Pergamon 0031-9422(95)00948-5 *Phytochemistry*, Vol. 42, No. 2, pp. 373–381, 1996

0031-9422/96 $15.00 + 0.00

VOLATILES EMITTED BY APPLE FRUITLETS INFESTED BY LARVAE OF THE EUROPEAN APPLE SAWFLY

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(*Received in revised form* 7 *November* 1995)

Key Word Index—*Malus domestica*; Rosaceae; apple varieties; *Hoplocampa testudinea*; Tenthredinidae; sawfly larvae; chemical ecology; terpenoids; volatiles; parasitic wasp.

Abstract—The effect of infestation by larvae of the European apple sawfly (*Hoplocampa testudinea*) on the emission of volatiles from apple fruitlets was investigated. Healthy apples emitted a blend of terpenoids and a few aromatic and short aliphatic compounds. Infested apples emitted the same compounds as healthy apples. However, *trans,trans*-α-farnesene, *trans*-β-ocimene and another terpenoid were emitted in significantly larger amounts, whereas the other volatiles showed no quantitative change. The results were obtained with picked apples and could be confirmed in the field with single apples left on the tree. Several non-terpenoid volatiles emanated from the frass and from epidermal glands of the sawfly larvae. The possible ecological significance of the increased emission of terpenoids by infested apples is discussed with reference to a parasitic wasp that attacks the sawfly larvae.

INTRODUCTION

Hundreds of volatiles have been identified from fruits, flowers and other plant parts [1, 2] and they can be implicated in insect–plant relationships. It has been shown that plants can emit volatiles from their leaves when infested by phytophagous insects and mites, and that invertebrate natural enemies of these herbivores are specifically attracted by these volatiles [3, 4]. Fruits, however, have never been analysed for volatiles induced by a phytophage. Apples are of great economic importance and, consequently, volatiles, especially those from apple juice and stored apples, have been well studied. About 350 volatiles have been listed and they are mainly esters, alcohols and acids [1].

In orchards of Europe and North America, the European apple sawfly *Hoplocampa testudinea* (Klug) (Hymenoptera, Tenthredinidae, Nematinae) is a pest insect. It is specialized on apple and has one generation per year. The larva has five instars, L1–L5, and feeds within the apple fruitlet in central Europe during May and June. L1 mines just under the apple epidermis. Later instars drill into the fruit and will attack several fruits. L5 leaves the fruit and pupates in the ground [5]. An infested apple is easily recognized when a late instar larva has made a hole that is usually surrounded by a 'crater' of accumulated frass (Fig. 1).

The present work studies the effect of sawfly larvae on apple *Malus domestica* Borkh. (Rosaceae) odours. Do the odours differ between healthy and infested apples? If a difference exists, which chemical compound(s) emitted by infested apples is/are actually produced by the fruit as a response to infestation? This is a crucial point since it is known that disturbed late instar larvae emit strong odours themselves [6]. Indeed, nematine larvae possess epidermal ventral glands which produce volatiles that function in defence against predators [7]. Here, we determined which odours are specifically emitted by the fruit and how these odours differ from those emitted by the larva itself or its frass (faeces). The study is to show what chemical cues are available to natural enemies to locate sawfly-infested apples.

RESULTS

Volatiles of picked apples

Healthy apples emitted a series of terpenoids and several aromatic and aliphatic compounds (Fig. 2). Among the terpenoids, three remained unidentified (terpenoids I–III; see Experimental). Chromatograms obtained from the analysis of volatiles of healthy apples of five varieties that were picked at five different dates, showed that *trans,trans*-α-farnesene, *trans*-β-ocimene and phenylacetonitrile were the major compounds. They were consistently present and relatively abundant

