The treatment of foliar extracts affects behavioural responses of ovipositing P. c-album females: effect of host-plant volatiles

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The treatment of foliar extracts affects behavioural responses of ovipositing P. c-album females: effect of host-plant volatiles

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Short title: Hop volatiles affect ovipositing females

Keywords: butterfly, Nymphalidae, Humulus lupulus, egg-laying, attractiveness, stimulant, synergism, EAG.
Abstract

For a long time the role of volatile cues for the search of host-plants was underestimated in the day-active butterflies compared to the closely related nocturnal moths, but it is now known that olfactory cues play an important role. The simultaneous acquiring of information from several senses during host-plant search, recognition and acceptance for oviposition requires appropriate design of experiments and sample preparation in order to investigate the effect of a selected type of cues while ensuring that the contribution of other sensory modalities will remain uniform. The goal of this study was to investigate the effect of host-plant volatiles initially contained in the foliar extracts on the behavioural responses of gravid Polygonia c-album L. (Lepidoptera: Nymphalidae) females with respect to changes in volatile cues after sample treatment, including concentration by rotavapor and solvent/solvent extraction. Eighty nine volatile compounds were detected in a non-concentrated methanol extract of Humulus lupulus L. (Rosales: Cannabaceae) by gas chromatography mass spectrometry, eleven of which elicited an electroantennographic response of gravid P. c-album females. Concentration of the crude extract significantly reduced the attractiveness of the sample due to loss of volatile compounds, but the oviposition response of gravid females was not affected. A mixture of 8 commercially available electroantennographically active volatiles attracted ovipositing females to their source but did not act as oviposition stimulants. Dividing the volatile compounds into two groups consisting of: i) hexanal, (E)-2-hexenal, octanal, nonanal, and decanal; and ii) sulcatone, humulene and benzyl alcohol obliterated attractiveness, revealing synergism between compounds. Although volatiles did not stimulate oviposition they significantly contributed to the distribution of eggs by increasing the attractiveness of the treated artificial leaves, hence, direct observation of behaviour by scoring female oviposition responses has to be used rather than methods based on egg distribution in bioassays for identification of oviposition stimuli.
Introduction

A number of sensory modalities are involved in the evaluation of plant-derived cues when searching and accepting a plant for oviposition. Visual characteristics, including shape, size, and colour, as well as olfactory cues comprised of attractants and repellents are more important when searching for host plants from a distance. After landing on the plant, gustatory cues (stimuli and deterrents), plant surface characteristics sensed by mechano-reception, and semi-volatile compounds perceived by olfaction become dominant cues (Schoonhoven et al., 2005). The simultaneous acquiring of information from several senses during host-plant search, recognition and acceptance for oviposition requires appropriate design of experiments and sample preparation in order to manipulate and investigate the effect of a selected type of cues while ensuring that the contribution of other sensory modalities will remain uniform (Städler, 2002).

In the day-active butterflies the role of olfactory cues for host search have long been underestimated, in particular in comparison to the their relatives, the nocturnal moths. However, it has recently been shown that volatile compounds also play an important role in attracting ovipositing butterfly females (e.g. Ikeura et al. 2010; Schäpers et al. 2015) and moreover that the brains of females seem to be adapted to distinguishing host odours from other odours (Carlsson et al., 2011). Thus the butterflies can now join those insect taxa where the presence or absence of volatile host plant fractions can be suspected to confound results from oviposition bioassays.

In this study we have investigated the effect of sample treatments which may selectively affect volatile compounds initially contained in the samples, including concentration by rotavapor and solvent/solvent extraction, on the behavioural responses of gravid Polygonia c-album females. We demonstrate the importance of actually observing oviposition behaviour
rather than just counting eggs, when critically evaluating host plant fractions for their activity in stimulating or deterring oviposition.

