiulus persimilis* Athias-Henriot (Acari: Phytoseiidae), was kept on spider mite-infested lima bean leaves in a climate cabinet at 23±1 °C, 50-70% r.h. and continuous light. To obtain females of the same age, we transferred about 5 gravid females from the stock colony onto a fresh *T. urticae*-infested lima bean or cucumber leaf in each of a series of Petri dishes (9 cm ∅). The females were allowed to oviposit for one or two days and were then removed. Fresh spider mite-infested leaves were provided every two days to feed the offspring. Offspring females were used in experiments eight days after initiation of the colonies, i.e. 1 to 2 days after their final moult. Petri dishes were kept under the same conditions as the stock colony.

Y-tube olfactometer

We analysed the choices of predatory mites between the volatiles from *T. urticae*-infested and *S. exigua*-infested lima bean in a closed-system Y-tube olfactometer (Takabayashi & Dicke 1992; chapter 2). Individual predatory mites were observed for a maximum of 5 min. Predators that had not made a choice for one of the two odour sources within 5 min were recorded as a “no-choice”. For comparison of predators of different treatments, predators of the different groups were introduced into the olfactometer alternately on the same day with the same odour sources. Each experiment was repeated on 4 to 10 different days with new sets of odour sources and new sets of about 8 to 20 predators per treatment on each day. Olfactometer experiments were carried out at 21±1 °C.

Odour sources

Odour sources consisted of four lima bean leaves, infested with *T. urticae* or *S. exigua* 3 days before an experiment. The two primary leaves of each plant were infested with adult *T. urticae* females from the greenhouse culture on lima bean, or with newly hatched *S. exigua* larvae. The latter were put in a clip cage. Clip cages were supported with sticks to prevent the leaves from bending down and incurring damage by the weight of the cages. Clip cages without caterpillars were placed on *T. urticae*-infested plants as a control. Plants from the different treatments were incubated separately in plastic cages in a climate room at 24±1 °C, 50-70 % r.h.

and 16L:8D. Just before an experiment, leaves were cut from the plant and their petioles were wrapped in wet cotton wool and aluminium foil to prevent wilting. Numbers of herbivores per leaf are specified in the figure legends for each experiment.

Predatory mite treatments

–Experience during rearing–

During rearing, predators acquired experience with volatiles induced by their prey, spider mites. They experienced these volatiles during development and the first days of the adult phase. Two groups of predators were used, one group was reared on spider mites on cucumber (CU-predators), the other on spider mites on lima bean (LI-predators). In addition, a third group of predators was reared as follows: Predatory mites were reared on spider mites on cucumber (which does not emit methyl salicylate, chapter 3) in the presence of synthetic methyl salicylate emanating from a filter paper with 0.2±0.05 mg of this compound (Merck, 99% pure). The filter papers were replaced every two days. The control treatments for this third group of predators consisted of CU-predators and LI-predators that had been reared on cucumber or lima bean with a filter paper without methyl salicylate in the Petri dish.

–Experiences during the adult phase–

The following descriptions of experiences that predatory mites acquired during the adult phase specifically relate to experiences with the odour sources that we offered in the olfactometer, i.e. a non-rewarding experience involved being exposed to the volatiles from *S. exigua*-infested lima bean in the absence of prey, and a rewarding experience involved being exposed to the volatiles from *T. urticae*-infested lima bean in the presence of prey.

A non-rewarding experience involved a period of 24 h in absence of prey while being exposed to the volatiles from *S. exigua*-infested lima bean. To this end, a group of predators was transferred to a Petri dish (15 cm ∅) with a lima bean leaf that had been infested 2 days before with 2 *S. exigua* larvae (in the same way as described in "*Odour sources*"); at the moment of introducing the predatory mites another 2 caterpillars were added. The control treatments for this non-rewarding experience consisted of 24 h in absence of prey, and without being exposed to *S. exigua*-induced lima bean volatiles, either as a group in a Petri dish with an uninfested lima bean leaf or individually in an Eppendorf vial.

A rewarding experience involved a period of 24 h in the presence of prey while being exposed to the volatiles from a *T. urticae*-infested lima bean leaf. To this end, a group of predators was transferred to a Petri dish (15 cm ∅) with a lima bean leaf that had been infested 2 days before with spider mites by attaching a heavily infested leaf from the greenhouse culture to an uninfested plant. The control treatment for this rewarding experience consisted of 24 h in the presence of prey without being exposed to *T. urticae*-induced lima bean volatiles, as a group in a Petri dish with a *T. urticae*-infested cucumber leaf.

A multiple experience involved a non-rewarding experience with *S. exigua*-induced lima bean volatiles followed by a rewarding experience with *T. urticae*-induced lima bean volatiles according to the procedures described above.

Experiments

The experiments were done to investigate the effect of experiences on the choices of *P. persimilis* between the volatiles from lima bean plants infested with *T. urticae* or *S. exigua*. The specific predatory mite treatments that we used per experiment are indicated in the figure legends.

–Experience during rearing–

Before testing, all predators were kept for about 24 h individually in Eppendorf vials without food but with a small droplet of water, at 23±1 °C.

Experiment 1A: We tested the hypothesis that experience acquired during rearing affects the choices of predatory mites between the volatiles from *T. urticae*-infested and S. *exigua*-infested lima bean leaves at different densities of *S. exigua* and a fixed density of *T. urticae*. Our expectation was that LI-predators have a stronger preference for lima bean volatiles induced by their prey than CU-predators. To this end, we tested the attractiveness of volatiles from three sets of leaves infested with 1, 2, or 4 *S. exigua* larvae per leaf versus the volatiles from one set of leaves infested with 20 *T. urticae* within the same experiment, and the attractiveness of volatiles from leaves infested with 10 *S. exigua* larvae per leaf versus the volatiles from leaves infested with 20 *T. urticae* per leaf in a separate experiment.

Experiment 1B: We tested the hypothesis described in experiment 1A, but here we used different densities of *T. urticae* and a fixed density of *S. exigua*. The attractiveness of the volatiles from three sets of leaves infested with 10, 20 or 40 *T. urticae* per leaf versus the volatiles from one set of leaves infested with 2 *S. exigua* per leaf was tested within one experiment, while the attractiveness of the volatiles from leaves infested with 100 *T. urticae* per leaf versus the volatiles from leaves infested with 2 *S. exigua* was tested in a separate experiment.

Experiment 1C: We previously showed that methyl salicylate can be used by *P. persimilis* to discriminate between the volatiles from *T. urticae*-infested and *S. exigua*-infested lima bean plants, and that the predators have to learn to respond to methyl salicylate (chapters 5 and 6). In this experiment we compared the choices of LI-predators, CU-predators, and predators that had been reared on cucumber in the presence of synthetic MeSA, between the volatiles from *T. urticae*- and *S. exigua*-infested lima bean leaves. We expected that experience with methyl salicylate results in a stronger preference for volatiles induced by their prey.

–Experiences during the adult phase–

Before testing, all predators (except for treatment E in experiment 3, see below) were kept for about 1-3 h individually in Eppendorf vials without food but with a small droplet of water, at 23±1 °C.

Experiment 2: We tested the hypothesis that a non-rewarding experience with *S. exigua*-induced volatiles from lima bean reduces the proportion of predatory mites choosing for *S. exigua*-induced volatiles in a choice test between the volatiles from *T. urticae*-infested or *S. exigua*-infested lima bean plants. The control groups consisted of predators that had been kept without food for 24 h but had not been exposed to *S. exigua*-induced volatiles.

Experiment 3: We tested the hypothesis that experiences with both of the odour sources offered in the choice test, i.e. the volatiles from lima bean leaves infested with *T. urticae* or *S. exigua*, during the adult phase have a stronger impact on the choices of predatory mites than an experience with only one, or none, of the odour sources used in the experiment. We did two sub-experiments, one with CU-predators, the other with LI-predators. Per sub-experiment we used four groups of predators that had acquired different experiences during the adult phase, besides the experience that they had acquired during rearing: (A) multiple experiences, (B) non-rewarding experience, (C) rewarding experience, (D) no additional experiences during the adult phase with the odour sources used in the experiment. In the experiment with CU-predators, we tested a fifth group of predators: (E) multiple experiences (like treatment A), prior to the experiment these predators were kept for about 24 h in Eppendorf vials, instead of 1-3 h. The fifth group of predators was tested on different days and with different odour sources than groups A to D.

Experiment 4: We tested the hypothesis that the impact of a rewarding experience is larger for starved predators than for satiated predatory mites. LI-predators were fed for 24 h on spider mite-infested cucumber or lima bean after they had been starved for 24 h in the absence of volatiles (in an Eppendorf vial), or directly after the rearing procedure.

Statistical analysis

Predatory mites that did not make a choice (7 % of 2652 predators) were excluded from the statistical analysis. We analysed the pooled choices of the predators between the odour sources in the olfactometer with a two-sided binomial test for each experiment, under the null-hypothesis that the distribution over the two odour sources was 50:50. The effect of predator treatment (including rearing, non-rewarding, and rewarding experiences) on the foraging responses of *P. persimilis* was analysed with a generalised linear model under the null-hypothesis that there was no effect of treatment (GLM, GenStat 6.1, McCullagh & Nelder 1989). In addition, in experiments 1A and 1B we analysed the effect of herbivore density on the responses of predatory mites. GLM's with a binomial distribution and logit-link function were used. The number of predatory mites choosing for *T. urticae*-induced volatiles per day of the experiment was set as the response variate. The total number of mites that made a choice per day was set as the binomial total. The predicting factors that we used to analyse each experiment are specified in the figure legends.

RESULTS

Experience during rearing

Figure 1 shows the responses of LI-predators and CU-predators to the volatiles from lima bean plants infested with prey or non-prey herbivores at different densities of herbivores.

When the densities of *S. exigua* were varied, LI-predators chose more frequently for *T. urticae*-induced volatiles than CU-predators, i.e. compared to CU-predators, the overall percentages of LI-predators choosing *T. urticae*-induced volatiles were larger in all tests (Figure 1A). However, the effect of the experience acquired during rearing on the choices of predators was not significant ($P \geq 0.16$, GLM). Neither CU-predators nor LI-predators discriminated between the volatiles from *T. urticae*-infested or *S. exigua*-infested lima bean leaves at densities of 1, 2 or 4 non-prey caterpillars per leaf ($P \geq 0.09$, binomial test). In contrast, both groups of predators preferred the volatiles from *S. exigua*-infested leaves at a density of 10 *S. exigua* larvae per leaf ($P < 0.001$, binomial test).

When the densities of *T. urticae* were varied, LI-predators also had a stronger preference for *T. urticae*-induced lima bean volatiles than CU-predators, i.e. compared to CU-predators, the overall percentages of LI-predators choosing *T. urticae*-induced volatiles were larger in all tests (Figure 1B). The effect of the experience acquired during rearing on the preference of predatory mites was significant at densities of 10, 20 or 40 *T. urticae* per leaf ($P < 0.001$, GLM), and almost significant at a density of 100 *T. urticae* per leaf ($P = 0.08$, GLM). The density of 100 spider mites per leaf was analysed separately because the experiments were carried out using different sets of *S. exigua*-infested lima bean leaves. At the low densities of spider mites (10 or 20 per leaf) CU-predators preferred the volatiles from *S. exigua*-infested leaves ($P \leq 0.01$, binomial test), whereas LI-predators did not discriminate ($P \geq 0.09$). At densities of 40 or 100 spider mites per leaf CU-predators did not discriminate ($P \geq 0.16$, binomial test), while LI-predators preferred the volatiles from *T. urticae*-infested lima bean to those from *S. exigua*-infested lima bean leaves ($P \leq 0.006$). Significantly more predatory mites responded to *T. urticae*-induced volatiles at increasing densities of spider mites within the experiment with densities of 10, 20 or 40 *T. urticae* per leaf ($P<0.001$, GLM).

In conclusion, this experiment demonstrates that the volatile blend experienced during rearing significantly influences the choices of predatory mites between lima bean volatiles induced by their prey *T. urticae* or by the non-prey caterpillar *S. exigua* ($P<0.001$, GLM on the pooled results of the eight different experiments, with experiment and experience acquired during rearing as predicting factors).

Experience with methyl salicylate during rearing

To test whether experience with methyl salicylate during rearing increases the preference of predatory mites for *T. urticae*-induced lima bean volatiles (Figure 2), we compared the responses of (1) LI-predators (that experienced methyl salicylate emitted by *T. urticae*-infested lima bean), (2) CU-predators (that did not experience methyl salicylate because *T. urticae*-infested cucumber does not emit methyl salicylate), and (3) CU-predators that had been reared in the presence of synthetic methyl salicylate. Predators of the third group did not discriminate between the volatiles from *T. urticae*-infested or *S. exigua*-infested lima bean plants (Figure 2, $P=0.09$, binomial test), while control CU-predators strongly preferred *S. exigua*-induced volatiles ($P<0.001$). However, the responses of the two CU-predator groups (methyl salicylate-exposed and control) differed significantly from the responses of LI-predators ($P\leq0.001$, GLM), which preferred *T. urticae*-induced to *S. exigua*-induced lima bean volatiles ($P=0.004$, binomial test). This experiment shows that the difference in the responses of LI-predators and CU-predators may be partly explained by the difference in experience of these groups with methyl salicylate but that other factors also play a role.

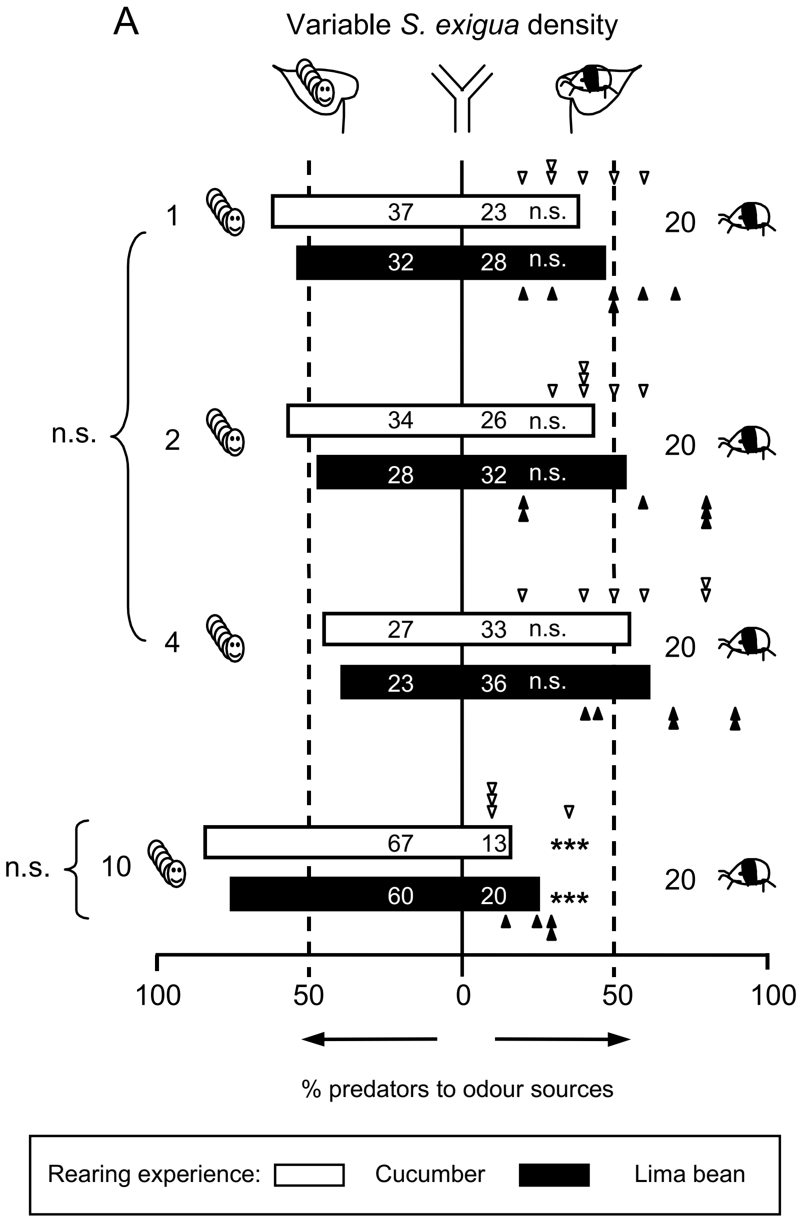
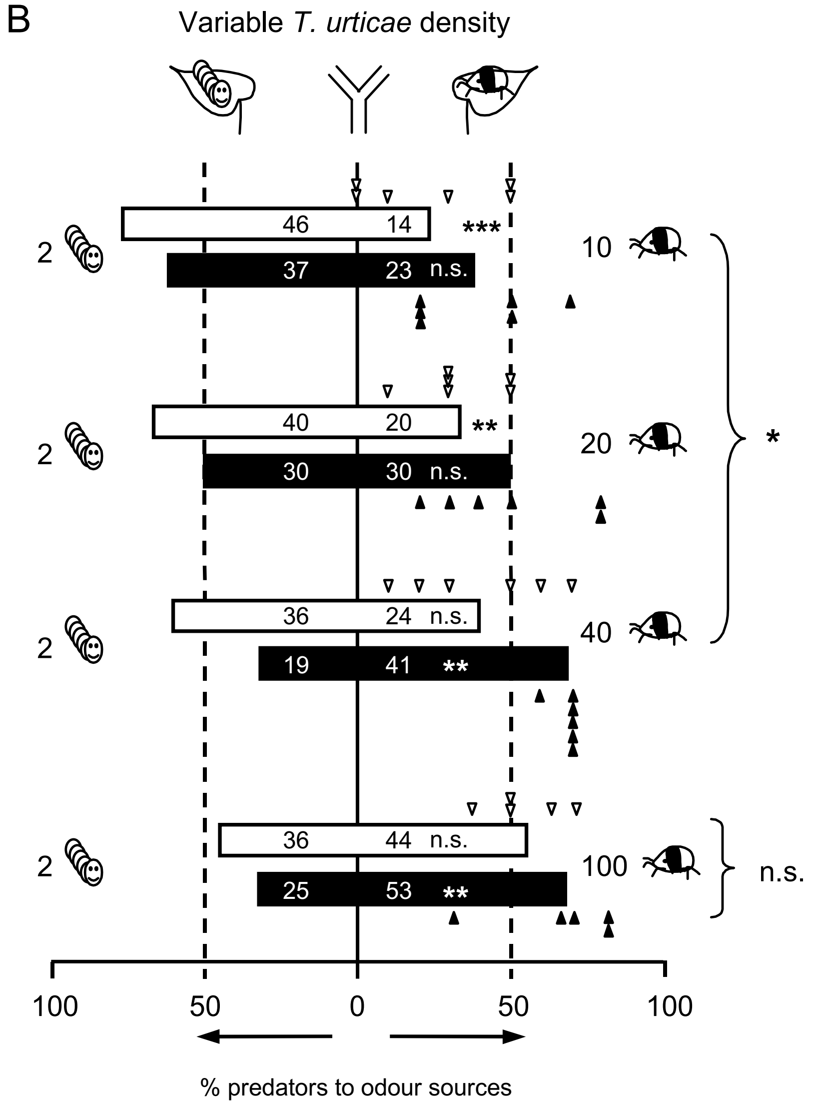


Figure 1: Effect of experience acquired during rearing on the choices of *Phytoseiulus persimilis* between the volatiles from lima bean plants infested with prey or non-prey herbivores at different herbivore densities (variable *Spodoptera exigua* density in A, variable *Tetranychus urticae* density in B). Numbers in front of the herbivore symbols indicate the number of herbivores per lima bean leaf. Bars present the overall percentages of CU-predators (white) and LI-predators (black) choosing for each odour source; triangles present the percentages predatory mites choosing for the volatiles from *T. urticae*-infested leaves per day of the experiment. Before testing, predators were kept without food and odours for 24 h. Numbers in bars are the total numbers of predators choosing for each odour source. Choices between odour sources were analysed with a two-sided binomial test. The effect of the experience acquired during rearing was analysed with a GLM, the significance of which is indicated in front or behind the brackets. Note that in Figure 1A the first three densities of *S. exigua* were tested against the same set of *T. urticae*-infested leaves (GLM: predictors: replicate [factor] + rearing experience [factor] + caterpillar density [variate]), while



(Figure 1 continued)

the density of 10 *S. exigua* per leaf was tested against another set of *T. urticae*-infested lima bean (GLM: predicting factors: replicate + rearing experience). Similarly in Figure 1B for *T. urticae* densities 10, 20 and 40 (GLM: predictors: replicate [factor] + rearing experience [factor] + spider mite density [variate]), and density 100 (GLM: predicting factors: replicate + rearing experience). (n.s. P>0.05; * P<0.05; ** P<0.01; *** P<0.001). Data of LI-predators for *S. exigua* densities 1, 2, and 4 in Figure 1A, and *T. urticae* densities 10, 20, and 40 in Figure 1B were published previously in chapter 5.

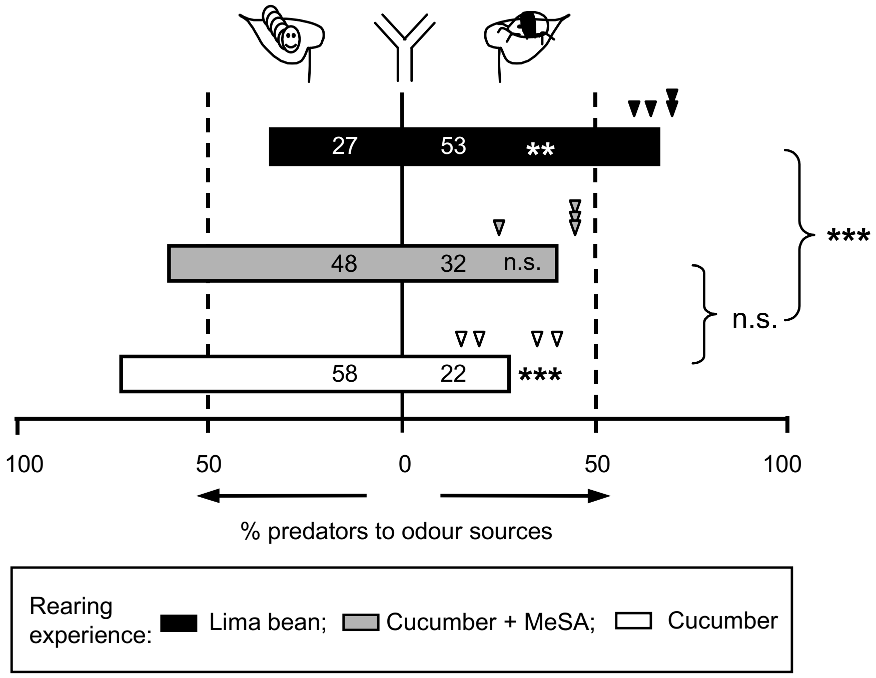


Figure 2: Effect of experience with methyl salicylate acquired during rearing on the choices of predatory mites between the volatiles from lima bean leaves infested with 40 *Tetranychus urticae* or 2 *Spodoptera exigua* per leaf. Before testing, predators were kept without food and odours for 24 h. Bars show the overall percentages of LI-predators (black bar), CU-predators reared in the presence of synthetic methyl salicylate (grey bar), and CU-predators (white bar). Numbers in bars are the total numbers of predators responding to the odour sources and triangles present the percentages predators choosing for *T. urticae*-induced volatiles per day of the experiment. Choices between odour sources were analysed with a two-sided binomial test; the effect of experience with methyl salicylate was analysed with a GLM, the significance of which is indicated behind the brackets (predicting factors: replicate + rearing experience). (n.s. P>0.05; * P<0.05; ** P<0.01; *** P<0.001).