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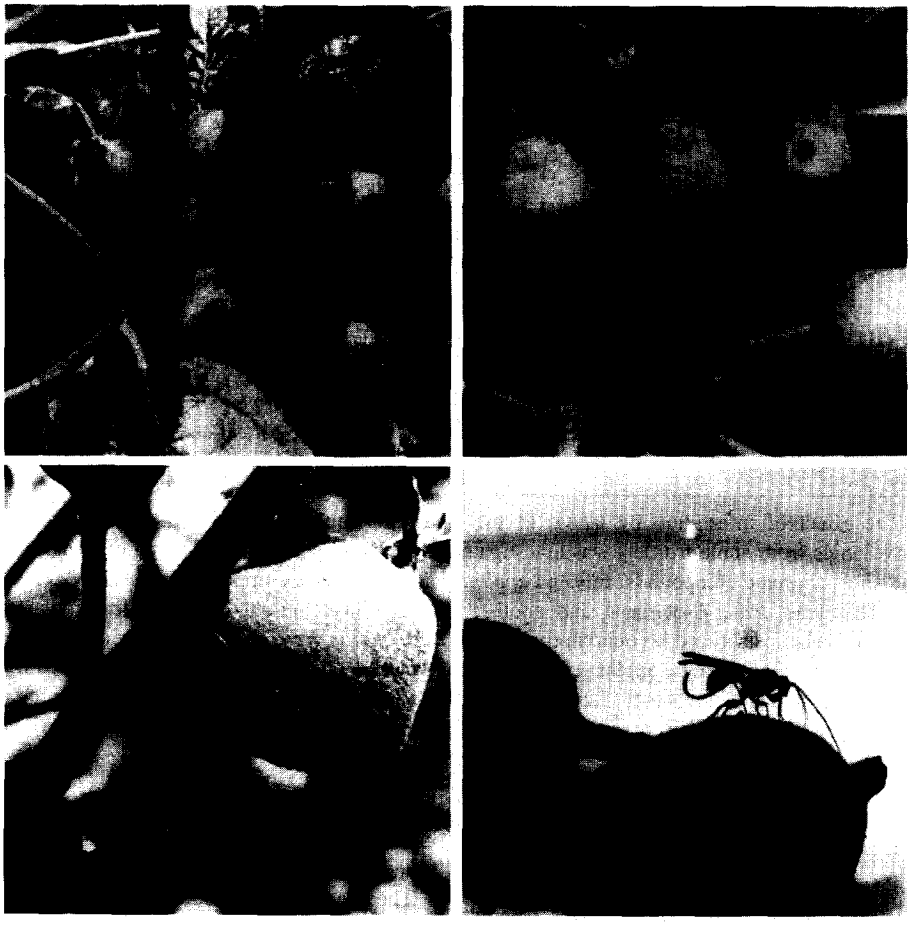

Fig. 1. A cluster of healthy apple fruitlets (above, left), an apple infested by a second instar (above, right) and fifth instar (below, left) sawfly larva, and a female of *Lathrolestes ensator* during egg-laying (below, right).

(typically ranging from 1–11, 1–6 and 1–6 ng apple^{-1} h^{-1}, respectively). Infested apples emitted *trans,trans*-α-farnesene, *trans*-β-ocimene and terpenoid II in significantly larger amounts than healthy apples ($P \leq 0.05$ for each compound at each date, randomization test for matched pairs, one tailed; $n = 5$ varieties; Fig. 3). Phenylacetonitrile did not follow this trend (Fig. 3). As the apples grew larger, no significant increase was found in the amount of the three terpenoids, neither for healthy apples ($P > 0.05$, Friedman two-way analysis of variance; $n = 5$ varieties; Fig. 3), nor for infested apples ($P > 0.05$). Although there is a trend for infested apples of several varieties to emit more volatiles at the later collection dates (Fig. 3), this could not be confirmed by the Friedman two-way analysis of variance. Apple diameter increased from *ca* 8 mm at the first collection date to 26 mm at the last date. So a single apple emitted similar amounts of volatiles, independent of its size, during the study period. Slight differences between the five varieties occurred in the amount of *trans,trans*-α-farnesene emitted by healthy apples ($P < 0.05$; Friedman two-way analysis of variance; $n = 5$ dates) and of *trans*-β-ocimene emitted by infested apples ($P < 0.05$). These differences were principally due to the relatively small amounts of volatiles produced by cv. Liberty (Fig. 3).

The following compounds (given as a range in ng apple^{-1} h^{-1}, for at least 47 of the 50 samples of apples) were detected in relatively small amounts and the amounts were similar for healthy and infested apples: *trans*-4,8-dimethyl-1,3,7-nonatriene (0.5–2), linalool, together with terpenoid I (0–2), *trans*-2(3)-epoxy-2,6-dimethyl-6,8-nonadiene (0–1), β-bourbonene (0–2), *trans*-β-caryophyllene (0–1), terpenoid III (0–4), *trans*-2-hexenal (0–1), *cis*-3-hexenyl acetate (0.1–5) and methyl salicylate (0–3). 2-Phenylethanol was detected in small amounts (0–1) at the first collection dates in both healthy and infested apples, whereas it increased drastically (reaching over 100) only for infested apples during the last two dates.

