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Oviposition by butterflies on young leaves: investigation of leaf volatiles

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Summary. Various butterflies select young foliage on which to lay their eggs; volatiles emitted by young and old leaves have been compared (by sorption enrichment, followed by GC-MS) to gauge possible qualitative and quantitative differences between the two age groups. The plants investigated are cabbage (*Brassica oleracea*), two milkweeds (*Asclepias syriaca* and *A. curassavica*), the bitter orange (*Citrus aurantium*) and the lime (*C. aurantiifolia*). The chemical compounds identified belong to three classes, isoprenoids, fatty acid derivatives and benzenoids. Quantitative differences were found between young and old leaves, of which a few may be characteristic of young leaves only. Thirty-four single trials with *Danaus plexippus* exposed to volatiles from young and old leaves are recorded.

Key words. oviposition sites – leaf volatiles – gas chromatography-mass spectrometry (GC-MS) – head-space adsorption – isoprenoids – fatty acid derivatives – benzenoids – *Asclepias* – *Brassica* – *Citrus*

Introduction

The defensive and alluring mechanisms of animals and plants are so bewilderingly varied that it is always agreeable to find some simple and easily interpreted trait common to many unrelated species and which appear to ignore phylogenetic boundaries. Thus the colour red serves as an alerting signal throughout the animal and plant kingdoms, and pyrazine functions in a similar manner and is present in numerous totally unrelated organisms (Woolfson & Rothschild 1990) ranging from mice and monarch butterflies to sea anemones, poppies and peas.

It is striking that numerous unrelated species of butterflies distinguish young from old leaves and consistently select them for oviposition. It is generally assumed that young foliage and shoots or flower buds are chosen because they provide tender food for freshly hatched, delicate young larvae (Jones 1991; Tyler *et al.* 1994), which thrive on them, but the nature of this subtle, inter-specific oviposition cue has, up to now, been ignored (see Renwick & Chew 1994; Nishida 1995).

We considered that specific volatiles, or an additive combination emitted from young leaves might, in conjunction with visual stimuli, direct the female to these particular oviposition sites. There might also be volatiles in old leaves which act as mild deterrents.

Relatively few studies have hitherto been made on the volatiles of young leaves, but they indicate there are at present a rather limited number of substances which seem to be widespread in many different plants. Some of these compounds are glucosidically bound in the tissues and then released (Stahl-Biskup *et al.* 1993). Some nitrogen- and sulphur-containing compounds and others like monoterpenes are derived from larger, non-volatile substances and can be emitted after hydrolysis, such as the formation of isothiocyanates from glucosinolates, especially during active growth (Bernays & Chapman 1994).

As a model system for studying this plant/butterfly relationship, we have chosen three unrelated species of plants, of which the specific non-volatile oviposition cue for host-plant selection by the butterflies is known (Nishida 1990, 1995; Chew & Renwick 1995; Haribal & Renwick 1996). These insects lay preferentially on young leaves:

Brassica oleracea, ovipositor: *Pieris brassica*
Asclepias curassavica and *A. syriaca*, ovipositor: *Danaus plexippus*
Citrus aurantium and *C. aurantiifolia*, ovipositor: *Papilio xuthus*

The volatile substances we have attempted to record are small enough to possess a vapour pressure which enables them to evaporate at normal pressure and temperature. They can be transported for longer or shorter distances through the air. This volatility requires molecules with a maximum molecular weight around 500, corresponding to a maximum of about 35 carbon atoms. Most of the common natural odour substances occur in the interval between C_5–C_{20}. Volatiles which emanate from larger molecules are produced biochemically or by decomposition in the air. Larger, in principle non-volatile compounds, may be transferred through air, transported by micro-droplets of water and other micro-particles. Some volatiles are produced by the leaf only after damage.

It should be noted that our technique involves an 'analytical window' (Fig. 1) where combined techniques of isolation of volatiles by adsorption/desorption, followed by separation and identification by (primarily) gas chromatography/mass spectrometry, function best. Problems can arise with extremely volatile (A), and

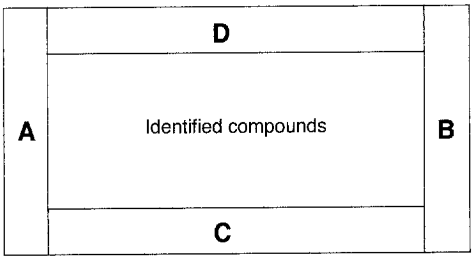

Fig. 1 The 'window' of optimal chemical analysis, *i.e.* the area where we are quite confident that we can analyse most volatile compounds; in the other four areas, there are specific uncertainties. **A** area of highly volatile compounds, including permanent gases; **B** area of very low-volatile compounds; **C** compounds occurring in very small amounts, in principle below the detection limit (GC-MS); **D** compounds which are not adsorbed on, or desorbed from the adsorbent and compounds which change chemically or decompose during the analytical process

very low-volatile compounds (B). They may not show up at all, or be quantitatively misrepresented in a blend of volatiles. There is also always the risk that a biologically active compound can be present in a very small amount – below the practical detection threshold (C), or it may be sensitive to the analytical procedure leading to decomposition or other chemical change (D). Consequently, there is a possibility that compounds like pyrazines and isothiocyanates, if they occur in small amounts, could be missed.

Materials and methods

Plants

The plants were grown at Ashton, Peterborough, UK. The seeds of *Asclepias curassavica* came *via* Clive Farrel from several different areas in Central and North America. *A. syriaca* had been grown for 15 years outside in the garden at Ashton from seed grown in T. Reichstein's garden in Basle, Switzerland. A small *Citrus aurantium* was obtained in Israel, a larger *C. aurantiifolia* was grown under glass in the Cambridge Botanic Garden. The cabbage, *Brassica oleracea*, "Greyhound" cultivar, was also grown under glass at Ashton. The seedlings used in extractions represented approximately the same leaf area as old leaves, but were not measured or weighed.

Butterflies

Observations on live butterflies were made in a large greenhouse at Ashton (10 m in length and 3 m in height). The seedlings and/or leaves for sprays were liquified in a domestic blender, tap water was added and the mixture strained through muslin. The leaves selected for testing the effect of the spray were maximum sized old leaves of *Asclepias syriaca*. The white strips of paper were cut to the same size as the old leaves. They were placed across the tops of the two empty glass jam-jars. Monarch pupae were obtained from Fort Lauderdale, Florida, and Costa Rica. The large whites were reared from wild stock obtained at Ashton.