Materials and methods

Insects

The comma butterfly, *Polygonia c-album* L. (Lepidoptera: Nymphalidae) were reared under laboratory conditions at the Royal Institute of Technology. Eggs were obtained on stinging nettle, *Urtica dioica* L (Rosales: Urticaceae) and were kept at a photoperiod of L12:D12 hour and about 18 °C. After the caterpillars reached the third instar, the light phase of the photoperiod was prolonged to L20:D4 hour and the temperature was increased to around 22 °C. In this way, adults of the “summer morph”, which develops directly to sexual maturation without adult diapause, were produced (Nylin 1989). After emergence, adults of both sexes were moved to the mating cages and provided with 20% honey solution in water. One-week-old mated females were used for oviposition experiments. It has been shown that the plant species used for rearing does not affect the preferences of the resulting adult females (Janz et al., 2009).

Extraction and fractionation of plant material

In the behavioural experiments to test attractiveness and egg-laying activity of extracts artificial leaves of 10 cm² were used, and this square was counted as the size of one leaf equivalent. Number of leaf equivalents was calculated as follows: about 400 cm² of *Humulus lupulus* L. (Rosales: Cannabaceae) leaves were weighed and the weight of one leaf equivalent (10 cm² of leaf) was calculated. This procedure was repeated four times and the average weight of one leaf equivalent was determined. The hop leaves collected for extractions were
weighed, and based on the data for weight of one leaf equivalent the number of leaf equivalents were calculated.

It has been shown that methanol extracts and fractions with the most polar phytochemicals stimulates egg-laying in comma butterflies (Mozuraitis et al., 2012). Based on that knowledge, around 4000 leaf equivalents of hop foliage were extracted in 3 l of methanol (99.9% Carlo Erba Reactifs-SDS, France) for 5 days. The extract obtained was filtered to remove mechanical particles. A part of the extract corresponding to 500 leaf equivalents was transferred to a bottle, closed and stored in a freezer at -14 °C. The rest of the extract was concentrated in vacuo below 50 ºC to 150 ml and another portion of the sample corresponding to 500 leaf equivalents was taken and stored at -14 °C. Methanol was removed from the remaining extract and an aliquot of the concentrate, after being dispersed in 150 ml of Millipore purity water, was extracted successively twice with 150 ml of chloroform (99.9% Carlo Erba Reactifs-SDS, France) followed by 150 ml of isobutanol (99+% Alfa Aesar, UK) to give three fractions, two organic and one aqueous. The aqueous fraction was stored at -14 °C.

Chemicals

All chemicals used in bioassay tests were obtained from Sigma-Aldrich Sweden AB (Stockholm, Sweden) including hexanal CAS# 66-25-1, (E)-2-hexenal CAS# 6728-26-3, octanal CAS# 124-13-0, nonanal CAS# 124-19-6, sulcatone CAS# 110-93-0, decanal CAS# 112-31-2, humulene CAS# 6753-98-6, benzyl alcohol CAS# 100-51-6.

Sampling of volatiles

Two leaf equivalents of extract and 100 ng of pentadecane as internal standard were added on 10 cm² of filter paper and when methanol was used as a solvent it was allowed to evaporate
before beginning the sampling. The solid phase micro-extraction (SPME) technique (Supelco, Pennsylvania, USA) was used to collect compounds released from treated filter paper placed in a 20 ml autosampler vial. Before each collection period, routine purification of SPME fibers coated with polydimethylsiloxane-divinylbenzene was conducted at 225 °C for about 5 min in a GC injector. Sorption was carried out at 22 °C for 0.5 hour and collected volatiles were thermally desorbed in the GC injector for 1 min.