Volatiles of apples on the tree

In an orchard, volatiles were collected from single apples on trees. Due to the small amounts released by a single apple, volatiles were not always detected from

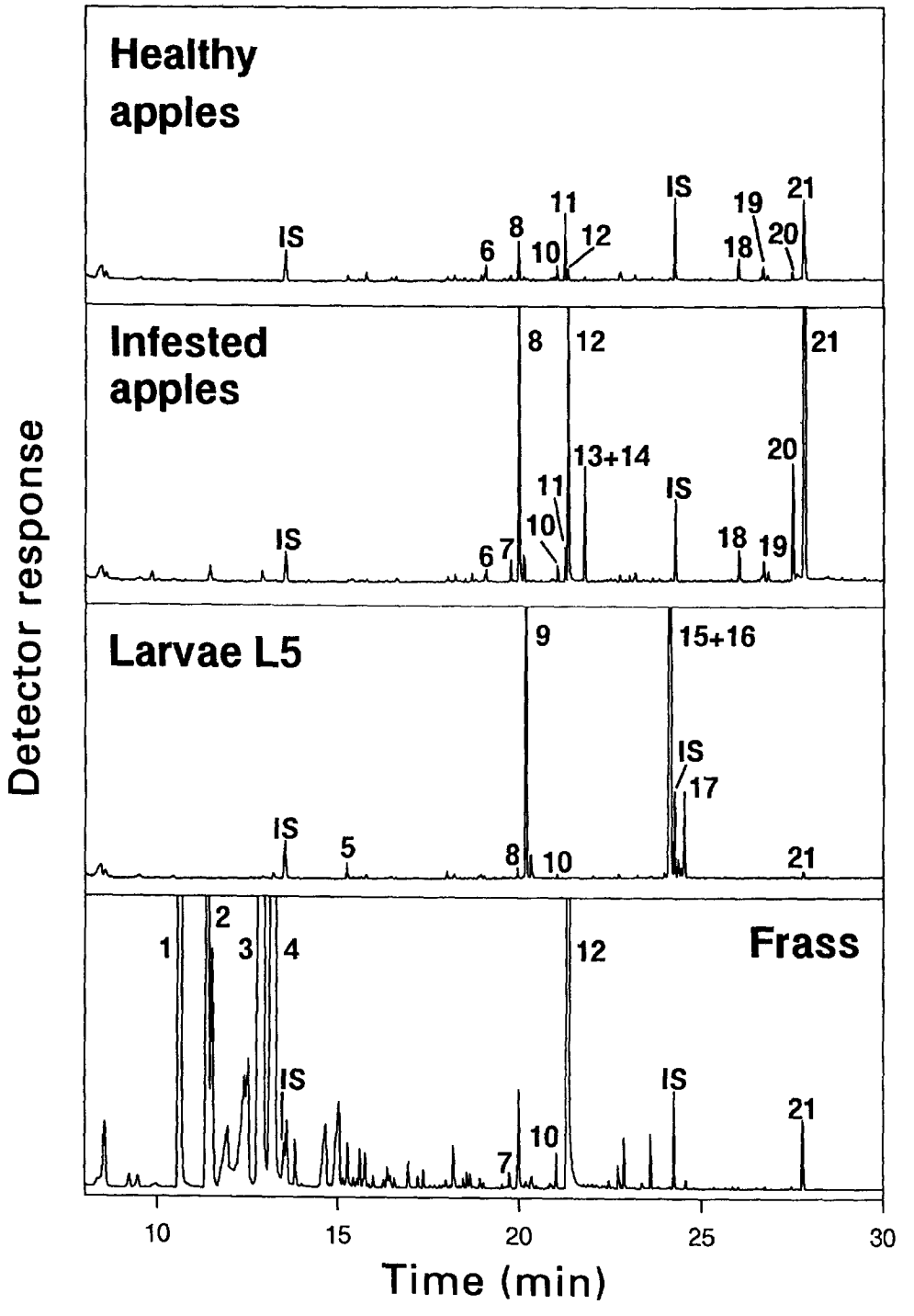

Fig. 2. Chromatograms of the volatiles emitted by healthy apples, by apples infested by *Hoplocampa testudinea* larvae, by isolated fifth instar larvae, and by the frass of larvae. Apples cv. Maigold. The identified compounds are: 3-hydroxy-2-butanone (1), 3-methyl-1-butanol (2), 2,3-butanediol (3), 1,2-butanediol (4), *trans*-2-hexenal (5), *cis*-3-hexenyl acetate (6), phenylacetaldehyde (7), *trans*-β-ocimene (8), *trans*-2-octenal (9), *trans*-4,8-dimethyl-1,3,7-nonatriene (10), phenylacetonitrile (11), 2-phenylethanol (12), linalool (13), the unidentified terpenoid I (14), *trans,cis,cis*-2,4,7-decatrienal (15), *trans,cis*-2,4-decadienal (16), *trans,trans*-2,4-decadienal (17), β-bourbonene (18), *trans*-caryophyllene (19), the unidentified terpenoid II (20), *trans,trans*-α-farnesene (21). *trans*-2(3)-Epoxy-2,6-dimethyl-6,8-nonadiene and the unidentified terpenoid III were not detected in the chromatograms shown here, but in others (see text).