Chemical analyses

The chemical analysis was made in Göteborg, Sweden. Odour collections were carried out in a greenhouse at Ashton.

Volatiles were collected from the chemo-sphere around the respective plants by headspace adsorption using an airflow onto Porapak Q (60 mg, 80–100 mesh) Supelco or Tenax GR (80 mg, 60–80 mesh) Chrompack, packed into teflon cartridges (3 mm i.d.) sealed with polypropylene wool (Aldrich) July 1994: Tenax GR; April 1995 Porapak Q; July 1995: Porapak Q or Tenax GR, were used. The material was enclosed in a polyacetate "roasting bag" (trade-mark Pingvin); the adsorbent plug was placed inside the bag and air was drawn through the plug by suction, using a mini-pump (air flow 130 ml/min for, typically, 5–7 h).

The adsorbent plugs were wrapped in aluminium foil and kept first at +4°C for 2–10 d, then at −20°C for 2 d. The volatiles were eluted with 300 µl high grade diethyl ether. The samples were gently concentrated to 10–40 µl. The chemical analyses were made by combined gas chromatography-mass spectrometry (GC-MS) on an HP 5890 gas chromatograph connected to an HP 5972 mass selective detector. The GC was equipped with an OV-351 fused silica column (25 m x 0.25 mm ID, coating 0.25 µm Chrompack). Helium was used as carrier gas at a constant flow of 0.6 ml/min (32 cm/s at 50°C). Injector temperature was 220°C. After sample injection the GC oven was kept at 50°C for 5 min then increased by 8°C/min to 230°C, and then isothermal for 10 min.

Volatiles were identified by comparing their GC retention times and mass spectra with those of known compounds in our possession. The composition of volatiles in each sample, expressed in percent representation of individual volatiles, was based on the relative peak areas of each compound. To determine the total amount of each sample, 500 ng methyl octadecanoate or 500 ng furfuryloctanoate was added as a standard to each sample prior to the concentration steps. The quantity of the total amount was calculated by comparing the total area with that of the standard.

Of the three collections of volatiles, in July 1994, April 1995 and July 1995, we have principally relied on the latter because we have then had the best control over quantities and of the material and the analytical process. The two earlier series generally speaking confirmed the presence of the compounds identified here with some distinct exceptions. Most compounds which occur in less then a few tenths of one percent have been omitted, especially when there is no indication of differences in amounts between young and old leaves.

Note: the numbers after each chemical substance mentioned in the text refer to compounds given in the Tables 1–5 and 6 and in Chart 1 of molecular formulas. The enantiomers of chiral compounds have not been determined.

Results

The large white (Pieris brassicae)

Behaviour

Kirby & Spence (1860) were so impressed with the care and skill exhibited by the ovipositing large white, particularly how she distinguished "some plant of the cabbage tribe from the surrounding vegetables", that they concluded she was "taught of God"! On surer ground they noted in passing, she avoided plants preoccupied by eggs of other butterflies. Rothschild & Schoonhoven (1977) went further and decided the large white could assess her own egg load and, after depositing a certain number, would pass on to a less favoured non-cruciferous host, such as *Reseda*, for further oviposition, rather than overload the cabbage of her initial choice. It was also suggested the butterfly oviposited only when she had gauged the presence of a suitable number of adjacent plants on which her offspring could find adequate sustenance (Rothschild 1987). This was suggested by the fact that the female tested an average of 16 suitable host plants before deciding on which to lay, even if they had been protected from herbivores or other damage.

Various authors have shown that vision, odour perception, contact evaluation and experience play their part in host selection. The butterfly responds to the so-called green volatiles, (E)-2-hexenal, (*Z*)-3-hexenyl

Table 1 Volatile compounds isolated from the "chemosphere" around old and young leaves of *Brassica*. The figures in each column are percentages of single collections. The compounds are arranged after their appearance in the gas chromatogram (after retention time). (The figures 1A, 1B *etc.* at the top of each column refer to the different collections.)

Comp. no.	**Compound**	1B y. leaves	1A old leaves	3B y. leaves	3A old leaves
2	α-pinene	3.9	5.4	2.9	3.5
3	α-thujene	7.0	20.2	12.9	13.0
5	β-pinene	tr	4.1	2.7	3.2
6	sabinene	33.8	36.9	29.0	31.1
8	myrcene	7.4	7.7	7.8	9.1
9	limonene	13.9	12.2	15.1	17.3
10	1,8-cineole	11.5	8.7	15.0	13.7
12	*trans*-β-ocimene			1.0	0.9
14	terpinolene			0.4	0.4
16	tridecane			0.4	
17	(Z)-3-hexenyl acetate	3.5	2.2	0.2	1.7
20	(Z)-3-hexenol	tr	tr		0.2
22	sqt. unknown	7.0	0.7	3.3	tr
23	tetradecane			0.3	0.3
25	*cis-para*-menth-2-en-1-ol*	3.5	1.1	2.0	1.8
29	sqt. unknown	3.0		0.7	
33	pentadecane			0.3	0.2
35	*trans-para*-menth-2-en-1-ol*			0.5	0.4
37	linalool	2.1		1.3	0.9
41	4-terpineol			0.3	0.2
44	hexadacane		0.3	0.3	0.2
49	linalyl propanoate*	tr	0.3	1.4	1.2
56	*trans*-α-farnesene	3.3	0.3	1.7	0.8
58	methyl salicylate	tr		0.3	
	Total %	100	100	100	100
	Total amounts	0.55 µg	2.33 µg	5.13 µg	7.09 µg

