The challenge of locating an endophytic host plant chemical cues and learning strategies in a parasitoid of apple leafminers

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The challenge of locating an endophytic host: plant chemical cues and learning strategies in a parasitoid of apple leafminers

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1. Summary

Upon landing on an infested leaf parasitoids of leafminers are faced with the challenge of locating their concealed host. Although studies have shown that visual, acoustic (Sugimoto et al., 1988b) and vibrational cues (Meyhöfer et al., 1994; Meyhöfer et al., 1997b) are involved in orienting leafminer parasitoids to their endophytic host, the significance of chemical cues has scarcely been studied. This is in contrast to the wealth of knowledge on the function and identification of host-derived chemical cues used by parasitoids attacking free-living herbivores (Rutledge, 1996). For the successful foraging of a parasitoid of an endophytic host, the key mechanisms underlying host location remain to be elucidated. The first aim of this study was to investigate the role of chemical cues in the interspecific relationship between the apple leafminer *Phyllonorycter pomonella* Zeller (Lepidoptera: Gracillariidae) and its parasitoid *Pholetesor bicolor* Ness (Hymenoptera: Braconidae).

In chapter 4 we elucidated the source of chemical cues mediating host location once the parasitoid has found an infested patch. The damaged leaf epidermis (mine), the larva, and the frass (faeces and silk) from *P. pomonella* were examined as likely sources of behavioural stimuli. The mine elicited ovipositional probing behaviour of parasitoid females. Probing on larvae or frass was seldom observed. Solvent extracts of the different components were offered to parasitoids in contact behavioural bioassays. Only hexane extracts of mines elicited an ovipositional probing response. No response was observed with hexane extracts of larvae or frass, or with methanol and diethyl ether extracts. Unlike parasitoids attacking free living herbivores which use chemical cues from the host, this leafminer parasitoid uses plant-derived semiochemicals for ovipositional probing behaviour and therefore in host location. This unexpected finding does not support the hypothesis that host-derived cues are reliable cues in host location of parasitoids (Vet et al., 1995). In this leafminer-parasitoid system plant- and not host-derived cues are involved in host location
possibly because the parasitoid has no immediate contact with the endophytic host upon landing or walking on the leaf.

Based on this finding, in chapter 5 we investigated the association between the chemical response of apple leaves to herbivory and parasitoid behaviour. Chemical analyses of solvent extracts from healthy and leafminer-infested leaves were performed together with behavioural bioassays. Results revealed that leafminer herbivory increased the amount of the triterpene squalene \((C_{30}H_{50})\) in apple leaves. Moreover, squalene elicited the characteristic ovipositional probing behaviour of naïve \(P.\ bicolor\) females demonstrating that this substance is innately recognised and used by the parasitoid for host location after landing on a host infested patch. Subsequent tests were performed with this substance to determine if the parasitoid used it in habitat location. In a Y-tube olfactometer bioassay, female parasitoids did not respond to this substance, while they were attracted to herbivore-induced plant volatiles. This result suggests that \(P.\ bicolor\) uses contact chemical cues for host location and volatile cues for habitat location. In both cases the cues are of plant origin.

\(P.\ bicolor\) is not only confronted with the difficult task of locating its endophytic host but this specific host has two host stages with different feeding habits (i.e., sap- and tissue-feeder larvae). The host feeding habits result in mine with different characteristics. The second aim of this work was to determine whether \(P.\ bicolor\) is able to find, accept and parasitize both host stages of the leafminer. In addition we investigated if the ability of this parasitoid to discriminate between the host stages depends on a previous experience with each host stage. For this purpose we conducted preference tests for parasitisation and behavioural bioassays. Although in laboratory bioassays \(P.\ bicolor\) can parasitize both host stages, parasitizing tissue-feeders is very time consuming and in most cases females failed to parasitize this host stage. In contrast female parasitoids were always able to parasitize sap-feeder hosts. Upon first contact with sap- or tissue-feeder hosts, naïve females were not able to discriminate between the two host stages. However, a previous experience
with the sap-feeder host enhanced the ability of the parasitoid to locate the most successfully parasitized hosts, namely the sap-feeder. An experience with tissue-feeders did not enhance the ability of females to locate tissue-feeder hosts. Although experience has been shown to contribute to more efficient foraging behaviour for parasitoids in habitat and microhabitat location, this is the first report of experience contributing to host stage discrimination. This ability to discriminate between the two host stages seems to be highly adaptive for *P. bicolor* given that in the field leafminer generations tend to overlap resulting in both host stages occurring simultaneously.
2. Zusammenfassung


Es wurde untersucht, welches die Quelle von chemischen Reizen ist, die dem Parasitoiden nach Auffinden eines befallenen Blattes zur Lokalisierung des Wirtes verhilft. Als mögliche Quellen von verhaltensbeeinflussenden Stimuli wurden die durch den Wirt verletzte Blattpidermis (Mine), die Wirtslarve selbst sowie Kot und Seide von *P. pomonella* untersucht. Es wurde festgestellt, dass die Mine bei Parasitoidenweibchen Eiablageverhalten auslöste, während sich die Parasitoiden kaum auf der Wirtslarve oder auf dem Kot oder der Seide aufhielten. In einem Kontakt-Versuch wurden den Parasitoiden mit Lösungsmitteln extrahierte Proben der verschiedenen potentiellen Duftquellen angeboten. Eiablageverhalten von *P. bicolor* wurde nur bei Hexan-Extrakten der Mine beobachtet. Die Parasitoidenweibchen reagierten weder auf Hexan-Extrakte von Larven, Kot und Seide noch auf Methanol- und Diethylether-


Eine weitere Schwierigkeit für *P. bicolor* ist neben der Lokalisierung des endophytischen Wirts die Tatsache, dass *P. pomonella* Larven in der Entwicklung zwei Typen mit unterschiedlichen Ernährungsweisen durchlaufen:
3. General Introduction

Biological control as a component of pest management in agroecosystems gains importance due to increasing ecological demands. More sustainable approaches for food production require reduction of pollution, food contamination, and resistance management. Insect behaviour and factors that mediate behaviour have to be understood to assure the success of biological control (Dorn, 1995). The role of semiochemicals which mediate parasitoid foraging behaviour has received increasing attention in the last twenty years, even though only a limited number of insect species has been studied. Attention to the fundamental mechanisms of foraging behaviour of parasitoids does not only provide an understanding of plant-insect interactions but it can also provide tools for improvements of pest management.

Recent ecological research focuses on multitrophic systems in which natural enemies (parasitoids or predators) are classified as the third, their hosts or prey as the second and the food of their host (plant) as the first trophic level. In contrast to the bitrophic approach of the past, it is now commonly accepted that to understand interactions between parasitoids and their hosts it is important to recognise the influence that plants have. Induced responses of plants to herbivory and the influence of these plant responses for parasitoid host location have only recently received attention (Karban & Baldwin, 1997). Parasitoids use both olfactory stimuli and visual stimuli from the plant during host-habitat location (Godfray, 1994; Vinson, 1985), and plant characteristics influence the ability of parasitoids to find and parasitize hosts (Price, 1997; Romeis et al., 1998). In addition, the host food-plant might provide a source of food in the form of floral nectar and pollen for parasitoids or may influence parasitism by masking attractive odours from the host plant (Price et al., 1980).

A sequence of responses to different information sources brings the searching parasitoid into close vicinity of its host. Female parasitoids go through
a series of host selection processes which are divided as habitat location, host location, and host acceptance (Doutt, 1964; Vinson, 1984). Within each of these host selection steps, chemical information from the plant-host complex plays an important role. Plants can attract parasitoids in the absence of hosts (Elzen et al., 1983), but parasitoids respond as well to chemical cues released by herbivore damaged plants (Mattiacci et al., 1994; Potting et al., 1995; Turlings & Tumlinson, 1992; Udayagiri & Jones, 1992). As the parasitoid gets closer to its host, host-derived stimuli, e.g. faeces, silk, cuticle, etc., seem to influence successful host location, acceptance and oviposition (Vinson, 1985).

Abundant information on the sources of chemical cues and the behaviour that they elicit in parasitoids is available (Vinson, 1985; Vinson, 1986; Vinson, 1991). In contrast the knowledge on the identity of the chemicals involved in plant-insect interactions is so far very limited (Rutledge, 1996). Although sophisticated methods for separating and identifying chemicals are available, many difficulties are encountered in attempting to identify the chemicals used by insects. Many semiochemicals are produced and detected in nanogram or subnanogram quantities. In several cases semiochemicals are comprised of multiple compound blends in which the quantities and ratios are important. Minor components of mixtures may comprise only a small percent of the total blend, however they may be critical in eliciting insect responses. Compounds may also be unstable upon extraction or analysis. For successful identification effective behavioural bioassays are also required. These behavioural bioassays have to be performed during each step of the extraction, separation and identification.

Vet and co-authors (Vet et al., 1995; Vet et al., 1991) argue that the ecological value of chemical information for searching parasitoids depends on two factors: 1) the reliability in indicating the presence of hosts, and 2) the detectability of the stimulus. They suggest that host-related chemical cues are reliable but not detectable, and used at close range host location. Moreover, due to their reliability, host-derived stimuli are likely to evoke strong innate responses that are not substantially modified by learning (Vet et al., 1995). On
the other hand, plant and other environmental cues are expected to be more variable in nature and to have lower reliability, thus being used in habitat location. These stimuli are likely to become the most useful cues in foraging once the parasitoid has experienced them.

Learning is a common phenomenon in insect parasitoids (Vet & Groenewold, 1990). Both pre-adult and adult experience influence the searching behaviour of parasitoids. Pre-adult learning occurs as a result of development in a certain host or a certain food substrate (Cortesero et al., 1995; Hérard et al., 1988). Adult learning occurs during host searching of female. Different types of adult learning have been defined; e.g. 1) habituation, the waning of the response to a stimulus with repeated exposure to that stimulus, 2) sensitisation ('priming'), a general increase in responsiveness as a result of exposure to an innately recognised stimulus, 3) associative learning, where the parasitoid associates innately recognised stimuli (unconditioned stimuli) with new stimuli (conditioned stimuli) to which they become responsive in their succeeding foraging behaviour (Turlings et al., 1993; Vet et al., 1995). Priming as well as associative learning may be key factors in improving the host-searching behaviour of biological agents in the field (Hare et al., 1997).

Parasitoid host-searching behaviour is not only influenced by previous experience but also by the physiological state of the parasitoid, such as mating, egg load, hunger (Drost & Carde, 1992; Lewis et al., 1998; Wäckers, 1994), its ‘motivational state’ (Vinson, 1998), and even by genetically fixed intraspecific characteristics (Gu & Dorn, 2000). Once a parasitoid has encountered a host it has to decide whether to accept the host for oviposition. Although parasitoids can develop successfully in different larval instars of the same host, the cost of parasitism (van Alphen & Jervis, 1996) and the suitability may vary between different host instars or different host species (Brodeur et al., 1998; Petitt & Wietlisbach, 1993; Slansky, 1986; Vinson & Iwantsch, 1980). The availability and detectability of a host in a habitat (Sait et al., 1997) may modify the value (Mackauer et al., 1996) of the host in terms of its suitability or the time required to parasitize. In the case of parasitoids attacking concealed hosts, host
accessibility and acceptance can be difficult and even risky (Mattiacci et al., 1999; Potting et al., 1997).

The system studied and thesis outline

Phyllonorycter leafminers are of world wide economic significance in apple (Malus domestica) orchards (Balázs et al., 1996; Blommers & Vaal, 1996; Ciampolini et al., 1988; Dorn, 1993b; Pottinger & LeRoux, 1971; Reissing et al., 1982). Although a proper classification of Phyllonorycter species is missing, those species attacking apple trees seem to have similar biological and morphological characteristics. A general description of the biology of P. blanda, a closely related species to P. pomonella is given by Pottinger & LeRoux (1971). Adult moths deposit single eggs on the lower side of leaves. First instar larvae bore into the leaf and start making a mine. Larvae go through five larval instars; the first three so called sap-feeders and the last two tissue-feeders. The two hypermetamorphic stages are distinguished by the different types of larval mouthparts that are adapted to the two developmental periods with specialised mining habits. The flat-shaped sap-feeders make a U-shaped mine between the lower epidermis and the palisade mesophyll which is only visible with the naked eye upon careful examination. The cylindrical-shaped tissue-feeders chew out palisade mesophyll cells resulting in green spotted appearance on the upper side of the leaf. The larva spins silk threads on both sides of the mine which cause the mine to arch (tent form). The pupae remain in the mine until emergence.

Economic losses due to Phyllonorycter leaf miners are attributed to premature leaf fall, reduction in terminal bud growth, reduction in fruit size, premature ripening, fruit drop and reduction in fruit set the following season (Kappel & Procor, 1986; Pottinger & LeRoux, 1971; Reissing et al., 1982). Whole ecosystem studies (Dorn, 1993b; Dorn et al., 1999) and long term ecological studies (Balázs, 1984; Balázs, 1989; Balázs et al., 1996; Weiss & Vogt, 1994) have demonstrated that selective means of control such as the insect growth regulator fenoxycarb or an insecticide together with a combination
of parasitoid species can keep *Phyllonorycter* leafminers at acceptable economical levels.