Gas-chromatography-electroantennography (GC-EAG) and chemical analyses

GC-EAG experiments were performed by a Clarus 500 gas chromatograph (PerkinElmer, Waltham, MA, USA) equipped with an DB-Wax capillary column (30 m; 0.25 mm i.d., 0.25 µm film-thickness, Agilent Technologies, Santa Clara, CA, USA) and a 1:1 effluent splitter that allowed a simultaneous flame ionization and EAG detection of the separated volatile compounds. Hydrogen was used as carrier gas (2.5 mL min⁻¹). The injector and the detector temperatures were set at 240°C. The oven temperature was programmed from 40°C for 1 min, then 5°C min⁻¹ to 200°C, then 10°C min⁻¹ to 240°C and held at 240°C for 12 min. The column outlet for the EAG was held in a humidified air-stream (room temperature) flowing at 0.5 m s⁻¹ over the *P. c-album* female antennal preparation. Antennae were prepared by cutting a single antenna at the base of the head of a female leaving a club of antenna undamaged. The cut antenna was attached to an EAG probe (Syntech, Hilversum, the Netherlands) by pushing the base and tip into droplets of electrically conductive gel (Spectra 360, Parker Laboratories, Fairfield, NJ, USA). The EAG probe was connected to an IDAC-4 data acquisition controller (Syntech). The signal was stored and analysed on a PC equipped with an IDAC-card using the program GC-EAD V. 4.4 (Synthech). A pasteur pipette containing a filter paper strip (10 mm x 5 mm) soaked with 10µl amount of hexanol in
hexane (1mg/ml) as control stimuli was used to test the sensitiveness of antennae. Three
antennae from three females were each used to test the EAG activity of the plant extract.
The qualitative analyses were performed using a Varian 3400 gas chromatograph coupled
with a Finnigan SSQ 7000 mass spectrometer (Termo-Finnigan, San Jose, CA, USA). The
GC was equipped with the same type of capillary column and operated under the same
condition as described in the EAG experiment except that Helium was used as the carrier gas
with an inlet pressure of 70 kPa. Electron ionization mass spectra were determined at 70 eV
with the ion source at 150°C. The EAG active compounds were identified by comparison of
their mass spectral data and GC-retention times with those of authentic synthetic standards.

Bioassay of oviposition response
Green Sponge Cloth (Wettex, Sweden) was used to make 10 cm² surrogate leaves. Each
artificial plant had two such surrogate leaves. In total two leaf equivalents of extract, one leaf
equivalent on each leaf side, were applied and when methanol solvent was used it was
allowed to evaporate before the beginning of bioassays. After evaporation of methanol,
surrogate leaves were moistened by gently spraying deionised water on the surface of the leaf
from both sides.
Female responsiveness was checked prior to the bioassay by placing them in a cage with two
artificial plants: one treated with 2 leaf equivalents of not concentrated methanol extract and
the second one with only solvent. Females which did not discriminate between the leaves
treated with extract and solvent or did not "drum" with their forelegs in order to taste
chemical cues were discarded from tests. Our previous pilot study revealed that Millipore
water and methanol did not affect the egg-laying of females.
Four multiple-choice experiments were carried out to test the attractiveness and stimulation of egg-laying behaviour of samples. The design of experiments 1-3 is presented in figure 2 A-C.

Experiment 4 was carried out to test the activities of selected EAG active compounds, which as single compounds were applied to surrogate leaves treated with two leaf equivalents of water fraction. The experiment comprised of: i) (E)-2-hexenal typical green leaf volatile; ii) octanal; iii) humulene; and iv) pure pentane as a solvent. No significant differences were found among landings, oviposition responses and number of eggs, hence data are not presented in the figure.

Four artificial plants were placed in the corners of a cage (70x70x70 cm) equipped with a transparent roof, in the centre of which air sucking was arranged at the flow rate 30 l min\(^{-1}\). The side walls of the cage were covered by a green fabric while the back and front were made from net. The cage was externally illuminated with a quartz metal halide lamp, HPI-T Plus 400 W (Philips, Holland). The location of the model plant in a cage was changed by clockwise moving of the plant to the location of the neighbouring one every 15 min, to eliminate corner biases and to prevent learning of the positions of particular extracts from having an impact on the experiment. The artificial plants were exchanged by new ones every half an hour. In total, a bioassay lasted for 2 hours. Only those landings followed by drumming behaviour were taken into the protocol. The behaviour response of an individual female to egg laying stimulants was scored as 100% for an actual egg-laying, 50% for curling the abdomen towards surrogate leaf and continuing irregular drumming but no oviposition observed, and 0% for drumming only. An average score of at least three replicates per female responding to a certain stimulus was calculated.