tr trace amounts; * identified by their mass spectra only; **sqt** sesquiterpene

acetate *etc.* (Visser 1986), and, when choosing a plant for oviposition, is particularly sensitive to different shades of green and specular reflectance (shine, or glare or waxy bloom). She also responds to the chemical contents of the host, both attractants and repellents (Ilse 1937; Rothschild *et al.* 1988), and is aware of shape and texture of leaves, the size of the plant, its situation relative to other species, and the presence of con-specific eggs and other herbivores, such as aphids and snails or damage by Lepidoptera larvae. Laying on variegated or virus-infected foliage is avoided. The butterfly is also influenced by various physical conditions such as light and shade, noise or movement engendered by hymenopterous predators, birds and so forth. She is also influenced by the quality of sunlight at different times of the day or the seasons, and of changes in atmospheric pressure, or the approach of rain or wind. Myers (1985) has shown that the small white (*P. rapae*) can differentiate between the physiological condition of plants which have been artifically fertilized and selects them for ovipostion as early as one day after application. Ives (1978) noted that this species, although it selected young in preference to old plants, chose the older, larger leaves of the specimen selected, but could also show preference for different varieties. Such factors may affect oviposition in *P. brassicae* but almost all observations on *P. rapae* have been carried out in the laboratory or in cultivated fields and gardens. It is striking that in the case of *P. brassicae* in wild undeveloped country, untouched by horticulture or agriculture, the ovipositing female searches for suitable host plants, *near the ground*, situated in relatively sparse vegetation, and preferably selects seedlings or young plants.

We found, if liberated in a greenhouse and offered a choice between a young and older cabbage leaf, carefully matched for shape, size and position on the stalk, and shielded from known deterrents, the large white will lay first – without hesitation – on the young leaf. The same applies if she has the choice of a young plant of 12 weeks old, or a well grown plant of 20 weeks.

The young foliage is identified without examining the surface of the leaves before making her choice (either by drumming after landing, or with antennae or tarsi without landing), and the female flies directly towards the younger plant. We agree with Chew & Renwick (1995) that she responds to visual rather than volatile cues, but possibly a combination of both.

Cabbage (Brassica oleracea)

Leaf volatiles

In Table 1, the 24 volatile compounds found in young and old leaves of *Brassica* are given as percentages of the whole isolate. Several monoterpenes, hydrocarbons, some alcohols and one monoterpene ester, is the main group of compounds found. In addition there are a few sesquiterpenes, one benzenoid compound methyl salicylate (58), and a few fatty acid derivatives.

Table 2 Volatile compounds identified from young leaves of *Brassica oleracea*, without and with eggs

Comp. no.	**Compound**	23B y. leaves wout eggs	23A y. leaves with eggs	10B y. leaves wout eggs	10A y. leaves with eggs
2	α-pinene	1.0	0.8	4.5	7.0
3	α-thujene	3.3	9.4	4.9	5.0
5	β-pinene	tr	0.2		tr
6	sabinene	36.7	34.1	28.3	26.4
8	myrcene	7.4	10.1	7.1	6.6
9	limonene	19.0	17.5	18.3	17.9
10	1,8-cineole	16.3	13.7	14.2	13.8
12	*trans*-β-ocimene	tr	1.1		
14	terpinolene	tr	0.6		
16	tridecane			5.8	
17	(Z)-3-hexenyl acetate	1.0	2.6		
20	(Z)-3-hexenol		1.2		
23	tetradecane			2.8	
25	*cis-para*-menth-2-en-1-ol*	7.3	4.6		
35	*trans-para*-menth-2-en-1-ol*	1.4	0.7	3.6	1.9
37	linalool	0.7	1.3	tr	
41	4-terpineol	tr	0.7		
44	hexadecane	1.5	0.7		
49	linalyl propanoate*	0.7	0.2	3.5	4.1
56	*trans*-α-farnesene			7.1	12.7
58	methyl salicyate	3.7	0.6	tr	tr
64	unknown				4.7
	Total %	100	100	100	100
	Total amounts	0.99 μg	2.65 μg	0.6 μg	0.55 μg

tr trace amounts; * identified by their mass spectra only

Of the latter, as anticipated, the two "green odour" substances, (Z)-3-hexenyl acetate (17) and (Z)-3-hexen-1-ol (20), are present. Tollsten & Bergström (1988) showed that in damaged leaves of other *Brassica* species isothiocyanates, nitriles and sulphides were present.

The compounds which differ in young and old leaves do so mainly quantitatively. The unidentified sesquiterpenes: "unknown 1" (22) and "unknown 2" (29), linalool (37) and *trans*-α-farnesene (56), occur in decidely larger amounts in young leaves. In the April 1995 runs, we also identified appreciable amounts of a series of ketones: 2-octanone, 2-nonanone and 2-undecanone, which occurred predominantly in young leaves. There are also a few compounds, notably α-pinene (2) and α-thujene (3), which appear in old leaves in higher quantities than in young ones. The total amounts of volatiles isolated in the four sets of trials (1B/3B and 1A/3A) are 0.55/5.13 and 2.33/7.09 μg, respectively.

Table 2 shows the results of analysis of young cabbage leaves with and without eggs of *Pieris brassicae*. Two young leaves carried eleven batches of eggs, laid over a period of 7 d (sample 23A). One young leaf from the same plant carried 20 eggs laid only 2 d before the analysis was made (sample 10A). Although the material is very small there are some indications of a difference between these samples and young "clean" leaves. The isomers identified by their mass spectra as *para*-menth-2-en-1-ols (25)(35) and methyl salicylate (58) seem to occur in relatively high percentages from clean young leaves whereas α-thujene (3) and the green odour compounds (*Z*)-3-hexenyl acetate (17) and (*Z*)-3-hexenol (20), were obtained in larger amounts from the young leaves carrying eggs. Eighteen volatile compounds were collected from the leaves on which eggs had been laid for 7 d (Table 2, 23A) and only 10 (with two additional traces), from a leaf off the same plant, on which eggs had been laid for only 2 d (Table 2, 10A). The total amount of volatiles isolated are in the order of 0.5 to 2.7 μg per sample.