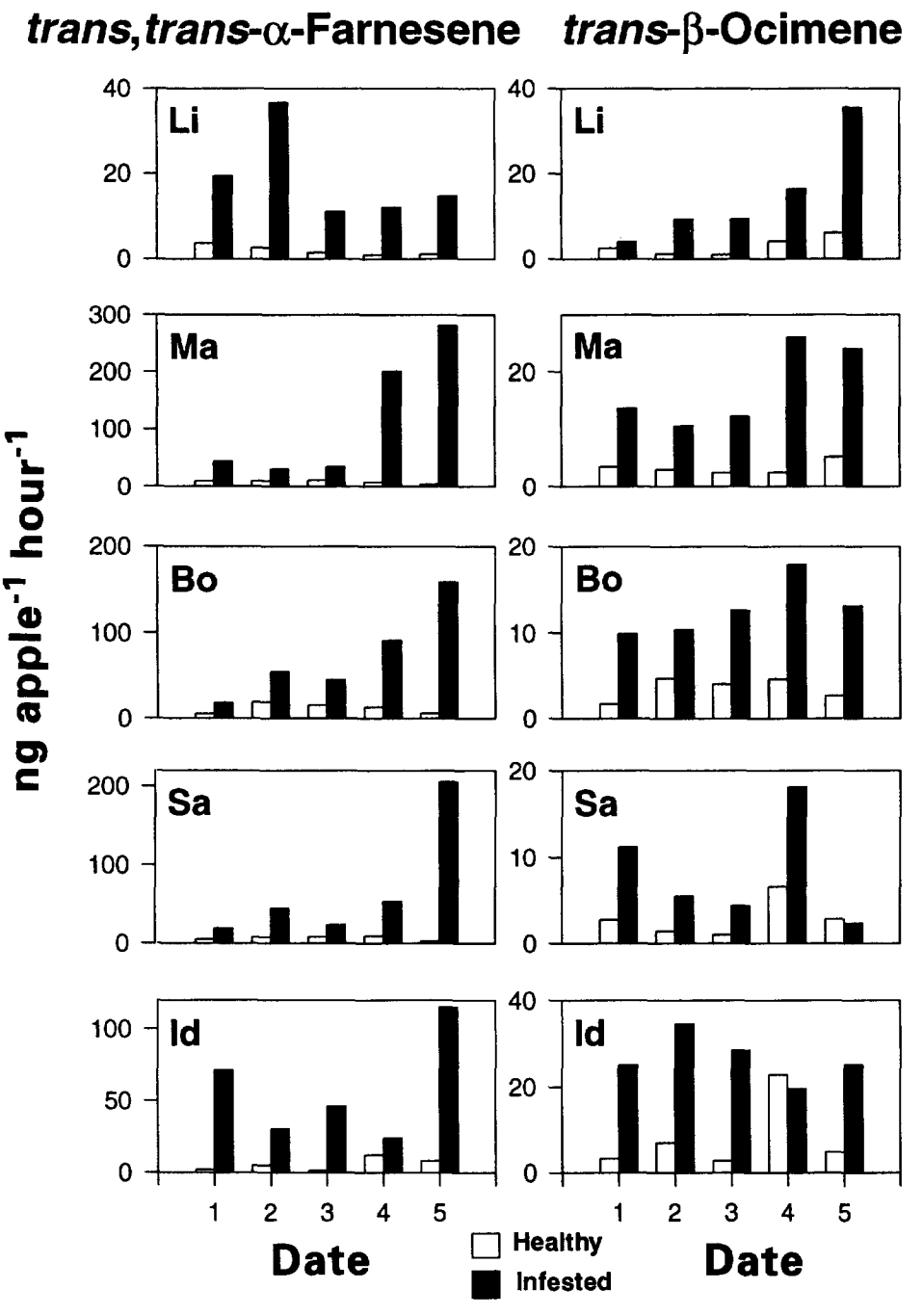

Fig. 3. Amounts of volatiles collected during 22 hr from healthy and infested apples on five dates (1–5) from the middle of May to the middle of June. Varieties: Liberty (Li); Maigold (Ma); Boskoop (Bo); Sauergrauech (Sa); Idared (Id).

every apple (Table 1). However, we did find that, in general, *trans,trans*-α-farnesene and *trans*-β-ocimene were emitted in larger amounts by infested than by healthy apples, as was observed in the laboratory with picked apples. Compared to the latter, apples on the tree emitted roughly similar amounts of volatiles. The presence of *trans,cis*-2,4-decadienal from healthy apples is surprising since it is also a major compound of the secretion of the sawfly larvae (Fig. 2).