Non-rewarding experience during the adult phase

Figure 3 shows the responses of LI-predators and CU-predators with or without a non-rewarding experience with *S. exigua*-induced volatiles. We tested whether a non-rewarding experience with *S. exigua*-induced volatiles reduced the proportion predators responding to *S. exigua*-induced volatiles and thus increase the preference for *T. urticae*-induced volatiles when offered a choice between the volatiles from *T. urticae*-infested or *S. exigua*-infested lima bean plants. Predatory mites with a non-rewarding experience on *S. exigua*-infested lima bean indeed had a slightly stronger preference for *T. urticae*-induced volatiles than predators that had been starved on an uninfested lima bean leaf; the effect of a non-rewarding experience was almost significant (P=0.052, GLM). None of the groups of predatory mites discriminated between the volatiles from *T. urticae*-infested and *S. exigua*-infested lima bean plants (P≥0.06, binomial test). The experience that the predators acquired during rearing (cucumber versus lima bean) did not affect the

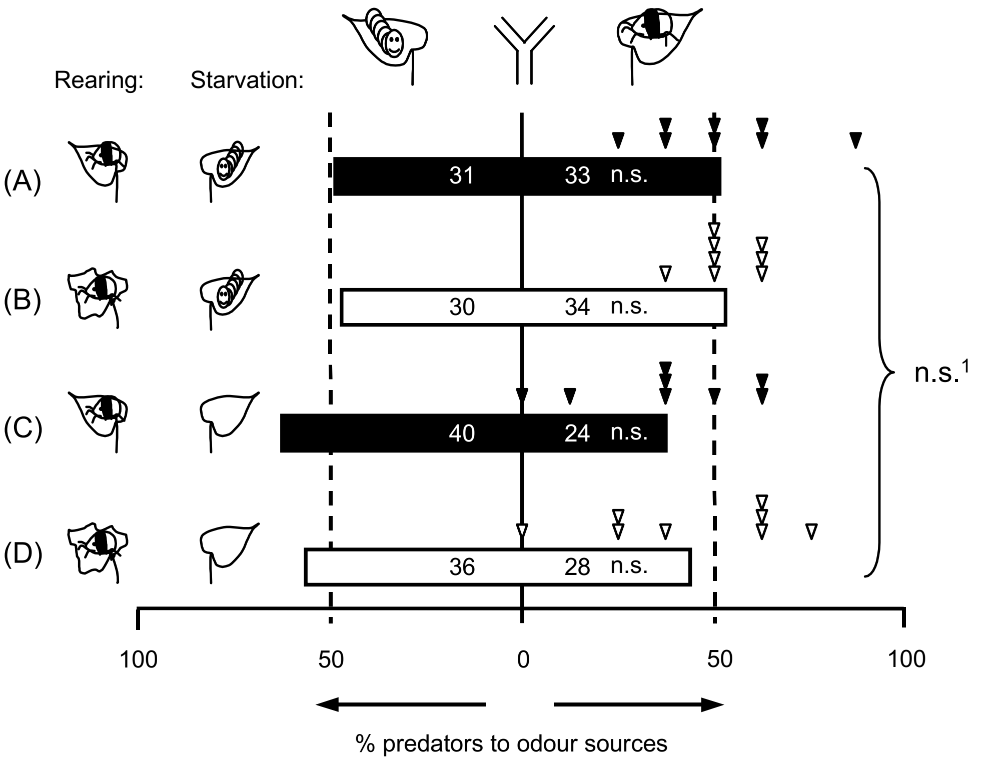


Figure 3: Effect of a non-rewarding experience with *Spodoptera exigua*-induced lima bean volatiles on the choices of the predatory mite *Phytoseiulus persimilis* between the volatiles from lima bean leaves infested with 40 *Tetranychus urticae* or 2 *S. exigua* per leaf. Bars present the overall percentages of LI-predators (black) and CU-predators (white) choosing for each odour source. Numbers in bars are the total numbers of predators choosing for each odour source; triangles indicate the percentage of predators choosing for *T. urticae*-induced volatiles per day of the experiment. Predatory mite treatments: (A) LI-predators with a non-rewarding experience (starvation on *S. exigua*-infested lima bean); (B) CU-predators with a non-rewarding experience; (C) LI-predators control (i.e. starvation on uninfested lima bean); (D) CU-predators control. Before testing, predators were kept without food and odours for 1-3 h. Choices were analysed with a two-sided binomial test; effects of experience acquired during rearing and non-rewarding experience were analysed with a GLM, the significance of which is indicated behind the bracket (predicting factors: replicate + rearing experience + non-rewarding experience). (n.s. $P>0.05$; n.s.[1] $P=0.052$).

foraging responses in this experiment ($P=0.53$, GLM). We conclude that a non-rewarding experience alone has a small effect on the preference of *P. persimilis* for *T. urticae*-induced volatiles.

Multiple experiences during the adult phase

In this experiment, we tested whether predatory mites with experiences during the adult phase with both of the odour sources that were offered in the olfactometer, i.e. the volatiles from lima bean leaves infested with *T. urticae* or *S. exigua*, in addition to the experience that they acquired during rearing have a stronger preference for the volatiles induced by their prey than predators with an experience with only one, or none, of the odour sources that were offered in the choice test. We did two sub-experiments, one with CU-predators, the other with LI-predators (Figures 4 and 5 respectively).

Indeed, the CU-predators only preferred the volatiles from *T. urticae*-infested lima bean over those from *S. exigua*-infested lima bean when they had experienced the volatiles from *S. exigua*-infested lima bean in a non-rewarding context (starvation) *and* the volatiles from *T. urticae*-infested lima bean in a rewarding context (feeding) (Figure 4A, P=0.002, binomial test). Predators that had only experienced one of the odour sources during the adult phase – i.e. the volatiles from *T. urticae*-infested lima bean plants in a rewarding context (Figure 4B), or the volatiles from *S. exigua*-infested lima bean in a non-rewarding context (Figure 4C) – or had not been exposed to experiences with either of the odour sources (Figure 4D) did not discriminate between the volatiles from *T. urticae*-infested or *S. exigua*-infested lima bean (P≥0.15, binomial test). Statistical analysis showed that the experience with *T. urticae*-induced lima bean volatiles in a rewarding context had a significant impact on the responses of CU-predators (Figures 4A and B vs. C and D, P=0.002, GLM): feeding on *T. urticae* on lima bean resulted in a stronger preference for *T. urticae*-induced volatiles from lima bean than feeding on *T. urticae* on cucumber. In contrast, the non-rewarding experience with *S. exigua*-induced volatiles did not significantly increase the preference of predatory mites for the volatiles from *T. urticae*-infested lima bean plants (Figures 4C and D, P=0.30, GLM). The effect of multiple experiences was retained for at least 24 h; predators that had been starved for 24 h in the absence of volatiles after acquiring multiple experiences also strongly preferred the volatiles from *T. urticae*-infested over those from *S. exigua*-infested lima bean plants (Figure 4E, P<0.001, binomial test). In conclusion, although multiple experiences resulted in the strongest preference of CU-predators for *T. urticae*-induced versus *S. exigua*-induced volatiles from lima bean, only the effect of a rewarding experience was significant while the effect of a non-rewarding experience was not.

When LI-predators were exposed to experiences during the adult phase with the odour sources offered in the choice test, predators with a rewarding experience on *T. urticae*-infested lima bean strongly preferred the volatiles from *T. urticae*-infested over those from *S. exigua*-infested lima bean plants (Figures 5A and B, P<0.001, binomial test), whereas the control groups that had fed on *T. urticae*-infested cucumber did not discriminate (Figures 5C and D, P≥0.38). Thus, the rewarding experience with the volatiles from *T. urticae*-infested lima bean significantly affected the responses of the LI-predators (Figures 5A and B vs. C and D, P<0.001, GLM), just as in the case of the CU-predators (Figure 4). A non-rewarding experience with *S. exigua*-induced volatiles from lima bean that preceded feeding on *T. urticae*-infested lima bean or cucumber did not influence the preferences of LI-predators (Figure 5, P=0.60, GLM), as was also found for CU-predators (Figure 4).

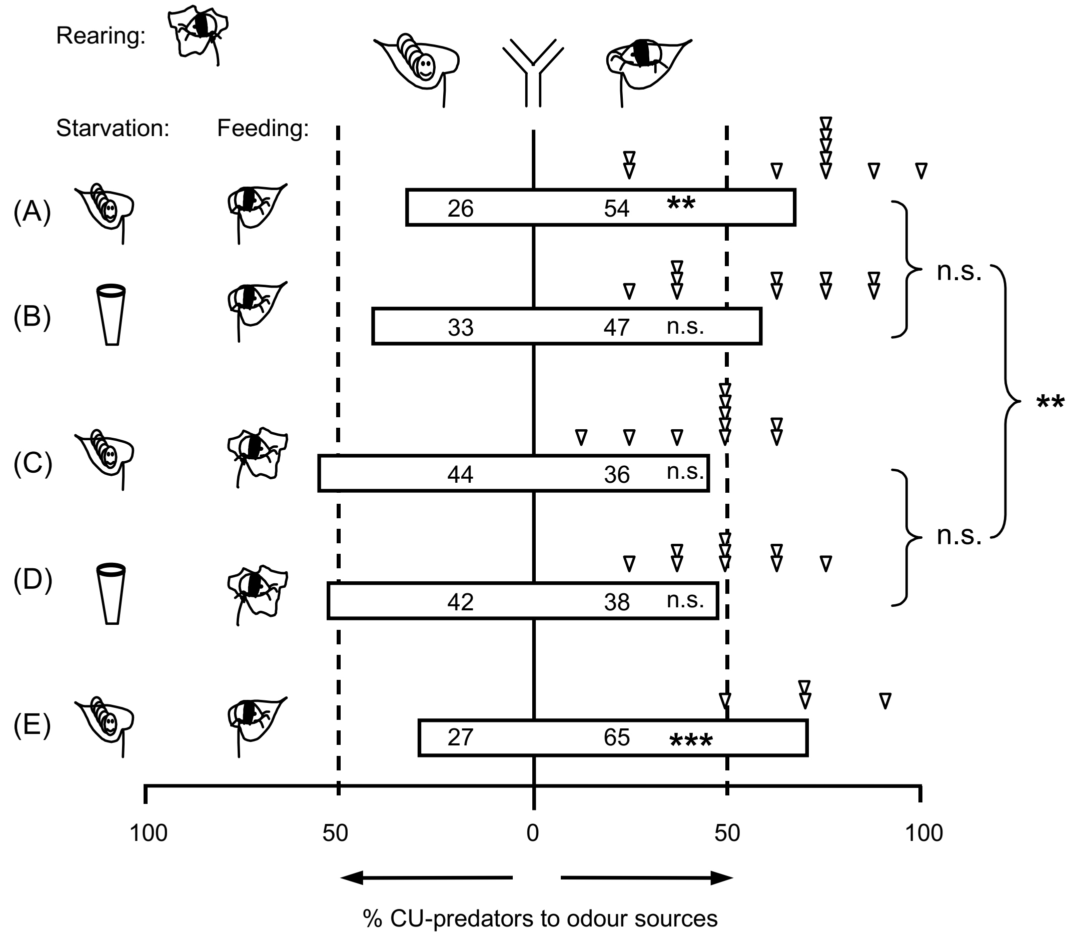


Figure 4: Effect of multiple experiences on the choices of CU-predators between the volatiles from lima bean leaves infested with 40 *Tetranychus urticae* or 2 *Spodoptera exigua* per leaf. Predatory mite treatments: (A) Multiple experiences (starved on *S. exigua*-infested lima bean, then fed on *T. urticae* on lima bean); (B) Rewarding experience (starved in Eppendorf vial, then fed on *T. urticae* on lima bean); (C) Non-rewarding experience (starved on *S. exigua*-infested lima bean, then fed on *T. urticae* on cucumber); (D) control (starved in Eppendorf vial, then fed on *T. urticae* on cucumber); (E) Multiple experiences (starved on *S. exigua*-infested lima bean, then fed on *T. urticae* on lima bean). Before testing, predators from groups A-D were kept without food and odours for 1-3 h, while predators from group E were kept without food and odours for 24 h. Bars show the overall percentages of CU-predators choosing for each odour source, numbers in bars are the total numbers of predators responding to the odour sources and triangles present the percentages predators choosing for *T. urticae*-induced volatiles per day of the experiment. Choices between odour sources were analysed with a two-sided binomial test. Among predator treatments A-D, effects of experiences were analysed with a GLM, the significance of which is indicated behind the brackets (predicting factors: replicate + rewarding experience + non-rewarding experience). (n.s. P>0.05; ** P<0.01).

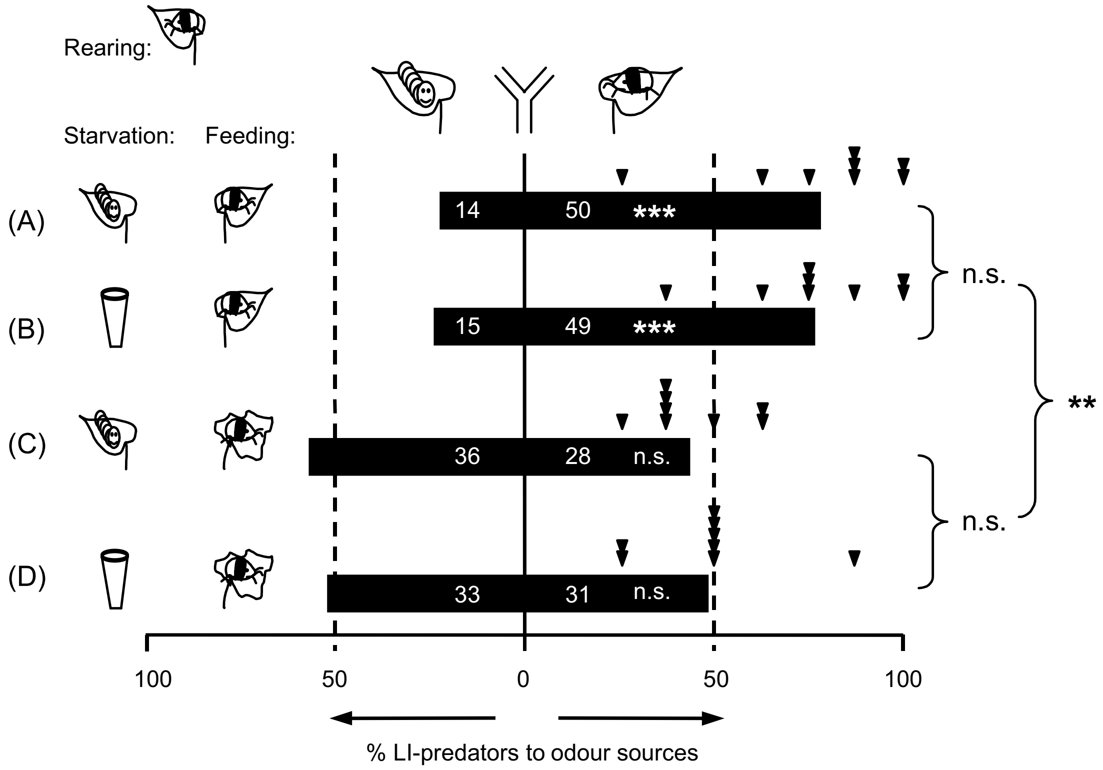


Figure 5: Effect of multiple experiences on the choices of LI-predators between the volatiles from lima bean leaves infested with 40 *Tetranychus urticae* or 2 *Spodoptera exigua* per leaf. Predatory mite treatments: (A) Multiple experiences (starved on *S. exigua*-infested lima bean, then fed on *T. urticae* on lima bean); (B) Rewarding experience (starved in Eppendorf vial, then fed on *T. urticae* on lima bean); (C) Non-rewarding experience (starved on *S. exigua*-infested lima bean, then fed on *T. urticae* on cucumber); (D) control (starved in Eppendorf vial, then fed on *T. urticae* on cucumber). Before testing, predators were kept without food and odours for 1-3 h. Bars show the overall percentages of LI-predators choosing for each odour source, numbers in bars are the total numbers of predators responding to the odour sources and triangles present the percentages predators choosing for *T. urticae*-induced volatiles per day of the experiment. Choices between odour sources were analysed with a two-sided binomial test. Effects of experiences were analysed with a GLM, the significance of which is indicated behind the brackets (predicting factors: replicate + rewarding experience + non-rewarding experience). (n.s. P>0.05; ** P<0.01; *** P<0.001).

Influence of starvation level on the impact of a rewarding experience

In the last experiment (Figure 6) we tested the effect of a starvation period of 24 h in the absence of volatiles prior to feeding on *T. urticae*-infested lima bean or cucumber on the choices of LI-predators between the volatiles from *T. urticae*-infested versus *S. exigua*-infested lima bean plants. Predators with a rewarding experience on *T. urticae*-infested lima bean after starvation for 24 h preferred *T. urticae*-induced over *S. exigua*-induced volatiles (Figure 6A, P=0.046, binomial test), while the control group that fed on cucumber after starvation for 24 h preferred *S. exigua*-induced volatiles (Figure 6C, P=0.03). Although feeding on

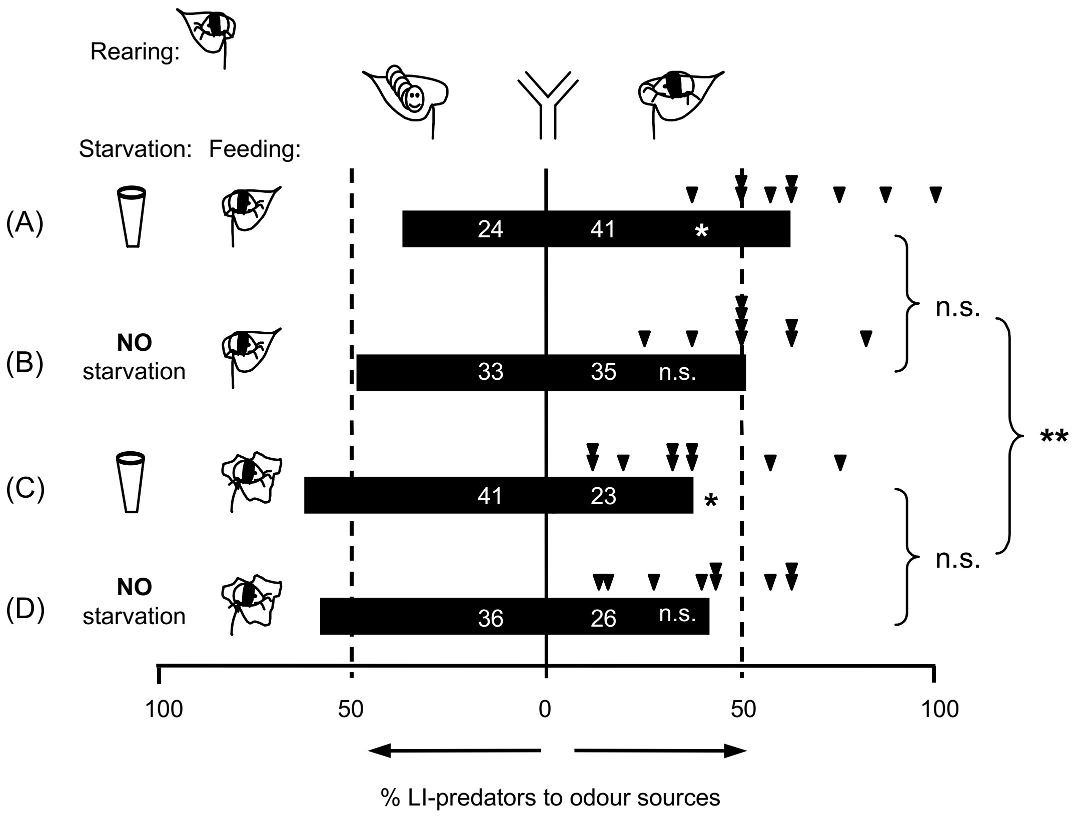


Figure 6: Effect of starvation prior to a rewarding experience on the choices of LI-predators between the volatiles from lima bean leaves infested with 40 *Tetranychus urticae* or 2 *Spodoptera exigua* per leaf. Predatory mite treatments: (A) 24 h starvation in Eppendorf vial, then 24 h feeding on *T. urticae* on lima bean; (B) 0 h starvation, then 24 h feeding on *T. urticae* on lima bean; (C) 24 h starvation, then 24 h feeding on *T. urticae* on cucumber; (D) 0 h starvation, then 24 h feeding on *T. urticae* on cucumber. Before testing, predators were kept without food and odours for 1-3 h. Bars show the overall percentages of LI-predators choosing for each odour source; numbers in bars are the total numbers of predators responding to the odour source and triangles present the percentages predators choosing for *T. urticae*-induced volatiles per day of the experiment. Choices between odour sources were analysed with a two-sided binomial test. Effects of starvation level and rewarding experience were analysed with a GLM, the significance of which is indicated behind the brackets (predicting factors: replicate + starvation + rewarding experience). (n.s. $P>0.05$; * $P<0.05$; ** $P<0.01$).

lima bean or cucumber directly after rearing did not result in discrimination between the volatiles from *T. urticae*-infested or *S. exigua*-infested lima bean plants (Figures 6B and D, $P \geq 0.25$, binomial test), the preceding starvation period did not significantly affect the responses of predatory mites (GLM, $P=0.66$). The plant species used for the feeding period again had a significant effect on the responses of predatory mites ($P=0.003$, GLM), similar to the effect shown in Figures 4 and 5.