The monarch (Danaus plexippus)

Behaviour

The female monarch resembles the ovipositing large white in her preferene for young plants and leaves (Figs. 2, 3) on which to lay and, in natural situations in the field, searching near the ground for suitable seedlings or young hosts. Both species drum the surface of the selected leaf with their forelegs after landing but the monarch first dips her antenna over the area, (Fig. 3) whereas the large white only does so rarely. The monarch generally lays on the first leaf of the host plant on which she alights, or on an adjacent leaf, while the large white appears dissatisfied with the first suitable host she tests, and drums on an average 16 cruciferous plants before deciding to oviposit (Rothschild 1987). Both species have become less 'choosy' after laying a large number of eggs. The monarch may select older leaves and then appears 'reckless' and will choose un-

Fig. 2 *Danaus plexippus* ovipositing on young leaves at the base of a plant of *Asclepias curassavica*

Fig. 3 *Danaus plexippus* ovipositing on young shoots at the apex of a full-grown plant of *Asclepias curassavica*. Note the typical position of the antenna

suitable vegetation on the ground in the vicinity of an *Asclepias*. In the greenhouse, she will then lay hurriedly on a glass vase or flower pot holding a host plant, while the large white, at all stages oviposits exclusively on vegetation containing glucosinolates and does not 'dump' eggs if deprived of them. In the garden and greenhouse when only tall, fully grown *Asclepias* are available, the monarch lays on the young leaves surrounding the flower heads or on a cluster of buds (Fig. 4), and less frequently on the petals of open flowers. Urquart (1987) carried out a count on *A. syriaca* in the field. He found no eggs but a single larva on 400 full grown plants bearing flowers or seed pods, but on 400 young plants, 20 cm high, he collected 74 eggs and 12 larvae.

Fig. 4 Eggs of *Danaus plexippus* deposited on the flower buds of *Asclepias syriaca*

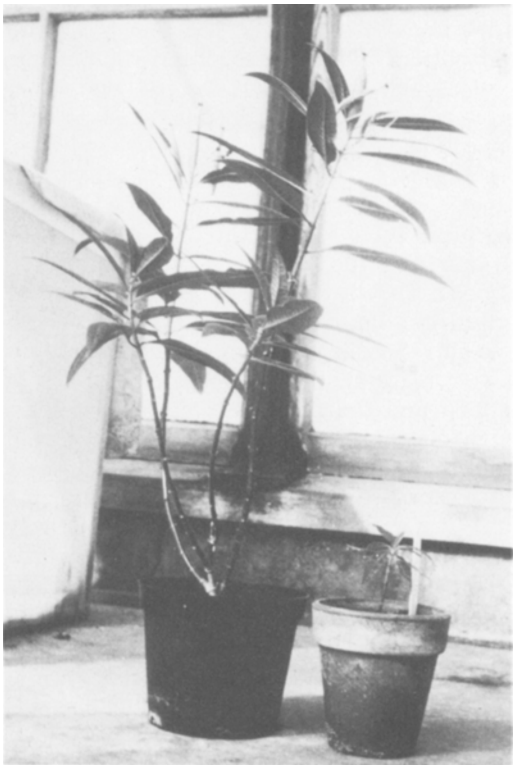

Fig. 5 Full-grown plant and seedling of *Asclepias curassavica*: the female *Danaus plexippus* selected the seedling for oviposition despite the proximity of several similar fully-grown plants

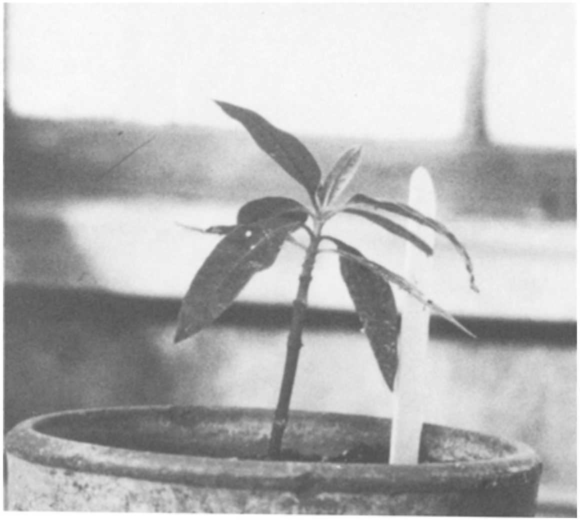

Fig. 6 Close-up of seedling selected in preference to full-grown plants, shown in Figure 5

In the greenhouse, a female may land on a full grown *Asclepias* but without a pause drop down onto a seedling 10–12 cm tall and lay immediately (Figs 5, 6). Given a choice of matched old and young leaves placed in adjacent jars, she will unhesitatingly select the latter for oviposition.

Young leaves of *A. syriaca* and new shoots on old plants are bright, light green in colour and almost transparent with the sun behind them. They also lack reflected light. Urquart (1960) believes that in the field, vision plays the decisive role in the selection of a plant on which the butterfly lays. Close observation in the greenhouse and a few experiments suggest that other factors, especially odours, probably play a significant part in the selection of seedlings.

Experiments

Maximum sized (23 cm × 10 cm) paired old leaves of *A. syriaca* were cut from the base of a plant 1.5 m tall, bearing seed pods. These were placed each in a glass jar one metre apart, in a large greenhouse (10 m in length and 3 m in height). Seedlings grown alongside the full-sized plants were macerated in a domestic blender, tap water was added and the fluid, pale green in colour, passed through muslin. Fluid was produced from old leaves by a similar process. At 11.30 am, one of the old leaves was then sprayed with the seedling 'soup', the other with tap water. Ten *D. plexippus* were actively flying around and feeding on flowers. Copulation had occurred previously. Out of 27 separate trials the leaf sprayed with seedling 'soup' was selected initially 24 times for egg laying. When this leaf became covered with eggs the female occasionally moved onto the old water sprayed leaf. We also constructed similar shape and sized leaves cut out of white, slightly absorbent paper. In this case, one "leaf" was sprayed with seedling 'soup' the other with old leaf 'soup'. The fluid gave a pale green tinge to the white paper. The 'leaf' sprayed with seedling 'soup' was visited first on 7 occasions (out of 8 trials) and 11 eggs were laid on the paper leaf and two on the container. One visit only was paid to the leaf sprayed with old leaf fluid and a single egg laid. The potential egg-laying females flapped around the greenhouse close to the substrate and abandoned their usual long sweeping flights and aerial combat, but more often than not settled on a flower and resumed feeding. They did not immediately find the white sprayed leaves. It is worth noting that females were also attracted to the white bottle containing the seedling 'soup' and settled on the nozzle. Unlike the large white, this butterfly only rarely alights and rests on objects in the greenhouse such as watering cans, jars, flower pots, or flowered material *etc.* placed on shelves.