Volatiles of sawfly larvae and their frass

The volatiles collected so far from infested apples could have been emitted by the fruitlet, the sawfly

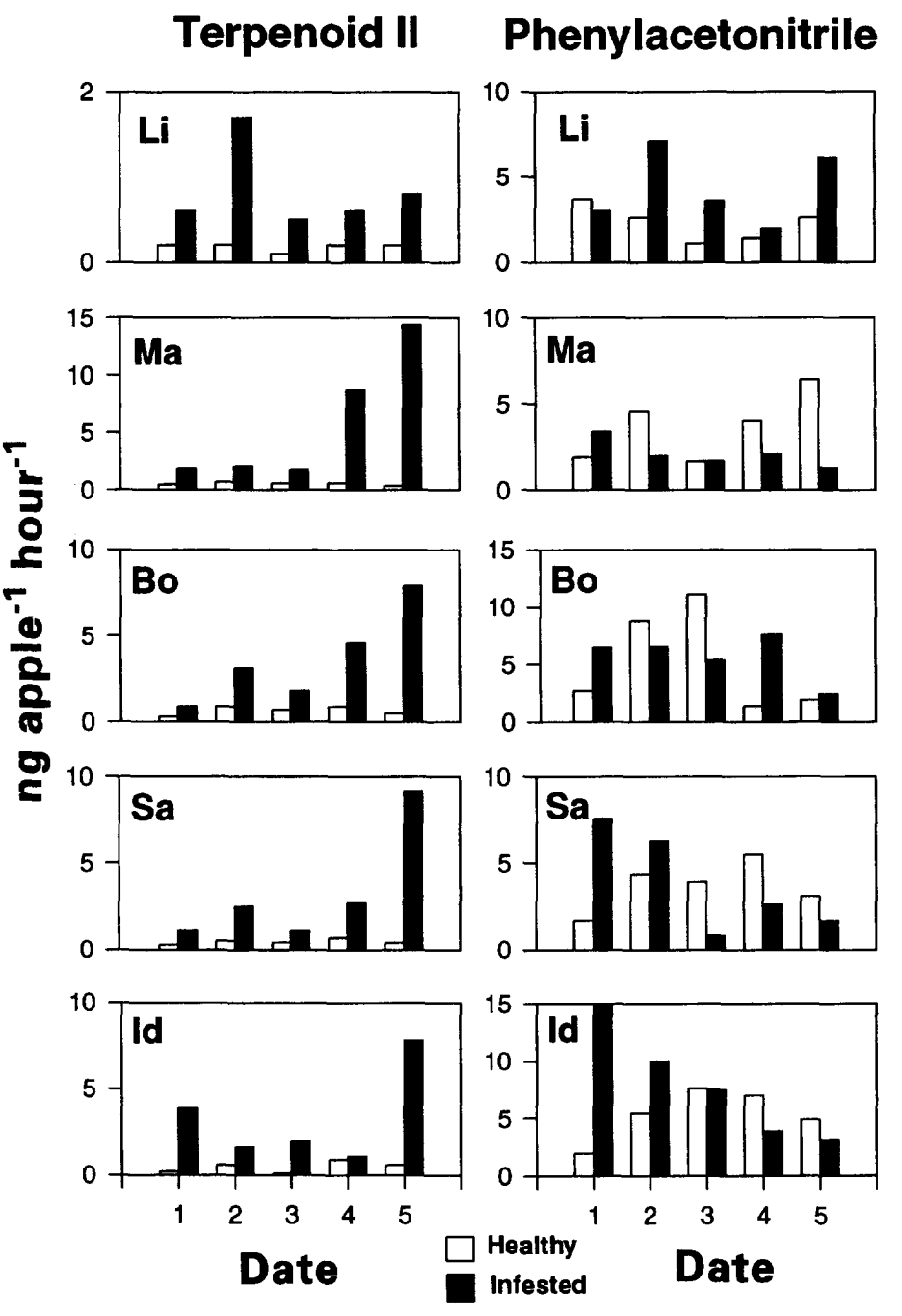

Fig. 3. *Continued.*

larva, or by its frass. To determine the exact source of the volatiles, larvae and frass were isolated from the fruit. Isolated larvae were mechanically disturbed to collect volatiles emitted by ventral glands which the larva can use as a chemical defence. A series of aliphatic compounds were detected (Fig. 2), which is in accordance with the presence of the same compounds in pentanolic extracts of dissected glands (Boevé, J.-L. *et al.*, unpublished results). Volatiles collected over a longer period from crushed larvae were the same as those from disturbed larvae in Fig. 2. All these results concerned L5 which, once disturbed, emits a strong odour. In contrast, no single compound was detected with living or crushed L4 analysed under the same conditions. By disturbing a L3 or L4, nothing was perceived by human olfaction.