The *Phyllonorycter* population supports a parasite fauna that is biologically variable and very rich in species (Balázs, 1989; Casas & Baumgärtner, 1990; Gagné & Barrett, 1994). However, only a few studies have looked into the possible mechanisms responsible for the control of *Phyllonorycter* leafminers by their natural enemies. A limited amount of behavioural research has been done with two of the most commonly found parasitoid species in European apple orchards, *Sympiesis sericeicornis*, Nees (Hym. Eulophidae), and *Pholetesor bicolor* Nees (Hym. Braconidae) (Balázs, 1989; Balázs et al., 1996; Dorn, 1993a; Dorn et al., 1999). Previous work of our group investigated the role of vibrational communication for host location by *S. sericeicornis* (Casas, 1989; Dorn et al., 1999; Meyhöfer et al., 1994; Meyhöfer et al., 1997a; Meyhöfer et al., 1997b). Work has also been conducted on the role of chemical stimuli for habitat location of *P. bicolor*, showing that this parasitoid upon previous exposure to infested plants uses herbivore-induced plant volatiles for habitat location (Dorn et al., 1999; Lengwiler et al., 1994). However, nothing is known on the role that chemical stimuli have on host location once the parasitoid has landed on an infested leaf. In a system where the host is concealed and the parasitoid has no direct contact with its host the source of chemical cues might differ to that of non-concealed hosts. In the first part of this thesis (Chapters 4 and 5) we elucidate the source of chemical cues, identified the compounds mediating host location and reported on the chemical response of apple seedlings to leafminer herbivory.

*P. bicolor* is a solitary koinobiont larval endoparasitoid with a relatively broad host range (Papp, 1988). In apple orchard this parasitoid is faced with the choice between different leafminer host stages, which have different mine characteristics (sap- and tissue-feeders). Until now no biological studies were available for this species. For example, the host stage preference, acceptance and suitability of the parasitoid are unknown. In the second part of this thesis (Chapter 6) these gaps on the biology of the species were investigated, here we
focused on host stage discrimination and the influence of experience on host stage selection.
4. Plant derived semiochemicals as contact host location stimuli for a parasitoid of leafminers¹

4.1. Abstract

Semiochemicals mediating host location of parasitoids after landing on a host-infested patch are most often produced by the host or they are host by-products. In this study we elucidated the source of chemical cues in a system where the host is concealed and the parasitoid has no direct contact with the host larvae or its frass. Behavioural bioassays with *Pholetesor bicolor*, a larval parasitoid of the apple leafminer, *Phyllonorycter pomonella*, showed that the herbivore damaged leaf epidermis (mine) elicited ovipositional probing of parasitoid females. Probing on larvae or frass was seldom observed. Hexane extracts of mines elicited the same ovipositional probing behaviour while no response was observed with hexane extracts of larvae or frass or with methanol and diethyl ether extracts. In addition, gas chromatographic analyses showed qualitatively and quantitatively different profiles of these three components of the host-plant complex. By far the highest quantities and also the highest number of compounds were recovered from mine extracts. Identified compounds in the mine included six n-alkanes (n-C₂₇ to n-C₃₃) and squalene (C₃₀H₅₀). A synthetic blend of the seven compounds was slightly less active in biotests than the equivalent natural blend, as shown by a time delay in female response. We conclude that this leafminer parasitoid does not rely on host derived kairomones but instead uses plant derived semiochemicals for host location and ovipositional probing behaviour.

¹ Based on: Dutton, A., Mattiacci, L. & Dorn, S., Plant derived semiochemicals as contact host location stimuli for a parasitoid of leafminers. (Submitted to Journal of Chemical Ecology).
4.2. Introduction

It is well known that chemical signals play an important role in the interaction between plants, phytophagous insects, and their natural enemies. Foraging parasitoids use chemical cues in the various stages of the host selection process, which is divided into habitat location, host location, host acceptance and oviposition (sensu Vinson, 1984). These semiochemicals have been shown to arise from the food of the host, the host itself, host by-products, associated organisms or interactions among sources (Godfray, 1994; Vinson, 1988). The source and function of various semiochemicals in different parasitoid-host systems have been determined. Some of these chemicals have been extracted, identified (see Rutledge, 1996 for a review) and experimentally used for rearing or for improving parasitism (Jones et al., 1973; Lilley et al., 1994). Elucidation of the chemicals utilised in each step of parasitoid foraging not only offer an understanding of plant insect interactions, but can provide tools for improvements in biological control (Hare & Morgan, 1997).

Although identification of over 47 different kairomones and synomones involved in the various stages of habitat and host location (Horikoshi et al., 1997; Ohara et al., 1996; Rutledge, 1996) have been accomplished, none of these studies has dealt with parasitoids of leafminer hosts. In five leafminer-parasitoid systems (including the one in study), it has been demonstrated that herbivore induced plant-related semiochemicals are used for habitat location (Dicke & Minkenberg, 1991; Finidori-Logli et al., 1996; Keller & Horne, 1993; Lengwiler et al., 1994; Petitt et al., 1992; Sugimoto et al., 1988a), but no identification of the active synomone(s) is available. Furthermore, the role of non-volatile chemical cues for host location, acceptance or oviposition has not been addressed in concealed host systems where the parasitoid has no direct immediate contact with the host upon landing or walking into a host infested patch. So far only visual, acoustic (Sugimoto et al., 1988b) and vibrational cues (Meyhöfer et al., 1994; Meyhöfer et al., 1997b) have been investigated for parasitoids foraging for concealed leafminer hosts. Given the unique situation of a concealed host, we are interested in determining to what extent chemical
cues influence the parasitoids searching behaviour once it has landed on a mined leaf.

The leafminer system studied here consists of Pholetesor bicolor Ness (Hymenoptera: Braconidae), a solitary koinobiont endoparasitoid with a relatively broad host range (Papp, 1988). One of its hosts is the apple leafminer Phyllocnistis pomonella Zeller (Lepidoptera: Gracillariidae). This parasitoid uses volatiles from leafminer-infested apple plants for habitat location (Dorn et al., 1999; Lengwiler et al., 1994) and possibly also visual cues. After landing on an infested leaf, female wasps start searching on the lower side of the leaf. Upon contact with a mine females follow the cascade of host acceptance events described by Vinson (Vinson, 1984), which starts with a) encounter and examination, b) thrust with the ovipositor, c) ovipositor insertion and finally d) oviposition. The number of thrust bouts with the ovipositor into the mine ranges from 2 to 490 according to the host-stage larva present in the mine (Chapter 6). This thrust with the ovipositor into the mine may have the function of host location or host examination (Vinson, 1991); we refer to it using the more descriptive term ovipositional probing behaviour. The aim of this study was to determine the source of chemical cues involved in the ovipositional probing behaviour of P. bicolor attacking the apple-leafminer. The lower leaf epidermis damaged by the herbivore (from now on referred to as the mine), the larvae and the frass of P. pomonella were examined as likely sources of behavioural stimuli. We further identified the chemicals through solvent extraction, isolation and gas chromatography coupled with mass spectrometry (GC-MS).

4.3. Materials and methods

**Plants and insects**

Two-months-old apple seedlings (Malus sylvestris c.v. Golden Delicious, Rosaceae) were used for the rearing of insects and for all experiments. Plants were grown in climatic chambers 25°C and 17°C (L:D), 70 % r.h. and a
The leafminer, *P. pomonella* (det. Deschka) colony originated from individuals collected in apple orchards in South Tyrol (Northern Italy). Insects were maintained at 23 ± 2°C, L16: D8 photoperiod and 50 ± 10% r.h. In order to obtain infested plants for rearing and for experiments, thirty 2-3 day old adult moths were released on 6 apple seedlings in a cage. Moths were allowed to oviposit for 24 h, after which they were removed and plants were kept until the leafminer had reached the desired larval instar.

The *P. bicolor* (det. Papp) colony originated from individuals collected in 1995 from the same area in Northern Italy. Insects were maintained at 20 ± 2°C, 50 ± 10% r.h and a photoperiod of L16:D8. In each rearing cage, two to three *P. bicolor* females were provided with 3-4 leafminer infested apple seedlings and allowed to parasitize for four days. Parasitoids were removed and plants with parasitized leafminers were kept until pupation. Subsequently, parasitized leafminers were separated from non-parasitized leafminers, and kept in a cage until emergence. Emerged parasitoids were provided with honey and water. Three to six day old naïve female parasitoids were used for all experiments.

**Bioassays**

*Bioassay arena.* All behavioural tests were performed in a single choice Petri dish arena (10 cm Ø). Parasitoids were placed individually in glass vials (2 cm Ø and 4 cm high) and the open end of the vial was positioned over the cue-sources or an extract-treated filter paper (1.3 cm Ø) placed on the Petri dish. The extract-treated filter paper was kept at a 45° angle to the surface of the Petri dish using a rolled piece of Teflon. Based on preliminary observations parasitoids appeared to be more responsive towards the filter paper when placed in this manner, than when placed flat. Parasitoids were observed for 20 or 90 min according to whether they were offered the biological cues (source or solvent extract) or the synthetic compounds. The observation time was increased because parasitoid response appeared to be slower when offered the
synthetic compounds than when offered the biological extract. However, for all experiments, we provided the number of individuals which responded within the initial 20 min. In the extract bioassays females which were observed probing on the Petri dish surface below the filter paper or the filter paper itself for more that 5 continuous seconds were considered to respond positively to the treatment. Behavioural observations were conducted under laboratory conditions; 23 ± 2 °C, 50 ± 10 % r.h. 1000 ± 500 lux. Twenty to 30 females were tested for each treatment and a control was always tested simultaneously. For statistical analysis a chi-square test was performed between treatments and controls to test differences in parasitoid response.

Cue sources. Parasitoids were offered the following individual components (cue source) of the plant-leafminer complex:

a) one leafminer larva at developmental instar 4th-5th (LARVA)

b) frass from one larva at developmental instar 4th-5th (FRASS)

c) the lower leaf epidermis damaged by one larva (approx. 1.2 cm²) (MINE)

e) empty Petri dish (CONTROL).

Three behavioural parameters were recorded, that is time spent walking, standing and probing on the cue source. The parameters were continuously recorded over a 20 minute period using the computer software The Observer (Noldus, 1991). Each treatment was replicated 10 times. The duration of each behavioural parameter was analysed by Kruskal-Wallis ANOVA followed by the Student-Newman-Keuls for pairwise multiple comparisons between treatments (Sokal & Rohlf, 1981)

Extraction and isolation. Larvae, frass and mine extracts were obtained by soaking these cue sources individually in solvents of increasing polarity (hexane, diethyl ether and methanol) at 4°C for 24 h. The extracts were concentrated so that 10 µl extract was equivalent to one larva (approx. 0.64 mg), the frass of one larva (approx. 0.54 mg) or one mine (approx. 1 mg). For bioassays 10 µl of extract was applied to the filter paper and offered to
parasitoids. Only the extract that elicited significant probing behaviour was further filtered through silica gel (Supelco LC-Si, no bonded phase) and tested. This was done to ensure that non-polar compounds were indeed responsible for the parasitoids behaviour and furthermore to produce cleaner extracts for analysis with GC-MS. Solvent extracts of larva, frass, and mine or the pure solvents (hexane, diethyl ether or methanol; purity 99.5-99.8%) were offered to parasitoids on a filter paper.

**Chemical analyses**

**Extracts**

*Mine.* Five hundred mines (lower leaf epidermis) of tissue-feeder larvae were removed from the infested leaves with tweezers, weighed and soaked in 5 ml hexane (purity 99.7) for 24 h at 4°C (1 mg mine = 1 mine / 10 µl hexane). The solvent was transferred to a clean vial and to remove all residues from mines, an additional 5 ml of solvent was applied to the mines and immediately extracted. The two extracts were combined and stored at -60°C. Subsequently, the extract was centrifuged, filtered through silica gel (100 mg silica gel/1 ml extract) packed into a 13 mm Swinny syringe holder (Millipore) at room temperature. The silica gel was initially conditioned with 200 µl hexane. After the extract was filtered, a similar quantity of hexane was applied to remove all residues. The extract was concentrated by evaporating excess solvent under a stream of nitrogen (purity 99.96 %) to a final concentration of 14 mines (14 mg) equivalent/µl solution.

* Larvae and frass.* The larvae and frass obtained from the dissected mines were removed, weighed and the same extraction as used above for the mines was conducted separately. Each extract to be analysed was equivalent to 14 larvae (9 mg/µl) or the frass from 14 larvae (7.5 mg frass/µl).