Milkweed (Asclepias curassavica)

Leaf volatiles

The compounds isolated from *A. curassavica*, 11 in all, are given in Table 3. There are remarkably few compounds from the leaves of this plant, and they are present in small amounts, 0.4, 1.0 and 3.6 µg from young leaves, old leaves and shoots, respectively. The main difference between young and old leaves is the occurrence, in the former, of *trans-β*-farnesene (47). Prominent compounds were three methyl-branched polyunsaturated hydrocarbons (homoterpenes) (15) (59) (60) a total of 79.2, 66.2 and 60.2% occurred in the three samples, and methyl salicylate (58), 13.3, 24.9 and 26.0% in the three samples. The three methyl branched poly-unsaturated hydrocarbons, the homoterpenes (15) (59) (60) are present in many plant species (Kaiser 1991). They can be synthesised in higher plants, (without damage), as shown by Boland & Gäbler (1989). They found how these compounds could be produced from nerolidol and from geranyl linalool. Their mass spectra were easily misinterpreted as perillene and dendrolasin. Two of the "green odour" substances and two monoterpenes were also present. In the 1994 analyses, two other compounds showed up in larger amounts in young leaves: 2-hydroxy-acetophenone (about 10 and 20%, respectively, in two different samples) and 6-methyl-5-hepten-2-one (about 3 and 6% in two samples), the latter also present in the controls. In the April 1995 material we found a large amount of methyl salicylate (58): 55.8% in young leaves and 17.5% in old ones. The other compounds occurring in *A. curassavica* in the July 1995 material were also present in the two earlier collections.

Milkweed (Asclepias syriaca)

The leaf volatiles

A. syriaca produces many more volatiles than *A. curassavica* (Table 4). Forty compounds are present in appreciable quantities. The amounts isolated from seedlings, old leaves, top leaves and top leaves + buds were 18.5, 15.0, 46.9 and 49.1 µg, respectively. The *A. syriaca* odour compound isolates contain many mono- and sesquiterpene hydrocarbons, 6 and 11, respectively, many in large amounts, and four benzenoid com-

Table 3 Volatile compounds from young and old leaves, and from shoots, of *Asclepias curassavica*

Comp. no.	**Compound**	4B y. leaves	4A old leaves	19A shoots
12	*trans*-β-ocimene	2.3	4.5	4.2
15	4,8-dimethyl-1,(E)3,7-nonatriene	59.2	30.3	20.2
17	(Z)-3-hexenyl acetate	2.9	1.8	6.5
20	(Z)-3-hexenol	tr	1.6	0.8
24	1,3,8-*para*-menthatriene*		tr	0.6
47	*trans*-β-farnesene	2.4		
58	methyl salicylate	13.3	24.9	26.0
59	4,8,12-trimethyl-1,(Z)3,(E)7,11-tridecatetraene		1.5	1.2
60	4,8,12-trimethyl-1,(E)3,(E)7,11-tridecatetraene	20.0	34.4	38.8
66	unknown		1.1	1.2
67	nerolidol isomer	tr	tr	0.5
	Total %	100	100	100
	Total amounts	0.4 µg	1.0 µg	3.6 µg

tr trace amounts; * identified by their mass spectra only

pounds. There are also the C_6 "green odour" compounds, two of them in quite large amounts, and two of the polyunsaturated hydrocarbons (homoterpenes). Differences between the emission from young and old leaves can be perceived in the larger amounts of *trans*-β-ocimene (12) and *trans*-α-farnesene (56) in young leaves, and in larger amounts in all the C_6-compounds and methyl salicylate (58) in old ones. Large amounts of germacrene D (50) and caryophyllene (43) in top leaves and top leaves + buds should be noted. The occurrence of larger amounts of *trans*-β-ocimene (12) in young leaves is consistent with the 1994 and April 1995 collections. In the former there was 16% *vs* 0% for young/old, and in the latter we found 87% *vs* 16%. In both these collections germacrene D (50) was present in larger amounts in the young leaves.

The swallowtail butterfly (Papilio xuthus)

Behaviour

The Swallowtail butterfly uses a wide variety of *Citrus* larval food plants. Although Nishida (1990, 1995) demonstrated that no less than 10 compounds constituted the oviposition stimulant of *Papilio xuthus*, none of these chemicals were volatile. *P. xuthus* also lays on the young leaves (Nishida 1990) of the host plant. This characteristic is reported for a number of swallowtails (Tyler *et al.* 1994) and one of us (M.R.) watched various species in the grounds of the Archbold Biological Station (Florida, US) ovipositing with remarkable precision on the young, bright green leaves of *Citrus* trees. These were apparently selected while the butterflies were on the wing, fluttering round the perimeter of the trees.

Feeny *et al.* (1989) have shown that the black swallowtail (*Papilio polyxenes*) lays more eggs on a model plant treated with the host plant carrot volatiles than without. Subsequently, they identified electrophysiologically active compounds in carrot and parsnip volatiles (Baur *et al.* 1992).

The bitter orange (Citrus aurantium)
The lime (C. aurantiifolia)

Leaf volatiles

In *Citrus aurantium*, and in *C. aurantiifolia* from Cambridge, we found 42 larger compounds (Table 5). The two *Citrus* are quite different in their chemical composition. Like in *A. syriaca* the mono- and sesquiterpene hydrocarbons occur frequently, especially in the bouquet of volatile components from *C. aurantium*. There are 7 monoterpene hydrocarbons and, at least, 9 sesquiterpene hydrocarbons in this species. In addition we recorded 3 monoterpene alcohols and, at least, 5 sesquiterpene alcohols, from *C. aurantium*. *C. aurantiifolia* has dominating amounts of oxygen-containing monoterpenes: linalyl acetate (38) and linalool (37). There are two benzenoid compounds and two polyunsaturated hydrocarbons, in both species. Possible differences between young and old leaves are larger amounts in the former of *trans*-α-farnesene (56), caryophyllene (43) and linalool (37), a common characteristic for both species, and larger amounts of β-pinene (5) and perhaps methyl salicylate (58) in *Citrus aurantium*.