Table 1. Amount (in ng apple^{-1} hr^{-1}) of volatiles collected in the field during 4 hr from five healthy and 13 infested apples on trees

Compounds	Healthy apple	Infested apple
trans-β-Ocimene	3.0	22.9±18.7 (6)
trans-4,8-Dimethyl-1,3,7-nonatriene	3.4±0.2 (3)	8.3±8.3 (3)
Linalool + terpenoid I	—	5.0±1.3 (3)
β-Bourbonene	12.8±10.9 (4)	4.4±2.2 (4)
trans-Caryophyllene	5.6±2.0 (2)	3.2 (1)
Terpenoid II	—	4.0±1.4 (2)
trans,trans-α-Farnesene	5.7 (1)	25.1±18.8 (7)
Phenylacetaldehyde	—	9.5 (1)
Phenylacetonitrile	3.0 (1)	14.4±8.1 (3)
2-Phenylethanol	—	73.8±76.8 (4)
3-Hydroxy-2-butanone	—	88.3±35.7 (2)
3-Methyl-1-butanol	—	32.8±11.4 (2)
2,3-Butanediol	—	61.3±24.7 (2)
1,2-Butanediol	—	59.6±3.2 (2)
trans-2-Hexenal	—	10.3 (1)
cis-3-Hexenyl acetate	4.6±2.1 (2)	—
trans,cis-2,4-Decadienal	3.9±1.4 (3)	3.7 (1)

Each value ±SD is based on those apples (number in parentheses) for which the given compound was detected. (–) Compound never detected. Apples cv. Maigold between 16 and 26 mm in diameter. Larvae L4 and L5. The four butanoic compounds and most of 2-phenylethanol emanated from the frass (see also Fig. 2).

Frass contained as major compounds a series of butanoic compounds and 2-phenylethanol (Fig. 2). Small amounts of *trans,trans*-α-farnesene were also detected.

DISCUSSION

The compounds *trans,trans*-α-farnesene, *trans*-β-ocimene and terpenoid II were emitted in significantly larger amounts by infested than by healthy apples (Fig. 3). The question arises, if the apple itself increases its production of these compounds under infestation. This seems to be likely, based on the following arguments. Firstly, almost no terpenoids were detected in the secretion of the larvae (Fig. 2). Secondly, only L5 were an obvious source of chemicals, whereas younger instars were chemically much more hidden. The infested apples emitted terpenoids in large amounts, however, throughout larval development (Fig. 3). This excludes the larvae as a source for the three terpenoids. Thirdly, only minute amounts of *trans,trans*-α-farnesene, but large amounts of four butanoic and two aromatic compounds, emanated from frass; reverse proportions were observed for infested apples. It is likely that frass deposited by the larva on the apple surface was somewhat contaminated with apple volatiles. Clearly, the large amounts of farnesene in our samples of infested apples cannot be derived from frass. Finally, healthy apples already produced those terpenoids, which were detected in larger amounts from infested apples. Each of these facts show that the terpenoids originated directly from the plant and not from the larvae or their products.

In the present study, only terpenoids and a few aromatic and aliphatic compounds were detected as volatiles emitted by apples. Others have described a much larger diversity of volatiles [1]. Compared to almost all previous studies, ours was different since it involved very young apples that were not mechanically damaged before or during collection of volatiles. The large diversity of volatiles observed in other studies is only produced during fruit ripening [8]. However, the necessary enzymes must already be present in fruitlets since Drawert *et al.* [9] showed that diverse volatiles are produced when 50-day-old fruitlets are picked and then stored for another 50 days prior to volatile collection. Apple varieties differ in the major types of chemicals they emit. They can be described as 'alcohol type' or 'ester type' [9]. We detected mainly terpenoids since we used fruitlets rather than fruits. The biosynthesis of volatiles appears similar for very young apples and apple leaves [9]. This is confirmed in our samples by the presence of *trans*-β-ocimene and β-bourbonene, for instance, which were not previously described from apples [1], but from apple leaves [10].

Phytophagous insects can elicit volatile production in plants [3, 4]. This effect is distinct from mechanical damage and we assessed it for apple fruitlets in preliminary experiments using sawfly larvae. During one day, healthy apples were either: (1) left undamaged; (2) mechanically damaged by drilling them once with a metal drillbit (diameter: 2.5 mm); (3) same mechanical damage with a larva crushed into the hole, to let a possible elicitor act; or (4) infested by a larva. The emission of *trans,trans*-α-farnesene and *trans*-β-ocimene increased in the last situation only. This

increase might be due to the continuous damage inflicted by the larva and/or to an elicitor coming from the larva.

2-Phenylethanol was found in large amounts in infested apples during late infestation. The presence in minute amounts of this compound in healthy apples may have been due to minor infestation by pathogens that we overlooked. 3-Hexenyl acetate was one of a series of compounds emitted in similar amounts by healthy and infested apples. It is known to be emitted by apple seedlings when leaves are subjected to mechanical stress (Lengwiler, U., unpublished results).