**Gas chromatographic quantification (GC).** Four separate extracts of each treatment (larva, frass and mine) were analysed with a HP 5890 Series II Plus gas chromatograph, equipped with a flame ionisation detector set at 300°C and a non-polar HP-1 (Crosslink Methyl Silicone Gum) capillary column (30m x 0.25 mm x 0.25 µm film thickness). The oven temperature was held at 100°C for 2
min, then increased to 300°C at a rate of 6°C/min and held at 300°C for 15 min. The split/splitless injector was operated in splitless mode throughout the analyses. Injector temperature was set at 250°C. The carrier gas was helium (purity 99.96 %) at a constant pressure of 108 kPa. Samples of 1μl were injected with 100 ng of octylbenzene as internal standard.

Quantitative differences among treatments for each of the identified compounds were analysed with Kruskal-Wallis ANOVA followed by the Student-Newman-Keuls test to make pairwise multiple comparisons between treatments.

Identification through coupled gas chromatography-mass spectrometry (GC-MS). GC analyses revealed that samples from mines contained the same substances as present in whole leafminer infested leaves, but in higher concentrations. Given the laborious work of removing leaf epidermis from mined leaves we decided to make whole leaf hexane extracts for identification. Leaf extracts were analysed using a gas chromatograph HP 6890 Series connected to an electron-impact (EI) HP 5973 MS. The chromatographic conditions were set as described above. The MS temperature parameters were: 230°C at the source, 150°C at the quadruple, and 280°C at the interface. The masses were swept at 23-600 mass units at a rate of 2.6 scans/sec. The identity of the compounds was confirmed by co-injections with authentic samples. Synthetic compounds were obtained from Fluka Chemie AG, Buchs (CH) and were ≥ 99.5% pure.

Behavioural activity of synthetic mixture. A mixture of the synthetic n-alkanes (n-C_{27}, n-C_{29}, n-C_{30}, n-C_{31}, n-C_{32}, n-C_{33}), and squalene (C_{30}H_{50}) (Fluka Chemie AG) at the concentration identified in the mined leaf extract, and the control solvent (hexane) were offered to parasitoids in the filter-paper Petri dish bioassay as explained above.
4.4. Results

**Cue sources.** Surprisingly, parasitoid females spent more time standing on mines or larvae than on frass (Figure 4.1, P < 0.05, Kruskal-Wallis ANOVA). In addition, ovipositional probing occurred for the longest period of time on mines, on the average 45 sec, significantly exceeding the time spent probing on other components of the plant-host complex or the control (P < 0.05, Kruskal-Wallis ANOVA). Frass did not elicit any behavioural response of the parasitoid. These results indicate that the leaf mine is the major source of stimuli for ovipositional probing behaviour of the parasitoid.

![Figure 4.1](image)

Figure 4.1. Behavioural activity of the parasitoid *P. bicolor* on various components (mine, larva, frass) of the herbivore-plant complex (*Phyllonorycter pomonella-Malus sylvestris*). Statistical differences in time duration spent for each activity on different treatments are shown with different letters (Kruskal-Wallis ANOVA, P < 0.05).

**Extraction and isolation.** Extracts with solvents of decreasing polarity of the mine, the larva and the frass were tested for their bioactivity on the parasitoid females (Figure 4.2). It was the hexane extract from the mine that
Figure 4.2. Ovipositional probing response of *P. bicolor* females to mine, larvae and frass solvent extracts of increasing polarity. Parasitoid response on a treated filter-paper with extract (e) or control solvent (C) (**${\chi}^2 = 13.3$, P < 0.001** for the only significant combination hexane extract from mines vs. hexane).
triggered significant ovipositional probing behaviour of *P. bicolor* \( \chi^2 = 13.3, P < 0.001 \). As many as 75 % of the females were stimulated to probe on this
extract. Some probing activity was observed as well for the diethyl ether extract of the mine, but it was not significantly different from the effect of the control solvent. No probing at all occurred on the methanol mine extract of the mine.

**Chemical identification and quantification.** A dramatic quantitative difference was found among the compounds extracted from the mine as compared to the compounds extracted from the larvae and the frass (Figure 4.3 and Table 4.1).

**Table 4.1.** Compounds identified from hexane extracts of larvae, frass and mines from *P. pomonella*. N = number of samples analysed in each treatment; + to ++++ = presence of compounds in one or more of the replications.

<table>
<thead>
<tr>
<th>Peak</th>
<th>Compound</th>
<th>Amounts (ng) in Larva† (N = 4)</th>
<th>Amounts (ng) in Frass‡ (N = 4)</th>
<th>Amounts (ng) in Mine§ (N = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Heptacosane</td>
<td>4.2 ± 1.4 ++++ a†</td>
<td>1.6 ± 1.4 ++ a‡</td>
<td>28.3 ± 5.3 ++++ b§</td>
</tr>
<tr>
<td>2</td>
<td>Squalene</td>
<td>0.9 ± 0.6 ++ a</td>
<td>0.1 ± 0.1 + a</td>
<td>37.4 ± 13.3 ++++ b§</td>
</tr>
<tr>
<td>3</td>
<td>Nonacosane</td>
<td>1.4 ± 0.6 +++ a</td>
<td>1.1 ± 0.8 ++ a</td>
<td>127.2 ± 30.4 ++++ b§</td>
</tr>
<tr>
<td>4</td>
<td>Triacontane</td>
<td>0.4 ± 0.4 + a</td>
<td>0.4 ± 0.4 + a</td>
<td>20.1 ± 5.5 ++++ b§</td>
</tr>
<tr>
<td>5</td>
<td>Hentriacontane</td>
<td>0.7 ± 0.7 + a</td>
<td>0.6 ± 0.6 + a</td>
<td>242.7 ± 93.6 ++++ b§</td>
</tr>
<tr>
<td>6</td>
<td>Dotriacontane</td>
<td>-</td>
<td>-</td>
<td>28.9 ± 5.5 ++++</td>
</tr>
<tr>
<td>7</td>
<td>Tritriacontane</td>
<td>5.4 ± 3.1 + a</td>
<td>-</td>
<td>82.6 ± 10.3 ++++</td>
</tr>
<tr>
<td></td>
<td><strong>Total</strong></td>
<td><strong>13.0</strong></td>
<td><strong>3.7</strong></td>
<td><strong>567.1</strong></td>
</tr>
</tbody>
</table>

† Average (ng ± SE) found in one larva (approx. 0.64 mg raw material), frass from one larva (0.54 mg) and one mine (1.0 mg)
‡ the synthetic mixture used for behavioural bioassays was composed of these substances in the respective quantities
§ Letters represent significant difference between treatments (Student-Newman-Keuls method)

Seven compounds from the herbivore damaged leaf epidermis were identified. These compounds reaching the highest quantities in the mine extract, totalling to 567 ng. The identified compounds consisted of six long chain hydrocarbons (*n*-C27 to *n*-C33), and a terpene, squalene (*C30H50*). In the larva and frass extracts, only 13 and 4 ng of these compounds were recovered, respectively. Qualitative differences among the treatments were also found for the different...
compounds identified. In fact compounds such as tritriacontane and dotriacontane were not recovered from the larva or the frass. However, there were other peaks detected in the frass and larva extracts and not in the mine extract. The identification of these compounds was not obtained.

*Behavioural activity of synthetic mixture.* The mixture of the seven synthetic compounds identified from the mine elicited significant ovipositional probing of *P. bicolor* at a concentration equivalent to that of one mine ($\chi^2 = 26.3$, $P < 0.001$).

![Figure 4.4. Comparison of probing response of *P. bicolor* to filter paper treated with mine extract or with synthetic mixture. Symbols indicate the cumulative percentage of females probing every 10 min. (Asterisks representing $\chi^2$ difference at $P < 0.05, 0.01$ and $0.001$, tested sample against the solvent control).](image)

However, the female response to the synthetic blend of compounds identified from the mine was slower than that observed with the biological mine extract (Figure 4.4).
4.5 Discussion

Previous work in other parasitoid-host-plant systems has shown that oviposition related behaviour such as probing can be stimulated by frass (Vinson et al., 1976), scales (Jones et al., 1973; Millar & Hare, 1993), silk (Weseloh, 1977), eggs (Kainoh, 1988), mandibular gland secretions (Corbet, 1973), host hemolymph (Eller et al., 1990; Kainoh & Brown, 1994) and even host-pheromone (Mattiacci et al., 1993). Unlike all of these studies showing that host-derived kairomones influence parasitoid host location, in our system we demonstrate that *P. bicolor* females responded to plant tissue damaged by the host (mines) with a characteristic ovipositional probing behaviour.

Only a few studies have shown that damaged plant material has an influence on host location of parasitoids after landing on an infested plant (Elzen et al., 1984; Horikoshi et al., 1997; Mattiacci & Dicke, 1995b). This effect is limited to contact responses such as antennation on freshly damaged plant tissue (Mattiacci et al., 1999; Potting et al., 1997). In these studies plant related substances elicited only a klinotactic response but not an ovipositional probing behaviour, as is the case in our system. This novel observation might be explained by the fact that the leafminer parasitoid in study has no direct contact with the larva and cannot perceive chemical cues from it. Vinson (1991) hypothesised that in the case of a leafminer, the mined leaf may act as an analogue to the surface of the host. Hence the chemical and physical characteristics of the mined leaf area act as the host integument and are involved in eliciting ovipositor insertion. To our knowledge such an effect in a leafminer parasitoid was found only once, without any indication on whether the cues were plant or host related. The parasitoid *Orgilus lepidus* continued to probe the mined leaf infested by the potato moth larva even after removal of the larva (Keller & Horne, 1993). Our study demonstrates that plant-derived compounds elicit the same innate behaviour on a parasitoid as host-derived compounds in a parasitoid of a non-concealed host (Vet et al., 1991).
The compounds identified in the mine are long chain alkanes and a terpene. Long chain hydrocarbons are commonly encountered in both the plant and animal kingdom. In fact n-alkanes are amongst the commonest constituents of all plant waxes (Baker, 1982; Jeffree, 1986). They have been found to play a role in several bitrophic herbivore-plant interactions: as attractant for oviposition, or as attractant or deterrent for feeding (reviewed by Bernays & Chapman, 1994; Udayagiri & Mason, 1997). They have also been found to play a role in parasitoid-herbivore (Rutledge, 1996) and predator-herbivore (Yasuda, 1997) interactions, as well as in insect chemical mimicry (Liepert & Dettner, 1996). In parasitoid-herbivore interactions long chain hydrocarbons are used both for habitat and for host location. However, in all available studies these compounds originate from the host or its by-products and not from the plant. Although in our case we detected some of these compounds from *P. pomonella* larvae and frass, *P. bicolor* did not respond to the larva or to frass extracts, possibly because of the low concentration. A similar concentration-dependent response to chemical cues was observed for this parasitoid in a habitat location study (Dorn et al., 1999).

The identified hydrocarbons are commonly found in apple leaves with *n*-C27 (nonacosane) and *n*-C31 (hentriacontane) as the most abundant (Hellmann & Stoesser, 1992), which is confirmed by our results. These compounds are known to constitute the texture of the epicuticular leaf surface (Baker, 1982). The amount and composition of alkanes in apple leaves changes strongly depending on the season, developmental age of the leaves and on apple tree varieties (Hellmann & Stoesser, 1992). In addition quantity of these compounds increases as a result of leafminer herbivory (Chapter 5). This increase might result in a different plant texture, which may contribute to the observed response of the female parasitoids. Indeed in several parasitoid species texture has been shown to be an important physical cue for host location (Schmidt, 1991; Vinson, 1985).

Squalene, the only terpene identified, is an important intermediate compound in the sterol biosynthesis (Simmen & Gisi, 1995). Although squalene
is considered not to be synthesised by insects and thus is required in dietary uptake (Downer, 1985), it has been isolated from the cuticle of blood-feeder arthropods such as *Triatoma infestans* (Heteroptera) (Juarez & Blomquist, 1993) and *Dermacentor* ticks (Yoder et al., 1993). The later species uses it as an ant-defence secretion. In our analysis squalene was detected in the larvae and frass of *P. pomonella* in amounts lower than 1 ng, possibly a contaminant from plant material. Squalene had not been previously reported to occur in apple leaves.

The synthetic mixture of the compounds identified elicited ovipositional activity of *P. bicolor*, however, this behaviour was delayed when compared to the natural extract. This suggests that the natural extract possibly contained other compound(s) in quantities not detected by the method used. Non-detected compounds can either contribute to the ovipositional response directly or have a synergistic effect with the compounds identified.

This work demonstrates that the source of chemical cues used by this leafminer parasitoid to locate its concealed host after landing on an infested patch is plant-derived and not insect-derived unlike in other tritrophic systems. The ecological function of the identified compounds and their occurrence on non-infested as compared to leafminer-infested leaves is the subject of another publication.