**Statistical analysis**
The nonparametric Quade test for dependent samples (Conover, 1999) was used to determine significant differences between the percentages of response type within each experiment. Values found to be significant at the $P<0.05$ level were marked with different letters. The Quade test was performed using the computer programme package StatXat version 9.

**Results**

Analysis of the chromatographic records obtained from 3 types of samples and a blank revealed that the non-concentrated methanol extract from *H. lupulus*, a preferred host plant of *P. c-album*, contained 89 volatile compounds which were either exclusively present or occurred at large amounts compared with those in the blank sample. Concentration of the sample resulted in loss of 36 compounds. Most of the volatiles were lost by evaporation and some sesquiterpenes were oxidised. The water fraction produced by solvent/solvent extraction was odourless and was composed of non-volatile phytochemicals (Figure 1).

Behavioural tests revealed that the significantly largest landing rate was observed on artificial leaves treated with not concentrated extract, compared to those recorded for substrates impregnated with concentrated extract, deodorised sample and control. Oviposition responses were in contrast similar to all sample types except control. Nevertheless, the proportion of eggs laid on artificial leaves treated with not concentrated extract differed significantly from the other three types of samples, and control leaves had the significantly lowest proportion of eggs (Figure 2A).

EAG test revealed 11 active compounds, over half of them representing aldehydes (Figure 1). A mixture of 8 EAG active commercially available compounds applied to artificial leaves treated with a deodorised water fraction bearing non-volatile oviposition stimulants attracted females to the source significantly, while dividing these volatile compounds into two groups:
the first one consisting of 5 aliphatic aldehydes namely hexanal, (E)-2-hexenal, octanal, nonanal, and decanal and the second group comprised of sulcatone, humulene and benzyl alcohol, obliterated attractiveness. Oviposition response did not differ significantly among the four types of treated leaves (Figure 2B).

The attractiveness of the leaves bearing synthetic odours were larger than that of the control but still significantly lower compare to the crude methanol extract, indicating that some active volatile compounds present in the crude extract were missing in the synthetic blend.

Female oviposition responses to the artificial leaves bearing host-plant contact chemicals differed significantly from those without nonvolatile phytochemical cues despite the presence of volatile compounds (Figure 2C). The significantly largest proportion of eggs was recorded on leaves treated with crude methanol extract, while control leaves and those containing 8 EAG active commercially available volatiles resulted in the significantly lowest proportion of eggs laid.

In conclusion, the results of the second and the third experiment demonstrated that volatile cues attracted females searching for host plants, as evidenced by tasting behaviour after landing, to their source but did not act as oviposition stimulants.

There were no significant differences detected in attractiveness ($DF=1.6$, $P=0.67$), oviposition responses ($DF=3.11$, $P=0.38$) or number of eggs laid ($DF=0.39$, $P=0.92$) among (E)-2-hexenal, octanal, and humolene as single compounds applied together with the water fraction as well as artificial leaves treated with only the deodorized water fraction.

**Discussion**

Data about foliar volatiles luring butterflies to their host-plants are scanty and reported mostly for papilionids (Vaidya, 1969; Saxena & Goyal, 1978; Feeny et al., 1989; Haribal &
Feeny, 1998; Heinz, 2008; Mercader et al., 2008; Pinto et al., 2009; Li et al., 2010), a few nymphaid species (Tang et al., 2013; Schäpers et al., 2015) as well as pierids (Ikeura et al., 2010). It is well known that butterflies require contact chemical cues to lay eggs (Huang & Renwick, 1994; Nishida, 1995; Honda et al., 2012), while host-plant volatiles primarily attract females, however, in some papilionid species volatile chemical cues applied on artificial substrate with no host-plant contact chemicals available elicit curl of abdomen, and oviposition of a few eggs (Saxena & Goyal, 1978; Heinz, 2008). In our experiments, oviposition response scores did not differ significantly among all substrates bearing host-plant contact chemicals regardless of the presence or absence of volatile compounds, hence, it was the observed enhancement of landing rates that resulted in the significantly larger proportions of eggs recorded on artificial leaves containing extract that had not been concentrated and contained the largest number and amount of volatiles, compared to those impregnated with concentrated extract or the deodorised water fraction.