Since infested apples emitted more volatiles than healthy ones, they could be more attractive to natural enemies of the sawfly *H. testudinea*. The parasitic wasp *Lathrolestes ensator* (Brauns) (Hymenoptera, Ichneumonidae) is a specialized endoparasitoid of larvae of this sawfly species [11]. It probably lays eggs into young sawfly larvae. In the field, the presence of adults of the parasitic wasp is synchronized with the presence of young larvae [12]. After landing on an infested apple, the parasitic female wasp walks on the surface of the apple and frequently inserts its ovipositor into the apple, in order to locate the sawfly larva (personal observation, Fig. 1). The ovipositor is about 3 mm long and, consequently, a L2 or older larva may escape parasitism by feeding deep inside an apple. Thus, the seasonal occurrence of the parasitoid and the location of its host suggest that especially young larval instars of the sawfly are susceptible to being parasitized. Volatile differences in apples, which may provide the parasitoid with chemical cues indicating the presence of a potential host, were already observed during early infestation (Fig. 3). Parasitic wasps and other entomophagous arthropods are known to be attracted to plant terpenoids induced by herbivores [3, 4, 13]. The chemical differences between healthy and infested apples were quantitative, whereas qualitative differences are known for other plant species that are damaged by herbivores [4]. As apples are not infested by other insects when sawfly larvae occur, the increase in terpenoids may be a sufficiently reliable indication of the presence of sawflies. Apple volatiles are known to attract phytophagous insects that are in search of a host plant [14–16]. It remains to be determined if the parasitic wasps exploit the chemical differences.

The glandular secretion of the L5 (Fig. 2) might constitute a defence against predators such as ants [7]. They are less likely to play a role in host location by the parasitoid because early larval instars do not emit these secretions. The interactions mediated by the apple-produced volatiles deserve further investigation involving the apple plant, the European apple sawfly and its natural enemies.

EXPERIMENTAL

The orchard. In a biologically managed orchard of 1 ha (altitude 440 m, Canton Aargau, Switzerland), *ca* 15 apple varieties were distributed in 25 lines with 70–120 dwarf trees per line. The trees of the 5 studied varieties (see later) were 4–20 years old, *ca* 2–3 m high, and had as rootstock M9, except cv. Idared that had as rootstock M26. The European apple sawfly is regularly an important pest species in this orchard. Five apple varieties were used in experiments for which apples were either picked or left on the tree.

Volatiles of picked apples. For each of the varieties Liberty, Boskoop, Maigold, Sauergrauech and Idared, healthy and infested apples were picked in the orchard, and their transversal diameter was measured. A healthy apple was picked from a cluster of 3 or more healthy apples. The infested apple was then picked in close vicinity, mostly on the same tree. At 5 dates, namely every 6 days from 15 May 1994, a decreasing number of apples were picked: 14, 10, 6, 4 and 4 healthy and as many infested apples. Thus, a total of 50 samples of healthy and infested apples from 5 varieties at 5 dates were obtained for volatile collection. At the beginning of the study period, apples were infested by L1 and L2 and at the end by L5. The same day the apples were picked, volatile collections were started in the laboratory from 22.00±1 hr for 22±1 hr.

A volatile collection system as described in ref. [17] was set up in a climatic chamber (25°, light from 6.00–22.00). It consisted of the following elements (Fig. 4): an air flow produced by a compressor was

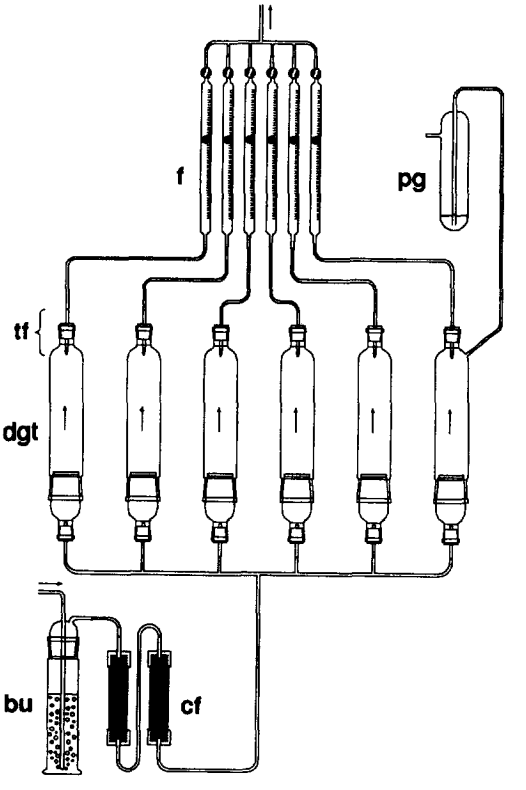

Fig. 4. Diagram of the volatile collection system. The elements are: bubbler (bu), charcoal filter (cf), dilated glass tube (dgt), trapping filter (tf), pressure gauge (pg), flow meter (f). Air flow (arrows).

pushed successively through a bubbler filled with H_2O, a set of 2 charcoal filters, and then in parallel through a max. of 6 dilated glass tubes. These glass tubes, of 720 ml each, contained the biological material. Downstream of each tube, a hand-made trapping filter made from a glass tube containing 50 mg adsorbent (Super Q, 80–100 mesh, Alltech) was fixed. The air was pulled through the trapping filter by a vacuum pump. There was a flow meter set at 0.5 l min^{-1} between each filter and the pump. A pressure gauge connected to one of the dilated glass tubes was used to control pressure inside the system.