**4.6. Acknowledgements**

We are particularly grateful to R. Amadò, S. Kürsteiner-Laube, A. Dutly and A. Hern for their valuable advice and help in the chemical identification as well as C. Fornallaz for technical assistance, to R. Bosshard, P. Egli, M. Krebs and T. Christoffel for helping with the maintenance of the insect colonies, and to F. Wäckers for his comments on an earlier draft of the manuscript.
5. A novel function of the triterpene squalene in a tritrophic system

5.1. Abstract

Upon insect herbivory plants often produce chemical cues that are used by parasitoids for host habitat location. We investigated the consequences of leafminer herbivory of *Phyllonorycter pomonella* on apple leaf chemistry and the subsequent effect on the generalist parasitoid *Pholetesor bicolor* on host and habitat location. Chemical analysis of leaf solvent extracts from healthy and leafminer damaged leaves revealed that herbivory increased the amount of the triterpene squalene (C$_{30}$H$_{50}$) whereas the other compounds identified were found in equal amounts in both plant treatments. To assess the use of the identified compounds in host location, a series of contact bioassays were conducted with female parasitoids. Parasitoids were exposed to plant material, hexane plant extracts, and the synthetic mixture of compounds identified. Results indicate that squalene elicits an innate contact response of *P. bicolor* during host location. This response was characterised by an ovipositional probing behaviour. To assess the use of the identified compounds in habitat location, Y-tube olfactometer experiments were conducted. Results showed that squalene is not involved in habitat location and has no priming effect on *P. bicolor*. These results show that squalene mediates host location but not habitat location of *P. bicolor*. Although other triterpenes are used by parasitoids in host location, this is the first report of this plant triterpene mediating host location of a parasitoid. The biological and ecological functions of squalene on all three trophic levels are discussed.

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5.2. Introduction

Upon insect herbivory plants generally activate biochemical defenses. These defenses have been shown to act directly against their herbivores (reviewed by Karban and Baldwin (1997)) and/or to elicit chemical signals that may attract natural enemies of the herbivore (reviewed by Price (1997)). A large number of plant compounds induced by herbivore feeding have been identified, and have been shown to be used by parasitoids during the initial stages of host selection, before landing, i.e. for habitat location (see review Rutledge (1996)). Plant chemicals used by parasitoids after landing onto an infested plant are relatively less studied. These later stages of host selection, including host location, host acceptance and oviposition (sensu Vinson (1984)) are reported to be most often mediated by semiochemicals produced by the arthropod host (Vet & Dicke, 1992; Vinson, 1991). There seem to be exceptions where plant derived chemical cues mediate host location of parasitoids. This includes foraging of parasitoids for non-concealed (Horikoshi et al., 1997) as well as concealed hosts (Mattiacci et al., 1999; Keller & Horne, 1993).

Endophytic hosts such as leafminers impose a particular challenge for natural enemies. For example parasitoids while searching on a leaf for concealed hosts do not have direct contact with the host. This investigation deals with the chemical signals involved in host location of *Pholetesor bicolor* Ness (Hymenoptera: Braconidae). This solitary koinobiont endoparasitoid, among other natural enemies, parasitizes apple leafminers of the genus *Phyllonorycter* (Lepidoptera: Gracillariidae) which are known to cause economic damage in several major apple growing regions of the world (Dorn et al. 1999). In Hungarian orchards *P. bicolor* is considered a primary parasitoid of apple leafminers (Balázs et al., 1996). Because *P. bicolor* is well synchronized with its host (Balázs, 1992), it can be considered a potential biological control agent.
Host location

Focusing on the behaviour of *P. bicolor* after landing on a leaf infested with the leafminer *P. pomonella* (det. Deschka), we recently obtained results indicating the significance of semiochemicals from the plant (Chapter 4). Female parasitoids responded innately to the herbivore-damaged plant tissue, but not to host-derived material (larva or frass). The response could be elicited by exposing females to a synthetic mixture of *n*-alkanes and squalene, which were identified from the herbivore-damaged mine area. *N*-alkanes have been shown to be important chemicals in both habitat and host location for various parasitoid species (Rutledge, 1996). However in this system we do not know 1) whether these compounds are produced by the plant after leafminer herbivory, 2) which specific compound(s) are used by the parasitoid for host location, and 3) whether they are used by the parasitoid as chemical cues for habitat location. These questions are addressed in this study.

Habitat location

Being a generalist parasitoid *P. bicolor* may use experience gained from a previous contact exposure with its endophytic host for the subsequent habitat location of the same host species. Indeed, experience is known to increase parasitoid responses towards certain stimuli (i.e. priming) or even generate new ones (i.e. associative learning) (Vet, 1996). For example, previous contact exposure to innately recognised semiochemicals enhances the searching ability of parasitoids used for biological control in greenhouses and the field (Grasswitz, 1998; Hare *et al.*, 1997). Previous experiments using *P. bicolor* showed that volatile chemical cues from leafminer infested leaves are used by this parasitoid for habitat location. However, the preference towards these plant volatiles could be observed only after prior parasitoid exposure to leafminer damaged plants (Lengwiler *et al.*, 1994). Given that *P. bicolor* responds innately to plant-derived cues for host location we test whether a previous contact exposure with those cues could affect the subsequent location of a habitat with potential hosts.
This study was designed to elucidate the chemical response of apple leaves to leafminer herbivory, and its consequences for both the host and habitat location of the leafminer parasitoid *P. bicolor*.

5.3. Materials and methods

**Plant and insect rearing**

Two-month-old apple seedlings (*Malus sylvestris* c.v. Golden Delicious, Rosaceae), were used for both rearing of insects and for leaf chemical extracts. Plants were grown in climatic chambers at 25°C and 17°C (L:D), 70% r.h. and 16 h/8 h light:dark.

Insects were collected in apple orchards in South Tyrol (Northern Italy) and reared as described in the previous chapter. For all bioassays we used 3-6 d old honey-fed female parasitoids. Host location bioassays were conducted with naïve females, whereas habitat location experiments were conducted with females that were given experience (see details below).

**Plant response to herbivory**

Leaf extracts were obtained by soaking 45-50 apple leaves with 100 mines (2 mines per leaf) in 200 ml hexane overnight. These herbivore-infested apple leaves will be from now on referred to as *mine-damaged leaves*. The hexane extract was filtered through 10 g silica gel (Supelco), which had been previously conditioned with 50 ml hexane, and concentrated first with a rotary evaporator (32 ± 3°C) and then under a stream of nitrogen (purity 99.96 %) to 10 µl. The same extraction procedure was applied to healthy leaves that were of similar age and had the same leaf area surface as those with mines. Leaf area was measured with an area meter instrument (Licor 300A).
Ten separate sample extracts of each treatment (mine-damaged and healthy) were analysed with a HP 5890 Series II Plus gas chromatograph, equipped with a flame ionisation detector (300°C) and a non-polar HP-1 (Crosslink Methyl Silicone Gum) capillary column (30m x 0.25 mm x 0.25 µm film thickness). The oven temperature program was held at 100°C for 2 min, then increased to 300°C at a rate of 6°C/min and held at 300°C for 15 min. The split/splitless injector (250°C) was operated in splitless mode throughout the analyses. The carrier gas was helium (purity 99.96 %) at a pressure of 108 kPa (constant pressure). Samples of 1 µl were injected. To evaluate quantitative differences between treatments for the individual compounds identified, peak areas were analysed using a t-test with the appropriate Bartlett's test for homogeneity and Kolmogorov-Smirnov test for normality (Sokal & Rohlf, 1981). To quantify the compounds found in mine-damaged leaves and healthy leaves, three additional extracts of each treatment were analysed with an internal standard (100 ng octylbenzene/µl). The quantities of each compound were calculation based on peak areas relative to the internal standard. Identification of compounds by co-injection with authentic standards was performed with gas chromatography-mass spectrometry (GC-MS) (for details refer to previous chapter).

Host location – Parasitoid response to contact chemical cues

All behavioural contact assays were performed in a single choice Petri dish (10 cm Ø) arena. Parasitoids were placed individually in glass vials (2 cm Ø and 4 cm high) and the open end of the vial was positioned over the plant cue sources or over an extract treated filter-paper (1.3 cm Ø). Females which were observed performing the characteristic thrusting of the ovipositor, from here on referred to as probing, on the filter-paper for more that 5 s were considered to respond positively to the treatment. Observations were conducted under laboratory conditions of 23 ± 2 °C, 50 ± 10 % r.h. and 1000 ± 500 lux. Each treatment was bioassayed simultaneously with a control, and for statistical analysis a \( \chi^2 \) test was performed between treatments and controls.
Experiment 1. Response to healthy and mine-damaged leaf material

In order to elucidate whether the source of active compounds eliciting ovipositional probing of *P. bicolor* are specific to herbivore damage, female parasitoids were offered the following cue sources: a) a 1.2 cm² piece of mechanically removed healthy lower-leaf epidermis (*HEALTHY*), b) a 1.2 cm² piece of lower-leaf epidermis damaged by one larva (*MINE-DAMAGED*), from which the larva and its by-products were carefully removed or c) an empty Petri dish (*CONTROL*). The leaf area offered corresponded to the size of a fully developed mine. This reference area was used throughout the work for quantifying the compounds extracted from leaves and for calculating the concentrations of the synthetic mixture used in subsequent experiments.

Behavioural parameters recorded were; time spent *walking, standing* and *probing* on the cue source offered. The parameters were recorded over a 20 min period using the computer software The Observer (Noldus, 1991). Each behavioural parameter was analysed by Kruskal-Wallis ANOVA followed by pairwise multiple comparisons between treatments using the Student-Newman-Keuls method (Sokal & Rohlf, 1981).

Experiment 2. Response to healthy and mine-damaged leaf extracts

In order to examine whether host location is elicited by chemical and not physical characteristics of leaves, extracts from both healthy and mine-damaged leaves were tested. Leaf epidermis from each treatment were carefully removed with tweezers and soaked separately in hexane (purity 99.7%) at 4°C for 24 h. The extract concentration applied to the filter-paper was made so that 10 μl were equivalent to 1.2 cm² mine-damaged or healthy leaf. Hexane alone was applied to the filter-paper as a control.
Experiment 3. Response to a mixture of n-alkanes and/or squalene

A mixture of the synthetic n-alkanes \( n-C_{27}, n-C_{29}, n-C_{30}, n-C_{31}, n-C_{32}, n-C_{33} \), and squalene \( (C_{30}H_{50}) \) (Fluka Chemie AG, purity \( \geq 99.5\% \)) at quantities equivalent to 1.2 \( cm^2 \) mine-damaged leaf (see Table 5.1) was tested against a control (hexane). A dose response test was carried out with the same reference mixture using a dilution factor of 10. The mixture of n-alkanes without squalene was tested, and subsequently a dose response of squalene was performed.

Habitat location – Parasitoid response to host location cues for habitat location

Two Y-tube olfactometer bioassays were conducted to examine: 1) whether the identified chemical cues identified in mine-damaged leaves are used by \( P. \ bicolor \) as olfactory stimuli for habitat location, and 2) whether a previous contact exposure to innately recognise plant-derived cues enhance habitat location of \( P. \ bicolor \) through priming (Hare et al., 1997).

The olfactometer consisted of a glass Y-shaped tube. Odour sources were placed in glass chambers (27 cm long, 2.5 cm \( \Theta \)) which directly fitted to each of the two Y-tube arms. Humidified air (300 ml/min) entering the odour source chamber was filtered through a charcoal filter. Parasitoids were individually introduced in the central tube of the olfactometer and given a maximum of 10 min to make a choice for one of the two olfactometer arms. A choice was recorded when the female had crossed a line marked 20 cm after the junction of the two arms. Responses of \( P. \ bicolor \) in the olfactometer were analysed with a \( \chi^2 \) test with null hypothesis of a 50:50 distribution over the two odour sources. The environmental conditions in the bioassay room were 23 ± 2°C, 50 ± 5 % r.h. and 1200 ± 50 lux provided with artificial light.
**Experiment 4. Squalene and n-alkanes as olfactory stimuli**

In the Y-tube olfactometer females were given the choice between the *SYNTHETIC MIXTURE* of squalene and *n*-alkanes identified in the mine-damaged leaves and pure solvent as control (*BLANK*). The mixture of compounds was tested at a concentration equivalent to 1.2 cm² mined-damaged leaf surface and at three higher concentrations (dilution factor of 10). The compounds in hexane were applied to a Teflon septum (1.3 cm Ø) and placed in one arm of the Y-tube. A similar septum with hexane (*BLANK*) was placed in the other arm of the Y-tube.

Prior to being tested, wasps were given 25 ± 5 min contact experience on leafminer-infested plants. Preliminary experiments showed that naïve parasitoids are not attracted to mine-damaged plants. Experienced wasps were placed individually in vials for 30 min before they were tested.