As far as we know, behavioural activity of synthetic analogues to foliar volatiles has only been reported for the two aldehydes nonanal and decanal, which were found to be EAG-active and exhibited attractiveness towards Graphium sarpedon nipponum (Papilionidae) when tested individually (Li et al., 2010). Our previous study showed that hexanal, octanal and nonanal evoked Ca$^{2+}$ activity in the antennal lobes of comma butterflies (Carlsson et al., 2011) and the present experiments demonstrated that nonanal and decanal also elicited EAG activity of P. c-album females. However, no significant attractiveness was observed when the compounds were tested in the aldehyde mixture, and the presence of other phytochemicals was required to elicit behavioural responses. The synergism between volatiles mediating attraction of gravid females that we have observed is a common phenomenon among phytophagous insects (Schoonhoven et al., 2005), making tests of any single compound’s contribution to activity more complex.
Identification of oviposition stimulants is usually carried out by bio-guided fractionation followed by comparison of fraction effectiveness with those of the sample before fractionation, either by direct observation of female behaviour and scoring her oviposition responses (Honda et al., 1997), which requires more time, or by simply counting the number of eggs laid on treated substrates after the experiment is finished. Our results showed that the concentration of the crude extract significantly reduced the attractiveness of the sample due to loss of volatile compounds, while the oviposition response of gravid females was not affected. Hence, in order to avoid biased results (if some of the treatments contain attractants or repellents for host search) direct observation of butterfly behaviour by scoring female oviposition responses has to be used rather than methods based on egg distribution as a measure of oviposition efficiency for identification of oviposition stimuli.

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References


Figure legends

Figure 1. Total ion chromatogram record of volatiles sampled from 2 leaf equivalents of *H. lupulus* (A) non-concentrated crude methanol extract, (B) concentrated and re-diluted methanol extract, and (C) water fraction of the extract.

Intensity values presented on the Y axis are numbers of counts related to the abundance of the ions formed and corresponds to the amount of compound registered; DB-Wax fused silica capillary column (30 m x 0.25 mm i.d., 0.25 µm film thickness); the EAD active peaks correspond to: 1 – hexanal; 2 – (Z)-3-hexenal; 3 – (E)-2-hexenal; 4 – octanal; 5 - sulcatone; 6 – unidentified, 7 – nonanal; 8 – decanal; 9 – humulene; 10 – unidentified; 11 – benzyl alcohol; IS – pentadecane used as internal standard; OS – oxygenated sesquiterpene not tested for EAG activity.

Figure 2. Responses of gravid *P. c-album* females to extract, fractions, synthetic compounds and their mixtures.

Values are the means in percent. (A) The experiment consisted of artificial plants which surrogate leaves were treated with two leaf equivalents of: i) E - not concentrated methanol extract; ii) T - concentrated methanol extracts; iii) W - water fraction; and iv) K - control consisting of artificial leaves treated only with solvent. (B) The experiment consisted of artificial plants which leaves were treated with two leaf equivalents of water fraction: i) G – hexanal, (E)-2-hexenal, octanal, nonanal, and decanal; ii) R – sulcatone, humulene and benzyl alcohol; iii) M - mix of 8 commercially available EAG active compounds; and iv) W - pure pentane as solvent. (C) The experiment was comprised of artificial plants type: i) E; ii) M; iii) K and iv) S - surrogate leaves treated only with synthetic analogues of EAG active compounds. Each compound was applied at 0.1 µg/leaf dosage except humulene which dosage was 1 µg/leaf. The nonparametric Quade test was used to determine significant
differences between the percentages of response type within each experiment. The values found to be significant at the $P<0.05$ level were marked with different letter