Discussion

We failed to find a volatile factor peculiar to young leaves, one which was unquestionably absent from old leaves. Instead we discovered certain quantitative differences between several compounds recorded from both age groups (Tables 1–5, 6). The first candidate, in larger amounts in young leaves was *trans*-α-farnesene (56), 3.3/0.3 and 1.7/0.8 in *Brassica*; 5.9/0.5 in *A. syriaca*; 28.3/18.9 and 9.6/2.3 in *Citrus*; (Tables 1, 4, 5). The closely related *trans*-β-farnesene (47) is only present in young leaves of *A. curassavica*, 2.4/0. Linalool (37) is also found in larger amounts in young

Table 4 Volatile compounds from seedlings, old leaves, top leaves and top leaves + buds, of *Asclepias syriaca*

Comp. no.	Compound	6A seedlings	7B old leaves	6B top leaves	16A top + buds
1	unknown	1.7	3.5	2.0	1.2
4	unknown		0.8		
6	sabinene	0.1		0.1	0.1
7	unknown	0.3	1.0	0.9	1.2
8	myrcene	0.3	0.3	0.3	0.3
9	limonene	0.6	0.1	0.6	0.3
11	*cis*-*β*-ocimene	0.5	0.1	0.3	1.1
12	*trans*-*β*-ocimene	44.1	13.8	26.6	26.3
13	hexyl acetate	0.1	0.2	tr	0.1
14	terpinolene	0.2		0.2	0.1
15	4,8-dimethyl-1,(E)3,7-nonatriene	14.7	17.9	5.9	5.7
17	(Z)-3-hexenyl acetate	3.2	12.6	1.6	2.8
18	hexanol	0.4	0.8	0.8	1.3
19	(E)-3-hexenol	tr	0.3	0.2	0.3
20	(Z)-3-hexenol	19.8	38.5	29.4	32.0
22	sqt. unknown	tr	0.7	0.2	
24	1,3,8-*para*-menthatriene*	1.4	0.9	2.0	2.3
26	*α*-cubebene	tr	0.1	0.2	0.1
28	(Z)-3-hexenyl isovalerate	tr	0.1	0.1	tr
30, 31	*trans*-ocimene epoxide + sqt. unknown	0.2	0.4	0.1	0.1
32	*α*-copaene	0.2	tr	0.4	0.3
33	pentadecane		1.0		
34	*β*-bourbonene	tr	0.2	0.8	0.3
36	*β*-cubebene	tr	0.1	0.4	0.2
37	linalool	0.5	0.1	0.4	0.5
40	*β*-gurjunene	0.1	0.2	0.6	0.3
43	caryophyllene	0.9	0.4	3.2	2.7
45	*allo*-aromadendrene			0.1	
46	*α*-humulene	0.3	0.7	1.1	1.0
50	germacrene D	1.4	0.7	12.1	10.3
53	sqt unknown	0.3	0.1	0.4	0.3
54	sqt. unknown	0.1		0.7	1.0
56	*trans*-*α*-farnesene	5.9	0.5	6.9	5.3
57	*δ*-cadinene	0.3	0.3	0.7	0.4
58	methyl salicylate	0.5	1.2	0.1	0.1
60	4,8,12-trimethyl-1,(E)3,(E)7,11-tridecatetraene	0.3	0.3	0.3	0.7
61	benzyl alcohol	0.1	0.2	0.2	0.5
63	2-phenyl ethanol	0.1	0.1	0.1	0.2
67	nerolidol isomer	0.1	0.1	0.1	0.1
71	(Z)-3-hexenyl benzoate	1.5	1.9	0.2	0.5
	Total %	100	100	100	100
	Total amounts	18.5 μg	15.0 μg	46.9 μg	49.1 μg

tr trace amounts; * identified by their mass spectra only; **sqt** sesquiterpene

leaves of *Brassica*, 2.1/0 and 1.3/0.9; *Citrus aurantium* 1.0/0.3; *C. aurantiifolia* 20.9/5.5; and in *A. syriaca* 0.5/0.1 (Tables 1, 4, 5). Because these two compounds display a quantitative difference in the same three species of plants, it is tempting to speculate on a possible linked "dual effect", so that together they might function as a young-leaf signal. In order to elucidate such a possibility further investigation is required; not only because of the lack of consistency in our own results but because the distribution of chemical compounds in plants varies so enormously, not only from season to season but from hour to hour and from one specimen to another. No better illustration can be found than in the apparently capricious distribution of cardenolides in *A. syriaca* (Rothschild *et al.* 1975) and of the volatiles from intact and damaged cabbage described by Agelopoulos & Keller (1994). (See also Chew & Renwick 1995, p. 216.)

An interesting aspect of the problem concerns those compounds found in larger quantities in old leaves. Methyl salicylate (58) is an example in *A. curassavica*, *A. syriaca* and *Citrus* and also monoterpene hydrocarbons in *Brassica*, *A. curassavica* and *Citrus* (Tables 1, 3, 4, 5). Individual compounds such as *α*-thujene (3) in *Brassica*, *trans*-*β*-ocimene (12) in *A. curassavica* and *β*-pinene (5) in *Citrus* and green volatiles in *Brassica* are

Table 5 Volatile compounds from young and old leaves of *Citrus*, from two different cultivations (Ashton and Cambridge: *Citrus aurantium* and *C. aurantiifolia*)