**Experiment 5. Squalene and n-alkanes as priming stimuli**

In the Y-tube olfactometer females were given the choice between one infested apple leaf containing five mines (*MINED LEAF*) or clean air (*BLANK*). Wasps were given either no experience (naïve) or a 25 ± 5 min experience on one of the following treatments a) a leafminer infested plant, b) the hexane extract of mine-damaged leaf, c) the synthetic mixture of squalene and *n*-alkanes or d) a healthy plant. The contact experience with leafminer infested or healthy plants was provided as in the previous experiment (see above). Experience on the leaf extract or the synthetic mixture was provided by offering the solution in a filter-paper as in the host location bioassays.
5.4. Results

*Plant response to herbivory*

Eight major compounds were identified by GC-MS in extracts from apple leaves infested with the leafminer *P. pomonella* and from healthy apple leaves (Table 5.1). Among them, only squalene (C₃₀H₅₀) was found in significantly larger quantities in the mine-damaged leaves than in the healthy leaves (Student t-test, t-value = 2.6; df = 9; P = 0.03). Only one minor peak was not identified, and all other compounds consisted of long chain hydrocarbons (n-C₂₇, n-C₂₉, n-C₃₀, n-C₃₁, n-C₃₂, n-C₃₃).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Herbivore-damaged (ng ± SE)</th>
<th>Healthy (ng ± SE)</th>
<th>P ¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heptacosane</td>
<td>n-C₂₇ 10.7 ± 0.8</td>
<td>8.2 ± 0.7</td>
<td>n.s</td>
</tr>
<tr>
<td>Unknown</td>
<td>- 6.3 ± 1.1</td>
<td>4.0 ± 0.9</td>
<td>n.s</td>
</tr>
<tr>
<td>Squalene</td>
<td>C₃₀H₅₀ 32.3 ± 13.1</td>
<td>12.2 ± 4.7</td>
<td>*</td>
</tr>
<tr>
<td>Nonacosane</td>
<td>n-C₃₀ 91.3 ± 8.9</td>
<td>77.4 ± 11.3</td>
<td>n.s</td>
</tr>
<tr>
<td>Triacontane</td>
<td>n-C₃₀ 8.7 ± 1.2</td>
<td>7.3 ± 0.8</td>
<td>n.s</td>
</tr>
<tr>
<td>Hentriacontane</td>
<td>n-C₃₁ 251.6 ± 36.3</td>
<td>228.8 ± 18.8</td>
<td>n.s</td>
</tr>
<tr>
<td>Dotriacontane</td>
<td>n-C₃₂ 5.9 ± 0.9</td>
<td>5.6 ± 0.6</td>
<td>n.s</td>
</tr>
<tr>
<td>Tritriacontane</td>
<td>n-C₃₃ 34.0 ± 5.5</td>
<td>34.2 ± 1.7</td>
<td>n.s</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>440.8 ± 67.6</td>
<td>377.8 ± 39.5</td>
<td>n.s</td>
</tr>
</tbody>
</table>

¹ t-test between treatments based on peak areas (n = 10), * P < 0.05, n.s. not significant

*Host location – Parasitoid response to contact chemical cues*

*Experiment 1. Response to healthy and mine-damaged leaf material*

The behaviour of *P. bicolor* offered mine-damaged or healthy leaf material is depicted in Figure 5.1. Wasps spent significantly more time *walking,*
standing and probing on the mine-damaged leaf (P < 0.05, Kruskal-Wallis ANOVA) than on the healthy leaf. In addition, no significant differences for probing and standing were observed between the healthy leaf and an empty arena (control). Only walking was shown to be similar on the mine-damaged leaf and the control.

Figure 5.1. Walking, standing, and probing activity of the parasitoid *P. bicolor* on mine-damaged and healthy leaf epidermis. Statistical differences in time duration spent for each activity on the different treatments are shown with different letters (Kruskal-Wallis ANOVA, P < 0.05).

**Experiment 2. Response to healthy and mine-damaged leaf extracts**

The ovipositional probing behaviour of *P. bicolor* was only triggered by the hexane extract from mine-damaged leaves ($\chi^2 = 14.7; \text{df} = 1; P < 0.001$) (Figure 5.2). In contrast, the response of females to the extract from healthy leaves was similar to that of the control solvent, and significantly different from the mine-damaged leaves ($\chi^2 = 12.2; \text{df} = 1; P < 0.001$).
Figure 5.2. Ovipositional probing response of *P. bicolor* females to hexane extracts of mine-damaged and healthy leaf epidermis. (Mined-damaged vs. control $\chi^2 = 14.7$, df = 1 $P < 0.001$).

**Experiment 3. Response to a mixture of n-alkanes and/or squalene**

The percent of females responding to the mixtures of synthetic compounds identified in the mine-damaged leaves in the contact bioassay is shown in Table 5.2. The mixture of n-alkanes and squalene elicited significant ovipositional probing behaviour of *P. bicolor* at concentrations corresponding to quantities found in 1.2 cm$^2 = 1x$ ($\chi^2 = 20.1$, $P < 0.001$), 12 cm$^2 = 10x$ ($\chi^2 = 11.8$, $P < 0.001$), and 120 cm$^2 = 100x$ ($\chi^2 = 11.1$, $P < 0.001$) of mine-damaged leaf. The mixture of n-alkanes (1x) devoid of squalene did not trigger a significant response ($\chi^2 = 2.5$, $P > 0.05$). In contrast, squalene alone triggered a response over a range of concentrations from 30 ng to 3.1 μg. These quantities corresponded to those found in 1.2, 12 and 120 cm$^2$ surface area of mine-damaged leaves. The strongest response was obtained when females were offered 30 ng of squalene. This amount of squalene elicited a significantly higher female response than the solvent control, already within the first 20 min of the bioassay.
Table 5.2. Ovipositional probing response of *Pholetesor bicolor* to mixture of $n$-alkanes and/or squalene. Concentration 1x corresponds to that given in Table 5.1 (herbivore-damaged) offered on a 1.3 cm² filter-paper. Each female observed for a total of 90 min.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>20 min</th>
<th>(\chi^2)</th>
<th>P</th>
<th>90 min</th>
<th>(\chi^2)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>synthetic mixture (all compounds)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>0.1 x</td>
<td>30</td>
<td>10</td>
<td>0.8</td>
<td>n.s.</td>
<td>23</td>
<td>2.9</td>
<td>n.s.</td>
</tr>
<tr>
<td>1 x</td>
<td>30</td>
<td>30</td>
<td>5.1</td>
<td>*</td>
<td>70</td>
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</tr>
<tr>
<td>10 x</td>
<td>30</td>
<td>20</td>
<td>2.6</td>
<td>n.s.</td>
<td>50</td>
<td>11.8</td>
<td>***</td>
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<tr>
<td>100 x</td>
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<td>13</td>
<td>0.2</td>
<td>n.s.</td>
<td>57</td>
<td>15.1</td>
<td>***</td>
</tr>
<tr>
<td>1000 x</td>
<td>30</td>
<td>7</td>
<td>0</td>
<td>n.s.</td>
<td>27</td>
<td>2.9</td>
<td>n.s.</td>
</tr>
<tr>
<td>synthetic mixture (1x) (excluding squalene)</td>
<td>30</td>
<td>3</td>
<td>0</td>
<td>n.s.</td>
<td>30</td>
<td>2.5</td>
<td>n.s.</td>
</tr>
<tr>
<td>Squalene amount (ng)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>30</td>
<td>7</td>
<td>0</td>
<td>n.s.</td>
<td>27</td>
<td>2.9</td>
<td>n.s.</td>
</tr>
<tr>
<td>15</td>
<td>30</td>
<td>7</td>
<td>0</td>
<td>n.s.</td>
<td>33</td>
<td>3.5</td>
<td>n.s.</td>
</tr>
<tr>
<td>30</td>
<td>30</td>
<td>30</td>
<td>4.0</td>
<td>*</td>
<td>63</td>
<td>16.1</td>
<td>***</td>
</tr>
<tr>
<td>310</td>
<td>30</td>
<td>13</td>
<td>0.2</td>
<td>n.s.</td>
<td>57</td>
<td>15.1</td>
<td>***</td>
</tr>
<tr>
<td>3100</td>
<td>30</td>
<td>20</td>
<td>2.3</td>
<td>n.s.</td>
<td>57</td>
<td>12.7</td>
<td>***</td>
</tr>
<tr>
<td>31000</td>
<td>30</td>
<td>7</td>
<td>0</td>
<td>n.s.</td>
<td>27</td>
<td>2.9</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

\(\chi^2\) performed between treatment and control (hexane)

* P ≤ 0.05, *** P ≤ 0.001, n.s. not significant

**Habitat location - Parasitoid response to host location cues for habitat location**

*Experiment 4. Squalene and $n$-alkanes as olfactory stimuli*

In the Y-tube olfactometer bioassay no response of females towards the mixture of squalene and $n$-alkanes was observed (Figure 5.3), regardless of the concentration offered. This indicates that squalene is not used by *P. bicolor* as a chemical cue in habitat location.

*Experiment 5. Squalene and $n$-alkanes as priming stimuli*

Only females which had been given a previous contact experience on leafminer damaged plants preferred, in the Y-tube, the volatiles emitted by the
Figure 5.3. Response of *P. bicolor* to the synthetic mixture of squalene and *n*-alkanes in the Y-tube olfactometer. Synthetic mixture concentrations are indicated on the left side (dilution factor of 10). 1x corresponds to 1.2 cm² leaf area surface of mine-damaged leaf (see Table 5.1 for quantities). Percent females making no choice is indicated on the right side.

Figure 5.4. Response of *P. bicolor* females to compounds from mine-damaged leaf in Y-tube olfactometer. Type of experience is indicated on left side. Percent females making no choice is indicated on the right side. Asterisks indicate a significant difference within a choice test: $\chi^2 *** P < 0.001$. 
mine-damaged leaf versus the control ($\chi^2 = 15; P < 0.001$) (Figure 5.4). Neither a previous contact experience with the mine-damaged leaf extract or with the innately recognised synthetic blend mixture, induced a significant preference for the odour of the mined leaf.

5.5. Discussion

Apple leaves infested by leafminers produce the triterpene squalene ($C_{30}H_{50}$) in higher quantities than non-infested leaves. Squalene elicits a characteristic ovipositional probing behaviour of naïve $P$. bicolor which demonstrates that this chemical is innately recognised and used by the parasitoid for host location after landing on a host infested patch. Given that squalene cannot be synthesised by arthropods (Downer, 1985), we conclude that this compound mediating parasitoid host location is of plant origin. Its increased production seems to be induced by Phyllonorycter pomonella’s herbivory. Previous reports have documented herbivore induction of terpenes in plants, and the use of these substances by parasitoids and predators for habitat location (for reviews see Rutledge (1996) and Dicke (1994)). However, this specific triterpene has not been reported as a plant-derived semiochemical used by parasitoids. Squalene in this system can be defined as a contact plant synomone for the parasitoid (sensu Dicke and Sabelis (1988)).

To our knowledge this is the first record of squalene being extracted from apple leaves. The more closely related plant species from which squalene has been extracted is Prunus persica (L.) Batsch (Lin & Lin, 1992). Squalene is widely found in both plant and animal tissue, being the basic intermediate metabolite for the biosynthesis of triterpenes (Bonner, 1965). From the genesis of squalene through the mevalonate $\rightarrow$ geranyl pyrophosphate $\rightarrow$ farnesyl pyrophosphate pathway, and the subsequent steps leading to sterol production (squalene epoxidase $\rightarrow$ squalene 2,3-oxide), it is evident that squalene is an essential compound regulating plant growth (Simmen & Gisi, 1995; Yates et al., 1991). The increase of squalene in organisms such as plants, fungi and
mammals has been correlated to the exogenous inhibition of squalene epoxidase (Petranyi et al., 1984; Ryder & Dupont, 1985; Simmen & Gisi, 1995; Yates et al., 1991). The inhibition of squalene epoxidase in these organisms is shown to constrain the biosynthesis of sterol and consequently leading to growth inhibition. In our apple plant system, we observed both an increase of squalene (Table 5.1), and a decrease of plant growth in mine-damaged seedlings as compared to healthy seedlings (A. Dutton, personal observation). We can postulate a possible direct effect of squalene on the first trophic level.

Given that squalene regulates the biosynthesis of other triterpenoids and sesquiterpenoids, it might indirectly affect the second trophic level. In fact, different plant species produce some of these sesquiterpenoids and triterpenoids as defence compounds against pathogens (phytoalexins) and/or herbivores (Gershenzon & Croteau, 1991; Threlfall & Whitehead, 1988; Zook & Kuc, 1991). Although squalene itself has not been reported as a phytoalexin or as a toxin to herbivores, its involvement in phytoalexin biosynthesis in plant cell cultures after fungal infection is known (Van Der Heijden et al., 1989).

Our results indicate a behavioural effect towards squalene on the third trophic level. The almost three fold increase of squalene in mine-damaged leaves, with a mean value of 32 ng/1.2 cm² (Table 5.1) was responsible for the observed ovipositional probing behaviour of the parasitic wasp P. bicolor (Table 5.2). A slightly higher amount of squalene was extracted from the mined leaf area (\(\bar{X} = 37\) ng/1.2cm², Chapter 4) as that extracted from the whole leaf (\(\bar{X} = 32\)). This indicates a possible localised concentration of this substance at the site of herbivory. This finding coincides with our behavioural observation that parasitoids probe on the mined leaf area and not on healthy tissue. A similar localised response to herbivory was recently shown for both the plant and parasitoid in the crucifer-Pieris-Cotesia tritrophic system (Horikoshi et al., 1997).