DISCUSSION

Experiences acquired during rearing

Predatory mites generally complete their development in prey patches on a single plant. Females mate before dispersal because it is difficult to find mates outside the prey patch. In exterminated prey patches, juvenile predatory mites may even cannibalise on conspecifics and develop into adults and mate before dispersing (Pels & Sabelis 1999). In this way predatory mites acquire experience with the volatiles from the plant on which they develop and this experience may influence the foraging responses of adult predatory mites. Indeed, Drukker *et al.* (2000a) demonstrated that predatory mites that had been reared in the absence of herbivore-induced plant volatiles were only weakly attracted to these prey-location cues. In addition, several studies have shown an effect of experience with the plant species used for rearing on the responses of predatory arthropods to volatiles induced by their prey or volatiles from uninfested plants (Takabayashi & Dicke 1992; Krips *et al.* 1999; Drukker *et al.* 2000a, b), but some other studies have shown that responses can be independent of rearing history (Takabayashi *et al.* 1994a; Shimoda & Dicke 2000; Van den Boom *et al.* 2002). Our study clearly demonstrates that the experience acquired during rearing affects the choices of *P. persimilis* between the volatiles from *T. urticae*-infested and *S. exigua*-infested lima bean plants; LI-predators had a stronger preference for *T. urticae*-induced volatiles than CU-predators at all the densities of herbivores that we tested ($P<0.05$, sign test, Figure 1).

Since the composition of the volatile blends from spider mite-infested lima bean and cucumber are known, an explanation for the observed differences in responses between LI-predators and CU-predators may be found (Table 1). Although LI-predators but not CU-predators have experienced methyl salicylate during rearing, and predators can use this compound to discriminate between *T. urticae*-infested and *S. exigua*-infested lima bean plants (chapter 5), experience with methyl salicylate cannot completely explain the different responses of LI-predators and CU-predators (Figure 2). The intermediate response of CU-predators that had been exposed to methyl salicylate during rearing shows that other differences between the volatile blends from spider mite-infested cucumber or lima bean that the predators experienced also play a role. Genetic selection during the period of experience cannot explain the different responses of LI-predators, CU-predators, and CU-predators reared in the presence of synthetic MeSA, because all predators originated from the same population, the same numbers of offspring were produced in the different treatments, and we did not observe differential mortality between the different treatments. Compounds that may explain the different responses of LI-predators and CU-predators could be the terpenoid (*E*)-β-ocimene, the green leaf volatile (*Z*)-3-hexen-1-ol acetate, and 3-methylbutanal-O-methyl oxime (Table 1). These compounds are present in large amounts in the volatile blends of *T. urticae*-infested cucumber and *S. exigua*-infested lima bean, while the amount in *T. urticae*-infested lima bean is small. CU-predators are thus most likely more experienced with these compounds than LI-predators. This experience may affect the role of these compounds in discrimination between lima bean plants infested with *T. urticae* or *S. exigua* by the two groups of predators.

Table 1: Volatile compounds emitted by lima bean infested with *T. urticae*, or *S. exigua*, and cucumber infested with *T. urticae*, attractiveness of single compounds to *P. persimilis* reared on lima bean or cucumber, and role of volatile compounds in choice of lima bean-reared predators between *T. urticae*- and *S. exigua*-infested lima bean volatiles.

	Volatile emission[1]			Attraction[2]		Discrimination[3]
Compound[4]	*LT*	*LS*	*CT*	*Pp-LI*	*Pp-CU*	*Pp-LI*
methyl salicylate	++	–	–	+	–	+
α-(*E*,*E*)-farnesene	–	–	++	nt	nt	nt
(*E*)-β-ocimene	–	+++	+++	+	nt	nt
(*E*)-DMNT	++	++	+++	+	nt	nt
(*E*,*E*)-TMTT	++	–	+	+/–	nt	+
2-butanone	++	++	–	+	nt	–
(*Z*)-3-hexen-1-ol acetate	–	+++	++	–	nt	nt
(*Z*)-3-hexen-1-ol	+	+++	+	–	nt	nt
oxime	–	+	+++	nt	nt	nt

[1] Volatile production as published in chapters 3 and 5; *LT*=*T. urticae*-infested lima bean, *LS*=*S. exigua*-infested lima bean, *CT*=*T. urticae*-infested cucumber; Compounds are not produced (–), or produced in minor (+), moderate (++), or major (+++) amount.

[2] Attraction of predatory mites to the single compound as published in chapters 4, 5 and 6, and by Dicke *et al.* (1990b); *Pp-LI*=lima bean reared predatory mites, *Pp-CU*=cucumber-reared predatory mites; Predatory mites are not attracted (–), attracted (+), attracted depending on starvation level (+/–), or the compound has not been tested (nt).

[3] Effect of the compound in discrimination of lima bean-reared predatory mites between *T. urticae*- and *S. exigua*-infested lima bean as published in chapter 5; the compound plays a role (+), does not play a role (–), or has not been tested (n.t.).

[4] (*E*)-DMNT=(3*E*)-4,8-dimethyl-1,3,7-nonatriene; (*E*,*E*)-TMTT=(3*E*,7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene; oxime=3-methylbutanal-O-methyl oxime

Adult experiences

The influence of experiences of adults with herbivore-induced volatiles on foraging responses has been extensively studied for parasitoid wasps (e.g. Du *et al.* 1997; Geervliet *et al.* 1998b; Fukushima *et al.* 2002). Most studies have concentrated on learning by positive reinforcement, i.e. rewarding experiences, for example by exposing the parasitoid wasps to herbivore-induced plant volatiles during an oviposition in a host. However, non-rewarding experiences, such as unsuccessful foraging, could also play a role in shaping behaviour (Papaj *et al.* 1994), and when combined with a rewarding experience, the effect on behaviour may become more pronounced (Vet *et al.* 1998). The fruit fly parasitoid *Leptopilina heterotoma* learned to discriminate between two substrates that differed in the amount of only one compound by experiencing one of the substrates with suitable hosts (reward) and the other one without hosts (no reward); in contrast, the wasps did not discriminate after a rewarding experience alone (Vet *et al.* 1998). Our experiments show that, having experienced the presence and the absence of prey in combination with *T. urticae*-induced and *S. exigua*-induced volatiles from lima bean respectively, multiple experienced predators had the strongest preference for the lima bean volatiles induced by their prey (Figures 4 and 5). However, their response was not significantly different from that of predators with a rewarding experience with the *T. urticae*-induced volatiles from lima bean only.

The plant species on which the prey was offered to the predators during the adult phase had a strong effect on the responses of *P. persimilis*; in CU-predators feeding on *T. urticae*-infested lima bean resulted in a preference for *T. urticae*-induced lima bean volatiles, while LI-predators lost their preference for *T. urticae*-induced lima bean volatiles after feeding on *T. urticae*-infested cucumber (Figures 4 and 5). Effects of a rewarding experience during the adult phase on the foraging behaviour of predatory arthropods have also been found in other studies but in most cases more than one day of feeding experience was needed before responses were changed (e.g. Dicke *et al.* 1990b; Dwumfour 1992; Krips *et al.* 1999; Drukker *et al.* 2000a). Most predatory mites start searching for a novel prey patch only when their current prey patch is wiped out completely, which implies that the predators are starved when they start to acquire experiences in a novel prey patch (Drukker *et al.* 2000a). This led to the hypothesis that a rewarding experience has a larger impact after a starvation period, but our data provide only weak support for this hypothesis (Figure 6). Although a rewarding experience on *T. urticae*-infested lima bean only resulted in a preference for lima bean volatiles induced by *T. urticae* after 24 h of starvation, and feeding on *T. urticae*-infested cucumber only resulted in preference for volatiles induced by *S. exigua* after 24 h of starvation, the influence of the starvation period was not statistically significant. This suggests that the effect of a previous starvation is weak compared to the effect of the rewarding experience that follows, or that starvation is only effective in the presence of volatiles (Drukker *et al.* 2000a).

A non-rewarding experience with *S. exigua*-induced volatiles had an almost significant effect on the choices of *P. persimilis* between the volatiles from plants infested with *T. urticae* or *S. exigua* (Figure 3), but it did not result in a preference of the predators for *T. urticae*-induced volatiles. In contrast, Drukker *et al.* (2000a) had previously shown that starvation of *P. persimilis* in the presence of *T. urticae*-induced volatiles resulted in repellence of the predators from this odour source and thus concluded that predatory mites could learn from a non-rewarding experience. A possible explanation for the different effects of non-rewarding experiences may be the rearing methods used in the two studies: i.e. in the absence of volatiles (Drukker *et al.* 2000a), or in the presence of volatiles (this study). Previous experiences with volatiles can inhibit or block the acquisition of experiences with other volatiles, an effect that has been demonstrated for olfactory learning in honeybees (Smith & Cobey 1994). Whether similar effects influence the acquisition of experiences by predatory mites remains to be investigated. The fact that a rewarding experience had a larger effect on foraging by predatory mites than a non-rewarding experience seems ecologically relevant. Whereas a rewarding experience ensures the presence of prey, a non-rewarding experience may not be a good indicator of the absence of prey, especially not on a larger spatial scale (Papaj *et al.* 1994).

Conclusions

The role of herbivore-induced plant volatiles in prey-location by predatory mites has been demonstrated not only in a Y-tube olfactometer in the laboratory, but also in more realistic semi-field set-ups (e.g. Janssen 1999; Agrawal *et al.* 2002; chapters 2 and 3). Under natural conditions, predatory mites have to locate their prey on many different host plant species and in a complex environment. Plant communities consist of uninfested plants, and plants infested with herbivores that may differ in their suitability as prey. In addition, variation in herbivore-induced plant volatiles is caused by other biotic factors, such as pathogens, and abiotic factors (e.g. Takabayashi *et al.* 1994b; Cardoza *et al.* 2002; Gouinguené & Turlings 2002). Our experiments demonstrate that learning enables *P. persimilis* to discriminate between the volatiles from plants infested with their prey or caterpillars that are not suitable prey, even when their prey occurs on a plant species different from the one experienced during development. During the adult phase, rewarding experiences have a large effect on the foraging responses of predatory mites while the non-rewarding experiences seem to have a small influence. The ecological significance of the learning abilities of predatory arthropods found in the laboratory needs to be evaluated under natural field conditions (Papaj & Lewis 1993).

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CHAPTER 8

Rearing history affects the behavioural responses of a predatory mite to the volatiles from plants infested with a non-prey herbivore

Abstract

Upon attack by herbivores plants emit herbivore-induced volatiles that can be used by carnivores to locate their prey. The composition of herbivore-induced volatile blends displays temporal and spatial variation, and it is expected that such environmental variation favours phenotypic plasticity in the foraging responses of carnivores. In this paper we show that the responses of the predatory mite *Phytoseiulus persimilis* – a specialised natural enemy of spider mites – to volatiles from plants infested with a non-prey herbivore are influenced by the plant species on which the predators had been reared. Predators that had been reared on cucumber were attracted significantly more to the volatiles from lima bean or cucumber plants infested with the non-prey caterpillar *Spodoptera exigua* than predators that had been reared on lima bean. In contrast, rearing history did not influence the responses of *P. persimilis* to the volatiles from plants infested with spider mites, or plants infested simultaneously with spider mites and caterpillars. We also investigated the effect of mixing *S. exigua*-induced volatiles from lima bean or cucumber plants with volatiles from spider mite-infested lima bean, on discrimination by the predators between plants infested with prey and non-prey herbivores. This odour mixing did not change the responses of lima bean-reared predators to volatiles induced by their prey but it did change the responses of cucumber-reared predators. We discuss the influence of rearing history on the responses of predatory mites in relation to foraging for prey in an environment with different plant species, and herbivore species that differ in their suitability as prey.

INTRODUCTION

Upon herbivore damage plants start to emit a bouquet of herbivore-induced plant volatiles that are known to attract a range of carnivorous arthropods, such as parasitoid wasps, and predatory bugs and mites (e.g. Dicke *et al.* 1990a; Turlings *et al.* 1990; Scutareanu *et al.* 1997). In general, this plant-derived information is more detectable than information from the prey themselves because plants have a much larger biomass than arthropod herbivores (Vet & Dicke 1992). Moreover, plants can benefit in terms of fitness from the attraction of natural enemies of herbivores by the emission of herbivore-induced plant volatiles (Dicke & Sabelis 1989; Van Loon *et al.* 2000a; Fritzsche Hoballah & Turlings 2001). However, herbivore-induced volatiles are a highly variable source of information. The composition of the odour bouquet varies with plant and herbivore species, and with other biotic and abiotic conditions in the environment (e.g. Takabayashi *et al.* 1994b; DeMoraes *et al.* 1998; Gouinguené & Turlings 2002; Schmelz *et al.* 2003a). As such, information about the presence of the same prey on a plant can vary within and between generations of carnivores, for example when the prey herbivores use different host plant species throughout the year that emit volatile blends of different composition. Temporal and spatial variation in information is thought to favour phenotypic plasticity or learning in the foraging responses of carnivores (Via 1987; Dukas 1998). Indeed, many studies have demonstrated that parasitoid wasps can learn to associate a specific blend of herbivore-induced plant volatiles with the presence of hosts (reviewed by Turlings *et al.* 1993a; Vet *et al.* 1995). Similarly, it has been shown that the responses of predatory arthropods to herbivore-induced plant volatiles are influenced by previous experiences with volatiles (e.g. Dicke *et al.* 1990b; Krips *et al.* 1999; Drukker *et al.* 2000a,b; chapters 6 and 7).

To date, most studies on phenotypic plasticity in the foraging behaviour of predators have focused on the responses of predators to herbivore-induced volatiles from plants of a single species infested with a single herbivore species (but see Perfecto & Vet 2003). However, in the field, carnivores are confronted with volatiles from different plant species, and the blends of herbivore-induced volatiles from two or more conspecific or heterospecific plants may mix. Moreover, an individual plant may be infested simultaneously with more than one herbivore species. To understand the role of phenotypic plasticity in the foraging behaviour of natural enemies of herbivorous arthropods, it is important to study how previous experiences with volatiles influence the foraging responses of predators in the more complex and realistic conditions that they can encounter in the field.
We previously showed that the choices of the predatory mite *Phytoseiulus persimilis* – a specialised natural enemy of spider mites – between plants infested with prey

or non-prey herbivores are influenced by their previous experiences with herbivore-induced volatiles (chapter 7). In the present chapter, we investigate the effect of the plant species on which the predatory mites have been reared, on their responses to:

(i) The volatile blends emitted by plants infested with: (A) the prey herbivore *Tetranychus urticae*, (B) the non-prey caterpillar *Spodoptera exigua*, and (C) the combination of *T. urticae* and *S. exigua* on the same individual plant.

(ii) Mixtures of the volatile blends from *T. urticae*-infested plants and *S. exigua*-infested conspecific or heterospecific plants.

MATERIAL AND METHODS

Plants and herbivores

Lima bean plants (*Phaseolus lunatus* L. cv Sieva) and cucumber plants (*Cucumis sativus* L. cv Lange Groene Giganten) were grown in a greenhouse at 20-30 °C, 50-70 % r.h. and 16 h of light. Lima bean plants were used when their primary leaves had just expanded (and were about 8-12 cm wide), 10 to 15 days after sowing. Cucumber plants were used when their first two leaves were about 8 to 12 cm wide, 15 to 35 days after sowing. Two separate colonies of two-spotted spider mites, *Tetranychus urticae* Koch (Acari: Tetranychidae), were maintained on lima bean plants and cucumber plants in a greenhouse-compartment under the same conditions as uninfested plants. Eggs of the beet armyworm *Spodoptera exigua* Hübner (Lepidoptera: Noctuidae) were obtained from a stock colony reared on artificial diet at the laboratory of Virology, Wageningen University, The Netherlands (Smits *et al.* 1986). Two to three egg batches were placed in a Petri dish together with a lima bean or cucumber leaf, and kept in a climate cabinet at 23±1 °C. Larvae were used to infest plants within 24 h of hatching.

Predators

A stock colony of predatory mites, *Phytoseiulus persimilis* Athias-Henriot (Acari: Phytoseiidae), was maintained on spider mite-infested lima bean leaves in a climate cabinet (23±1 °C, 50-70 % r.h. and continuous light). To obtain predators of the same age with a rearing history on lima bean or cucumber, we transferred about five gravid females from the stock colony to each of a series of Petri dishes with a piece of spider mite-infested lima bean or cucumber leaf. The females were removed after one or two days, and fresh spider mite-infested leaves were supplied every two days to feed their offspring. Adult females were used in experiments one to two days after the final moult. Prior to testing their behavioural responses, females were kept individually in Eppendorf vials without food but with a small droplet of water, for 22-26 h.

Experiments

We studied the behaviour of predatory mites in a closed system Y-tube olfactometer (Takabayashi & Dicke 1992; chapter 2). The behaviour of an individual female was observed for a maximum of 5 min; if a choice for one of the two odour sources had not been made within 5 min, we recorded a "no choice". Every experiment was repeated on 4 different days, using new odour sources and new sets of predatory mites on each day. To investigate the effect of rearing history (i.e. the plant species on which prey was offered during development from egg to adult) on the behaviour of predators, we alternately tested the responses of females that had been reared on lima bean or cucumber within each replicate of an experiment, using the same odour sources. The number of leaves and herbivores that we used per odour source are specified in the figure legends.

(i) Responses to the volatiles from plants infested with *T. urticae* or *S. exigua*, or infested simultaneously with *T. urticae* and *S. exigua*

Odour sources were prepared by infesting plants with spider mites, with caterpillars, with the combination of both herbivore species, or leaving them uninfested. To this end, the herbivores were transferred to the two oldest leaves of a plant. Caterpillars were placed in a clip cage that was attached to a leaf to prevent them from dropping off the leaves; 2 to 5 caterpillars were placed in one cage. Clip cages were supported with sticks to prevent the leaves from bending down and incurring damage by the weight of the cages. In the treatment of simultaneous infestation with spider mites and caterpillars, both herbivore species were placed on each of the two oldest leaves of a plant. Spider mite-infested leaves or uninfested leaves were treated in the same way with empty clip cages. Clip cages were moved daily to a new position on the same leaf. Plants were incubated in a climate room at 24±1 °C for three days, using separate plastic cages for each treatment. Just before an experiment, leaves were cut from the plant and wrapped with their petiole in wet cotton wool and aluminium foil to prevent wilting.

(ii) Mixtures of volatiles from *T. urticae*-infested and *S. exigua*-infested plants

Plants were infested with spider mites or caterpillars three days before an experiment as described above. To test the effect of mixing spider mite-induced volatiles with caterpillar-induced volatiles from conspecific or heterospecific plants we compared the responses of predatory mites first when offered volatiles from four *T. urticae*-infested lima bean leaves versus volatiles from four *S. exigua*-infested cucumber (or lima bean) leaves. Subsequently, we placed an odour container with eight *S. exigua*-infested cucumber or lima bean leaves upwind of these odour sources. The air stream from this container was split into two and was led through each of the containers with the original odour sources. This resulted in the second choice situation: volatiles from four *T. urticae*-infested lima bean leaves plus four *S. exigua*-infested cucumber (or lima bean) leaves versus volatiles from eight *S. exigua*-infested cucumber (or lima bean) leaves. In both choice-situations a set of 16 to 18 predatory mites was tested on each day of the experiment.

Statistical analysis

Predatory mites that had not made a choice in the Y-tube olfactometer (in total 3 % of 2015 tested predators) were excluded from the statistical analysis. Choices between odour sources in the olfactometer were analysed with a two-sided binomial test, to investigate whether the distribution differed from 50:50. We used the pooled data from the replicates carried out on different days.

To comparatively analyse the effect of rearing history on the behaviour of predatory mites within experiment (i), we used generalised linear models, with the null hypothesis that rearing history had no effect (GLM, GenStat 6.1, McCullagh & Nelder 1989). The number of predatory mites choosing for one of the odour sources per day of the experiment was taken as the response variate. The total number of predatory mites that made a choice per day was taken as the binomial total. To check the validity of the model, we subjected the deviance of the residual to a χ^2-test. We used replicate and rearing history as predicting factors for each sub-experiment. Similarly, in experiment (ii), the effect of mixing the volatiles from *T. urticae*-infested and *S. exigua*-infested plants on the responses of predators was analysed with a GLM, using replicate and odour source as predicting factors for each sub-experiment and the null hypothesis that the responses of predators were not different between the two situations (no mixing versus mixing).

RESULTS

Responses to the volatiles from plants infested with prey, non-prey, or the combination of prey and non-prey

Rearing history did not affect the responses of predatory mites to the volatiles from plants infested with suitable prey herbivores (Figure 1A, $P \geq 0.31$, GLM). Both predatory mites that had been reared on cucumber (CU-predators) or on lima bean (LI-predators) were significantly attracted to the volatiles from lima bean or cucumber plants infested with *T. urticae* ($P \leq 0.03$, binomial test).

In contrast, rearing history did have a significant influence on the responses of predatory mites to the volatiles from plants infested with the non-prey herbivore *S. exigua* (Figure 1B, $P \leq 0.008$, GLM). CU-predators were strongly attracted to the volatiles from lima bean or cucumber plants infested with *S. exigua* ($P < 0.001$, binomial test), while LI-predators were not significantly attracted to the volatiles from *S. exigua*-infested lima bean or cucumber plants ($P \geq 0.06$).