Comp. no.	**Compound**	15A y. leaves Ashton	15B old leaves Ashton	20A y. leaves C-bridge	20B old leaves C-bridge
2	α-pinene	0.6	2.0		
5	β-pinene	5.2	17.0		
6	sabinene	1.5	4.6		
8	myrcene	0.6	0.9		
9	limonene	0.3	0.8		
10	1,8-cineole	0.6			
11	*cis*-β-ocimene	0.8	1.2		
12	*trans*-β-ocimene	26.2	38.9	4.4	6.8
15	4,8-dimethyl-1,(E)3,7-nonatriene	1.2	1.0	5.1	2.7
17	(Z)-3-hexenyl acetate	0.7	0.4	0.9	
21	2-nonanone		0.4		
27	δ-elemene	0.2			
30	*trans*-ocimene epoxide	0.1	0.3		
34	β-bourbonene	0.2			
37	linalool	1.0	0.3	20.9	5.5
38	linalyl acetate			50.5	58.7
39	*trans*-α-bergamotene	1.6	0.9		
42	2-undecanone		0.4		
43	caryophyllene	4.2		2.3	
46	α-humulene	0.3			
47	*trans*-β-farnesene	0.3			
48	α-terpineol	0.1			
50	germacrene D	9.9			
51	unknown			0.8	16.5
52	sqt. unknown	1.6	0.4		
55	*cis*-α-farnesene	1.4	0.9	0.6	
56	*trans*-α-farnesene	28.3	18.9	9.6	2.3
58	methyl salicylate	2.2	5.0	2.3	2.2
60	4,8,12-trimethyl-1,(E)3,(E)7,11-tridecatetraene	0.7	0.7	1.4	
61	benzyl alcohol	0.4	1.3		
62	unknown	0.2	0.7		
65	unknown			1.1	1.2
67	nerolidol isomer	0.5			
68	elemol	1.6	0.7		
69	γ-eudesmol*	0.5			
70	sqt. unknown	0.6			
72	sqt. unknown	0.5			
73	sqt. unknown	1.1	1.1		
74	α-eudesmol	0.9			
75	β-eudesmol	2.7	1.2		
76	unknown	0.7			
77	sqt. unknown				4.1
	Total %	100	100	100	100
	Total amounts	32.6 μg	5.2 μg	4.4 μg	1.2 μg

tr trace amounts; * identified by their mass spectra only; **sqt** sesquiterpene

also found in larger amounts in old leaves (Tables 1, 3, 5), and it is not impossible that they function as mild deterrents which help steer the ovipositing female to young leaves. Takabayashi *et al.* (1994) also noted that old cucumber leaves infected with spider mites emitted more volatiles, namely, 3-methylbutanal *O*-methyloxime and an unknown oxime, than young leaves.

We also found that young cabbage leaves on which *P. brassicae* had laid eggs emitted larger amounts of α-thujene (3) than young 'clean' leaves (Table 2), which is precisely that compound most characteristic of the contrast between old and young cabbage leaves. Blaakmeer (1994) suggested that plants responded to the presence of *P. brassicae* eggs by producing a leaf-surface repellent that inhibited further oviposition. Consequently, small though they be, our observations seem worth following up.

Urquart (1960) believed that visual cues are paramount in the choice of host plants for the monarch. Haribal & Renwick (1996) in their excellent paper on the flavonol glycoside oviposition stimulants for the monarch says "the involvement of host volatiles in recognition of milkweed by monarch butterflies has not yet been demonstrated". In the greenhouse, as we have

Table 6 Sum of volatile compounds from young and old leaves etc. of all the plants investigated, arranged after classes of chemical compounds.

Compound classes	*B. oleracea*					*B. oleracea*		
	1B y. leaves	1A old leaves	3B y. leaves	3A old leaves	23B y. leaves wout eggs	23A y. leaves with eggs	10B y. leaves wout eggs	10A y. leaves with eggs
ISOPRENOIDS								
Monoterpenes								
Hydrocarbons (2,3,5,6,8,9,11,12,14,24)	66.0	86.5	71.8	78.5	67.4	73.8	63.1	62.9
Oxygen containing mt (10,25,30,35,37,38,41,48,49)	17.1	10.1	20.5	18.2	26.4	21.2	21.3	19.8
Sesquiterpenes								
Hydrocarbons (22,26,27,29,31,32,34,36,39,40,43, 45,46,47,50,52,53,54,55,56,57)	13.3	1.0	5.7	0.8			7.1	12.7
Oxygen containing sqt (67,68,69,70,72,73,74,75,77)								
Irregular terpenes								
C11; C16 (15,59,60)								
FATTY ACID DERIVATIVES								
Hydrocarbons (16,23,33,44)		0.3	1.3	0.7	1.5	0.7	8.6	
Oxygen containing fad (21,42)								
C6-"green" (13,17,18,19,20,28)	3.5	2.2	0.2	1.9	1.0	3.8		
BENZENOIDS (58,61,63,71)	tr		0.3		3.7	0.6	tr	tr
UNKNOWNS (1,4,7,51,62,64,65,66,76)								4.7

tr trace amounts; * identified by their mass spectra only; **mt** monoterpene; **sqt** sesquiterpene; **fad** fatty acid derivative

pointed out, the butterfly gives every indication that volatiles are concerned with host plant identification but we were surprised our experiments showed the butterfly could distinguish, before landing to oviposit, between those old leaves sprayed with 'soup' or tap water, or strips of white paper which had been sprayed with liquidised young leaves, and selected them in preference to those sprayed with old leaf extract or tap water.

It is probable that crushed leaves emit additional volatiles from those given off by entire leaves at the leaf surface. Nevertheless, the fact remains that the butterflies could, before contact, differentiate the young from the old leaf extracts and were attracted by them. It is evident that our quest for the youthful factor-quantitative or qualitative-has not yet ended.

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Numbers in parenthesis refer to individual compounds (see Tables 1–5)

A. curassavica				*A. syriaca*			*C. aurantium*		*C.* sp.	
4B	4A	19A	6A	7B	6B	16A	15A	15B	20A	20B
y. leaves	old leaves	shoots	seedlings	old leaves	top leaves	top + buds	y. leaves Ashton	old leaves Ashton	y. leaves C-bridge	old leaves C-bridge
2.3	4.5	4.8	47.2	15.2	30.1	30.5	35.2	65.4	4.4	6.8
			0.6	0.3	0.5	0.6	1.8	0.6	71.4	64.2
2.4			9.6	4.2	27.9	22.3	48.1	21.1	12.5	2.3
tr	tr	0.5	0.1	0.1	0.1	0.1	8.4	3.0		4.1
79.2	66.2	60.2	15.0	18.2	6.2	6.4	1.9	1.7	6.5	2.7
				1.0						
								0.8		
2.9	3.4	7.3	23.5	53.5	32.1	36.5	0.7	0.4	0.9	
13.3	24.9	26.0	2.2	3.4	0.6	1.3	2.6	6.3	2.3	2.2
	1.1	1.2	2.0	5.3	2.9	2.4	0.9	0.7	1.9	17.7