Several terpenes are known to be involved in habitat and host location of parasitoids (Rutledge, 1996). To our knowledge, this is the first report of squalene involved in host location of a parasitoid. Behavioural effects of
squalene in interactions among other organisms are known. For example, ticks of the genus *Dermacentor* ingest squalene from mammal blood and use the substance as an allomone to protect themselves against ant attack (Yoder et al., 1993). Furthermore, pollinating bats use squalene as a flower attractant (Ecroyd et al., 1995).

Enhanced responsiveness of parasitoids after a brief exposure to innately recognised cues has been demonstrated previously (Hare et al., 1997; Lewis & Martin, 1990; Turlings et al., 1989). In our system, exposing *P. bicolor* females to innately recognised cues such as squalene did not result in a subsequent attraction to leafminer infested leaves (Figure 5.5). However, we cannot rule out the possibility that this substance is used in associative learning (Vet et al., 1995). If such were the case the parasitoid would need a previous exposure to the contact cue in association with plant volatiles. This can be supported by the fact that parasitoids which were exposed to contact and volatile cues (i.e. females given a contact experience on leafminer infested plants) subsequently were attracted to herbivore-plant volatiles.

In the Y-tube olfactometer experiments we could not demonstrate a habitat location response to the mixture of squalene and *n*-alkanes. However both in a previous study (Lengwiler et al., 1994) and in the present experiments (Figure 5.4) we observed a significant attraction of parasitoids to herbivore induced leaf volatiles. It seems therefore evident that the volatile and contact cues used by *P. bicolor* for habitat and host location are different. In both cases the cues are of plant origin.

5.6. Acknowledgements

This project was supported by a TH grant (ETH Zurich). We thank A. Hern, C. Fornallaz and A. Dutly for technical support with GC-MS analysis; R. Bosshard, P. Egli for assistance with behavioural bioassays, M. Krebs, T.
Christoffel for helping in rearing of plants and insects; R. Amadó, and A. Hern for their comments on an earlier draft of the manuscript.
6. Learning used as a strategy for host stage location in an endophytic host-parasitoid system

6.1. Abstract

The relationship between host stage selection and foraging behaviour of *Pholetesor bicolor* Nees (Hymenoptera: Braconidae), a larval parasitoid of *Phyllonorycter* spp. (Lepidoptera: Gracillariidae), was investigated under laboratory conditions. The endophytic host develops through two larval stages with different feeding habits, accordingly named sap- and tissue-feeders. The parasitoid was able to find and parasitize both larval stages, even though it is most successful in parasitizing the sap-feeder stage. The influence of experience in the parasitoid’s searching behaviour was observed in a choice bioassay. Searching activity increased when either contact experience with the sap- or the tissue-feeder host was given. Furthermore, the ability of the parasitoid to locate a sap- or a tissue-feeder infested plant was influenced by the type of experience given prior to the bioassay. Naïve females were less active, and were observed with equal frequency on sap-feeder, tissue-feeder and non-infested plants. In contrast, females that were given previous contact experience with sap-feeders (i.e., the host stage that provided the most successful parasitism) were observed foraging more often on plants infested by the sap-feeders, than on those infested by tissue-feeders or on non-infested plants. Experience with a tissue-feeder host had no detectable effect on host stage location and only enhanced *P. bicolor*’s foraging activity. The advantages of learning in this tritrophic system are discussed.

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6.2. Introduction

It is known that parasitoids can develop successfully in different larval instars of the same host but both the cost of parasitism (*sensu* van Alphen & Jervis, 1996) and the suitability may vary between different host instars (Petitt & Wietlisbach, 1993; Slansky, 1986; Vinson & Iwantsch, 1980). In addition, the spatial and temporal availability and detectability of a host in a certain habitat (Vet & Dicke, 1992; Sait et al., 1997) may modify the value (*sensu* Mackauer et al., 1996) of the host itself and, hence, the propensity of the parasitoid to attack a particular instar.

For parasitoids attacking species that are concealed within their food plant, such as leafminers, host accessibility and acceptance can further be complicated by its particular feeding behaviour and/or by the shape of the mine. For example, leafminers of the genus *Phyllonorycter* spend their larval life inside a mine and the larvae develop through two morphologically and behaviourally distinct stages. The first three instars, the sap-feeders, are dorso-ventrally flattened and feed with their sucking mouthparts on the sap exuded by damaged cells in the spongy layer. The first two sap-feeder instars produce a narrow linear U-shaped mine between the lower and upper epidermal cells. The third instar larvae feed within the U-shaped mine previously bored, forming a flat blister-like leafmine visible only from the underside of the leaf. During the third larval moult, hypermetamorphosis occurs. The fourth and fifth instar larvae, the tissue-feeders, become cylindrical and have chewing mouthparts that allow them to feed on whole parenchyma cells. The tissue-feeders do not enlarge the mine but increase its depth by removing parenchyma cells and spinning silk threads on both surfaces of the mine. These threads create a cavity (tentiform mine) which allows for free movement of the larvae (Pottinger & LeRoux, 1971; Ridgway, 1986).

*Pholetesor bicolor* Nees is a primary solitary koinobiont endoparasitoid of the apple leafminer *Phyllonorycter pomonella* Zeller (Lepidoptera: Gracillariidae) (det. G. Deschka). At present, limited information is available on both host
location and host stage selection. Furthermore, with the exception of a few studies (Mattiacci & Dicke, 1995a; Takabayashi et al., 1995; van Alphen & Janssen, 1982), little is known about the relationship between host location and host age/stage preference. The laboratory rearing of this parasitoid is optimally carried out with sap-feeder larvae (personal observation). Laboratory studies on the host searching behaviour show that *P. bicolor* uses plant-induced chemical cues for the long range searching behaviour (Dorn et al., 1999; Lengwiler et al., 1994) and that these volatile stimuli are emitted by the plant infested by the tissue-feeder stage (U. Lengwiler, unpubl.). However, it is not known whether this parasitoid is able to parasitize tissue-feeder leafminers. This could be possible, given that in the field second and third generations tend to overlap allowing for both stages to occur together (Pottinger & LeRoux, 1971). Given the lack of information on this particular species, we initiated a study on the relationship between host location and host stage preference. In the first and second part of this paper our goal was to determine if *P. bicolor* is able to find, accept and parasitize both host stages of the leafminer. For this purpose we conducted behavioural observations and parasitisation preference tests. We considered percent pupal parasitoid mortality and sex ratio as host stage suitability parameters.

Prior experience with the host or with cues associated with the plant-host complex are also known to influence the behavioural responses of parasitoids (Lewis & Martin, 1990; Petitt et al., 1992; Turlings et al., 1993; Vet & Groenewold, 1990). However, it is not known if experience plays a role in parasitoid host age/stage selection. In the third part of this work we examined the influence of experience on the searching behaviour of *P. bicolor*. Our aim was to determine the occurrence of host stage discrimination during host location and the influence of parasitoid experience on location of the two leafminer host stages.
6.3. Materials and Methods

**Plants.** Two month old apple seedlings (*Malus sylvestris* cf. Golden Delicious) were used for the rearing of insects and for all experiments.

**Insects.** The leafminer species reared at our institute was *P. pomonella*. The colony originated from individuals collected in apple orchards in South Tyrol (Northern Italy). Insects were maintained at 23 ± 2°C, L16: D8 photoperiod and 50 ± 10% r.h. In order to obtain infested plants for rearing and for experiments, thirty three to four day old adult moths were released on six apple seedlings in a cage. Moths were allowed to oviposit for 24 h, after which they were removed and plants were kept until the desired host stage. The time from egg to first instar larvae (sap-feeding stage) is approximately nine to eleven days and eight additional days are needed to reach the tissue-feeding stage. The number of mines per plant ranged from 15 to 45.

The braconid parasitoid *P. bicolor* (det. J. Papp) was collected in 1995 from the same area in Northern Italy. The culture was kept at 21 ± 2 °C, L16: D8 photoperiod and 50 ± 10% r.h. Parasitoids were reared on sap-feeder leafminers. In each rearing cage two to three *P. bicolor* females were provided with three to four heavily leafminer infested apple seedlings and allowed to parasitize for four days. Parasitoids were removed and plants with parasitized leafminers were kept until pupation. Upon pupation parasitized leafminers were separated from non-parasitized leafminers, and kept in a cage until emergence. Emerged parasitoids were provided with honey and water and allowed to mate over a three to six day period, before they were used in the experiments.

**Experiment 1. - Host location and parasitisation**

The experiments were carried out under the insectary conditions, described above. A leafminer infested apple seedling with either sap-feeder or tissue-feeder leafminers was introduced in a 25x25x25 cm Plexiglas cage. At the start of each observation a single female was placed on the lower side of a leaf infested with one mine. The following parameters were recorded with the software programme ‘The Observer’ (Noldus, 1991): 1) time to find and
recognise the mine (from release to first ovipositor insertion), 2) the time spent attempting oviposition (probing with the ovipositor) and 3) the total time spent on the leaf. Recording stopped when the parasitoid left the leaf either by flying or walking. Females which remained standing for more than 5 min or flew away immediately after being placed on the leaf were discarded. Twenty females were tested with each host stage. Plants were labelled and kept in the insectary until pupation when parasitism could be recorded.

Statistical analysis. The Mann-Whitney test was used for pairwise comparisons of the behavioural parameters described above for the two host stage treatments (Sokal & Rohlf, 1981).

**Experiment 2. - Host stage selection**

Two apple seedlings (height 25 cm) infested with sap-feeders only (1st and 2nd instar), tissue-feeders only (4th and 5th instar) or a mixture of sap- and tissue-feeders (3rd and 4th instar) were introduced in a 25x25x25 cm Plexiglas cage. We chose to offer 3rd instar hosts in the later treatment for two reasons: first, we wanted to determine the acceptance of the sap-feeder stage which has the same mine surface area as that of a tissue-feeder larvae and, second, to standardise the number of mines of the two host stages offered (1st and 2nd sap-feeders are not visible and cannot easily be quantified). In this treatment mines were marked in order to monitor the different instars.

For the bioassay, a single female was placed on the lower side of an infested apple leaf. Only females that were observed probing a mine with the ovipositor within the first few minutes were allowed to parasitize for a 24 h period. After this time, parasitoids were offered two new infested plants (with the same host instars) for another 24 h period. We decided to provide hosts in two blocks of 24 h, in order to avoid superparasitism and, at the same time, to compensate for the slow oviposition time we normally observed with this species (Experiment 1). The number of mines offered to each individual could not be standardised due to the small size of the moth eggs and varied between 50 and 90 mines/day. This number provided at least double the number of hosts
that *P. bicolor* is able to parasitize in one day (preliminary observations). After the bioassay, the individual females were removed from the cage and preserved at -60°C for later dissection to determine egg availability. Plants were labelled and kept in the insectary until pupation when parasitism could be recorded. Leafminers were categorised as alive and unparasitized, dead but not parasitized, and parasitized. Parasitism of dead tissue-feeders was determined by dissection. Sap-feeders that died during the experiment could not be dissected because the mines often 'heal over' and the larvae cannot be recovered. The number of parasitized hosts that did not emerge (parasitoid pupal mortality) was quantified and the sex ratio of those that emerged was recorded.

**Statistical analysis.** ANOVA was used for normally distributed data and the Kruskal-Wallis one way analysis of variance on ranks was used for data which failed the normality and/or equal variance test. One way analysis of variance (ANOVA) was performed to determine if parasitisation differed between the first and the second day. Since the number of hosts offered could not be standardised, a Spearman rank correlation test between parasitism (data taken only from the females which parasitized at least one host) and number of hosts offered for each treatment was performed to determine host density dependency. A contingency chi-square test was performed to determine if the number of females that successfully parasitized at least one host differed among host-stage offered. Percent parasitism and number of parasitized hosts were analysed using Kruskal-Wallis one way analysis of variance on ranks and Dunn's test for comparison among host stages (Sokal & Rohlf, 1981). In the treatment where both host stages were offered simultaneously a Wilcoxon's signed rank test was performed to compare percent parasitism and the number of parasitized 3rd and 4th instar hosts (Sokal & Rohlf, 1981). Data were tested statistically only for females that parasitized at least one host. Percent pupal mortality (parasitoids that did not emerge) and sex ratios for the two different host stages were analysed using Kruskal-Wallis one way analysis of variance on ranks to determine if host quality differs between treatments.
Experiment 3. – Effect of experience on host stage location

Bioassay. The bioassay was performed in a 25x25x25 cm Plexiglas cage where three apple seedlings were placed 12 cm apart from each other. Female parasitoids in groups of three were released from individual vials at the centre of the cage. The locations on one of the three plant treatments and the activity of the parasitoids were recorded every 5 min over a 2 h period. This method of observation was chosen based on previous observations, to compensate for the slow responsiveness of the parasitoid and therefore to increase the acquisition of testable data within a workable time frame. The three plant treatments (locations) were as follows:

a) S-plant: a sap-feeder (1\textsuperscript{st} and 2\textsuperscript{nd} instar) infested plant (20-25 mines),
b) T-plant: a tissue-feeder (4\textsuperscript{th} and 5\textsuperscript{th} instar) infested plant (20-25 mines),
c) N-plant: a non-infested plant.