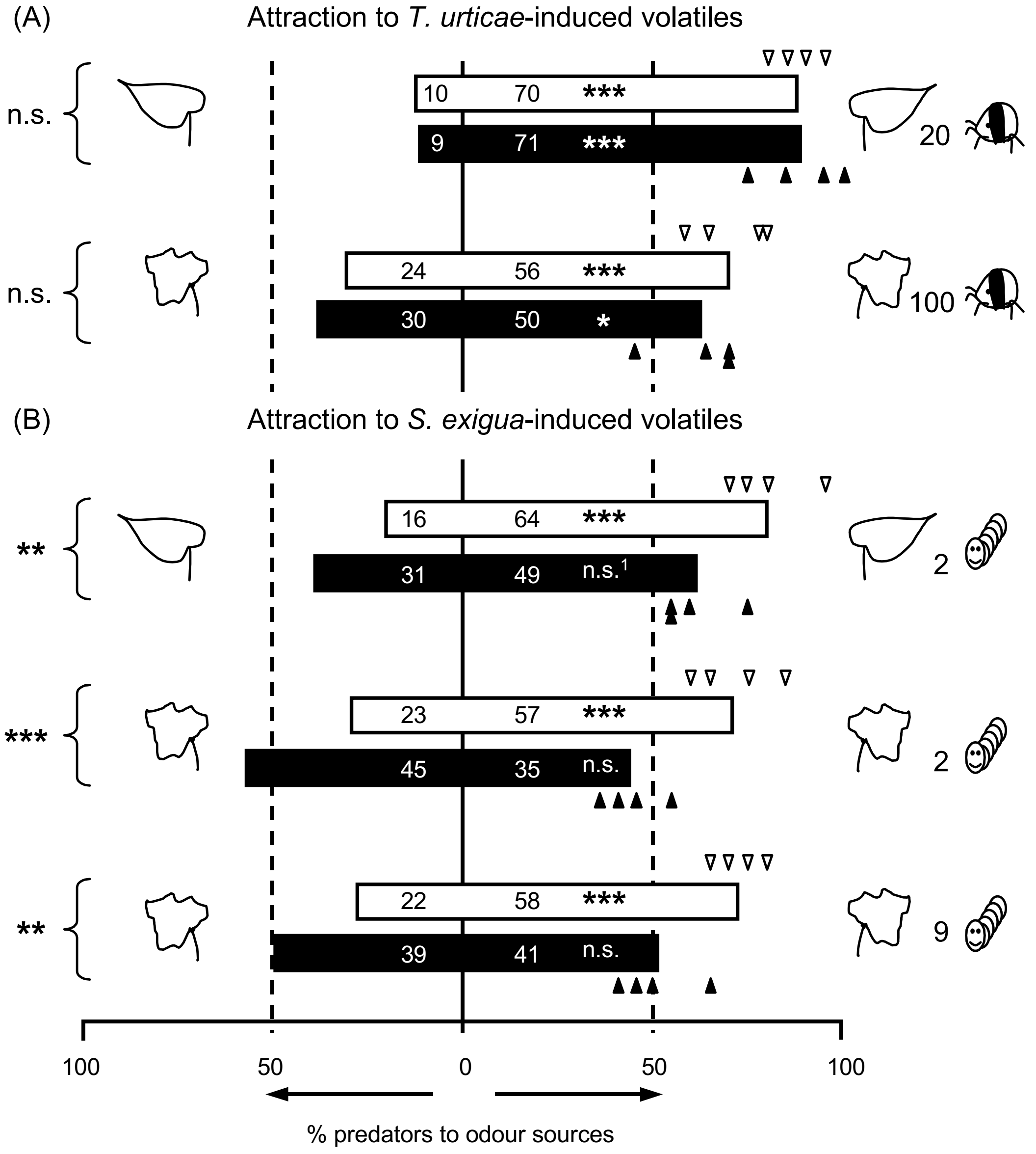

Figure 1: Attraction of the predatory mite *Phytoseiulus persimilis* to volatiles induced by (A) its prey *Tetranychus urticae*, or (B) the non-prey herbivore *Spodoptera exigua* on lima bean or cucumber plants. Four leaves were used per odour source, the number of herbivores per leaf is indicated in front of the herbivore-symbols. Bars present the percentages of the total numbers of cucumber-reared (white) and lima bean-reared (black) predatory mites choosing for each odour source. Numbers in bars are the total numbers of predators responding to the odour sources; triangles indicate the percentage predators choosing for *T. urticae*-induced (A) or *S. exigua*-induced (B) volatiles per day of the experiment. Choices between odour sources were analysed with a two-sided binomial test. The effect of rearing history (cucumber or lima bean) was analysed with a GLM, the significance of which is indicated in front of the bracket (predicting factors: replicate + rearing history). (n.s. $P>0.05$; n.s.[1] $P=0.056$; * $P<0.05$; ** $P<0.01$; *** $P<0.001$). Data of the lima bean-reared predators were published previously in chapter 3.

Rearing history did not influence the choices of predatory mites between the volatiles from plants infested with *T. urticae* or *S. exigua* alone versus the volatiles

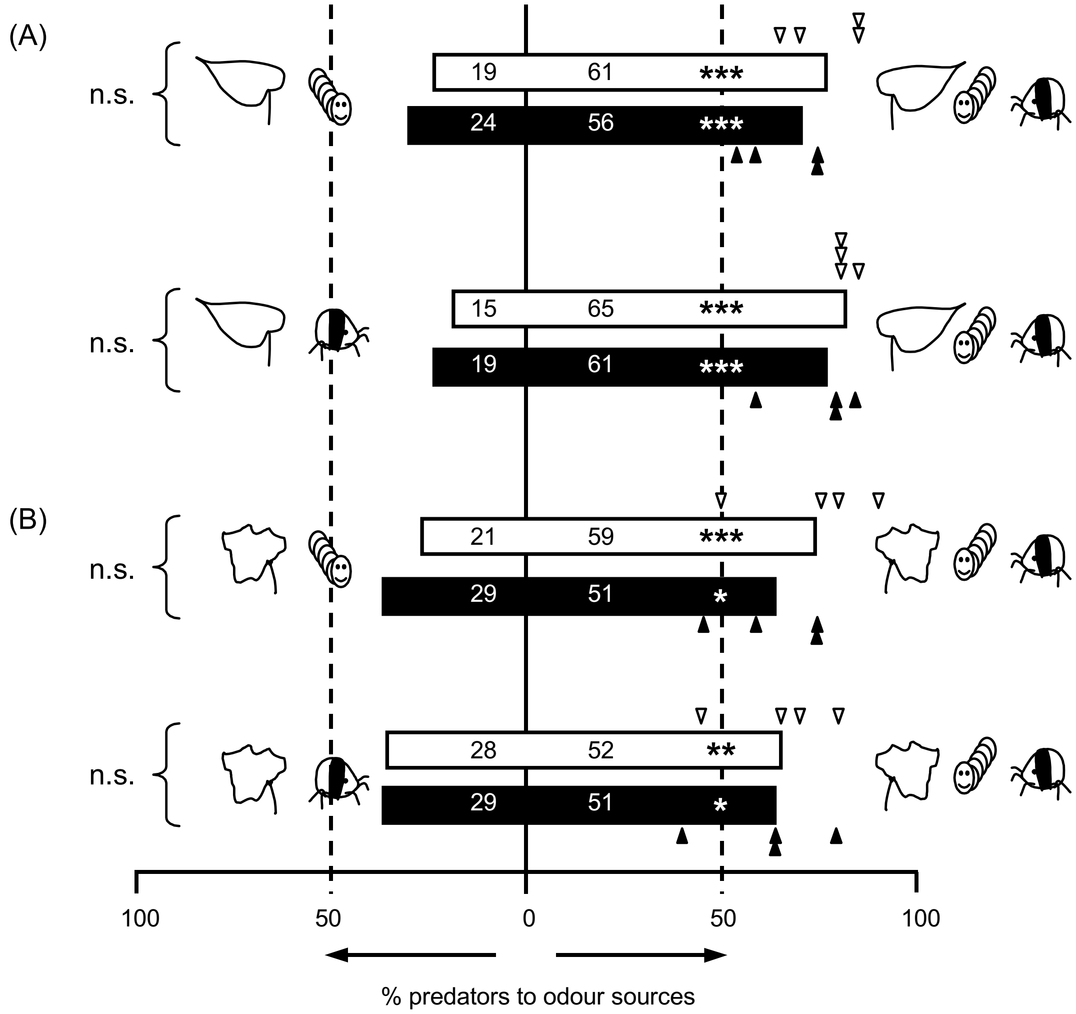

Figure 2: Choice of the predatory mite *Phytoseiulus persimilis* between volatiles from lima bean (A) or cucumber (B) plants simultaneously infested with the prey *Tetranychus urticae* and the non-prey *Spodoptera exigua* versus plants infested with one of the herbivore species alone. In experiments with lima bean nine leaves were used per odour source, and 20 spider mites and/or 2 caterpillars were used per leaf. In experiments with cucumber four leaves were used per odour source, and 100 spider mites and/or 2 caterpillars were used per leaf. Bars present the percentages of the total numbers of cucumber-reared (white) and lima bean-reared (black) predators choosing for each odour source. Numbers in bars are the total numbers of predators responding to the odour sources; triangles indicate the percentage predators choosing for the volatiles from multiple-herbivore infested plants per day of the experiment. Choices between odour sources were analysed with a two-sided binomial test. The effect of rearing history (cucumber or lima bean) was analysed with a GLM, the significance of which is indicated in front of the bracket (predicting factors: replicate + rearing history). (n.s. $P>0.05$; * $P<0.05$; ** $P<0.01$; *** $P<0.001$). Data of the lima bean-reared predators were published previously in chapter 3.

from plants simultaneously infested with both herbivore species (Figure 2, $P \geq 0.16$, GLM). Both CU-predators and LI-predators significantly preferred the volatiles from lima bean or cucumber plants simultaneously infested with the combination of *T. urticae* and *S. exigua* to the volatiles from plants infested with one of the herbivore species alone ($P \leq 0.02$, binomial test).

Rearing history did not affect the choices of *P. persimilis* between the volatiles from cucumber plants infested with the prey *T. urticae* or the non-prey *S. exigua* either (Figure 3, $P=0.87$, GLM). Neither CU-predators nor LI-predators discriminated between the volatiles from *T. urticae*-infested or *S. exigua*-infested cucumber plants ($P \geq 0.43$, binomial test).

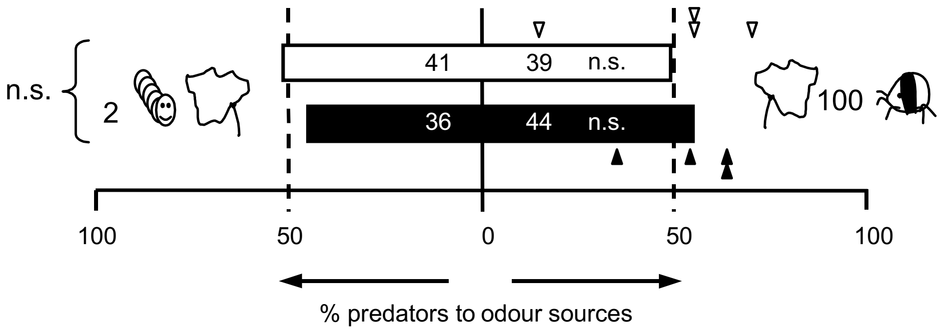

Figure 3: Choices of the predatory mite *Phytoseiulus persimilis* between cucumber plants infested with the prey *Tetranychus urticae* or the non-prey *Spodoptera exigua*. Four leaves were used per odour source, infested with 100 spider mites or 2 caterpillars per leaf. Bars present the percentages of the total numbers of cucumber-reared (white) and lima bean-reared (black) predatory mites choosing for each odour source. Numbers in bars are the total numbers of predators responding to the odour sources; triangles indicate the percentage predators choosing for the volatiles from prey-infested plants per day of the experiment. Choices between odour sources were analysed with a two-sided binomial test. The effect of rearing history (cucumber or lima bean) was analysed with a GLM, the significance of which is indicated in front of the bracket (predicting factors: replicate + rearing history). (n.s. $P>0.05$).

Mixing the volatiles from T. urticae*-infested and* S. exigua*-infested plants*

This experiment was done with CU-predators only; for data on LI-predators see chapter 3. To test if mixing volatiles from *T. urticae*-infested lima bean plants with the volatiles from conspecific or heterospecific plants infested with *S. exigua* had an effect on the foraging behaviour of CU-predators, we carried out four sub-experiments with different combinations of plant species and different numbers of herbivores. Whether or not the responses of CU-predators were influenced by odour mixing depended on the density of herbivores used in the different sub-experiments (Figure 4).

CU-predators preferred the volatiles from lima bean plants infested with *S. exigua* to those of lima bean infested with *T. urticae* at a density of 20 spider mites per leaf (Figure 4A, $P<0.001$, binomial test). When both odour sources were mixed with the volatiles from *S. exigua*-infested lima bean plants, this response shifted significantly ($P=0.003$, GLM) towards no discrimination between the mixture of

S. exigua-induced *and T. urticae*-induced lima bean volatiles versus *S. exigua*-induced lima bean volatiles (P=0.71, binomial test).

In contrast, at a density of 40 spider mites per leaf, we did not find an effect of mixing *T. urticae*-induced volatiles from lima bean plants with *S. exigua*-induced volatiles from lima bean on the responses of CU-predators (Figure 4B, P=1, GLM). CU-predators did not discriminate between the volatiles from *T. urticae*-infested lima bean plants versus those from *S. exigua*-infested lima bean at a density of 40 spider mites per leaf (P=0.38, binomial test), and neither did they discriminate between the mixture of *T. urticae*-induced *and S. exigua*-induced lima bean volatiles versus *S. exigua*-induced lima bean volatiles (P=0.38).

CU-predators preferred the volatiles from *T. urticae*-infested lima bean plants to the volatiles from *S. exigua*-infested cucumber plants at a density of 2 or 9 caterpillars per leaf (Figures 4C and D, P≤0.003, binomial test). Mixing these odour sources with the volatiles from *S. exigua*-infested cucumber plants significantly changed the responses of predatory mites at a density of 2 caterpillars per leaf (Figure 4C, P=0.049, GLM), but not at a density of 9 caterpillars per leaf (Figure 4D, P=0.56, GLM). CU-predators significantly preferred the mixture of *T. urticae*-induced lima bean volatiles *and S. exigua*-induced cucumber volatiles versus *S. exigua*-induced cucumber volatiles at both densities of caterpillars (P≤0.03, binomial test).

DISCUSSION

Responses of predators to volatiles from plants infested with prey or non-prey herbivores

We demonstrated that rearing history has an effect on the attraction of *P. persimilis* to the volatiles from *S. exigua*-infested lima bean or cucumber plants, but not on the attraction of the predators to the volatiles from *T. urticae*-infested plants. Although both predatory mites that had been reared on spider mites on cucumber (CU-predators) or on lima bean (LI-predators) were attracted to *T. urticae*-induced volatiles, only CU-predators were significantly attracted to *S. exigua*-induced volatiles (Figure 1). This may explain why CU-predators did not prefer the volatiles from *T. urticae*-infested lima bean plants over the volatiles from *S. exigua*-infested lima bean plants, whereas LI-predators had a preference for *T. urticae*-induced to *S. exigua*-induced volatiles when spider mite densities were sufficiently large (chapter 7). However, no effect of rearing history on the choices of *P. persimilis* between *T. urticae*-induced and *S. exigua*-induced volatiles from cucumber was recorded (Figure 3).

Most likely, CU-predators were more attracted to *S. exigua*-induced volatiles because the volatile blend that they had experienced during rearing (from *T. urticae*-infested cucumber) is more similar to that of *S. exigua*-infested lima bean or

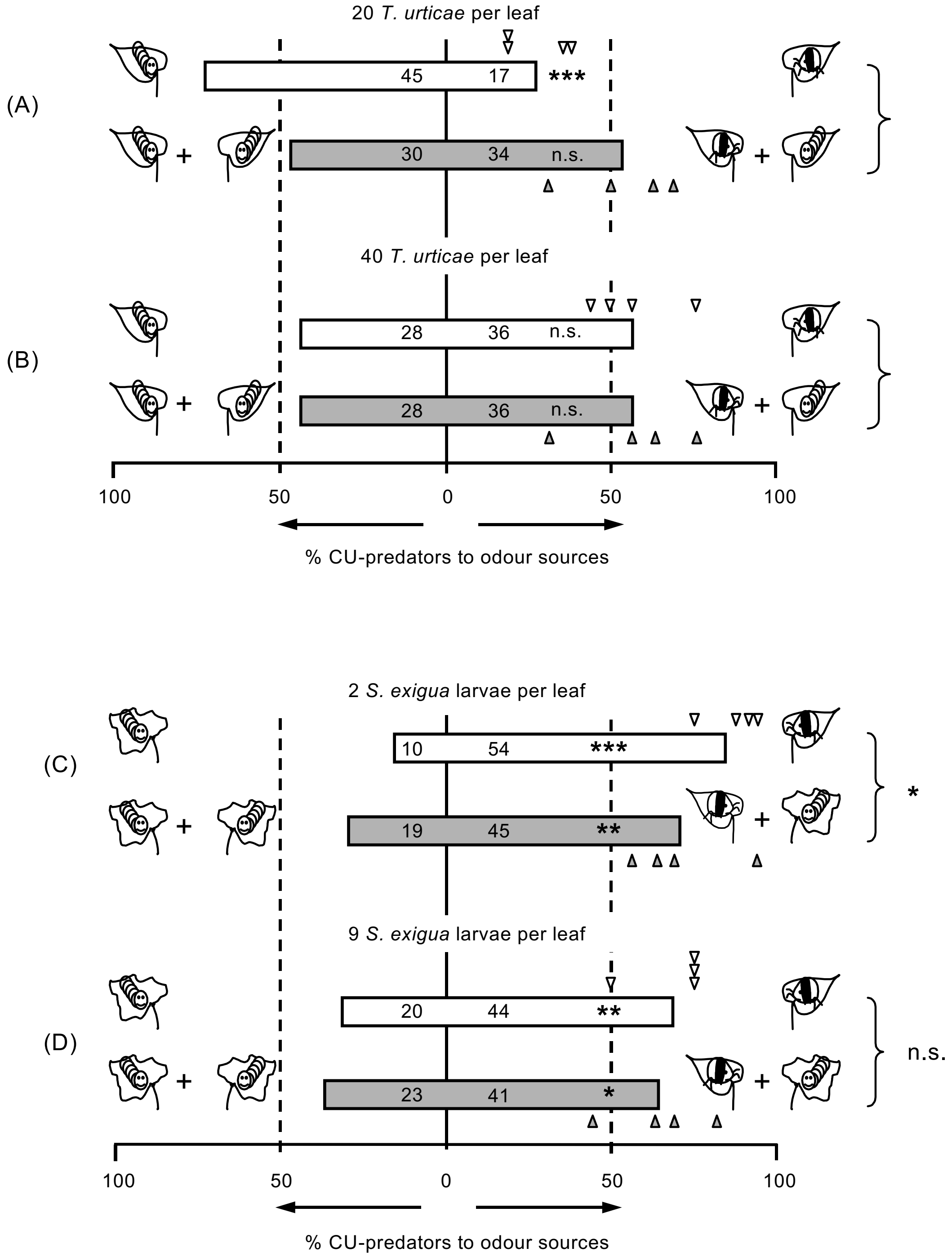

Figure 4: Effect of mixing the volatiles from *Tetranychus urticae*-infested lima bean plants with the volatiles from *Spodoptera exigua*-infested lima bean (A and B) or cucumber (C and D) plants on the choices of *Phytoseiulus persimilis*. All predatory mites had been reared on cucumber. In A and B, white bars present the percentages predators choosing between the volatiles from four *T. urticae*-infested lima bean leaves (20 or 40 spider mites per leaf) versus the volatiles from four *S. exigua*-infested lima bean leaves (2 caterpillars per leaf); grey bars present the percentages predators choosing between the volatiles from four *T. urticae*-infested lima bean leaves (20 or 40 spider mites per leaf) ánd four *S. exigua*-infested lima bean leaves (2 caterpillars per leaf) versus the volatiles from eight *S. exigua*-infested lima bean leaves (2 caterpillars per leaf). In C and D, white bars

cucumber plants than is the volatile blend experienced by LI-predators during rearing (from *T. urticae*-infested lima bean); this explanation is supported by the chemical analyses of the composition of these volatile blends (chapter 3). For example, in contrast to LI-predators, CU-predators have experienced large amounts of (*E*)-β-ocimene, (*Z*)-3-hexen-1-ol acetate and 3-methylbutanal-*O*-methyl oxime, and these compounds are also present in a large amount in the volatile blends from *S. exigua*-infested cucumber or lima bean plants. Similarly, LI-predators, but not CU-predators, have experienced a large amount of methyl salicylate and this compound is absent in *S. exigua*-induced cucumber or lima bean volatiles. Methyl salicylate has been shown to play an important role in the foraging behaviour of LI-predators (chapters 4 and 5) and experience with this compound influences the choices of *P. persimilis* (chapter 6).

Our data suggest that the attraction of predatory mites to prey-related information is less influenced by previous experiences than the attraction to information that is not related to prey. This is supported by other studies that have also shown attraction of *P. persimilis* to *T. urticae*-induced volatiles independent of rearing history (e.g. Takabayashi *et al.* 1994a; Shimoda & Dicke 2000; Van den Boom *et al.* 2002; but see Krips *et al.* 1999, and Drukker *et al.* 2000a). However, most studies on phenotypic plasticity in the responses of predators and parasitoids to herbivore-induced plant volatiles relate to volatiles induced by herbivores that are suitable as prey or hosts. Whether the responses of predators to volatiles induced by prey herbivores are indeed less subject to variation, than responses to volatiles induced by herbivores that are not suitable prey, remains to be further investigated.

(Figure 4 continued)

present the percentages predators choosing between the volatiles from four *T. urticae*-infested lima bean leaves (20 spider mites per leaf) versus the volatiles from four *S. exigua*-infested cucumber leaves (2 or 9 caterpillars per leaf); grey bars present the percentages predators choosing between the volatiles from four *T. urticae*-infested lima bean leaves (20 spider mites per leaf) ánd four *S. exigua*-infested cucumber leaves (2 or 9 caterpillars per leaf) versus the volatiles from eight *S. exigua*-infested cucumber leaves (2 or 9 caterpillars per leaf). Numbers in bars are the total numbers of predators responding to the odour sources; triangles indicate the percentage predators choosing for the volatiles from prey-infested lima bean plants per day of the experiment. Choices between odour sources were analysed with a two-sided binomial test. The effect of mixing prey-induced with non-prey-induced volatiles was analysed with a GLM, the significance of which is indicated behind the bracket (predicting factors: replicate + odour source). (n.s. $P>0.05$; * $P<0.05$; ** $P<0.01$; *** $P<0.001$).