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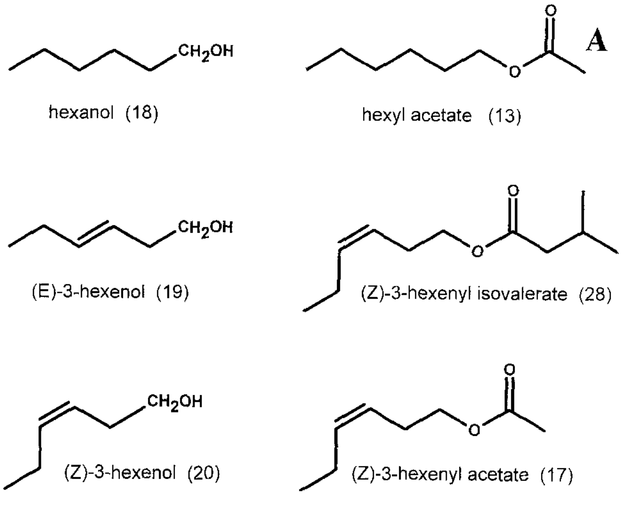


hexanol (18)

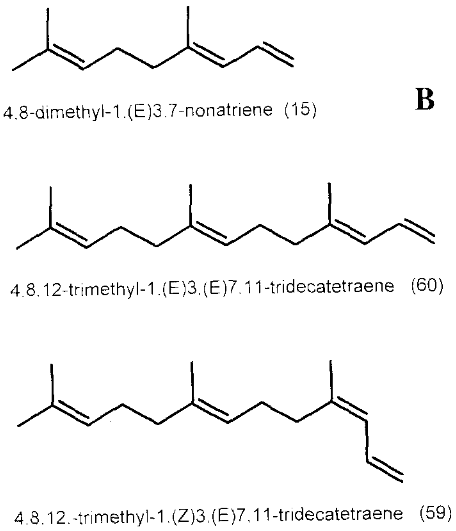
hexyl acetate (13)

(E)-3-hexenol (19)

(Z)-3-hexenyl isovalerate (28)

(Z)-3-hexenol (20)

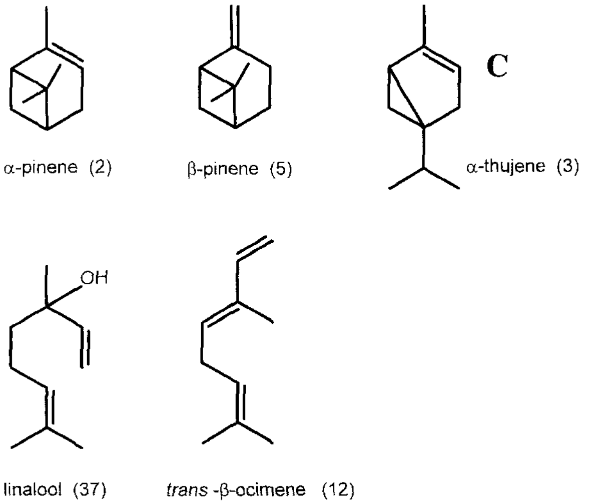
(Z)-3-hexenyl acetate (17)



4,8-dimethyl-1,(E)3,7-nonatriene (15)

4,8,12-trimethyl-1,(E)3,(E)7,11-tridecatetraene (60)

4,8,12,-trimethyl-1,(Z)3,(E)7,11-tridecatetraene (59)



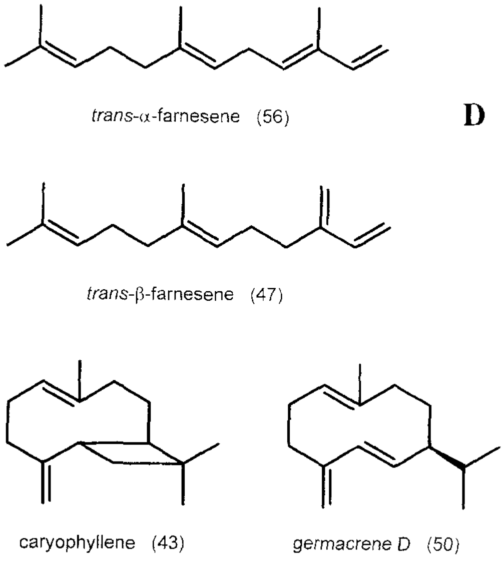
α-pinene (2)

β-pinene (5)

α-thujene (3)

linalool (37)

trans-β-ocimene (12)

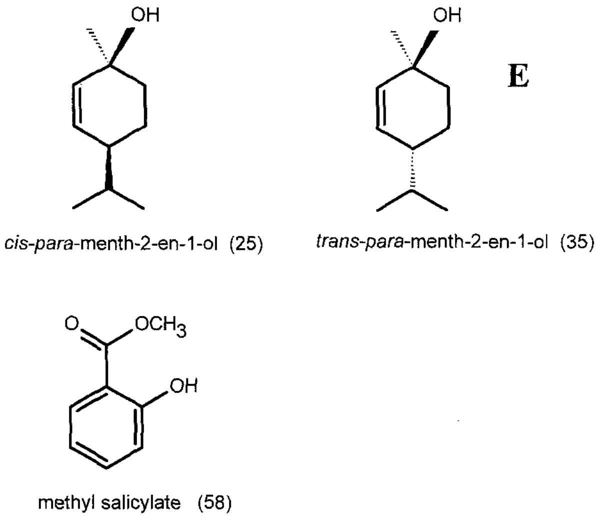


trans-α-farnesene (56)

trans-β-farnesene (47)

caryophyllene (43)

germacrene D (50)



cis-para-menth-2-en-1-ol (25)

trans-para-menth-2-en-1-ol (35)

methyl salicylate (58)

Chart 1 shows of 21 out of totally 77 compounds tabelled in this study of leaf volatiles. (The 21 substances represent those which have been especially commented upon in the text.) **A** The "green" ("grass") volatiles identified. Components number: 18, 13, 19, 20, 17 and 28; **B** the three polyunsaturated hydrocarbons identified, components 60, 59 and 15. **C** four prominent monoterpene hydrocarbons, component numbers 2, 5, 3 and 12 and one important alcohol, component 37; **D** four important sesquiterpene hydrocarbons, component numbers 56, 47, 43 and 50. The two former are acyclic, the two later di- and monocyclic, respectively; **E** two terpenoid, monocyclic alcohols, component numbers 25 and 35, and one prominent benzenoid compound, number 58