Activities were recorded as follows:

a) stand (motionless or while grooming),
b) search (walking and/or oviposition behaviour).

For each replicate, the position of the different plants was changed in the cage. Ten replicates were performed, for a total of 30 females/treatment. Parasitoid treatments (explained below) were tested simultaneously in separate cages, to compensate for daily variability in responsiveness.

Parasitoid treatments. Three different experience groups were compared.
1) NAÏVE: parasitoids that had no previous contact with the host or plant. 2) S-FEM: females who were given experience with sap-feeder hosts. 3) T-FEM: females who were given experience with tissue-feeder hosts. Experience was given 5 h before experiments, by placing the parasitoid on a leaf with either the sap- or the tissue-feeding host. When the parasitoid found the mine and initiated the characteristic oviposition behaviour (probing the mine with the ovipositor) she was removed and placed in a vial until used in the experiment. Probing in this case was not considered as ovipositional experience, since the female was not given sufficient time to oviposit. From personal observations we know that at least one ovipositional bout of 9 s is required in order for the female to lay an egg. Ovipositional experience was not given since we know
(Experiments 1 and 2) that successful parasitism in tissue-feeder hosts is very low and difficult to ascertain.

Statistical analysis. ANOVA and Kruskal-Wallis one way analysis of variance on ranks were applied as described in Experiment 2. In order to analyse the data, the total frequency of parasitoids at each plant treatment throughout the 2 h period was calculated. These frequencies were compared within female treatments by Kruskal-Wallis one way analysis of variance on ranks. For pairwise multiple comparison the Student-Newman-Keuls method was used. The total frequencies of each activity parameter (search and stand) were calculated and compared among female treatments using ANOVA and for pairwise multiple comparison the Student-Newman-Keuls method was used. Furthermore, data were analysed at each time interval for each treatment to determine the position of the parasitoids within the 2 h time period. We used the method of least squares (Zar, 1984) to determine 1) time dependence on parasitoids' response to S-plant and 2) influence of experience on the number of parasitoids recorded on the S-plant.

6.4 Results

Experiment 1. – Host location and parasitisation

The time allocated by *P. bicolor* females to find and recognise the mine was similar for both host stages, the sap-feeder and the tissue-feeder of *P. pomonella* (Table 6.1). The number of females successfully parasitizing a host within the duration of the bioassay was low with only one female parasitizing a tissue-feeder, and five females parasitizing a sap-feeder larva. The amount of time required for parasitizing the tissue-feeder host was 1015 s, whereas the average time required for parasitizing a sap-feeder host was 297 s. No statistical analysis to compare the time required for parasitizing between host stages was possible due to the low number of females which were successful in parasitizing a host.
Table 6.1. Host location and parasitisation of *Pholetesor bicolor* with different *Phyllonorycter pomonella* host stages

<table>
<thead>
<tr>
<th>Host stages</th>
<th>Females tested</th>
<th>Females which encountered mine and initiated probing</th>
<th>Time (s)(^1) to first ovipositor insertion</th>
<th>Time (s)(^1) probing</th>
<th>Residence time (s)(^1) on leaf</th>
<th>Number of successful females</th>
<th>Average time (s)(^1) attempting parasitism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sap-feeder</td>
<td>20</td>
<td>13</td>
<td>253 ± 234 a(^2)</td>
<td>122 ± 192 a(^2)</td>
<td>887 ± 964 a(^2)</td>
<td>5</td>
<td>297 ± 222</td>
</tr>
<tr>
<td>Tissue-feeder</td>
<td>20</td>
<td>12</td>
<td>435 ± 395 a</td>
<td>195 ± 303 a</td>
<td>1034 ± 1035 a</td>
<td>1</td>
<td>1015 ± 0</td>
</tr>
</tbody>
</table>

\(^1\) average and SD \(^2\) standard deviation

identical letters within same column indicate no significant differences between host stages (Mann-Whitney rank sum test)
**Experiment 2.- Host stage selection**

The average number of hosts parasitized on the two experimental days did not differ for each of the host stages offered, thus all data were pooled for further analysis. No host-density dependency was observed for the treatments where we offered tissue-feeders alone or the combination of sap- and tissue-feeders. In contrast we found a significant negative correlation (P < 0.02, Spearman rank test) between parasitism and number of hosts offered for the treatment where only sap-feeders were available. This was probably due to the fact that hosts were offered in excess. Dissection of female ovaries to determine egg availability showed that all individuals had an egg load which exceeded by far the number of hosts offered during the bioassay (>200 mature eggs).

The host stage preference of the parasitoid *P. bicolor* is depicted in Table 6.2. The number of females that successfully parasitized at least one leafminer was lower for those offered tissue-feeder larvae than for those offered sap-feeder larvae (P < 0.001, Chi-square test). In fact only 53% of the females which were offered tissue-feeders parasitized at least one host, while 88% and 89% of the females which were offered sap-feeders or the combination of sap- and tissue-feeders, respectively, parasitized at least once. Percent parasitism was higher for sap-feeders and the combination of sap- and tissue-feeders than for tissue-feeders (P < 0.01, Mann-Whitney rank test). Within the choice treatment, where both stages were offered, we observed a significantly higher number of sap-feeders parasitized than tissue-feeders (P < 0.001, Wilkoxon signed rank test). The average number of hosts parasitized per female confirms this preference for the sap-feeder host also.

The sex ratio and pupal mortality of parasitoids obtained from the different host stages are also shown in Table 6.2. A high male progeny bias was observed for all host stages. This male bias is highest, although not statistically different, for the tissue-feeder host (81%) and lowest for the sap-feeder hosts (60%). For all host stages a similar high proportion (26 to 31%) of parasitoids died at the pupal stage.
Table 6.2. Parasitisation preference by *Pholetesor bicolor* and suitability of different host stages of the apple leafminer *Phyllonorycter pomonella*

<table>
<thead>
<tr>
<th>Host stages</th>
<th>Total females tested</th>
<th>No. females parasitising ≥ 1 host</th>
<th>Percent parasitism (^1) (no. parasitised hosts/total no. host offered)</th>
<th>Average number of hosts parasitised / female (^1)</th>
<th>Progeny sex ratio (% females)</th>
<th>Parasitoid pupal mortality (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sap</td>
<td>25</td>
<td>22 a(^2)</td>
<td>40.7 ± 20.7 a(^3)</td>
<td>45.6 ± 15.3 a(^3)</td>
<td>40 a(^3)</td>
<td>28 a(^3)</td>
</tr>
<tr>
<td>1st + 2nd instar</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sap and tissue</td>
<td>18</td>
<td>16 a</td>
<td>28.6 ± 22.5 ab</td>
<td>12.0 ± 8.9 b</td>
<td>35 a</td>
<td>31 a</td>
</tr>
<tr>
<td>3rd + 4th instar</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(3rd)</td>
<td>(24.7 ± 18.6 x(^4))</td>
<td></td>
<td></td>
<td>(9.4 ± 7.7 x(^4))</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(4th)</td>
<td>(3.9 ± 6.0 y)</td>
<td></td>
<td></td>
<td>(2.6 ± 3.2 y)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tissue</td>
<td>28</td>
<td>15 b</td>
<td>2.5 ± 22.2 b</td>
<td>4.3 ± 3.6 b</td>
<td>19 a</td>
<td>26 a</td>
</tr>
<tr>
<td>(4th + 5th instar)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^1\) average and SD, \(^2\) different letters within same column indicate significant differences among host stages (Chi-square test) 
\(^3\) different letters within same column indicate significant differences among host stages (Mann-Whitney rank sum test) 
\(^4\) different letters within same column indicate significant differences between host stages (Wilcoxon signed-rank test)
Experiment 3. - Effect of experience on host stage location

The influence of experience on host location in *P. bicolor* is shown in Figure 6.1. The total frequency of female parasitoids for every plant treatment was recorded over a 2 h period, giving the insects multiple choice between plants with sap-feeders, plants with tissue-feeders and non-infested plants. Naïve females were observed on all three plants in equal proportion. In contrast, females experienced with leafminers of either stage were found more often on infested plants than on non-infested plants (*P* < 0.01, Kruskal-Wallis). Furthermore, females that were given an experience on sap-feeder hosts were recorded significantly more frequently with sap-feeder infested plants than on tissue-feeder infested plants (*P* < 0.01, Kruskal-Wallis). On the other hand, a previous experience with tissue-feeder hosts did not increase the frequency of parasitoids on tissue-feeder infested plants.

The influence of experience on the level of searching activity in *P. bicolor* is shown in Figure 6.2. Naïve females were observed to be active (searching) significantly less often than experienced females (*P* < 0.05, ANOVA). In contrast they were recorded often while standing. No significant differences in the activity level where recorded for the two types of experience, that is exposure to sap-feeders or tissue-feeders.
Figure 6.1. Average frequency of *P. bicolor* females observed on each plant treatment in the host location bioassay (S = sap-feeder; T = tissue-feeder; N = non-infested). Arrows indicate on which type of host the parasitoids were given contact experience prior to the bioassay. Letters show statistical differences within parasitoid treatments (*P* < 0.01, Kruskal-Wallis one way analysis of variance on ranks and Student-Newman-Keuls test for pairwise multiple comparisons).
As the bioassay progressed, an increased number of parasitoids was recorded only on the plants infested with sap-feeders (relationship fits a linear regression). This temporal dynamics of parasitoid abundance on sap-feeder infested plants is shown in Figure 6.3. This time-dependency applies for all female treatments, the experienced and the naïve females (least square analysis S-FEM: $F = 13.1$; d.f. = 1, 238; $P < 0.001$; T-FEM: $F = 11.6$; d.f. = 1, 238; $P < 0.001$; NAÏVE: $F = 9.1$; d.f. = 1, 238; $P < 0.01$). The total number of parasitoids recorded on sap-feeder infested plants over the 2 h bioassay period was higher for experienced than for naïve females ($F = 16.88$; d.f. = 4, 714; $P < 0.001$) and higher for sap-feeder experienced females than for tissue-feeder experienced females ($F = 25.1$; d.f. = 2, 476; $P < 0.001$).
6.5. Discussion

Our experiments on host location and host stage selection provide conclusive evidence that *P. bicolor* is capable of finding and parasitizing both larval stages of its concealed host, the apple leafminer *P. pomonella*. A previous contact experience on any of the two host stages increases the searching activity of *P. bicolor* when compared to no experience. An experience with sap-feeders enhances the ability of the parasitoid to locate sap-feeder infested plants (Figure 6.1). In contrast, an experience with tissue-feeders does not enhance its ability to locate tissue-feeder infested plants. In fact, sap-feeder larvae are more successfully parasitized than tissue-feeders. Thus, the host
location and selection strategy of this parasitoid is shaped by the unique two-stage larval development, the endophytic lifestyle of its host, and by experience.

Differences in detectability, accessibility and suitability of the two host stages may account for the observed differences in searching activity and parasitisation. The time allocated by parasitoids to localise and attempt parasitisation of both host stages are comparable, indicating similar detectability of both host stages. In contrast, different accessibility could explain the finding that the leafminer larvae in the tissue-feeding stage are less successfully parasitized by *P. bicolor* than the leafminer larvae in the 1st and 2nd instars sap-feeder stage. In fact behavioural defences of the larvae (Bacher *et al.*, 1996; Conner & Cargain, 1994) and morphological barriers of the mine (Gross, 1993) are known to prevent parasitoids from parasitizing their host. In our tritrophic system the size and tentiform structure of the mine give the tissue-feeding larva the possibility to move and escape parasitism. In addition the accessibility of late sap-feeder larvae (3rd instar) is comparable to that of tissue-feeders, since the size of the mine is similar. Indeed in our experiments 3rd instar sap-feeders were parasitized less than 1st and 2nd instar sap-feeders (Table 6.2).

The host suitability parameters we measured, provided only partial information on host quality and did not indicate any differences between sap- and tissue-feeders. Pupal mortality and sex ratio were similar for both host stages. Since the age-related size of the host can influence the capability of the immune system to defend itself against parasitoid attack (Bauer *et al.*, 1998; Godfray, 1994) we cannot exclude encapsulation as a possible reason for the low parasitism obtained in tissue-feeders.