Foraging in an environment with non-prey: odour mixing and multiple infestation

We expected that mixing *T. urticae*-induced volatiles with *S. exigua*-induced volatiles would reduce the detectability of the prey-related signal and thus the proportion of predatory mites choosing for the volatiles from plants infested with their prey. However, the choices of LI-predators between *T. urticae*-induced and *S. exigua*-induced volatiles were not affected by a background of volatiles from *S. exigua*-infested conspecific or heterospecific plants (chapter 3). In contrast, our present data show that for CU-predators mixing *S. exigua*-induced volatiles from lima bean or cucumber with *T. urticae*-induced volatiles significantly influenced the foraging responses in two out of four experiments (Figure 4). The different impact of odour mixing on the responses of CU-predators and LI-predators can most likely be explained by the fact that the *S. exigua*-induced volatiles were attractive to CU-predators, whereas they represented a neutral odour source to the LI-predators (Figure 1B). A similar finding has been reported for parasitoid wasps of stemborers (Gohole *et al.* 2003a,b). Attraction of the larval parasitoid *Cotesia sesamiae* to volatiles from stemborer-induced maize was hampered in a background of volatiles from uninfested molasses grass, whereas the responses of the pupal parasitoid *Dentichasmias busseolae* were not hampered. The volatiles from uninfested molasses grass strongly attracted *C. sesamiae* but repelled *D. busseolae*.

To date, only one other study had addressed the role of learning in foraging of carnivores in relation to odour mixing. Learning improved the foraging efficiency of the parasitoid wasp *C. glomerata* in a system of brussels sprouts plants infested with its host *Pieris rapae* surrounded by potato plants infested with the non-host beetle *Leptinotarsa decemlineata* (Perfecto & Vet 2003). We have previously shown that experiences during the adult phase can improve the foraging responses of CU-predators such that they learn to prefer the volatiles from *T. urticae*-infested lima bean plants to those of *S. exigua*-infested plants (chapter 7). Assessing whether such experiences can also improve the responses of *P. persimilis* to mixtures of volatiles induced by herbivores that differ in their suitability as prey will improve our understanding of how predators use herbivore-induced plant volatiles to locate their prey under natural conditions.

Independent of rearing history, predatory mites preferred the volatiles from plants that had been simultaneously infested with their prey *T. urticae* and the non-prey caterpillar *S. exigua* to the volatiles from plants infested with one of the herbivore species alone (Figure 2). The responses of carnivorous arthropods to the volatiles from plants infested with more than one herbivore species have only been studied in one other tritrophic system, consisting of cabbage plants, caterpillars, and parasitoid wasps, but learning was not included in these studies (Shiojiri *et al.* 2001, 2002; Vos *et al.* 2001). It seems likely that learning influences the responses of parasitoids and predators to plants infested with more than one herbivore species because multiple herbivory may affect the fitness of carnivores, for example by reducing the quality of the prey or host. The presence of non-host caterpillars had a negative influence on the parasitoid wasp *C. plutellae*, which parasitised fewer hosts on plants infested with both host and non-host caterpillars than on plants infested with hosts alone (Shiojiri *et al.* 2002). The responses to the volatiles from plants infested with multiple herbivore species may only be changed after experiences with the specific volatile blend from such plants, and not by the experiences acquired on plants infested with prey alone as had been tested in the present study.

In conclusion, our data demonstrate that the volatile blend experienced by predatory mites during their development can influence their foraging responses in an environment where non-prey caterpillars are present. This can result in the attraction of predators to the volatiles from plants infested with non-prey, to "mistakes" in their choices between volatiles from plants infested with prey or non-prey herbivores, and to changes in their choices between volatiles from plants infested with prey or non-prey herbivores in a background of volatiles induced by non-prey herbivores.

Studying the behavioural responses of carnivorous arthropods to herbivore-induced plant volatiles in situations that they may encounter in the field, such as odour mixing and multiple infestation (Shiojiri *et al.* 2000, 2001, 2002; Vos *et al.* 2001; Gnanvossou *et al.* 2002; Gohole *et al.* 2003a,b; chapters 2 and 3), and the role of phenotypic plasticity in responding to such variable information in these situations (Perfecto & Vet 2003; the present study) can narrow the gap between laboratory experiments and the situation in which carnivores forage for their prey in the field, and thereby our understanding of information use by natural enemies of herbivorous arthropods.

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CHAPTER 9

Summarising discussion

Information use by natural enemies of herbivorous arthropods

Introduction

Information from plants can mediate interactions between the herbivores that attack plants and the natural enemies of these herbivores. Upon damage by herbivorous insects or mites plants can release a bouquet of volatiles, which are used by many natural enemies of herbivorous arthropods as the main source of information during prey location (e.g. Turlings *et al.* 1991; Vet & Dicke 1992; Du *et al.* 1996). Several characteristics of blends of herbivore-induced plant volatiles may influence how carnivores use this information:

(i) Information related to the presence of suitable prey is not always the same. Many carnivores prey on more than one herbivore species. Most of these herbivore species, in turn, feed on several host plant species. Because both plant and herbivore species are major determinants of the composition of volatile blends, the information that relates to prey can differ for each plant-herbivore combination (e.g. Takabayashi & Dicke 1996; Van den Boom *et al.* 2004). Moreover, the availability of plants and herbivore species can vary within and between years or habitats.

(ii) The composition of a volatile blend from a specific plant-herbivore combination is not always the same; extra variation can be caused by simultaneous infestation of the plant with a second herbivore species or a pathogen, or by abiotic factors, such as soil humidity or nitrogen availability (Shiojiri *et al.* 2001; Cardoza *et al.* 2002; Gouinguené & Turlings 2002; Schmelz *et al.* 2003a).

(iii) In a plant community, information – i.e. volatile blends – from different plants can mix and thereby increase the background noise in which carnivores have to detect the volatiles induced by their prey.

When foraging for prey, carnivores are thus confronted with a diversity of herbivore-induced volatile blends that may or may not indicate the presence of suitable prey herbivores feeding on a plant. An important aspect of information use is discrimination between cues related to prey – signal – and cues that are not related to prey – noise. Despite the extensive amount of research on the production of herbivore-induced volatiles by plants and on the responses of carnivores to these volatiles, it remained unknown which parts of volatile blends signal the presence of prey to carnivores, and which parts are noise (e.g. Chadwick & Goode 1999; Dicke & Vet 1999; Vet 1999b; Dicke & Van Loon 2000). The aim of this thesis was to fill this gap in the knowledge on information use by natural enemies of herbivorous arthropods. To do so, I addressed the following questions:

- How do predators respond to volatiles induced by prey herbivores in a background of volatiles induced by non-prey herbivores?
- Which parts of volatile blends can predators use to discriminate between two volatile blends?
- What is the role of learning in foraging behaviour of predators in relation to variation in herbivore-induced volatiles?

These questions were studied for a tritrophic system consisting of lima bean plants, the two-spotted spider mite *Tetranychus urticae*, and the predatory mite *Phytoseiulus persimilis*. Because the spider mite is a highly polyphagous herbivore (Van de Vrie *et al.* 1972), *P. persimilis* is potentially confronted with a large variation in prey-location cues (Dicke *et al.* 1998).

This summarising discussion synthesises the main results and conclusions of all the previous chapters and discusses the use of herbivore-induced volatiles during prey location by carnivorous arthropods.

Foraging in an environment with non-prey herbivores

To date most studies on the responses of predators to herbivore-induced plant volatiles have been carried out in the laboratory in an odour-free background (Hunter 2002; but see Drukker *et al.* 1995; Shimoda *et al.* 1997; De Moraes *et al.* 1998; Thaler 1999; Geervliet *et al.* 2000; and Kessler & Baldwin 2001 for field studies). Under natural conditions, however, predators have to locate their prey in an environment where a diversity of other information is available as well. As mentioned in the introduction, the volatile blends from two or more conspecific or heterospecific plants can mix, or a single plant can be infested with more than one herbivore species at the same time. In the field, such background noise of volatiles induced by non-prey herbivores may reduce the detectability of the signal that is used by carnivores to locate their prey, or hamper the ability of carnivores to discriminate between relevant and irrelevant information. Hence, the probability that carnivores are able to locate their prey may be influenced by background noise, which could have several consequences for the interactions in a tritrophic system: (1) Herbivore-induced volatiles might have a different benefit to both plants and carnivores in environments with different degrees of background noise; (2) Herbivores themselves are expected to select plants on which they run a low risk of predation and thus altered responses of carnivores may influence the distribution of herbivores on their food plants; (3) Other species in the food web may indirectly be influenced by a changed probability that a certain carnivore species locates its prey. For example when two carnivore species share the same herbivorous prey, one species could benefit from a disturbed prey location behaviour of the other species. Given these possible consequences, it is important to study the responses of carnivores to herbivore-induced plant volatiles under the more complex and realistic conditions that they can encounter in the field.

A study on the effects of odour mixing on the searching behaviour of the colorado potato beetle showed that attraction of the herbivorous beetle to its host plant was reduced when the odour of its host plant was mixed with the odour of non-host plants (Thiery & Visser 1986). Adding specific synthetic compounds from the odour of the host plant to the complete blend emitted by the host plant also reduced the attraction of the beetle, demonstrating that its searching behaviour was hampered because the original ratios between compounds of the host plant odour were changed (Visser & Avé 1978). Similarly, we hypothesise that mixing volatiles induced by prey herbivores with compounds that identify the presence of prey to carnivores hampers prey location by carnivores.

However, currently little is known about the parts of herbivore-induced volatile blends that signal the presence of prey to carnivores (but see part II of this thesis), and therefore it is difficult to predict in which situations odour mixing will lead to a reduced foraging success of carnivores. However, we can predict which odour blends are most similar in terms of chemical composition. In general, volatile blends from heterospecific plants infested with the same herbivore species are *qualitatively* different (i.e. they consist partly of different compounds), whereas volatile blends from conspecific plants infested with different herbivore species are *quantitatively* different (i.e. they consist of the same compounds in different ratios) (e.g. Dicke 1999a; Van den Boom *et al.* 2004). This means that prey-related information is most likely more similar to volatiles induced by a non-prey herbivore on a conspecific plant than on a heterospecific plant (Figures 1A and B). Similarly, the volatile blend from plants simultaneously infested with two herbivore species may be even more similar to each of the volatile blends induced by the two herbivore species alone than are the volatile blends from different individual plants infested by either of the two herbivore species alone (Figures 1B and C). In part I of this thesis we tested whether the responses of predatory mites to volatiles induced by their prey were influenced by volatiles induced by non-prey caterpillars. Based on the expected overlap in the composition of volatile blends (Figure 1), we expected that the effect of non-prey herbivores on the foraging behaviour of predatory mites increases in the following order: non-prey herbivores feeding on heterospecific plants, on conspecific plants, or on the same individual plant.

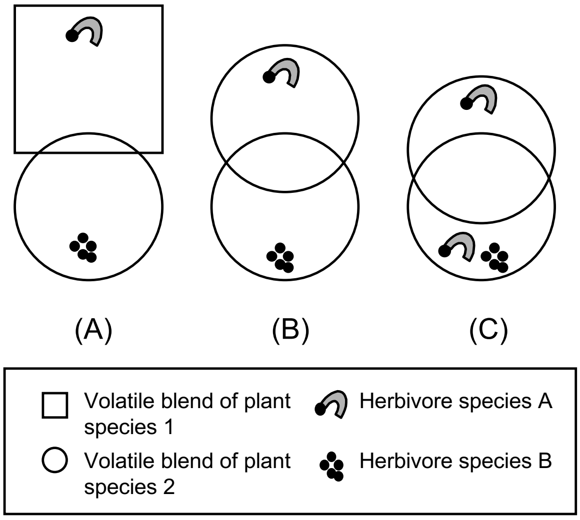

Figure 1: Overlap in volatile blend composition of two heterospecific (A) or conspecific (B) plants infested with different herbivore species, or two conspecific plants infested with two herbivore species simultaneously, or with one of the herbivore species alone (C).

– Odour mixing –

First, we investigated how the attraction of *P. persimilis* to volatiles induced by their prey was influenced by volatiles from *Pieris brassicae*-infested brussels sprouts plants (chapter 2). *Pieris brassicae* is not suitable as prey for *P. persimilis*, and brussels sprouts plants are poor host plants for spider mites. The volatile blend emitted by *P. brassicae*-infested brussels sprouts plants is qualitatively very different from that of *T. urticae*-infested lima bean plants (Mattiacci *et al.* 1994; Dicke *et al.* 1999). Mixing the volatiles from *T. urticae*-infested lima bean plants with the volatiles from *P. brassicae*-infested brussels sprouts did not result in a reduced attraction of *P. persimilis* to *T. urticae*-induced volatiles in the Y-tube olfactometer, or in a lower number of predatory mites recovered on *T. urticae*-infested plants in a greenhouse set-up that resembled the situation in the field more closely.

Second, we studied the foraging responses of predatory mites to mixtures of volatiles from *T. urticae*-infested lima bean plants and *Spodoptera exigua*-infested cucumber plants (chapter 3). The caterpillar *S. exigua* is not suitable as prey for *P. persimilis*. Based on the composition of volatile blends of *T. urticae*-infested cucumber plants (Takabayashi *et al.* 1994a), we expected that the volatiles induced by the non-prey caterpillar *S. exigua* on cucumber were more similar to those induced by *T. urticae* on lima bean than were the volatiles from *P. brassicae*-infested brussels sprouts. Thus we also expected a larger effect on the responses of *P. persimilis*. However, mixing the volatiles from *T. urticae*-infested lima bean plants with the volatiles from *S. exigua*-infested cucumber plants did not have a negative influence on the prey location behaviour of *P. persimilis* either, both in the Y-tube olfactometer and in the greenhouse. The same results were found for mixing *T. urticae*-induced volatiles from lima bean with *S. exigua*-induced volatiles from lima bean (chapter 3).

Analysis of the chemical composition of the volatile blends from *T. urticae*-infested plants and heterospecific or conspecific plants infested with the non-prey caterpillars *P. brassicae* or *S. exigua* support the prediction of an increasing degree of overlap between the volatile blends from heterospecific plants and conspecific plants (Figure 1; Mattiacci *et al.* 1994; chapter 3). However, this did not result in an increasingly negative effect on the ability of *P. persimilis* to locate its prey. This finding may be explained by the fact that *P. persimilis* was not attracted to the volatiles from *P. brassicae*-infested brussels sprouts plants, and *S. exigua*-infested cucumber or lima bean plants, when tested against the volatiles from uninfested brussels sprouts, cucumber, or lima bean plants respectively (chapters 2 and 3). Results from chapter 8 and from Gohole *et al.* (2003a,b) support the idea that the attractiveness of the odours used for mixing influence the foraging responses of carnivores to mixtures of volatiles. In contrast to predatory mites that had been reared on lima bean, cucumber-reared predators were strongly attracted to the volatiles from *S. exigua*-infested cucumber or lima bean plants when tested versus the volatiles from uninfested cucumber or lima bean plants (chapters 3 and 8). The responses of cucumber-reared predators were indeed influenced more than the responses of lima bean-reared predators by mixing the volatiles from *T. urticae*-infested lima bean plants with the volatiles from *S. exigua*-infested cucumber or lima bean plants (chapter 8). Gohole *et al.* (2003a,b) reported similar results for the attraction of two parasitoid wasps to volatiles induced by their hosts. The volatiles from uninfested molasses grass strongly attract *Cotesia sesamiae*, while *Dentichasmias busseolae* is repelled (Khan *et al.* 1997; Gohole *et al.* 2003a,b). The responses of *C. sesamiae* to stemborer-induced maize volatiles were hampered in a background of molasses grass odour, while the responses of *D. busseolae* were not.

To conclude, it appears that the effect of odour mixing on the foraging responses of carnivores cannot be predicted merely from the degree of overlap between the volatiles from the prey-infested plant and the odour used for mixing. Instead, the effect of odour mixing may depend on the degree of overlap between the odour used for mixing and the signal that is used by the carnivore to locate its prey. This, in turn, depends on the carnivore species used (Gohole *et al.* 2003a,b; Perfecto & Vet 2003), and its previous experiences with volatiles (Perfecto & Vet 2003; chapters 3 and 8), and on the plant species infested with the prey herbivore (Gohole *et al.* 2003a).

– Multiple herbivore infestation –

We also investigated the responses of *P. persimilis* when the non-prey caterpillar *S. exigua* infested the same individual plant as the prey herbivore *T. urticae* (chapter 3). Predatory mites had a significant preference for the volatiles from plants infested with the combination of *T. urticae* and *S. exigua* (multiple-herbivore-infested) versus those from plants infested with *S. exigua* or *T. urticae* alone. The preference of predatory mites for multiple-herbivore-infested plants over *S. exigua*-infested plants clearly benefits the predator but it is less clear why the predators preferred multiple-herbivore-infested plants over *T. urticae*-infested plants. Although plants simultaneously infested with two herbivore species are most likely easier to detect, there may also be disadvantages to the predator. The caterpillars may pose a direct danger to the predatory mites, by accidentally feeding on their eggs, or caterpillars may indirectly reduce the quality or quantity of prey available to *P. persimilis*, either through competition for food with the spider mites, or by accidentally eating spider mite eggs. Shiojiri *et al.* (2002) reported that the parasitoid wasp *C. plutellae* parasitised fewer hosts on cabbage plants that were simultaneously infested with a non-host caterpillar than on plants infested with hosts alone. This reduction in fitness may explain the preference of *C. plutellae* for the volatiles from plants infested with hosts alone over those from plants simultaneously infested with host and non-host caterpillars (Shiojiri *et al.* 2000).

Not only the effects of multiple infestation on the fitness of predatory mites need further investigation, but also the effects of multiple infestation on spider mites. Several herbivore species, including *T. urticae*, are known to respond to herbivore-induced volatiles in their selection of food plants (e.g. Dicke 1986; Loughrin *et al.* 1996; Pallini *et al.* 1997; Bernasconi *et al.* 1998; Shiojiri *et al.* 2002; Shiojiri & Takabayashi 2003). Herbivores are expected to select food plants where the performance of their offspring is maximised, this includes a high nutritional quality, a low level of competition, a low level of direct defence, and a low risk of predation (Dicke 2000). Spider mites are thus expected to avoid plants infested with *S. exigua* if the caterpillars decrease the quality of the host plant for spider mites, and/or spider mites run a risk from being eaten by a caterpillar, and the probability that the natural enemies of spider mites locate this plant is higher. Females of the moth *Plutella xylostella* preferentially oviposited on plants infested with *P. rapae* caterpillars over uninfested plants or *Pl. xylostella*-infested plants (Shiojiri *et al.* 2002). This preference might be a response to a low risk of parasitisation by its natural enemy, the parasitoid *C. plutellae* (see above).

The studies by Shiojiri *et al.* show that volatiles induced by multiple infestation can mediate interactions in a tritrophic system, which possibly affects the distribution of carnivores and herbivores in systems with non-host or non-prey herbivores. Clearly, more studies are needed to assess the effects of multiple-herbivore-infestation on the interactions in the tritrophic system of plants, spider mites and predatory mites that was studied in this thesis.

–Synopsis–

In short, our studies on odour mixing and multiple infestation imply that herbivore-induced plant volatiles can benefit spider mite-infested plants by attracting predatory mites, and can benefit predatory mites in prey location, in more complex situations than investigated so far, i.e. in systems with non-prey caterpillars. This may not come as a surprise considering that both the foraging responses of carnivores and the emission of herbivore-induced volatiles by plants have presumably evolved in a system with background noise. If predators that are attracted to prey-related information in a background of volatiles induced by non-prey herbivores have a fitness advantage compared to predators that can not locate their prey in an environment with non-prey herbivores, predators are expected to be able to cope with such background noise. Similarly, plants are expected to emit a signal that attracts predators in an environment with background noise if the carnivores are effective in reducing the level of herbivory. Nevertheless, before it can be concluded that herbivore-induced volatiles indeed benefit both plants and predators under field conditions, several aspects need to be investigated in more detail. First, we showed that the effects of odour mixing depend on the previous experiences of predatory mites with herbivore-induced volatiles (chapter 8). Although "mistakes" of the predators, i.e. attraction to plants infested with non-prey herbivores, may be overcome by learning during the adult phase (see part III of this thesis) little is yet known about the role of learning in the foraging behaviour of carnivores under field conditions (e.g. Papaj & Lewis 1993). Second, the direct effects of multiple-herbivore-infestation on the fitness of predatory mites remain to be investigated. Third, the prey and the non-prey herbivores that we used in our studies differed much in terms of the damage that they inflict on the plant: spider mites feed in a piercing-sucking way whereas caterpillars feed in a biting-chewing way. Feeding style most likely has an effect on the composition of volatile blends (see *Defence pathways and herbivore-induced plant volatiles*), and we expect that two volatile blends induced by herbivores with the same feeding style will be more similar than two volatile blends induced by herbivores with a different feeding style. Consequently, the overlap between the volatile blend used for mixing and the prey-related signal used by carnivores may be larger when the feeding styles of the prey and non-prey herbivores are the same. Moreover, different species of non-prey herbivores may vary in the direct and indirect effects that they have on the prey herbivores, for example by inducing direct defences when simultaneously infesting the same plant.

Discrimination between volatile blends: the role of volatile blend composition

An important aspect of information use is the discrimination between relevant and irrelevant information. Natural enemies of herbivorous arthropods are expected to discriminate between volatiles that reveal the presence of suitable prey herbivores on a plant and volatiles that are not related to the presence of prey, such as volatiles that are induced by non-prey herbivores or by abiotic stress factors. Herbivore-induced plant volatiles can be complex blends of many different compounds – even more than 100 – and it is not expected that carnivores use the complete composition of such blends to identify the presence of prey on a plant because part of the volatile blend is not herbivore-specific. This noise-part of the volatile blend may for example consist of volatile compounds that are released upon any type of stress to which the plant is exposed. Furthermore, a specific plant-herbivore combination may emit different volatile blends depending on the environmental conditions, e.g. light intensity or nitrogen availability (Gouinguené & Turlings 2002; Schmelz *et al.* 2003a).