Volatiles of apples on the tree. In the orchard, the volatiles of single apples cv. Maigold were collected starting at 12.00±1 hr for 4 hr, using a volatile collection system adapted to field conditions. Instead of a dilated glass tube, a transparent polyethylene terephthalate (PET) bag was used that contained an apple on a tree. The inlet air flow was generated by an aquarium pump and passed through a bubbler and then through a flow meter set at 1.2 l min^{-1} and through charcoal filters; all these elements were fixed on an aluminium stake, which was planted in the ground in the vicinity of a tree. The PET bag had two openings and was tied on one side around the air inlet tubing and on the other side around the apple pedicel as well as around the trapping filter. The outlet air flow was generated by a vacuum pump and passed through the trapping filter and a flow meter set at 0.8 l min^{-1}.

Volatiles of sawfly larvae and their frass. Five L4 or three L5 were collected from the field and placed in a dilated glass tube of 65 ml in the laboratory. Volatiles were collected for 1 hr. The tube also contained a small iron screw which could be moved from the outside with a magnet. Thus, the larvae were disturbed by 'attacking' them with the screw and we could concurrently collect the volatiles they emitted. In other experiments, five L4 or three L5 were crushed in a dilated glass tube and their volatiles collected for 10 hr.

Frass agglomerates were collected in the field on numerous apples cv. Maigold infested by L4 and L5. A 12-ml dilated glass tube was loosely packed with frass. Collection in the laboratory of the frass volatiles lasted 1 hr.

Chemical analyses. After trapping volatiles, the filters were eluted with CH_2Cl_2. Known amounts of int. standards, *n*-octane and nonyl acetate, dissolved in CH_2Cl_2, were added to the samples. On the same day volatile collection and filter elution were performed, the volatile samples were analysed on-column on an apolar methyl silicone capillary column (HP-1, 60 m × 0.25 mm). Temp. programme: 4 min at 40°, 5° min^{-1} to 80°, 10° min^{-1} to 190°, 12 min at 190°. Temp. were 180° for the injector and 280° for the FID. Chromatographic information was integrated with HP ChemStation software.

Several samples were analysed by GC-MS. A gas chromatograph (Fisons GC 8065, Carlo Erba) was equipped with the capillary column described above and connected to a mass spectrometer (MAT SSQ 710, Finnigan) set on EI at 70 eV. No effluent splitting occurred during the first 10 sec following the injection of a 2 μl sample. Temp. programme: 4 min at 40°, 4° min^{-1} to 200°, 5 min at 200°. The injector temp. was 180°.

Compounds were identified by comparing their mass spectra with those in a spectral library (Interactive Chemical Information System, vers. 7.0, Finnigan) and with those from authentic samples. Furthermore, R_ts were matched with those from authentic samples on the apolar column described above and on a polar one (HP-Innowax, 30 m × 0.25 mm). MS data for *trans*-2(3)-epoxy-2,6-dimethyl-6,8-nonadiene have been described [18]. For the unidentified terpenoids from apples, MS data by EIMS 70 eV [*m/z* (%)] were as follows. Terpenoid I: 150 (14) $[M]^+$, 81 (17), 79 (15), 69 (100), 53 (14), 41 (71), 39 (20); terpenoid II: 204 (1) $[M]^+$, 119 (86), 107 (41), 93 (100), 79 (37), 69 (47), 55 (42), 41 (83); terpenoid III: 204 (13) $[M]^+$, 161 (43), 133 (45), 120 (32), 93 (75), 79 (35), 69 (100), 55 (25), 41 (96). By comparing their R_ts with authentic samples it was determined that the terpenoids I-III were different from the already reported compounds *trans*-α-bergamotene, *cis*-α-farnesene, *trans*-β-farnesene and β-bisabolene.

Acknowledgements—We thank R. Kaiser (Givaudan-Roure, Dübendorf, Switzerland) for supplying us with several terpenoids as authentic samples, and R. Amadó and D. Wechsler (ETH, Zürich) for their support and for carrying out mass spectrometry. We thank L.H.M. Blommers and J.-P. Zijp (IPO-DLO, Kesteren, The Netherlands), K. Carl and A. Kählert (IIBC, Delémont, Switzerland) and B. Graf (FAW, Wädenswil) for providing cocoons and larvae of the sawfly, and H. Vock for the permission to work in his orchard. S. Bacher, U. Schaffner, M. Bernasconi and K. Tschudi-Rein provided us with useful comments on the manuscript.

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