Experience played an important role in the host searching behaviour of *P. bicolor* as the multiple choice host location test demonstrated (Figures 6.1, 6.2 and 6.3). Behavioural changes in both responsiveness and preference upon experience are generally referred to as learning (Turlings *et al.*, 1993; Vet *et al.*, 1995). Through selective increase in responsiveness and induction of preferences, learning can change initial random search to directed search
resulting in increased host-encounter rate (Vet, 1996). In our system naïve females were equally distributed on infested and non-infested plants (which we interpret as a consequence of random searching) and were less active than experienced females. Females experienced with tissue-feeders were more often recorded on infested plants than on non-infested ones. Furthermore, females experienced with sap-feeder mines were recorded more often on plants infested by the preferred host stage (sap-feeders) than on plants infested by a less preferred host stage (tissue-feeders) or on the non-infested ones. Therefore, experience on hosts of different value (i.e., high or low accessibility) affected both the parasitoid’s searching direction and their level of searching activity. This behavioural increase in activity, after an experience with the host-plant complex, has been observed for many parasitoids, including several braconid species (Vet & Groenewold, 1990). The effect of directional searching activity after experience has also been demonstrated for parasitoids in habitat and micro-habitat location (Geervliet et al., 1998; Papaj & Vet, 1990; Vet & Papaj, 1992; Vet & Schoonman, 1988; Vet & van Opzeeland, 1984) but to our knowledge not for parasitoid host-stage/age selection. The closest work is an investigation of the influence of experience on host quality assessment of host aphid species (and not host-stage or age) by Monoctonus paulensis (Michaud, 1996). In this system the parasitoid abandons quickly a low-quality host species, after a previous encounter (i.e., experience) with a high-quality host.

This ability of learning to search more efficiently a host which can be parasitized more successfully is highly adaptive for *P. bicolor* in this tritrophic system. In fact this parasitoid is not able to rely on long range chemical cues to discriminate between volatiles emitted by plants infested by sap- and tissue-feeder hosts (U. Lengwiler, unpubl.). This contrasts with another braconid, *Cotesia kariyai*, which is able to discriminate between herbivore-induced synomones from corn plants damaged by the different instars of the host, and prefers volatiles emitted by the suitable ones (Takabayashi et al., 1995). In addition upon first contact with the mine, *P. bicolor* does not seem able to discriminate between sap- and tissue-feeder, but attempts probing all available mines, including those with less accessible hosts. This again contrasts with the
strategy of the braconid *Cotesia glomerata*, which uses age-specific contact cues to select the most suitable instar of its host, *Pieris brassicae*, even before contacting the host itself (Mattiacci & Dicke, 1995a; Mattiacci & Dicke, 1995b). Remarkably, *P. bicolor* utilises a third strategy to locate its preferred host stage and to increase its chances of successful parasitism: learning.

Unrewarding experience could also explain the results observed. In our system the experience with a sap-feeder host has a stronger effect on searching than the experience with a tissue-feeder host (Figure 6.1). This could be due to the fact that the latter does not provide a reward (Papaj *et al.*, 1994) in terms of contact with the larva. In fact, females given experience with a sap-feeder had a higher chance of contacting directly the host during experience due to a smaller mine. In contrast, the likelihood that females contact a tissue-feeder larva during experience is reduced, due to the tentiform shape of the mine and the ability of the larva to move. Thus, the parasitoid's experience with tissue-feeders would only be an exposure to odour cues from the host-plant complex and not a direct contact with the host.

Parasitoid directional foraging was not only dependent on experience, but also on the duration of the searching activity. In particular, the number of parasitoids recorded on sap-feeder infested plants showed a positive correlation with time (Figure 6.3). This could be due to the fact that individuals which landed directly on a patch of preferred hosts remained there and continued to parasitize the available hosts, as observed in other host parasitoid systems (Dethier *et al.*, 1960; Vet & Schoonman, 1988). In contrast, parasitoids that first landed on a patch of less preferred hosts or a non-infested plant spent initially some time foraging in that area, before moving on to the plant with preferred hosts. Since attempts to parasitize a tissue-feeder host are very time consuming (Table 6.2), it is expected that with time, the cost of foraging, in terms of energy expenditure and physical fitness, is so high that abandoning an unrewarding host patch would be more profitable (Papaj *et al.*, 1994).
The type and nature of the cues used and learned by *P. bicolor* for close range host location and host acceptance remain to be elucidated. Ongoing experiments reveal that chemical cues from the mine are strongly involved, possibly in association with tactile, visual and vibrational stimuli.

### 6.6. Acknowledgements

We thank T. Christoffel, H. Simmler, M. Probst and M. Krebs for the rearing of plants and insects; C. Fornallaz for computer and graphic support; H. Gu for statistical help; G. Deschka (Steyr, Austria) and J. Papp (Budapest, Hungary) for the identification of the species; T. Webb and K. Tschudi-Rein for their editorial comments and F. Wäckers for his valuable suggestions.
7. General Discussion and Outlook

The foraging behaviour of the leafminer parasitoid *P. bicolor* once it has landed on a leafminer-infested leaf was investigated. In the first part (Chapters 4 and 5) we elucidated the source of chemical cues, identified the compounds mediating parasitoid host location and we analysed the chemical response of apple seedlings to leafminer herbivory. The compounds identified were subsequently tested to determine their role for parasitoid habitat location and their possible use as priming stimuli (Chapter 5). In the second part (Chapter 6) the host stage preference, acceptance and suitability were studied. Here we focused on host stage discrimination and the influence of experience on host location. The findings are discussed regarding the possible reasons why plant-derived rather than host-derived chemical cues are used by a leafminer-parasitoid for locating its host, the ecological function of the identified induced compound mediating host location, and the adaptive value of learning for host-stage discrimination. Possible future research perspectives are integrated in the discussion.

**Plant-derived versus host-derived chemical cues**

The results of this study reveal that plant-derived chemical cues are used by the leafminer parasitoid *P. bicolor* for locating its host, *P. pomonella*, once having landed on an infested patch. These plant derived chemical cues trigger an innate ovipositional probing behaviour, which was not observed when other components derived from the host complex were offered, such as larvae or frass (Chapter 4). This result is in contrast to what is generally found for parasitoids attacking free living herbivores which use host-derived chemical cues for host location after contacting a host infested patch. Based on studies on parasitoids of free living hosts, the current reliability-detectability hypothesis on parasitoid use of semiochemicals (Vet *et al.*, 1991) assumes that parasitoids should respond innately to host or host by-products. In the case of a leafminer...
parasitoid the unexpected innate contact response to a plant- rather than a host-derived cue could be due to the fact that the parasitoid has virtually no immediate direct contact with the concealed host once on a leaf. In such a system therefore, the chemical and physical characteristics of the mined leaf might act as an analogue of the surface of a free-living herbivore which elicits an ovipositional behaviour (Vinson, 1991).

For the leafminer producing no chemical cues might be a mechanism of predator avoidance. Leafminers are easily discovered by a large number of parasitoid species over evolutionary time because they are relatively immobile and are only partially protected by their mine. In fact leafminers in the average have a much richer parasitoid complex when compared to external free feeder herbivores (Hawkins, 1993). The opportunities that leafminers have to protect themselves against parasitoids are quite limited as compared to free feeders which for example can bite or move away from the parasitoid by dropping off the plant (Gross, 1993). Producing no chemical cues is perhaps a mechanism by which leafminers, which are under strong selective pressure, make themselves less conspicuous to foraging parasitoids. Studies on the behavioural interactions of leafminers with other parasitoid species should be carried out to obtain a more detailed insight on whether leafminer parasitoids rely on other cues such as vibrational and visual cues rather than host-derived chemical cues for finding their concealed hosts.

**Ecological function of the induced compound mediating host location**

The induced production of the triterpene squalene in apple leaves following leafminer herbivore attack was demonstrated in Chapter 5. Furthermore, its involvement in the ovipositional probing behaviour and therefore in host location of the leafminer parasitoid was shown in Chapters 4 and 5. The induction of volatile mono- and sesquiterpene compounds has been shown in crops such as corn, cotton, lima beans and Brussels sprouts, following herbivore attack and their involvement in habitat location of parasitoids has
been documented (Dicke et al., 1990; Mattiacci et al., 1994; McCall et al., 1993; Turlings et al., 1990).

Whether plants have specifically evolved the ability to release compounds that attract natural enemies of a herbivore that is attacking them remains a matter of debate, especially when the compound is involved in other plant functions or when the compound is not herbivore specific. Taking the example of squalene, this terpene is the basic intermediate metabolite for the biosynthesis of triterpenoids including phytosterol, cardenolides, limonoids, saponins, quassinoids, to mention only a few of those that have been considered as ecologically significant in plant-herbivore interactions. The increased production of squalene upon herbivory in apple seedlings could lead, with time, to the production of other compounds with various other plant functions. For example, an increase of squalene could be involved in repairing damaged plant parts, or in the biosynthesis of compounds acting as phytoalexins, toxins, digestibility reducers and/or repellents against the herbivore feeding on the plant or against other herbivores. Squalene could also be involved as a precursor of volatile mono- and sesquiterpenes used by natural enemies. Long term chemical studies would need to be conducted, particularly on perennial plants, in order to understand the possible direct and indirect defence mechanisms of the plant and the functions that squalene might have in relation to insect herbivory.

Investigating the specificity of the plant response with respect to the damaging agent could also provide some insight as to whether the production of squalene is an induced defence response to leafminer herbivory. Although the data shows that fresh mechanical damage to apple leaf tissue does not trigger an ovipositional response in *P. bicolor* (Chapter 5); no chemical analysis was performed with such leaves. To mechanically mimic the exact way (site and process) in which leafminers damage a leaf poses some difficulties; first because the larvae feed within the tissue, and second because this specific leafminer has two distinct feeding habits (sap- and tissue-feeder). Nevertheless, examining the chemical response of apple leaves to other leafminer species
that are not hosts of *P. bicolor* would provide further understanding on the response specificity.

**Adaptive value of learning**

The ability of parasitoids to learn has now been demonstrated for many different species (Turlings *et al.*, 1993) and it seems to be the rule rather than the exception. In Chapter 5 as well as in previous studies (Lengwiler *et al.*, 1994) we observed that a contact experience of the parasitoid to infested plants was needed for *P. bicolor* to locate leafminer infested leaves. This result strongly supports the hypothesis that learning plays an important role in habitat location of a generalist parasitoid such as *P. bicolor*.

*P. bicolor* is not only confronted with various host species but when searching for hosts in apple orchards it is confronted with two host stages of the apple leafminers of the genus *Phyllonorycter*. A difference in the ability of *P. bicolor* to parasitize these two host stages was shown in Chapter 6. For example attempts to parasitize a tissue-feeder host were very time consuming and in most cases females failed to parasitize this host stage, while they were always successful in parasitizing sap-feeder hosts. For *P. bicolor* females which contain an unlimited number of eggs (> 200), reproductive success is most likely limited by the time available for host location and oviposition. Hence, for this parasitoid the ability to discriminate between the two host stages would optimise the foraging behaviour and consequently the reproductive fitness. In this work we observed that naïve females were not able to discriminate between the two host stages. In contrast previous contact with any of the two host stages of the leafminer increased searching activity of *P. bicolor*, and experience with sap-feeder hosts enhanced the ability of the parasitoid to locate sap-feeder hosts. An experience with tissue-feeders did not enhance the ability of females to locate tissue-feeder hosts.

The ability of learning to search more efficiently a host that can be parasitized more successfully is highly adaptive for *P. bicolor* in this tritrophic
system. First because this parasitoid is not able to rely on long-range chemical cues to discriminate between volatiles emitted by plants infested by sap- and tissue-feeder hosts (U. Lengwiler, unpubl.). This contrasts with another braconid, *Cotesia kariyai*, which is able to discriminate between herbivore-induced synomones from corn plants damaged by the different instars of the host, and prefers volatiles emitted by the suitable ones (Takabayashi *et al.*, 1995). Second, upon first contact with the mine, *P. bicolor* does not seem able to discriminate between sap- and tissue-feeder, but attempts probing all available mines, including those with less accessible hosts (Chapter 6). This again contrasts with the strategy of the braconid *Cotesia glomerata*, which uses age-specific contact cues to select the most suitable instar of its host, *Pieris brassicae*, even before contacting the host itself (Mattiacci & Dicke, 1995a; Mattiacci & Dicke, 1995b). *P. bicolor* utilises instead learning as a third strategy to locate its preferred host stage and to increase its chances of successful parasitism.

Learning is not only used as a strategy by which *P. bicolor* can successfully find a habitat with potential hosts, as known for several other systems, but also as a strategy to recognise the preferred host stage. This ability to learn might contribute to the observed success of this parasitoid in the field and could have applications for *Phyllonorycter* leafminer management. Further work to elucidate the stimuli involved in learning need to be done in order to determine its potential use in the field. In the laboratory we have observed that priming female parasitoids with the innately recognised plant derived squalene did not result in a subsequent attraction to leafminer infested plants. This demonstrates that squalene is not a priming stimulus, but it is likely that this substance is used in associative learning. This means that the parasitoid needs an exposure to the innately recognised stimuli (unconditioned stimuli) together with other novel stimuli (conditioned stimuli). For this system this could mean that an experience with squalene should be associated to volatile plant cues or to an oviposition in order to be effective.
8. References


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10. Curriculum Vitae

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1994-1996  Research assistant at the Swiss Federal Research Station for Agroecology and Agriculture Zurich-Reckenholz in the Dept. of Plant Protection, Entomology with Dr. Franz Bigler and Plant Pathology Division with Dr. Hans Rudolf Forrer.

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