In part II of this thesis, we addressed the question which parts of herbivore-induced volatile blends contain relevant information for foraging predatory mites. Given the type of information, i.e. blends of volatile compounds, we can think of two possible ways for carnivores to identify the presence of prey on a plant:

(i) The quantity of one or more specific compounds, and/or
(ii) The ratios between two or more specific compounds

In identifying which specific compounds play a role in the foraging responses of carnivores, a comparison of the behavioural preferences of carnivores with the chemical composition of volatile blends can pinpoint potentially bioactive compounds. However, in doing so, two problems can arise. First, the sensitivity of the analytical equipment used to determine the composition of a blend of herbivore-induced plant volatiles is much lower than the sensitivity of the chemoreceptors of the arthropods themselves (e.g. Pickett *et al.* 1998). The absence of a compound in a volatile blend as measured with GC–MS (gas chromatography–mass spectrometry) is thus no proof that this specific compound is not emitted by a plant. This problem can partly be solved by a coupled analysis of volatile blend composition and electrophysiological recording of the perception of compounds by chemoreceptors of an arthropod (gas chromatography–electroantennography, or GC–EAG). This technique can elucidate whether the plant emits compounds that are not detected by the GC but that are detected by the arthropod (Pickett *et al.* 1998). GC–EAG can also be used to narrow down the range of potentially bioactive compounds in the volatile blend by measuring the sensory responses of the arthropod to the different compounds in the volatile blend. However, although this technique has been used successfully for a long time to identify bioactive compounds for herbivores, it has been applied to carnivores only recently (Du *et al.* 1998; Van Loon & Dicke 2000; Weissbecker *et al.* 2000; Smid *et al.* 2002). It may be particularly difficult with small carnivorous arthropods, such as egg parasitoids or predatory mites (De Bruyne *et al.* 1991). A second problem arises when the compounds that are potentially important in carnivore foraging are not commercially available or have not been identified yet. This obviously complicates further experiments.

Recently, two solutions to both problems were proposed. Dicke & Van Loon (2000) proposed the use of genetically modified plants (transgenic or mutant) that lack the ability to produce one or more specific compounds upon herbivore damage, and Van den Boom (2003) developed a GC-based fractionation method which makes it possible to remove one or more specific compounds from the total herbivore-induced volatile blend. Although both solutions seem highly valuable in identifying the role of specific compounds in carnivore foraging, they are not yet fully developed and they were not available during the research described in this thesis. Therefore, in our experiments on the discrimination between volatile blends by predatory mites we used the following method: We induced plants to emit volatile blends of different composition by treatment with a plant hormone that elicits volatile emission, or infestation with different herbivore species, and we manipulated the composition of these volatile blends by adding specific synthetic volatiles.

First we investigated the role of methyl salicylate (MeSA) in the foraging behaviour of *P. persimilis*. We showed that predatory mites were attracted to the single compound MeSA in a dose-dependent way (chapter 4). The effect of a *quantitative* difference in MeSA was tested by offering predators a choice between two *T. urticae*-induced volatile blends from lima bean, one of which was combined with an extra dose of synthetic MeSA. A quantitative difference in the amount of MeSA did not affect the choice of the predators between these odour sources. Volatile blends that differed *qualitatively* in the presence of MeSA were obtained by infesting lima bean plants with *T. urticae* – which induces the emission of MeSA – or treating lima bean plants with jasmonic acid (JA) – which induces a rather similar volatile blend without MeSA (Dicke *et al.* 1999). A qualitative difference in the presence of MeSA did have a significant effect on the responses of *P. persimilis*: predators preferred the volatiles from *T. urticae*-infested lima bean plants to the volatiles from JA-treated lima bean. After adding synthetic MeSA to the JA-induced volatile blend, the predators lost their preference for the volatiles from *T. urticae*-infested lima bean plants.

Second, we investigated how predatory mites discriminate between the volatiles from *T. urticae*-infested lima bean and *S. exigua*-infested lima bean plants (chapter 5). We compared the behavioural preferences of predators with the chemical analyses of the volatile blends. This revealed a difference in the amounts of several compounds in the *T. urticae*-induced volatile blends at different spider mite densities that did or did not result in discrimination by *P. persimilis*. We hypothesised that the large amounts of MeSA and (3*E*,7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene [(*E*,*E*)-TMTT] in the volatile blend from *T. urticae*-

infested plants at a high density of spider mites enabled predatory mites to discriminate between plants infested with *T. urticae* or *S. exigua*. Supplementing the volatile blend from *S. exigua*-infested plants with MeSA or with (*E*,*E*)-TMTT influenced the behaviour of the predators so much that they lost their preference for the volatiles induced by their prey, thereby demonstrating a significant role for these two specific compounds within a natural volatile blend. Predatory mites did not discriminate between synthetic MeSA and the natural volatile blend induced by *T. urticae* on lima bean, thus supporting the important role of MeSA in the foraging behaviour of *P. persimilis*. We also identified 2-butanone as an attractive compound for *P. persimilis*, but it did not play a role in discrimination between plants infested with prey and non-prey herbivores because the amount of 2-butanone was equal in the volatile blends from lima bean plants infested with *T. urticae* or *S. exigua*.

Our experiments demonstrated important roles for MeSA and (*E*,*E*)-TMTT in discrimination between volatile blends by predatory mites. It remains to be investigated whether this finding applies to our specific tritrophic system of lima bean plants, the herbivores *T. urticae* and *S. exigua*, and the predatory mite *P. persimilis*, or whether it applies to a broader range of plant species and non-prey herbivore species, or even to other tritrophic systems. To date, the volatile blends released upon *T. urticae*-infestation from at least 16 plant species from 8 different families have been analysed, *all* of them emitted MeSA and/or (*E*,*E*)-TMTT (Dicke *et al.* 1990a; Takabayashi *et al.* 1991; Takabayashi *et al.* 1994a; Krips *et al.* 1999; Ozawa *et al.* 2000a,b; Maeda & Takabayashi 2001; Van den Boom *et al.* 2004; chapter 3). However, MeSA and/or (*E*,*E*)-TMTT are also commonly found in the volatile blends of plants infested with other herbivore species that are not suitable as prey for *P. persimilis* (e.g. Van Poecke *et al.* 2001; Fritzsche Hoballah *et al.* 2002). It thus seems unlikely that predatory mites can always identify the presence of prey on a plant using MeSA and/or (*E*,*E*)-TMTT only. In addition to these two compounds, the predators most likely use other compounds as well, possibly compounds that have not been detected yet. Experiments in chapter 3 comprise choice situations in which *P. persimilis* most likely used other compounds than MeSA and (*E*,*E*)-TMTT:

–Predatory mites clearly preferred the volatiles from lima bean plants infested with the combination of *T. urticae* and *S. exigua* to the volatiles from *T. urticae*-infested plants, even though the amounts of MeSA and (*E*,*E*)-TMTT in both volatile blends were equal.

–Predatory mites preferred the volatiles from cucumber plants infested with the combination of *T. urticae* and *S. exigua* to the volatiles from cucumber infested with one of the herbivore species alone although the emission of MeSA was not recorded from cucumber plants, and the quantity of (*E*,*E*)-TMTT was not significantly influenced by herbivore species.

Nevertheless, the data presented in part II are the first evidence to demonstrate that specific compounds can play a significant role in enabling predatory mites to discriminate between two odour sources. Several other studies have identified carnivore-attracting compounds from herbivore-induced volatile blends by testing their attractiveness as single compounds, as mixtures of synthetic compounds, or as fractions of the total volatile blend (e.g. Dicke *et al.* 1990a; Turlings *et al.* 1991; Whitman & Eller 1992; Scutareanu *et al.* 1997; Du *et al.* 1998; Turlings & Fritzsche Hoballah 1999; Drukker *et al.* 2000b; Birkett *et al.* 2003). However, these studies have not investigated the role of specific volatile compounds *within* a natural volatile blend and have neither demonstrated a role in enabling carnivorous arthropods to *discriminate* between two odour blends.

Defence pathways and herbivore-induced plant volatiles

We have analysed the chemical composition of blends of herbivore-induced plant volatiles in chapters 3 and 5. Besides helping to explain the responses of predators to these blends, the composition of herbivore-induced volatile blends contains information on the mechanisms of induced production of volatiles by plants. Induced plant defence requires that defence pathways are activated upon attack. Several plant hormones play a role in the emission of herbivore-induced volatiles and the attraction of carnivores (Dicke & Van Poecke 2002). Treatment with jasmonic acid (JA) resulted in the emission of carnivore-attracting volatiles in a range of plant species, including *Arabidopsis*, ginkgo, lima bean, maize, tomato, and gerbera (Dicke *et al.* 1999; Gols *et al.* 1999; Ozawa *et al.* 2000a; Thaler *et al.* 2002b; Van Poecke & Dicke 2002; Van den Boom *et al.* 2002, 2004; Schmelz *et al.* 2003b). Similarly, treatment of lima bean plants with salicylic acid (SA) or MeSA resulted in the emission of a range of volatiles that were attractive to a predatory beetle (Ozawa *et al.* 2000a; Shimoda *et al.* 2002). Treatment of lima bean leaves with 12-oxo-phytodienoic acid (OPDA) resulted in the emission of the terpene (*E*,*E*)-TMTT and in the attraction of the predatory mite *P. persimilis* (Koch *et al.* 1999; Dicke & Van Poecke 2002), and ethylene was shown to play a role in volatile emission by lima bean and maize plants (Horiuchi *et al.* 2001; Schmelz *et al.* 2003a).

In lima bean, the volatile blend induced by *T. urticae* includes large amounts MeSA and (*E*,*E*)-TMTT and a low amount of (*E*)-β-ocimene, whereas the volatile blend induced by *S. exigua* comprises low amounts of MeSA and (*E*,*E*)-TMTT, and a large amount of (*E*)-β-ocimene (Ozawa *et al.* 2000a; Horiuchi *et al.* 2003; chapters 3 and 5). Ozawa *et al.* (2000a) demonstrated that the *T. urticae*-induced volatile blend from lima bean plants was regulated by both MeSA and JA. The volatile blend from *S. exigua*-infested lima bean, on the other hand depended mainly on JA alone. This suggests that in lima bean (1) the JA- and the SA-pathways can function synergistically in the induction of blends of volatiles, and (2) the regulation of defence pathways and the composition of the induced volatile blends partly depends on the feeding style of the attacking herbivore species: piercing-sucking herbivores induce higher levels of SA, and chewing-biting herbivores induce higher levels of JA (Ozawa *et al.* 2000a; Walling 2000; Thaler *et al.* 2002a). Whether this latter effect of the feeding style of the attacking herbivore species is a general phenomenon has not yet been established. However, our analysis of the volatile blends from cucumber provides little support for this phenomenon. Although the data show a trend for a larger amount of (*E,E*)-TMTT induction by *T. urticae*-infestation than by *S. exigua*-infestation, which may suggest a larger induction of SA by *T. urticae* than by *S. exigua*, no MeSA was emitted upon any of the herbivore treatments. In contrast to lima bean, the emission of (*E*)-β-ocimene was not differentially induced by *T. urticae*-infestation or *S. exigua*-infestation in cucumber (chapter 3), which may suggest that the two herbivore species induced JA similarly. Our results suggest that the induction of plant hormones that lead to the production or emission of volatiles differs between cucumber and lima bean plants, or that the induction of hormones is the same but the two plant species respond differently. How the absence of MeSA in the volatile blends of cucumber relates to the regulation of the SA-dependent defence pathway in this plant species also needs to be investigated.

Our analysis of the volatile blends from lima bean or cucumber plants upon infestation with *T. urticae*, *S. exigua*, or the combination of both herbivore species showed that the volatile blend released upon multiple infestation was almost equal to the sum of the single species-induced volatile blends (chapter 3). This may indicate that upon multiple infestation there is little or no interaction between the pathways induced by *T. urticae* or *S. exigua*. Comparable results have been found for cabbage plants infested with the caterpillars *Pl. xylostella* and/or *P. rapae*, and for peanut plants infested with the caterpillar *S. exigua* and/or a fungal pathogen (Shiojiri *et al.* 2001; Cardoza *et al.* 2002). However, Rodriguez-Saona *et al.* (2003) reported that cotton plants simultaneously infested with *S. exigua* and the whitefly *Bemisia argentifolii* emitted a lower amount of volatiles than plants infested with *S. exigua* alone, which suggests that infestation with *B. argentifolii* induces pathways that inhibit the volatile production induced by *S. exigua* in cotton.

Phenotypic plasticity in the foraging behaviour of predatory mites

The composition of blends of herbivore-induced plant volatiles can be highly variable, both in space and time. Carnivores are expected to be able to cope with such variation by phenotypic plasticity (Via 1987; Dukas 1998). There is ample evidence for phenotypic plasticity in the foraging behaviour of parasitoid natural enemies of herbivores (e.g. Turlings *et al.* 1993a; Vet *et al.* 1995). These parasitoid wasps can learn to associate a specific herbivore-induced volatile blend with the presence of their hosts (e.g. Lewis & Tumlinson 1988; Du *et al.* 1997; Geervliet *et al.* 1998a). They can do so after short moments of exposure to the volatiles, for example during an oviposition that may take only 20 seconds. Studies to investigate what parasitoids can learn have been initiated recently. Meiners *et al.* (2003) showed that by exposure to a mixture of three synthetic compounds in the presence of host frass the parasitoid *Microplitis croceipes* learned to respond to two of the individual components but not to the third one. When the parasitoid was exposed to the third component as a single compound it did learn to respond, suggesting that the other components of the mixture inhibited or suppressed learning of the third component. Moreover, *M. croceipes* females could learn to discriminate between alcohols with a different chain length or position of the alcoholic group, but generalisations to structurally similar compounds also occurred (Meiners *et al.* 2002). Such generalisations have also been reported for the fruitfly parasitoid *Leptopilina heterotoma* (Vet *et al.* 1998). Furthermore, Vet *et al.* showed that to differentiate between two odour sources that differed in the amount of only one compound, parasitoids had to experience one odour in a rewarding context and the other odour in a non-rewarding context. Associative learning enables parasitoids to temporarily specialise on the odours from the plants that their hosts use in a certain place and time.

In contrast to the studies on learning in parasitoid wasps, studies on learning of herbivore-induced plant volatiles by arthropod predators are still scarce (e.g. Bernays 1993; Dicke *et al.* 1998). However, several studies have shown an effect of previous experiences with volatiles on the subsequent foraging responses of predators (e.g. Dicke *et al.* 1990b; Takabayashi & Dicke 1992; Dwumfour 1992; Krips *et al.* 1999). Moreover, Drukker *et al.* (2000a,b) showed that predators that had been reared in the absence of volatiles were only weakly attracted to prey-related information. In part III of this thesis, we investigated how the foraging responses of the predatory mite *P. persimilis* are influenced by previous experiences with volatiles. Several aspects of the biology and ecology of predatory mites are likely to determine the role of learning in their foraging behaviour, and the type of experiences that influence their responses. In contrast to many parasitoid species that develop inside their host, predatory mites are exposed to volatiles induced by their prey during their entire development. Moreover, the development of predatory mites is hemimetabolous which may mean that the olfactory systems of immature stages and the adult phase resemble each other more than for different developmental stages of holometabolous carnivores, such as parasitoid wasps or flies. In fact, one study showed that the protonymphs of two species of predatory mites (including *P. persimilis*) were attracted to herbivore-induced volatiles (Dong & Chant 1986), demonstrating that immature predatory mites can indeed perceive these cues and respond to them. Unlike parasitoid wasps, predatory mites are also predacious during the adult phase. This means that adults respond to volatiles in search of both oviposition sites and food. Once they have found a new prey patch, they will stay – and thus will be exposed to the volatiles emitted by the prey-infested plant – for relatively long periods (compared to an oviposition by a parasitoid wasp) (Drukker *et al.* 2000a).

We showed that the responses of *P. persimilis* were influenced by the plant species that had been used for rearing (chapters 6, 7 and 8). In contrast to predators that had been reared on lima bean, cucumber-reared predators were not attracted to MeSA (chapter 6). Cucumber plants do not emit MeSA upon spider mite-infestation whereas lima bean plants do, and thus, unlike lima bean-reared predators, cucumber-reared predators have not experienced MeSA (Takabayashi *et al.* 1994a; Dicke *et al.* 1999; chapter 3). We explicitly demonstrated that experience with MeSA caused the difference in attraction of lima bean-reared and cucumber-reared predators to MeSA by rearing a third group of predators on cucumber in the presence of synthetic MeSA. These MeSA-experienced predators were indeed attracted to MeSA (chapter 6). There was also some effect of exposing the cucumber-reared predators to MeSA for three days during the adult phase on their response to MeSA. Experience with MeSA during rearing also influenced the choices of *P. persimilis* between two volatile blends that differed in the presence of MeSA: lima bean-reared predators preferred the volatiles from *T. urticae*-infested lima bean (including MeSA) to those of JA-treated lima bean (lacking MeSA), whereas cucumber-reared predators preferred the volatiles from JA-treated lima bean (chapter 6). Predators that had been reared on cucumber in the presence of synthetic MeSA did not discriminate between these volatile blends. Furthermore, the plant species on which predatory mites had been reared influenced their choices between the volatiles from *T. urticae*-infested and *S. exigua*-infested lima bean plants: lima bean-reared predators had a stronger preference for volatiles induced by their prey than cucumber-reared predators (chapter 7). This was explained by the stronger attraction of cucumber-reared predators than of lima bean-reared predators to *S. exigua*-induced volatiles. The responses of both groups of predators to *T. urticae*-induced volatiles were equal (chapter 8). The chemical composition of the volatile blends showed that the overlap between the blend from *T. urticae*-infested cucumber and the blends from *S. exigua*-infested lima bean or cucumber plants is larger than the overlap between the blend from *T. urticae*-infested lima bean and the *S. exigua*-induced volatile blends (chapter 3). This may explain the stronger attraction of cucumber-reared than of lima bean-reared predators to *S. exigua*-induced volatiles (chapter 8).

During the adult phase the responses of predatory mites were influenced by a non-rewarding experience with *S. exigua*-induced volatiles (i.e. starvation in the presence of these volatiles) and/or a rewarding experience with *T. urticae*-induced volatiles (i.e. feeding in the presence of these volatiles) (chapter 7). A non-rewarding experience had a small effect on the choices of *P. persimilis*: predators with a non-rewarding experience chose more frequently for *T. urticae*-induced volatiles than predators without a non-rewarding experience. A rewarding experience had a large effect on discrimination between plants infested with prey and non-prey herbivores: the response of cucumber-reared predators shifted towards a preference for *T. urticae*-induced lima bean volatiles after feeding on *T. urticae* on lima bean, while lima bean-reared predators lost their preference for *T. urticae*-induced volatiles after feeding on *T. urticae* on cucumber. Predatory mites with a non-rewarding experience on *S. exigua*-infested lima bean followed by a rewarding experience on *T. urticae*-infested lima bean had the strongest preference for *T. urticae*-induced versus *S. exigua*-induced volatiles from lima bean plants (chapter 7).

At present we can not conclude which parts of the volatile blends to which the predators were exposed during rearing or during the adult phase influenced their subsequent foraging responses. We demonstrated that MeSA is important in enabling predators to discriminate between *T. urticae*-induced and *S. exigua*-induced volatiles (chapter 5), and because lima bean-reared predators but not cucumber-reared are attracted to this compound (chapter 6), we hypothesised that experience with MeSA could result in a preference of predators for *T. urticae*-induced volatiles. However predators that had been reared on cucumber in the presence of synthetic MeSA did not discriminate between *T. urticae*-induced and *S. exigua*-induced volatiles either (chapter 7), which suggests that experience with other compounds has most likely played a role as well.

Besides using herbivore-induced plant volatiles in long-distance searching of a new prey patch, predatory mites can also use these volatiles to remain in a prey patch by responding to the steep odour gradient at the border of the patch (Sabelis *et al.* 1984). The learned response to the volatiles emitted by the plant on which they develop probably helps predatory mites to optimise this response that prevents them from leaving a prey patch that has not been exterminated yet. However, when the predators start searching for a new prey patch, they may encounter prey on another plant species than the one on which they developed, and this novel plant species is likely to emit a different volatile blend in response to spider mite infestation. This may result in attraction to plants infested with non-prey herbivores, and thus in a non-rewarding experience. A non-rewarding experience can then lead to modified responses to the odour of the novel plant species and it may increase the chance of locating a prey patch. Once a predator locates a new prey patch, a rewarding experience with the volatiles induced by the prey on the novel plant species can quickly adjust the responses of the predator to the spider mite-induced odours from this plant. This learned response probably helps the predatory mite to stay in the novel prey patch until it has been exterminated (Sabelis *et al.* 1999).

Thus, even though the plant species on which *P. persimilis* has been reared has a large impact on its behavioural responses to herbivore-induced volatiles, the responses of predatory mites remain flexible during the adult phase and learning can modify their responses. We expect that these learning abilities of predatory mites play a significant role in a natural environment where prey-location cues vary in time and space, and where non-prey herbivores are also present. Clearly, field experiments are needed to evaluate the role of learning under such natural conditions (see below)

Future perspectives

Although the work described in this thesis led to important new insights in the understanding of information use by the specialised natural enemy of spider mites, *P. persimilis* (see *Main conclusions* below), a complete answer to the question which parts of volatile blends signal the presence of suitable prey to carnivores, and which parts are noise, cannot yet be given. This thesis provides helpful leads for future research on the role of herbivore-induced plant volatiles in tritrophic interactions. An important next step is to investigate whether the findings described in this thesis apply to the foraging behaviour of *P. persimilis* in systems with other plant and herbivore species than we used, or whether they apply also to other tritrophic systems.

To understand how carnivores use herbivore-induced plant volatiles, a good starting point could be to investigate the effect of the feeding styles of herbivores on the emission of volatiles for a broader range of herbivore and plant species. This should then show whether there is a general pattern of hormone induction and volatile emission by plants, and of information use by carnivores in relation to the feeding styles of the attacking herbivore species. If such a pattern exists this could mean that plants have been selected to respond differentially to herbivores with a different feeding style and/or that carnivores have been selected to respond to the specific parts of volatile blends that are related to the feeding style of their prey.

Another issue that needs to be investigated in more detail is the effect of multiple-herbivore-infestation on the interactions in a tritrophic system. Multiple infestation may influence the probability that carnivores locate their prey and the effectiveness of predation on herbivores. This, in turn, may affect selection of host plants by herbivores (Shiojiri *et al.* 2002). Furthermore, the responses of one carnivore species may indirectly affect other organisms linked to the tritrophic system, for example carnivore species that prey on the same herbivore species or that prey on the carnivore itself (Janssen *et al.* 1998; Dicke & Van Loon 2000). Studying the responses of plants to simultaneous infestation with more than one herbivore or pathogen species can also increase the understanding of the regulation of defence pathways in plants.

To investigate what the role of learning is in the foraging behaviour of natural enemies of herbivorous arthropods, it is important to understand the situations that foraging carnivores encounter in the field. Questions that need to be answered with respect to the role of learning in the foraging behaviour of the predatory mite *P. persimilis* include: How many generations of predatory mites can develop in a single prey patch? How many decisions do predators make during their lifetime? Which experiences are dispersing females likely to acquire? What is the range of host plant species that spider mites use? Does this range change throughout the year? What type of non-prey herbivores are present in the same environment?

Finally, predatory mites are important biocontrol agents in a range of crops and knowledge on their responses to volatiles from plants can contribute to the development of methods to manipulate their behaviour in the crop, and thereby to a more efficient control of pests (e.g. Powell & Pickett 2003; Degenhardt *et al.* 2003). For example, crops may be engineered that emit a larger amount of induced volatiles that are important in prey-location by the predators, or prior to releasing the predators in the crop they could be exposed to specific compounds that improve their searching efficiency.

Main conclusions

The work described in this thesis has increased the understanding of the use of herbivore-induced plant volatiles as foraging cues by natural enemies of herbivorous arthropods for a tritrophic system consisting of lima bean plants, spider mites and the predatory mite *P. persimilis*. To summarise, the main conclusions are:

(i) Mixing volatiles from spider mite-infested plants with volatiles from conspecific or heterospecific plants infested with non-prey caterpillars has only a weak influence on the attraction of predatory mites to plants infested with their prey (Figure 2A). This effect depends on the plant species on which predatory mites have been reared (Figure 3).

(ii) Lima bean and cucumber plants simultaneously infested with spider mites and non-prey caterpillars attract predatory mites (Figure 2B). The fitness consequences of simultaneous infestation with more than one herbivore species for the predators remain to be investigated.

(iii) Lima bean and cucumber plants simultaneously infested with spider mites and caterpillars emit a volatile blend that is qualitatively very similar to the volatile blends emitted by plants infested with one of the herbivore species alone.

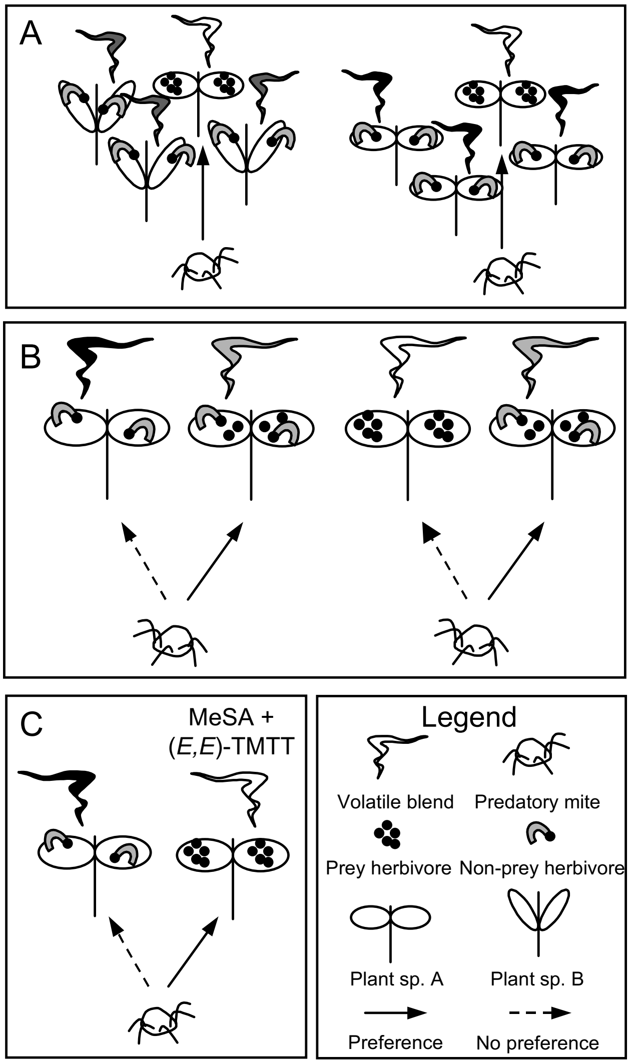

Figure 2: Summary of the main results obtained in this thesis: (A) Predatory mites can locate their prey when neighbouring heterospecific or conspecific plants are infested with non-prey caterpillars; (B) Predatory mites prefer plants infested with the combination of prey and non-prey herbivores to plants infested with one of the herbivore species alone; (C) Predators can use MeSA and (*E,E*)-TMTT to discriminate between two volatile blends.

(iv) The phenolic methyl salicylate and the terpenoid (3*E*,7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene play an important role in the foraging behaviour of *P. persimilis* (Figure 2C).

(v) The foraging behaviour of predatory mites is influenced by experiences acquired during rearing, and by non-rewarding and especially rewarding experiences during the adult phase (Figure 3). These learning abilities may enable the predators to handle variation in volatiles induced by prey herbivores in the field.

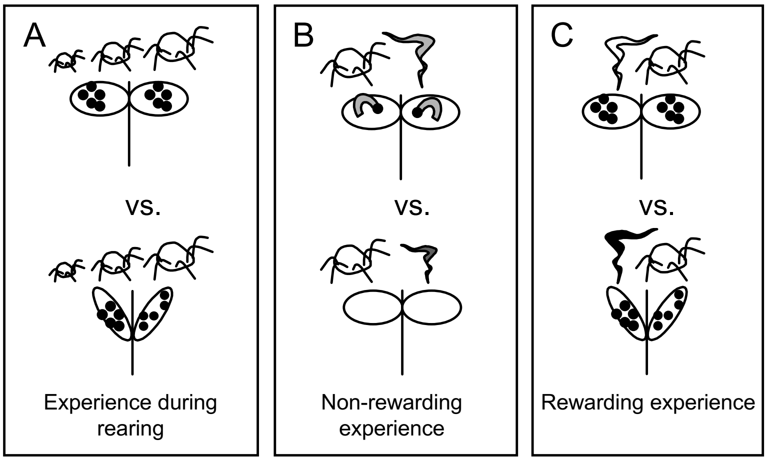

Figure 3: The responses of *Phytoseiulus persimilis* to herbivore-induced plant volatiles are influenced by the plant species on which predatory mites have been reared (A), or during the adult phase by a non-rewarding experience with volatiles induced by a non-prey herbivore (B), or a rewarding experience with volatiles induced by a prey herbivore (C). For legend, see Figure 2.

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NEDERLANDSE SAMENVATTING

Deze 'Nederlandse samenvatting' is geschreven voor niet-vakgenoten. Het is een vertaling van delen van de inleiding en de samenvattende discussie van dit proefschrift, waarbij vaktaal zoveel mogelijk is vervangen door algemeen taalgebruik.

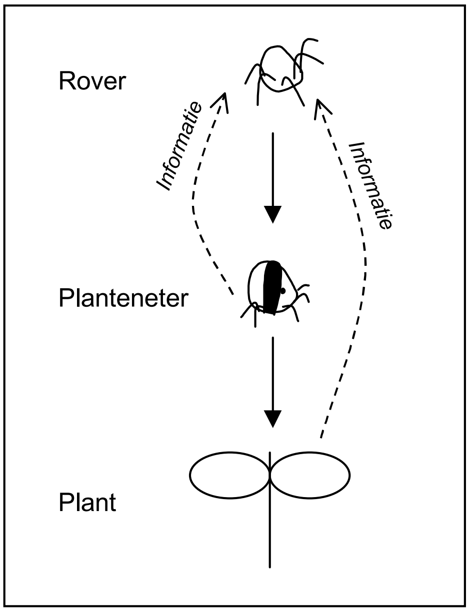
Informatie

Dit proefschrift gaat over hoe roofmijten hun prooi vinden. Alle roofdieren die naar hun prooi zoeken hebben informatie nodig over *waar* ze hun prooi kunnen vinden. Deze informatie kan visueel zijn, denk bijvoorbeeld aan een slechtvalk die zijn prooi vanaf zeer grote hoogte uit de lucht kan waarnemen. Een andere vorm van informatie is geluid. Geluid wordt bijvoorbeeld gebruikt door een uil die in het donker van de nacht op muizen jaagt. Binnen de wereld van insecten en mijten (die beiden onder de geleedpotigen vallen) spelen geuren een grote rol. Geuren kunnen informatie geven over de aanwezigheid en plaats van seksuele partners, prooien, vijanden en concurrenten.

Een belangrijk aspect van informatiegebruik is het maken van onderscheid tussen relevante signalen en achtergrondruis. Dit is gemakkelijk te begrijpen voor geluid: het is moeilijker om iemand te verstaan in een rumoerige discotheek dan in een rustig restaurant. Dit komt omdat de verhouding tussen de signalen die je op wilt vangen en de ruis in de discotheek veel lager ligt dan in het restaurant. Hetzelfde geldt voor geuren. In hun zoekgedrag naar prooien moeten rovers onderscheid maken tussen geuren die gerelateerd zijn aan hun prooi (signalen) en geuren die niet gerelateerd zijn aan hun prooi (ruis).

Herbivoor-geïnduceerde plantengeuren

Het onderwerp van mijn proefschrift is het gebruik van geuren door roofmijten in een simpel voedselweb bestaande uit planten, plantenetende en rovende geleedpotigen. Zo'n eenvoudig voedselweb wordt ook wel een tritroof systeem genoemd omdat het uit organismen van drie verschillende voedselniveaus bestaat. In een tritroof systeem kunnen rovers gebruik maken van informatie van hun prooi zelf, of van de planten waar hun prooi van eet (Figuur 1). Informatie van de prooi zelf is betrouwbaar maar vaak moeilijk te detecteren. Plantenetende geleedpotigen zijn namelijk kleine onderdelen van de omgeving. Bovendien is het voor hen ongunstig om ontdekt te worden omdat ze dan het gevaar lopen opgegeten te worden. De planten waar de planteneters van eten zijn veel groter en kunnen dus meer informatie afgeven. Misschien nog een belangrijkere reden is dat het voor planten wél gunstig is om ontdekt te worden door de natuurlijke vijanden van planteneters. Deze natuurlijke vijanden kunnen er namelijk voor zorgen dat het aantal planteneters op de plant kleiner wordt en dus minder schade aan de plant toebrengt. Dit kan er uiteindelijk toe leiden dat deze plant een groter aantal nakomelingen krijgt dan een plant die de natuurlijke vijanden van zijn belagers niet naar zich toe kan lokken.



Figuur 1: Een simpel voedselweb bestaande uit 1 soort plant, 1 soort planteneter, en 1 soort rover. De pijlen geven de relaties in het voedselweb weer.

Een jaar of 25 geleden werd een nieuwe manier van samenwerken ontdekt tussen rovers en planten ten koste van plantenetende geleedpotigen. Deze samenwerking is gebaseerd op plantengeuren die afgegeven worden door de plant in reactie op vraat door herbivoren. Deze geuren worden herbivoor-geïnduceerde plantengeuren genoemd. Mengsels van herbivoor-geïnduceerde plantengeuren kunnen bestaan uit wel meer dan 100 verschillende chemische verbindingen. De samenstelling van de geurmengsels wordt beïnvloed door een aantal factoren, waaronder bijvoorbeeld de plantensoort en de soort planteneter, maar ook het type plantenweefsel en de leeftijd van de plant, tijd van de dag, en factoren zoals lichtintensiteit, temperatuur en beschikbaarheid van water en voedingsstoffen in de omgeving van de plant.

Herbivoor-geïnduceerde plantengeuren kunnen dus voor zowel de plant als voor de rover een voordeel opleveren. Planten worden immers van hun aanvallers verlost, en voor rovers neemt de kans toe dat ze hun kleine prooien vinden in een ingewikkelde omgeving. Daarom wordt verwacht dat planten de productie van herbivoor-geïnduceerde geuren zullen optimaliseren, en dat rovers aanpassingen zullen ontwikkelen waardoor ze beter in staat zijn om te reageren op de plantengeuren.

"Bugs in odour space"

In werkelijkheid ziet een voedselweb er niet zo simpel uit als het voorbeeld dat in Figuur 1 staat. In een natuurlijke omgeving zullen rovers blootgesteld zijn aan vele verschillende geuren tegelijkertijd. Ze bevinden zich als het ware in een omgeving vol geuren, vandaar de titel van dit proefschrift "*Bugs in odour space*". Voor rovers is het van het grootste belang dat ze reageren op de plantengeuren die de aanwezigheid van een geschikte prooi verraden. Ze hebben namelijk prooien nodig om nakomelingen te kunnen krijgen en zelf te overleven.

Vanwege de grote variatie in de samenstelling van plantengeuren lijkt het onwaarschijnlijk dat de samenstelling van het complete herbivoor-geïnduceerde geurmengsel van belang is voor rovende geleedpotigen om hun plantenetende prooi te vinden. De aanwezigheid van één bepaalde prooisoort kan namelijk samenhangen met verschillende plantengeuren, bijvoorbeeld als de prooisoort zelf van verschillende plantensoorten kan eten. We verwachten daarom dat de informatie die door de rovers gebruikt wordt voor een deel uit signaal bestaat (gerelateerd aan de aanwezigheid van een geschikte prooi), en voor een deel uit ruis (niet gerelateerd aan de aanwezigheid van een geschikte prooi). Ondanks het jarenlange onderzoek aan de reactie van rovers op plantengeuren, is nog steeds niet bekend welke componenten van plantengeuren het signaal bevatten dat de aanwezigheid van een geschikte prooi aan de rover verraadt. Het analyseren van signaal en ruis, en de wijze waarop rovers daarmee omgaan vormde het doel van het promotie-onderzoek dat beschreven is in dit proefschrift.

Hieronder beschrijf ik eerst het tritrofe systeem waaraan ik onderzoek heb gedaan en de methoden die ik gebruikt heb voor dit onderzoek. Vervolgens komen in drie paragrafen de belangrijkste resultaten en conclusies van mijn proefschrift aan de orde.

Tritroof systeem

Ik heb onderzoek gedaan aan een tritroof systeem bestaande uit limaboonplanten (in het Latijn *Phaseolus lunatus*), spintmijten (*Tetranychus urticae*), en de roofmijt *Phytoseiulus persimilis* (dit beestje heeft geen Nederlandse naam). Op pagina 8 zijn tekeningen te zien van deze drie soorten. Spintmijten zijn planteneters die op zeer veel verschillende plantensoorten kunnen leven. Ze vormen een belangrijke plaag in allerlei land- en tuinbouwgewassen. Een van de belangrijkste natuurlijke vijanden van spintmijten is de roofmijt *P. persimilis*. Deze roofmijt wordt veelvuldig ingezet als biologische bestrijder van spintmijt-plagen. Volwassen vrouwtjes van de roofmijt zijn ongeveer 0.8 mm groot. Ze zijn blind en daarom afhankelijk van geuren om hun prooi te vinden. Dat ze dit uitstekend kunnen werd al in 1985 door mijn beide promotoren beschreven. In een twee-keuze experiment hebben roofmijten een voorkeur voor de geur van planten die door spintmijten aangevreten zijn ten opzichte van de geur van planten die niet aangevreten zijn. Dit is inmiddels voor meer dan 15 verschillende plantensoorten aangetoond.

Methoden

In het onderzoek is alleen gewerkt met volwassen vrouwtjes van de roofmijt. Het zijn namelijk deze vrouwtjes die op zoek gaan naar nieuwe prooihaarden om daar eitjes te leggen. Voor deze vrouwtjes is het dus van zeer groot belang dat ze een plant weten te vinden waar zich een geschikte prooi op bevindt. Om de reactie van roofmijten op plantengeuren te bestuderen, heb ik gebruik gemaakt van een zogenaamde Y-buis olfactometer. Dit is een Y-vormige glazen buis waarin een roofmijt de keuze krijgt tussen twee verschillende geuren. Omdat dit een nogal kunstmatige situatie is waarbij de twee geuren netjes van elkaar gescheiden zijn, heb ik het gedrag van roofmijten ook bestudeerd in een opstelling in een kas. Hierbij werden 100 roofmijten tegelijkertijd losgelaten in het midden van een cirkel van 6 planten (zie Figuur 2 op pagina 22). Van deze 6 planten waren er telkens 3 aangevreten door spintmijten. De behandeling van de andere 3 planten verschilde per experiment. Na 24 uur werd het totale aantal roofmijten bepaald dat op de 3 planten met prooien terug gevonden werd. Op deze manier werd het zoeksucces van roofmijten vergeleken voor verschillende combinaties van planten in de opstelling.

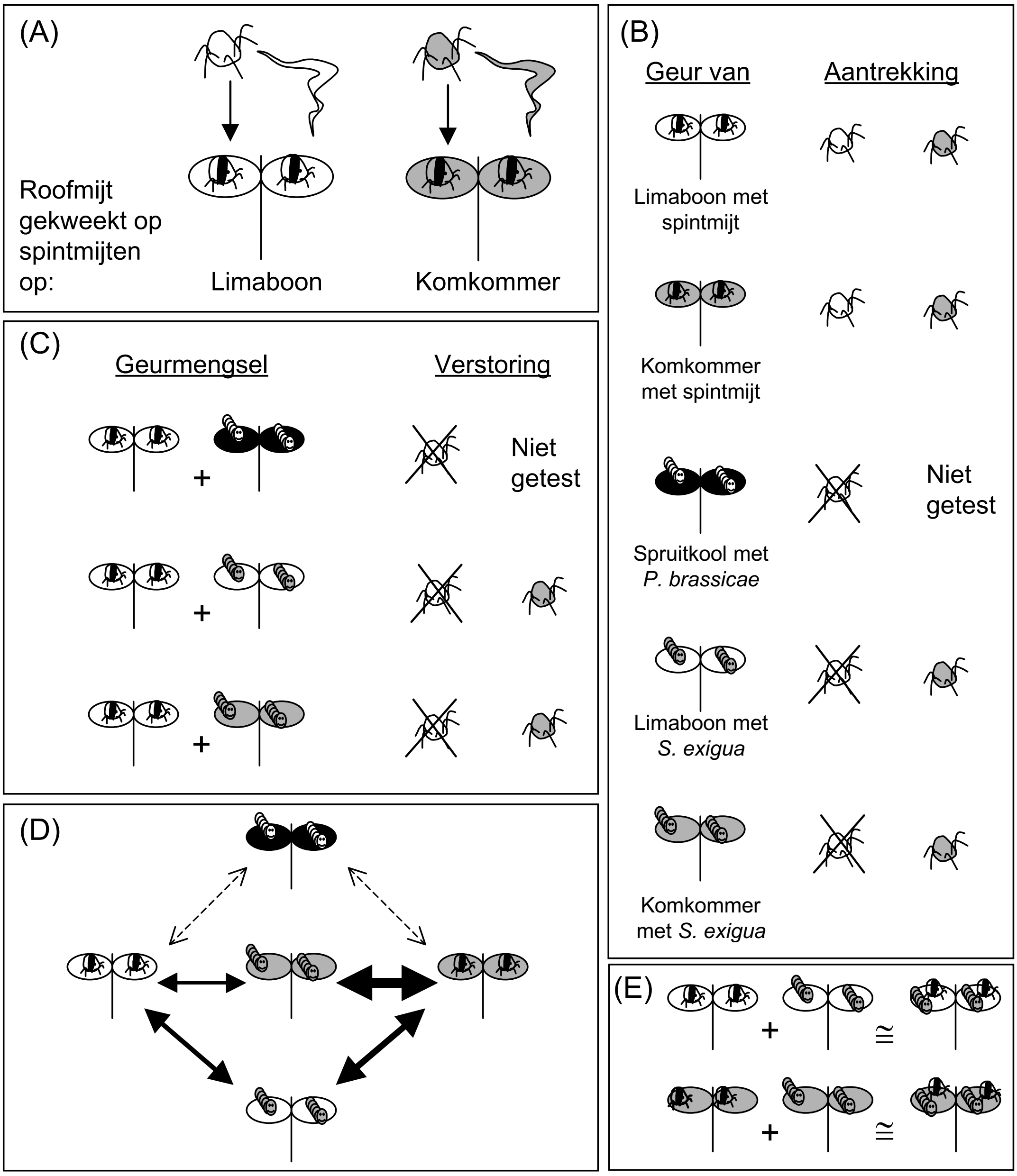
Naast het bestuderen van het gedrag van roofmijten heb ik ook chemische analyses van de samenstelling van plantengeuren gedaan. Het is mogelijk om plantengeuren op te vangen en vast te houden op een materiaal dat “tenax” heet. De buisjes met tenax en plantengeuren werden vervolgens bij het laboratorium voor Organische Chemie in Wageningen of het Nederlands Instituut voor Oecologisch Onderzoek in Nieuwersluis gebracht. Hier werd met gekoppelde gaschromatografie en massaspectrometrie onderzocht welke geurcomponenten zich in elk monster bevonden.

Zoeken in een omgeving met niet-prooi herbivoren

Tot nu toe is het onderzoek aan de reacties van rovende geleedpotigen op plantengeuren voornamelijk in het laboratorium uitgevoerd. Hierbij wordt geprobeerd in een zo schoon mogelijke omgeving te werken. Dit betekent dat er geen “achtergrondgeuren” aanwezig zijn. Deze situatie lijkt niet erg op de natuurlijke situatie waarin rovers hun prooi moeten vinden. Immers, een rover bevindt zich in een wereld vol geuren. In een natuurlijke omgeving maakt een plant met een geschikte prooi onderdeel uit van een plantengemeenschap. De andere planten in die gemeenschap kunnen ook aangevreten zijn door planteneters en dus ook herbivoor-geïnduceerde geuren afgeven. De wind kan de geuren van verschillende planten mengen en hierdoor zou het voor rovers lastiger kunnen zijn om de geur te herkennen die de aanwezigheid van hun prooi verraadt.

Bovendien kan één plant tegelijkertijd aangevallen worden door meerdere soorten planteneters, bijvoorbeeld een soort die geschikt is als prooi én een soort die niet geschikt is als prooi. Deze dubbele aanval op de plant zou ervoor kunnen zorgen dat de plant een ander mengsel van geurstoffen afgeeft. Ook hierdoor zou de rover in verwarring gebracht kunnen worden.

In hoofdstuk 2 en 3 heb ik geprobeerd om meer inzicht te krijgen in de reactie van roofmijten op geuren van planten met spintmijten in een omgeving met geuren van planten die aangevreten zijn door rupsen. Rupsen zijn geen geschikte prooi voor de roofmijt *P. persimilis*. Roofmijten reageerden uitstekend op spintmijt-geïnduceerde geuren van limaboonplanten wanneer deze geur werd gemengd met de geur van spruitkoolplanten met rupsen van het grote koolwitje (*Pieris brassicae*) (Figuur 2C). Dit werd zowel aangetoond met Y-buis olfactometer experimenten als in de kasopstelling (hoofdstuk 2). Het resultaat was niet erg verrassend omdat de geur van spruitkoolplanten met koolwitjes rupsen sterk verschilt van de geur van limaboonplanten met spintmijten (Figuur 2D). De geur van aangevreten spruitkoolplanten was niet aantrekkelijk voor roofmijten in een Y-buis experiment met de keuze tussen de geur van aangevreten spruitkoolplanten en onaangetaste spruitkoolplanten (Figuur 2B). Vervolgens werd de geur van limaboonplanten met spintmijten gemengd met de geur van komkommerplanten die aangevreten waren door rupsen van de floridamot (*Spodoptera exigua*). Hetzelfde experiment werd gedaan met rupsen van de floridamot op limaboonplanten. De chemische analyse van de geurmengsels liet zien dat de geuren van komkommer- en limaboonplanten met rupsen van de floridamot meer leken op die van limaboonplanten met spintmijten dan de geur van spruitkoolplanten met *P. brassicae* (hoofdstuk 3) (Figuur 2D). Desondanks leidde het mengen met deze geuren niet tot een verminderd percentage roofmijten dat aangetrokken was tot de geur van limaboonplanten met hun prooi (hoofdstuk 3) (Figuur 2C). Dit komt misschien omdat ook de geuren van komkommer- en limaboonplanten met rupsen van de floridamot niet aantrekkelijk waren voor roofmijten (Figuur 2B). Dit zou kunnen betekenen dat de geurcomponenten die afgegeven worden door spruitkoolplanten met *P. brassicae*, of komkommer- en limaboonplanten met *S. exigua* niet overeenkomen met de geurcomponenten die de aanwezigheid van een geschikte prooi (spintmijten) verraden aan de roofmijt *P. persimilis*. Een aanwijzing hiervoor werd gevonden in hoofdstuk 8. In dit hoofdstuk hebben we onderzocht hoe roofmijten die opgekweekt zijn op spintmijten op verschillende plantensoorten (limaboon of komkommer) reageren op mengsels van geuren. Tijdens het opkweken van ei tot volwassen stadium worden de roofmijten blootgesteld aan de geur van de plant waarop hun prooi eet.



Figuur 2: Overzicht van de experimenten beschreven in de paragraaf "*Zoeken in een omgeving met niet-prooi herbivoren*". (A) Roofmijten gekweekt op spintmijten op limaboon- of komkommerplanten zijn gebruikt. Deze roofmijten hebben een verschillende ervaring met herbivoor-geïnduceerde plantengeuren, respectievelijk met het witte geurmengsel of met het grijze geurmengsel. (B) Aantrekking van de roofmijten naar de verschillende geurmengsels. De kleur van de roofmijt komt overeen met de geur waarmee ze ervaring hebben gekregen tijdens het opkweken (zie A). Een kruis betekent dat dit type roofmijten niet aangetrokken was tot een geur. (C) Effect van het mengen van twee herbivoor-geïnduceerde geurmengsels op het gedrag van roofmijten. Een kruis betekent geen verstoring. (D) De overeenkomst in samenstelling van de geurmengsels van de verschillende plant-herbivoor combinaties die gebruikt zijn in de experimenten. De dikte van de pijlen tussen twee plant-herbivoor combinaties geeft de mate van overeenkomst aan. (E) Samenstelling van de geurmengsels van limaboon- en komkommerplanten aangevreten door spintmijten en/of rupsen van de floridamot *S. exigua*.

Deze geur is verschillend voor komkommer- en limaboonplanten (Figuur 2A). We vonden dat roofmijten die opgekweekt waren op komkommer wél aangetrokken waren tot de geuren van limaboon- en komkommerplanten met rupsen van de floridamot (Figuur 2B). Het zoekgedrag van deze roofmijten werd ook licht verstoord door het mengen van één van deze geuren met de geur van limaboonplanten met spintmijten (Figuur 2C).

In hoofdstuk 3 heb ik de reactie van roofmijten op de geur van limaboon- of komkommerplanten bestudeerd wanneer deze tegelijkertijd aangetast werden door spintmijten én rupsen van de floridamot. Roofmijten bleken in de Y-buis olfactometer een voorkeur te hebben voor de geur van deze dubbel-aangetaste planten boven de geur van planten die alleen door spintmijten of alleen door rupsen aangetast waren. Dit zou kunnen komen door de grotere hoeveelheid geur die vrijkomt bij deze dubbele aantasting. De chemische samenstelling van de geuren van dubbel-aangetaste planten werd ook geanalyseerd. De resultaten laten zien dat dubbele aantasting leidt tot de productie van veelal dezelfde geurcomponenten die geproduceerd worden na aantasting door alleen spintmijten of alleen rupsen (Figuur 2E). Deze resultaten kunnen niet alleen helpen om het gedrag van de roofmijten te verklaren maar bevatten ook informatie over de manier waarop een plant zich verdedigt tegen een aanval door meerdere soorten belagers tegelijkertijd.

Uit de experimenten in hoofdstuk 2 en 3 kunnen we concluderen dat het zoekproces van de roofmijt *P. persimilis* nauwelijks verstoord wordt door de aanwezigheid van plantengeuren geïnduceerd door niet-prooi rupsen. Het is mogelijk dat andere resultaten gevonden worden als andere plantensoorten of andere niet-prooi planteneters gebruikt worden, bijvoorbeeld bladluizen of witte vliegen. Bovendien moet nog verder uitgezocht worden wat het effect van ervaring van roofmijten is op hun gedrag in zulke situaties (zie ook de paragraaf "*De rol van leren in het zoekgedrag van roofmijten*")

Geurcomponenten die een belangrijke rol spelen

In het tweede deel van dit proefschrift (hoofdstukken 4 en 5) heb ik onderzocht welke componenten een belangrijke rol spelen in het zoekgedrag van *P. persimilis*. Dit is niet zo gemakkelijk te doen omdat herbivoor-geïnduceerde plantengeuren uit zeer veel componenten kunnen bestaan. Bovendien zijn geleedpotigen over het algemeen veel gevoeliger voor deze geurcomponenten dan de apparatuur die gebruikt wordt om de chemische samenstelling van de mengsels te onderzoeken (gaschromatografie-massaspectrometrie). Ik heb dit probleem opgelost door met natuurlijke plantengeuren van een bekende chemische samenstelling te werken. De samenstelling van deze mengsels heb ik vervolgens gemanipuleerd door één of meerdere kunstmatige geurcomponenten toe te voegen.

In hoofdstuk 4 heb ik de rol van de geurcomponent methyl salicylaat onderzocht. Methyl salicylaat is één van de componenten van de geur van limaboonplanten met spintmijten. De roofmijt *P. persimilis* was aangetrokken tot de enkelvoudige component methyl salicylaat. Deze aantrekking was afhankelijk van de dosis methyl salicylaat. Bij een lage dosis (0.002 µg) was er geen aantrekking. Bij een dosis van 0.2 µg vond ik een optimale aantrekking: 71 % van de roofmijten verkoos methyl salicylaat ten opzichte van geen geur in de olfactometer. Echter bij een hoge dosis methyl salicylaat (200 µg) werden de roofmijten afgestoten door deze component. Vervolgens heb ik bestudeerd of roofmijten onderscheid kunnen maken tussen geurmengsels die verschillen in de hoeveelheid methyl salicylaat maar verder van gelijke samenstelling zijn. Hiertoe voegde ik een bepaalde hoeveelheid methyl salicylaat toe aan de geur van limaboonplanten met spintmijten. De keuze van roofmijten tussen twee geuren bleek niet beïnvloed te worden door een verschil in de hoeveelheid methyl salicylaat. Een verschil in de aan- of afwezigheid van methyl salicylaat speelde wel een rol. Limaboonplanten werden behandeld met het plantenhormoon jasmonzuur om ze een geur te laten afgeven die géén methyl salicylaat bevat. Jasmonzuur speelt een belangrijke rol in de verdediging van planten tegen planteneters. De samenstelling van het geurmengsel van jasmonzuur-behandelde planten lijkt verder op dat van limaboonplanten met spintmijten. Roofmijten hadden een voorkeur voor het geurmengsel *met* methyl salicylaat (van limaboonplanten met spintmijten) boven het geurmengsel *zonder* methyl salicylaat (van planten die met jasmonzuur behandeld zijn). Het toevoegen van methyl salicylaat aan de geur van jasmonzuur-behandelde planten had zo'n sterke invloed op het gedrag van *P. persimilis* dat deze haar voorkeur verloor voor de geur van planten met prooien. De experimenten in hoofdstuk 4 tonen aan dat methyl salicylaat een belangrijke rol speelt in het zoekgedrag van de roofmijt *P. persimilis*. Dit werd bevestigd in hoofdstuk 5. In hoofdstuk 5 laat ik eerst zien dat de keuze van roofmijten tussen de geuren van limaboonplanten met rupsen van de floridamot of limaboonplanten met spintmijten afhangt van het aantal spintmijten per blad. Roofmijten hadden een voorkeur voor limaboonplanten met spintmijten ten opzichte van limaboonplanten met rupsen bij een hoog aantal spintmijten (d.w.z. 40 volwassen spintmijt vrouwtjes per blad), maar niet bij een laag aantal (d.w.z. 10 of 20 spintmijten per blad). De chemische samenstelling werd onderzocht van de geur van limaboonplanten met lage en hoge aantallen spintmijten, of met rupsen van de floridamot. De hoeveelheid van de geurcomponent methyl salicylaat die afgegeven werd door limaboonplanten met 40 spintmijten per blad was veel groter dan de hoeveelheid afgegeven door planten met rupsen. Hetzelfde resultaat werd gevonden voor een andere geurcomponent: (*E*,*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraeen (afgekort TMTT). Bij lage aantallen spintmijten was de hoeveelheid methyl salicylaat en TMTT ongeveer hetzelfde in de geurmengsels van planten met spintmijten of planten met rupsen. Het gedrag van de roofmijten en de chemische analyse gaven hiermee een aanwijzing dat methyl salicylaat en TMTT van belang zouden kunnen zijn voor de het keuzegedrag van *P. persimilis*. Vervolgexperimenten met de Y-buis olfactometer toonden aan dat deze twee componenten inderdaad gebruikt kunnen worden door *P. persimilis* om onderscheid te maken tussen de geuren van limaboonplanten aangevreten door spintmijten of rupsen. Verder werd in hoofdstuk 5 de geurcomponent 2-butanone geïdentificeerd als aantrekkelijke component. Deze stof wordt afgegeven door planten met 40 spintmijten per blad en door planten met rupsen. Omdat de hoeveelheid 2-butanone ongeveer gelijk is in beide geurmengsels konden roofmijten deze geurcomponent niet gebruiken om onderscheid te maken tussen de geur van planten met spintmijten of planten met rupsen.

Uit de experimenten van hoofdstuk 4 en 5 kan geconcludeerd worden dat methyl salicylaat en TMTT een belangrijke rol spelen in het zoekgedrag van de roofmijt *P. persimilis*. Het lijkt niet waarschijnlijk dat de aanwezigheid van methyl salicylaat en TMTT altijd de aanwezigheid van spintmijten verraden aan de roofmijt. Er zijn uit de literatuur en uit dit proefschrift namelijk ook situaties bekend waarin roofmijten wel onderscheid maken tussen twee geuren maar waarin geen verschil in de hoeveelheid methyl salicylaat en TMTT aangetoond kan worden. Andere, misschien nu nog onbekende, geurcomponenten spelen dus waarschijnlijk ook een rol in het zoekgedrag van *P. persimilis*. Niettemin is de ontdekking van de rol van methyl salicylaat en TMTT belangrijk. Het is namelijk de eerste keer dat zo'n duidelijke rol aangetoond wordt voor specifieke geurcomponenten als onderdeel van een natuurlijke plantengeur in het maken van onderscheid tussen plantengeuren door natuurlijke vijanden van plantenetende geleedpotigen.

De rol van leren in het zoekgedrag van roofmijten

Zoals al eerder gezegd is, is de geur van planten die door spintmijten aangevreten worden niet altijd gelijk. Deze geur kan verschillen tussen planten van verschillende soorten maar bijvoorbeeld ook tussen planten van één soort die in een andere omgeving opgroeien of van ongelijke leeftijd zijn. De verwachting is dat dieren in een variabele omgeving hun gedrag aan kunnen passen aan de heersende omstandigheden in de omgeving. Dit kunnen dieren door te leren. Ik definieer 'leren' hier als een verandering in gedrag die veroorzaakt wordt door een ervaring met de omstandigheden in de omgeving. In het geval van roofmijten kan dit een ervaring met een plantengeur zijn die de aanwezigheid van geschikte prooien verraadt. De rol van leren in de reactie van de roofmijt *P. persimilis* op plantengeuren werd onderzocht in hoofdstuk 6, 7 en 8.

Tijdens het opkweken van ei tot en met het volwassen stadium ervaren roofmijten de geur van de plant waarop zij hun prooien verorberen. De plantensoort waarop roofmijten opgekweekt worden kan een groot effect hebben op de reactie van volwassen roofmijt vrouwtjes op plantengeuren (zie Figuur 2A). In hoofdstuk 6 werd de reactie van drie groepen roofmijten op methyl salicylaat vergeleken. De eerste groep werd opgekweekt op spintmijten op limaboon (waarvan het geurmengsel methyl salicylaat bevat), de tweede groep werd opgekweekt op spintmijten op komkommer (waarvan het geurmengsel geen methyl salicylaat bevat), de derde groep was gelijk aan de tweede maar tijdens het opkweken werden de roofmijten blootgesteld aan methyl salicylaat. Roofmijten van de eerste en de derde groep waren aangetrokken tot methyl salicylaat, maar roofmijten van de tweede groep niet. Dit experiment toont aan dat ervaring met methyl salicylaat van belang is voor de aantrekking van de roofmijt *P. persimilis* naar deze component. Ervaring met methyl salicylaat tijdens het opkweken had ook een effect op de keuze van roofmijten tussen twee geurmengsels: één *met* methyl salicylaat (afkomstig van limaboonplanten met spintmijten) en één *zonder* methyl salicylaat (van jasmonzuur-behandelde limaboonplanten, zie ook vorige paragraaf).

In hoofdstuk 7 heb ik onderzocht of roofmijten kunnen leren onderscheid te maken tussen geuren van limaboonplanten met hun prooi of planten met rupsen van de floridamot. Ook hier speelde de plantensoort waarop de roofmijten hun prooi verorberd hadden gedurende de ontwikkeling van ei tot en met het volwassen stadium een belangrijke rol. Roofmijten die op spintmijten op limaboon opgekweekt waren, kozen vaker voor de geur van limaboonplanten met spintmijten dan roofmijten die op komkommer opgekweekt waren. Dit komt waarschijnlijk door de sterke aantrekking van roofmijten die op komkommer opgekweekt zijn naar de geuren van planten met *S. exigua* rupsen (hoofdstuk 8). Roofmijten die op limaboon opgekweekt zijn, worden niet aangetrokken door deze geuren (Figuur 2B). Het verschil in aantrekking kan verklaard worden door de geuren waar de roofmijten aan blootgesteld zijn tijdens het opkweken. De geur van komkommer met spintmijten lijkt namelijk meer op de geuren van limaboon- of komkommerplanten met rupsen dan de geur van limaboonplanten met spintmijten (hoofdstuk 3) (Figuur 2D).

Ook tijdens het volwassen stadium werd het keuzegedrag van de roofmijten nog beïnvloed door ervaringen met plantengeuren. Een ‘negatieve’ ervaring (d.w.z. de afwezigheid van geschikte prooien) in aanwezigheid van de geur van limaboonplanten met rupsen, leidde tot een kleine toename in het percentage roofmijten dat voor de geur van limaboonplanten met spintmijten koos in de Y-buis olfactometer. Een ‘belonende’ ervaring had echter een veel groter effect. Een ‘belonende’ ervaring bestond uit het blootstellen van roofmijten gedurende één dag aan de geuren van komkommer of limaboonplanten met spintmijten in de aanwezigheid van hun prooi. Eén dag voeden op spintmijten op komkommer leidde tot een verlies van aantrekking van limaboon-opgekweekte roofmijten naar de geur van limaboonplanten met spintmijten. Eén dag voeden op spintmijten op limaboon leidde tot de aantrekking van komkommer-opgekweekte roofmijten naar deze geuren. Een ‘negatieve’ ervaring gevolgd door een ‘belonende’ ervaring had het grootste effect op de keuze van roofmijten tussen de geur van limaboonplanten met spintmijten en de geur van planten met rupsen.

Het derde deel van dit proefschrift leidt tot de conclusie dat ‘leren’ de roofmijt *P. persimilis* kan helpen om te gaan met de variatie in plantengeuren die gerelateerd kunnen zijn aan de aanwezigheid van geschikte prooien in een natuurlijke omgeving. Hierbij is de ervaring die roofmijten opdoen tijdens de ontwikkeling van ei tot en met het volwassen stadium van groot belang. Deze ervaring zorgt er waarschijnlijk voor dat de reactie van de roofmijten op de geur van de aangevreten plant waarop ze opgroeien optimaal word. Dit voorkomt dat roofmijten een prooihaard verlaten waarin nog voedsel aanwezig is. Het kan gebeuren dat roofmijten tijdens het volwassen stadium op zoek gaan naar prooien op een andere plantensoort dan diegene waarop ze opgegroeid zijn. De ervaring die opgedaan is tijdens de ontwikkeling kan aanvankelijk leiden tot aantrekking naar planten met planteneters die niet geschikt zijn als prooi. Echter, ‘negatieve’ en/of ‘belonende’ ervaringen kunnen de kans vergroten dat roofmijten hun prooi vinden op ‘nieuwe’ plantensoorten.

Tot slot

Met dit proefschrift heb ik een bijdrage geleverd aan het beter begrijpen van de rol van plantengeuren in het zoekgedrag van natuurlijke vijanden van plantenetende geleedpotigen. Hiermee is een belangrijke stap gezet in het ontrafelen van de samenwerking tussen planten en rovers met behulp van plantengeuren. Dit proefschrift is niet alleen interessant vanuit fundamenteel oogpunt. Plantenetende insecten en mijten vormen namelijk een belangrijke bedreiging van verschillende voedselgewassen. Roofvijanden kunnen zulke plagen effectief voorkomen en bestrijden. Fundamenteel onderzoek aan het zoekgedrag van de natuurlijke vijanden van insecten en mijten kan daarmee mogelijk een bijdrage leveren aan de ontwikkeling van efficiëntere biologische bestrijding van plagen. Zo zouden plantenrassen geselecteerd kunnen worden die een grotere hoeveelheid van bepaalde geurcomponenten afgeven na vraat door plaaginsecten of –mijten. Ook zou bijvoorbeeld de rovers aangeleerd kunnen worden op bepaalde geurcomponenten te reageren.

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Ik heb het de afgelopen vier jaar bij Entomologie prima naar mijn zin gehad. En dat komt niet alleen omdat ik het zo leuk vind om roofmijten te kweken, Y-buis experimenten te doen, en plantengeuren op te vangen ☺. Heel veel mensen hebben tijdens mijn promotietijd het werk interessanter, gemakkelijker en leuker gemaakt, en het leven aangenamer. Daarom is het nu tijd voor een dankwoord.

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Samen met jou kan ik de wereld aan!

Jetske
Wageningen, December 2003

CURRICULUM VITAE

Op 16 augustus 1976 werd Jetske Gudrun de Boer geboren in Culemborg. Binnen een jaar verhuisde ze naar Niewerkerk a/d Ijsel. Toen Jetske 6 jaar was verhuisde ze naar Schoonhoven, waar ze naar de lagere en middelbare school ging. Na het behalen van het VWO diploma op de "Willem de Zwijger" scholengemeenschap in 1994, begon ze aan de studie 'Plantenveredeling en gewasbescherming' aan de toenmalige Landbouw Universiteit in Wageningen. Tijdens het eerste afstudeerproject bij de vakgroep Entomologie bestudeerde ze onder begeleiding van Joop van Loon en Marcel Dicke het effect van parasitering van rupsen van het kleine koolwitje door de sluipwesp *Cotesia rubecula* op de zaadproductie van Arabidopsis planten. Hiermee werd de modelplant *Arabidopsis thaliana* geïntroduceerd in de studie naar indirecte verdediging van planten tegen herbivore insecten. Vervolgens vertrok ze naar Zwitserland om vier maanden stage te lopen bij de 'Forschungsanstalt für Agrarökologie und Landbau' in Zürich. Terug in Wageningen deed ze een afstudeerproject bij Dick Peters van de vakgroep Virologie, aan de transmissie van verschillende tospovirussen door verschillende thrips soorten. In een derde afstudeerproject werd gewerkt aan het effect van transgene insect-resistente appelbomen op de roofmijt *Phytoseiulus persimilis*, een natuurlijke vijand van niet-doel herbivoren. Dit afstudeerproject werd uitgevoerd op het Rothamsted 'Institute for Arable Crop Research' in Harpenden, Engeland, en voor dit werk werd een C.T. de Wit scriptieprijs toegekend. In september 1999 werd de Ingenieurstitel 'cum laude' behaald. Van november 1999 tot december 2003 werkte ze aan een promotie project bij de vakgroep Entomologie van Wageningen Universiteit onder begeleiding van Marcel Dicke en Maurice Sabelis (Universiteit van Amsterdam). In oktober 2002 werd een 'Storm van der Chijs' stipendium uitgereikt voor veelbelovende vrouwelijke promovendi van Wageningen Universiteit. Haar onderzoek aan de respons van de roofmijt *P. persimilis* op herbivoor-geïnduceerde plantengeuren is beschreven in dit proefschrift.

LIST OF PUBLICATIONS

JJA van Loon, **JG de Boer** & M Dicke (2000) Parasitoid-plant mutualism: parasitoid attack of herbivore increases plant reproduction. Entomologia Experimentalis et Applicata 97 (2): 219-227

M Dicke, **JG de Boer**, M Höfte & MC Rocha-Granados (2003) Mixed blends of herbivore-induced plant volatiles and foraging success of carnivorous arthropods. Oikos 101:38-48

M Dicke, RMP van Poecke & **JG de Boer** (2003) Inducible indirect defence of plants: from mechanisms to ecological functions. Basic and Applied Ecology 4:27-42

JG de Boer & M Dicke (2004) The role of methyl salicylate in prey searching behavior of the predatory mite *Phytoseiulus persimilis*. Journal of Chemical Ecology, in press

JG de Boer & M Dicke (2004) Experience with methyl salicylate affects behavioural responses of a predatory mite to blends of herbivore-induced plant volatiles. Entomologia Experimentalis et Applicata, in press

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